DEVELOPMENT OF RECOMBINANT scFv ANTIBODY FOR THE DETECTION OF ZEARALENONE BY PHAGE DISPLAY ANTIBODY TECHNOLOGY

Pensuda Sompunga

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การพัฒนาแอนติบอดีสายเดี่ยวปรับแต่งพันธุกรรมสำหรับตรวจสอบซีราลีโนนโดยใช้เทคโนโลยีการแสดงแอนติบอดีบนผิวเฟจ

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

มหาวิทยาลัยเทคโนโลยีสุรนารี

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

Thesis Examining Committee

(Prof. Dr. Neung Teaumroong)

Chairperson

(Prof. Dr. Montarop Yamabhai)

Member (Thesis Advisor)

(Asst. Prof. Dr. Parinya Noisa)

Member

(DI Barbara Cvak)

Member

(Prof. Dr. Santi Maensiri)

Acting Vice Rector for Academic Affairs and Internationalization

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เพ็ญสุดา สมภูงา : การพัฒนาแอนติบอดีสายเดี่ยวปรับแต่งพันธุกรรมสำหรับตรวจสอบซีราลีโนนโดยใช้เทคโนโลยีการแสดงแอนติบอดีบนผิวเฟจ (DEVELOPMENT OF RECOMBINANT scFv ANTIBODY FOR THE DETECTION OF ZEARALENONE BY PHAGE DISPLAY ANTIBODY TECHNOLOGY) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร. มณฑารพ ยมาภัย, 97 หน้า.

ซีราลีโนนจัดเป็นสารที่ออกฤทธิ์คล้ายฮอร์โมนเอสโตรเจน เป็นสารพิษที่ถูกผลิตขึ้นจากเชื้อราในตระกูลฟูซาเรียม (*Fusarium* spp.) สามารถเข้าสู่ห่วงโซ่อาหาร มนุษย์และสัตว์ จากธัญพืชที่ปนเปื้อนและตกค้างในสิ่งปฏิกูลหรือน้ำธรรมชาติ จึงเป็นภัยคุกคามต่อสัตว์เลี้ยง สัตว์ป่าและมนุษย์ ดังนั้นการตรวจสอบซีราลีโนนจึงเป็นเรื่องที่สำคัญมากในด้านความปลอดภัยในอาหาร วิทยานิพนธ์นี้เป็นการประยุกต์ใช้เทคโนโลยีเฟจในการการพัฒนาแอนติบอดีชนิดเส้นเดี่ยวที่จำเพาะต่อซีราลีโนน โดยได้ทำการคัดหาจากคลังแอนติบอดีมนุษย์แบบปฐมภูมิ ซึ่งเป็นคลังเฟจที่แสดงแอนติบอดีแบบเส้นเดี่ยว (เอสซีเอฟวี) บนผิวเฟจ (คลังย่าโม) และคลังแอนติบอดีกระต่ายที่ถูกกระตุ้นด้วยสารพิษจากเชื้อรา โดยในการคัดเลือกแอนติบอดีจากทั้งสองคลังได้ทำการสลับชนิดของโปรตีนที่เชื่อมต่อกับสารพิษ คือใช้ ซีราลีโนนเชื่อมกับ Bovine Serum Albumin (BSA) และซีราลีโนนที่เชื่อมกับ ovalbumin (OVA) เพื่อเพิ่มโอกาสของการคัดหาให้ได้โคลนที่สามารถจับจำเพาะกับสารพิษจากเชื้อราในรูปแบบอิสระที่ปนเปื้อนอยู่ในธรรมชาติ ผลการคัดหาจากคลังแอนติบอดีกระต่าย พบว่าสามารถคัดหาเฟจได้หนึ่งโคลน ตั้งชื่อว่า bZD2B4 สามารถตรวจสอบปริมาณสารพิษจากเชื้อราซีราลีโนนที่ในช่วงสมการเชิงเส้น 50-5,000 ng/ml ค่า IC_{50} คือ 50 ng/ml ส่วนคลังแอนติบอดีมนุษย์ย่าโม ๑ สามารถคัดหาเฟจ ที่แสดงแอนติบอดีเอสซีเอฟวี ที่จำเพาะเจาะจงกับสารพิษจากเชื้อราซีราลีโนนได้ 1 โคลน ตั้งชื่อว่า yZA8B2 จากนั้นได้ทำการตัดชิ้นส่วนของยีนเอสซีเอฟวีไปไว้ในพลาสมิดที่เหมาะสม เพื่อนำไปพัฒนาให้อยู่ในรูปของ scFv และ scFv-AP แล้วได้ทำการตรวจสอบความจำเพาะต่อสารพิษจากเชื้อราซีราลีโนนโดยวิธีอีไลซ่าแบบยับยั้ง พบว่า ค่า IC_{50} ของ yZA8B2 scFv และ scFv-AP คือ 100 และ 20 ng/ml ตามลำดับ จากนั้นได้ทำการปรับสภาวะการทำปฏิกริยาอิไลซ่าให้เหมาะสมขึ้นจนทำให้ ความไวของแอนติบอดี yZA8B2 scFv และ scFv-AP ได้รับการปรับปรุงให้ดีขึ้น 2 เท่าและ 40 เท่า ตามลำดับ ผลการทดสอบความจำเพาะเจาะจงของแอนติบอดีโดยการทดสอบการข้ามไปจับกับสารพิษจากเชื้อราชนิดอื่นๆ ได้แก่ อะฟลาท็อกซินบี 1, โอคราท็อกซิน เอ, ดีออกซีนิวาลีนอล และฟูโมนิซิน พบว่าไม่จับกับสารพิษจากเชื้อราเหล่านั้น นอกจากนั้นแล้วได้นำแอนติบอดีทั้งสองรูปแบบมาตรวจสอบความสามารถในการตรวจซีราลีโนนที่ปนเปื้อนในตัวอย่างข้าวโพดและข้าวสาลี ซึ่งจากการตรวจสอบพบว่า แอนติบอดีทั้งสองรูปแบบสามารถตรวจสอบการปนเปื้อนได้ในทางเชิงคุณภาพ จากการศึกษาแบบจำลองโครงสร้างการจับกันระหว่าง

โมโนโคลนอลแอนติบอดีที่ปรับแต่งพันธุกรรมกับสารพิษจากเชื้อราซีราลีโนน ได้รับการยืนยันว่าโมโนโคลนอลแอนติบอดีของมนุษย์โคลน yZA8B2 สามารถจับแบบจำเพาะเจาะจงกับสารพิษจากเชื้อราซีราลีโนน โดยมีแอนติบอดีส่วนเส้นหนักมีบทบาทสำคัญ ผลงานวิจัยทั้งหมด สรุปได้ว่าสามารถใช้เทคโนโลยีการแสดงแอนติบอดีบนผิวเฟจในการสร้างโมโนโคลนอลแอนติบอดีที่ปรับแต่งพันธุกรรมที่สามารถจับกับสารพิษจากเชื้อราซีราลีโนนได้อย่างจำเพาะเจาะจง และสามารถตรวจหาสารพิษจากเชื้อราซีรารีโนนที่ปนเปื้อนในผลิตภัณฑ์เกษตรและธัญพืช ภายใต้ข้อบังคับตามมาตรฐานของประเทศไทย จึงอาจใช้แอนติบอดีที่ได้จากการวิจัยเป็นต้นแบบในการพัฒนาให้ดียิ่งขึ้นเพื่อให้สามารถตรวจวิเคราะห์ปริมาณการปนเปื้อน ด้วยวิธีการทางอิมมูนอื่นๆ อีกทั้งแอนติบอดีที่พัฒนาขึ้นมาได้นี้ยังอาจเป็นประโยชน์สำหรับการประยุกต์ใช้ทางการแพทย์ในอนาคต เนื่องจากแอนติบอดีนี้มีต้นกำเนิดมาจากมนุษย์

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา เพ็ญจิดา สัมฤาชา

ปีการศึกษา 2560 ลายมือชื่ออาจารย์ที่ปรึกษา

PENSUDA SOMPUNGA : DEVELOPMENT OF RECOMBINANT scFv ANTIBODY FOR THE DETECTION OF ZEARALENONE BY PHAGE DISPLAY ANTIBODY TECHNOLOGY. THESIS ADVISOR : PROF. MONTAROP YAMABHAI, Ph.D., 97 PP.

RECOMBINANT scFv ANTIBODY/ZEARALENONE/ELISA/PHAGE DISPLAY ANTIBODY TECNOLOGY

Zearalenone (ZEN) is a nonsteroidal estrogenic mycotoxin, produced as a secondary metabolite of *Fusarium* spp. It enters the food and feed chains from contaminated cereals and infiltrates into sewage or natural waters, posing a potential threat to exposed livestock, wildlife and humans. Therefore, establishing sensitive and specific methods to detect ZEN has become very important for food and feed safety reasons. This thesis involved identification, engineering and characterization of recombinant single chain variable fragment (scFv) antibodies against ZEN from the human phage display antibody library (Yamo I) and the immunized rabbit mix library. The antibodies were selected from these two libraries by switching the conjugated proteins, i.e., bovine serum albumin (BSA)-ZEN and ovalbumin (OVA)-ZEN, to increase the chance of obtaining clones that can bind to free toxin. For the immunized scFv rabbit antibody library, one phage clone, designated bZD2B4, was selected. This phage-displayed scFv could be inhibited by soluble ZEN at the linear range of 50-5,000 ng/ml by competitive ELISA. The detection limit of this clone was approximately 50 ng/ml. However, the antibody in the soluble scFv format could not bind to free ZEN. For the Yamo I library, one phage-displayed scFv clone specific to

free ZEN, designated yZA8B2, was isolated. The yZA8B2 soluble scFv format could bind to free ZEN; therefore, this clone was subcloned into the pET21d+ and pKP300 delta III vectors to express scFv and scFv-AP antibody formats. Competitive ELISA indicated that the median inhibition concentration (IC_{50}) of recombinant yZA8B2 scFv antibody and scFv-AP fusion were 100 and 20 ng/ml. After ELISA optimization by the checkerboard titration method, the sensitivity of recombinant yZA8B2 scFv antibody and scFv-AP fusion was improved approx. 2-fold and 40-fold, respectively. No cross-reactivity to other mycotoxins; i.e., (aflatoxin B1 (AFB1), ochratoxin A (OTA), deoxynivalenol (DON), and fumonisins B1 (FUM)), was observed. Finally, the ability of recombinant yZA8B2 scFv and scFv-AP antibodies for the detections of ZEN contamination in corn and wheat samples were investigated. The yZA8B2 scFv and scFv-AP can be used to qualitatively detect zearalenone contamination in corns and wheat. Homology modeling study illustrated specific binding of the recombinant antibody to ZEN and confirmed the better fit of human yZA8B2 antibody as well as the role of the variable heavy chain in the binding. In conclusion, the recombinant monoclonal antibody specific to zearalenone could be obtained from phage display technology and detected zearalenone contamination in certain agricultural and cereal products, according to the Thai's regulations. This antibody can be used as a template to further develop the quantification of contaminated ZEN in food and feed using different immuno-based methods. Moreover, this antibody could be useful for medical applications in the future because of its human origin.

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Pensuda Sompunga

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

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)
Amp	=	Ampicillin
AP	=	Alkaline phosphatase
bp	=	Base pairs
BSA	=	Bovine serum albumin
cfu	=	Colony forming units
CDR	=	Complementary determining region
DNA	=	Deoxyribonucleic acid
E.coli	=	Escherichia coli
ELISA	=	Enzyme-linked immunosorbent assayxvi
Fc	=	Constant region of an antibody molecule
HRP	=	Horse radish peroxidise
IgG	=	Immunoglobulin class
IMAC	=	Immobilised metal affinity chromatography
Kan	=	Kanamycin
KLH	=	Keyhole limpet hemocyanin
MW	=	Molecular weight
OD	=	Optical density
OVA	=	Ovalbumin

LIST OF ABBREVIATIONS (Continued)

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
V_H	=	Variable region of heavy chain of antibody
V_L	=	Variable region of light chain of antibody
µg	=	microgram
kDa	=	(kilo) Daltons
µl	=	microlitre
µM	=	micromoles
^oC	=	degrees Celcius
g	=	grams
h	=	hours
L	=	litre
m	=	metre
M	=	molar
mg	=	milligram
min	=	minute

LIST OF ABBREVIATIONS (Continued)

ml	=	millilitre
nM	=	nanomolar
nm	=	nanometer
rpm	=	revolutions per minute
sec	=	seconds
w/v	=	weight per unit volume

CHAPTER I

INTRODUCTION

1.1 Significance of this study

Zearalenone (ZEN) is a non-steroidal estrogenic mycotoxin produced by *Fusarium* species. The exposure risk to humans and animals is the consumption of contaminated food and animal feeds. This toxin has been proved to be hepatotoxic, hematotoxic, immunotoxic, genotoxic, teratogenic and carcinogenic to a number of mammalian species (Zinedine et al., 2007). Many types of immunoassays have been developed for ZEN contamination including immunosensors, quantum dot-based detection, fluorescence polarization and a large number of ELISA-based assays (Yuan et al., 1997). The key advantages offered by immunoassays, over more classical analytical techniques (HPLC, TLC and LC-MS/MS), are rapidity and ease-of-use. ELISA technique requires high sensitive and specific polyclonal, monoclonal, or recombinant antibody against target antigens for detection. Recombinant antibodies may be used as an immunoanalytical agent in analytical or screening assays based on various formats of ELISA and/or as an active compound of biosensor surfaces (Brichta J. et al., 2005).

Conventionally, mycotoxin or hapten-specific antibodies are produced from hyperimmunised animals or hybridoma cell lines. However, monoclonal antibody production is technically demanding and sometimes not straightforward enough (Yau et al., 2003). Therefore, recombinant DNA technology is one of the ways to complement and even partly replace hybridoma technology in the production of monoclonal antibodies. Production of antibodies has been revolutionized by the development of modern molecular biology methods for the expression of recombinant DNA. Phage display technology represents one of the most powerful tools for the production and selection of recombinant antibodies and has been

recognized as a valuable alternative way for the preparation of antibodies of a desired specificity.

This thesis involved engineering and characterization of single chain variable fragment (scFv) antibodies against ZEN isolated from human phage display antibody libraries (Yamo. II) (Potjamas et al, 2009) and immunized rabbit mix (Bmab-OTA&ZEN) library. Binding properties of antibodies selected from these two libraries were evaluated. The scFv gene was cloned into the expression vector for expression in *E.coli* SHuffle strain and vector containing an alkaline phosphatase (AP) gene for the production of scFv-AP fusion protein upon expression. This scFv-AP might be used as one-step detection probe for developing a rapid and affordable immunoassay for the detection of ZEN in food and feedstuffs.

1.2 Research objectives

1. To select specific phage scFv monoclonal antibody against zearalenone (ZEN) from immunized rabbit mix (OTA&ZEN) and Yamo I library

2. To engineer scFv and scFv-alkaline phosphatase fusions (scFv-AP) antibodies for the detection of free Zearalenone

3. To optimize competitive ELISA assay for the detection of zearalenone in commodities and agricultural products

1.3 Scope and limitation of the study

In this study, the phage scFv monoclonal antibody was selected from immunized rabbit mix (OTA&ZEN) and Yamo I library by biopanning. Zearalenone was used as a target antigen. Enzyme-Linked Immunosorbent assay (ELISA) was used to confirm the binding of scFv phage positive clone. Selected clones were engineered to create scFv and scFv-alkaline phosphatase fusions (scFv-AP). The sensitivity and cross-reactivity of scFv-AP antibody was determined by competitive inhibition ELISA. Finally, application of this antibody for the

detection of ZEN from agricultural products was tested by spike experiments on Corn and Wheat samples.

CHAPTER II

LITERATURE REVIEWS

2.1 Zearalenone

2.1.1 Production and occurrence

Agricultural products are often contaminated with fungi that can produce toxic metabolites referred to as mycotoxins. Mycotoxins are highly toxic secondary metabolites found during pre-and post-harvest, storage, or during food processing periods of almost all agricultural commodities worldwide. Some of the mycotoxins considered to be of importance to human health are: aflatoxins (AF) produced by *Aspergillus* spp., ochratoxin A (OTA) produced by *Aspergillus* spp. and *Penicillium* spp., deoxynivalenol (DON), zearalenone (ZEN) and fumonisins (FB) produced by *Fusarium* spp. (Binder et al., 2007).

This thesis was focused on zearalenone. Worldwide several studies have reported high ZEN contamination in a wide variety of important agricultural products such as maize, barley, oats, wheat, sorghum, millet and rice. In addition, this toxin has been detected in cereals products like flour, malt, soybeans and beer (Edupuganti, Edupuganti, and O'Kennedy., 2013). No international harmonised maximum limit for zearalenone in foodstuff exists (CCFAC, 2000). However, the maximum limit of zearalenone in various products are shown in Table 2.1. The dietary intake of ZEN may also occur through consumption of meat, milk and eggs from animals exposed to this toxin. ZEN has been found in maize extraction from agricultural samples in

various countries such as Europe, North America, Philippines, Thailand, and Indonesia (Xiaoqian et al., 2014). Moreover, this toxin was also detected in soils, drainage water, wastewater influents and effluents, rivers and lakes of the United States, Italy and Poland and ranged up to 220 ng/L (Bakos et al., 2013).

Table 2.1 Regulation limits of zearalenone in various countries.

Countries	Maximum limit (ppb)	Food and feed commodities	Refs
Japan	1000	Compound feeds	(Anukul et al., 2013).
South Korea	200	Grains and processed grain foods	(Anukul et al., 2013).
	50	Confectionaries	(Anukul et al., 2013).
	20	Baby foods	(Anukul et al., 2013).
Thailand	30-1000	All foods	(Anukul et al., 2013).
	2000	Cereal and product	Standard Criteria for Animal Feed Quality Control Act, 2017.
	3000	Corn and product	Standard Criteria for Animal Feed Quality Control Act, 2017.
	100	Concentrate feed and complete feed for gilts pigs	Standard Criteria for Animal Feed Quality Control Act, 2017.
	250	Concentrate feed and complete feed for sow and breeding pigs	Standard Criteria for Animal Feed Quality Control Act, 2017.

Table 2.1 Continued.

Countries	Maximum limit (ppb)	Food and feed commodities	Refs
Thailand	530	Concentrate feed and complete feed for calf ,cattle, sheep, lamb	Standard Criteria for Animal Feed Quality Control Act, 2017.
China	60	Wheat, corn and their products in food	Worldwide Mycotoxin Regulations - Romer Labs
	≤500	Complementary and complete feeding stuffs, corn	Worldwide Mycotoxin Regulations - Romer Labs
Iran	200	Maize, wheat	Regulations - Biomin.net - Mycotoxins.info
	400	barly	Regulations - Biomin.net - Mycotoxins.info
Republic of Korea	1000	All other feeds	Regulations - Biomin.net - Mycotoxins.info
	3000	All plant originated materials	Regulations - Biomin.net - Mycotoxins.info
Central and South America	100	Wheat flour, cereals and cereals products except wheat and malted barley	Regulations - Biomin.net - Mycotoxins.info

Table 2.1 Continued.

Countries	Maximum limit (ppb)	Food and feed commodities	Refs
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	≤500	Complementary and complete feeding stuffs, corn	Worldwide Mycotoxin Regulations - Romer Labs
Iran	200	Maize, wheat	Regulations - Biomin.net - Mycotoxins.info
	400	barly	Regulations - Biomin.net - Mycotoxins.info
Republic of Korea	1000	All other feeds	Regulations - Biomin.net - Mycotoxins.info
	3000	All plant originated materials	Regulations - Biomin.net - Mycotoxins.info
Central and South America	100	Wheat flour, cereals and cereals products except wheat and malted barley	Regulations - Biomin.net - Mycotoxins.info

Table 2.1 Continued.

Countries	Maximum limit (ppb)	Food and feed commodities	Refs
	400	Rice, processed and derived	Regulations - Biomin.net - Mycotoxins.info
	600	Brown rice	Regulations - Biomin.net - Mycotoxins.info
	150	Corn, canjiquinha, canjica, corn products and by-products	Regulations - Biomin.net - Mycotoxins.info
Central and South America	200	Whole wheat, whole wheat flour, wheat bran	Regulations - Biomin.net - Mycotoxins.info
Chile	200	All foods	Regulations - Biomin.net - Mycotoxins.info
Canada	3000	feed for gilts and sows	Regulations - Biomin.net - Mycotoxins.info
Colombia	1000	sorghum	Regulations - Biomin.net - Mycotoxins.info
Uruguay	200	corn, barley	Regulations - Biomin.net - Mycotoxins.info
European Union	200	unprocessed maize	(Anukul et al., 2013).
	100	unprocessed cereals	(Anukul et al., 2013).

Table 2.1 Continued.

Countries	Maximum limit (ppb)	Food and feed commodities	Refs
	50	cereal snacks, breakfast cereals and processed cereal-based foods	(Anukul et al., 2013).
	20	Baby food	(Anukul et al., 2013).
Morocco	200	cereals, vegetable oils	Regulations - Biomin.net - Mycotoxins.info
South Africa	5000	Feeding stuffs on full ration basis for sow and pigs	Regulations - Biomin.net - Mycotoxins.info
	3000	Piglets	Regulations - Biomin.net - Mycotoxins.info
	500	Calves and dairy cattle	Regulations - Biomin.net - Mycotoxins.info

2.1.2 Structure

Zearalenone (ZEN; 6-(10-hydroxy-6-oxo-trans-1-undecyl)-b-resorcylic acid lactone) is a lactone derivative of resorcylic acid and a nonsteroidal estrogenic mycotoxin produced by several species belonging to the genus *Fusarium*, a common field and storage fungus (Tanaka et al., 1988). The molecular weight of ZEN is around 318 Da. ZEN was given the trivial name zearalenone as a combination of *G. zeae*,

resorcylic acid lactone, -ene (for the presence of the C-1’ to C-2 double bond), and -one, for the C-6’ ketone (Bennett and Klich, 2009). In mammals, the keto group at C-8 is reduced to two sterioisomeric metabolites of ZEN (α- and β-isomers) (Figure 2.1). These metabolites are also produced by the fungi, but at much lower concentrations than for ZEN (CCFAC, 2000). The ZEN derivatives (α-zearalenol (α-ZEN), β-zearalenol (β-ZEA), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), zearalanone (ZAN)) can be detected in corn stems infected with *Fusarium* spp. in the field and in rice culture (Bravin et al., 2009). Mycotoxins including ZEN are considered as hapten as their molecules are small.

Haptens are incomplete immunogens but can be made fully immunogenic by coupling them to a suitable carrier molecule. Some of the more common carrier proteins include keyhole limpet hemocyanin (KLH; 350 kDa), bovine serum albumin (BSA; 67 kDa) and ovalbumin (OVA; 45 kDa). The number of conjugated ZEN molecules on BSA and KLH are 35 and 94, respectively while number of conjugated ZEN molecules on OVA is unknown (Edupuganti, Edupuganti, and O'Kennedy., 2013). Haptens and corresponding hapten-carrier conjugates have been essential to the development of sensitive, quantitative and qualitative immunoassays. The amount of hapten attached to the carrier influences the strength of the immune response directed toward the newly created antigenic determinant. Hence the haptenic density of the conjugate is also important in the development of immunoassays.

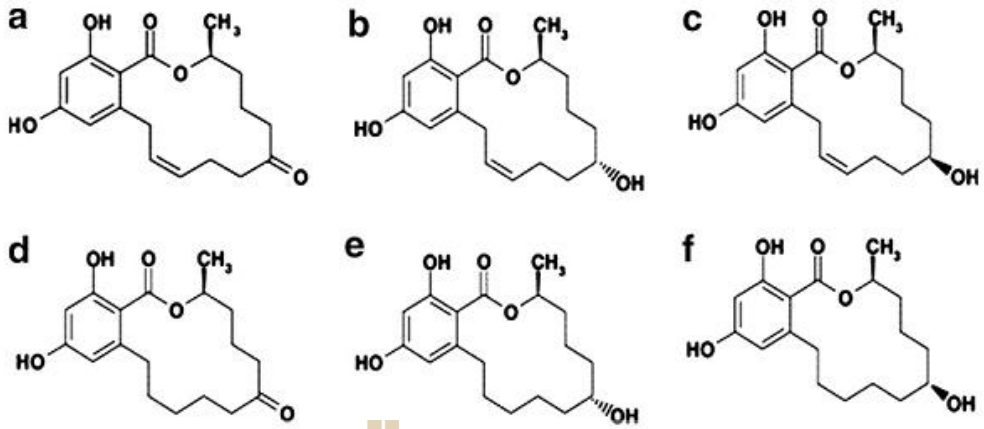


Figure 2.1 Chemical structures of ZEN and its derivatives: (a) zearalenone (ZEN), (b) α-zearalenol (α-ZEN), (c) β-zearalenol (β-ZEN), (d) zearalanone (ZAN), (e) α-zearalanol (α-ZAL) and (f) β-zearalanol (β-ZAL).

2.1.3 Toxicity

ZEN is classified as a type-III carcinogenic agent (IARC, 2002), plays a major role in inducing reproductive toxicological effects like precocious puberty (Edupuganti, Edupuganti, and O'Kennedy., 2013). ZEN and its metabolites are able to bind to estrogen receptors (ER) resulting in estrogenicity that exceeds the activity of most of other naturally occurring nonsteroidal estrogens (phytoestrogens) (Bakos et al., 2013). In fact, the occurrence of ZEN in food has been related to the early onset of puberty in children from Puerto Rico (Schoental, 1983). ZEN and its metabolites have become of great interest because evidence suggests that they may play an important role in increasing the risk of hormone-dependent tumors (Bakos et al., 2013), such as α-zearalanol (α-ZAL) has been suggested to increase the risk of developing breast cancer in human (Belhassen et al., 2015). However, it is unclear whether α-ZAL derives from the metabolism of ZEN or from consumption of contaminated meat because α-ZAL can be detected in the final product when used as a

growth promoter for livestock (Coulombe et al., 2014). The effect of ZEN in animal varies. Pigs are especially sensitive; while poultry and cows show little sensitivity. The effects of ZEN on animals include feminization of male animals, disrupts conception, ovulation, and fetal development in female animals. ZEN has been shown to affect the male reproductive system and trigger reactive oxygen species (ROS) generation. Lipid metabolism changed significantly after low-dose ZEN exposure, resulting in two alterations. One is the increase in energy production through promoted fatty acid uptake and β-oxidation, along with excessive oxidative stress; the other is an inhibition of steroidogenesis and esterification, possibly resulting in reduced hormone secretion (Li. et al., 2014).

2.1.4 Methods of Detection

Conventional methods for the detection of ZEN include Thin-layer chromatography (TLC), High performance liquid chromatography (HPLC) and Gas-liquid chromatography (GLC). These techniques are usually high cost and time-consuming. In recent years, many immunoassay methods have been developed for rapid detection of ZEN, such as enzyme-linked immunosorbent assay (ELISA), fluorescence polarization immunoassay, electrochemical microfluidic chips, immunochemical test, electrochemical magnetic bead-based inmunosensor, immunochromatographic strip, and so on. Among these immunoassay methods, ELISA has been extensively used as a cost- and time-saving, sensitive, quantitative, and high-throughput method (Xiaoqian et al., 2014). One key advantage of antibodies is the ease by which they can be incorporated into multiplexed assay formats for detecting multiple antigens.

2.2 Phage display antibody technology

2.2.1 Phage display technology

Phage display was first described by George P. Smith in 1985. Ultimately, phage display technologies, recombinant and combinatorial gene techniques merged into a powerful technology for displaying diverse libraries (Mccafferty et al., 1990). The main advantage of phage display technology is the direct link between genotype and phenotype of the antibody being displayed. This technique refers to the display of functional foreign proteins or antibody fragments on the surface of a bacteriophage (Clackson et al., 1991). Filamentous bacteriophages have been used to display single-chain variable fragment (scFv) and the fragment antigen binding (Fab) antibody fragments by fusion to the phage minor coat protein pIII (Hoogenboom et al., 1991; McCafferty et al., 1990) (Figure 2.2). As the molecular understanding of the filamentous bacteriophage lifecycle and structure evolved, it became clear that the simplicity of its single-stranded DNA (ssDNA) genome and the close coupling of viral genotype with phenotype offered means for manipulating and presenting proteins for binding selections (Smith, 1985). scFv stands for single-chain variable fragment and is one of the smallest units capable of binding an antigen. The minimized format has several advantages in clinical, diagnostic and research applications due to its small size and reduced immunogenicity (Yuan et al., 1997). The procedure for selecting phage is simple and inexpensive. Using recombinant DNA technology and microbiological methods, peptides or proteins are fused to one of the phage capsid proteins by genetic engineering into appropriate vector (plasmid or phagemid) (Figure 2.3). The proteins that displayed on the phage coat proteins are accessible to various molecular interactions. Affinity selection of antibodies is accomplished by exposing the phage

library to immobilized antigen molecules (binding clones are captured and non-binding clones are washed away). The captured phage particles are eluted from antigen, amplified by infecting *Escherichia coli* host cells and used in a subsequent round of affinity selection. After the final round of affinity selection phage particles are amplified in order to prepare and characterize their displayed antibodies individually (Petrenko and Vodyanoy, 2003). Finally, the monoclonal phage population with the desired binding specificities can be isolated (Willats, 2002). The antibodies phage display library can be used to select antibodies against any antigen, including foreign antigen, non-immunogenic antigens and toxic antigens (Marks et al., 1991). The advantages of phage display are easy to manipulate, rapid, inexpensive and appropriate for large scale production. The main application of hapten-specific recombinant antibodies may be as an immune-analytical agent in analytical or screening assays based on various formats of ELISA and/or as an active compound of biosensor surfaces. Currently, there are companies that manufacture assays affected by the toxins in the haptens agricultural country (Brichta J. et al., 2005).

2.2.2 Structure of antibody

Antibodies can identify and neutralize foreign objects like bacteria and viruses. Each of them could recognize a specific antigen unique to its target as they possess the antigen-binding sites, a paratope (a structure analogous to a lock) located at the upper tips of the "Y" shaped antibody molecules. This paratope is specific for one particular epitope (analogous to a key), displayed on the particular antigen, allowing these two structures to specifically bind together (Ahmad et al., 2012). So, Antibodies represent a powerful weapon system in defending our body against non-self-agents.

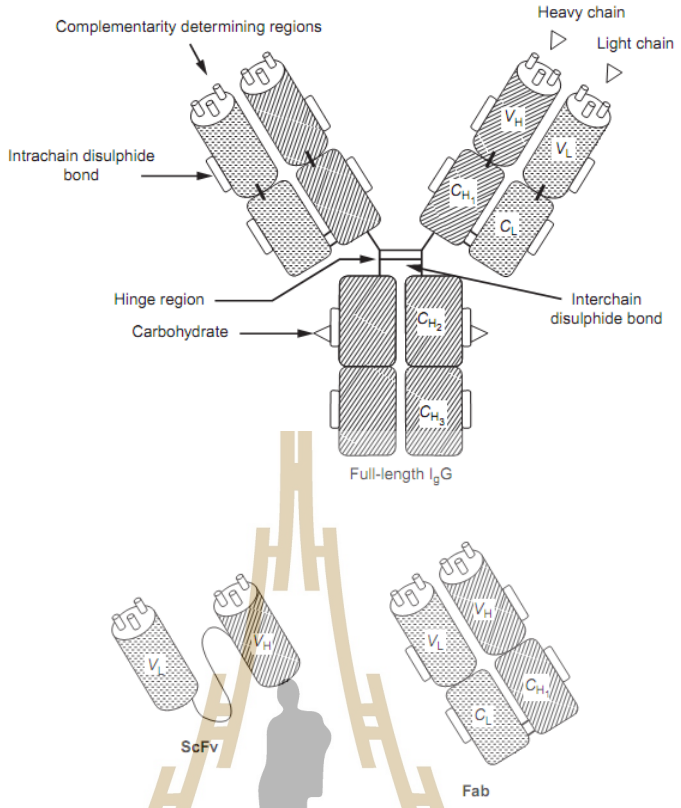


Figure 2.2 A schematic representation of a full-length immunoglobulin G (IgG) antibody comprising two heavy and light chains. For IgG, carbohydrate elements are attached via the asparagine 297 amino acid residue. Key: V_H, variable heavy; V_L, variable light; C_H, constant heavy; C_L, constant light. Recombinant antibody fragments, namely, the single-chain variable fragment (scFv) and fragment antigen binding (Fab), are also illustrated. These are produced using phage display technology, as outlined in the text (Byrne et al., 2015).

The structure of antibody consists of four heavy (H) polypeptide chains whose molecular weights are approximately 50 kDa and two light (L) chains (25 kDa) covalently attached by disulphide bonds. The heavy chains are encoded by variable heavy (V_H), diversity (D), joining heavy (J_H), and constant heavy (C_H) genes, while the

corresponding light chain is encoded by variable light (V_L), J_L, and C_L segments. With emphasis on immunodetection, antigen binding (e.g., to an intact, pathogenic bacterial cell) is mediated by the hypervariable complementarity-determining regions (CDRs) found in the variable regions of heavy and light chains. With emphasis on immunodetection, antigen binding is mediated by the hypervariable complementarity-determining regions (CDRs) found in the variable regions of heavy and light chains.

2.2.3 Recombinant antibody

Advances in recombinant antibody technology have greatly facilitated the genetic manipulation of antibody fragments. The genetic manipulation of recombinant antibodies thus improved our understanding about the structure and functional organization of immunoglobulins (Brichta J. et al., 2005). Further, these advances have led the development of a large variety of engineered antibody molecules for research, diagnosis, and therapy with specificities out of reach of conventional antibody technology.

In 1975, biological research was revolutionized by the landmark discovery that the fusion of B cells with myeloma cells yields an immortalized ‘‘hybridoma’’ cell that continuously produces monoclonal immunoglobulin from an immunized animal to immortal myeloma cells (Fields et al., 2013). The generation of recombinant antibodies from hybridoma cell lines is not easily accomplished due to high levels of aberrant mRNA molecules from rearranged, nonfunctional heavy- and light-chain genes. scFv antibodies directly cloned from monoclonal hybridoma cells often have lower binding affinities than their parent mAbs (Hua et al., 2015). In comparison to poly- and monoclonal antibodies, recombinant antibodies using the phage display technology can be prepared faster, in more automatic process and with reduced consumption of laboratory animals (J. brichta, 2005).

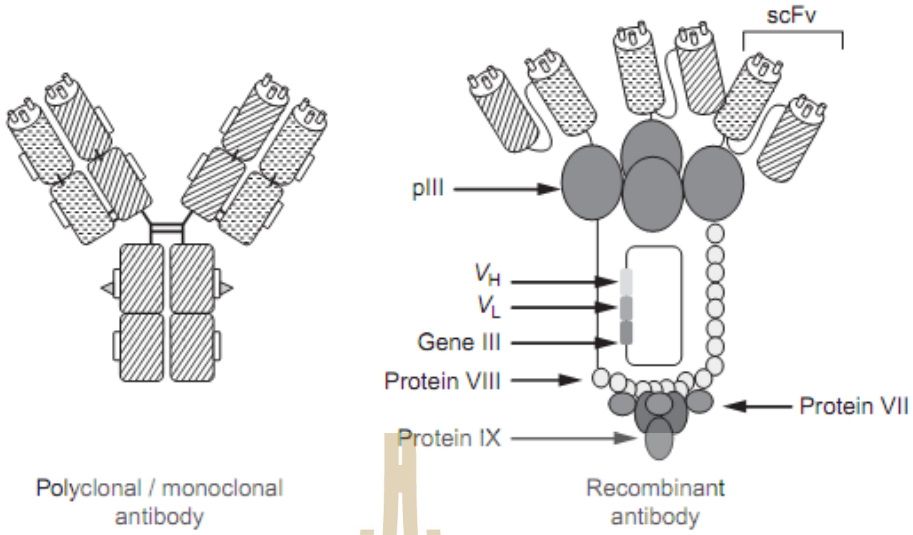


Figure 2.3 Structure of monoclonal (mAb), polyclonal (pAb), and recombinant antibodies (rAb). For recombinant antibodies, a phage particle displaying three scFv antibody fragments is also shown. For soluble expression, as outlined in the text, the phage nucleocapsid containing proteins III, VII, VIII, and IX is not expressed (Byrne et al., 2015).

2.3 Recombinant antibody against ZEN

Recently, there has been an increasing amount of literature on polyclonal, monoclonal and recombinant scFv antibodies against ZEN by immunization of Balb/c mice (Burmistrova et al., 2009; Xiaoqian et al., 2014; Yuan et al., 1997), rabbits (A. A. Burkin et al., 2002), phage display technology (Edupuganti, Edupuganti, and O'Kennedy., 2013), transgenic *Arabidopsis* plants (Yuan et al., 2000) and *Pichia pastoris* (Chang et al., 2008) (Table 2.2).

However, the development of an antibody which is essential for immunoassay using animals and the hybridoma cell line is usually expensive and time-consuming. When a hybridoma is used as an mRNA source for construction of scFv antibody, it is

most desirable to use a cell line that produces a high-affinity antibody for the target hapten or antigen (Brichta J. et al., 2005). Single chain antibodies fragments are small engineered antibodies and are potential alternatives to monoclonal antibodies. The expression level of single chain antibody is the direction of the variable heavy chain and light chain in both yeast and the *E. coli* expression system V_H - V_L demonstrated better expression than V_L - V_H in *Pichia pastoris* (Chang et al., 2008).

One report combined hybridoma and phage display technology to screen for the generation of a scFv from a monoclonal antibody-secreting hybridoma to ZEN (Yuan et al., 1997). They reported that pQY1.5 clone in HB2151 secreted a soluble scFv antibody (QY1.5) with a high zearalenone-binding affinity (concentration required for 50% inhibition of binding (IC_{50}), 14 ng/ml), similar to that of parent monoclonal antibody in a competitive indirect enzyme-linked immunosorbent assay. But, this scFv exhibited higher cross reactivity with other structurally similar ZEN derivatives than the original monoclonal antibody. On the other hand, when soluble scFv antibody (QY1.5) was used to detect ZEN by competitive direct-ELISA method, and 50% inhibition of binding around 50 mg/L (Wang et al., 2008). Moreover, generation of anti-zearalenone scFv by only phage display showed that the IC_{50} value of scFv-P2A2 is higher than the scFv generated earlier by Yuan et al. 1997. However, scFv-P2A2 exhibits minimal cross reactivity in comparison to the already reported antibodies for detection of ZEN (Edupuganti, Edupuganti, and O'Kennedy., 2013). Thus, the generation of anti-ZEN scFv antibodies from cDNA libraries provides scope for the development of a range of scFvs with highly selected specificities.

Table 2.2 Sensitivity of assays developed to-date using previously reported anti-ZEN antibodies. The IC_{50} value is a 50% inhibition of binding.

Type of antibody	IC_{50} value	Method	Reference
Single chain antibody fragment (scFv)	14 ng/ml	Competitive indirect ELISA	(Yuan et al., 1997)
Monoclonal antibody	17 ng/ml	Competitive indirect ELISA	(Yuan et al., 1997)
Single chain antibody fragment (scFv)	11.2 ng/ml	Competitive indirect ELISA	(Yuan et al., 2000)
Single chain antibody fragment (scFv) (Yuan et al., 1997)	50 µg/ml	Competitive direct ELISA	(Wang et al., 2008)
Monoclonal antibody	0.8 ng/ml	Competitive direct ELISA	(Burmistrova et al., 2009)
Single chain antibody fragment (scFv)	80 ng/ml	Competitive direct ELISA	(Edupuganti, Edupuganti, and O'Kennedy, 2013)
Monoclonal antibody	20 ng/ml	Competitive indirect ELISA	(Xiaoqian et al.,2014)

Normally, the test kit use polyclonal or monoclonal antibodies antibody as detection reagents that have high price such as Neogen veratox kit and AgraQuant® Zearalenone Assay. However, there have been no test kits that used recombinant antibody by phage display technology available in the market. The most commonly

used enzyme immunoassays for analytic detection are carried out with primary or secondary antibodies that are labeled with enzymes, such as horseradish peroxidase (HRP) or alkaline phosphatase (AP), which are used to generate the reporter signal. Interestingly, the sensitivity of scFv-AP fusion was better than the scFv form (Rangnoi et al., 2011). The fusion protein is bifunctional, retaining both antigen binding specificity and AP enzymatic activity.

CHAPTER III

METERIALS AND METHODS

3.1 Materials

The immunized rabbit mix (OTA&ZEN) and Yamo I library (Potjamas et al, 2009) were constructed in our laboratory. KM1307 helper phage NEB (Vieira and Messing, 1987) was propragated as described in the MRC phage display protocols. Standard Zearalenone (ZEN), Zearalenone conjugated with Bovine albumin serum (BSA), ovalbumin (OVA) and keyhole limpet hemocyanin (KLH) was prepared from *Fusarium* species (Dokin, Germany). Bovine albumin serum (BSA) was natural purified protein obtained from Fluka, USA. Keyhole limpet hemocyanin (KLH) was natural purified protein obtained from Merck, Germany. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and p-Nitrophenyl phosphate disodium salt hexahydrate (pNPP) were obtained from Amresco (USA). Mouse anti-M13-HRP, His probe-HRP and Protein L-HRP were purchased from Amersham-Pharmacia Biotech (Uppsala, Sweden), Thermo Fisher Scientific, USA and Sigma, Germany, respectively. *E. coli* strains were TG1TR (suppressor) and *E. coli* HB2151 (non-suppressor) were obtained from the MRC, Cambridge, UK. *E. coli* SHuffle B cell was obtained from New England Biolabs, NEB (Massachusetts, United States).

3.2 Methods

3.2.1 Biopanning of Phage display scFv library against ZEN

For the immunized rabbit mix (OTA&ZEN) library, four rounds of biopanning were performed with alternate selection stringency through the antigen conjugated with bovine albumin serum (BSA), ovalbumin (OVA), a concentration of 25 and 20 µg/ml in phosphate buffer saline (PBS), respectively and incubated at 4ºC for overnight, followed by blocking with 2% fat-free powdered milk (MPBS) for 1 h at room temperature. After three washings with 1xPBS, 100 µl of phage library (10^{13} pfu/µl) was added and incubated at room temperature for 1 h with rotation and standing on bench for 1 h. For Yamo.II library, two rounds of biopanning were performed with alternate selection stringency through the antigen conjugated with OVA and BSA, a concentration of 20 and 15 µg/ml in phosphate buffer saline (PBS), respectively and incubated at 4ºC for overnight, followed by blocking step. After three washings with 1xPBS, 100 µl of phage library (10^{11} pfu/µl) was added and incubated at room temperature for 1 h with rotation and standing on bench for 1 h. Another method of biopanning of both libraries were immobilized 200 µg/ml of ZEN conjugated BSA in 1xPBS. After adding the library, the unbound phages were removed by washing from 10 to 20 times with both 0.05% PBST and 1xPBS in the each round of biopanning. The phages binding to antigen-coated wells were eluted by 1 µg/ml trypsin buffer, followed by 0.2M glycine-HCl, pH 2.0. The eluted phage was infected into *E.coli* TG1 to obtain individual phage clones as previously described (Rangnoi et al., 2011). After each round of selection, the binding specificities of individual phage scFv clones were identified by enzyme-link immunosorbent assay (ELISA).

3.2.2 Amplification of bound phage

One hundred fifty µl of elute phage was added into 2 ml of log phase growing culture of TG1 and incubated at 37°C without shaking for 30 minutes. 10 µl of infected *E.coli*. TG1 was made 10-fold serial dilutions. Then, the serial dilution were spread 100 µl on to 2xYT agar plate containing 100 µg/ml of ampicillin and 1% glucose and incubated overnight at 37°C. Next day, colonies were counted the total number of eluted phage.

3.2.3 Monoclonal phage ELISA

Single colony of each round of biopanning was picked randomly and cultured in 96-deep well plate followed by super infection by KM13 helper phage. Phage supernatants were collected after centrifugation and subjected to ELISA for screening of monoclonal anti-ZEN phage scFv. For immunized rabbit mix (OTA&ZEN) library, the immune 96 micro well™ plate (Nunc, Denmark), for first, second and fourth rounds using 2 µg of ZEN-OVA in PBS and third round of biopanning using 2 µg of ZEN-BSA in PBS as a target were immobilized and used 1% BSA as a negative control. For Yamo.II library, two rounds of biopanning using 2 µg of ZEN-KLH in PBS as a target were immobilized and used 1% BSA and 1% keyhole limpet hemocyanin (KLH) as a negative control. And, then the plate was incubated at 4ºC for overnight. The phage supernatant was added to immune 96 micro well™plate and the binding phage scFv was detected with horseradish peroxidase (HRP)-conjugated anti-M13 antibody (1:5000). The color of reaction was developed with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) reagent (Ameresco, USA). The reaction was quantified by measuring the absorbance at 405 nm.

3.2.4 Confirmation of binding activity of monoclonal phage clones by ELISA

The positive clones of each round of biopanning were determined expression levels and specificity of phage produced scFv fragments using Enzyme-linked immunosorbent assay (ELISA). For immunized rabbit mix (OTA&ZEN) library, the immune 96 micro well™ plate (Nunc, Denmark) were coated with 2 µg of ZEN conjugated BSA and OVA using 1% BSA as a negative control. For Yamo.II library, the immune 96 micro well™ plate (Nunc, Denmark) were coated with 2 µg of ZEN conjugated BSA, OVA and KLH by using 1% BSA and 1% KLH as a negative control. The remaining binding site was blocked. Detection of the binding phage antibody was determined with HRP-conjugated anti-M13 antibody (1:5000). The color of reaction was developed with ABTS reagent (Ameresco, USA) and measured an absorbance at 405 nm.

3.2.5 DNA sequence and analysis

After, the positive clones were confirmed by phage ELISA. The positive phage clones were extracted using DNA miniprep kit (Qiagen) and confirmed by automated DNA sequence (Macrogen, Korea). DNA sequence was analyzed with IgBLAST and data of complementarity determining regions (CDRs) 1, 2, 3 with Imgt software. The 3D structure was predicted by Phyre2 software.

3.2.6 Production of soluble scFv antibody

The selected recombinant phages were used to infect *E. coli* HB2151 for the production of soluble scFv antibodies. Briefly, the infected *E. coli* HB2151 cells were cultured in 2xYT medium supplemented with 100 mg of ampicillin per ml and 0.1% glucose at 30ºC with 250 rpm shaking until they reached an A_{600} of 0.9. Isopropyl-b-dthiogalactopyranoside (IPTG) was then added to 1 mM final

concentration, and the cells were incubated on a shaker at 30ºC overnight. Supernatants containing the extracellular soluble scFv antibodies were separated from the cell pellets by centrifugation at 4000 rpm for 15 min. The affinities of soluble scFv antibodies were detected with the scFv ELISA described 3.2.8.

3.2.7 Cloning of single-chain variable fragment (scFv) antibody against zearalenone

The bZD2B4 clone from immunized rabbit mix (OTA&ZEN) library and yZA8B2 clone from Yamo.II library were engineered to create scFv antibody. The pET21d+ vector was brought from New England Biolabs (NEB, USA). The scFv fragment was inserted into pET21d+vector with *Nco*I and *Not*I sticky ends. The DNA of scFv framents and pET21d+ vector was digested with the *Nco*I (20U/µl, NEB, USA) and *Not*I (20U/µl, NEB, USA) enzymes. The digestion reactions of scFv fragments and pET21d+ vector were performed separately, each in a total volume of 80 and 100 µl. The reaction mixtures consist of 5 µg of insert DNA, 2.5 µg of vector DNA, 1xCutsmart buffer, 20U of *Nco*I (20U/µl, NEB, USA) and 10U of *Not*I (20U/µl, NEB, USA). The reactions were incubated at 37ºC for 3 hours and heat inactivated at 80ºC for 20 min. Then, DNA of vector was dephosphorylated by adding 0.5 µl of CIP enzyme (10U/µl, NEB, USA) and incubated at 37ºC for 1 hour. The inserts and vector were separated by gel electrophoresis and followed by Wizard clean up kit (Promega, USA). The DNA of scFv antibodies were ligated into pET21d+ vector at a 3:1 ratio and incubated at 25ºC for 2 hours. The final ligation reaction was transformed into *E*.coli TG1 by chemical transformation. After that the transformed cells were spread on LB plate containing 100 µl/ml of ampicillin and incubated overnight at 37ºC. The individual colony was picked and analyzed by double digestion with of *Nco*I and *Not*I sites. The positive clones were shown 2 bands in approximately size 5.4 kb and 800 bp.

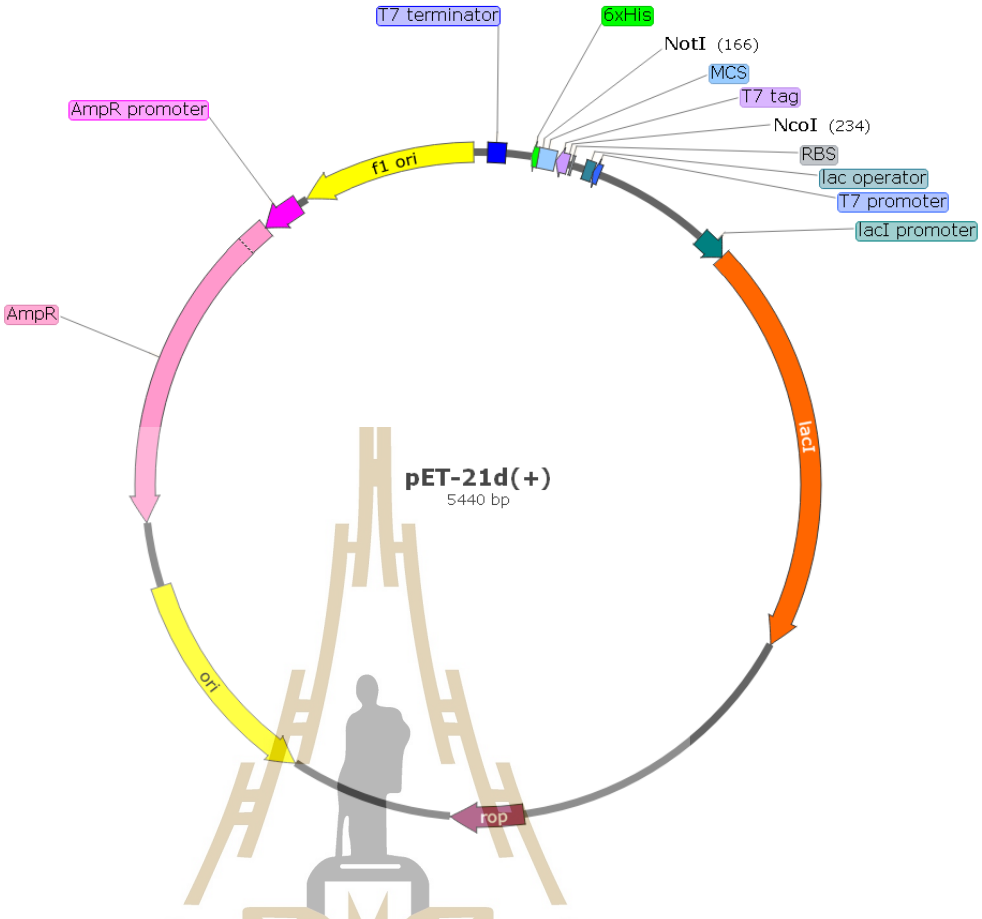


Figure 3.1 Map of pET21d+vector. The arrow shows the position of T7 promoter and ampicillin resistance genes.

3.2.8 Production of anti-ZEN scFvs

The bZD2B4 and yZA8B2 clones were transformed into *E.*coli SHuffle B cell and spread on LB plate containing 100 µl/ml of ampicillin and incubated for overnight at 30ºC. A single colony containing the bZD2B4 and yZA8B2 plasmids were inoculated in 5 ml LB media containing 100 µl/ml of ampicillin and cultured at 30ºC for overnight. Four mL of overnight culture were used to inoculate 400 ml of LB medium containing 100 µl/ml of ampicillin next day. Cells were cultured at 30ºC to OD_{600}=0.9, followed by induction by adding 0.4 mM isopropyl-b-D-thiogalactopyranoside (IPTG) at 25ºC for 16 h before cell harvest. The cell pellets were

harvested by centrifugation at 8000 rpm for 10 min, which were re-suspended in 20 ml cold binding buffer (20 mM sodium phosphate, 500 mM NaCl and 20 mM imidazole, pH 7.4) with 1 mg/ml lysozyme. Cells were disrupted by intermittent sonication for 5 mins on ice using 30 s pulse and 30 s break for cooling, followed by centrifugation at 4ºC for 30 mins at 10,000 g. The retained soluble fractions were further processed for protein purification. The supernatant was applied to the Ni-NTA column pre-equilibrated with binding buffer (20 mM sodium phosphate, 500 mM NaCl and 20 mM imidazole, pH 7.4). Elution by a 250 mM imidazole in elution buffer (pH 7.4) was performed. Fractions containing bZD2B4/yZA8B2 were pooled of each clone and exchanged by dialysis into PBS buffer, respectively at 4ºC. The samples were collected and kept at 4ºC. The soluble fraction and purity of the samples were assessed by SDS-PAGE. Then, 1 M $MgCl_2$ was added to give a final concentration of 1 mM $MgCl_2$ and kept at 4ºC. Total protein concentrations were determined with the Nanodrop 2000 spectrophotometer (thermo scientific). The affinities of soluble scFv antibodies were detected with the scFv ELISA described in 3.2.8.

3.2.9 scFv ELISA

The immune 96 micro wellTM plate (Nunc, Denmark) was coated with 2 µg of ZEN conjugated BSA, OVA and KLH using 1% BSA, 1% KLH and skim milk as a negative control. Detection of the bound scFv antibody was determined with HRP-conjugated anti-HIS antibody (1:5000). The color of reaction was developed with ABTS reagent (Amresco) and measured an absorbance at 405 nm.

3.2.10 Enginerring of scFv-AP (alkaline phosphatase) fusions

The bZD2B4 clone from immunized rabbit mix (OTA&ZEN) library and yZA8B2 clone from Yamo.II library were engineered to create scFv-AP fusion. The pKP300 ΔIII vector was generated from Brain, Kar Lab (UIC, USA). It is an

expression vector that containing alkaline phosphatase gene. The expression of this gene is under pho A promotor that is inexpensively induced when the culture is starved for inorganic phosphate. The scFv fragment was inserted into pKP300ΔIII vector with *Nco*I and *Not*I sticky ends. The DNA of scFv framents and pKP300ΔIII vector were digested with *Nco*I (20U/µl, NEB, USA) and *Not*I (20U/µl, NEB, USA) enzymes. The digestion reactions of scFv fragments and pKP300ΔIII vector were performed separately, each in a total volume of 50 µl. The reaction mixtures consist of 5 µg of insert DNA, 3.5 µg of vector DNA, 1xCutsmart buffer, 20U of *Nco*I (20U/µl, NEB, USA) and 10U of *Not*I (20U/µl, NEB, USA). The reactions were incubated at 37ºC for 3 hours. The reactions were heat inactivated at 80ºC for 20 min. Then, DNA of vector was dephosphorylated by adding 0.5 µl of CIP enzyme (10U/µl, NEB, USA) and incubated at 37ºC for 1 hour. The inserts and vectors were separated by gel electrophoresis and followed by Wizard clean up kit (Promega, USA). The DNA of scFvs were ligated into pKP300ΔIII vector at a 3:1 ratio and incubated at 25ºC for 2 hours. The final ligation reaction was transformed into *E.coli* TG1 by chemical transformation. After that the transformed cells were spread on LB plate containing 100 µl/ml of ampicillin and incubated overnight at 37ºC. The individual colony was picked and analyzed by double digestion with of *Nco*I and *Not*I sites. The positive clones were shown 2 bands in approximately size 5 kb and 800 bp.

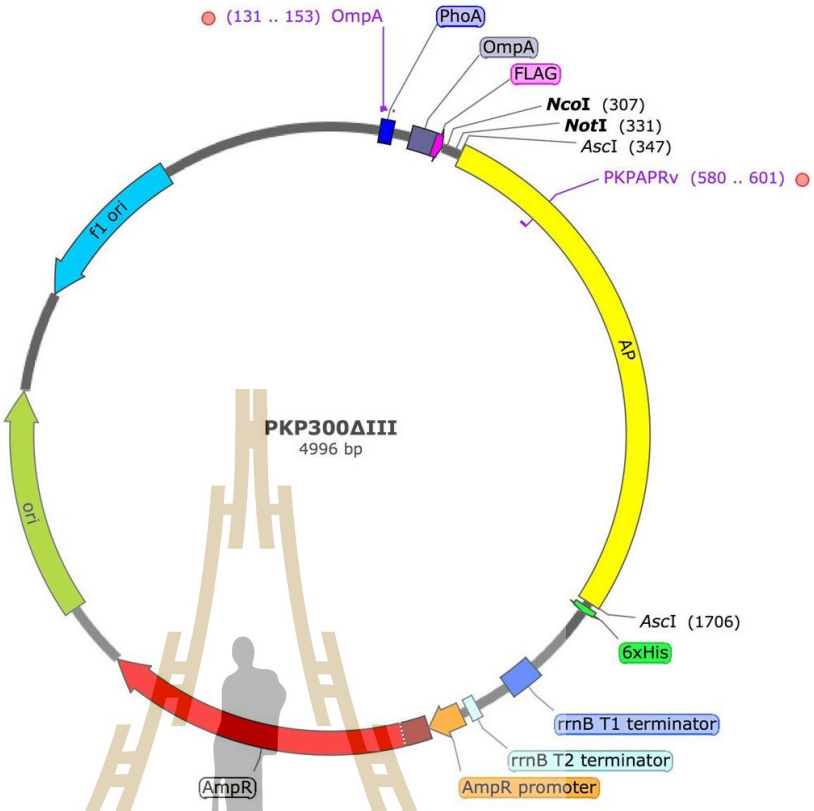


Figure 3.2 Map of pKP300ΔIII (Krittika, personal contact). The arrow shows the position of PhoA promotor, alkaline phosphatase and ampicillin resistance genes.

3.2.11 Expression and detection of anti-ZEN scFvs-AP

The bZD2B4 and yZA8B2 clones were transformed into *E.coli* TG1 (Chicaco) and spread on LB plate containing 100 µl/ml of ampicillin and incubated for overnight at 37ºC. The next day, individual colony was picked into 5 ml of LB broth containing 100 µl/ml of ampicillin and incubated overnight at 37ºC. Then, the overnight culture of positive clones was grown into 200 ml of low phosphate media (1:1000 dilution) containing 1 M $MgSO_4$ and 1 M glucose at 30ºC, 250 rpm for 20

hours. The culture was centrifuged at 8,000 rpm for 10 min at 4ºC. The cell pellet was lysed on ice with binding buffer (15 mL). Composition of lysis reagent: BugBuster® 10× reagent (Novagen, Madison, WI) diluted to 1× final concentration using 1× Tris Buffered Saline (Amresco, Solon, OH). The lysed cells were incubated at room temperature on a shaking platform at 40 rpm for 20 min, followed by centrifugation at 8,000 rpm for 10 min, at 4°C, to pellet the cell debris. The clarified cell lysate will be used for protein purification by immobilized metal affinity chromatography (IMAC) according to the manufacturer's protocol (Qiagen, Germany). The resins were washed twice with washing buffer 1 (20 mM Tris; pH 7.4, 500 mM NaCl, 20 mM imidazole) and washing buffer 2 containing 20 mM imidazole before scFv fragments were collected with elution buffer (20 mM Tris; pH 7.4, 500 mM NaCl, 250 mM imidazole). To monitor antibody purify and determine the efficiency of purification, the flow through fraction, wash faction and elution fraction were compared by SDS-PAGE. Fractions containing bZD2B4/yZA8B2 were pooled of each clone and exchanged by dialysis into TBS buffer at 4ºC. Then, 1 M $MgCl_2$ was added to give a final concentration of 1 mM $MgCl_2$ and kept at 4ºC. Total protein concentrations were determined with the Nanodrop 2000 spectrophotometer (thermo scientific).

The immune 96 micro well™ plate (Nunc, Denmark) was coated with 2 µg of ZEN conjugated BSA, OVA and KLH, 1%BSA and 1%KLH as a control overnight at 4ºC. The ELISA plate was washed three times with Tris-buffered saline (TBS; 25 mM tris-HCl pH 7.5, 140 mM Nacl, 3mM KCl) supplemented with 0.05% tween 20 (TBST) and blocked with 2% MPBS for 1 hour at room temperature. The ELISA plate was washed 3 times with TBST and 2 times with TBS. After that, the ELISA plates were added 50 µl of TBST and 150 µl of scFv-AP. After incubated scFv-AP for 2 hours with rotating, the ELISA plate was washed 3 times with TBST and 2

times with TBS. Finally, 200 µl of p-Nitrophenyl phosphate disodium salt hexahydrate (pNPP; Ameresco) was added into each well and incubated for 1 hour at room temperature. The reaction was quantified by measuring the absorbance at 405 nm.

3.2.12 Competitive ELISA

To provide that the bZD2B4 and yZA8B2 scFv antibodies can be bound with free ZEN by competitive ELISA. Competitive ELISA was developed and used to determine the sensitivities and specificities of the scFv antibodies (phage scFv, scFv antibody and scFv-AP. Two µg of ZEN-BSA, ZEN-OVA and ZEN-KLH were coated on an ELISA plate. The scFv antibodies (phage scFv, scFv antibody and scFv-AP), at dilution previously determined by an antibody titer were pre-incubated with varying concentrations of soluble ZEN. After incubation at 37ºC, 300 rpm in dark for 30 min, the mixture was transferred to previously coated target and incubated for 1 h. The unbound antibodies were washed away 3 times with TBST/PBST and 2 times with TBS/PBS. The amount of bound phage scFv antibody was determined by the addition of 100 µl HRP-conjugated anti-M13 antibody diluted 1:5,000 in PBS. The amount of soluble scFv antibody bound was determined by incubation with 100 µl of HRP-conjugated anti-HIS antibody (1:5,000). The amounts of HRP-conjugated anti-M13 antibody and HRP-conjugated anti-HIS antibody bound were assessed by the addition of 200 µl of ABTS reagent (Ameresco, USA). Finally for scFv-AP fusion, the pNPP substrate was added into each well and incubated for 1 hour at room temperature. The reaction was quantified by measuring the absorbance at 405 nm.

3.2.13 Cross reactivity of scFv-AP fusion in competitive ELISA

To test the specific binding of recombinant yZA8B2 scFv-AP fusion. Recombinant yZA8B2 scFv-AP fusion was tested against a range of free AFB_1 (Alfatoxin B1) and OTA (Ochratoxin), FUM (Fumonisin), and DON (Deoxynivalenol).

Stock solution of AFB_1, OTA, FUM and DON were prepared in TBST. The assay was immobilized and blocked as described in 3.2.11. After washing step, the dilution antibodies were pre-incubated with varying concentrations of soluble AFB_1, OTA, FUM and DON from 5 µg to 0.004 µg/ml and dilution of recombinant yZA8B2 scFv antibody and scFv-AP fusion. After incubation at 37ºC, 300 rpm for 30 minutes, the mixture was transferred to the previously coated and blocked ELISA plate and then incubated for 1 hour. The unbound antibodies were washed was washed 3 times with TBST and 2 times with TBS. Finally, the pNPP substrate was added into each well and incubated for 1 hour at room temperature. The reaction was quantified by measuring the absorbance at 405 nm.

3.2.14 Optimization of ELISA

The optimum ZEN-BSA, ZEN-OVA and ZEN-KLH concentration required for coating onto the microtiter plate, and the best working concentration of the recombinant yZA8B2 scFv antibody and scFv-AP fusion were determined using checkerboard titration. In the titration procedure, appropriate concentrations of the coating antigen were prepared with varied concentration per well from 2 µg to 0.01 µg of ZEN-BSA, ZEN-OVA and ZEN-KLH in PBS. The recombinant yZA8B2 scFv antibody and scFv-AP fusion were serially diluted with 2-fold dilutions from 1:10 to 1:1,280 in TBST/PBST. A set of experimental parameters including concentration of ZEN-BSA, ZEN-OVA and ZEN-KLH and dilution of anti-ZEN antibody was optimized sequentially to improve the sensitivity of the immunoassay. The optimal condition was used to test affinities of anti-ZEN antibodies with free ZEN by competitive ELISA as described 3.2.11.

To optimize conditions for storage of each antibody, recombinant yZA8B2 scFv antibody and scFv-AP fusion were kept at 4ºC and -20°C for storage.

For increasing stability, glycerol was added to a final concentration of 50% and stored at -20°C. The antibody solutions were stored in small working aliquots to avoid repeated freeze-thaw cycles. Finally, recombinant yZA8B2 scFv antibody and scFv-AP fusion were checked stability at 2 and 4 weeks by scFv and scFv-AP dilution ELISA.

3.2.15 Detection of ZEN with contaminated corns and wheat samples

Corns and wheat spike or reference sample (TR-Z100) containing 0, 454.2 and 1085.5 ppb of ZEN were extracted for detection of ZEN contaminated in natural products. A 2 g of corns and wheat reference samples were taken and extracted with 10 mL of 70% of methanol in water mixture (70:30) or PBST or TBST with vortex for 5 min followed by the centrifugation at 4000 rpm for 5 min at 4ºC. The extract samples were through a Whatman #1 filter and collected the filtrate. For 0 ppb of corns and wheat reference sample, the extract samples were added vary concentration of ZEN (3000, 1000, 500, 300, 100, 50, 30, and 10 ng/ml). For 454.2 and 1085.5 ppb of corns and wheat reference samples, the extract samples were tested detection in natural samples. Extraction was performed with 70% methanol, the extract samples were diluted in PBST/TBST buffer (a ratio of 1:3 and 1:5 (w:v). Both spike and reference samples were performed in triplicate. Recombinant yZA8B2 scFv antibody and scFv-AP fusion were used optimal dilution of antibodies from procedure in 3.2.13. The efficiency of extraction procedure was evaluated through competitive ELISA.

3.2.16 Antibody modeling and molecular docking

Homology modeling of the three-dimensional (3D) structures of bZD2B4 and yZA8B2 scFv were generated from the amino acid sequences using the SWISS-MODEL website. The server chose the template by sequence identity analysis. After that, the sequence was processed by the server for modeling. Models

were visualized with the program PyMOL (www.pymol.org). To understand the molecular interaction between ZEN and the scFv antibody, GOLD software was used to analyze antigen-antibody docking. A ZEN molecule (PubChem accession nos.: 5281576) was obtained from PubChem compound database (www.pubchem.ncbi.nlm.nih.gov). All scoring functions that were available for GOLD at the time of the study (ChemPLP, GoldScore, ChemScore, and Astex Statistical Potential (ASP)) were tested in separate runs. The active site for docking was defined follow as homology model of scFv at the position of OH, Tyr224 incase of bZD2B4 antibody; NZ, Lys175 atoms in the case of yZA8B2 within 6. Å radius. The best scored solution (ChemPLP) was considered, and viewed in Discovery studio 2016 (BIOVIA, San Diego).

CHAPTER IV

RESULTS

4.1 Generation of recombinant Rabbit scFv antibody against Zearalenone by Phage Display Technology

4.1.1 Affinity selection of rabbit scFv library against ZEN

Two methods of biopanning were performed by switching the conjugated proteins and using 200 µg of ZEN conjugated BSA. However, enrichment of ZEN-specific phage after each round of affinity selection was observed for switching the conjugated proteins method only. A summary of bio-panning results are listed in Table 4.1.

4.1.2 Phage ELISA

After biopanning of both libraries were performed with two methods. The recombinant phage clones of rabbit scFv libraries were tested binding affinity with ZEN conjugated proteins by ELISA (Figure 4.1-4.4). A total of 6 positive clones from rabbit library were isolated. It has showed that the RB8, RC7, RC12, RH1, RH6 and bZB2D4 phage clones were bound specific with ZEN. Further, the positives clones were binding specific with ZEN conjugated KLH when compared with negative control. The result displayed that 6 positive clones were bound specific with ZEN conjugated KLH as shown in Figure 4.5.

Table 4.1 Overview of selection of antibodies against ZEN conjugated proteins from rabbit library.

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

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

4.1.3 Amino acid sequence analysis of selected scFv clones

The DNA sequences of the six positive phage clones were determined by automated DNA sequencing. The DNA sequences analysis indicated that RB8, RC7, RC12, RH1, RH6 and bZD2B4 scFv antibodies are the same clone and amino acid sequence as shown in Figure 4.6. These scFv antibodies contain the variable heavy chain (VH) from family 1, which was derived from germ line V1-S69*01 and variable light chain (VL) from family 1, which was derived from germ line V4-S4*01. The 3D structure was predicted by Phyre2 software (Figure 4.7).

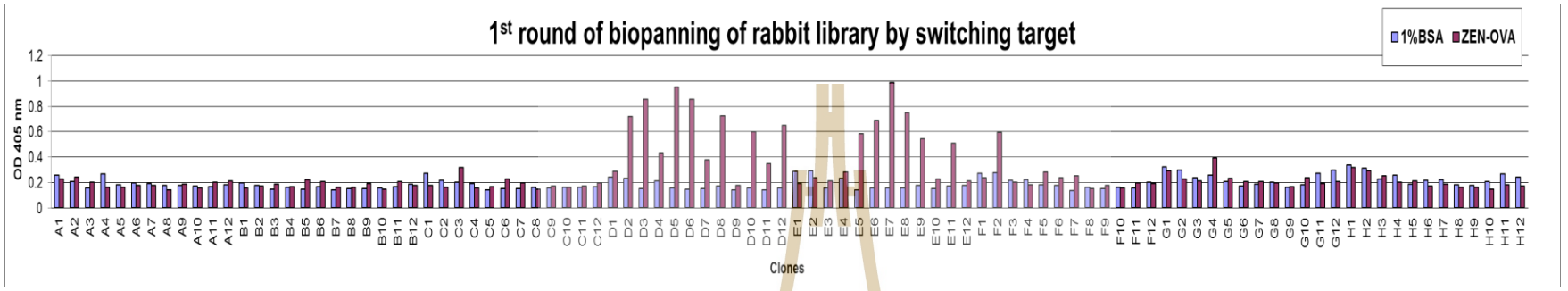


Figure 4.1 Binding of phage scFv antibody fragments to ZEN-OVA. Sixteen positive clones were induced and analyzed binding to ZEN-OVA by ELISA. Negative control is a 1% BSA. The data is expressed as absorbance at 405 nm.

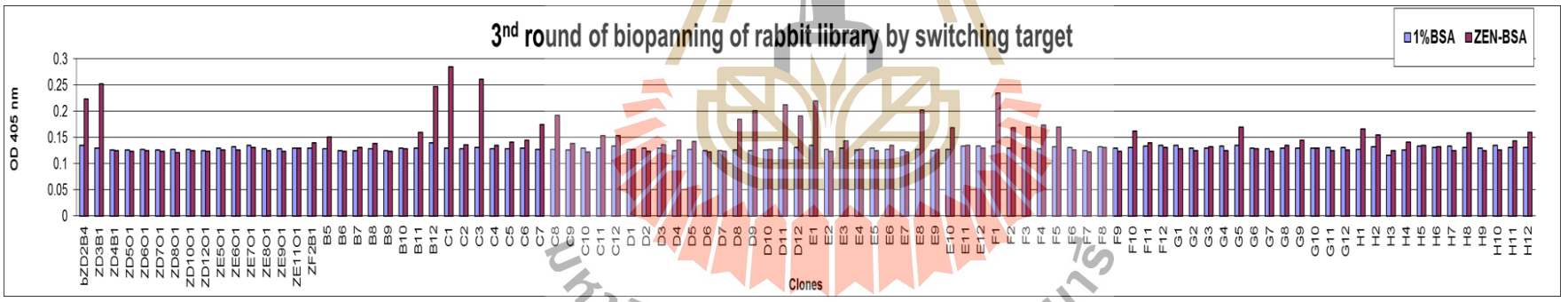


Figure 4.2 Binding of phage scFv antibody fragments to ZEN-BSA. Two positive clones from first round and four positive clones from third round were induced and analyzed binding to ZEN-BSA by ELISA. Negative control is a 1% BSA. The data is expressed as absorbance at 405 nm.

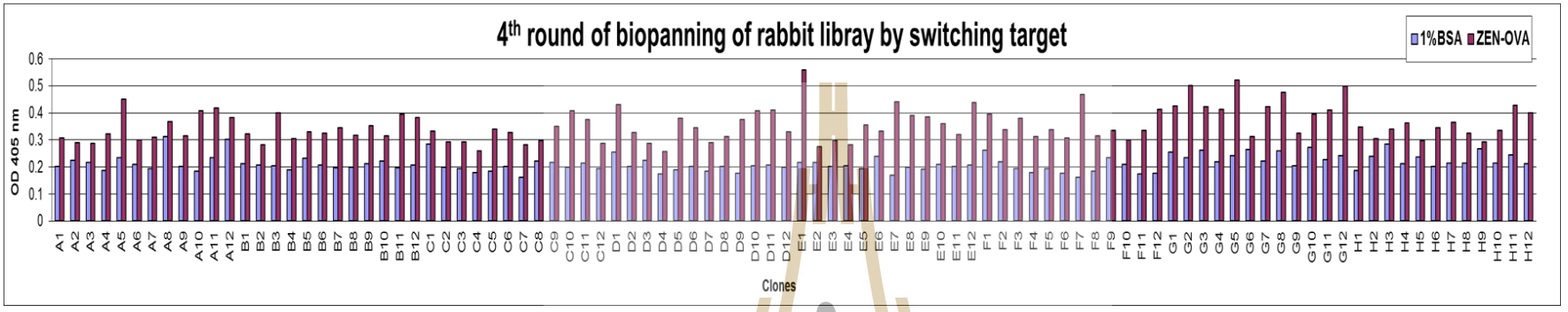


Figure 4.3 Binding of phage scFv antibody fragments to ZEN-OVA. Thirty positive clones were induced and analyzed binding to ZEN-OVA by ELISA. Negative control is a 1% BSA. The data is expressed as absorbance at 405 nm.

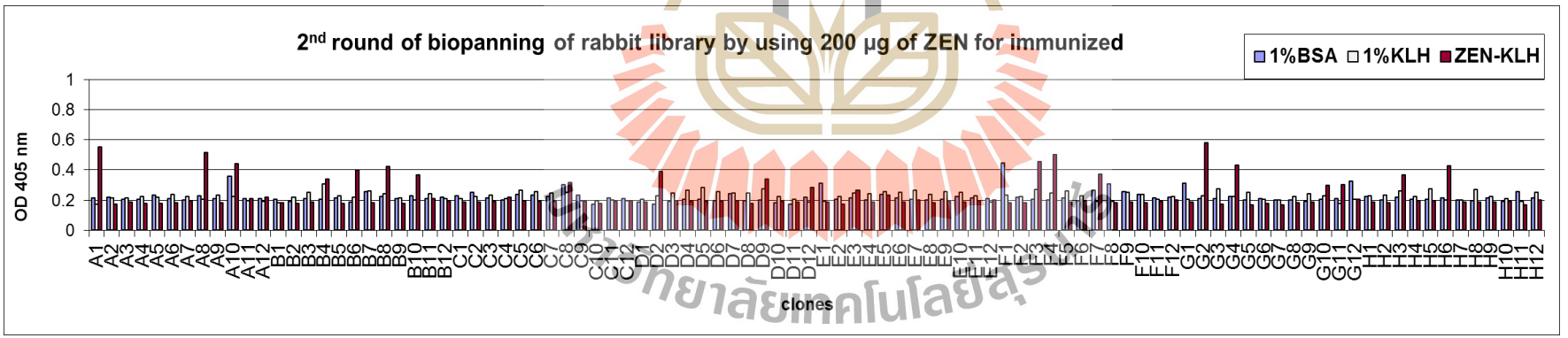


Figure 4.4 Binding of phage scFv antibody fragments to ZEN-KLH. Thirteen positive clones were induced and analyzed binding to ZEN-KLH by ELISA. Negative control is a 1% BSA and 1% KLH. The data is expressed as absorbance at 405 nm.

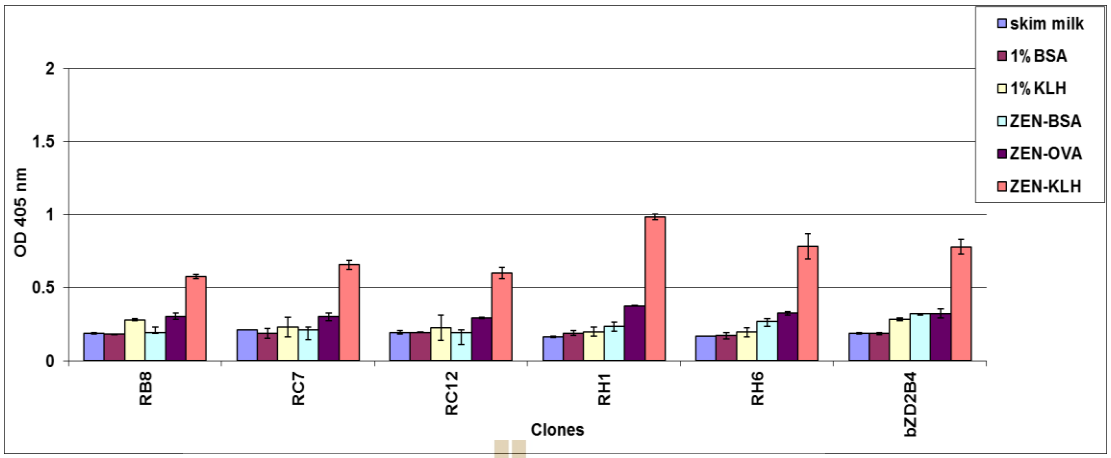


Figure 4.5 Binding of phage scFv antibody to ZEN-BSA, ZEN-OVA and ZEN-KLH. Negative control is a skim milk, 1% BSA and 1%KLH. The data is expressed as absorbance at 405 nm.

4.1.4 Characterization of soluble scFv produced in *E. coli* HB2151

In the nonsuppressor strain, *E. coli* HB2151 (supE), the amber stop codon between E tag and fd g3 in scFv clones is recognized as a stop codon and a soluble scFv-E tag fusion protein was produced as a consequence. Six individual recombinant phage clones were used to infect *E. coli* HB2151. The expression of scFv-E tag fusion protein in the resultant HB2151 clones was induced by IPTG, and soluble scFv antibodies were secreted into the culture supernatants. Confirmation of specific binding by scFv ELISA indicated that a total of 6 positive clones were not obtained (Figure 4.8).

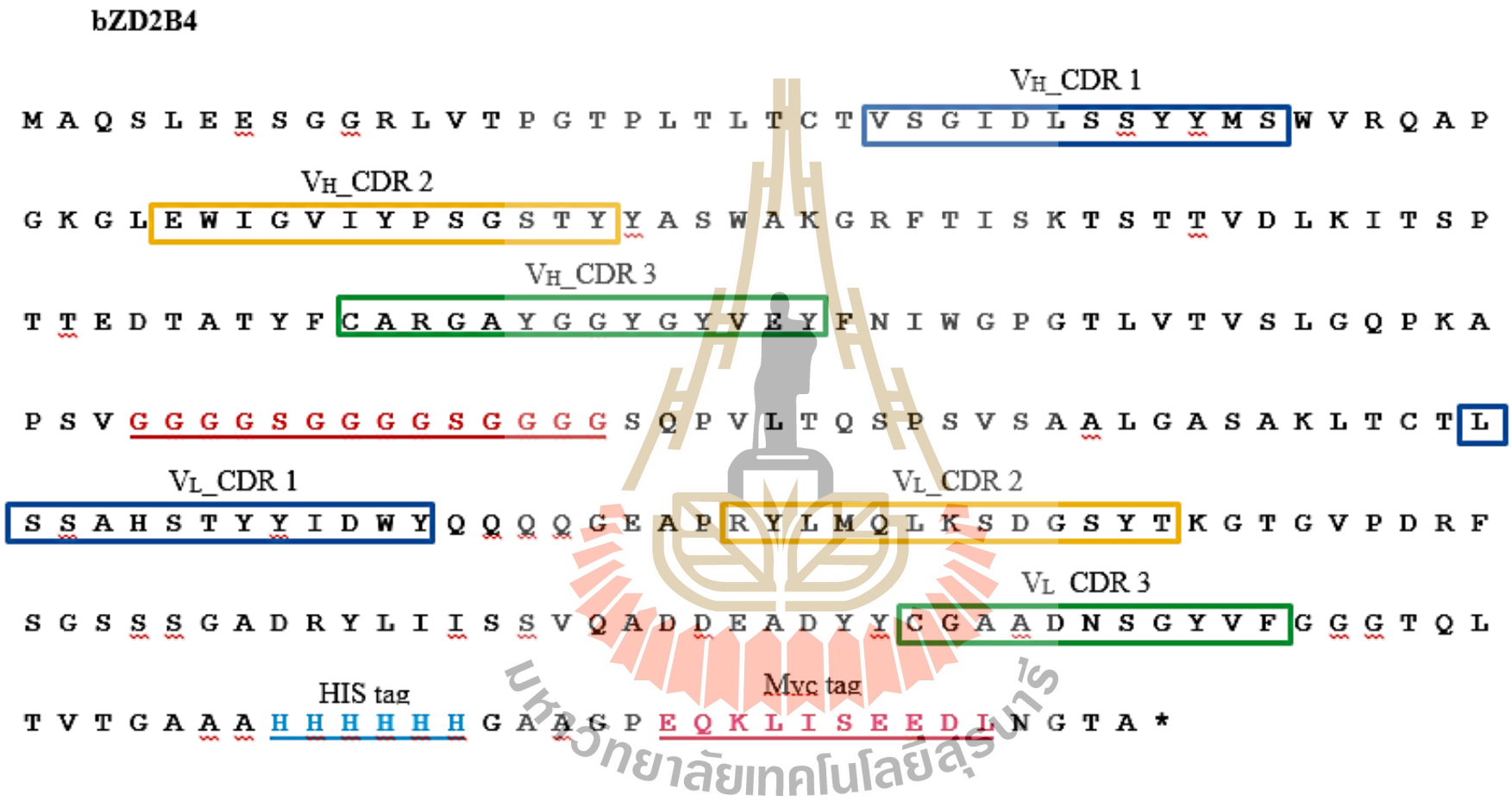


Figure 4.6 Amino acid sequence of clones selected from the rabbit library.

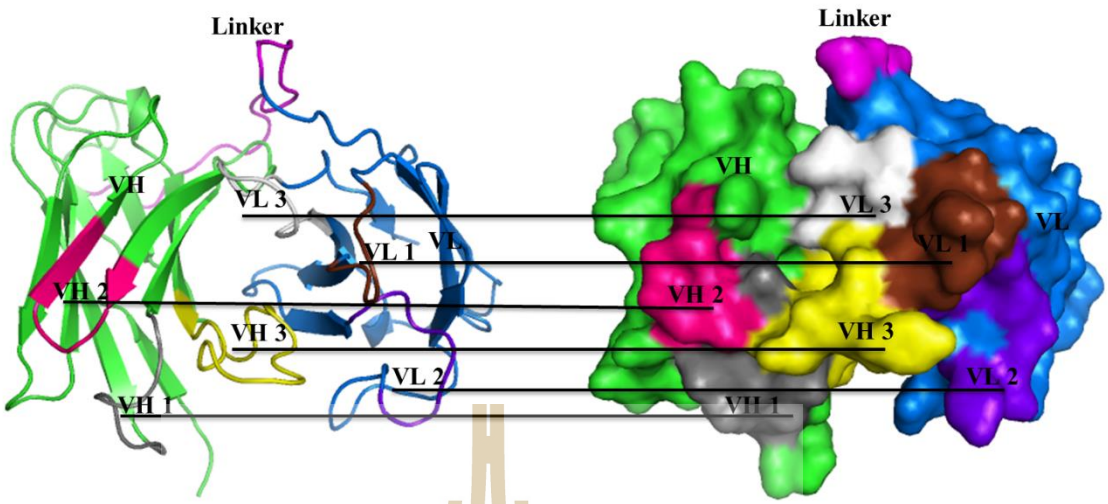


Figure 4.7 3D structure of bZD2B4, indicating the scFv binding site. The variable heavy chain of scFv is green, grey area is CDR 1, pink area is CDR 2 and yellow area is CDR 3. The variable light chain of scFv is light blue, brown area is CDR 1, purple area is CDR 2 and white area is CDR 3. Light pink area is linker. The hole is predicted to be a binding site of scFv with ZEN.

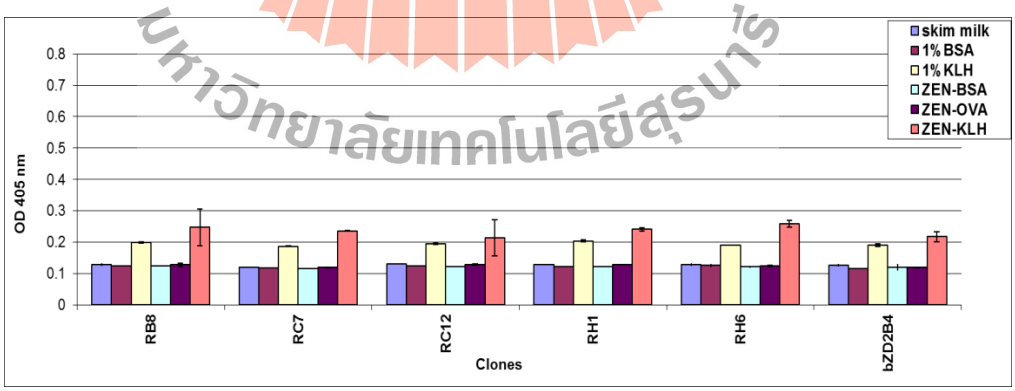


Figure 4.8 Binding of recombinant scFv antibody to ZEN-BSA, ZEN-OVA and ZEN-KLH. Negative control is a skim milk, 1% BSA and 1%KLH. The data is expressed as absorbance at 405 nm.

4.1.5 Cloning, expression and characterization of anti-ZEN scFvs

The scFv fragment of bZD2B4 clone was sub-cloned into pET-21d(+) to construct pET-21d(+)-bZD2B4 vector. The coding regions of the vectors are shown in Figure 4.9. The expression cassette for pET-21d(+)-bZD2B4 vector is driven by a T7 promoter and encoded ampicillin resistance. In pET-21d(+)-bZD2B4, the scFv gene was inserted in-frame between an N-terminal ribosomal binding site (RBS) sequence and a C-terminal hexahistidine tag of plasmid pET-21d(+). The positive clones showed 2 bands in approximately size 5.4 kb of pET-21d(+) vector and 800 bp of scFv fragment.

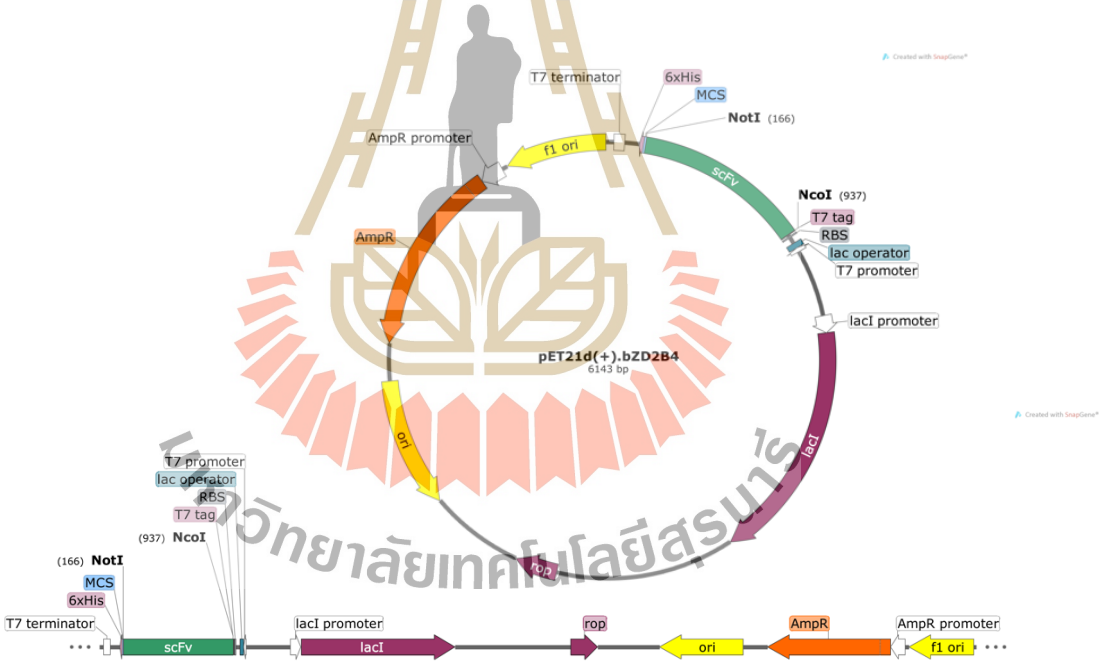


Figure 4.9 Schematic view of subcloning of scFv fragment into the pET21d(+) vector.

The SHuffle T7 Express strain is from New England Biolabs, which carries cytoplasmic expression of chaperone DsbC in addition to trxB/gor mutations.

After IPTG induction, most of the bZD2B4 protein was induced as inclusion bodies and purified by immobilized metal affinity chromatography (IMAC) from the insoluble fraction of flask cultures using the hexahistidine tail present at the C-terminus of the scFv. The purified 29 kDa of scFv was confirmed by SDS-PAGE analysis (Figure 4.10). Moreover, purified proteins were used for measuring the affinity against ZEN by enzyme-linked immunosorbent assay (ELISA).

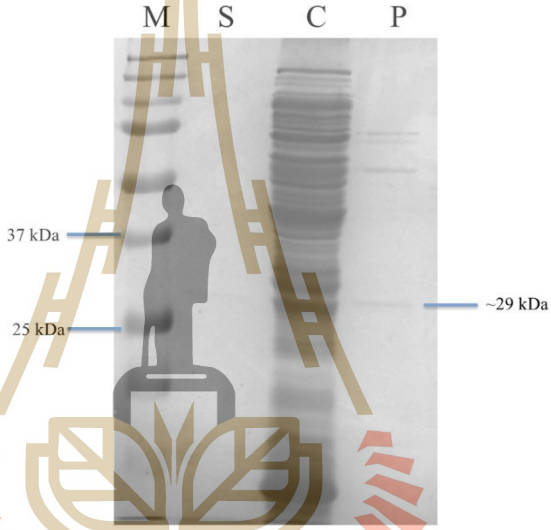


Figure 4.10 His affinity chromatography purified fractions from cell lyste expression of scFv of bZD2B4 in *E.coli* SHuffle B cell. M, Precision Plus Protein™ All Blue Standards; lane S, supernatant; lane C, cell lyste ; lane P, purified scFv antibody. Twenty microliters per lane of purified proteins were analyzed on 12% polyacrylamide gels and stained with coomassie blue.

For ELISA, bZD2B4 clone could be expressed scFv antibody and bound specifically with immobilized ZEN conjugated KLH (Figure 4.11).

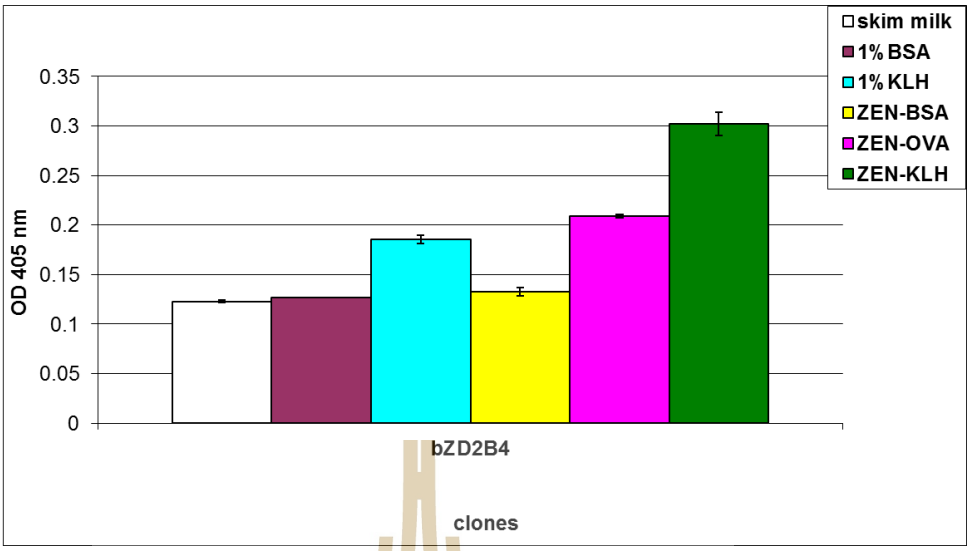


Figure 4.11 Binding of recombinant scFv antibody to ZEN-BSA, ZEN-OVA and ZEN-KLH. Negative control is a skim milk, 1% BSA and 1%KLH. The data is expressed as absorbance at 405 nm.

4.1.6 Cloning, expression and characterization of anti-ZEN scFvs-AP

The scFv fragment of bZD2B4 was sub-cloned into pKP300ΔIII to construct pKP300ΔIII-bZD2B4 as shown in Figure 4.12. The scFv fragment was inserted into pKP300ΔIII vector between *NcoI* and *NotI* sticky ends. The positive clone showed 2 bands in approximately size 5 kb of pKP300ΔIII vector and 800 bp of scFv fragment. pKP300ΔIII vector is an expression vector containing alkaline phosphatase gene. The expression of this gene is under the control of pho promoter, which can be inexpensively induced when the culture is starved for inorganic phosphate.

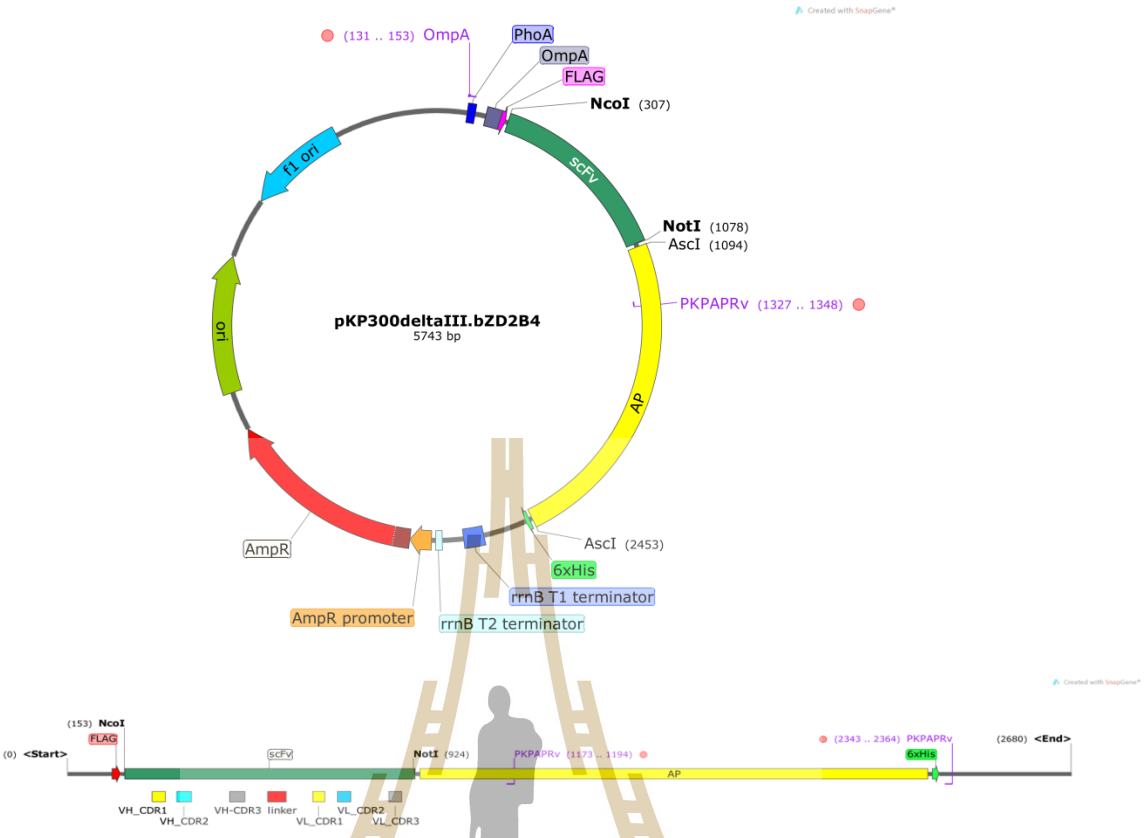


Figure 4.12 Schematic view of subcloning of bZD2B4 scFv fragment into the pKP300ΔII vector.

The purified 79 kDa scFv-AP fusion was observed by SDS–PAGE analysis (Figure 4.13). After that, the purified protein was used for measuring the affinity against ZEN by enzyme-linked immunosorbent assay (ELISA).

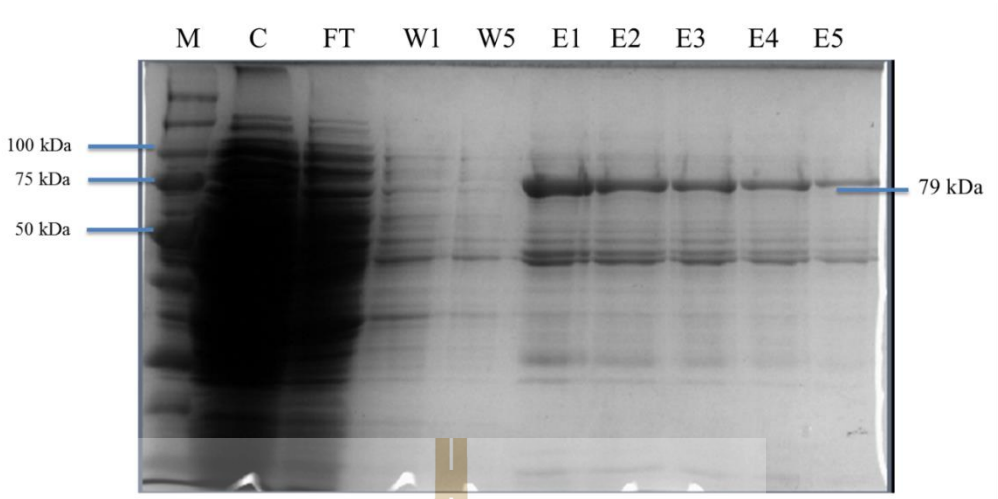


Figure 4.13 SDS-PAGE analysis of expression and purification of bZD2B4 scFv-AP. Lane M: protein marker (Precision Plus Protein Unstained Standards, Biorad), lane C: cell lysate, FT: flow-through, lane W1: wash 1, lane W5: wash 5, lane E1: elute 1, lane E2: elute 2, lane E3: elute 3, lane E4: elute 4 and lane E5: elute 5.

For ELISA, bZD2B4 clone could be expressed as scFv-AP fusion and bound specifically with immobilized ZEN conjugated KLH as shown in Figure 4.14.

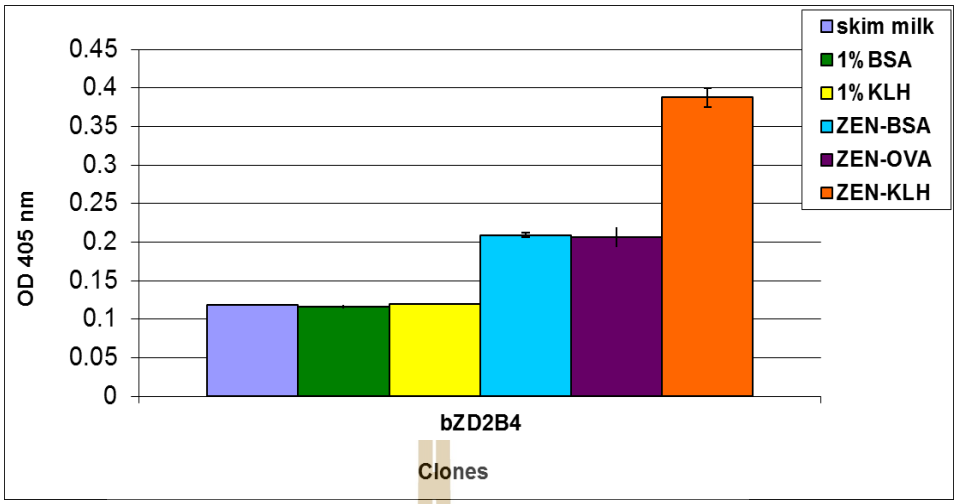


Figure 4.14 Binding of scFv-AP fusion to ZEN-BSA, ZEN-OVA and ZEN-KLH. Negative control is a skim milk, 1% BSA and 1%KLH. The data is expressed as absorbance at 405 nm.

4.1.7 Competitive ELISA

bZD2B4 clone was test binding specific with free ZEN by competitive ELISA. For phage scFv, the result indicated that bZD2B4 clone could be inhibited by soluble ZEN at the linear range of 50–5,000 ng/ml. The detection limit of this clone was approximately 50 ng/ml as shown in Figure 4.15. Moreover, bZD2B4 clone cannot bound specific with free ZEN in scFv and scFv-AP formats (Figure 4.16). Thus, the result indicated that scFv and scFv-AP format cannot bind specific with free ZEN.

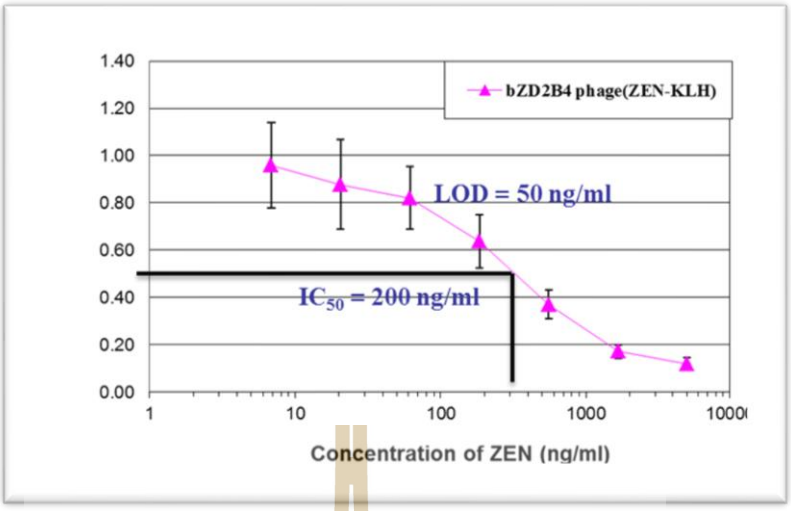


Figure 4.15 Competitive inhibition curves of bZD2B4 phage scFv and ZEN-KLH conjugate. The standard curves demonstrate the inhibition of bZD2B4 phage scFv binding to free ZEN with increasing concentrations of ZEN standard. Data are represented as an average standard deviation of three replicates.

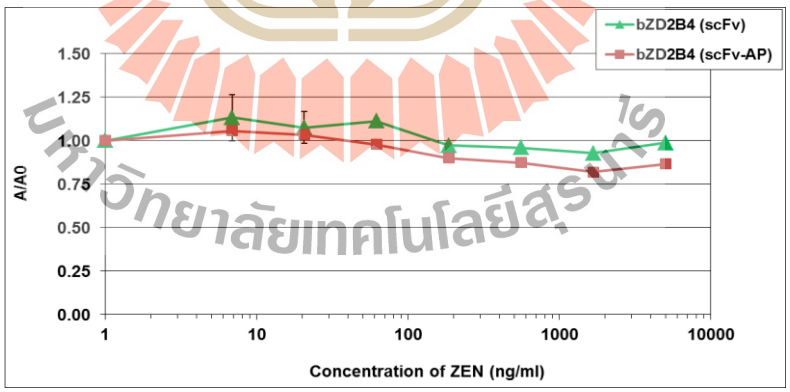


Figure 4.16 Competitive inhibition curves of bZD2B4 scFv and scFv-AP, and ZEN-KLH conjugate. Data are represented as an average standard deviation of three replicates.

4.2 Generation of recombinant human scFv antibody against Zearalenone by Phage Display Technology

4.2.1 Affinity selection of human scFv library against ZEN

Two methods of biopanning were performed by switching the conjugated proteins and using 200 µg of ZEN conjugated BSA. However, enrichment of ZEN-specific phage after each round of affinity selection was observed for switching the conjugated proteins method only. A summary of bio-panning results are listed in Table 4.2.

Table 4.2 Overview of selection of antibodies against ZEN conjugated proteins from human scFv library.

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

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

4.2.2 Phage ELISA

After biopanning of both libraries were performed with two methods. The recombinant phage clones of both libraries were tested binding affinity with ZEN conjugated proteins by ELISA (Figure 4.17-4.9). A total of 8 positive clones from rabbit library were isolated. It has showed that the YA1, YA7, YA9, YB1, YE11,

HYG9, HYH10 and yZA8B2 phage clones were bound specifically with immobilized ZEN conjugated proteins (ZEN-BSA, ZEN-OVA and ZEN-KLH) (Figure 4.20).

4.2.3 Characterization of soluble scFv produced in *E. coli* HB2151

In the nonsuppressor strain, *E. coli* HB2151 (supE), the amber stop codon between E tag and fd g3 in scFv clones is recognized as a stop codon and a soluble scFv-E tag fusion protein is produced as a consequence. Eight individual recombinant phage clones were used to infect *E. coli* HB2151. The expression of scFv-E tag fusion protein in the resultant HB2151 clones was induced by IPTG, and soluble scFv antibodies were secreted into the culture supernatants. Confirmation of specific binding by scFv ELISA indicated that a total of 3 positive clones were obtained from naïve human scFv library. There are yZA8B2, YB1 and HYH10 (Figure 4.21).

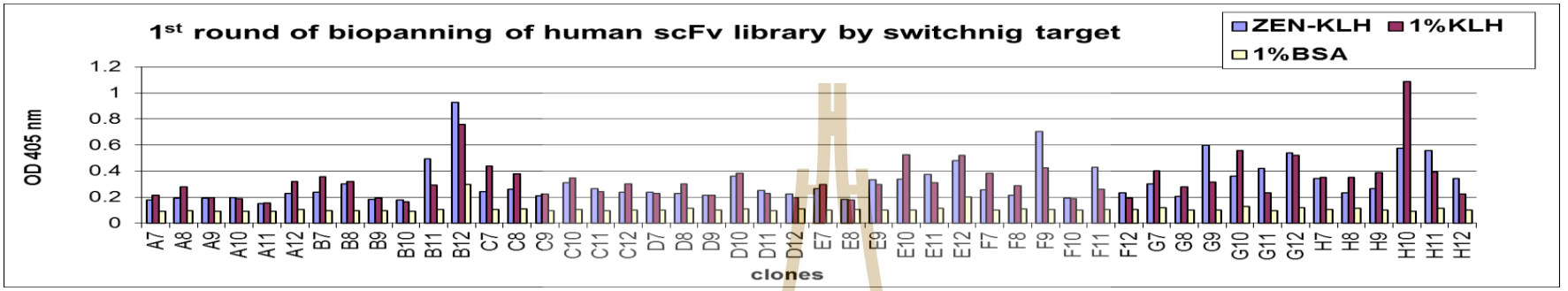


Figure 4.17 Binding of phage scFv antibody fragments to ZEN-KLH. Three positive clones were induced and analyzed binding to ZEN-KLH by ELISA. Negative control is a 1% BSA and 1% KLH. The data is expressed as absorbance at 405 nm.

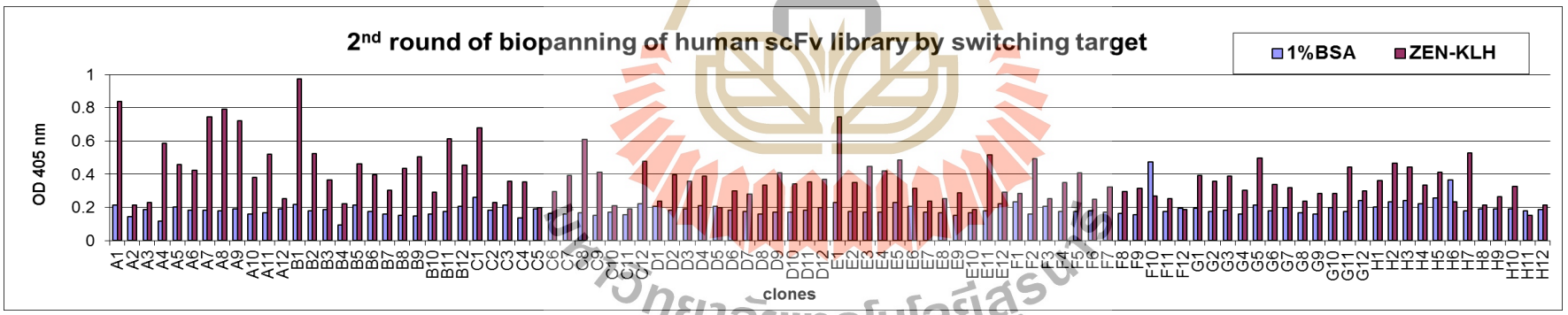


Figure 4.18 Binding of phage scFv antibody fragments to ZEN-KLH. Sixteen positive clones were induced and analyzed binding to ZEN-KLH by ELISA. Negative control is a 1% BSA. The data is expressed as absorbance at 405 nm.

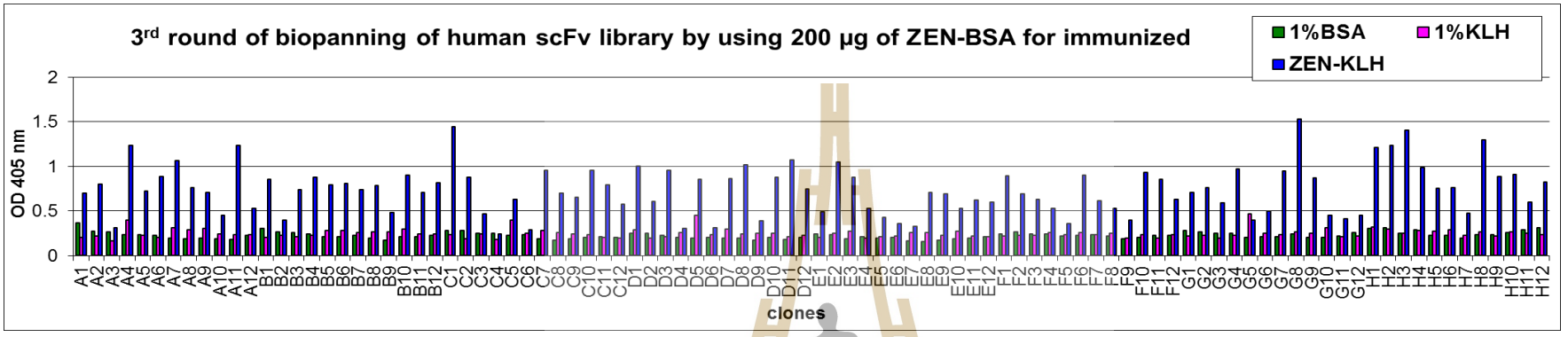


Figure 4.19 Binding of phage scFv antibody fragments to ZEN-KLH. Forty-four positive clones were induced and analyzed binding to ZEN-KLH by ELISA. Negative control is a 1% BSA and 1% KLH. The data is expressed as absorbance at 405 nm.

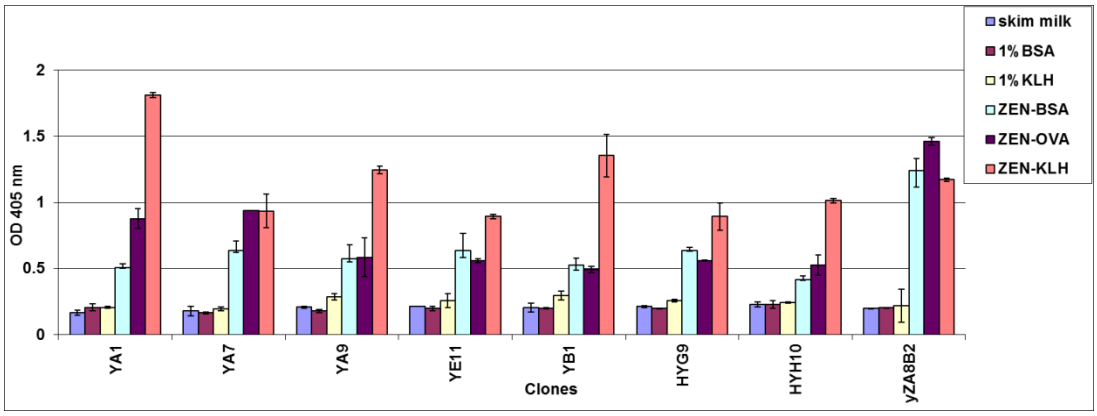


Figure 4.20 Binding of phage scFv antibody to ZEN-BSA, ZEN-OVA and ZEN-KLH. Negative control is a skim milk, 1% BSA and 1%KLH. The data is expressed as absorbance at 405 nm.

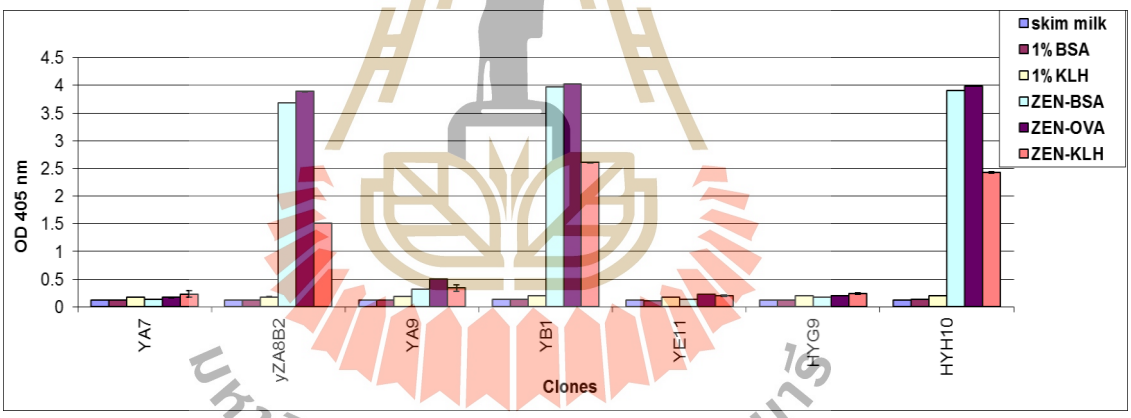


Figure 4.21 Binding of recombinant scFv antibody to ZEN-BSA, ZEN-OVA and ZEN-KLH. Negative control is a skim milk, 1% BSA and 1%KLH. The data is expressed as absorbance at 405 nm.

4.2.4 Amino acid sequence analysis of selected scFv clones

yZA8B2, YB1 and HYH10 clones, producing scFv with the highest affinity from human scFv library were chosen for DNA sequencing. The DNA

sequences analysis indicated that yZA8B2, YB1 and HYH10 scFv antibodies are the same clone and amino acid sequence as shown in Figure 4.22. These scFv contain the variable heavy chain (VH) from family 5, which was derived from germ line V5-51*01 and variable light chain (VL) from family 1, which was derived from germ line V1-44*01. The 3D structure was predicted by Phyre2 software as shown in Figure 4.23.

4.2.5 Cloning, expression and characterization of anti-ZEN scFvs

The scFv fragment of yZA8B2 clone was sub-cloned into pET-21d(+) to construct pET-21d(+)-yZA8B2. The coding regions of the vectors are shown in Figure 4.24. The expression cassette for pET-21d(+)-yZA8B2 vectors is driven by a T7 promoter and encoded ampicillin resistance. In pET-21d(+)-yZA8B2, the scFv gene was inserted in-frame between an N-terminal ribosomal binding site (RBS) sequence and a C-terminal hexahistidine tag of plasmid pET-21d(+). The positive clones were shown 2 bands in approximately size 5.4 kb of pET-21d(+) vector and 800 bp of scFv fragment.

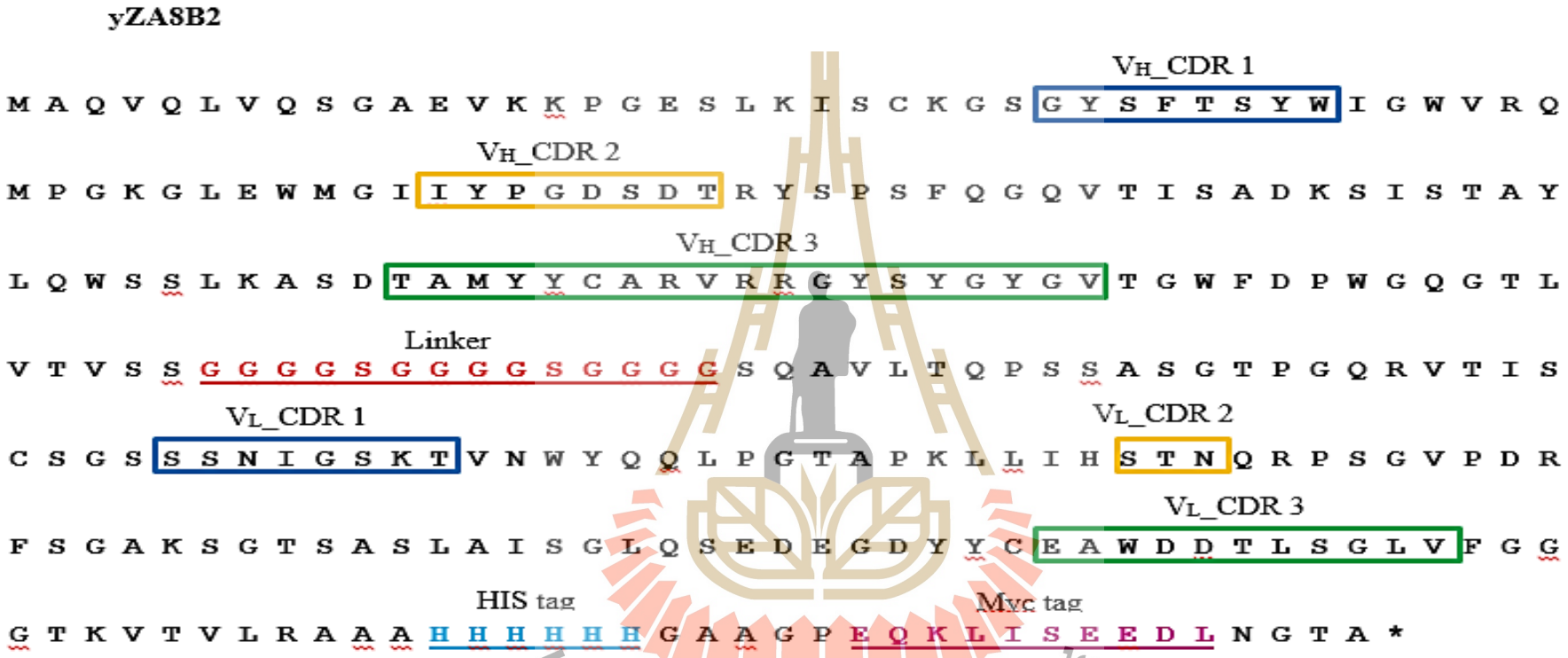


Figure 4.22 Amino acid sequence of clones selected from the human scFv library.

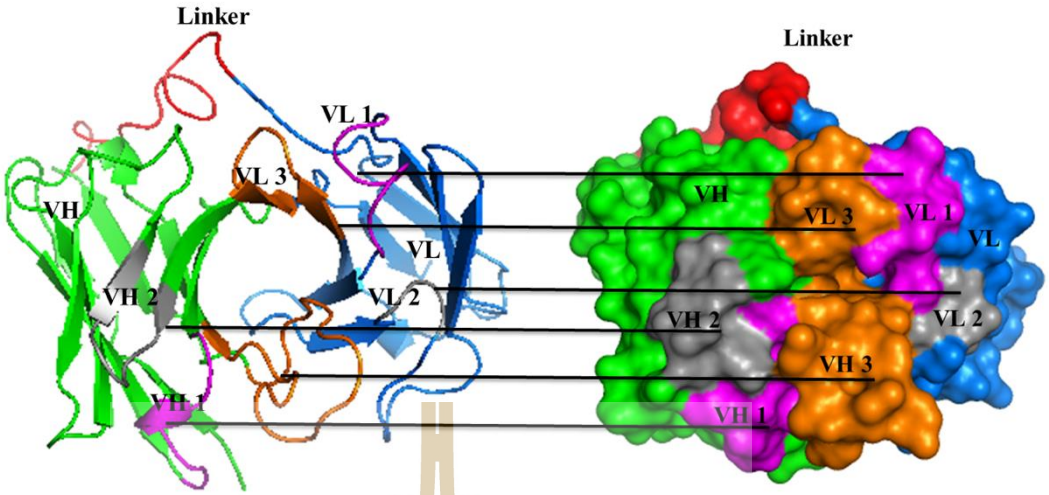


Figure 4.23 3D structure of yZA8B2, indicating the scFv binding site. The variable heavy chain and light chain of scFv are green and light blue, respectively. Pink areas are CDR 1, grey areas are CDR 2 and light brown areas are CDR 3. Red area is linker. The hole is predicted to be a binding site of scFv with ZEN.

4.2.5 Cloning, expression and characterization of anti-ZEN scFvs

The scFv fragment of yZA8B2 clone was sub-cloned into pET-21d(+) to construct pET-21d(+)-yZA8B2. The coding regions of the vectors are shown in Figure 4.24. The expression cassette for pET-21d(+)-yZA8B2 vectors is driven by a T7 promoter and encoded ampicillin resistance. In pET-21d(+)-yZA8B2, the scFv gene was inserted in-frame between an N-terminal ribosomal binding site (RBS) sequence and a C-terminal hexahistidine tag of plasmid pET-21d(+). The positive clones were shown 2 bands in approximately size 5.4 kb of pET-21d(+) vector and 800 bp of scFv fragment.

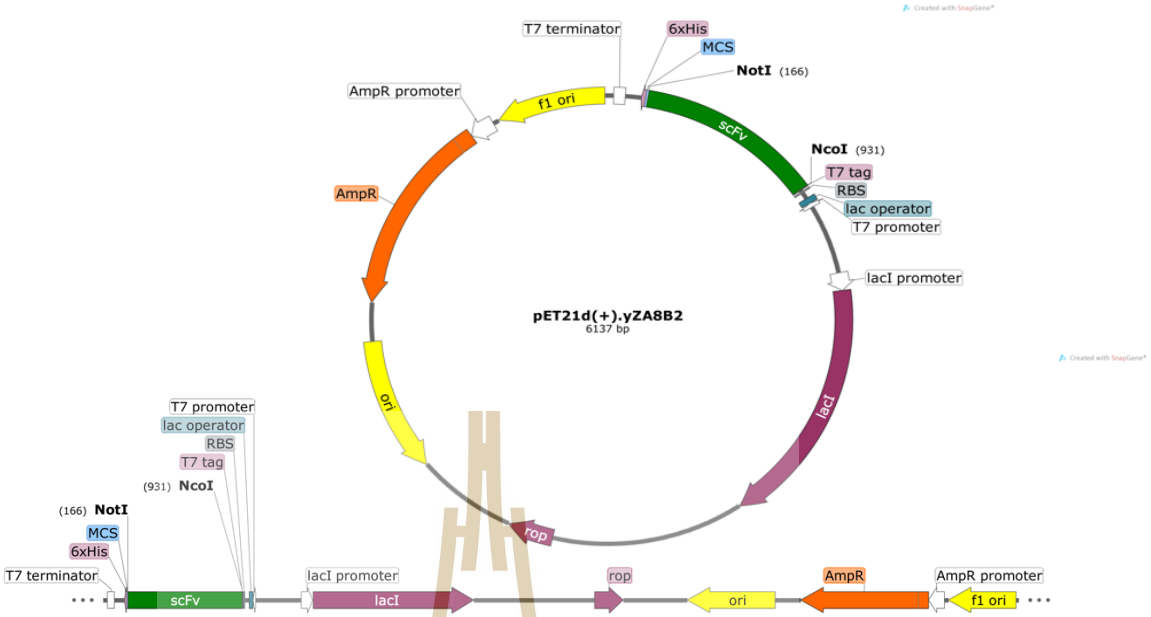


Figure 4.24 Schematic view of subcloning of scFv fragment into the pET21d(+) vector.

The SHuffle T7 Express strain is from New England Biolabs, which carries cytoplasmic expression of chaperone DsbC in addition to trxB/gor mutations. After IPTG induction, most of the yZD2B4 protein was induced as inclusion bodies and purified by immobilized metal affinity chromatography (IMAC) from the insoluble fraction of flask cultures using the hexahistidine tail present at the C-terminus of the scFv. The purified 29 kDa of scFv was confirmed by SDS–PAGE analysis (Figure 4.25). Moreover, purified proteins were used for measuring the affinity against ZEN by enzyme-linked immunosorbent assay (ELISA).

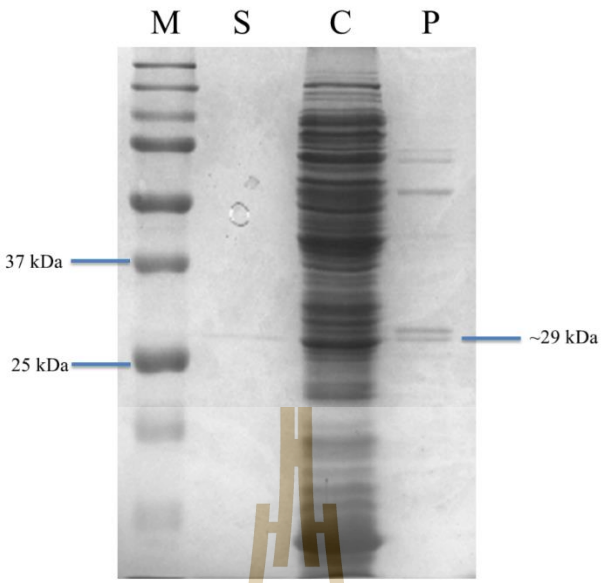


Figure 4.25 His affinity chromatography purified fractions from cell lyste expression of scFv of yZA8B2 in *E.coli* SHuffle B cell. M, Precision Plus Protein™ All Blue Standards; lane S, supernatant; lane C, cell lyste ; lane P, purified scFv antibody. Twenty microliters per lane of purified proteins were analyzed on 12% polyacrylamide gels and stained with coomassie blue.

For ELISA, yZA8B2 clone could be expressed and bound specifically with immobilized ZEN conjugated proteins (ZEN-BSA, ZEN-OVA and ZEN-KLH) Figure 4.26.

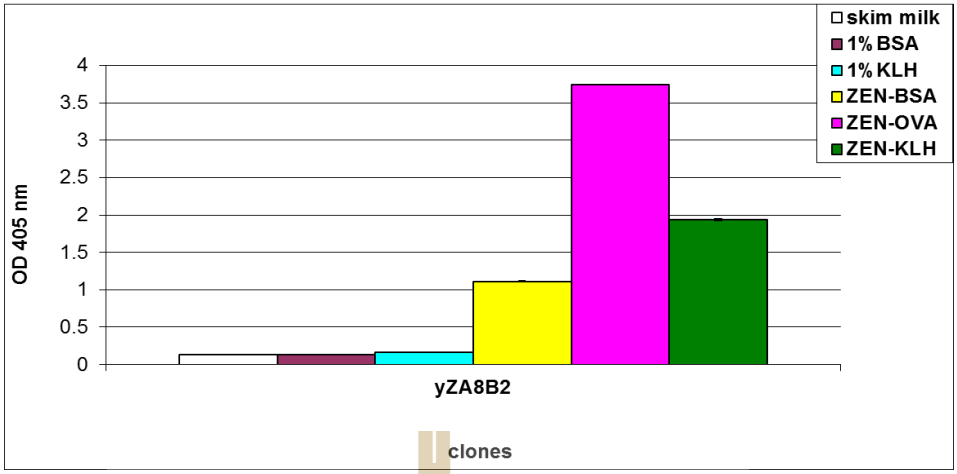


Figure 4.26 Binding of recombinant scFv antibody to ZEN-BSA, ZEN-OVA and ZEN-KLH. Negative control is a skim milk, 1% BSA and 1%KLH. The data is expressed as absorbance at 405 nm.

4.2.6 Cloning, expression and characterization of anti-ZEN scFvs-AP

The scFv fragment of yZA8B2 clone was sub-cloned into pKP300ΔIII to construct pKP300ΔIII-yZA8B2 (Figure 4.27). The scFv fragment is inserted into pKP300ΔIII vector between *NcoI* and *NotI* sticky ends. The positive clones were shown 2 bands in approximately size 5 kb of pKP300ΔIII vector and 800 bp of scFv fragment.

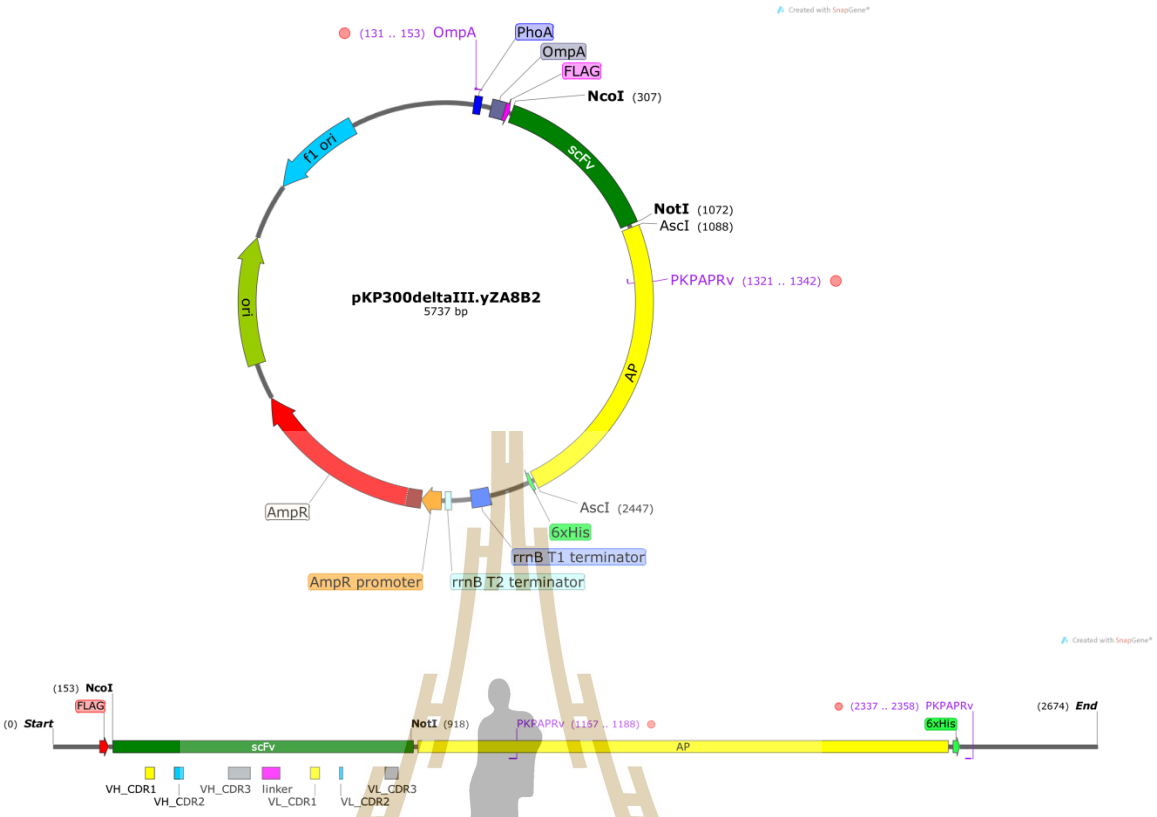


Figure 4.27 Schematic view of subcloning of yZA8B2 scFv into the pKP300ΔII vector.

The purified 79 kDa scFv-AP fusion was confirmed by SDS–PAGE analysis (Figure 4.28). Moreover, purified proteins were used for measuring the affinity against ZEN by enzyme-linked immunosorbent assay (ELISA).

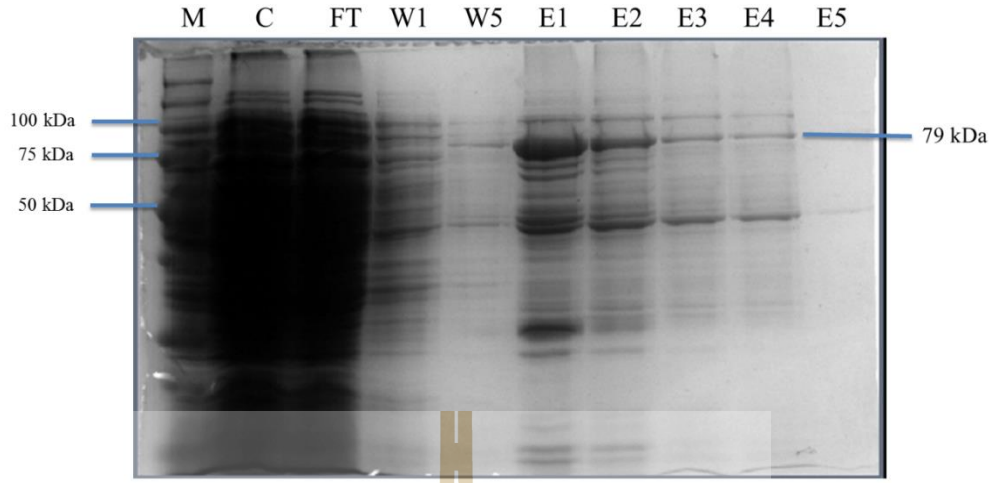


Figure 4.28 SDS-PAGE analysis of expression and purification of yZA8B2 scFv-AP. Lane M: protein marker (Precision Plus Protein Unstained Standards, Biorad), lane C: cell lysate, FT: flow-through, lane W1: wash 1, lane W5: wash 5, lane E1: elute 1, lane E2: elute 2, lane E3: elute 3, lane E4: elute 4 and lane E5: elute 5.

For ELISA, yZA8B2 clone could be expressed and bound specifically with immobilized ZEN conjugated proteins (ZEN-BSA, ZEN-OVA and ZEN-KLH) as shown Figure 4.29.

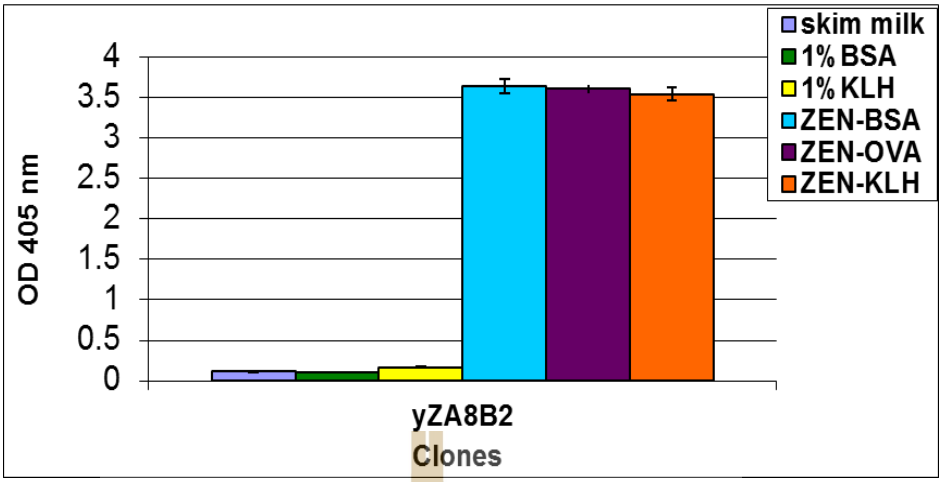


Figure 4.29 Binding of scFv-AP fusion to ZEN-BSA, ZEN-OVA and ZEN-KLH. Negative control is a skim milk, 1% BSA and 1%KLH. The data is expressed as absorbance at 405 nm.

4.2.7 Competitive ELISA

yZA8B2 clone was tested specific binding with free ZEN by competitive ELISA. For scFv antibodies, yZA8B2 clone of scFv antibodies in *E. coli* HB2151 and *E. coli* SHuffle T7 Express strain were indicated by the decrease in an absorbance when the concentrations of free ZEN were increased. The sensitivities of both scFv antibodies were tested in competitive ELISA as shown Figure 4.30. The result displayed that the limit of detection (LOD) of scFv antibodies in *E. coli* HB2151 was approximately 60 ng/ml at a linear range of 60-5,000 ng/ml. Meanwhile, yZA8B2 scFv antibody in *E. coli* SHuffle T7 Express strain, the IC_{50} was 200 ng/ml and detection limit of this scFv antibody was 20 ng/ml, with a linear range 20 to 5000 ng/ml. Thus, yZA8B2 clone of scFv antibody in *E. coli* SHuffle T7 Express strain was high sensitivity to detect free ZEN.

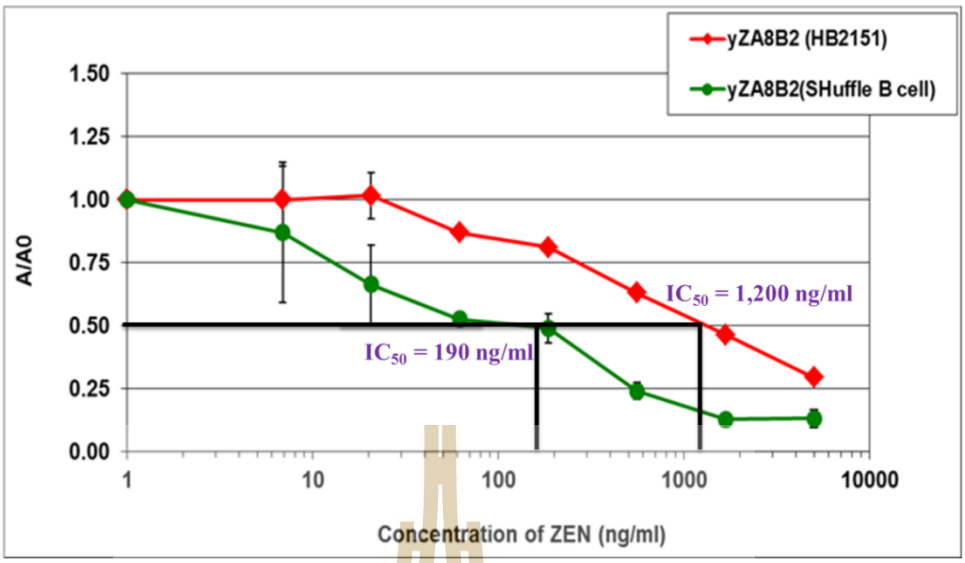


Figure 4.30 Competitive inhibition curves of yAZ8B2 scFv antibody and ZEN-KLH conjugate. yAZ8B2 scFv antibody in *E. coli* HB2151 (red line) was used to detect ZEN by comparison with yAZ8B2 scFv antibody in *E. coli* SHuffle B cell (green line).

For yZA8B2 scFv-AP fusion, this scFv-AP fusion had binding specific to free ZEN. The IC_{50} of this scFv-AP fusion was 800 ng/ml and detection limit of this scFv antibody was 100 ng/ml, with a linear range 100 to 5000 ng/ml (Figure 4.31).

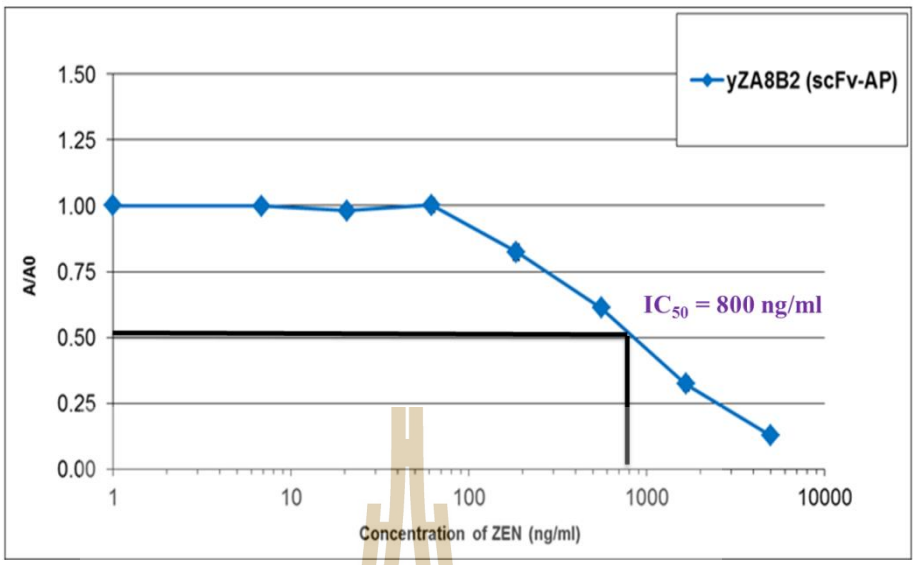


Figure 4.31 Competitive inhibition curves of yAZ8B2 scFv-AP fusion and ZEN-KLH conjugate.

4.2.8 Cross reactivity of scFv-AP fusion

To confirm that the yZA8B2 scFv-AP fusion recognizes free ZEN, competitive ELISAs were conducted with soluble Aflatoxins B1 (AFB1), Ochratoxin A (OTA A), Fumonisins (FUM) and Deoxynivalenol (DON) as competitors. The yZA8B2 scFv-AP fusion was not able to bind to soluble AFB_1, OTA, FUM and DON (Figure 4.32) when compared with soluble ZEN. Thus, yZA8B2 scFv-AP fusion had no cross-reactivity with AFB1, OTA, FUM and DON that are frequently present together with ZEN in agricultural samples.

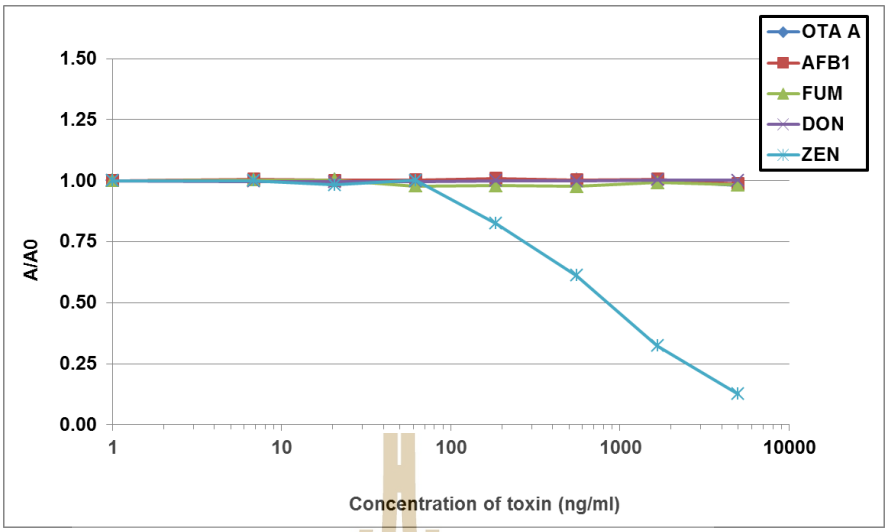


Figure 4.32 Cross reactivity of yZA8B2 scFv-AP fusion to soluble AFB_1, OTA A, FUM and DON.

4.2.9 ELISA Optimization

The optimal concentration of the immobilized ZEN conjugated proteins (ZEN-BSA, ZEN-OVA and ZEN-KLH) and recombinant yZA8B2 scFv antibody and scFv-AP fusion dilution were investigated. To optimize the dilution of the recombinant yZA8B2 scFv antibody and scFv-AP fusion and concentration of ZEN conjugated proteins were applied to competitive ELISA. The concentrations of the immobilized ZEN conjugated proteins were 0.5, 0.1, 0.05, 0.02 and 0.01 µg/well, respectively. The checkerboard titration result of scFv antibody and scFv-AP are shown in Figure 4.33 and 4.34, respectively.

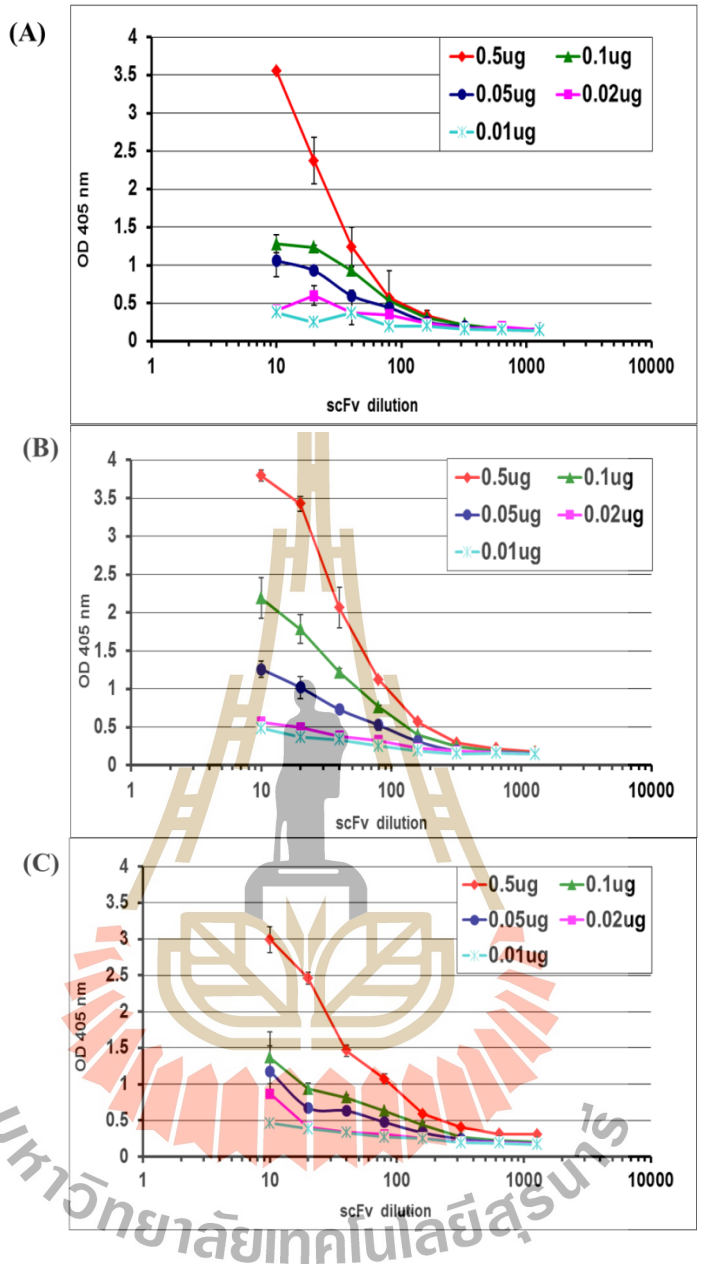


Figure 4.33 Checker broad of yZA8B2 scFv antibody by using ZEN-BSA (A), ZEN-OVA (B) and ZEN-KLH (C) as a target. The concentration of the immobilized ZEN conjugated proteins was 0.5 (red line), 0.1 (green line), 0.05 (blue line), 0.02 (pink line) and 0.01 µg/well (light blue), respectively.

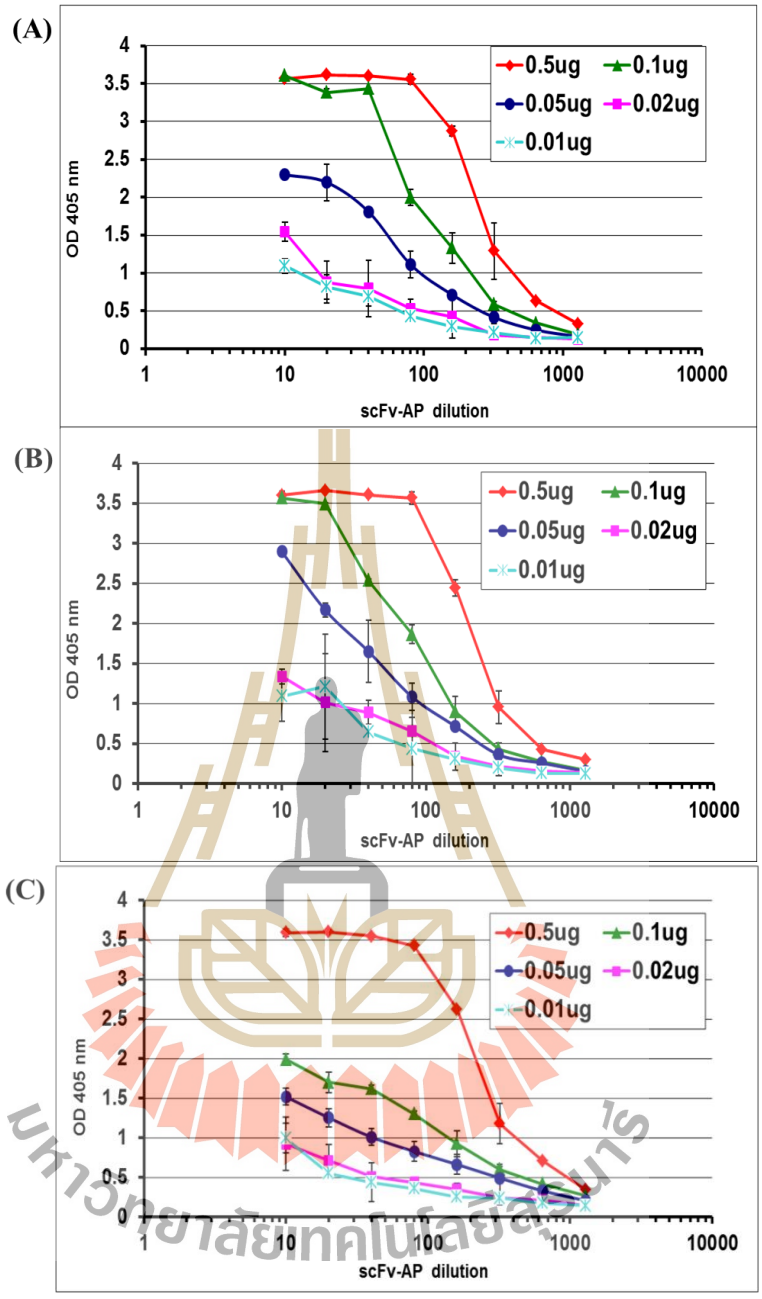


Figure 4.34 Checker broad of yZA8B2 scFv-AP fusion by using ZEN-BSA (A), ZEN-OVA (B) and ZEN-KLH (C) as a target. The concentration of the immobilized ZEN conjugated proteins was 0.5 (red line), 0.1 (green line), 0.05 (blue line), 0.02 (pink line) and 0.01 µg/well (light blue), respectively.

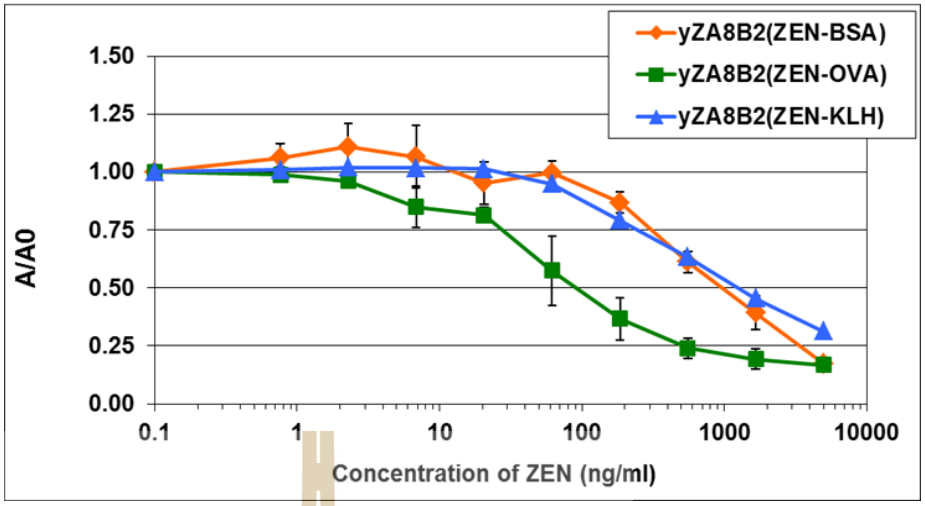
In addition, to confirm the accuracy of optimized ELISA conditions to quantify anti-ZEN antibodies were tested binding specific with free ZEN by competitive ELISA. The summary of immobilized ZEN conjugated proteins and anti-ZEN antibodies dilution were used in this method as shown in Table 4.3.

Thus, the result indicated that ZEN-OVA is the best of ZEN conjugated proteins when compared with ZEN-BSA and ZEN-KLH in inhibition assay (Figure 4.35). Because of molecular mass of OVA is smaller than BSA and KLH so, ZEN conjugated OVA can be immobilized on the ELISA plate more than amount of ZEN conjugated other protein. The IC_{50} of recombinant yZA8B2 scFv antibody and scFv-AP fusion were 100 and 20 ng/ml, with a linear range from 100 and 20 to 5000 ng/ml, respectively. The sensitivity of recombinant yZA8B2 scFv antibody and scFv-AP fusion was improved 2 -fold and 40-fold affinity to ZEN compared with previous result that used 2 µg/well of immobilized ZEN-KLH in competitive ELISA. Moreover, the result also indicated that appropriate dilution of anti-ZEN antibodies resulted in more efficient binding of the antibodies with free ZEN.

Table 4.3 Optimized conditions of immobilized ZEN conjugated proteins and yZA8B2 anti-ZEN antibodies dilution for competitive ELISA.

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

(A)



(B)

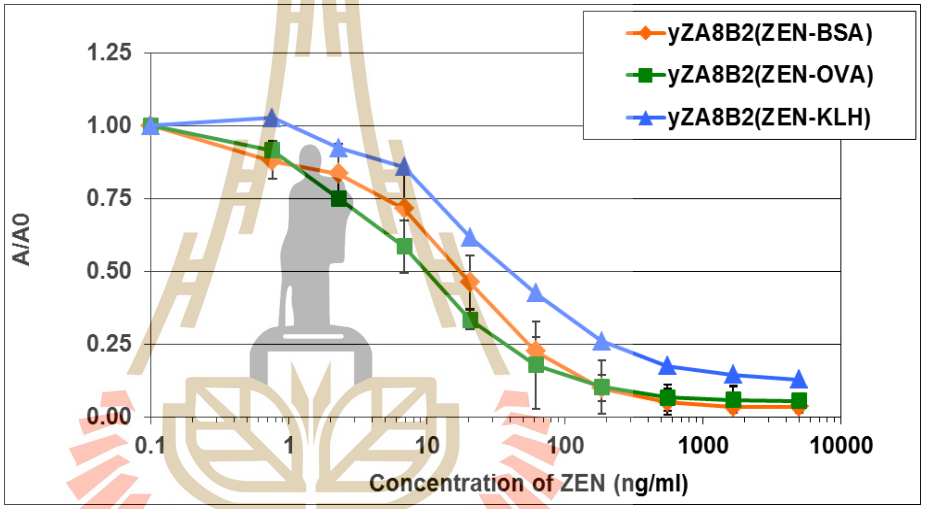


Figure 4.35 Optimal competitive inhibition of yAZ8B2 scFv antibody (A) and scFv-AP fusion (B) to ZEN performed with different of immobilized ZEN conjugated proteins.

To optimize conditions for storage of antibodies, recombinant yZA8B2 scFv antibody and scFv-AP fusion were kept at 4 ºC and -20°C (50% glycerol) for 2 and 4 weeks. Antibodies dilution was performed to check stability of both antibodies format (Figure 4.36). However, the binding activities of scFv and scFv-AP antibodies were bound to ZEN conjugated OVA when kept until 4 weeks. Therefore, it means

that the stability of both antibodies still working at both conditions. Moreover, binding activity of scFv and scFv-AP antibodies to ZEN were performed by competitive ELSA and show in Table 4.4.

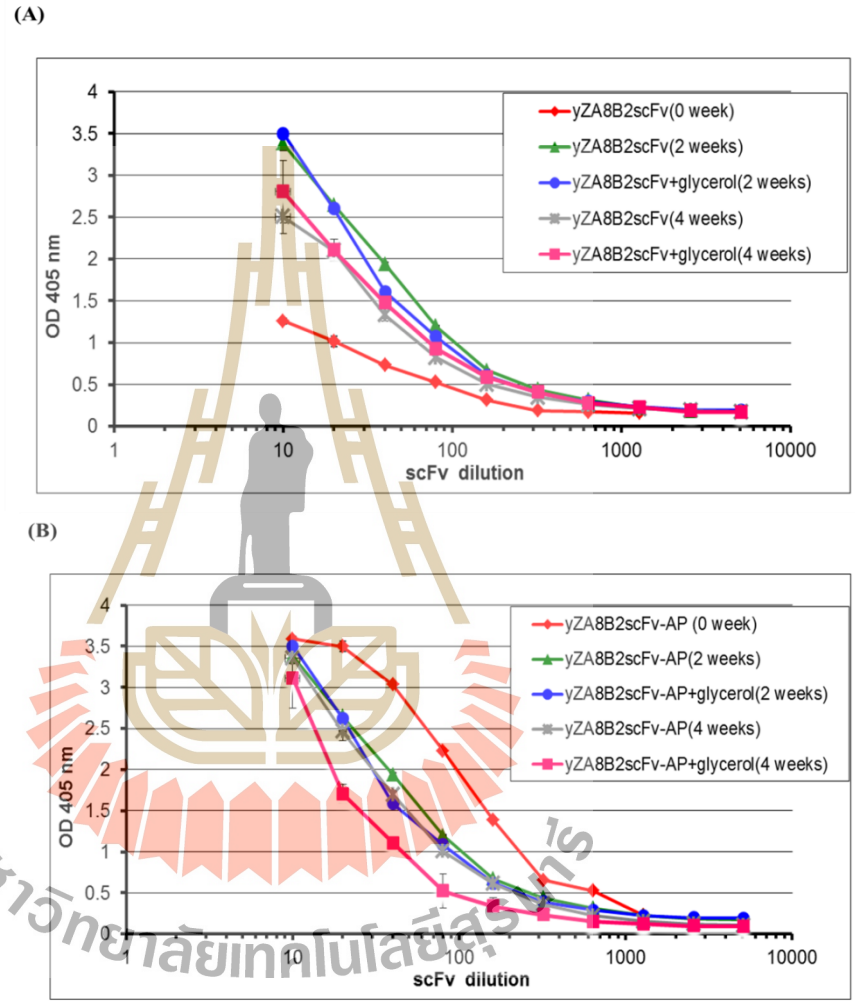


Figure 4.36 The stability of yAZ8B2 scFv antibody (A) and scFv-AP fusion (B) to ZEN conjugated OVA when kept different time.

Table 4.4 The summaries stability of scFv and scFv-AP by competitive ELISA.

Antibody	**Antibody dilution**	**IC_{50}**
scFv - Fresh	1:40	500 ng/ml
- 2 weeks (4ºC)	1:60	200 ng/ml
- 2 weeks (-20ºC)	1:60	200 ng/ml
- 4 weeks (4ºC)	N/A	N/A
- 4 weeks (-20ºC)	N/A	N/A
scFv-AP - Fresh	1:40	10 ng/ml
- 2 weeks (4ºC)	1:20	30 ng/ml
- 2 weeks (-20ºC)	1:20	50 ng/ml
- 4 weeks (4ºC)	N/A	N/A
- 4 weeks (-20ºC)	N/A	N/A

N/A that mean not available

4.2.10 Assay validation

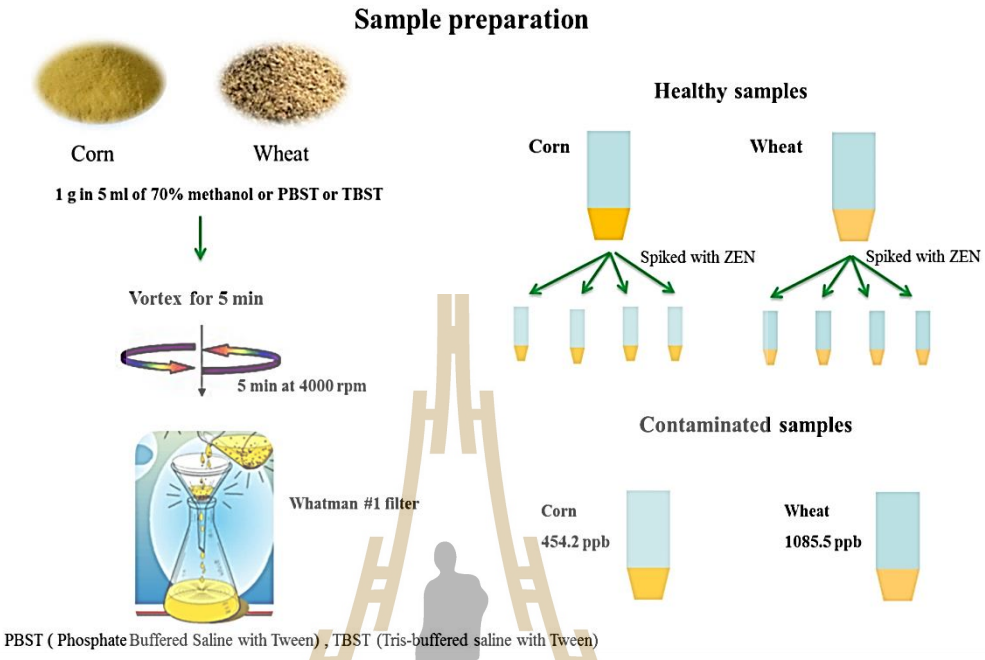


Figure 4.37 Experimental scheme of detection of Zearalenone in agricultural products.

Corns and wheat reference samples (TR-Z100) containing 454.2 and 1085.5 ppb of ZEN were investigated. Spiked extracts and contaminated extracts samples were compared by competitive ELISA. Standard curves were generated by 0 ppb of corns and wheat that spiked with a series of known concentrations of soluble ZEN (3000, 1000, 500, 300, 100, 50, 30 and 10 ng/ml) (Figure 4.38 (A) and 4.39 (A)). However, the standard curves of spiking soluble ZEN in corns and wheat extracts samples were calculated linearization using logit/log transformation by the two-parameter fit as show in Figure 4.38 (A) and 4.39 (B). Moreover, the concentration of contaminated extracts corns and wheat samples were calculated using logit/log transformation (Table S2 and S3 in appendix).

For scFv, the result indicated that when extract with PBST, this antibody can detect 83% of corn and wheat samples tested as presented in the Table 4.5. For scFv-AP, when corns samples extracted with 70% Methanol in 1:3 dilution, this antibody can detect 85%. When wheat samples extracted with 70% methanol in 1:5 dilution, this antibody can detect 91% while when extracted with PBST, this antibody can detect 100% of samples tested as presented in the Table 4.6. Thus, both formats can detect ZEN contaminated in samples only qualitative detection.

(A) **(B)**

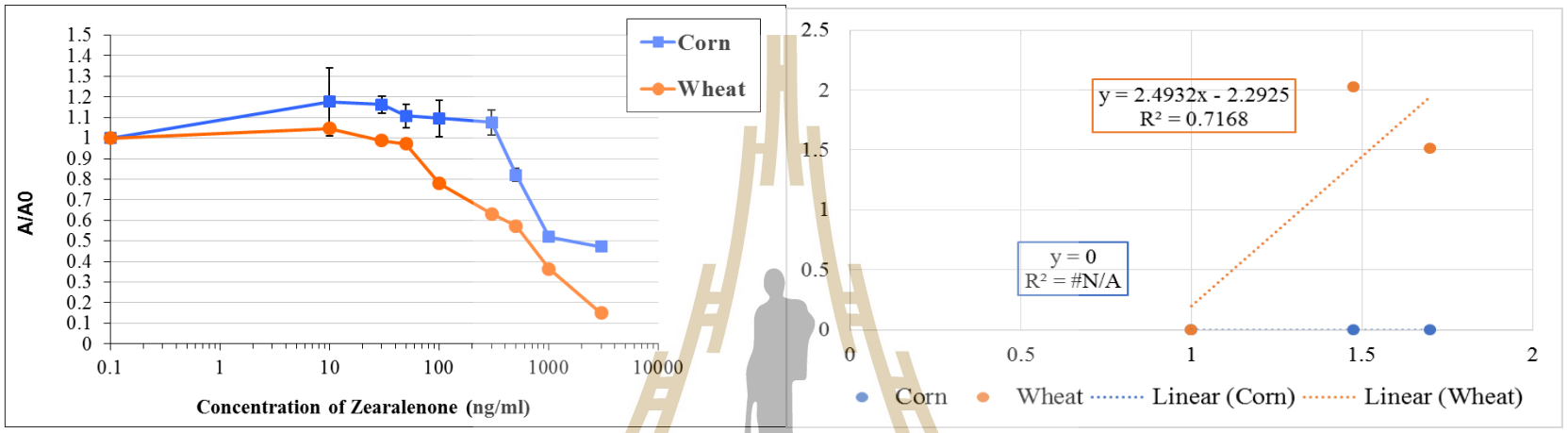


Figure 4.38 Standard curves of spiking soluble ZEN in corns (light blue line) and wheat (orange lines) extracts samples detected by scFv competitive ELISA (A), while calculated linearization using logit/log transformation by the two-parameter fit as show in (B).

(A) **(B)**

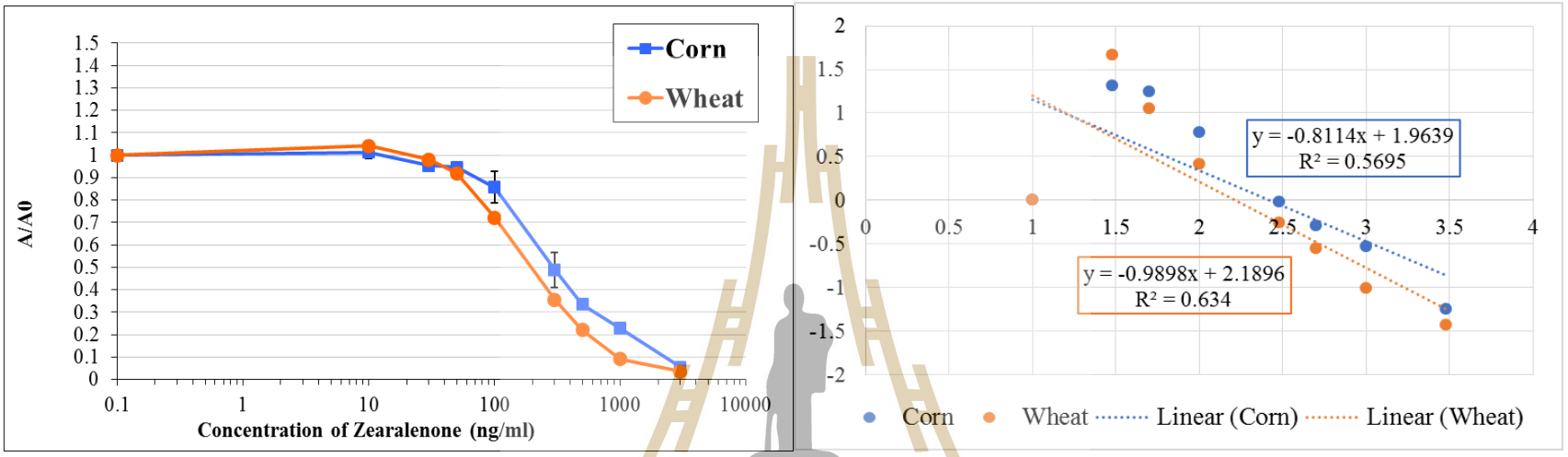


Figure 4.39 Standard curves of spiking soluble ZEN in corns (light blue line) and wheat (orange lines) extracts samples detected by scFv-AP competitive ELISA (A), while calculated linearization using logit/log transformation by the two-parameter fit as show in (B).

Table 4.5 Assays of contaminated samples by competitive scFv ELISA.

Date	scFv							
	Corn				Wheat			
	Methods							
	1:3 70%MeOH	1:5 70%MeOH	PBST	TBST	1:3 70%MeOH	1:5 70%MeOH	PBST	TBST
1/6/17	-	-	✓	-	-	-	✓	-
21/6/17	x	x	-	-	✓	x	-	-
30/6/17	✓	x	-	-	x	✓	-	-
	✓	✓	-	-	x	✓	-	-
3/7/17	-	-	✓	x	-	-	✓	x
4/7/17	-	x	-	-	-	x	-	-
13/7/17	✓	✓	-	-	✓	x	-	-
19/7/17	-	x	x	-	-	x	x	-
	-	✓	✓	-	-	✓	✓	-
31/7/17	-	x	✓	-	-	x	✓	-
	-	✓	✓	-	-	x	✓	-
8/8/17	x	x	-	-	x	✓	-	-
	✓	x	-	-	✓	x	-	-

(✓ indicated that the toxin could be detected and x indicated that the toxin couldn't be detected)

66.7%	**36.4%**	**83%**	**50%**	**36.4%**	**83%**

Table 4.6 Assays of contaminated samples by competitive scFv-AP ELISA.

Date	scFv-AP							
	Corn				Wheat			
	Method							
	1:3 70%MeOH	1:5 70%MeOH	PBST	TBST	1:3 70%MeOH	1:5 70%MeOH	PBST	
1/6/17	-	-	-	x	-	-	-	✓
21/6/17	✓	x	-	-	x	✓	-	-
3/7/17	-	-	x	x	-	-	✓	✓
4/7/17	✓	x	-	-	✓	x	-	-
13/7/17	✓	✓	-	-	✓	✓	-	-
14/7/17	-	-	✓	✓	-	-	✓	x
15/7/17	✓	✓	-	-	x	✓	-	-
	x	x	-	-	x	✓	-	-
19/7/17	-	✓	✓	-	-	✓	✓	-
	-	✓	✓	-	-	✓	✓	-
31/7/17	-	✓	x	-	-	✓	✓	-
	-	x	✓	-	-	✓	✓	-
8/8/17	✓	✓	-	-	✓	✓	-	-
	✓	x	-	-	✓	✓	-	-

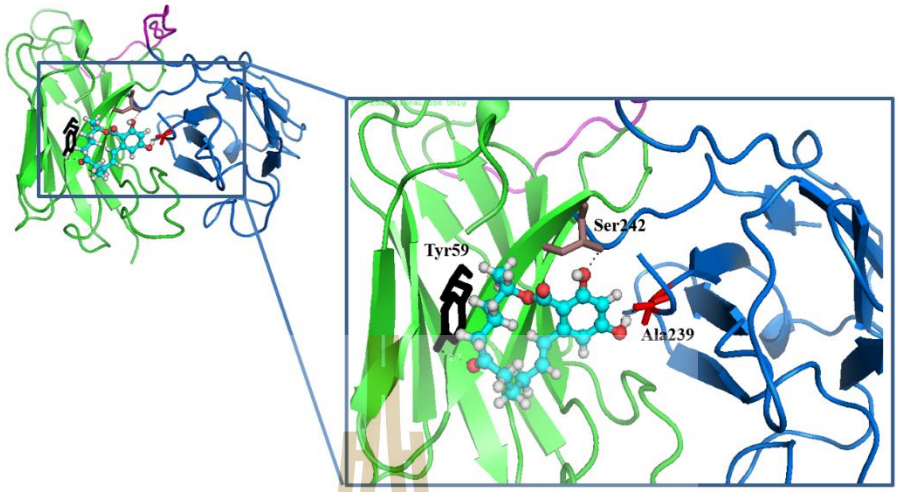
(✓ indicated that the toxin could be detected and x indicated that the toxin couldn't be detected)

85%	63.3%	67%	33.3%	57%	91%	100%

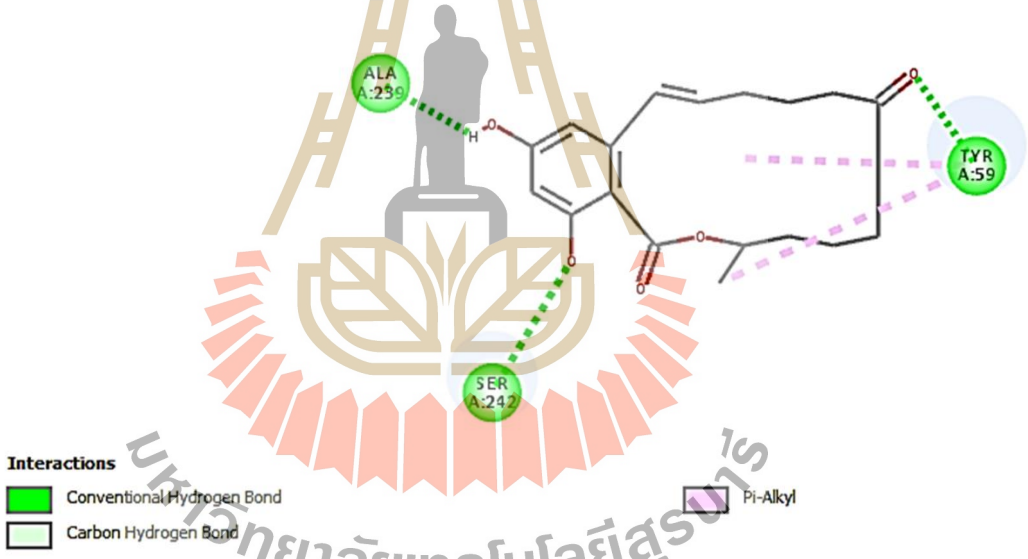
4.2.11 Molecular docking and putative rational for the binding of the bZD2B4 and yZA8B2 scFv antibody to Zearalenone

Structure simulation of the scFv antibodies were done by homology modeling and further molecular docking analysis was carried out. For bZD2B4 scFv antibody, the homology model was generated based on template (PDB code: 5i4f.1.A) with the solution of 1.55 Å. The percentage of a sequence identity with template of bZD2B4 was 47.11. For yZA8B2 scFv antibody, the homology model was generated based on template (PDB code: 4buh.1.A) with the solution of 1.30 Å. The percentage of a sequence identity with template of yZA8B2 was 80.08. These scFv antibodies with a peptide of 15 amino acids that linked VH and VL domains was composed of antiparallel folded β-sheets connected by loops (Figure 4.40 A and C), displaying the characteristic variable regions of immunoglobulin folding. For bZD2B4 scFv antibody (Figure 4.40 B), shows that a favorable binding surface is formed and that the CDRs of both the heavy and light chains are involved in antibody-antigen interactions. In addition, the hydrogen bond interactions analysis revealed the formation of three hydrogen bonds located in the CDRs; In CDRH2, one hydrogen bond is formed between the carboxyl group of ZEN and Tyr59; In CDRL3, two hydrogen bonds are formed between the carboxyls of ZEN and Ala239, while one is seen between the other carboxyl and Ser242. For yZA8B2 scFv antibody (Figure 4.40 D), two hydrogen bonds are formed between the carboxyls of ZEN and Arg103, while one is seen between the other carboxyl and Val111 in CDRH3; In CDRL3, one hydrogen bond is formed between the carboxyl of ZEN and Lys175 while, the alkyl branch chain (Trp235) in the scFv antibody has a favorable interaction with the hydrophobic domains of ZEN.

(A)



(B)



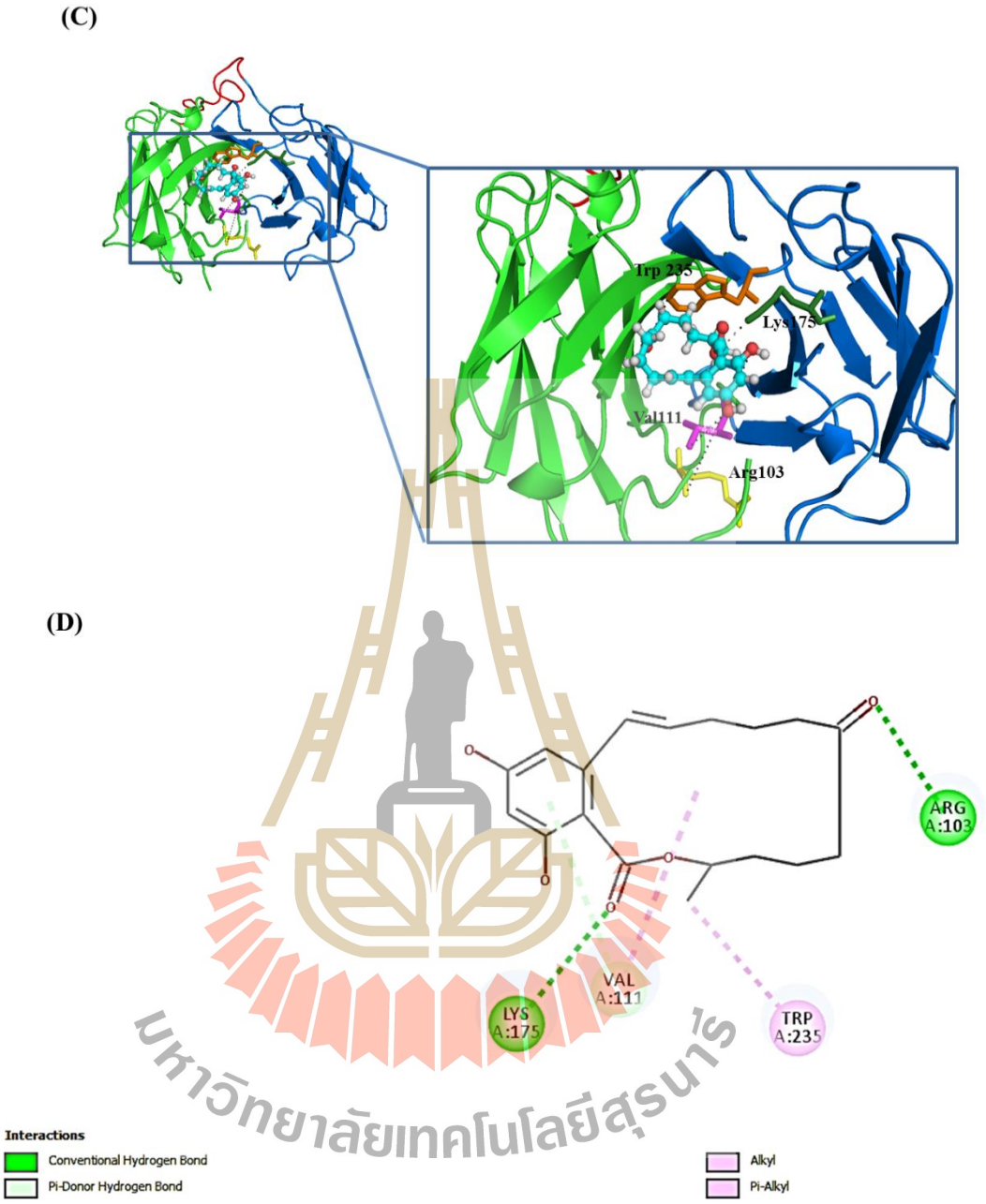


Figure 4.40 Comparison of the molecular docking models for the bZD2B4 and yZa8B2 scFv antibodies generated using PyMol and Discovery studio 2016. (A) Molecular docking model of bZD2B4 scFv with ZEN and close-up views of the antibody and hapten interactions. (B) Two dimensional diagram of ligand (ZEN) interaction to bZD2B4 scFv (C)

Molecular docking model of yZA8B2 scFv with ZEN and close-up views of the antibody and hapten interactions. (D) Two dimensional diagram of ligand (ZEN) interaction to yZA8B2 scFv. Amino acid residues involved in interactions with the ligand are displayed as capped sticks. The backbones of the light and heavy chains are shown in green and blue, respectively.

The differences between the structures of the complexes are subtle and the encoded binding pockets have very difference shapes. In binding pocket of bZD2B4 scFv antibody, ZEN could be bond above of binding pocket while binding pocket of yZA8B2 scFv antibody, a deep cavity, is highly complementary to the hapten ZEN. Both the light (green) and heavy (blue) chains contribute to the antigen-binding site (Figure 4.41). However, since, the percentage of a sequence identity with template of bZD2B4 scFv antibody was 47.11 so, the structure and binding pocket of this antibody might be difference from template.

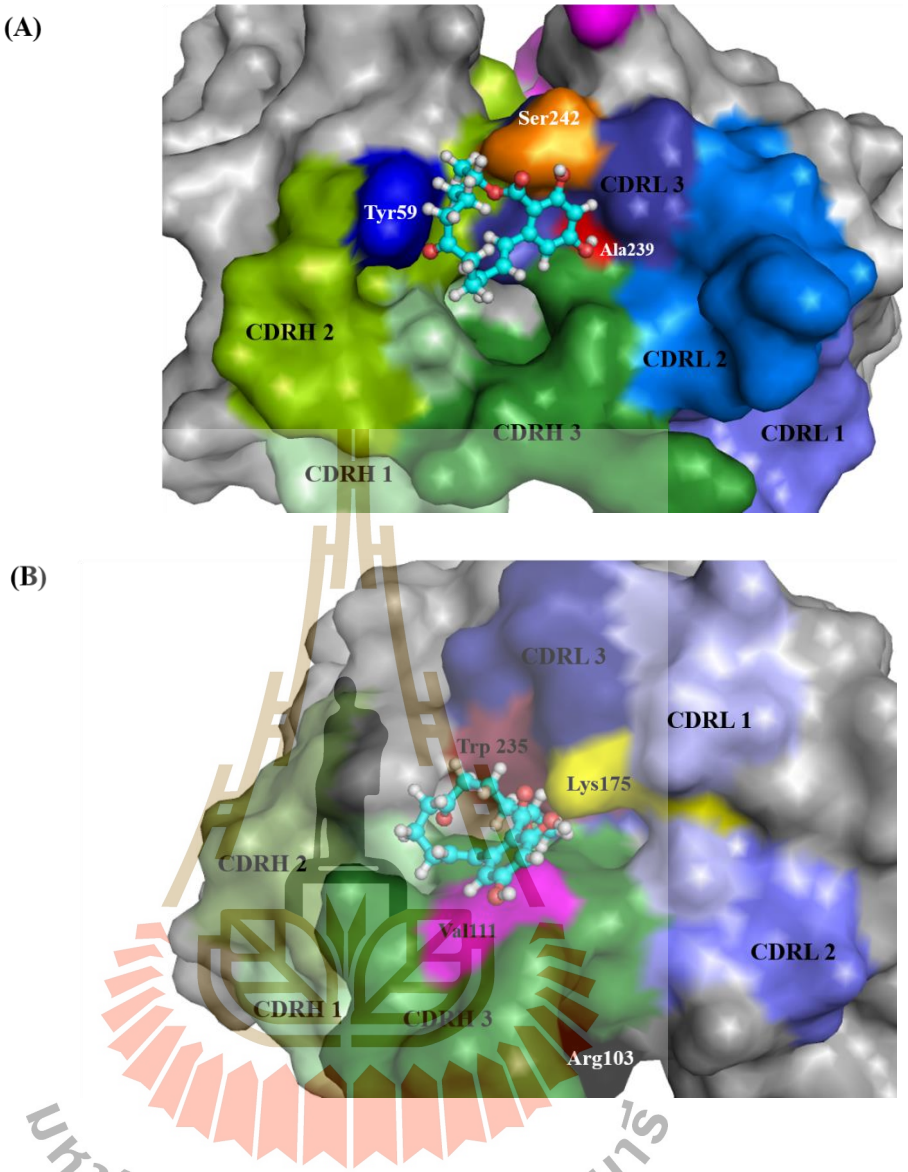


Figure 4.41 The binding pocket of molecular docking model with ZEN: (A) bZD2B4 and (B) yZA8B2 scFv antibodies. The figure was generated in PyMol.

Furthermore, when compare amino acids sequences of both scFv antibodies using Clustal Omega. The result showed that light chains of both antibodies have very similar amino acid more than heavy chain as shown in Figure 4.42. However, the binding activity of yZA8B2 scFv antibody to ZEN better than bZD2B4

scFv antibody, it might be related to amino acid differences in heavy chain of this antibody. Thus, heavy chain of antibody has important for the binding activity of antibody.

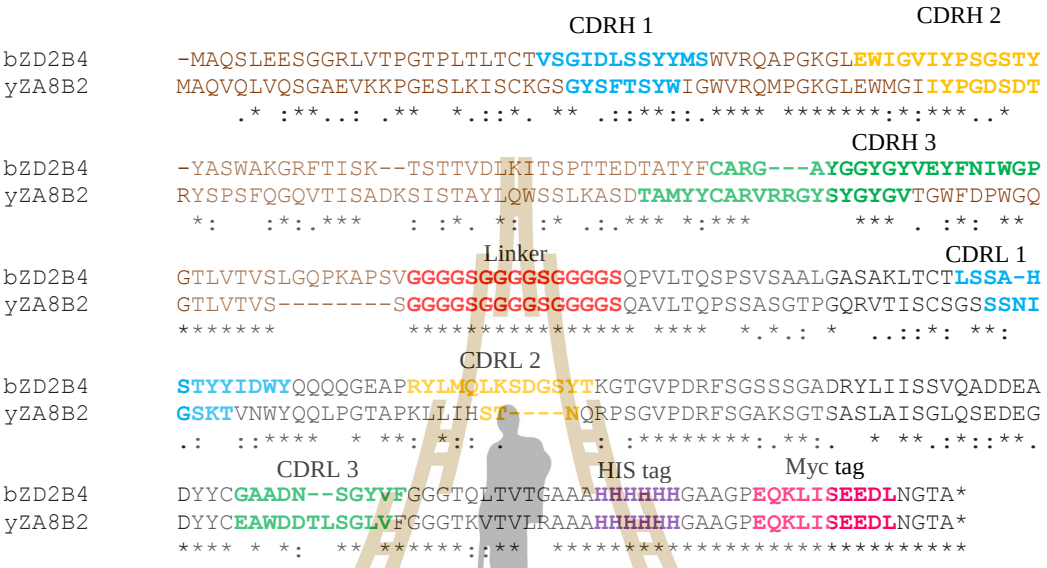


Figure 4.42 Comparison of amino acids sequence of bZD2B4 and yZA8B2 scFv antibodies. Brown colors represent the amino acid sequences of heavy chain, and black colors represent the amino acids sequences of light chain.

In summary, the yZA8B2 scFv antibody can shape an appropriate cavity to embed the entire ZEN molecule and a steady-state complex is formed through the interactions of hydrogen bonds and hydrophobic characteristics. Further investigation could focus on the improvement of the scFv antibody properties based on site-directed mutagenesis specific to the poor matched domains, coupled with DNA shuffling and phage display.

CHAPTER V

DISCUSSION AND CONCLUSION

Since obtaining specific antibody to free hapten is often very difficult with either naïve libraries or libraries derived from immunized animals, many strategies have been developed to increase the chance of selecting specific antibodies from a phage display antibody library (Rangnoi et al., 2011). In this study, we were able to isolate specific antibody by alternating the conjugates of the target during the biopanning procedure. The reason why all six positives clones were the same sequence could be because the library was quite small. The size of rabbit library was $3.0x10^{15}$ pfu/ml. Analysis of binding sensitivity by competitive ELISA revealed that the LOD of bZD2B4 phage scFv was approximately 50 ng/ml or 50 ppb. This antibody can bind ZEN conjugated KLH when display on phage coat protein or when express as soluble scFv or scFv-AP formats. However, only the phage displayed bZD2B4 format could bind to free ZEN as demonstrated by competitive ELISA. The scFv and scFv-AP format couldn't bind with free ZEN, this result suggested that the phage coat protein may help with the binding of the rabbit bZD2B4 scFv to the free toxin. Therefore, we decided to isolate recombinant antibody from human scFv library (YamoI) (Pansri P., et al, 2009), as we have found that it could be used to generate scFv antibody against free aflatoxin.

The reason why the three positives clones that were obtained from human scFv phage display library showed the same sequence could be because the biopanning

were performed for three rounds and the library is a compact size library. Therefore, only the phage that display the anti-ZEN scFv that could grow well were selected, and these are phage that display the same scFv antibody. Even if this library is relatively small but it was constructed from healthy people in Northeastern Thailand. It is highly possible that some of the healthy volunteers who have donated their blood for the construction of Yamo I library had previously exposed to ZEN, consequently a specific scFv antibody against ZEN could be obtained from this library. Moreover, in contrast to rabbit scFv human scFv clone, yZA8B2 clone from human library in both scFv and scFv-AP formats showed specific binding to free ZEN by competitive ELISA. After, ELISA optimization by checker-board titration was performed, the binding activity of scFv and scFv-AP to free ZEN could be improved by 2 and 40-fold, respectively. Furthermore, no cross-reactivity was observed even when the concentrations of non- related mycotoxins were as high as 5,000 ng/ml. These results indicated that the recombinant yZA8B2 scFv-AP fusion can efficiently detect ZEN, with high specificity.

To investigate whether yZA8B2 scFv and scfv-AP formats could be used to detect ZEN contaminationin agricultural and cereal products. Spike experiments of ZEN into wheat and corn extracts were analyzed by competitive ELISA. The results indicated that the IC_{50} of scFv and scFv-AP were 8 and 10 more than when the PBST was used in the competitive ELISA. This result indicated that extract of corn and wheat could slightly interfere with the binding of the scFv and scFv-AP to the toxin. Nevertheless, the result indicated that it is still possible to detect ZEN contamination in wheat and corn using the yZA8B2 antibodies in both formats. Finally to confirm that the antibody could be used to detect ZEN contamination in agricultural and cereal

products, corn and wheat samples with known amount of contaminated ZEN were used in the study. According to Thailand's regulation, the maximum allowance of contaminated corn and products were set at 3000 ppb. For cereal and product, 2000 ppb is the limit. However, we could only purchase corn contaminated with 454.2 ppb and wheat contaminated with 1085.5 ppb of ZEN. The assay indicated that antibody in scFv format could detect corn contaminated with 454.2 ppb and wheat contaminated with 1085.5 ppb at 83% accuracy when PBST were used as extraction buffer. For scFv-AP, when corns samples extract with 70% Methanol in 1:3 dilution, this antibody can detect 85% while wheat samples were extracted with 70% methanol, followed by 1:5 dilution, the antibody can detect 91% while when extract with PBST, this antibody can detect 100% of samples tested. In conclusion, the most optimal condition for the qualitative detection of contaminated agricultural products is to extract with PBST and used scFv-AP format of antibody for the detection.

Molecular modeling and docking analyses indicated that the yZA8B2 scFv antibody carries an appropriate binding structure and formed a steady complex with the ZEN molecule by antibody-ligand interactions. The difference in binding activity of bZD2B4 and yZA8B2 to ZEN might be partially due to the presence of hydrogen bonding, hydrophobic interactions and amino acid of heavy chain played important roles in mediating ZEN and antibody interactions.

In summary, the antibody could be used to detect ZEN the contamination in agricultural product; however, antibody engineering by various methods of affinity maturation (Sheedy et al., 2007) and ELISA optimization will be necessary to conduct in order to use this antibody for quantitative detection of ZEN contamination in agricultural products. In addition, the method used for the extraction of samples is

critical for the analysis. In our experiments, the amount of samples used in each study was very small because the cost of reference materials is very high. In the general protocol of the commercial available test kit, the company suggests to use a large scale of amount of sample. This could be one of the reasons why quantitative detection of ZEN couldn't be achieved. Moreover, another effect is from human error. Even if ELISA is a simple procedure but, the samples extraction method requires careful laboratory skill. Accordingly, the ELISA response could possibly be further enhanced by optimization of sample preparation.

In conclusion, a prototype of recombinant monoclonal antibody for specific detection of zearalenone within the limit of the Thai's regulations could be successfully developed by phage display technology. More development will be required for quantitative detection as well as for assembled into test kit or biosensors for commercialization as point-of-care diagnostic in the field. Since this antibody has human origin, it could be used for therapeutic purposes, such as in the case of acute toxicity of the toxin.

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APPENDIX

1. **Thai Patent Application No. 1701001529**

   Yamabhai M., and Sompunga, P.

   Title: Recombinant single chain fragment variable (scFv) antibody against Zearalenone

2. **Thai Patent Application No. 1701001530**

   Yamabhai M., and Sompunga, P.

   Title: Recombinant single chain fragment variable antibody fused with alkaline phosphatase (scFv-AP) for one-step detection of Zearalenone

3. **Proceeding; the 28th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2016)**

   Sompunga P., Srila W., and Yamabhai M.

   Title: Generation of Recombinant Rabbit scFv Antibody against Zearalenone by Phage Display Technology

4. **Proceeding; the 13rd The Asian Congress on iotechnology 2017 (ACB2017) "Bioinnovation and Bioeconomy",**

   Sompunga P., and Yamabhai M.

   Title: Generation of recombinant human scFv antibody against zearalenone using phage display technology

5. **Poster presentation; 45th International Exhibition of Inventions of Geneva, at Geneva, Switzerland. (Bronze medal award).**

   Yamabhai M., Rangnoi K., and Sompunga, P.

   Title: Mycotoxin test kits using recombinant antibodies

Table S1 Assays of contaminated samples by competitive scFv ELISA.

Date	scFv							
	Corn				Wheat			
	Methods							
	1:3 70%MeOH	1:5 70%MeOH	PBST	TBST	1:3 70%MeOH	1:5 70%MeOH	PBST	TBST
1/6/17	-	-	759.6268	-	-	-	115.5361	-
21/6/17	#NUM!	#NUM!	-	-	4.785286	#NUM!	-	-
30/6/17	377.22121	#NUM!	-	-	#NUM!	358.4776	-	-
	738.39551	2192.1891	-	-	#NUM!	1144.814	-	-
3/7/17	-	-	5.593	#NUM!	-	-	0.033623	#NUM!
4/7/17	-	#NUM!	-	-	-	#NUM!	-	-
13/7/17	21885.226	25901.089	-	-	9161.457	#DIV/0!	-	-
19/7/17	-	#NUM!	#NUM!	-	-	#NUM!	#NUM!	-
	-	4490.1134	296.7151	-	-	4878.962	31.41584	-
31/7/17	-	#NUM!	145.3474	-	-	#NUM!	0.88591	-
	-	2868.0275	11.07582	-	-	#NUM!	1.250848	-
8/8/17	#NUM!	#NUM!	-	-	#NUM!	7424.396	-	-
	35233.173	#NUM!	-	-	5267.227	#NUM!	-	-

(#NUM! indicated that the toxin couldn't be detected)

Table S2 Assays of contaminated samples by competitive scFv-AP ELISA.

Date	scFv-AP							
	Corn				Wheat			
	Method							
	1:3 70%MeOH	1:5 70%MeOH	PBST	TBST	1:3 70%MeOH	1:5 70%MeOH	PBST	TBST
1/6/17	-	-	-	#NUM!	-	-	-	117.8067
21/6/17	52.899524	#NUM!	-	-	#NUM!	376.5954	-	-
3/7/17	-	-	#NUM!	#NUM!	-	-	0.000282	0.128
4/7/17	340.12779	#NUM!	-	-	310.3032	#NUM!	-	-
13/7/17	1254.729	1062.1904	-	-	2832.415	1838.426	-	-
14/7/17	-	-	5.047944	0.0001	-	-	52.61082	#NUM!
15/7/17	230.6152	221.2209	-	-	#NUM!	1417.594	-	-
	#NUM!	#NUM!	-	-	#NUM!	424.1783	-	-
19/7/17	-	385.11933	10.46634	-	-	242.322	0.148697	-
	-	21.967469	51.93784	-	-	665.0695	2.954901	-
31/7/17	-	628.01771	#NUM!	-	-	507.888	0.251503	-
	-	#NUM!	3.257087	-	-	1.257516	20.52794	-
8/8/17	13.992	1522.662	-	-	1616.564	2881.321	-	-
	203.305	#NUM!	-	-	484.181	1476.549	-	-

(#NUM! indicated that the toxin couldn't be detected)

BIOGRAPHY

Miss Pensuda Sompunga was born on November 2, 1990 in Sisaket Province, Thailand. She received her Bachelor's Degree in Biomedical Scicence from Rangsit University of technology in 2005. After graduation, she has been employed research assistant for 1 year in Molecular Biotechnology Laboratory, Suranaree University of Technology. She continued with her graduate studies in School of Biotechnology, Institute of Agricultural Technology (IAT), Suranaree University of Technology. During her study, she had presented research work in The 28th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2016), November 28-30, 2016, The Empress Hotel, Chiang Mai, Thailand (Poster presentation; Generation of Recombinant Rabbit scFv Antibody against Zearalenone by Phage Display Technology). 45th International Exhibition of Inventions of Geneva, March 27-April 3, 2017, Palexpo, Hall 7, Geneva, Switzerland (Poster presentation; Mycotoxin test kits using recombinant antibodies). The 13rd The Asian Congress on iotechnology 2017 (ACB2017) "Bioinnovation and Bioeconomy", July 23-27, 2017, Pullman Khon Kaen Raja Orchid Hotel, Khon Kaen, Thailand (Poster presentation; Generation of recombinant human scFv antibody against zearalenone using phage display technology). The 5th School of Biotech International colloquium September 22, 2016, Suranaree University of Technology, Nakhon Ratchasima, Thailand (Oral presentation; Development of recombinant scFv antibody for the detection of zearalenone by phage display antibody technology).