



**SELECTION AND CHARACTERIZATION OF SINGLE CHAIN
F_v ANTIBODY SPECIFIC TO PROSTATE SPECIFIC ANTIGEN
USING PHAGE DISPLAY TECHNOLOGY**

**By
Pakkhapon Songthong**

**A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree
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Graduate School
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การคัดเลือกและศึกษาคุณลักษณะของ Single Chain Fv แอนติบอดีที่จำเพาะต่อโปรตีน
Prostate Specific Antigen โดยใช้เทคโนโลยีเฟจดิสเพลย์

โดย
นายภคพงศ์ ทรงทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเกษตรศาสตร์ชีวภาพ
บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
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The graduate school, Silpakorn University accepted thesis entitled “Selection and Characterization of Single Chain Fv antibody specific to Prostate Specific Antigen Using Phage Display Technology” by Pakkhapong Songthong in partial fulfillment of the requirements for the degree of Master of Pharmacy, program of Biopharmaceutical Sciences.

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49355201 : MAJOR : BIOPHARMACEUTICAL SCIENCES

KEY WORDS : PROSTATE SPECIFIC ANTIGEN/ PHAGE DISPLAY/ SC Fv ANTIBODY

PAKKHAPONG SONGTHONG : SELECTION AND CHARACTERIZATION OF SINGLE CHAIN Fv ANTIBODY SPECIFIC TO PROSTATE SPECIFIC ANTIGEN USING PHAGE DISPLAY TECHNOLOGY. THESIS ADVISOR: ASST.PROF. WISIT TANGKEANGSIRISIN, Ph.D. AND ASST.PROF. SIRIPAN LIMSIRICHAIKUL, Ph.D.. 105 pp.

Prostate specific antigen (PSA) level in serum correlates with prostate diseases; thus, it has been used as a marker for monitoring benign prostatic hyperplasia and prostate cancer. PSA screening method has been developed for more accurate detection. Monoclonal antibody (mAb) has been used to develop an enzyme-linked immunosorbent assay (ELISA). "Phage Display" is a technology for mAb selection; because, this technology is rapid, easy, low cost, and a high affinity antibody selection. The purpose of this study is to select single chain Fv (scFv) antibody from Tomlinson I+J library, which was developed to large diversity of antibody by MRC laboratory. The selected scFv from panning is used for further characterization.

The in vitro selection result shows that the positive clones are enriched in round 4 of panning. Four hundred clones are randomly screened for scFv expression. There are 11 clones that can express scFv antibody specific to PSA. The diversity analysis shows that all clones have an identical DNA fingerprint pattern; this result indicates that there are no variations of all clones. The scFv clone is additionally analyzed for DNA sequencing and purified by Ni²⁺ column. The DNA sequence result finds one amber stop codon (TAG) between V_H genes. The TAG is transferred to GAG by site-directed mutagenesis. The scFv purification using Ni²⁺ column shows the impurity of scFv antibody for further characterizations.

The transformation of scFv construct to Fab construct is considered for more stability and higher affinity of antibody. Fab is purified by Protein G chromatography; it shows the purity of Fab antibody for further studies, including detection limit, cross reactivity, and affinity determination. In conclusion, this study can select the potential scFv and Fab antibody against to PSA. In the future, the characterizations will be improved for more efficient diagnostic tool of PSA detection.

Program of Biopharmaceutical Sciences Graduate School, Silpakorn University Academic Year 2008

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คำสำคัญ : พีเอสเอ / เฟจคิสเพลย์ / sc Fv แอนติบอดี

นักพงศ์ ทรงทอง : การคัดเลือกและศึกษาคุณลักษณะของ Single Chain Fv แอนติบอดีที่จำเพาะต่อโปรตีน Prostate Specific Antigen โดยใช้เทคโนโลยีเฟจคิสเพลย์. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ผศ. ดร.วิสิฐ ตั้งเคียงศิริสิน และ ผศ. ดร.ศิริพรรณ ลิมศิริชัยกุล. 105 หน้า.

ระดับพีเอสเอ (Prostate Specific Antigen, PSA) ในซีรัมมีความสัมพันธ์กับการเกิดโรคในต่อมลูกหมาก พีเอสเอจึงถูกนำมาใช้เป็นตัวบ่งชี้ความเสี่ยงของการเกิดโรคต่อมลูกหมากโตและมะเร็งต่อมลูกหมาก ในปัจจุบันการตรวจระดับพีเอสเอในซีรัมถูกพัฒนาให้สามารถตรวจสอบระดับพีเอสเอได้แม่นยำมากขึ้นโดยโมโนโคลนัลแอนติบอดีถูกนำมาใช้เป็นตัวพัฒนาการตรวจสอบพีเอสเอด้วยวิธี Enzyme-linked immunosorbent assay (ELISA) ซึ่งเทคโนโลยีเฟจคิสเพลย์เป็นหนึ่งในวิธีที่ใช้ในการคัดเลือกโมโนโคลนัลแอนติบอดีเนื่องจาก รวดเร็ว สะดวก ลดค่าใช้จ่าย และสามารถคัดเลือกแอนติบอดีที่มีความจำเพาะสูงได้ จุดประสงค์ของงานวิจัยนี้คือการคัดเลือกโมโนโคลนัลแอนติบอดีรูปแบบ single chain Fv (scFv) ที่จำเพาะต่อพีเอสเอของมนุษย์ ในการศึกษาครั้งนี้ได้ใช้ Tomlinson I+J library ซึ่งถูกพัฒนาโดย MRC Laboratory ให้มี scFv แอนติบอดีที่หลากหลาย โดยแอนติบอดี scFv ที่ถูกคัดเลือกจากการ panning จะถูกนำไปศึกษาคุณลักษณะต่อไป

จากผลการคัดเลือกด้วยวิธี panning พบว่ามีการเพิ่มจำนวนของโคลนที่จำเพาะต่อพีเอสเอในรอบที่สี่ของการ panning จึงทำการสุ่มคัดเลือกกลุ่มตัวอย่างจำนวน 400 โคลนมาตรวจสอบการสร้างแอนติบอดี ผลการศึกษาพบว่าจำนวน 11 โคลนที่สร้างแอนติบอดี scFv ต่อพีเอสเอ และเมื่อศึกษาความหลากหลายของโคลนพบว่าโคลนที่คัดเลือกได้มีรูปแบบของ DNA fingerprint เหมือนกัน จึงคัดเลือกตัวแทนมาหนึ่งโคลนเพื่อศึกษาลำดับนิวคลีโอไทด์ รวมถึงการแยกบริสุทธิ์ของแอนติบอดี scFv ด้วยคอลัมน์ Ni²⁺ จากผลการศึกษาลำดับนิวคลีโอไทด์ พบว่ามี amber stop codon (TAG) ภายในฮิน V_H ผู้วิจัยจึงเปลี่ยน TAG ดังกล่าวเป็น GAG codon ด้วยวิธี site-directed mutagenesis อย่างไรก็ตามการแยกบริสุทธิ์ของแอนติบอดี scFv ด้วยคอลัมน์ Ni²⁺ พบว่าความบริสุทธิ์ไม่เพียงพอที่จะนำไปใช้ในการศึกษาต่อ

การเปลี่ยนโครงสร้างของ scFv ไปเป็น Fab เพื่อเพิ่มความคงตัว และความสามารถในการจับกับแอนติเจน ซึ่งแอนติบอดี Fab ที่ถูกแยกบริสุทธิ์ด้วย Protein G chromatography มีความบริสุทธิ์พอที่จะนำไปทดสอบหาความเข้มข้นต่ำสุดของพีเอสเอที่แอนติบอดีสามารถตรวจสอบ ความสามารถในการจับอย่างจำเพาะของแอนติบอดี และวัดแรงของการจับกันระหว่างแอนติบอดีและแอนติเจนได้ การศึกษาครั้งนี้สามารถคัดเลือกแอนติบอดี scFv และ Fab ที่จำเพาะต่อพีเอสเอ ซึ่งการศึกษาลักษณะเหล่านี้ของแอนติบอดี Fab เพื่อใช้ตรวจวัดพีเอสเอได้อย่างมีประสิทธิภาพ

สาขาวิชาเภสัชศาสตร์ชีวภาพ

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ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์.....

Acknowledgements

I wish to express my gratitude and grateful thanks to my thesis advisor Assistant Professor Dr. Wisit Tangkeangsirisin for his helpful, valuable advices and kind encouragement. I have learnt a lot from him.

I would like to thank Assistant Professor Dr. Thongchai Taechowisan, Assistant Professor Dr. Busaba Powthongchin, and Assistant Professor Dr. Sunee Techaarpornkul for their valuable suggestions and discussions.

This work was supported in part by National Research Council of Thailand (NRCT), which I was grateful thanks.

I would like to thanks my friends, Rutairat Putkam, Vijitra Jumpatong and Siwaporn Kamlanghan for their encouragement and friendship.

Finally, I would like to express my deep gratefulness to my family, my father, mother and sisters for their love and emotional support.

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List of abbreviations

A	adenine
ABTS	2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)]
ACT	alpha1-antichymotrypsin
AMG	alpha2-macroglobulin
bp	base pair
BPH	benign prostatic hyperplasia
BPSA	benign PSA
BSA	bovine serum albumin
C	cytosine
°C	degree celsius
C _H	constant region, heavy chain
C _L	constant region, light chain
C _κ	constant region, kappa light chain
Ca	calcium
CDRs	complementary determining regions
CFU	colony-forming unit
cm	centimeter
cPSA	complexed PSA

Cy3	cyanine 3
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DRE	digital rectal examination
ELISA	enzyme-linked immunosorbent assay
Fab	fragment antigen binding
fPSA	free PSA
FR	frame work region
Fv	fragment variable
G	guanine
HA	hemagglutinin
HAT	hypoxanthine aminopterin thymidine
HCl	hydrogen chloride
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HRP	horseradish peroxidase
Ig	immunoglobulin
IgG	immunoglobulin G
IGF-1	insulin-like growth factor 1
IGFBP-3	insulin-like growth factor binding protein-3
IGFBP-4	insulin-like growth factor binding protein-4
IGHV	immunoglobulin heavy variable group

IGHJ	immunoglobulin heavy joining
IGHD	immunoglobulin heavy diversity
IGKV	immunoglobulin kappa variable
IGK	immunoglobulin kappa locus
IMZ	imidazole
inPSA	intact PSA
IPTG	isopropyl thio- β -D-galactopyranoside
kDa	kilodalton
M	molar
mA	milliampere
mg	milligram
Mg	magnesium
mL	milliliter
mM	millimolar
M_w	molecular weight
NaCl	sodium chloride
ng	nanogram
Ni	nickel
nm	nanometer
nM	nanomolar
OD	optical density

pIII	protein III
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PEG	polyethylene glycol
pH	hydrogen-ion concentration
PSA	prostate specific antigen
PSAD	PSA density
PSADT	PSA doubling time
PSAV	PSA velocity
RNA	ribonucleic acid
rpm	revolutions per minute
scFv	single-chain Fv
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T	thymine
TRUS	transrectal ultrasonography
UV	ultraviolet
V _H	variable region, heavy chain
V _{H1}	upstream variable region
V _{H2}	downstream variable region

V_{κ}	variable region, kappa light chain
V_L	variable region, light chain
w/v	weight by volume
μg	microgram
μL	microliter
%	percent

Chapter I

Introduction

1. Statement and significance of the problem

Prostate cancer has a large increases in both incidence and mortality rate in many countries, especially the United State, Canada, Australia, and Europe (Hsing, Tsao, & Vanesa, 2000), and it can be found 3.8 in 100,000 men in Thailand (Vatanasapt, 1993). The prostate cancer is an adenocarcinoma of the prostate gland, which locates in front of rectum, under bladder, and surrounding urethra. The prostate gland is a male reproductive organ, producing and storing seminal fluid. Prostate cancer can affect in urination and ejaculation; it makes uncomfortable for men's life. In 2007, the National Comprehensive Cancer Network recommended that men age 40-50 should have Prostate Specific Antigen (PSA) test (Loeb & Catalona, 2007).

PSA, a serine protease, is a glycoprotein that mainly produced from epithelial cell in prostate gland. In normal men, the serum PSA is detected in a small amount, but it is detected at high concentration in prostate disease patient. Four ng/mL is a cut-off value of PSA concentration for

indication a risk and recurrence of prostate disease. Men, who have 4 to 10 ng/mL of serum PSA, will be confirmed by specific screening such as biopsy test (Thompson et al., 2004). Thus, the early detection of PSA can relieve prostate disease and reduce the cost of treatment. However, the variation of PSA measurement has been considered because the serum PSA exists in several different forms, including free PSA (fPSA) and complexed PSA (cPSA) (Buntion et al., 2003). In prostate cancer, the proportion of cPSA to total PSA was significantly higher than the proportion of fPSA to total PSA (Loeb & Catalona, 2007). Recent studies reported that the complexed PSA detection using monoclonal antibody can specifically discriminate between prostate cancer and benign prostatic hyperplasia (BPH) (Brawer et al., 2000; Kobayashi et al., 2003).

Currently, the monoclonal antibody has been used to detect PSA because of its high specificity and sensitivity. Hybridoma technology has been conventionally used for monoclonal antibody production; however, this technique is tedious, time-consuming, and very expensive. Alternatively, “Phage display technology” is a high-throughput selection technique that can solve the problems. The phage display technology has been successfully used to select the monoclonal antibody as a diagnostic and therapeutic tool (Yau, 2003). The small constructs of antibodies, including scFv and Fab, have been employed to generate monoclonal

antibody libraries, because it is easy to genetically manipulate and to display on the coat protein of phage. Krag and et al. successfully used the Tomlinson J library to select scFv antibody against tumour-targeting ligand (Krag et al., 2006).

In this study, Tomlinson I+J library (Cambridge, UK) was used to select the monoclonal antibody specific to PSA. The library provides the human single-chain Fv (scFv) antibody comprising one variable region of heavy chain and light chain conjugated with glycine-serine linker, and displays the scFv on protein pIII of phage. Because Fab construct has been considered for more stability and affinity of antibody (Quintero-Hernández et al., 2007); the selected scFv was transformed to the Fab construct, inserted into pComb3XSS-dIII (Putkam, 2007), and characterized by DNA sequencing and expression. The selected Fab can be developed as a diagnostic tool to detect prostate diseases.

2. Objectives of the study

The objective of this study is to select, characterize, and purify the scFv antibody specific to prostate specific antigen using phage display technology. The selected scFv is transformed to Fab construct and purified by Protein G chromatography.

3. Scope of the study

Tomlinson I+J library is used to select human scFv antibody specific to PSA by “panning” process. The selected scFvs are characterized of clone diversity, sequencing analysis, and antibody expression. The scFv construct is transformed to the Fab construct. The variable regions, V_H and V_K , of scFv are independently amplified and combined to constant region, C_H and C_K , bearing in pComb3XTT. The amber stop codon (TAG) between V_H fragment is mutated to glutamic acid codon (GAG) by site-directed mutagenesis during V_H amplification. The recombinant Fab is inserted into pComb3XSS-dIII, derived from clone Afl15 (Putkam, 2007). The identity of Fab is verified by DNA sequencing and antibody expression.

4.1 Prostate Specific Antigen (PSA)

4.1.1 Discovery of PSA

Rubin H. Flocks and colleagues were the first to identify PSA as an antigen specific to human (Flock et al., 1960). PSA was characterized as a semen-specific protein and used as a forensic marker for rape case (Hara, Koyanagi, & Inove, 1971). In 1980, the PSA was first detected in serum of advanced prostatic cancer patients using immunoelectrophoresis technique (Papsidero et al., 1980). Moreover, Stamey and et al. found the PSA in serum patient of prostate cancer, they showed the evaluated PSA in serum correlated with the advancing stage of prostate cancer (Stamey et al., 1987). In the present time, the PSA has been widely used to diagnose the risk of prostate diseases.

4.1.2 PSA production

PSA is a glycoprotein that mainly produced from prostatic epithelial cells. The majority of PSA is generated in the transitional zone (TZ) of prostate gland. The PSA production is regulated by androgen hormone (Riegman et al., 1991). In healthy men, the PSA can be found at high concentration, 0.5 to 2 mg/mL in seminal fluid (Wang et al., 1998) and at low concentration in serum, 4 ng/mL (Oesterling, Jacobsen, & Cooner, 1995). However, the PSA in serum is detected at high

concentration in prostate disease patients; because the PSA may leak from prostate gland into blood circulation. Although PSA is semen-specific protein, it could be found in the other fluid or tissues such as salivary duct (James et al., 1996), human endometrium (Clements & Mukhtar, 1994), and breast tissue (Yu & Diamandis, 1995).

4.1.3 Characteristic of PSA

PSA belongs to human kallikrein family of serine protease that is encoded by *KLK3* gene on human chromosome 19q13.4 (Balk et al., 2003). PSA is synthesized with a 17-amino acid with leader sequence (prepropeptide). Cleavage of N-terminal amino acids of proPSA generates the active enzyme (Fig. 2), which has a mass of 33 kDa (Balk et al., 2003).

N-glycosylation site on PSA was identified at amino acid residue 45 (Lundwall & Lilja, 1987). Moreover, Peracaula et al. studied the glycosylation pattern of PSA and can differentiate between normal and cancer cell. The PSA from normal prostatic cells was monosialyated and disialyated, while the PSA from cancer prostatic cells has a high fucose and N-actyl-D-galactosamine (Peracaula et al., 2003). The typical characteristic of serine proteases is the active-site composed of amino acid residue His, Asp, and Ser (Lundwall & Lilja, 1987).

The mature PSA is activated by proteolytic cleavage with trypsin, hK2, hK4, and prostin. In seminal fluid, PSA exists in the active and inactive form. Seventy percent of PSA in the seminal fluid is an enzymatically active, and the rest is an inactive PSA.

4.1.4 Biological function of PSA

The main function of PSA is the liquefaction of the seminal fluid by the degradation of seminogelin I and II, and of fibronectin. The enzymatic activity of PSA, it preferentially hydrolyzes at the carboxy-terminus of the hydrophilic residues, Lys and Arg; because, the negatively charged side chain of Asp at catalytic triads is able to attract the positively charged of Lys and Arg (Lundwall & Lilja, 1987). PSA can cleave a variety of proteins including insulin-like growth factor binding protein-3 (IGFBP-3) and IGFBP-4, making release of active insulin-like growth factor 1 (IGF-1), that can enhance tumor growth factor (Cohen et al., 1992; Rehaul et al., 2001). Moreover, the PSA also cleaves fibronectin and laminin (Webber, Waghray, & Bello, 1995) and activates latent TGF-beta (Killian et al., 1993), which resulting in progression of prostate cancer.

4.1.5 Molecular form of PSA

PSA in serum and seminal fluid has several forms (Fig. 1). Two main forms are complexed PSA (cPSA) and free PSA (fPSA). In human serum, 70-90% of PSA is cPSA and 10-30% is fPSA.

cPSA is a conjugated form of PSA and protease inhibitor in serum such as alpha2-macroglobulin (AMG) or alpha1-antichymotrypsin (ACT). cPSA in serum is higher in men with prostate cancer. Brawer and et al. showed that cPSA is more specific to prostate cancer than total PSA in 4 to 10 ng/mL of total PSA range (Brawer et al., 2000). In 2000, cPSA was approved by FDA for monitoring prostate cancer.

fPSA comprises of three forms, proPSA (pPSA), BPSA, and intact PSA (inPSA). The pPSA is a precursor of mature PSA. It was detected about 33% of serum fPSA. The pPSA contains 7, 2, and 5-amino acid pro-leader peptide that respectively represented in (-7), (-2), and (-5) PSA, the (-7) PSA is secreted into lumen of prostate gland and cleaved by human kallikrein-2 and -4 to active form (Fig. 2). The (-2) PSA has a high specificity in monitoring prostate cancer (Lein et al., 2005).

Twenty-eight percent of fPSA is BPSA, which is only form that correlates in benign prostate hyperplasia (BPH). BPSA is pPSA that internally cleaved at Lys-145 and Lys-146. Higher BPSA in serum has been found to correlate strongly with BPH and prostate volume, which

closely associated with progressive enlargement of prostate gland (Mikolajczyk et al., 2000). The third fPSA isoform is inPSA, which is enzymatically activated by hK2 to generate mature PSA; but, it is a misfolded form of mature PSA. The inPSA is also detected in serum similar to BPSA and correlates with the BPH pathology.

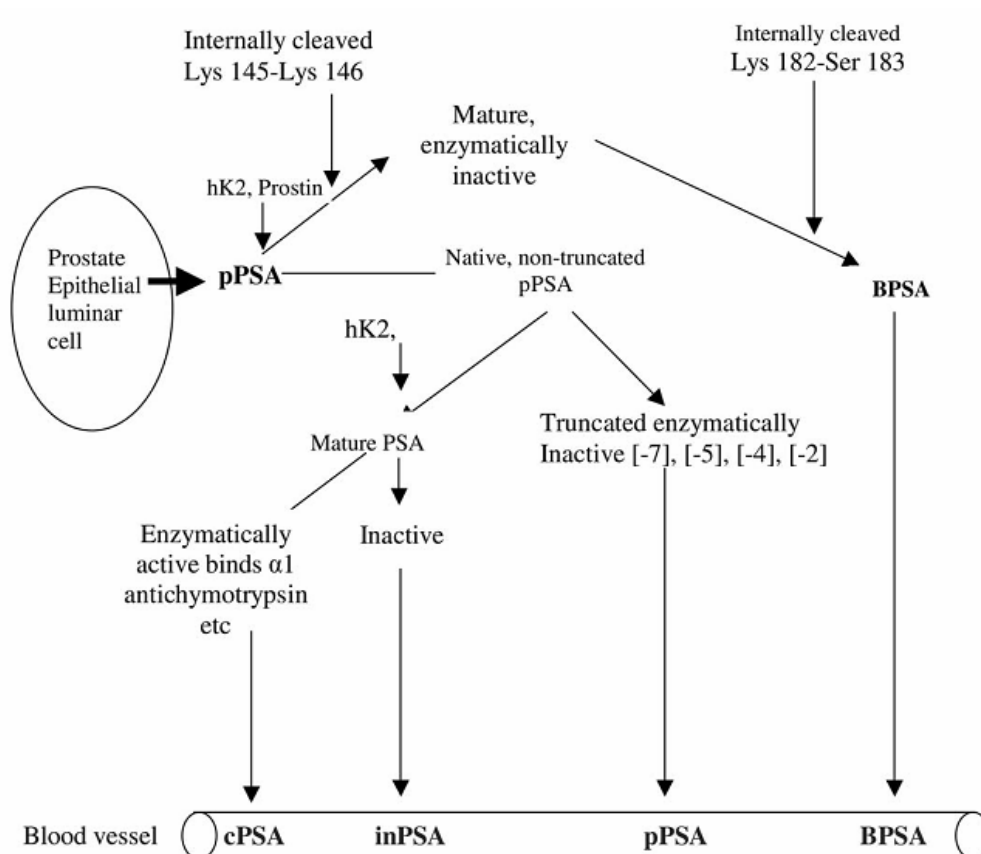


Fig. 1. The formation and distribution of PSA forms in blood vessel. hK2; human kallikrein 2, cPSA; complexed PSA, inPSA; inactive PSA, pPSA; precursor PSA, and BPSA; benign PSA (Naya et al., 2005).

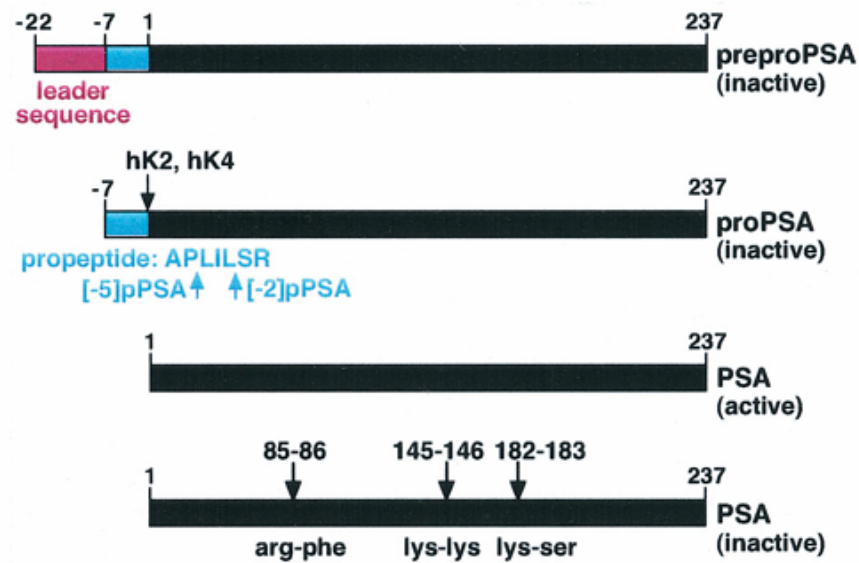


Fig. 2. Structure of PSA forms. The leader sequence of preproPSA is cleaved to generate proPSA (inactive form). Cleavage of the propeptide by hK2 generates the active mature PSA. Truncated form of proPSA can also be generated by cleavage within the propeptide, and these proPSA form are inactive, including (-2)pPSA and (-5)pPSA. Active PSA can be further cleaved at arg85-phe86, lys145-lys146, lys182-ser183 to generate inactive PSA (Balk, Ko, & Bublely, 2003).

4.1.6 PSA in clinical use

In 1980, Pepsidero and et al. were the first who detected the serum PSA in men with prostate cancer (Papsidero et al., 1980). In further study, the PSA showed immunological identity in prostatic tissue of advanced prostate cancer patients. However, the serum PSA can also be evaluated in a variety of clinical condition, including prostatic inflammation, BPH, and prostate cancer. In the first PSA testing, total PSA was approved by FDA in 1994 using 4.0 ng/mL as a cut-off value. There are variations on the total PSA measurement, because PSA in blood has two main forms, fPSA and cPSA. Many researches including Brawer et al. used the ratio of fPSA to total PSA for prostate cancer detection than cPSA to total PSA (Brawer et al., 2000). Furthermore, human kallikrein-2 (hK2) is a member of human kallikrein family. The ratio of hK2 to fPSA was also used to predict prostate cancer with PSA level 2.5 to 10 ng/mL (Magklara et al., 1999).

The detections of fPSA have been improved for the accuracy testing. The inPSA and BPSA correlate well to BPH; whereas, (-2)pPSA correlates to prostate cancer (Mikolajczyk et al., 2000). Catalona et al. showed that using percent of pPSA (i.e. $\text{pPSA}/\text{total PSA} \times 100$) can improve the specificity of PSA detection. From this report, the number of

unnecessary biopsy in men with total PSA between 2 to 4 ng/mL was decreased (Catalona et al., 2003).

However, other parameters of PSA kinetic have also been considered and used for more specific PSA testing. There are three parameters that have been commonly used, including PSA density (PSAD), PSA velocity (PSAV), and PSA doubling time (PSADT).

The detection of PSAD is described in Table 1. Veneziano et al. used PSAD for monitoring prostate cancer. They showed that this PSA/ prostate volume ratio was 1.73 in men with prostate cancer (Veneziano et al., 1990). The limitation of PSA is the measurement of prostate volume by transrectal ultrasonography (TRUS); because it is a time-consuming and expensive method. In addition, the actual prostate size is not perfectly measured and the different gland sizes affect the accuracy of PSAD calculation.

Smith and Catalona showed that the PSAV, longitudinal changes in PSA over time, was greater than 0.75 mg/mL/year in men with prostate cancer (Smith & Catalona, 1994). In 2006, the National Comprehensive Prostate Cancer Network recommends biopsy test when men has PSAV cut-off at 0.5 ng/mL/year (Loeb & Catalona, 2007). The limitation of PSAV is a physiologic fluctuation in serum PSA level within an individual (Roehrborn et al., 1996).

PSADT, the time required for the serum PSA level to double, has been used for post-treatment in prostate cancer patients. It can be made a decision as to whether local salvage radiotherapy or hormonal therapy. However, Sengupta and et al. studied about PSADT in pretreatment of prostate cancer. The 5.79-fold of PSADT showed the risk of prostate cancer (Sengupta et al., 2005).

In conclusion, the specificity of PSA testing has been improved; although, there are many variations of PSA measurement. The fPSA to total PSA ratio has been frequently used to monitor prostate cancer than the cPSA to total PSA ratio because of its high specificity. The fPSA, inPSA and BPSA forms are used to discriminate the prostate cancer from BPH. Additionally, PSAD, PSAV, and PSADT measurement has been improved for more specificity to predict the likelihood of prostate cancer.

Table 1. Definitions of variation on the PSA measurement (Loeb & Catalona, 2007).

Variation of PSA measurement	Definitions
Total PSA	The most commonly reported measurement of serum prostate-specific antigen; FDA approved for prostate cancer early detection in 1994 using a threshold of 4.0 ng/mL as the upper limit of normal; provides a continuum of prostate cancer risk across all levels; also useful in monitoring for recurrence after treatment.
Free PSA (fPSA)	The unbound isoform of PSA; the proportion of total PSA in the free form is generally greater in benign conditions.
Complexed PSA (cPSA)	The fraction of PSA that is bound to other molecules, such as alpha-1-antichymotrypsin; the proportion of PSA in the bound form is higher in malignancy.
PSA density (PSAD)	The serum prostate-specific antigen level divided by the prostate volume, as estimated by transrectal ultrasound; used as a means to control for gland size when interpreting PSA values.
PSA velocity (PSAV)	Measurement of the longitudinal change in PSA values over a specified time interval; has been used both to predict the presence and aggressiveness of prostate cancer prior to definitive therapy.

Table 1. (Continued)

Variation of PSA measurement	Definitions
PSAdoubling time (PSADT)	Measurement of the amount of time required for the PSA level to double; highly dependent of the initial PSA measurement; primarily useful in the predictive of metastatic and cancer-specific mortality.

4.1.7 The detection method of prostate cancer

There are several procedures for prostate cancer detection, including DRE (Digital Rectal Examination), TRUS (Transrectal Ultrasonography), and tumour marker PSA screening. In present time, The 2006 National Comprehensive Cancer Network recommends offering the PSA test and DRE for more specificity of detection (Loeb & Catalona, 2007). However the PSA detection method is comfortable and low cost procedure; thus, it has been generally used to screen a risk and a recurrence of prostate cancer.

The PSA screening using ELISA detection method has been developed for more specificity. Wan et al. developed ELISA method to discriminate benign and malignant prostatic disease. They used three different monoclonal antibodies recognizing three distinct PSA epitopes

(Wan et al., 2003). The PSA commercial test kit generally used the monoclonal antibody against fPSA, following GeneWay Biotech protocol. Anti-fPSA monoclonal antibodies are coated to the solid phase (micro titer plate) and rabbit anti-PSA antibody conjugated with HRP was used as secondary antibody. The fPSA molecules in serum samples can be sandwiched between the two antibodies. The HRP activity bound in wells are developed to colour and measured the intensity that is proportional to the concentration of fPSA present in serum samples.

4.2 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA is based on immunological reaction to detect antibodies and antigens in a sample. In 1970, Engvall and Perlmann were the first that developed the ELISA technique (Engvall & Perlmann, 1971). In principle, antigen is coated on solid phase, and an enzyme-linked antibody is incubated with the antigen. The unbound antibodies are washed out, and an enzyme substrate is added to develop colour. The intensity of colour is proportional to the amount of antigen.

There are several of ELISA methods, including direct, indirect, sandwich, and competitive ELISA.

A direct ELISA has been frequently used for antigen detection. In basic method, antigens coated on the solid phase directly bind to enzyme-

linked antibodies. After washing step, the enzyme substrate is added to well, making the colour development. The intensity of colour is measured, and it is a proportional to the amount of antigen. In the other hand, an indirect ELISA method has been frequently used for antibody detection. In principle of indirect ELISA, the coated antigens are bound by antibody presented in samples. The bound antibodies are detected by secondary antibody conjugated with an enzyme.

A sandwich ELISA method, the antibody specific to antigen of interest is coated on solid phase (microtiter well). The antigen in samples is added to well. The bound antigens are detected by secondary antibody conjugated with an enzyme. The colour intensity is proportional to amount of the bound antigens.

A competitive ELISA implies that two reactants try to bind to a third reactant. In the first step, the antibodies are incubated with free antigens. Then, the antibody/antigen complexes are added to antigen coated on well, the unbound antibodies (antibody bind to free antigen) are removed by washing step. The secondary antibodies conjugated with an enzyme are used to detect the antibody that bind to antigen coated on well. The last step, the enzyme substrate is added and the intensity of colour is measured. The high intensity indicates that the free antigen in sample is high amount.

In a nutshell, it is difficult to choose the ELISA methods for appropriate works. When the direct ELISA is used to detect serum or crude samples containing a high concentration of contaminating substances, the antigens coated on microtiter plate are competed by the contaminating in samples. The indirect ELISA offers advantage over the direct test; because, only secondary antibody conjugated with an enzyme is required to determine the primary antibody bound to antigen directly (Crowther, 1995). In sandwich ELISA, the procedure has been considered that an antigen has to contain at least two antigenic sites. It is difficult to use the same antibody in sandwich ELISA, when the detected antigen in samples has one antigenic epitope. In affinity determination, the indirect competitive ELISA has been used to detect the concentration of antigen at 50% inhibition of ELISA signal.

4.3 Antibody technology

4.3.1 Principle of antibody

Antibody is a glycoprotein which is important in an adaptive immunity. The antibody is a part of humoral immune system that found in blood or bodily fluid of vertebrates. It could be produced from plasma cells that are activated by foreign objects, called antigen. The main function of antibody is to neutralize antigens, such as virus and bacteria.

Antibody can also activate complement system and effector cells. There are five antibody isotypes known as IgG, IgM, IgD, IgE and IgA, which are categorized into the different isotypes based on heavy chain.

The structure of antibody comprises of two heavy chains and two light chains linked together by flexible disulfide bonds. Both have two regions, constant and variable regions (Fig. 3). The constant region of heavy chains is identical in the same isotype, but it differs from different isotypes. Each variable domain comprises a beta-sheet “Frameworks” and “Complementarity Determining Regions (CDRs). The framework is a conserved region of each species; whereas, the CDR is a hypervariable region, resulting from the VDJ in heavy chain or VJ in light chain combinations. The CDR is a region that recognizes antigen. The site of CDR recognizing antigen is called paratope, while the site of antigen recognized by antibody is called epitope.

Papain enzyme can cleave IgG into three fragments, comprising two Fab fragments and one Fc fragment. Each Fab consists of the variable and constant regions of the light chain (Fig. 3), providing one antigen-binding site. Pepsin digestion can cleaves the Fc fragment from the molecule of antibody, but the rest part of antibody is $F(ab')_2$ (Fig. 3).

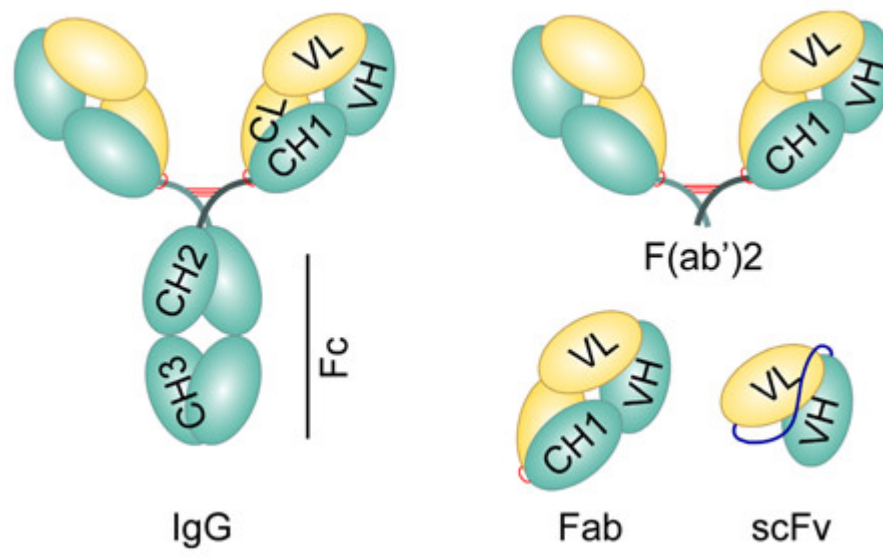


Fig. 3. The structures of antibody. The picture shows the four construct of antibody, IgG, F(ab')₂, Fab, and scFv (Peterson, Owens, & Henry, 2006).

4.3.2 Antibody technology

Accordingly, antibody can bind to antigen specifically, thus, it was applied to be diagnostic and therapeutic use (Yau, 2003). In 1980, monoclonal antibody (mAb) has developed into a beneficial and profitable group of product resulting in the high specificity and sensitivity, compared to polyclonal antibody. The original method for production of mAb is hybridoma technology.

Fig. 4 shows the principle of hybridoma technology (Abbas, Lichtman, & Pillai, 2007) that is the fusion between the murine plasma cells and murine myeloma cells (murine cancerous plasma cell). For the reason of plasma cells (antibody producing cell) can not survive for a long time in the in vitro environment, but the myeloma cell can be immortal growth cell. As a result, the fusion can make the hybrid cells that are able to produce mAb for a long period of time. After fusion, the hybrid cell could be selected by the selective medium, HAT (Hypoxanthine-Aminopterin-Thymidine). The HAT medium contains a drug aminopterin, which blocks the purine synthesis in salvage pathway using HGPRT enzyme. Thus, the myeloma cell and plasma cell can not growth because of lacking HGPRT enzyme and their limited life span, respectively. However, the hybridoma technology is a tedious work, time consuming, high cost, and low yield (industrial scale). Importantly, the technique is a

screening method, which rarely obtains the high affinity antibody. Thus, the in vitro selection techniques have been developed for acquiring the high affinity and specificity of mAb.

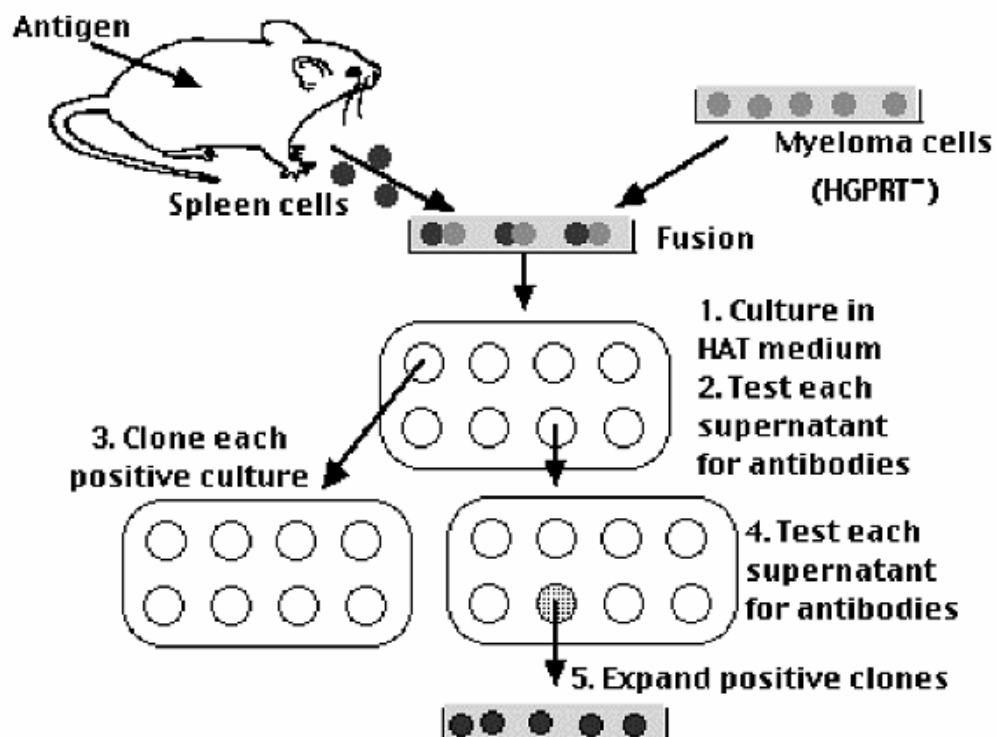


Fig. 4. The principle of hybridoma technology for monoclonal antibody production (Abbas, Lichtman, & Pillai, 2007).

4.4 Phage technology

4.4.1 Phage display technology

Phage display is a valuable molecular technology, which was first developed by George Smith in 1985 (Smith,1985). This technology links between phenotype and genotype. An antibody gene is inserted to a coat protein gene of phage. The antibody phage libraries were used to select mAb. The phages displayed mAbs of interest bind to the target molecule, whereas the unbound phages are washed out. Then, the bound phages are eluted and reamplified for further rounds of selection. The cycles of in vitro selection are called 'biopanning'. The selected clones were characterized by sequencing, ELISA, and purification. Phage display was used to generate the mAb because of its large diversity (10^{12} - 10^{14} pfu/mL), ease of manipulation with high throughput selection. Many applications of phage display are employed as a basic research tool in antibody engineering and molecular biology, it can also used to identify a novel receptor, ligand and mAb. Furthermore, this technology has been embraced in the biotechnology and pharmaceutical industries, the recombinant proteins and antibodies that were discovered or modified by phage display are now approved for clinical use (Yau, 2003). In 2003, HUMIRA[®] or Adalimumab approved by FDA was the first mAb derived from phage display technology. It was used as TNF-alpha blocker for

rheumatoid arthritis treatment (Putte et al., 2004). In a nutshell, phage display technology is a powerful tool to select the antibody specific to prostate specific antigen.

4.4.2 Filamentous phage biology

Filamentous phage is a bacterial virus in genus Inovirus. A circular single stranded DNA is encased in a long cylinder-shaped capsid that has a diameter and a length of about 6.5 nm and 930 nm, respectively. The Ff class of filamentous phage, f1, fd and M13, has 98 % homology in the DNA sequence. The phage attaches to the tip of F-conjugative pilus as a receptor for infection to gram negative bacteria such as *Escherichia coli*, *Xanthomonas spp.*, *Thermus spp.*, *Pseudomonas spp.*, *Vibrio spp.*, and *Salmonella spp.*

The structural proteins of phage were divided to minor and major coat proteins. One major coat protein is pVIII, and the other four minor coat proteins are pVII, pIX, pVI, and pIII (Fig. 5). Several thousand copies of pVIII have a positively charged amino acid at C-terminus. The positive amino acids on pVIII interact with phosphates of the viral genome. One end of the particle is pVII and pIX that are the first part of phage assembly and the other end is pVI and pIII. The pIII is the most commonly used protein for display. It is composed of three domains, N1,

N2 and CT that they integratedly interact to bacterial pilus for infection process.

The filamentous bacteriophage is a lysogenic phage (Fig. 6). The replication begins when the phage genome enters a bacterial cell. The host RNA/DNA polymerase and topoisomerase convert the single-stranded DNA (ssDNA) genome to a double-stranded replicative form (RF). The RF is employed as a template for the synthesis of new viral genomes and the phage coat protein. pII and pX are required for replication of phage. The replication continues until the phage genome and pV reach to critical quantity. In the first generation 1,000 phage particles are produced.

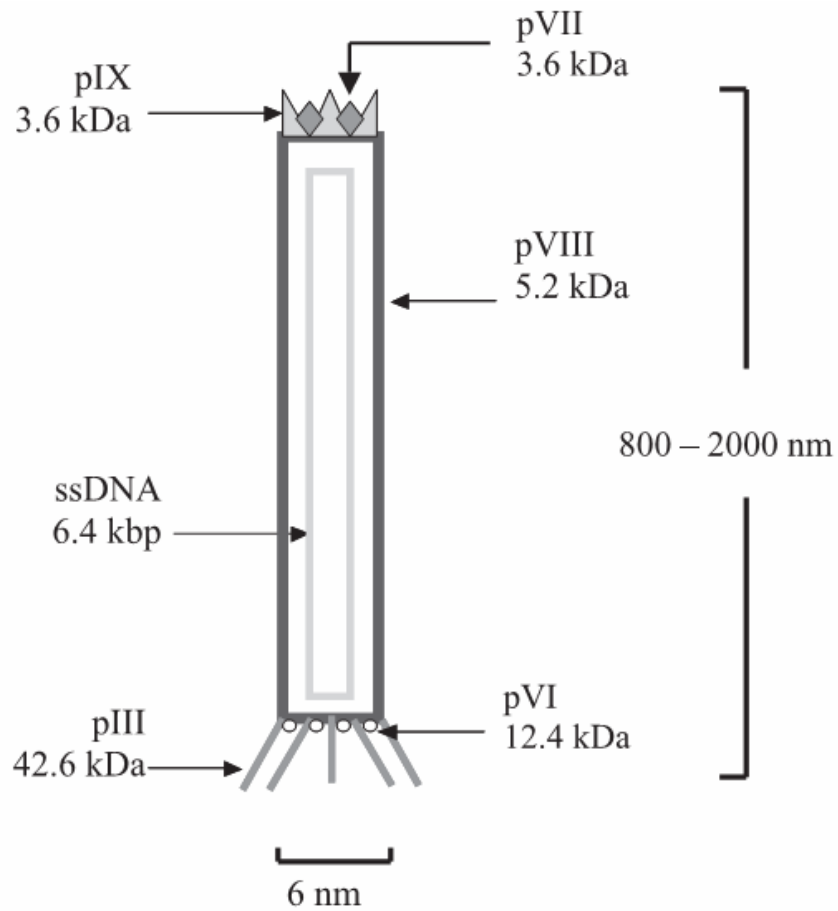


Fig. 5. Structure of filamentous bacteriophage. The coat protein of phage comprises of major coat protein (pVIII) and minor coat proteins (pVII, pIX, pVI, and pIII) (Yau, 2003).

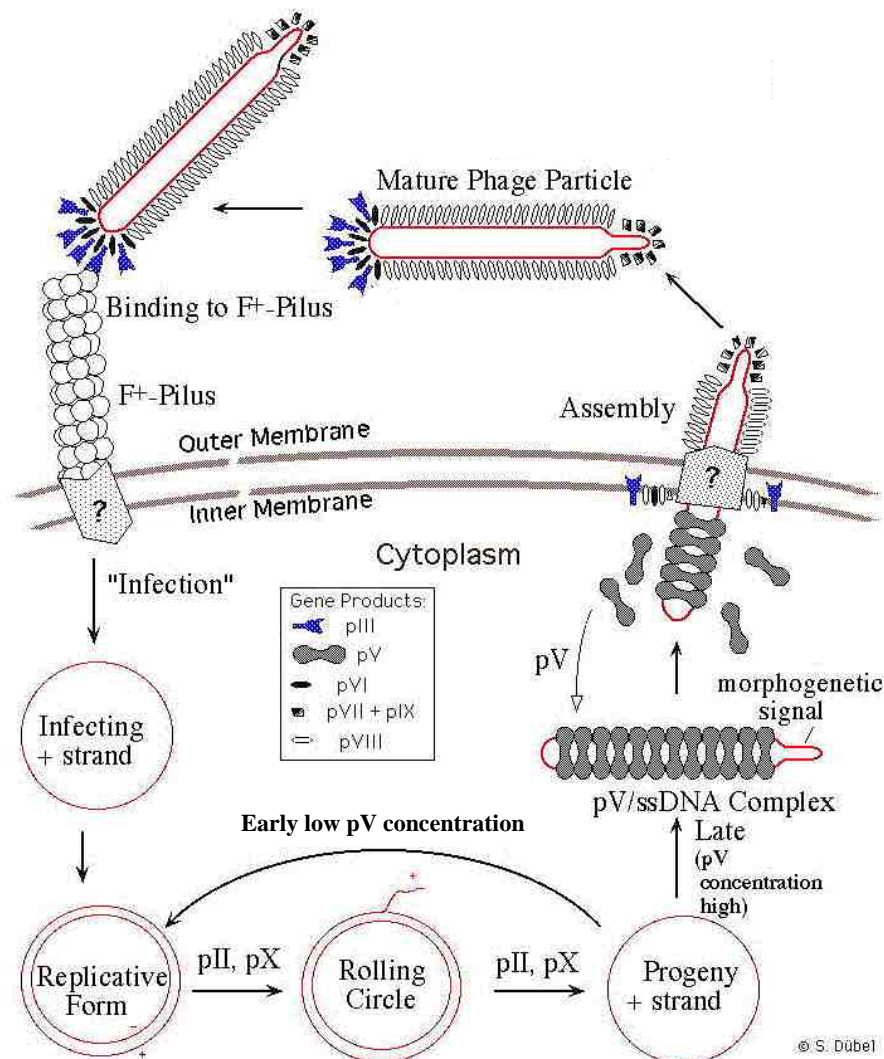


Fig. 6. The life cycle of filamentous phage. The phage infects the *E. coli* host cell through the sex pilus. The phage replicates its genome and produces the protein required for assembly. In a critical volume of one protein pV, phage ends the replication and translation then the assembly process starts (Creighton, 1999).

4.4.3 Phagemid vector system

Phagemid is a plasmid vector that contains only the fusion protein gene without other coat protein genes. Additionally, phagemid also contains a phage-derived origin of replication and antibiotic-resistance marker for selection and propagation as with other plasmids. Cells transfected with phagemid are superinfected with a helper phage. The helper phages provide all of the phage-derived proteins including the coat proteins that encapsulate the phagemid genome and enzymes required for phage replication. VCSM13 and KM13 are the most commonly used helper phages, which their packaging-signal is defective.

4.4.4 pIII used for display

pIII, existing five copies at the end of phage particle, is commonly used as a coat protein for phage display. pIII is more tolerant to substantial insertions, compared to pVIII, although the infectivity of phage can be reduced. The phage produced type 3+3 phagemid systems (Fig. 7) could potentially display from 0 to 4 or 5 copies of wild-type pIII on their surface. Two types of pIII, wild type from helper phage and fusion protein from phagemid, compete for incorporation into the virion during phage assembly in the bacterial inner membrane. The type 3+3 phagemid vector system is smaller than phage vector; thus, it is easier to

maintain and propagate (Mackenzic & To, 1998). Moreover, bacterial transformation efficiency is higher with phagemid vector than phage vector (Mackenzic & To, 1998), it can result the large library diversity. Furthermore, monovalent display can select based on pure affinity therefore the high affinity can be selected from type 3+3 phagemid system (Clackson & Lowman, 2004).

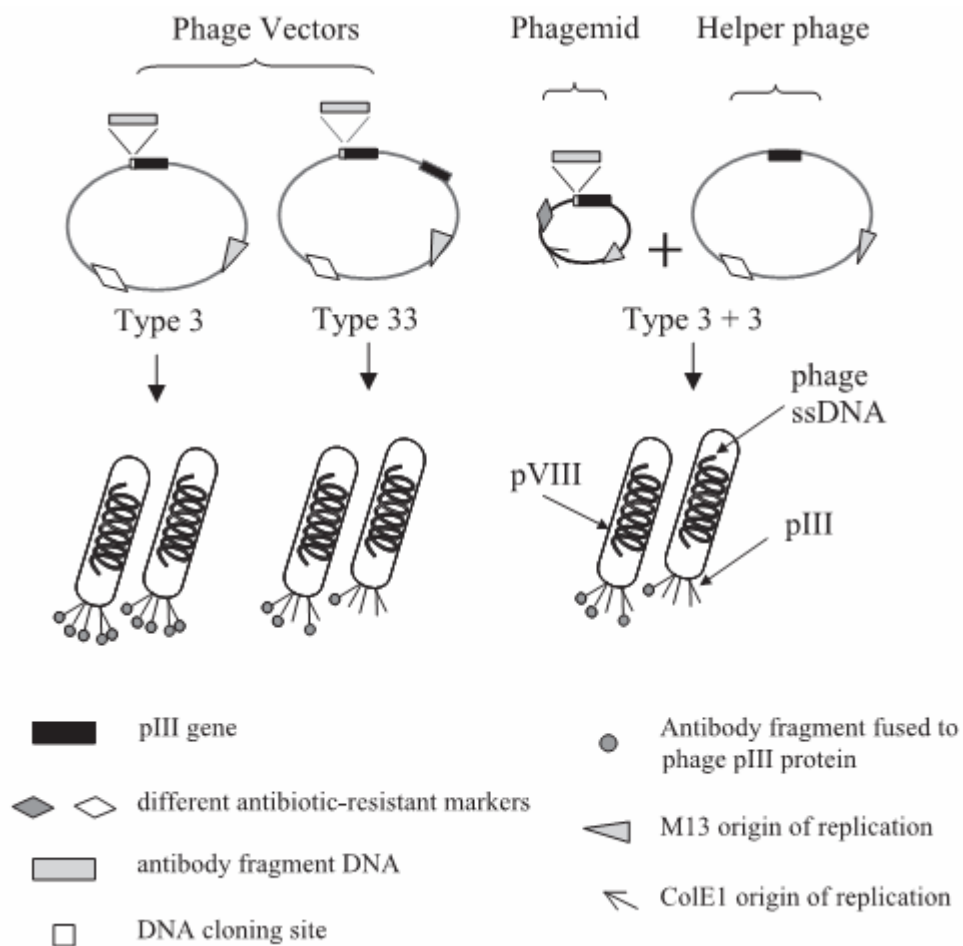


Fig. 7. The various vector system that used to display the antibody or peptide to the minor coat protein pIII (Yau, 2003).

4.4.5 Tomlinson I+J library

Tomlinson I+J library, is the naïve library or non-immune library that was developed by Greg Winter and colleagues (Wildt et al., 2000). This library exists over 100 million different scFv genes, and was cloned in a ampicillin resistant phagemid vector, pIT2 (Fig. 8). Tomlinson I and J are respectively based on DVT and NNK degenerative codon, which incorporate at position in the antigen binding site (CDR1 and CDR2). N represents a mixture of the 4 possible nucleotides A, C, G, and T; K is a mixture of G and T; D is a mixture of A, G and T; V is a mixture of A, C, and G. These degenerative codons can make high diversity of the libraries.

The scFv fragments in this library comprise of V_H and V_L domains, which are linked together by a flexible glycine-serine linker and expressed on protein pIII. The scFv antibody can be selected from “biopanning”. After each round of panning, the ratio of specific to non-specific binders increases following the number of panning. The monoclonal scFv can be screened by ELISA, and then used for further analysis, such as DNA sequencing, the diversity of selected clones, expression, and purification. The scFv in Tomlinson I and J library bind to the protein A and L, either of these secondary reagents can be used for

detection of scFv antibody. Moreover, the scFv antibody can also be detected and purified by c-myc and 6X histidine tag.

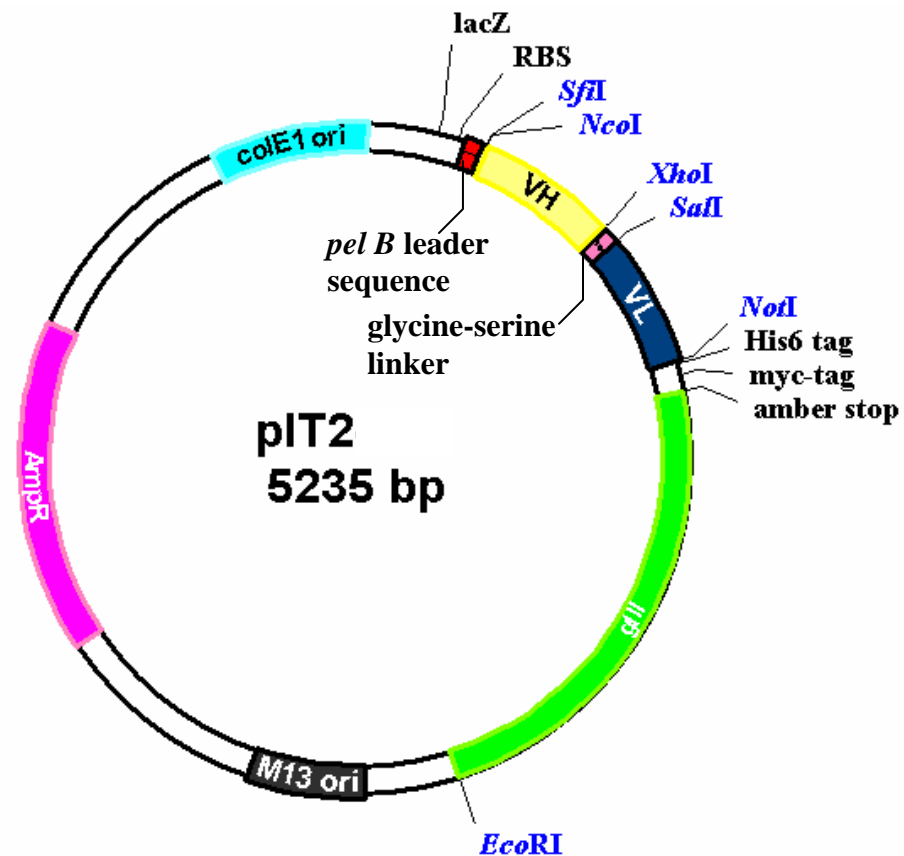


Fig. 8. The vector map of pIT2. The *pelB* leader directs secretion of scFv-pIII fusions to the periplasm. The scFv are clones into the vector at *SfiI*-*NotI* cloning site. The c-myc and 6x histidine tag are used to detect and purified of antibody. The amber stop codon allows simple swithching between displayed scFv and secreted native scFv (Clackson & Lowman, 2004).

Chapter II

Materials and methods

1. Affinity selection of single-chain Fv antibody (scFv antibody) specific to prostate specific antigen

1.1 Preparation of electrocompetent cell *Escherichia coli* (*E. coli*)

The stock culture of *E. coli* strain ER2738 or XL1-blue was streaked on the LB agar plate supplemented with 10 µg/mL tetracycline, and incubated at 37°C for overnight. Each colony was cultured in 5-mL SB or 2xTY medium, supplemented with 10 µg/mL tetracycline, incubated at 37°C, and shaken at 200 rpm for 16-18 hours. Five hundred milliliters of culture was added into 2-L flasks containing 500 mL SB or 2xTY medium, without adding tetracycline. The culture was grown until the OD₆₀₀ reaches 0.8 to 0.9. Then, the culture was transferred to 500-mL centrifuge bottles, 4°C pre-chilled on ice for 15 minutes. The culture was centrifuged at 4,500 rpm, 4°C for 15 minutes, and the supernatant was

removed. For washing, the bacterial pellet was resuspended in 250 mL of 4°C pre-chill 10% (v/v) glycerol and centrifuged at 4,500 rpm 4°C for 15 minutes. The supernatant was discarded, and the pellet was additionally washed for 3 times as described previously. After that, the pellet was resuspended with 2 mL of 10% glycerol and aliquoted 100 µL into each microcentrifuge tubes that were placed in the dried ice using snipped-off end of pipette tip, and the competent cells were stored at -80°C for competency test. The competency test was determined following Barbas protocol, and the acceptable competency should be above 1×10^9 colonies/µg plasmid (Barbas, 2001). Furthermore, the test for contamination of phagemids and helper phage are required for the newly prepared electrocompetent cells.

1.2 Preparation of helper phage

The VCSM13 helper phage (Stratagene, USA) containing kanamycin resistance gene was used to reinfect into the bacteria existing phagemid. One single plaque of VCSM13 was picked to infect 5 mL of pre-cultured *E. coli* strain ER2738 that OD₆₀₀ reaches 0.8-0.9, shaken at 200 rpm, and incubated at 37°C for 2 hours. The infected culture was transferred to 500 mL of pre-warmed SB medium supplemented with 70 µg/mL kanamycin and incubated overnight with shaking at 37°C. The

overnight-culture was centrifuged at 6,000 rpm, 4°C for 15 minutes. The supernatant was transferred to a 500-mL centrifuge bottle containing 4% (w/v) PEG-8000 and 3% (w/v) NaCl, placed on ice for 30 minutes, and centrifuged at 7,500 rpm 4°C for 20 minutes. The phage pellet was resuspended with 2 mL of 10% glycerol in PBS containing 1 % BSA, transferred into microcentrifuge tube, and centrifuged at 12,000 rpm 4°C for 5 minutes. The supernatant was filtered through a 0.2 µm filter and kept at -80°C. The phage titer was determined by plaque-forming assay.

1.3 Plaque-forming assay

One microliter of each 10-fold serial dilution of phage (10^{-6} , 10^{-7} , and 10^{-8}) was used to infect 50 µL of pre-cultured *E. coli* strain ER2738 or XL1-blue and incubated at room temperature for 15 minutes. Three milliliters of liquefied LB top agar (50°C) was added, mixed well, and poured on the plain LB agar plate. The plates were incubated overnight at 37°C. The plaque numbers were counted and calculated for the phage titer.

1.4 Library reamplification

Tomlinson I + J library, obtained from MRC laboratory of molecular biology and the MRC centre for protein engineering, was

E. coli existing phagemid stock. The phagemids in *E. coli* were reamplified by helper phage and precipitated phage to keep as phage library stock. Five microliters of phage library was used to infect 50 mL of *E. coli* strain ER2738 culture (OD₆₀₀ is approximately 0.8-0.9), incubated at room temperature for 15 minutes in the dark area. Ten microliters of 100 mg/mL ampicillin was added to the infected culture for selection of *E. coli* containing phagemid. To determine the size of library, the titer of phage infection can be determined by spreading an amount equivalent to 1 μ L and 10 μ L of 10⁻⁴ dilution of the infected culture on LB agar supplemented with 100 μ g/mL ampicillin, and the plates were incubated overnight at 37°C. The colonies were calculated the number of transformants by multiplying the number of colonies by the culture volume and dividing by the plating volume. After 200 rpm shaking at 37°C for 1 hour, the infected 50-mL culture was added with 15 μ L of 100 mg/mL ampicillin, an additional shaking for 1 hour. The culture was added with 10¹²-10¹³ pfu/mL of VCSM13 helper phage and 148 mL of 37°C-prewarmed SB medium with 70 μ L of 100 mg/mL ampicillin and 300 μ L of 5 mg/mL tetracycline, additional culturing for 2 hours. Following, the culture was supplemented with 280 μ L of 50 mg/mL kanamycin and shaken overnight at 37°C. The overnight-culture was centrifuged at 7,500 rpm at 4°C for 20 minutes, and the supernatant was

precipitated by adding 8 g (or 4% w/v) of PEG-8000 (Bio Basic INC, Canada) and 4 g (or 3% w/v) of NaCl. The phage pellet was resuspended with 2 mL of PBS containing 1% BSA and freshly used for panning process.

1.5 Panning

The microtiter plate's well (Costar EIA/RIA 96-well half area, USA) was coated with 200 ng PSA (Chemicon, USA) in 25 μ L of PBS buffer and incubated overnight at 4°C. In round 1-2 of panning, the PSA was coated for 2 wells, and in round 3-5 the PSA was coated for 1 well. Then, the coating reagent was removed, the well was blocked with 170 μ L of PBS containing 3% BSA, and incubated at 37°C for 2 hours. The blocking reagent was removed, and 50 μ L of freshly prepared phage library was added into the well for phage binding. After 2 hours of binding at room temperature, the phage pools were removed, and then the wells were vigorously pipetted 10 times with 170 μ L of washing buffer (PBS buffer containing 0.1% Tween20[®]), and waited for 5 minutes between each washing. The washing buffer was discarded and the washing steps were repeated 5 times in the first round and 10 times in the subsequent round. The washing step can eliminate non-specific binding of phage. The bound phages were eluted by adding 50 μ L of 10 mg/mL

trypsin in PBS and incubated at 37°C for 30 minutes. In the infection step, the trypsinized phages were vigorously pipetted for 10 times, transferred to 2 mL of pre-cultured *E. coli* strain ER2738 (OD₆₀₀ is approximately 0.8-0.9), and incubated in the dark at room temperature for 15 minutes. The infected culture was added with 6 mL of 37°C pre-warmed SB medium supplemented with 1.6 µL of 100 mg/mL ampicillin and 12 µL of 5 mg/mL tetracycline. The output titer was determined by following the method in titer determination. After 200 rpm shaking at 37°C for 1 hour, the culture was added with 2.4 µL of 100 mg/mL ampicillin, and further shaken for 1 hour. Then, the culture was superinfected with 1 mL of 10¹²-10¹³ pfu/mL of VCSM13 helper phage and transferred to 91 mL of 37°C pre-warmed SB medium supplemented with 46 µL of 100 mg/mL ampicillin and 184 µL of 5 mg/mL tetracycline. After 200 rpm shaking 37°C for 2 hours, the culture was added with 140 µL of 50 mg/mL kanamycin, shaken at 200 rpm, and incubated overnight at 30°C. The overnight-culture was centrifuged at 7,500 rpm at 4°C for 15 min, and the supernatant was transferred to new centrifuge bottle containing 4% w/v of PEG-8000 and 3% w/v NaCl for phage precipitation. The supernatant was placed on ice for 30 minutes and centrifuged at 12,000 rpm 4°C for 5 minutes. The phage pellet was resuspended with 2 mL of PBS containing 1% BSA and freshly used for panning in subsequent round.

1.6 Titering determination

The titer of eluted phage was determined by diluting 2 μL of the infected culture in 200 μL of SB medium and various amount of the diluted culture (1, 10, and 100 μL) were plated on LB agar supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin. The plates were incubated overnight at 37°C. The number of output colonies was used to calculate the number of transformants by multiplying the number of colonies by the culture volume and dividing by the plating volume.

1.7 Polyclonal phage ELISA

The wells of microtiter plate were coated with 200 ng PSA in PBS overnight at 4°C. The coating reagent was removed, and the wells were blocked with 3% BSA in PBS at 37°C for 1 hour. The PEG-precipitated phage solution of each rounds were diluted to two-fold in 3% BSA in PBS. The blocking reagent was removed, and the wells were added with the diluted phage solution and incubated at 37°C for 2 hours. The plate was washed with distilled water for 10 times, added with 1:5000 of HRP-conjugated anti-M13 antibodies (Amersham, UK) in 3% BSA in PBS, and incubated the microtiter plate at 37°C for 1 hour. The plate was washed as described before, and then added with the ABTS (Fermestas, USA) peroxidase substrate (as shown in the appendix). The reaction was

incubated at room temperature for 30 minutes and the colour intensity was measured at 405 nm by microplate-reader (Biohit Plc, Finland). The 3% BSA in PBS coated wells was used as a negative control. This experiment was performed in duplicate.

1.8 scFv expressing ELISA

The output-colonies in the panning round that showed the positive signal in phage ELISA were randomly selected. The colonies were cultured in 2 mL SB medium and on LB agar plate (used as a master plate) supplemented with 100 µg/mL ampicillin. The culture was grown at 37°C and shaken at 200 rpm, until the medium reaches the appropriate turbidity (OD_{600} is approximately 0.5). Subsequently, the antibody expression was induced with 1 mM IPTG (Fermentas, USA) final concentration, and the culture was grown additionally at 30°C for 24 hours. The overnight-culture was centrifuged at 4,000 rpm 4°C for 15 minutes. The supernatant of culture was used in ELISA.

The microtiter plate (Costar EIA/RIA 96-well half area, USA) was coated with 200 ng of PSA in 25 µL PBS, the plate was incubated overnight at 4°C. The coating reagent was removed, the wells were blocked with 170 µL of 3% BSA in PBS and incubated at 37°C for 2 hours. Fifty microliters of 1:2 supernatant in 3% BSA in PBS was added

to the wells after removing the blocking reagent. The plate was incubated at room temperature for 2 hours. The plate was washed 10 times by distilled water, then added with 50 μ L of 1:2,500 dilution of mouse anti-6x histidine tag antibody as a primary antibody (Amersham, UK), and incubated at room temperature for 1 hour. The plate was washed as described before, added with 50 μ L of 1:2,500 dilution of HRP-conjugated goat anti-mouse antibody as a secondary antibody, and incubated at room temperature for 1 hour. The plate was washed 10 times with distilled water, and then 50 μ L of ABTS peroxidase substrate was added. The ELISA plate was incubated at room temperature in the dark area for 30 minutes. The colour intensity was measured at 405 nm by microplate-reader (Biohit Plc, Finland). The 3% BSA binding was used as a negative control.

1.9 Diversity analysis of positive clones

The positive colonies determined by scFv ELISA were analyzed the diversity of these clones. The colonyPCR was performed to amplify the scFv fragment using LMB3 (forward) and pHENseq (reverse) primers, then the PCR-product (900 bp) were digested with restriction enzyme and analyzed DNA fingerprint pattern on agarose gel. Each colony was picked, resuspended into the PCR-tube, and added a PCR master mix

containing 2 μL of 10x PCR buffer, 2 μL of 2.5 mM dNTP, 1.6 μL of 25 mM MgCl_2 , 20 pmole of the primers, 0.25 μL of *i-Taq*TM DNA polymerase (5 units/ μL) (iNtRON, Korea), nuclease-free water to a final volume of 20 μL per reaction. PCR-tube was placed into a Thermal cycler (Biometra, Germany) using the following condition:

94°C for 2 minutes

Followed by 30 cycles of

94°C for 15 seconds

56°C for 30 seconds

72°C for 2 minutes and continued 72°C for 10 minutes

The PCR-product was digested with *AluI*, *BstNI*, *AluI/BstNI*, *MseI/BstNI*, *MseI/HhaI*, and *MseI/AluI* master mix containing 2.4 μL of 10x NEB buffer (Biolabs, UK), 0.24 μL of 10 mg/mL BSA solution, 2.5 units of enzyme (Biolabs, UK), and nuclease-free water to a final volume of 16 μL per reaction. the *AluI* and *BstNI* digestions were respectively performed at 37°C and 60°C for 3 hours. The clone diversity revealed the digested products on 10 % polyacrylamide gel electrophoresis (PAGE) in 1xTBE buffer with 50-base pairs DNA ladder for references marker. DNA bands were separated at 60 volts for 1.5 hours using vertical electrophoresis apparatus.

1.10 Sequence analysis of selected clones

The positive clones were cultured for 18 hours and extracted the plasmid following by HiYield Plasmid Mini Kit (Real Biotech Corp, USA). The purified phagemid DNA was sequenced using LMB and pHENseq primers (see appendix) for scFv fragment and ompseq and pelseq primers (see appendix) for Fab fragment.

2. Transformation of the scFv construct into Fab construct

The human variable regions (V_H and V_K) of scFv antibody that could specifically bind to PSA were amplified with primers including part of *SfiI* site, C_K , pelB-leader, and C_{H1} (Fig. 9). The C_{H1} and C_K were amplified from pComb3XTT phagemid vector. The PCR overlap extension was performed to combine variable region of scFv clone to constant region from pComb3XTT vector, and then combined the light chain to the heavy chain. These steps provided Fab fragments, which were clone to pComb3XSS-dIII.

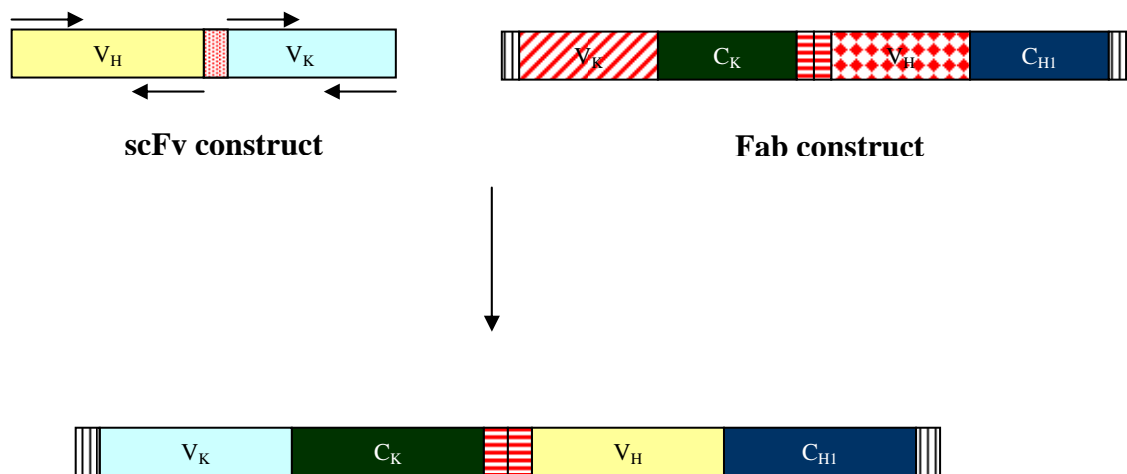





Fig. 9 Transformation of scFv construct to Fab construct. The variable regions of scFv specific to PSA were amplified and combined to human constant regions from pComb3XTT vector.  : *Sfi*I cloning site;  : pelB leader sequence;  : glycine-serine linker.

2.1 Amplification of variable regions of scFv construct and human constant regions from pComb3XTT vector

2.1.1 Amplification of V_K

The V_K of the scFv antibody fragment was amplified from plasmid of scFv clone (TL1) using TLVKF and TLVKR primers (as shown in the appendix). The TLVKF primer included part of *Sfi*I site and the TLVKR included part of C_K sequence. The reaction of V_K amplification containing the following components:

TL1 Plasmid	100 ng
TLVKF (forward primer)	60 pmole
TLVKR (reverse primer)	60 pmole
10x PCR buffer	10 μL
2.5 mM dNTP	10 μL
50 mM MgCl ₂	4 μL
High-fidelity Pyrobest DNA polymerase (5 units/μL)	0.2 μL
q.s. water to	100 μL

The PCR reaction was performed by a Thermal cycler (Biometra, Germany) using the following condition:

94°C for 30 seconds

Followed by 30 cycles of

94°C for 15 seconds

56°C for 30 seconds

72°C for 90 seconds and continued at 72°C for 10 minutes

The PCR products of V_K was resolved on 1.5% agarose gel and purified by Nucleospin[®] Extract II PCR clean-up gel extraction (MACHEREY-NAGEL, Germany). The target band is 324 base pairs. The purified PCR products were quantified by $OD_{260/280}$.

2.1.2 Amplification and Site-directed mutagenesis of V_H

The V_H was amplified by PCR overlap extension and introduced site-specific mutation at nucleotide 175 of V_H DNA fragment, changing TAG (amber stop codon) to GAG (glutamic acid). The mutagenesis was performed by specially designed oligonucleotide primers, VHMF and VHMR primers (as shown in the appendix). The VHMF and VHMR have a complementary sequence, making overlap site on PCR-product using these primers (Fig. 10). For the first PCR reaction, two fragment of V_H was amplified independently V_{H1} (upstream of V_H fragment) and V_{H2} (downstream of V_H fragment) using TLVHF:VHMR and VHMF:TLVHR (Fig. 10) pair of primers, respectively. The reaction of V_{H1} and V_{H2} amplification contained the following components:

TL1 Plasmid	100 ng
TLVHF or VHMF (forward primer)	60 pmole

VHMR or TLVHR (reverse primer)	60 pmole
10x PCR buffer	10 μ L
2.5 mM dNTP	10 μ L
50 mM MgCl ₂	4 μ L
High-fidelity Pyrobest DNA polymerase (5 units/ μ L)	0.2 μ L
q.s. water to	100 μ L

The PCR reaction was performed by a Thermal cycler (Biometra, Germany) using the following condition:

94°C for 30 seconds

Followed by 30 cycles of

94°C for 15 seconds

56°C for 30 seconds

72°C for 60 seconds and continued at 72°C for 10 minutes

The PCR products of V_H1 and V_H2 were resolved on 1.5% agarose gel and purified by Nucleospin[®]Extract II PCR clean-up gel extraction (MACHEREY-NAGEL, Germany). The target bands were 201 and 210 base pairs, respectively. The purified PCR products were quantified by OD_{260/280}.

The two fragments were fused together by PCR overlap extension using TLVHF and TLVHR. The overlap extension of V_H1 and V_H2 contained the following components:

V _H 1	100 ng
V _H 2	100 ng
TLVHF (forward primer)	60 pmole
TLVHR (reverse primer)	60 pmole
10x PCR buffer	10 µL
2.5 mM dNTP	10 µL
50 mM MgCl ₂	4 µL
High-fidelity Pyrobest DNA polymerase (5 units/µL)	0.2 µL
q.s. water to	100 µL

The PCR reaction was performed by a Thermal cycler (Biometra, Germany) using the following condition:

94°C for 30 seconds

Followed by 30 cycles of

94°C for 15 seconds

58°C for 30 seconds

72°C for 90 seconds and continued at 72°C for 10 minutes

The PCR products of V_H was resolved on 1.5% agarose gel and purified by Nucleospin[®] Extract II PCR clean-up gel extraction (MACHEREY-NAGEL, Germany). The target band is 354 base pairs. The purified PCR products were quantified by OD_{260/280}.

2.1.3 Amplification of human constant region

The C_K-pelB fragment and heavy chain constant region (C_{H1}) were amplified from pComb3XTT using HKC-F and lead-B primers and HIgGCH1F and dpseq primers (Fig. 11), respectively. The reaction of amplification contained the following components:

pComb3XTT Plasmid	100 ng
HKC-F or HIgGCH1F (forward primer)	60 pmole
Lead-B or dpseq (reverse primer)	60 pmole
10x PCR buffer	10 µL
2.5 mM dNTP	10 µL
25 mM MgCl ₂	8 µL
High-fidelity Pyrobest DNA polymerase (5 units/µL)	0.2 µL
q.s. water to	100 µL

The PCR reaction was performed by a Thermal cycler (Biometra, Germany) using the following condition:

94°C for 30 seconds

Followed by 30 cycles of

94°C for 15 seconds

56°C for 30 seconds

72°C for 90 seconds and continued at 72°C for 10 minutes

The PCR products of C_{H1} and C_K-pelB were resolved on 1.5% agarose gel and purified by Nucleospin[®]Extract II PCR clean-up gel extraction (MACHEREY-NAGEL, Germany). The target bands are 336 and 411 base pairs. The purified PCR products were quantified by OD_{260/280}.

2.2 PCR-overlap extension of light chain and heavy chain

The PCR-overlap extension was used to combine V_H to C_H and V_K to C_K using specified primers LeadV_H and dpseq primers and RSC-F and LeadB primers (Fig. 11), respectively. The resulting of the heavy chain (V_H to C_H) combined to the light chain (V_K to C_K-pelB) are approximately 690 and 753 base pairs, respectively. The reaction of amplification contained the following components:

Purified variable region fragment	100 ng
Purified constant region fragment	100 ng
RSC-F or Lead-V _H (forward primer)	60 pmole
Lead-B or dpseq (reverse primer)	60 pmole
10x PCR buffer	10 µL
2.5 mM dNTP	10 µL
50 mM MgCl ₂	4 µL
High-fidelity Pyrobest DNA polymerase (5 units/µL)	0.2 µL

q.s. water to 100 μ L

The PCR reaction was performed by Thermal cycle (Biometra, Germany) using the following conditions:

94°C for 30 seconds

Followed by 15 cycles of

94°C for 15 seconds

56°C for 15 seconds

72°C for 2 minutes and continued at 72°C for 10 minutes

The PCR products of heavy chain and light chain were resolved on 1% agarose gel electrophoresis and purified by Nucleospin[®] Extract II PCR clean-up gel extraction (MACHEREY-NAGEL, Germany). The target bands are 690 and 753 base pairs, respectively. The purified PCR products were quantified with OD_{260/280}.

2.3 Amplification of Fab fragment

The PCR-overlap extension is the combination of the light and the heavy chain using RSC-F and dp-EX primers (Fig. 11). The reaction contained the following components:

Purified light chain	100 ng
Purified heavy chain	100 ng
RSC-F (forward primer)	60 pmole

dp-EX (reverse primer)	60 pmole
10x PCR buffer	10 μ L
2.5 mM dNTP	10 μ L
50 mM MgCl ₂	4 μ L
High-fidelity Pyrobest DNA polymerase (5 units/ μ L)	0.2 μ L
q.s. water to	100 μ L

The PCR reaction was performed by a Thermal cycler (Biometra, Germany) using the following condition

94°C for 30 seconds

Followed by 10 cycles of

94°C for 15 seconds

56°C for 30 seconds

72°C for 3 minutes and continued at 72°C for 10 minutes

The pool of PCR products were resolved on 1% agarose gel electrophoresis and purified by Nucleospin[®] Extract II PCR clean-up gel extraction (MACHEREY-NAGEL, Germany). The target band is 1,443 base pairs. The purified PCR products were quantified with OD_{260/280}.

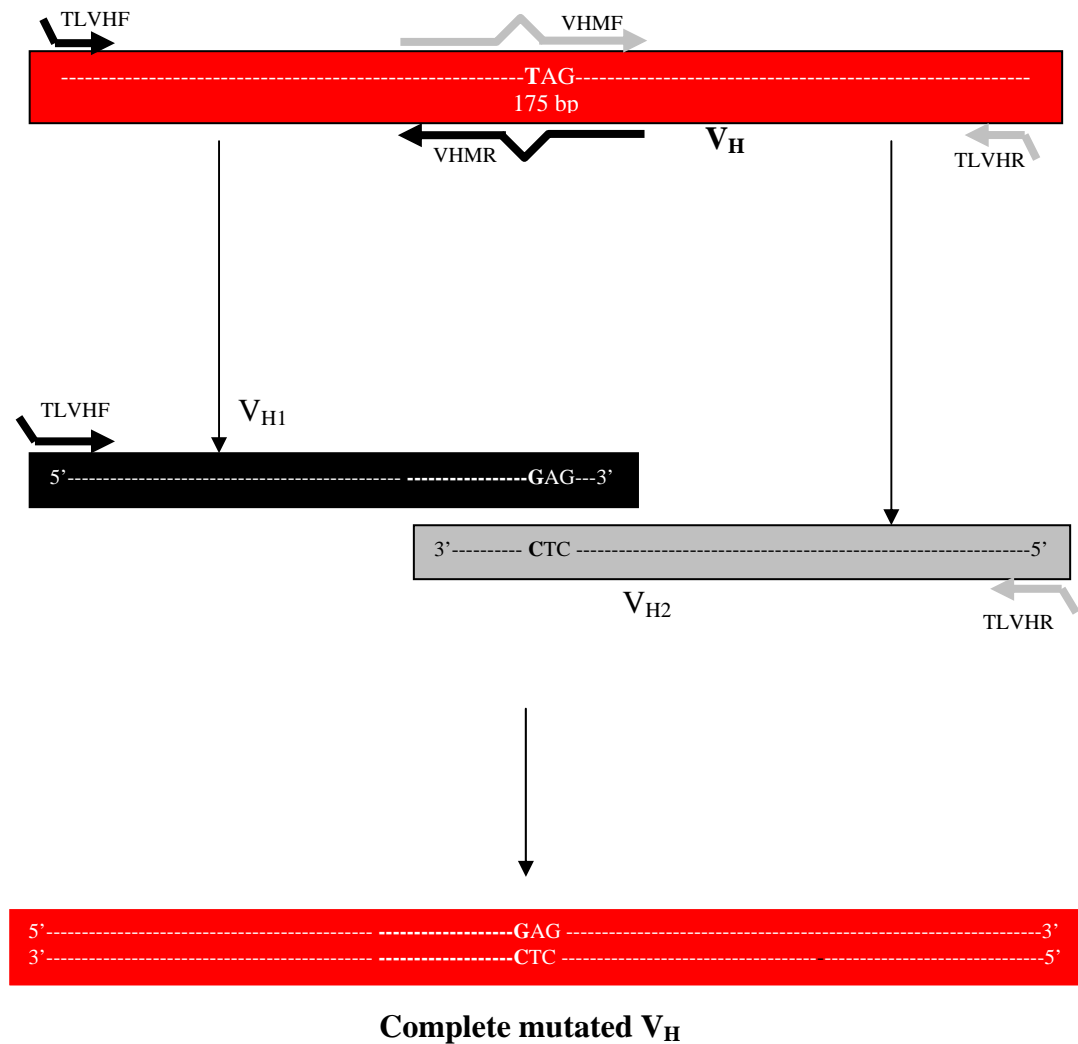


Fig. 10. A diagram shows the amplification of V_H region using PCR overlap extension. The site-directed mutagenesis using V_HMF and V_HMR primers introduces a mutation from T to G at nucleotide 175.

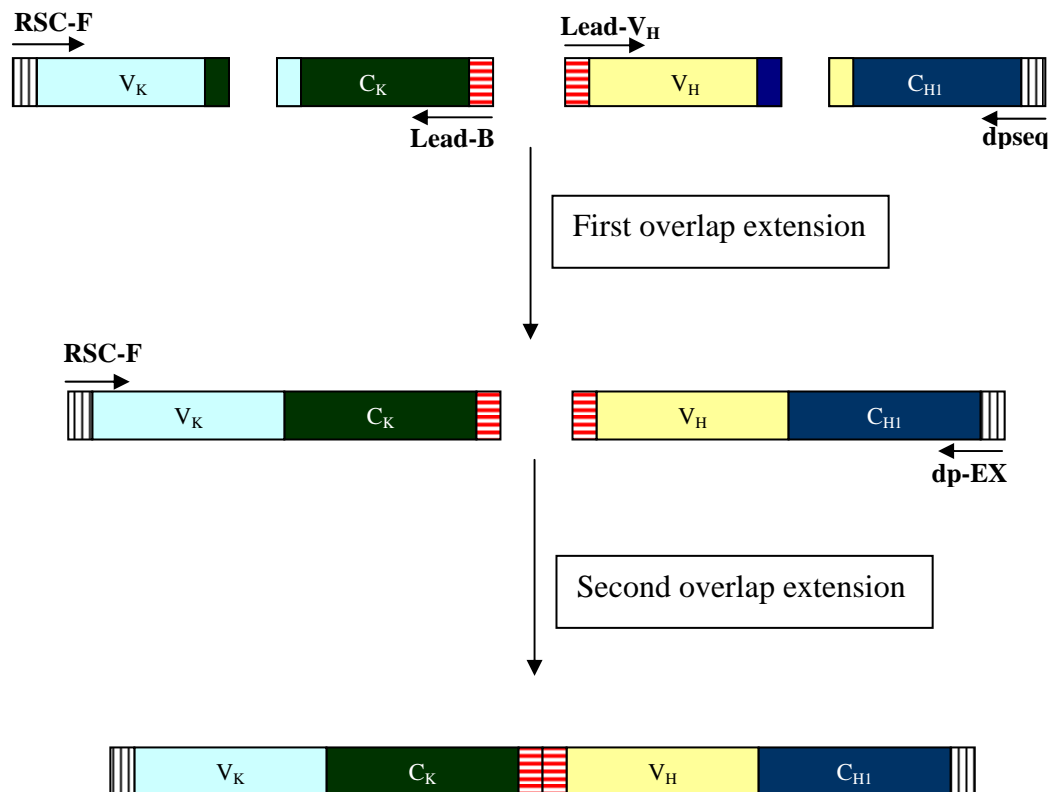


Fig. 11. A schematic depicts the steps of overlap extension using specific primers for Fab construct. V_K : variable region of light chain; V_H : variable region of heavy chain; C_K : constant region of light chain; C_{H1} : constant region of heavy chain; $\square\square\square$: *SfiI* cloning site; $\square\square\square$: pelB leader sequence.

2.4 Cloning of Fab into pComb3XSS-dIII vector

The Fab fragment and pComb3XSS-dIII (Fig. 12B) were digested by *Sfi*I restriction enzyme to clone the Fab gene to pComb3XSS-dIII vector obtained from Aflatoxin clone (Putkam, 2007), which the gene III encoding protein III of phage was deleted. Thus, the expression of Fab antibodies can be detected by either antibodies specific to human constant region or 6x histidine tag. The Fab fragments were directly cloned into the phagemid vector by *Sfi*I restriction site. The digestion reaction of the PCR-product contained the following components:

Purified Fab fragment	0.5 µg
10x Buffer G (Fermentas, USA)	10 µL
<i>Sfi</i> I (10 units/µL) (Fermentas, USA)	0.8 µL
q.s. water to	100 µL

The digestion reaction of pComb3XSS-dIII contained the following component:

pComb3XSS-dIII phagemid vector	1 µg
10x Buffer G (Fermentas, USA)	10 µL
<i>Sfi</i> I (10 units/µL) (Fermentas, USA)	0.6 µL
q.s. water to	100 µL

The digestion reactions were performed at 50°C for 3 hours, and then the Fab fragment (about 1,443 base pairs), the pComb3XSS-dIII vector (about

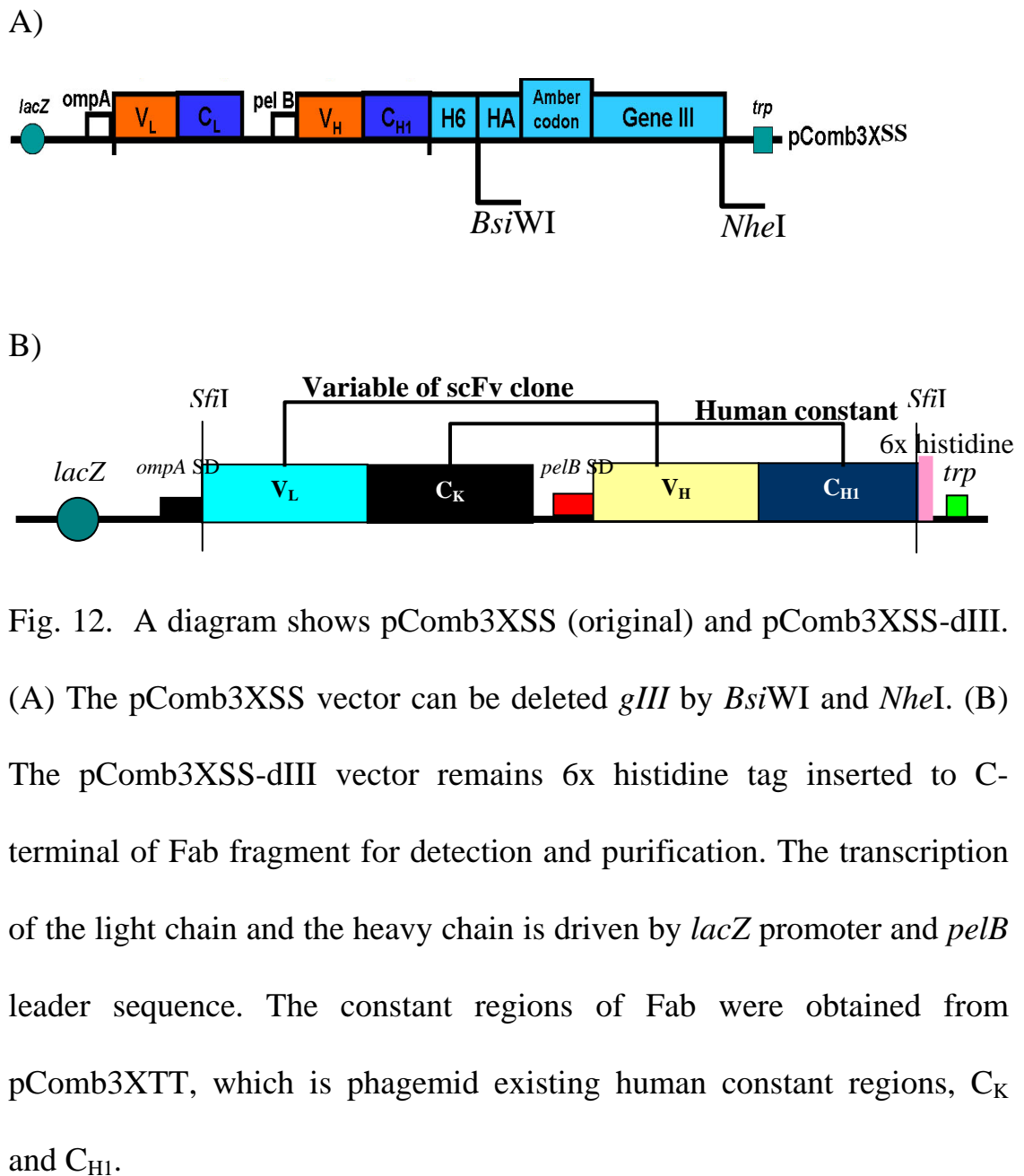
2,700 base pairs) and the stuffer (about 1,450 base pairs) were purified using 1% agarose gel and then followed by Nucleospin[®] Extract II. The purified products were quantified with OD_{260/280}.

The digested phagemid vector and Fab fragment were ligated and transformed to *E. coli* XL1-blue. The ligation reaction contained the following components:

The digested pComb3XSS-dIII vector	100 ng
The digested Fab fragment	100 ng
10x T4 DNA ligase buffer (Invitrogen, USA)	2 μ L
T4 DNA ligase (1 unit/ μ L) (Invitrogen, USA)	1 μ L
q.s. water to	20 μ L

The ligated product was incubated at room temperature overnight. One microliter of reaction was mixed to 50 μ L of electrocompetent XL1-blue cells and 4°C pre-chilled on ice for 15 minutes. Then, the mixture of DNA and electrocompetent cells was transferred to the pre-chilled 1 mm gap-Eppendorf[®] cuvette (Cole-Parmer, USA) and placed on ice for 2 minutes. The cuvette was electroporated by Eppendorf[®] electroporator (Cole-Parmer, USA) at 1.5 kV. The transformed cells were flushed with 3 mL of SOC medium, an additional 200 rpm shaking at 37°C for 1 hour. The culture was centrifuged and resuspended with 200 μ L of SOC, 10 and 100 μ L of culture was spreaded on LB agar supplemented with 100 μ g/mL.

The plates were incubated overnight at 37°C. The colonies were picked and validated for the inserted fragment by colony-PCR using RSC-F and TLVHR primers, resulting the target band 1,128 base pairs.



3. Production and Purification of antibody

3.1 Protein analysis with Western blotting

The supernatant and cell pellet of 24-hr IPTG-induced culture were monitored for protein expression using immunoblotting. One hundred microliters of cell pellet was lysed with 500 μ L of lysis buffer (0.06 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol). The lysed cell pellet was sonicated on ice at 40% amplitude and the samples were placed for a minute, additional sonicating for 4 times. The samples were centrifuged at 15,000 rpm at 4°C for 10 minutes. The supernatant were collected to analyze antibody by SDS-PAGE using 12% T polyacrylamide resolving gel and 4% T stacking gel. The samples were firstly mixed with lysis buffer and boiled for 5 minutes. The samples were then loaded on SDS-PAGE gel and resolved at 90 volts for 1.5 hours. The protein samples were transferred on Hybond™-LFP membrane (Amersham, UK) using wet transfer technique in 1x transfer buffer (1.5% (w/v) Tris-base, 1.8% (w/v) glycine and 10% (v/v) methanol). The buffer was cooled along 90 minutes using constant current 400 mA. The membrane was blocked with 3% BSA in PBS at room temperature for 1 hour. Then, the membrane was probed with 1:3,000 of mouse anti-6x histidine antibody (Amersham, UK), diluted by 1% BSA in PBST (0.05%

v/v Tween20 in PBS). The membrane was incubated at room temperature for 1 hour, and then it was washed 5 times with PBS containing 0.05% Tween20 in. After washing step, the membrane was probed with 1:5,000 of Cy3 goat anti-mouse IgG antibodies (Amersham, UK) in PBS containing 0.05% Tween20. Importantly, after this step the membrane should keep in the dark area. The membrane was then washed 5 times as described previously for 5 minutes and dried at 37°C for 1 hour. The fluorescence bands of 6x histidines tag protein were detected by Ettan™ DIGE Imager (Amersham, UK).

3.2 Affinity chromatography, protein G column

Five hundred milliliters of 24-hr IPTG-induced culture was centrifuged at 7,500 rpm, 4°C for 1 hour. The supernatant was transferred to a new centrifuge bottle and further centrifuged at 7,500 rpm, 4°C for 30 minutes. The supernatant was filtrated through 0.8, 0.45, and 0.2 µm filter membrane (Sartorius, Germany). Five hundred milliliters of filtrated supernatant was concentrated using Centricon® centrifugal filter unit (Millipore, USA), YM10 membrane (cut off 10 kDa). The Fab antibodies were purified from 10-mL of concentrated medium using 1-mL HiTrap® Protein G HP column (Amersham, UK). The column, performed by 50-mL syringe, was equilibrated with 10 column volumes (CV) of binding

buffer (phosphate buffer pH 7.4). After that, the concentrated medium was passed through the equilibrated column (0.5 mL/min) and the flow-through was collected in fraction tubes. The column was washed with 10 CV of binding buffer and the wash-out unbound was collected in fraction tubes. The elution of bound antibodies used 15 CV elution buffer (0.1 M glycine-HCl pH 2.7) and the eluted fractions were reserved in fraction tubes containing 90 μ L of neutralizing reagent (1 M Tris-HCl, pH 9.0). The eluted fractions were determined by ELISA, and others should also be analyzed to check the leakage of antibodies in binding and washing step. The positive fractions in ELISA were subjected the purity of antibodies using silver staining protocol. The purified positive fractions were combined together and determined the protein concentration by MicroBCATM assay (Pierce, USA).

3.3 Silver staining

The fractions of purified protein were monitored the purity using Sterling[®] rapid silver stain kit (National diagnostics, UK) on 12%T SDS-PAGE. The samples were mixed with sample buffer (0.06 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.025% bromphenol blue and 5% 2-mercaptoethanol), loaded, and resolved on 12% total acrylamide gel at 90 volts. After that, the gel was fixed with 100 mL standard mixture

(methanol: water: acetic acid; 5:5:1) at room temperature for 30 minutes and additionally fixed with 100 mL fixative solution (methanol: water: fixative reagent; 5:5:1) for 5 minutes. The gel was washed twice for 15 minutes with deionized water supplemented with 0.1% Tween20[®] and immersed into freshly prepared staining solution (50 mL of 1:2 diluted Reagent A in water and 50 mL of dissolved 2.8 g Reagent B in water). The protein bands will appear within 5-10 minutes.

3.4 Protein assay by MicroBCA

The protein assay was performed by MicroBCA[™] assay kit (Pierce, USA). Bovine serum albumin (BSA) standard solution (1 mg/mL) was diluted with water to 100, 200, 400, 600, 800 and 1000 ng/ μ L. Ten microliters of each standard dilutions and protein samples were added into a flat-bottom well microtiter plate (NUNC, Denmark) containing 40 μ L of water in duplicate. Fifty microliters of working reagent (A:B:C reagent; 25:24:1) was added to wells, the plate was incubated at 60°C for 45 minutes in hybridization oven. Subsequently, the plate was cooled down and the intensity colour was measured at 562 nm by microplate-reader (Biohit Plc, Finland).

The slope of the standard curve, plotted between an absorbance versus amount of standard proteins (1 mg/mL BSA), was used to calculate

the equation that was performed by Microsoft Office Excel version 2003.
The O.D. of samples should be in the range of the BSA standard curve.
The concentration of samples can be calculated from O.D. value in the equation from the standard curve.

Chapter III

Results

1. The in vitro selection of Tomlinson I + J scFv antibody (Panning)

The synthetic libraries, Tomlinson I + J scFv, were reamplified and used to select antibodies specific to PSA. The two wells of the microtiter plate were coated with 200 ng of PSA in each round. After binding of phage, the unbound phages were washed out, and then the bound phages were eluted and reinfected into the *E. coli* host to reamplify phage clones for the next round of panning. After five rounds of panning, the output titer gradually decreased from round 1 to round 3 and dramatically increased for 100 times higher in round 4 (Table 2). It means that non-specific binding phages existed in round 1 and 2. By increasing washing, the positive clones were selected in round 3 and enriched in round 4 and 5. The result showed that the clones specific to PSA were successfully enriched from the in vitro selection.

Table 2. The panning titers of the scFv against PSA using Tomlinson I+J library

Panning	Input ($\times 10^{10}$ CFU)	Output ($\times 10^4$ CFU)
Reamplification	1.2	-
I	10	34
II	6.5	2.4
III	15	0.8
IV	100	70
V	27	350

2. Analysis of selected antibodies

2.1 Polyclonal phage ELISA

The PEG-precipitated phage solutions from panning were used to monitor phages that displayed antibody specific to PSA using ELISA technique. The phages of interest could bind to PSA immobilized on ELISA plate; the unbound phages were washed out. Then, the bound phage clones were detected by mouse anti-M13 antibodies conjugated with HRP. The phage pools from round 4 and 5 showed the positive

signal (with PSA coated) that was measured at OD₄₀₅. In Fig. 13, the result showed that the O.D. values (subtracted from blank) of positive signal were about three times higher than that of the negative control (without PSA coated). The result indicated that non-specific binding phage existed in round 1 and 2. Increasing washing step in the subsequent round, the positive phage clones were selected in round 3 and enriched in round 4 and 5.

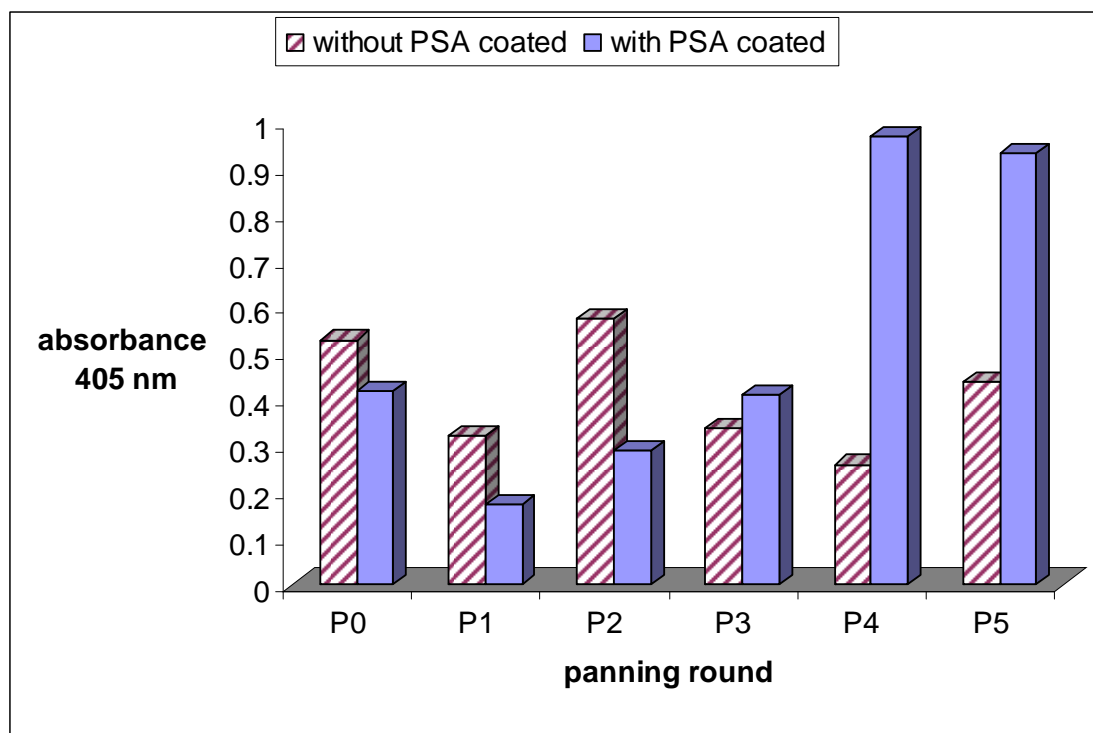


Fig. 13. The phage pool analysis using polyclonal phage ELISA. The bar graph depicted the absorbance OD₄₀₅ of ELISA following panning round (P0-P5). The positive signal represented by gray bars and the negative control represents on striped bars.

2.2 scFv expressing ELISA

Three hundred and 100 colonies from output plates of round 4 and 5, respectively, were screened using ELISA technique, each wells was coated with 200 ng of PSA, blocked, and bound with the supernatant of IPTG-induced culture. After washing, the bound antibodies were detected by using antibodies specific to mouse anti-6x histidine tag antibody and goat anti-mouse antibody conjugated with HRP as primary and secondary antibody, respectively. There were 10 positive clones (Fig. 14) from randomly screening 100 colonies of round 5. There was 1 positive clone (data not shown) from screening 300 colonies of round 4. However, the clone showed very low signal in ELISA; but, it was used to be the representative clone from round 4. Ten positive clones from round 5 show the high and low O.D. value. Nevertheless, the ten positive clones from round 5 have to analyze the DNA fingerprint pattern, which can prove the variation of those clones.

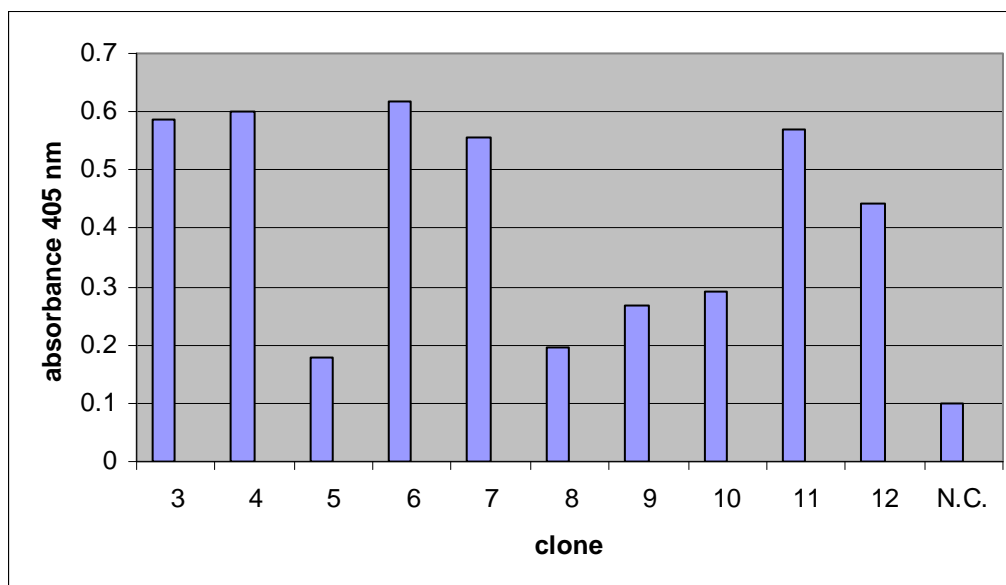


Fig. 14. The scFv antibody analysis using scFv expressing ELISA. The bar graph showed the absorbance (OD_{405}) of scFv expressing ELISA signal of clones number 3-12 of round 5. The absorbance values were the average of duplicate O.D. values. N.C. is the negative control competent cell ER2738.

2.3 Diversity analysis of positive clones

The 935 base pairs target band of positive clones obtained from scFv expressing ELISA were amplified (Fig. 15A) using LMB3 (specific to upstream of the V_H gene) and pHENseq (specific to downstream of the V_K gene) primers. The PCR-products were then digested by *AluI* (AG/CT), *BstMI* (CC/WGG), *AluI/BstNI*, *MseI/BstNI*, *MseI/HhaI*, and *MseI/AluI*. The digested PCR products were resolved on 10% PAGE stained by ethidium bromide and visualized under UV 340 nm. Ten clones from round 5 showed identical fingerprint patterns in every combination enzyme digestions (data not shown); thus, one clone was used as a representative clone from round 5. In Fig. 15B, TL1 (the representative from round 5) and TL3 (the representative from round 4) showed the same DNA fingerprint patterns. The results implied that there was no variation in all eleven selected clones from round 4 and 5; however, the sequence results can disclose the differences of those clones.

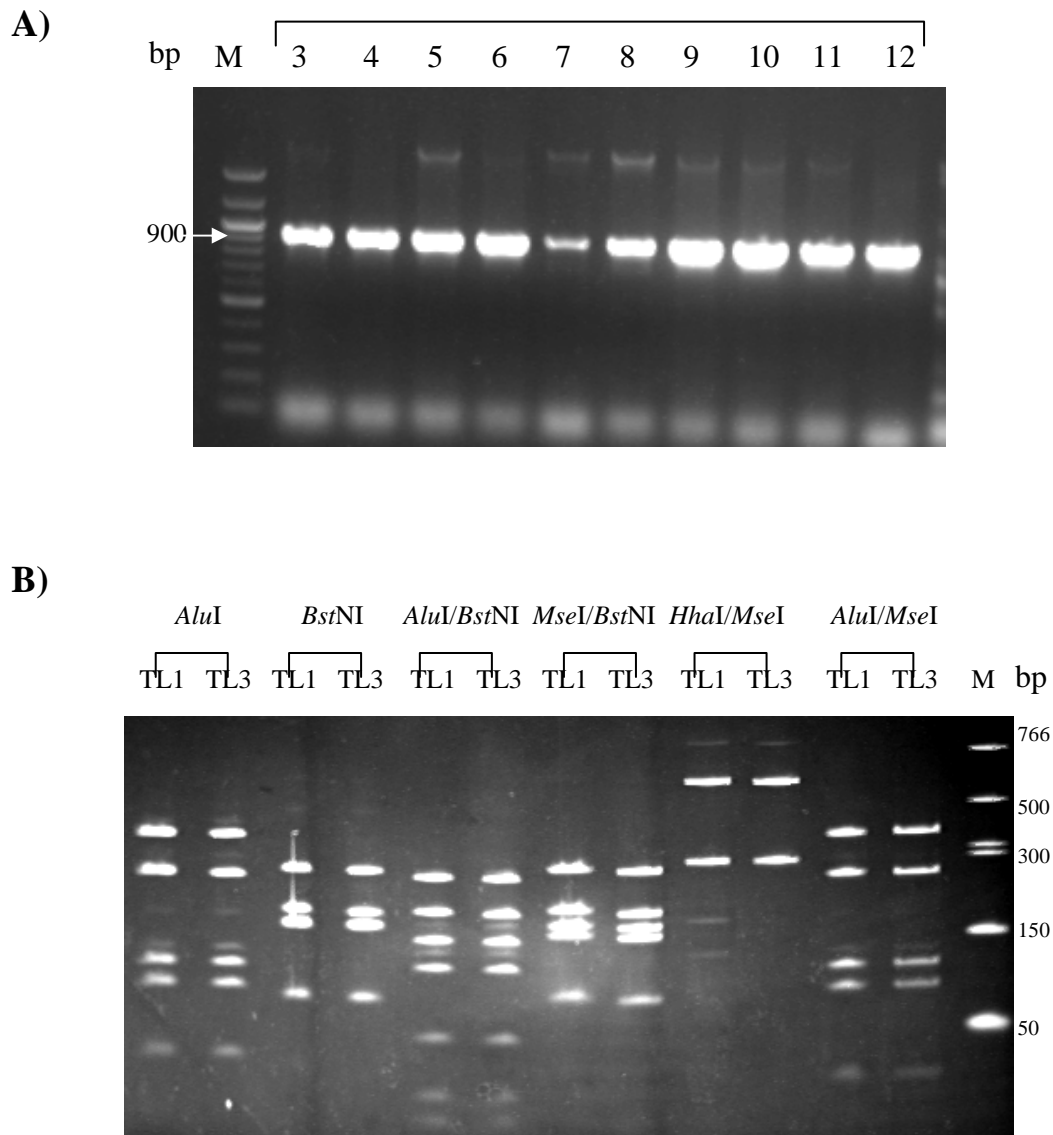


Fig. 15. The PCR amplification and the restriction analysis of scFv clones. (A) The 935 base pair of scFv clones was amplified by colony-PCR using LMB3 and pHENseq primers. (B) The DNA fingerprint of clone TL1 and TL3 showed the same pattern in all enzyme digestions.

2.4 Sequence analysis of scFv clones

Two clones, TL1 and TL3, were selected for DNA sequencing. The sequencing results showed that the two clones have 96% identity (10 codons differences on V_H and 5 codons differences on V_K). One nucleotide base of clone TL3 was found, making out-of-reading-frame in translation. Moreover, the antibody expression of clone TL3 was confirmed by ELISA and western blot; the results showed that there was no activity in ELISA and no antibody expression in western blot (data not shown). For clone TL1, one amber stop codon (TAG) was found within V_H fragment; thus, it could be problematic during translation in non-suppressor hosts (eg. TOP10 F⁺). Because the non-suppressor strain can read the stop codon on V_H fragment, resulting no scFv antibody expression.

Furthermore, the sequence result can be used to determine the V-D-J and V-J of V_H and V_K by IMGT/V-QUEST programme (Giudicelli, Chaume, & Lefrane, 2004). The data uncovered V-D-J (human V_H region) and V-J (human V_K region) that localized on locus group IGHV3-23, IGHD6-6, and IGHJ4 and IGKV1-39 and IGKJ1 group, respectively. Moreover, the data also revealed CDRs and frameworks of variable regions, the CDR regions showed that the majority of amino acid was neutral polar and non-polar group.

2.5 Expression and purification of soluble scFv

The scFv cloned into pIT2 phagemid vector was expressed by IPTG induction using the *E. coli* strain ER2738. The soluble scFv in medium was concentrated using Ultra centrifugation YM100 membrane (Amicon, USA). The concentrated medium was then purified by Ni²⁺ affinity chromatography. The purified fractions containing the recombinant scFv fragment were eluted by 20-500 mM imidazole with segmented gradient elution technique. The scFv antibodies were eluted in a range of 260-404 mM of imidazole (Fig. 17 and 18). The scFv fractions resolved on SDS-PAGE conditions showed a M_w of approximately 30 kDa (Fig. 16); however, it was not pure enough for further characterization (Fig. 17).

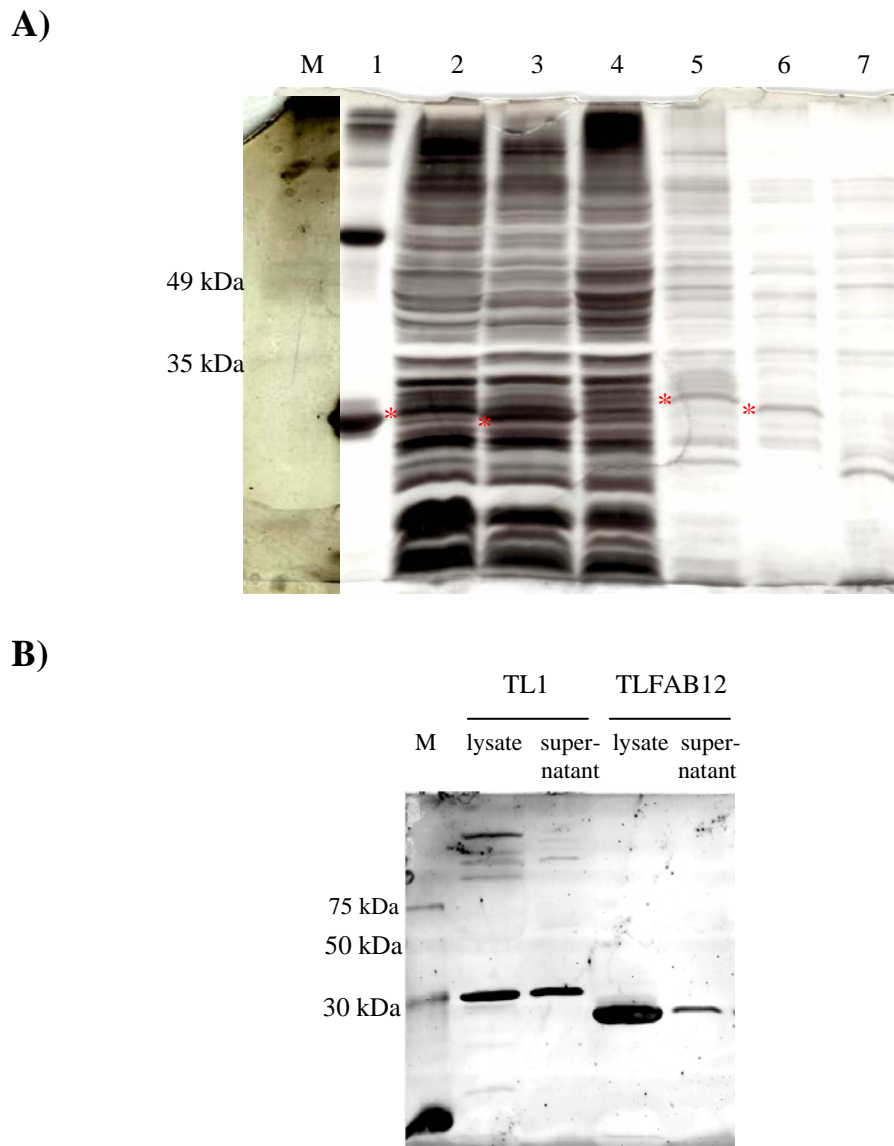


Fig. 16. The antibody expression of TL1 (scFv clone) and TLFAB12 (Fab clone). (A) The silver staining showed M: prestained marker, lane1: purified Fab containing BSA stabilizer protein, lane2: TL1 lysate, lane3: TLFAB12 lysate, lane4: ER2738 lysate (negative control), lane5: TL1 supernatant, lane6: TLFAB12 supernatant, lane7: ER2738 supernatant (*: indicates band of target proteins). (B) The immunoblotting analysis of clone TL1 and TLFAB12 lysate and supernatant.

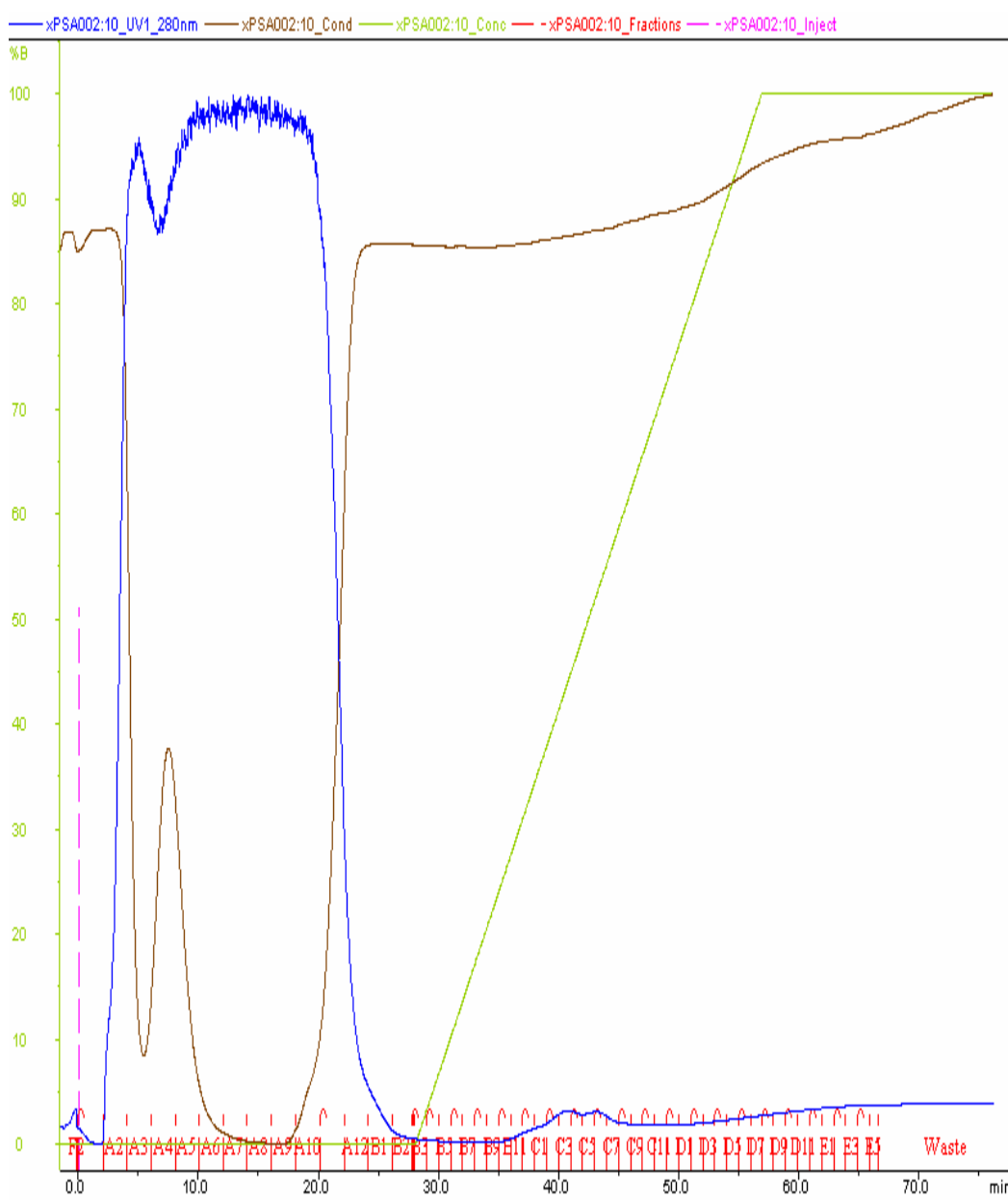


Fig. 18. Chromatogram shows the purification profile of TL1 clone (scFv antibody) in SB medium by Ni²⁺ column. The chromatogram was performed by UNICORN® programme (Amersham, UK) that provided with ÄKTA Explorer FPLC system.

3. The transformation of scFv construct into Fab construct

3.1 Construction of Fab fragment

The antigen-binding fragment (Fab) has been considered as a more functionally stable and higher affinity version of recombinant antibody than single chain antibody fragment (scFv). Moreover, the purification of Fab construct by Protein G column might be purer than Ni²⁺ column purification.

The variable region of light chain, V_L, was amplified by PCR using primers containing tag sequence for PCR-overlap extension. Whereas, the variable region of heavy chain, V_H, was amplified by PCR overlapping between V_{H1} and V_{H2}, and the amber stop codon (TAG) in V_H gene was changed to GAG during the PCR-overlap reaction between V_{H1} and V_{H2} (Fig. 10). The variable regions were resolved in 1.5% agarose gel. The target bands, V_H and V_K, were 354 and 324 base pairs (Fig. 19A) respectively, and then DNA fragments were purified by Nucleospin[®] Extract II gel extraction kit. The human constant regions were amplified from phagemid pComb3XTT bearing C_K-pelB and C_H. The target bands, approximately 411 base pairs for C_K-pelB and 336 base pairs for C_H (Fig. 19A), were purified as described previously (see method 2.1.3).

The light chain (V_K-C_K -pelB) and heavy chain (V_H-C_H) were constructed from the first step overlap extension. The 754 base pairs for light chain and 690 base pairs for heavy chain (Fig. 19A) were resolved on 1.2% agarose gel as described previously (see method 2.2). In the last PCR reaction, the Fab fragment was constructed from the second step overlap extension. The Fab fragments were 1,440 base pairs and then was purified as described in the method 2.3.

The Fab fragments were directionally cloned into pComb3XSS-dIII using *Sfi*I cloning site (Fig. 12B). The pComb3XSS-dIII obtained from clone Afl15 (Putkam, 2007) was derived from pComb3XSS, which the *gIII* was deleted by *Bsi*WI and *Nhe*I restriction enzyme (Fig. 12A). Fab fragment and pComb3XSS-dIII phagemid vector were digested with *Sfi*I restriction enzyme and then ligated together by T4 DNA ligase. The recombinant DNA was validated for correct insertion of the Fab fragment by colony-PCR using RSC-F and TLVHR primers. The PCR products showed positive target band (1,128 base pairs). The result showed that the Fab fragments were correctly inserted into pComb3XSS-dIII.

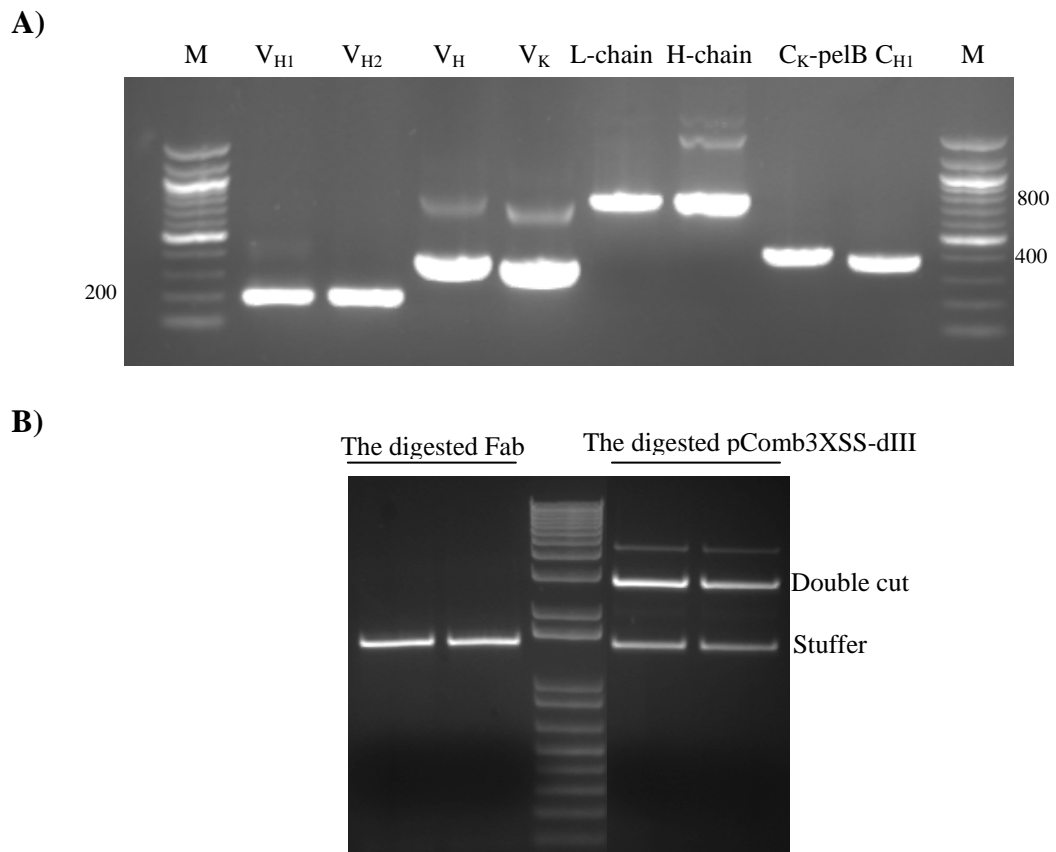


Fig. 19. Electrophoresis gel showed the PCR products stained by ethidium bromide and visualized under UV 340 nm. (A) The gel showed the PCR products obtained from first round of PCR (V_{H1} : 201 bp, V_{H2} : 210 bp, V_H : 354 bp, V_K : 324 bp, C_K -pelB: 411 bp, and C_{H1} : 336 bp) and the second round of PCR (light chain: 754 bp and heavy chain: 690 bp). (B) *Sfi*I digestion of Fab and pComb3XSS-dIII exhibited 1,440 bp (Fab fragment), 2,800 bp (pComb3XSS-dIII phagemid vector), and 1,450 bp (stuffer: Fab fragment of clone Afl15), respectively.

3.2 Transformation of Fab clone

The recombinant Fab fragment sub-cloned into pComb3XSS-dIII vector was efficiently electrotransformed into the *E. coli* strain XL1-blue. The colony-PCR was performed to ensure the existence of the inserted Fab fragment. The Fab clones showed 754 and 354 base pairs of the positive band of light chain and V_H fragment, respectively (data not shown).

3.3 Sequence analysis of Fab clone

Two clones, TLFAB12 and TLFAB13 that showed positive band, were selected for DNA sequence analysis. There was 100% sequence identity between clone TLFAB12 and TLFAB13. In V_H region, the thymine base of amber stop codon was absolutely changed to guanine base.

4. Production and purification of Fab antibody

The Fabs sub-cloned into pComb3XSS-dIII phagemid vector were expressed and induced by IPTG using the *E. coli* strain XL1-blue. The expression was observed by immunoblotting using the antibody that recognizes the histidine x6 tag at the C-terminus of the C_{H1} fragment. The Fab was purified by Protein G affinity chromatography.

4.1 Protein analysis on Western Blotting

The pellet and supernatant of IPTG-induced clone were used for Fab expression analysis. The bacterial cell pellet was dissolved in lysis buffer. The protein sample in lysate and supernatant was detected by immuno-blotting using mouse anti-histidine x6 antibody as primary antibody and goat anti-mouse antibody conjugated with Cy3 as secondary antibody. In SDS-PAGE method, Fab fragment was digested at disulfide bond (conjugates between heavy chain and light chain) by reducing agent. This result revealed only 27 kDa of heavy chain within cell lysate and supernatant (Fig. 16B), because the antibody recognized histidine x6 tag contained at C-terminus of heavy chain. Thus, the western blot in Fig. 16B showed that the heavy chain comprising of V_H and C_{H1} was lower than scFv fragment comprising of V_H and V_K.

4.2 Protein purification by Protein G column

To scale-up antibody production, the culture was induced with final concentration of 1 mM IPTG. After 24 hours induction, the supernatant was concentrated by Centricon[®] centrifugal filter unit (Millipore, USA), YM10 membrane (cut off 10 kDa). The concentrated medium was loaded into the equilibrated HiTrap[®] protein G column. The recombinant Fabs were eluted in an acidic condition, 0.1 M glycine-HCl, pH 2.7. The eluted fractions were determined for the activity of antibody by ELISA, and the purity of each fraction were observed by silver staining (Fig. 20A) and coomassie blue staining (Fig. 20B) on SDS-PAGE. The result showed the eluted fractions of Fab antibody were pure enough for further characterization. The concentration of Fab antibody was determined by Micro BCA assay and approximately 25% recovery yield was found by 1-mL HiTrap[®] protein G column.

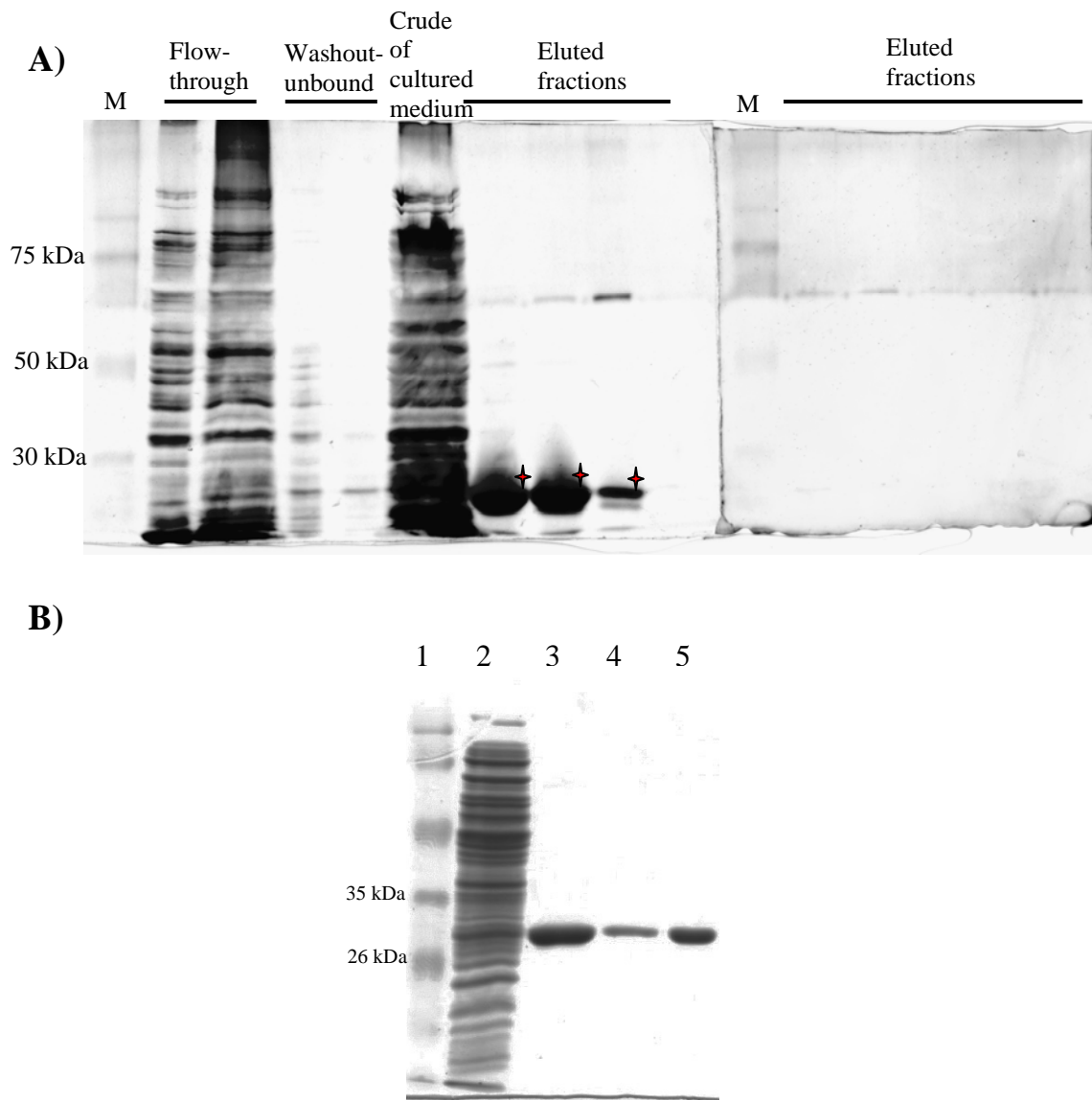


Fig. 20. SDS-PAGE of purified fractions of Protein G chromatography of TLFAB12 (Fab clone) was detected with silver staining (A) and coomassie blue staining (B). (A) the flow-through, washout-unbound, crude of cultured medium, eluted fractions were monitored the purity of SDS-PAGE († : indicates band of target protein). (B) lane1: prestain protein marker, lane2: concentrated medium, lane3: pool of positive fractions, lane4-5: the purified Fab antibody.

Chapter IV

Discussions

In 1985, George Smith conceived the idea of the linkage between genotype and phenotype (Smith,1985), “Phage Display”. Parmley and Smith showed that the gene encoding antibody can be inserted into phage genome encoding coat protein of phage, creating a fusion antibody that was incorporated into the virion of phage (Parmley & Smith, 1998). Over the past decade, the technique has been developed to select the monoclonal antibody used as diagnostic and therapeutic tool. In agriculture, the technology can select the high potential Fab antibody specific to Aflatoxin B1 (Putkam, 2007), the toxin contaminating in agricultural products and causing in hepatocarcinoma. The advantages of phage display technology are a high-throughput selection, low cost, and high yield. Furthermore in medical research, Julia smith and et al. employed the phage display to select the monoclonal antibody against known or novel endothelial cell marker, such as VEGFR, endoglin, and extracellular domain B (ED-B) domain of fibronectin (FN) for stimulating angiogenesis in angiogenic therapeutic strategies (Smith et al., 2005).

In this study, the immune libraries were firstly used to select the chimeric mouse/human Fab antibody against PSA. Several factors in panning process were thoughtfully concerned including the PSA dose of immunization, the library size, PSA coating, washing buffer, blocking reagent, and *etc.*; however, the in vitro selection was not successful from these immune libraries. The highest PSA dose for immunization was 30 μg of PSA; whereas, the aflatoxin antibody library used 50 μg for a dose of immunization. For this reason, the dose of antigen for immunization may affect the library size. In conclusion, these immune libraries were not successful for antibody selection. Therefore, the other larger diversity library was chosen to select the recombinant antibody against to PSA.

The scFv antibody against PSA was selected from Tomlinson I+J library, which is a synthetic and non-immune library based on human single framework, comprising heavy-chain germline gene V3-23/DP-47 and J_H4b and the κ light-chain gene O12/O2/DPK9 and J _{κ} 1 (Wildt et al., 2000). Tomlinson I and J are based on DVT and NNK side chain, which incorporate at position in the antigen binding site, making a high diversity of libraries. The library size of Tomlinson I and Tomlinson J were 1.47×10^8 and 1.37×10^8 CFU, respectively. This large diversity can increase a probability to select the scFv antibody efficiently.

From the *in vitro* selection, the coated PSA used for panning was purified PSA from human seminal fluid (Chemicon, USA), which the majority was fPSA. Therefore, it is most likely that scFv and Fab obtained in this study was specific to fPSA, unless the binding site is not the binding sites of protein inhibitor. However, the epitope of PSA binding to the selected mAb can be identified by epitope mapping method (Baumgart et al., 2005).

There were many factors that influence to successfully select scFv construct, including washing step and temperature in panning cycles. To increase washing step in the round 3 of panning can diminish non-specific binding phage; specific binding phages are selected from this round. The amount of Tween20 in PSA is considered in panning. The high concentration (0.5% Tween20 in PBS) of surfactant may diminish both unbound and bound phages, while the low concentration (0.05% Tween20 in PBS) may not eliminate the unbound phage. Here, several washing buffers, 0.5, 0.1, and 0.05% Tween20 in PBS, were tried out; the positive clones are successfully selected using 0.1% tween20 in PBS. The use of low temperature in cultivation affects structure and function of soluble recombinant protein (Vasina & Baneyx, 1997). Heo and et al. optimized the temperature for productivity of functional scFv antibody against c-Met. Their report showed that the use of 25°C and 30°C cultivation

temperature led to the efficient folding of scFv antibody (Heo et al., 2006).

The number of panning rounds depends on output titer. The output titers that indicated the successful selection should be lower after increasing washing step, and rise in the subsequent round. The panning can be terminated after the output titers increase for 1 to 2 rounds.

The characterizations of scFv clones were performed by scFv expressing ELISA, diversity analysis, DNA sequencing, CDR determination, and purification of scFv antibody. There were 10 positive clones from round 5. The clone diversity was determined by DNA fingerprint, which the V_H - V_L fragment of clones were digested by *AluI*, *BstNI*. Furthermore, the double digestion of *AluI/BstNI*, *MseI/BstNI*, *MseI/HhaI*, and *MseI/AluI* were additionally used to enhance the specificity of the diversity test. All clones had the same DNA fingerprint pattern in all enzyme digestions, it implied that one type of clone was selected in this panning. From this result, it indicated that the double digestions were not necessary for diversity analysis; in that, it can not distinguish more efficiently. Thus, only *AluI* or *BstNI* are adequate for diversity test of clone in the future.

The output of DNA sequencing revealed the amber stop codon (TAG) between CDR2 of V_H fragment of clone TL1 (scFv clone), this

scenario can explain that the Tomlinson J library uses the NNK side chain for segmental mutagenesis. N represents a mixture of the 4 positive nucleotides A, C, G, or T; whereas K is a mixture of G and T, making 32 codons and a single amber stop codon (Barbas, 2001). The amber stop can occur in the synthetic libraries due to the absence of the natural improvement. In suppressor host strains (eg. XL1-blue and ER2738), the TAG codon can be translated to glutamine; but, it will be problematic during translation as changing to non-suppressor host strains (eg. TOP10 F'). As a result, the V_H can not translate to complete fragment in the non-suppressor strain. Therefore, the thymine nucleotide in TAG was changed to guanine by site-directed mutagenesis protocol (Vallejo, Pogulis, & Pease, 2008), which introduces the desired nucleotide using specially designed primers. The transformation of TAG to GAG can not affect to binding of paratope; because, the TAG and GAG are a symnomymous codons that code the same amino acid, glutamic acid. In the PCR reactions, the DNA polymerase used *Pyrobest*TM (the high fidelity DNA polymerase), because it possesses an associated 3' → 5' exonuclease (proofreading) activity. Thus, the PCR products supplied with *Pyrobest*TM are high fidelity and good productivity. However, the site-directed mutagenesis condition is concerned about purity and concentration of template, primer concentration, magnesium concentration, and *etc.*

The complementarity determining regions (CDRs) of V_H and V_K are determined by IMGT/V-QUEST programme. The CDRs revealed that the majority of amino acid is the neutral polar and non-polar group, indicating that the antibody-antigen interaction employs hydrophobic bond.

The transformation of scFv to Fab construct was considered for more stability and higher affinity of antibody (Quintero-Hernández et al., 2007). The stability of Fab antibody depends on the mutual stabilization between the V_H/V_L and between the C_H/C_L heterodimers (Röthlisberger, Honegger, & Plückthun, 2005). Furthermore, the Fab construct was specifically and easily purified by protein G affinity chromatography. The Fab fragment is inserted to pComb3XSS-dIII obtained from clone Afl15 (Putkam, 2007). The pComb3XSS-dIII was derived from pComb3XSS phagemid vector, which HA tag and *gIII* is deleted by *BsiWI* and *NheI* restriction enzyme. The deletion of *gIII* fragment increases the efficiency of Fab production and remains the activity of expressions (Barbas, 2001; Putkam, 2007), because antibody fragment without pIII can be produced simply in a non-suppressor strain of *E. coli*. TLFAB12 and TLFAB13 (Fab clone) were sequenced for proving the correct inserted Fab fragment and the absolute change of TAG amber stop codon.

The Fab antibody purification by Protein G column is purer (Fig. 20A) than the scFv purification by Ni²⁺ column (Fig. 17). Furthermore, the percent recovery of the Protein G column (25-30% recovery) was better than the Ni²⁺ column. The results indicate that the use of affinity chromatography with the streptococcal Protein G, is more specific than metal chelate affinity chromatography. The Protein G can bind specifically to constant region of various mammalian heavy chain of antibody; whereas, the Ni²⁺ ion could recognize histidine x6 tag that the contaminated proteins may have histidine-rich regions.

For further study, the purified Fab antibody will be used to analyze detection limit, cross-reactivity, and affinity determination. The detection limit study, the PSA should be coated on microtiter well in a range of lower and higher cut-off value (4-10 ng/mL). The cross-reactivity determination can use protein of human Kallikrein family, including hK2 (Black et al., 1999); because, hK2 has 79% homology to PSA (Schedlich, Bennetts, & Morris, 1987). In affinity determination, the purified Fab antibodies are tested with different concentrations of PSA, creating a mixture of antigen/antibody complex. The unbound antibodies in the mixture are assayed in PSA-coated plates. The PSA concentrations, at 50% inhibition of ELISA signal (compared to the maximal binding in the

absence of competing PSA) are taken as an equilibrium constants for the dissociation reactions (Rojas et al., 2002).

Chapter V

Conclusions

The purposes of this study are to select, characterize, and purify scFv antibody specific to PSA. The in vitro selection from Tomlinson I+J library can be used to select the scFv antibody against to PSA. The phage ELISA results show the enrichment of positive clones after increasing washing step. The output colonies from the enriched round of panning are screened for antibody expression using scFv expressing ELISA.

The positive clones expressing scFv specific to PSA are further characterized by diversity analysis, DNA sequencing, and CDRs determination. The clone diversity analysis shows an identical DNA fingerprint pattern; this result implies that there is no variation in all selected clones. The DNA sequencing and CDRs determination reveal that there is one amber stop codon between CDR2 of V_H genes. The amber stop codon is changed to its synonymous codon that codes the same amino acid by site-directed mutagenesis. The purity of scFv by nickel-affinity column is inefficient and required for purification.

Transformation of scFv construct to Fab construct may increase stability and affinity of antibody. The Fab fragment is inserted into

pComb3XSS-dIII, which is derived from Afl15 clone. DNA sequencing result of Fab clone shows the correct inserted Fab gene in pComb3XSS-dIII.

The purification of Fab by Protein G chromatography shows the high efficiency for further characterizations, including detection limit, cross reactivity, and affinity determination. In the future, these characterization methods should be performed the quality of Fab antibody for developing the PSA test kit.

In this study, scFv against PSA is successfully selected from Tomlinson I+J library and the transformation of scFv construct to Fab construct can not influence to PSA binding. The antibodies can be further used to develop for more specificity of PSA detection method (e.g. ELISA), and will be a potential diagnostic tool for PSA detection in clinical practice that can rescue human life from prostate diseases.

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Appendix

ABTS developing substrate

17 μL of 60x ABTS (5 mg of ABTS in 367 μL of)

100 μL of 10x citrate buffer pH 4.0 (10.5 g Citric acid in 1 L of D.W. water)

1.7 μL of hydrogen peroxide (H_2O_2)

Adjust volume to 1 mL with D.W. water

***E. coli* strain ER2738** *tonA: F'proA⁺B⁺, lacI^q, Δ (lacZ)M15/ fhuA2(tonA)*

Δ (lac-proAB) supE thi-1 Δ (hsdMS-mcrB)5

***E. coli* strain XL1-blue** *F' proA⁺B⁺ lacI^q Δ (lacZ)M15 Tn10/ recA I endAI*

gyrA96 thi-I hsdR17 supE44 relA1 lac

LB agar plates

Dissolve 10 g of tryptone, 10 g of NaCl, 5 g of yeast extract, and 12 g of Bacto agar in 1 L of D.W. water. Adjust pH 7.4 with 10 N NaOH, and sterile at 121°C for 20 minutes. Let the medium cool down to 50°C, add antibiotics, and mix by magnetic stirrer. Pour the medium on Petri dishes and allow solidifying. Store at 4°C.

LB top agar

Dissolve 1g tryptone, 1 g of NaCl, 0.5 g of yeast extract, and 0.7 g of Bacto agar in 1 L of D.W. water. Sterile at 121°C for 20 minutes and store at 4°C. Before use, the medium is melt and cooled down.

Table of Primer Lists

Primer name	Nucleotide sequence (5'→3')
Primer for amplification of scFv antibody	
LMB3 (sense)	CGA CCC GCC ACC GCC GCT G
pHENseq (reverse)	CTA TGC GGC CCC ATT CA
Primer for sequencing of Fab antibody	
Ompseq (sense)	AAG ACA GCT ATC GCG ATT GCA G
pelseq (sense)	ACC TAT TGC CTA CGG CAG CG
Primer for amplification of V _K from scFv antibody	
TLVKF (sense)	GG GCC CAG GCG GCC GAG CTC GAC ATC CAG ATG ACC CAG TCT
TLVKR (reverse)	GAC AGA TGG TGC AGC CAC AGT CCG TTT GAT TTC CAC CTT GGT CCC
Primer for amplification and site-directed mutagenesis of V _H from scFv antibody	
TLVHF (sense)	GCT GCC CAA CCA GCC ATG GCC GAG GTG CAG CTG TTG GAG TCT
TLVHR (reverse)	CGA TGG GCC CTT GGT GGA GGC GCT CGA GAC GGT GAC CAG GGT TCC
VHMF (sense)	GCG GGT GCT GAG ACA CGG TAC
VHMR (reverse)	GTA CCG TGT CTC AGC ACC CGC
Primer for PCR assembly of V _L with human C _K	
RSC-F (sense)	GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC
Lead-B (reverse)	GGC CAT GGC TGG TTG GGC AGC

Table of Primer Lists (Continued)

Primer name	Nucleotide sequence (5'→3')
Primer for PCR assembly of V _H with human C _{H1}	
Lead-V _H (sense)	GCT GCC CAA CCA GCC ATG GCC
dpseq (reverse)	AGA AGC GTA GTC CGG AAC GTC
Primer for PCR assembly of light-chain and heavy chain	
RSC-F (sense)	GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC
dp-EX (reverse)	GAG GAG GAG GAG GAG GAG AGA AGC GTA GTC CGG AAC GTC

10x PBS buffer

Dissolve 21.7 g of Na₂HPO₄·7H₂O, 2 g of KH₂PO₄, 2 g of KCl and 80 g NaCl in 1 L of D.W. water. Adjust to pH 7.4 and sterile at 121°C for 20 minutes. Store at room temperature.

8x Phosphate buffer

Dissolve 2.14 g of Na₂HPO₄·7H₂O, 1.11 g of NaH₂PO₄·H₂O, and 23.38 g of NaCl in 100 mL D.W. water. Adjust to pH 7.4 with 10 N NaOH and sterile with 0.45 µm of filter. Store at 4°C.

SB medium (super broth)

Dissolve 10 g of MOPS (3(N-Morphilino) propanesufonic acid), 20 g of yeast extract, 30 g of tryptone in 1 L of D.W. water. Adjust to pH 7.4 and sterile at 121°C for 20 minutes. Store at 4°C.

SOC medium

Dissolve 20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, and 2.5 mL of 1 M KCl in 1 L of D.W. water. Adjust to pH 7.0 with 10 N NaOH and sterile at 121°C for 20 minutes. Store at 4°C. Before use, add 10 mL of sterile 1 M MgCl₂ and 20 mL sterile 1 M glucose.

2XTY medium

Dissolve 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl in 1 L of D.W. water. Adjust to pH 7.4 with 10 N NaOH, and sterile at 121°C for 20 minutes. Store at 4°C.

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