การสร้างและตรวจสอบคุณลักษณะของวัคซีนต้นแบบชนิดอนุภาคเสมือนไวรัสลูกผสม
ต่อเชื้อไวรัสเด็งกี่ซีโรไทป์ 2

นางสาวพัชรี แซ่หลิน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

Construction and Characterization of a Chimeric Viral Like Particle (VLP)-based Vaccine Candidate against Dengue Virus Serotype 2

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Science
Faculty of Medicine
Chulalongkorn University
Academic Year 2015

Thesis Title	Construction and Characterization of a Chimeric Viral Like Particle (VLP)-based Vaccine Candidate against Dengue Virus Serotype 2
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พัชรี แซ่หลิน : การสร้างและตรวจสอบคุณลักษณะของวัคซีนต้นแบบชนิดอนุภาคเสมือนไวรัสลูกผสม ต่อเชื้อไวรัสเด็งกี่ซีโรไทป์ 2 (Construction and Characterization of a Chimeric Viral Like Particle (VLP)-based Vaccine Candidate against Dengue Virus Serotype 2) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ชุติธร เกตุลอย, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. อลัน แจ็คเควท, 4 หน้า.

วัคซีนป้องกันโรคไข้เลือดออกชนิดเชื้อเป็นอ่อนกำลังที่ได้รับการขึ้นทะเบียนเมื่อไม่นานมานี้นั้น แสดงให้เห็นถึงความจำเป็นในการปรับปรุงการสร้างภูมิคุ้มกันต่อการติดเชื้อไวรัสเด็งกี่ให้เพิ่มมากขึ้น โดยเฉพาะต่อเชื้อไวรัสเด็งกี่ซีโรไทป์ 2 การศึกษานี้จึงมีวัตถุประสงค์ที่จะพัฒนาวัคซีนรูปแบบใหม่ที่เป็นวัคซีนอนุภาคเสมือนไวรัสลูกผสมต่อไวรัสเด็งกี่ซีโรไทป์ 2 โดยใช้แอนติเจนจากส่วนแกนของไวรัสตับอักเสบที่ก่อโรคใน woodchuck เป็นฐานของวัคซีน เพื่อนำเสนอส่วนของ fusion loop ร่วมกับ domain III ของโปรตีน E ของไวรัสเด็งกี่ซีโรไทป์ 2 (ทดลองใช้เป็นต้นแบบ) บนผิวของอนุภาค ซึ่งมีชื่อว่า WHcAg-FL-DIII DENV-2 ทั้งนี้มีการสร้างตัวควบคุม คือ WHcAg (อนุภาคเสมือนไวรัสแบบไม่ดัดแปลง) ทั้งวัคซีนอนุภาคเสมือนไวรัสลูกผสมต่อไวรัสเด็งกี่ซีโรไทป์ 2 และตัวควบคุมสามารถสร้างโปรตีนได้ใน *Escherichia coli* ในรูปแบบที่ละลายน้ำและจากการวิเคราะห์ด้วยกล้องจุลทรรศน์อิเล็กตรอนแสดงให้เห็นการรวมตัวเป็นอนุภาคเสมือนไวรัสอย่างไรก็ตาม โปรตีนส่วนใหญ่ที่สร้างขึ้นนั้นเกิดการรวมตัวกันเป็น inclusion bodies ซึ่งควรถูกทำให้ละลายเป็นรูปแบบโมเลกุลเดี่ยวภายใต้สภาวะการละลายที่เหมาะสม ก่อนที่จะสร้างอนุภาคเหมือนนี้อีกครั้งโดยวิธี dialysis ต่อไป

โดยสรุป งานวิจัยนี้แสดงให้เห็นว่าวัคซีนอนุภาคเสมือนไวรัสลูกผสม โดยการใช้แอนติเจนส่วนแกนของ woodchuck สามารถนำเสนอ fusion loop-EDIII ของไวรัสเด็งกี่ซีโรไทป์ 2 ได้ อย่างไรก็ตาม การหาสภาวะที่เหมาะสมของวิธีการที่ทำให้โปรตีนบริสุทธิ์นั้นมีความจำเป็นยิ่ง ก่อนที่จะนำวัคซีนที่พัฒนาได้นั้นไปทำการทดสอบประสิทธิภาพในการกระตุ้นภูมิคุ้มกันในสัตว์ทดลองต่อไป

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5574151130 : MAJOR MEDICAL SCIENCE
KEYWORDS: VIRAL LIKE PARTICLE / DENGUE VIRUS SEROTYPE 2 / WOODCHUCK HEPATITIS B VIRUS CORE ANTIGEN

PATCHAREE SAELIN: Construction and Characterization of a Chimeric Viral Like Particle (VLP)-based Vaccine Candidate against Dengue Virus Serotype 2. ADVISOR: ASST. PROF. CHUTITORN KETLOY, Ph.D., CO-ADVISOR: ASSOC. PROF. ALAIN JACQUET, Ph.D., 4 pp.

As a licensed live-attenuated vaccine for Dengue virus (DENV) currently indicates a necessity of improving the induction of protective immunity against diseases, especially dengue virus serotype 2 (DENV-2) infection. This study, therefore, aimed to develop a new chimeric virus-like particle (VLP) vaccine candidate against DENV-2 by using the woodchuck hepatitis virus core antigen (WHcAg) as a vaccine platform to expose the fusion loop and cooperating with the domain III of E protein of DENV-2 (as a prototype) on the particle surface, which was named WHcAg-FL-DIII DENV-2. As a control, the WHcAg (wild-type VLP) was also generated. Both of the chimeric WHcAg-FL-DIII DENV-2 vaccine candidate and the control could be expressed in *Escherichia coli* as soluble proteins, while electron microscopy showed the presence of VLP formations. Most of the produced proteins, however, are the inclusion bodies (IBs) which should be further solubilized into a monomeric form under appropriate extraction conditions and being reassembled by dialysis methods.

In conclusion, this research demonstrates that WHcAg-derived VLP can serve as a useful platform for the display of fusion loop-EDIII DENV-2. However, a further optimization of the protein purification process is required before evaluating its immunogenicity *in vivo*.

Field of Study: Medical Science
Academic Year: 2015

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

This thesis has been successfully completed because of kind support from my advisor, Assistant Professor Chutitorn Ketloy, Ph.D. and my co-advisor, Associate Professor Alain Jacquet, Ph.D. They provided me a plenty of helpful suggestions and other beneficial comments on my thesis. They also assisted me in solving many problems rising during the research and dedicated their time to review and revise my thesis as well as other reports. They also set up milestones for each phase of my research and papers in which the working schedules forced me very well to accomplish the thesis. Besides, they always encouraged me to overcome all obstacles I faced throughout the research. I am very much grateful for their support and kindness.

I would like to thank Professor Kiat Ruxrungthum, M.D., Head of Vaccine and Cellular Immunology Laboratory (VCI), who granted me a great opportunity to do the research at VCI, gave me many useful suggestions, guidelines and other trouble spots through our Monday lab meeting session, as always.

In addition, I would like to thank members of VCI including Dr. Sunee Sirivichayakul, Dr. Eakachai Prompetchara, Dr. Pinya Pulsawat, Miss Patrawadee Pitakpolrat, Miss Suwanna Mekprasan and Miss Pattraporn Satitsuksanoa, for teaching me several skills, giving me comments and helping me throughout my research.

I would like to thank chairman and committee for providing me guidance, suggestions and other observations which made me carefully carry out the thesis and I would also like to thank National Vaccine Institute, Thailand who kindly funded throughout this research study.

Finally, I must express my very profound gratitude to my parents and family for providing me unfailing support, education, assistance, love and understanding. I am gratefully indebted to their continuous encouragement throughout my years of study until I successfully graduate.

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LIST OF ABBREVIATIONS

aa	amino acid
ADE	Antibody-Dependent Enhancement
APC	Antigen Presenting Cell
BCR	B cell receptor
DC	Dendritic cell
DENV	Dengue Virus
DF	Dengue Fever
DHF	Dengue Hemorrhagic Fever
DSS	Dengue Shock Syndrome
Fc**γ**R	Fc-gamma Receptors
gor	Glutaredoxin Reductase gene
HBcAg	Hepatitis B virus core Antigen
IPTG	Isopropythiogalactoside
KCl	Potassium Chloride
kDa	kilo Dalton
MC	Macrophage Cell
MHC	Major Histocompatibility Complex
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulfate
ml	milliliter
mM	millimolar
$MnCl_2$	Manganese Chloride
NaCl	Sodium Chloride
trxB	Thioredoxin reductase
µg	microgram
µl	microliter
VLPs	Virus-like particles
WHcAg	Woodchuck core antigen

CHAPTER 1

INTRODUCTION

Dengue virus (DENV) is a member of genus *Flavivirus* in the family *Flaviviridae*, which is transmitted by *Aedes* mosquitoes. The DENV contains four different serotypes (DENV-1, -2, -3 and -4) which causes dengue fever (DF) and the severe clinical symptoms (dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)) (1). There are approximately 390 million new dengue infections every year in tropical and subtropical areas of which 96 million cases require hospitalization (2). The severity of disease is more frequent in patients with a subsequent heterotypic virus infection. A phenomenon named antibody-dependent enhancement (ADE) has been proposed to describe this mechanism, in which a recent virus can use suboptimal heterotypic antibodies (enhancing antibody) triggered by a previous heterotypic virus to gain entry to Fc receptor-positive cells (3). This immune enhancement would be a major obstacle in the dengue vaccine development. Therefore, an ideal dengue vaccine candidate should generate life-long protective immunity against all four serotypes at similar levels (4).

Despite the lack of a reliable animal model that mimics the dengue disease, the first dengue vaccine developed by Sanofi Pasteur, Dengvaxia® (CYD-TDV), has been licensed in the end of 2015. Moreover, five additional dengue vaccine candidates are in clinical trials, with two candidates (developed by Butantan and Takeda) expected to start phase III trials in early 2016. The most advance vaccine candidates are based on a chimeric live attenuated approach. Vaccine interference is a known problem which occurred by viral replication among the four serotypes in tetravalent formulation and caused an imbalance of immunity through the failure or the rising of immunogenicity of each serotype (5-8).

Recombinant subunit vaccines may have a number of potential advantages over the live attenuated vaccines. For example, they have no risk of pathogenicity because it cannot reproduce in the host cells and it is easier to be produced in

a large-scale along with engineering possibility which can be expressed in various systems including insect cells, yeast, bacteria or mammalian cells. Several dengue subunit vaccines are currently being evaluated in clinical studies and some are under evaluation in preclinical studies. Recombinant dengue E and its domain III (EDIII) proteins are the target antigens for subunit vaccine development which have shown to induce serotype-specific antibodies against DENV in mice or non-human primates (9-15). According to the literature, neutralizing antibodies directed against DENV E protein are the main mediators of protection against DENV infection. Studies using mouse and human mAbs demonstrated that EDIII is the primary target of the most potent neutralizing antibodies against dengue virus (16). DI/DII-specific mAbs were less potent in viral neutralization but showed an even higher degree of cross-reactivity. Indeed, fusion loop (aa 98 - 110) from domain II contains cross-reactive neutralizing epitopes which against all four dengue serotypes (17). However, the requirement of adjuvants and various doses to reach the greatest immunogenicity are the drawbacks of the monomeric antigen (18, 19). The immunogenicity of recombinant subunit vaccine can be improved by displaying the multiple copies of the foreign antigens on the surface of carrier. Virus-like particle (VLP) is one of the most popular approach for vaccine development which spontaneous self-assembly using only viral structural proteins. VLPs are greatly effective at mimicking natural viruses and inducing potent immune responses without a capability for infection.

This approach is currently used in hepatitis B virus (HBV) and Human Papillomavirus (HPV) vaccines (20). Hepatitis B virus core antigen (HBcAg)-based VLP vaccine candidates for malaria and influenza A have been evaluated in clinical trials (21, 22). Furthermore, there were the chimeric HBcAg VLPs which displayed the DENV-2 EDIII that shown its immunogenicity in mice (23, 24). The major concerns of HBcAg platform technology are the pre-existing immunity issue and the assembly crisis. The use of other hepadnavirus core protein, which infect all kinds of creature except for human beings such as woodchuck and duck hepadnaviruses are likely to avoid the pre-existing immunity problem. Additionally, the woodchuck-derived WHcAg has verified to be at least as immunogenic as the human-derived HBcAg and

it can be produced in *Escherichia coli* (*E. coli*), a cost-effective expression system (25) and demonstrated protection against malaria and influenza (26, 27).

Consequently, this vaccine candidate used WHcAg as a VLP platform by choosing the position amino acid 78 to insert the EDIII (serotype-specific antibodies) and the fusion loop of EDII (region comprehends many cross-reactive neutralizing epitopes against the entire dengue serotypes), which represents a protein fragment of 120 aa and produced in *E. coli*. The chimeric vaccine candidate, designated as chimeric WHcAg-fusion loop EDII + EDIII vaccine, was characterized its VLPs formation by electron microscopy analysis, sucrose density gradient ultracentrifugation analysis.

Research Questions

1. Whether a new chimeric VLP vaccine candidate based a Woodchuck Hepatitis core protein (WHcAg) carrying the fusion loop and the DIII of DENV-2 E proteins (WHcAg-FL-DIII), can be developed in the E. coli expression system.
2. Whether the new chimeric VLP vaccine can be self-assembled into VLPs.

Research Objectives

1. To develop a new chimeric VLP vaccine based on a WHcAg-FL-DIII in the E. coli expression system by using DENV serotype 2 as a prototype.

2. To characterize the VLP formation of the new chimeric VLP vaccine in comparison with particulate nature of a WHcAg alone (wild-type VLP) by using electron microscopic analysis.

Hypotheses

1. A new chimeric VLP vaccine candidate based on a WHcAg-FL-DIII can be developed in the *E. coli* expression system.
2. The new chimeric VLP can be produced as a VLP formation.

CHAPTER 2

LITERATURE REVIEWS

2.1 Dengue Virus and Epidemiology

Dengue Virus (DENV), a single-stranded, positive-sense RNA virus, is member of the *Flaviviridae* family and the *Flavivirus* genus. This kind of genus includes yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV) tick-borne encephalitis virus (TBEV), Zika virus and other viruses which may cause encephalitis (28). Dengue is the world's public health problem which appears widespread in more than 120 countries especially in tropical and subtropical areas, for example, Southeast Asia, South America and the West Pacific (Fig. 1). The DENV are transmitted via the bite of *Aedes* mosquitoes, primarily *Aedes aegypti* and *Aedes albopictus*. There are four distinct serotypes of DENV (DENV-1, DENV-2, DENV-3 and DENV-4) which differentiated by the sequence of the envelope gene (E). Each serotype may be subdivided to different phylogenetic genotypes which seem to be important to the increase of DENV among nature and the dengue fever among human beings.

The incidence of dengue has greatly increased around the world in recent decades. An estimated 3.9 billion people in more than 120 countries are at risk for dengue infection (29). Currently, it is estimated that 390 million dengue infections occur annually (2) and approximately 500,000 serious cases (most of them are children) require hospitalization with a 2.5% mortality rate (source: WHO, 2016 (30) and (31)).

Figure 1: The epidemiological map of tropical and subtropical areas (green) at risk of dengue infection (data from WHO Map, 2015 (32))

The disease outbreak in Thailand has been reported for over 50 years since 1958. Moreover, the highest number of individuals who had a hemorrhagic fever was shown in 1987. In 2015, approximately 143,000 people were infected with the dengue virus and 141 people died (Department of Disease Control, 2015). The disease is also found all over the country especially in the central and the southern part containing a large number of patients all years. From 2010 to 2015, the number of dengue fever patients is constantly increasing every year and reach the peak period during May through September rainy season (Fig. 2). The climate change including temperature, humidity, season and rainfall will definitely affect the abundance and distribution of mosquito vectors.

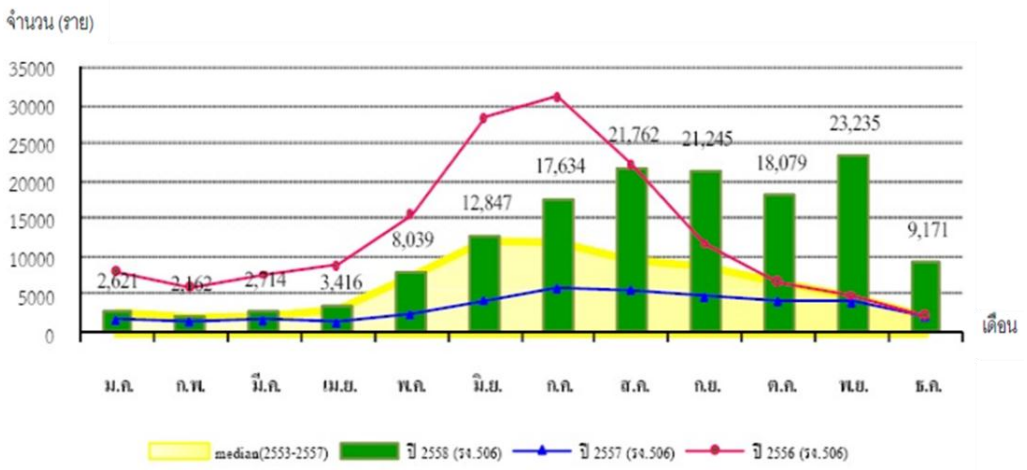

Figure 2: Number of reported dengue cases by month, Thailand, 2015 (data from Department of Disease Control (33))

According to the DENV serotype reports for Thailand from the Ministry of Public Health (MoPH) during 1973 - 2015, major dengue outbreaks have occurred sporadically every few years. During the last decade, DENV-1 and DENV-2 were the most commonly reported serotypes and the proportion of DENV-2 was decreased in 2014 but remained in circulation. DENV-4 peaked during 2005 and 2006, then declined and gradually increased again in 2013. DENV-3 was more commonly found during the whole decade. In 2015, the most commonly identified serotype was DENV-3, indicating over half of overall isolated, followed by DENV-1, DENV-4 and DENV-2 (Fig. 3). Thai people, therefore, are more likely to be encountered with high-risk recurrent infections since the various virus serotypes are extensively expanded all over the country at the same time.

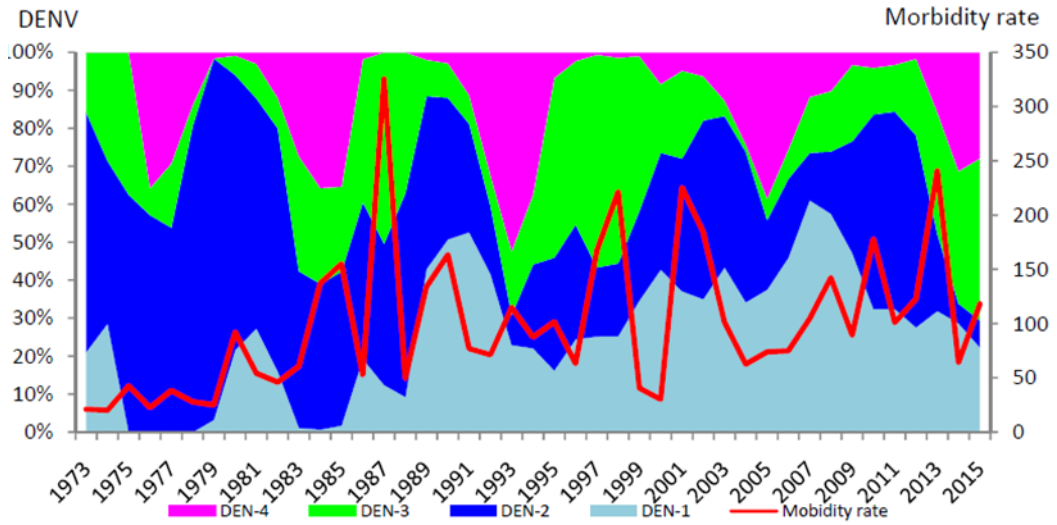

Figure 3: Change in pattern of circulating dengue virus serotypes by year in Thailand during 1973-2015 (data from Department of Medical Sciences (34))

2.2 Morphology and Genomic Properties of Dengue Virus

The DENV had a spherical shape with approximately 50 nm diameter. It consists of the viral genome and capsid proteins enclosed by a lipid bilayer envelope and a shell of proteins. DENV has a single stranded positive-sense RNA genome, ~11 kb, that encodes a long open reading frame (ORF), franked by 5' and 3' untranslated region (UTRs). The DENV enters the host cell by receptor mediated endocytosis. Upon virus uncoats intracellularly via a specific process, the genomic RNA which serves as mRNA is released into the cytoplasm. Translation of a single ORF produces a large polyprotein that is subsequently cleaved and post translationally modified into the mature proteins. There are three structural proteins (C, prM/M, E), the components of the virus particle, and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) involved in viral RNA replication.

2.2.1 Capsid Protein (C)

Capsid protein contains 113 amino acids with approximately 13 kDa in length (35). It is also the first protein that is synthesized by the sequence of the gene divided into two types. The first type is anchored C comprising 113 amino acids which are separated from long proteins by signalase. Most of them are hydrophobic amino acids which has C-terminus in the membrane of the endoplasmic reticulum (ER). After that, C-terminus is cut by NS2B-NS3 protease and become virion C, the second type, which is used in the assembly of particles (36, 37). Besides, this kind of protein is found in the nucleus and cytoplasm covering the RNA sequence of DENV, called the nucleocapsid, which is the core part of the particle (38).

2.2.2 Precursor Membrane (prM) and Membrane Protein (M)

The prM protein is a glycoprotein, consists of 166 amino acids, which found in immature virions. The prM protein forms a prM/E heterodimer with the E protein (39) which contribute to create the viral coat at the rough endoplasmic reticulum (RER). This also helps prevent membrane fusion from conformational changes while carry out of the cell (40). Since prM is unable to replicate in cells, the antibody against prM is poorly neutralizing and encourages the viral replication through Fc-gamma receptors (Fc**γ**R) on the cell surface, called the enhancing antibody (41, 42). The glycosylated parts of prM protein are cleaved by a furin becoming a M protein, approximately 75 amino acids, and produces mature virions. Its dimension also appears smaller and it is complete to replicate in new cells (43).

2.2.3 Envelope Protein (E)

E protein is N-linked glycosylation comprising of 495 amino acids. Its monomeric form as a homodimer which parallel to the surface of particle, each monomer is divided into three distinct domains including domain I, II and III (44). Domain I, structural domain, has an 8-beta-sheet and becomes a beta barrel when the formation occurs. It is the central domain between domain I and II. Domain II, a dimers domain links structural and binding domain. The end of this domain, the fusion loop area (residue 98 - 110), is highly conserved among *Flaviviruses* in lifted

under acidic condition, the fusion loop will be lifted which changes the DENVE dimer to trimer (45) and allows its fusion loop embed into lipid bilayer producing a membrane fusion between endosome and DENV. Then, viral RNA can be released in cytoplasm in order to replicate. This fusion loop is a cross-reactive epitope which seems to be recognized by all four DENV serotypes (17). Lastly, domain III, binding domain, is an immunoglobulin-like domain and contains the receptor-binding site to mediate virus attachment to host cell surface (16). Therefore, domain II and domain III are the main targets for neutralizing antibodies. In addition, there are two regions located in the remaining C-terminal 20% of the E protein (next to domain III), a stem region helps to generate the E homotrimer during the fusion process and is important for the stability of the prME heterodimer during virus assembly (39, 46). Another part is a transmembrane anchor which aids to signal for sending NS1 into ER (46).

2.2.4 Nonstructural Proteins (NS)

NS1 is a glycoprotein and exists in different forms when located in distinct cellular compartments. In virus-infected cells, NS1 appears as a highly stable dimeric form which play a role in viral replication (47). In addition, NS1 is found on the infected cell surface as a membrane-anchored form (48) and secreted from, the infected cells as a soluble hexameric form (49). Both membrane-anchored and hexameric form, known as the soluble complement fixing antigen, can induce the production of NS1-specific antibodies results in a complement activation (50). Furthermore, the attachment of a soluble form with a heparan sulfate makes a change of a capillary permeability leading to plasma leakage (51). NS2A is a hydrophobic protein, which required for the processing of NS1 protein through a protease activity of cells (52). Additionally, it is generally involved in RNA replication and viral assembly (53, 54). NS2A also inhibits the host-mediated interferon signaling (55, 56).

NS2B, work as a part of the NS2B-NS3 protease, is essential for protease activity (57, 58). It is required for the cleavage of NS2A/NS2B, NS2B/NS3 and NS3/NS4A (58, 59).

NS3 is the second largest viral protein having 3 types of enzymatic activities at C-terminus end: (i) a RNA-5'-triphosphatase (RTPase), (ii) a nucleoside-5'-triphosphatase (NTPase) and (iii) a RNA helicase (60). NS3 has a multi-functional role in RNA replication and polyprotein processing.

NS4A and NS4B are transmembrane proteins that can inhibit the host-mediated interferon responses (56). NS4A is supposed to act as a part of RNA replicase complex which involved in RNA replication (53, 61). NS4B was found in both cytoplasm and nucleus which may be required for viral replication.

NS5, the largest protein, appears to play a role in RNA replication due to the presence of RNA-dependent RNA polymerase (RdRP) activity and a methyltransferase (MTase) activity on the capped RNA (62).

2.3 Immunopathogenesis of Dengue Virus Infection

The immune response has an important role in the pathogenesis of dengue virus infection. When the primary infection occurs to a person, it may show asymptomatic or undifferentiated fever or may cause dengue fever (DF) with mild symptoms. In addition, people with dengue infection can have severe symptoms due to dengue hemorrhagic fever (DHF) which can cause symptoms in the same way as in DF. However, the symptoms can be more severe as it leads to a disorder encounter of hemostasis and vascular leakage. Moreover, it is possible to find the hypovolemic shock conditions; they can cause the level of hematocrit (Hct) to clearly arise which can detect pleural effusion as classified in dengue shock syndrome (DSS). Some patients may die if not properly treated. The DHF/DSS can be found in secondary infection for 15 - 80 times rather than in primary infection (63). In a normal state of dengue virus infection, it is commonly found both the cellular and humoral immunities. To illustrate, the virus will enter such as macrophages and monocytes through the receptor on the cell surface and multiply in the cells. There are responses to the invasion by producing antiviral substances such as any cytokines, IFN-γ, α, β, and free-radical. During the primary infection, neutralizing antibodies can be found. They can neutralize and inhibit the increase of the viruses. These are

specific to the serotype received and it persists a long time or lifelong in the host while it gives a short-term prevention for different serotypes (cross-reaction).The neutralizing antibody, which is mainly specific to outer proteins of virus (E protein) acts as a protective antibody that can prevent infection and disease. It is, therefore, the goal of developing a vaccine for choosing merely some antigen, such as subunit vaccine and DNA vaccine.

When leaving it long enough, the neutralizing antibody contained in a lower number is not enough to neutralize or inhibit across serotypes in the secondary infection. In contrast, it is to urge more infection; this is known as enhancing antibody. In fact, it is like a bridge which connects DENV to Fc-receptor and allows viruses to get into the target cells and multiply quickly. This mechanism is called antibody-dependent enhancement. When the infected cells are increasing and viruses are more produced in the blood, it stimulates the cells in the immune system much more than usual. This causes a large amount of quantity and type of cytokine production, complement activation, and histamine secretion. This can increase vascular permeability until the plasma leakage occurs without causing the death of vascular endothelial cells; however, it can lead to severe symptoms (DHF/DSS) (3, 64-66). Thus, the problem in developing vaccine is a cause of enhancing antibody of vaccines, in which that need a long-term study. Additionally, secondary infection with viruses in different serotype is generating antibody from the memory B cell rapidly. When the antibody occurs during the high levels of viruses in blood, it causes immune complex which can stimulate complement system (67). This mechanism may have a role in the leak of fluid because the vascular leakage leads to a decrease in blood volume and shock, which is the main pathogenesis of DHF/DSS. Also, it causes T-cell activation and cytokine secretion, which was found that level of cell-mediated immunity and some cytokines correlated with disease severity. It is therefore believed that the function of T cell, which is specific to the antigen of viruses, may involve in coming up pathogenesis of DHF/DSS (68). As each serotype has 70% sequence homology (69), at the same time, memory B cells make the immune system respond quickly to the known antigen, while the response to the new antigen which is presenting will occur more slowly. So, it is found that at the

beginning phase of the secondary antibody, the immune system responded to the antigen of the old virus serotype, which had been already infected, more than responding to serotype, which is being infected, called Original antigenic sin. Although it is a common mechanism that immunity is being created as soon as possible, it might not be effective enough to get rid of the infection. It can finally cause inflammation and tissue damage resulting in plasma leakage (70). For this issue, vaccine development is very important to stimulate antibodies against the four serotypes.

2.4 Dengue Vaccine Development

An ideal dengue vaccine should have a good safety profile with an ability to produce life-long protective immune response against all four serotypes. There are several obstacles to the successful development of dengue vaccines, including

- The lack of an animal model that mimics patterns of human disease. Although, non-human primates can show a result of dengue virus infection and antibody production, they do not develop the diseases (71, 72).

- The development of antibody dependent enhancement (ADE) that leads to the severe dengue during secondary infection from different serotype or after vaccination.

- The lack of information regarding the correlation of immunity that causes the disease protection. According to the WHO guidelines for the clinical evaluation, the neutralizing antibodies against dengue envelop proteins are considered to be a reliable indicator for evaluating the vaccine efficacy.

Despite the challenges for an ideal dengue vaccine, several approaches of dengue vaccine candidates have been developed and evaluated such as live attenuated vaccine, chimeric live attenuated vaccine, inactivated vaccine, DNA vaccine and subunit vaccine. The summary of current dengue vaccine candidates in clinical trial is shown in table 1.

2.4.1 Live Attenuated Dengue Vaccines (LAV)

Live attenuated vaccine is the first vaccine that has been developed, and also the most advanced in pipeline. LAVs are capable of producing robust, broad and long-lasting immune response that closely similar to the natural infection by wide-type viruses. However, there are some limitations including the high rate of adverse events and the awareness of vaccination in immunocompromised people. There are two types of attenuated vaccine classified by the methods of producing live attenuated viruses include attenuation by serial passage in cell culture (classic LAV) and targeted mutagenesis combining with the chimeric viruses construct (chimeric LAV).

2.4.1.1 Classic Live Attenuated Vaccines

The development of live attenuated vaccine was initiated by Mahidol University, Bangkok, Thailand. The viruses were attenuated by serial passages in primary dog kidney (PDK) cells or primary African green monkey kidney cells and then fetal rhesus lung cells, the combination of each serotype to the tetravalent formulation failed to induce a balance immune response to all serotypes which mainly related to the adverse reactions of DENV-3 vaccine strain.

A group of Walter Reed Army Institute of Research (WRAIR) in collaboration with GlaxoSmithKline (GSK) produced the tetravalent LAVs by serially passage the candidate strains in PDK cells and fetal Rhesus monkey lung cells. They started phase II clinical trials in 2007 by administered 2 doses of the vaccine at 0 and 6 months. the WRAIR-GSK LAVs had shown to meet the standard safety profiles with a seroconversion rate of approximately 60% in each serotype (73). However, it was appearing no antibody titer rose after giving the third dose, and when one more test had done, the vaccine eventually stopped developing (74).

2.4.1.2 Chimeric Live Attenuated Vaccines

Chimeric virus was made from the engineer chimeric approach, which works by replacing genes in some position of DENV (prM and E) into the backbone of

attenuated viruses which derived from the *Flavivirus* genus (Yellow fever) and the dengue virus.

Dengvaxia, the chimeric yellow fever-tetravalent dengue vaccine (CYD-TDV), is the first licensed dengue vaccine by Sanofi Pasteur. The prM and E proteins from each dengue serotype are substituted into the yellow fever (YF) 17D vaccine backbone. Two phase III clinical trials, CYD14 in Asia and CYD15 in Latin America, showed overall efficacy (to reduce symptomatic virologically confirmed dengue) around 60% at 25 months following the three-dose schedule (0, 6 and 12 months) (75, 76). The vaccine efficacy also altered by age groups and serostatus at baseline vaccination (pre-existing dengue immunity). It has been shown a greater efficacy in people with pre-existing dengue immunity. Moreover, CYD-TDV had been shown to decrease two-thirds of hospitalizations and 88.5% of severe diseases caused by dengue infection.

The Centers of Disease Control and Prevention (CDC) developed a tetravalent chimeric vaccine by the substitution of prM and E gene from DENV-1, -3 and -4 into the attenuated DENV-2 backbone which derived from the Mahidol University-Sanofi Pasteur vaccine (DENV-2 strain 16681 PDK53). A tetravalent chimeric vaccine candidate, DENVax, was then licensed to Takeda. Phase I studies in Colombia and US evaluated the dose and administration routes revealed the safety and sufficient immunogenicity (77, 78). An ongoing phase II study of DENVax in different age ranges and in endemic area is being assessed.

Lastly, the National Institute of Allergy and Infectious Disease (NIAID), National Institutes of Health (NIH), USA developed a live attenuated vaccine by based on a site directed mutagenesis approach (79). Deletion of 30 nucleotides at the 3 'UTR of the full length cDNA clones of DENV-1 to DENV-4 was used to produce attenuated strains designated as DEN1Δ30 to DEN4Δ30, respectively. However, due to the lack of attenuation in Δ30 deletion of DENV-2 and DENV-3 strains, the chimeric viruses were modified by replacing M and E genes with those from DENV-2 and DENV-3 into an attenuated DEN4Δ30 backbone and designated as DEN2/4Δ30 and DEN-3/4Δ30. Five different tetravalent formulations (TV001-TV005) were assessed immunogenicity and safety for a single dose administration. Although TV003 induced the most balanced

antibody response, the seroconversion rate was low in DENV-2 (50%) compared with the others (85 - 100%) (80). In the follow-on trial, a second formulation of TV005 containing 10-time increased dose of DEN-2/4 was compared with TV003. A single dose of TV005 induced overall tetravalent response in 90% of volunteers and increased the seroconversion rate to DENV-2 from 76% (TV003) to 97% (TV005) (80, 81). A phase II study of TV003 and TV005 is currently being evaluated in Thailand in collaboration with the Butantan Institute.

2.4.2 Purified Inactivated Vaccine (PIV)

From the achievement of licensed purified inactivated Japanese encephalitis vaccine, PIV dengue vaccines are being developed by Walter Reed Army Institute of Research (WRAIR) in collaboration with Glaxo-SmithKline (GSK). In comparison to live-attenuated vaccine, PIV, a non-replicating vaccine, are less likely to exhibit interference among DENV serotypes. However, the immunity induced by PIV showed an incompetent and short-term immune response. To achieve acceptable immunogenicity, the combinations of PIV with different adjuvants were tested and showed a tolerant and persistent neutralizing antibody response to all serotypes and the phase I trial is currently underway (NCT01666652 and NCT1702857) (82). In addition, a prime-boost strategy with PIV prime and live attenuated vaccine boost, resulted in the complete protection in non-human primate and is under evaluation in a phase II trial (83).

2.4.3 DNA Vaccine

DNA vaccine composed of antigen encoding genes cloned into the plasmid vector is taken into cells and generated the antigen proteins. By the association with major histocompatibility complex (MHC) class I and class II, DNA vaccine can induce both humoral and cell-mediated immune responses. There are several advantages of DNA vaccine include the ease of design and large scale production, the longer shelf life (thermostable) and low-cost production. In dengue, DNA vaccines encoding the prM and full-length E proteins were shown to be immunogenic in mice and non-human primate. Since DNA vaccine is poorly immunogenic due to inadequate

cellular uptake, several strategies are being evaluated include the delivery systems (needle-free injection, *in vivo* electroporation, etc.) and the addition of intrinsic adjuvants into the plasmid vector (immunocostimulatory CpG motifs, t-PA (secretory signal sequence of human tissue plasminogen), granulocyte macrophage-colony stimulating factor (GM-CSF), (84-87)). Naval Medical Research Center (NMRC) has developed a monovalent D1ME100. The proof-of-concept phase I trial did not show adequate antibody response (42%) (88). The subsequently study by adding an adjuvant, Vaxfectin, into tetravalent DNA vaccine is being evaluated in phase I trial (NCT01502358).

2.4.4 Subunit Vaccine

Subunit vaccines are vaccines that use only part of the targeted antigen to stimulate the immune response. In dengue, the main target proteins are E and EDIII, which is the most diverse domain in the level of amino acid sequence between serotypes (4, 17, 89). These proteins can be easily expressed in different expression systems including insect cells, baculovirus vectors, mammalian cells, yeast and *E. coli*. Although subunit vaccines, a non-living vaccine, can be given to an immune-compromised people and circumvented the interference between serotype, several doses should be given for the life-long immunity.

Hawaii Biotech, Inc produced a recombinant subunit vaccine based on the dengue prM and truncated envelope glycoproteins (DEN-80E) of each serotype via expression in the *Drosophila* S2 cells (90). A phase I study revealed that a monovalent formulation of DEN1-80E component (HBV-001 D1) was safe and immunogenic. This candidate was then transferred to Merck which known as V180. The current phase I trial is being assessed in a combination with and without adjuvants ISOCOMATRIX.

There are several approaches to develop subunit proteins based on EDIII (Table 2). In one, the EDIII is fused to the Maltose Binding Protein (MBP), the meningococcal p64k for each serotype. These monovalent candidates were immunogenic in NHPs (91, 92). The second approach uses EDIII fused to capsid protein. The tetravalent formulation of EDIII-capsid is currently evaluated in NHPs.

The last approach is a single tetravalent subunit vaccine which used the consensus EDIII from different 4 serotypes. The administration of consensus EDIII with alum can induce neutralizing antibody responses to all serotype in mice (93) while induce only neutralizing antibody against DENV-2 in NHP (9).

2.4.4.1 Viral Like Particle (VLP)

VLP is an approach using only the structure protein of virus to make a self-assembly and the protein can be produced in both prokaryotic and eukaryotic systems under the process of bioengineering (94). As compared to the live-attenuated virus (LAV), a VLP displays some advantages. First, the particulate structure of VLP which exposes multi-copies of the antigen is still maintained, however, the VLP does not contain any DENV genetic materials which prevent reactogenicity from the risk of reversion of the live-attenuated to wild-type (95). In addition, one more important advantage is that the production process to attenuate the viruses in infected cells normally is never easy. Besides, the problem at the level of reproducibility might ultimately arise to get the same batch of the viruses. Here, it is different; the VLP can be produced more easily than the recombinant proteins in *E. coli* (96).

VLP, in a form of particulate antigen, has various advantages compared to monomeric antigen; it can stimulate immunogenicity more highly as the size and receptor interact better with antigen presenting cell (APC) (96). The size of the antigen is a very important factor for the effective uptake of the APC (97). Dendritic cells (DCs) and probably macrophage cells (MCs) are significant APCs in stimulating T cell responses. DCs like viral size ranges (20 - 100 nm), while MCs like bacterial size (μm) and the monomeric or soluble antigens with the size less than 10 nm are not efficient in uptake and presentation by APCs (96). Thus, it needs to be formulated with a suitable adjuvant for the larger particles. While VLP and nanoparticle which is a particulate antigen is 20 - 200 nm, it is likely to be itself an adjuvant (96). Moreover, the particulate antigens arrange their repetitive antigens on the surface of the particles in which that increases phagocytosis. From the consideration, pathogen-associated molecular patterns (PAMPs) induce the mature DC from up-regulation of

DC maturation markers and then cause antigen cross-presentation, which presents peptide (degraded by the lysozyme in the endosome) to native $CD4^+$ and $CD8^+$T cells via the MHC class II and I, respectively. The stimulation of B cell occurred by the particulate antigen size which can directly lead to the lymphatic system since the antigen carrier is being protected from degradation. Also, this repetitive arrangement can cause the stimulation through cross-link B-cell receptor while the soluble antigen is firstly being degraded by the proteolytic enzyme before being transported to the lymph nodes (96). For the mechanism of B cell, it is described through the B cell receptor (BCR) which can recognize and respond to the antigen in the form of protein. It can move anytime because the component of B cell membrane is a fluid-phase lipid bilayer. When it catches the antigen alone, it would be bound with the ratio of 1: 1. In contrast, BCR is bound to antigen (epitope) on the surface of the VLP, which is well-organized. With high density presented on the particles, it creates a high avidity to BCR and makes some move on the surface area with VLP presentation. Then the number of these epitopes is going to be presented by B cell. Finally, that causes the secretion of cytokine in a large quantity and stimulates B cell to switch into the plasma cells, which create and release neutralizing antibodies (98).

VLP has been developed in the dengue vaccine candidate as well, by using HBsAg as a carrier to present EDIII of DENV-2 which it was accessible to the VLP formation (99). Moreover, VLP has been created from prM and E protein of DENV-2, and it was being tested in monkeys (13). The vaccine candidate was most often created as a monovalent formulation, which is related to this study. During the test, DENV-2 was built as a prototype first, to test the possibility of producing a tetravalent formulation. Some VLP-based human vaccines are already available on the market such as hepatitis B virus (HBV) vaccine based on HBsAg (100) and human papillomavirus vaccine (HPV) based on L1 (20). The HBcAg can also self-assemble to produce VLP which is similarly to HBsAg.

HBcAg is like a promising of VLP, which was reported (101, 102), and it is superior to other VLPs due to its high competence in self-assembly; with 120 homodimers (one monomer is 183 aa) (103), it forms approximately 36 nm diameter in a heterologous expression system including yeast and *E. coli*. Particularly in *E. coli*, recombinant proteins are produced at a large-scale by using fast and cheap process. Moreover, the part of C-terminus can be cut around at the amino acid 150 - 183, which contains nucleic acid binding domain (positively charged area) as it was found unnecessary for particle assembly (117). Another outstanding advantage is that it can be inserted by a foreign antigen at the immunodominant B-cell epitope or c/e1 loop (residue 76 - 82) and can be organized to expose on the particles effectively without losing their ability to achieve self-assembly (98, 104) as shown in figure 4. The largest size of the antigen that can be put is 255 aa from Outer Surface Protein A (OspA) of *B.burgdorferi,* followed by the green fluorescent protein (GFP) 238 aa (105, 106). In addition, it can also be produced in *E. coli* by remaining VLP structure in which that causes HBcAg to be a cost-effective strategy Therefore, HBcAg was applied as a vaccine platform for infectious diseases. It was tested in both preclinical and clinical trials. It is being used as a carrier for foreign B cell epitopes to get high immunogenicity which is called chimeric VLP or HBcAg-based VLP vaccine candidates such as Malarivax developed for a malaria vaccine, which specific immune response against malaria as found in phase I trial (22). Besides, there is a development of influenza A vaccine candidate which also showed well immunogenicity and safety in phase I clinical trial (21, 107, 108). While, a chimeric HBcAg VLP which exposed the DENV-2 EDIII was developed and shown a low titer of neutralizing antibody in mice (23, 24). However, the position of insertion in c/e1 loop should be considered for appropriately exposing of epitopes. The last 5 years, HBcAg-based VLP vaccine candidates were able to generate both cellular and humoral immune responses (21, 23, 24, 109-114) (Table 3). However, the limitation on pre-existing immunity is that it will be found in a person who has the anti-HBc antibodies from the previous infections which may lead negative effects to immunogenicity and the assembly problem as well. Firstly, this HBcAg is derived from a human pathogen, anti-HBc antibodies are existing in individuals previously exposed to HBV infection which may

adversely affect the HBcAg-based vaccine immunogenicity. In addition, the anti-HBc antibodies elicited by the HBcAg-based vaccine will compromise the usefulness of the anti-HBc assay currently employed as a diagnostic for current or recent HBV infection. Most importantly, T-cell immune tolerance towards HBcAg is present in individuals chronically infected with HBV (350 million globally (source: WHO, 2002)). In Thailand 2014, it is estimated that approximately 2,222,540 are HBV carrier (115).

Secondly, the assembly problem is demonstrated by the observation that less than 50% of the VLP-subunits are successfully accommodated by the HBcAg platform (116). This failure might be due to destabilization of particle assembly caused by inserting foreign sequences. Very often, the length of the insert appears to be the bottleneck for proper assembly with usually a typical length of insertions of 120 aa. Flexible linkers based on glycine residues can improve the proper insertion of polypeptides into the HBcAg to maintain the VLP formation.

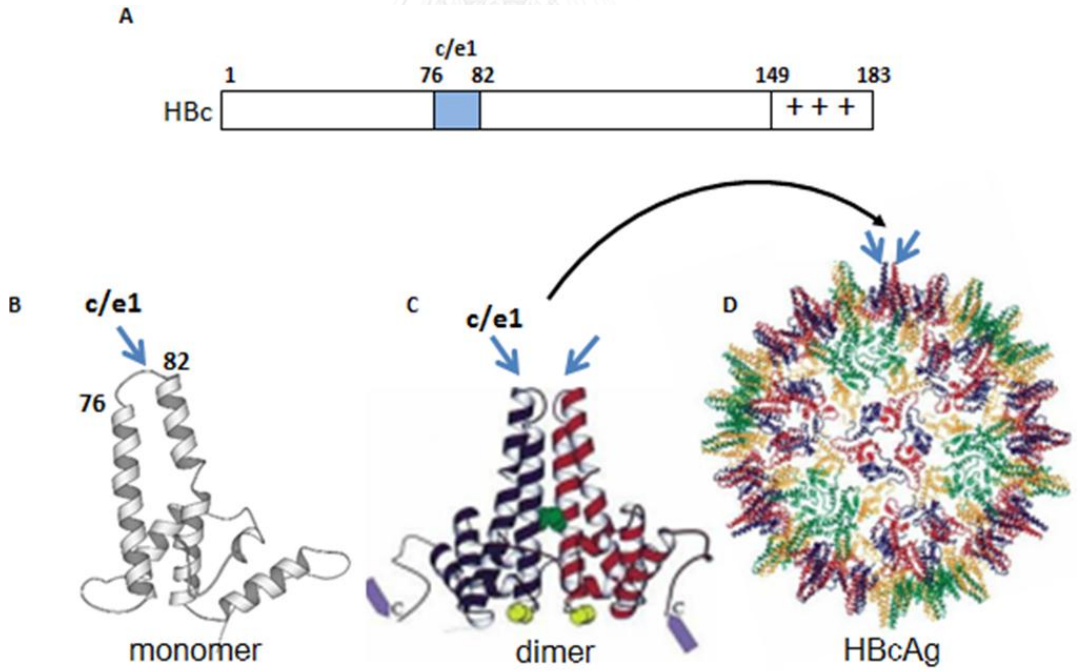

Figure 4: Self-assembling chimeric HBcAg VLP by inserting foreign antigen in the c/e 1 loop. (A) Scheme of chimeric HBc gene, (B) HBc assembly domain, (C) dimerization of HBcAg and (D) VLP formation (modified from (117, 118)

When using the core protein from the virus that is a member of hepadnavirus genus, which is closely related to human hepatitis B virus but causing diseases in animals, such as the woodchuck and duck hepatitis viruses, it can avoid anti-HBc antibody against human hepatitis B virus originating from earlier infection (25, 27, 119). Moreover, the part of C-terminus contains nucleic acid binding domain (aa 150-188) as it is not associated with the particle assembly (27, 120).

Interestingly, woodchuck core antigen (WHcAg) and HBcAg are actually 67% identity and 78% similarity at the amino acid level, but it occurs cross-reactive to the antibody insignificantly (27). According to the literatures, woodchuck-derived WHcAg has proven to be at least as immunogenic as the human-derived HBcAg and such VLP can still be produced in *E. coli*, a cost-effective expression system (25). The WHcAg-based VLP vaccines demonstrated protection against malaria and influenza (26, 27) as shown in table 3.

Table 1: The most advance of dengue vaccine candidates in clinical trials

Vaccine type/Developer	Approach	Status	Ref.
Live attenuated vaccine (LAV)			
Classic LAV (TDEN)/WRAIR and GSK	Attenuated DENV 1-4 were made by serial passage in PDK cells	Phase 2	(73, 121)
Chimeric LAV (CYD-TDV)/Sanofi Pasteur	prM/E genes from each of 4 serotypes were substituted in individual attenuated YFV 17D backbones	Phase 3	(75, 76, 122, 123)
Chimeric LAV (Δ30 3'UTR)/NIH	Deletion of 30 nucleotide at 3'UTR of DENV1-4 genome and DENV-2 formulation requiring a backbone of DENV-4 as a chimeric virus	Phase 1	(80)
Chimeric LAV (DENVax)/Takeda	Attenuated DENV-2 by serial passage (53 passages) in PDK as a backbone for prM/E from each serotype	Phase 2	(78)
Purified inactivated vaccine (PIV)/GSK	Purified inactivated of the four dengue serotypes	Phase 1	(82)
Recombinant subunit vaccine/Merck	A monovalent formulation form 80%E protein of DENV-1	Phase 2	(14)
DNA tetravalent/WRAIR	prM and E from each of four serotypes containing adjuvant (Vaxfectin)	Phase 1	(88, 124)

Table 2: Currently dengue EDIII-based vaccine candidates within last 3 years

Approach	Developer/ Year/Ref.	Details/ Expression host	Methodology	Immunogenicity results
DENV 3 ED III	Chiang C.Y. 2016 (10)	Recombinant lipidated DENV3 EDIII (LD3ED III) The lipid moiety of the bacterial-derived lipoprotein; lipid signal peptide using pET22b as a vector (Novagen, Madison, WI)/ *E. coli*	Two times immunization at a 2-week interval (S.C.) in mice (Balb/C) 10 µg/0.2 mL per dose Gr.1 LD3EDIII Gr.2 D3EDIII + Alum Gr.3 D3EDIII Gr.4 PBS 20 weeks after first immunization, mice were challenged with DENV3	$FRNT_{70}$ titers against DENV3 Gr.1 = 1:12.7 Gr.2, 3 and 4 < 1:8 The viremia level of LD3EDIII group quickly reduced after challenging for 22 ($p<0.01$) and 23 h ($p<0.05$)

Table 2: Currently dengue EDIII-based vaccine candidates within last 3 years (Cont.)

<table>
<tr><th>Approach</th><th>Developer/
Year/Ref.</th><th>Details/ Expression host</th><th>Methodology</th><th>Immunogenicity results</th></tr>
<tr><td>Dengue serotype 1-4 E domain III</td><td>Qin C.F.
2014
(15)</td><td>Tetravalent subunit vaccine based on domain III; tandem EDIIIs (amino acid residues 298-400 of the E) of two serotypes (1-2 and 3-4) of DENV connected by Gly-Ser linker ($(Gly_4Ser)_3$). Thioredoxin (Trx) was fused at the N-terminal (MixBiEDIII) + FA using pBAD-D12 and pBAD-D34 as a vector (Invitrogen) /E. coli</td><td>Two times immunization at a 2-week interval (S.C.) in mice (Balb/C) 100 µg per dose containing 50 µg of D12-EDIII and D34-EDIII (MixBiEDIII group) Priming (day 0) in complete Freund s adjuvant (CFA) Boost (day 14) in incomplete Freund s adjuvant (IFA)
Gr.1 MixBiEDIII+FA
Gr.2 Trx protein</td><td>$PRNT_{50}$ titers against DENV1-4
Gr.1 MixBiEDIII+FA
Gr.2 Trx protein (control) <1:8 Protection rate :
<table><tr><td>Virus</td><td>D1</td><td>D2</td><td>D3</td><td>D4</td></tr><tr><td>$PRNT_{50}$</td><td>45</td><td>29</td><td>57</td><td>22</td></tr></table>DENV1, 2, 4 Challenged = only partial protective
DENV3 Challenged = 100%</td></tr>
</table>

Table 2: Currently dengue EDIII-based vaccine candidates within last 3 years (Cont.)

<table>
<tr><th>Approach</th><th>Developer/
Year/Ref.</th><th>Details/ Expression host</th><th>Methodology</th><th>Immunogenicity results</th></tr>
<tr><td>DENV1-4
EDIII</td><td>Izquierdo A 2014
(125)</td><td>Four individuals of tetravalent formulation (TF) was comprised of E domain III (DENV-1, -3, -4; aa 286-426) fused with P64k from N. meningitidisas as well as DENV-2 E domain III fused with DENV-2 Capsid protein using pD10, 18 and 24 plasmid/E. coli</td><td>Three times immunization (I.P.) in mice (Balb/C) at day 0, 15, 30
Gr.1 80 µg of TF + Alum
Gr.2 Alum
Gr.3 Infectious DENV1-4
and were challenged (I.C.) with live neuroadapted DENV1-4</td><td>$PRNT_{50}$ titers against DENV1-4
Gr.1 = TF + Alum
Gr.2 = 0
Gr.3 = As Gr.1 has similar results
<table><tr><td>Virus</td><td>D1</td><td>D2</td><td>D3</td><td>D4</td></tr><tr><td>$PRNT_{50}$</td><td>50</td><td>181</td><td>19</td><td><10</td></tr></table>
Protection rate:
(P< 0.05)
Gr.1, 2 and 4 = 70%
Gr.3 = 100%</td></tr>
</table>

Table 2: Currently dengue EDIII-based vaccine candidates within last 3 years (Cont.)

Approach	Developer/ Year/Ref.	Details/ Expression host	Methodology	Immunogenicity results
DENV1-4 EDIII	Chen H.W. 2013 (9)	Tetravalent subunit vaccine containing a consensus domain III prepared by aligning amino acid sequences from different isolates of the four serotypes of DENV (cEDIII)+ Alum/*E. coli*	Two times immunization at 8-weeks interval (I.D.) in monkeys (n=3) (200 µg per dose) Gr.1 cEDIII Gr.2 cEDIII + Alum	$FRNT_{50}$ titers against DENV1-4 (cutoff value 1:16) Gr.1 No significant neutralizing antibody activities Gr.2 Two out of three monkeys developed neutralizing antibodies against DENV1-4
DENV2 EDIII	Chiang C.Y. 2013 (11)	Recombinant lipidated DENV-2 EDIII (LD2EDIII) The lipid moiety of the bacterial-derived lipoprotein; lipid signal peptide using pET22b as a vector (Novagen, Madison, WI)/ *E. coli*	Two time immunization at a 2-week interval (S.C.) in mice (Balb/C) 10 µg/0.2 mL per dose Gr.1 LD2ED III Gr.2 D2ED III Inoculation (I.P) on the same schedule	$FRNT_{50}$ titers against DENV-2 Gr.1 = 1176 Gr.2 = 16 Gr.3 = 223

Table 3: HBcAg and WHcAg-based vaccine candidates

VLP genus	Displayed antigen (pathogen)	host	Immunogenicity results	Status	Ref.
HBcAg	CSP epitope (*P.falciparum*)	*E. coli*	Protection (monkey)	Phase 1	(126, 127)
	M2e epitope (influenza virus)	*E. coli*	Protection (mice)	Phase 1	(108, 127, 128)
	SP55/SP70 epitopes (EV71)	*E. coli*	Neutralization and protection (mice)	Preclinical trial	(113)
	epitopes (HCV)	*E. coli*	-	Preclinical trial	(129)
	HVR1 epitope (HCV)	*E. coli*	Neutralization (mice)	Preclinical trial	(130)
	EDIII antigen (DENV-2)	*E. coli*, Yeast *E. coli*	Neutralization (mice)	Preclinical trial	(23, 24)
	E1 epitope (rubella virus)	*E. coli*	-	Preclinical trial	(112)
	OspA antigen (*B.burgdoferi*)	*E. coli*	Protection (mice)	Preclinical trial	(106, 117)
	VP2 five-mimotope (IBDV)	*E. coli*	Protection (chicken)	Preclinical trial	(131)
	CFP-10 antigen (MTB)		-	Preclinical trial	(110)
WHcAg	M2e epitope (influenza virus)	*Salmonella*	Neutralization and protection (mice)	Preclinical trial	(27)
	CSP epitope (*P.falciparum*)	*E. coli*	Neutralization and protection (mice)	Preclinical trial	(114)

CHAPTER 3

MATERIALS AND METHODS

3.1 Creation of Consensus Sequence for Exposing on WHcAg

One hundred twenty eight sequences of truncated E sequences including fusion loop (E aa 98 - 110) of EDII and EDIII (E aa 295 - 395) of DENV-2 that isolated in Thailand during 1974 - 2004 were selected from GENBANK database. Then a consensus amino acid sequence was created from alignment by using BioEdit Sequence Alignment Editor Program version 7.0.5.3, in which each sequence had details as follows:

NCBI accession number:

- AB194882
- AF195032 - AF195043 12 sequences
- AF264053
- AF410377
- AY577435 - AY577438 4 sequences
- DQ181797 - DQ181901 105 sequences
- NC001474
- NP739582.2
- NP739583.2
- NP739592.2

Total nucleotide = 342 bp

Total amino acid = 114 amino acids

3.2 Modification of WHcAg Amino Acid Sequence

Woodchuck hepadnaviral core antigen (WHcAg) sequence (2009) is from GENBANK database in 2009. Then it was modified to truncated WHcAg which was left with 150 amino acids by cutting C-terminus in amino acid at 150-188. Then cysteine (Cys) residue was added at C-terminus has been previously shown to stabilize truncated HBcAg (132).

NCBI accession number:

- NC004107

Total nucleotide sequence = 567 bp

Total amino acid = 188 amino acids

3.3 Generation of Chimeric VLP Constructs

Consensus sequence was placed in the middle of fusion loop (FL) and EDIII by $(Gly_4Ser)_3$ linker and enclosed by the same linker at N and C-terminus of consensus sequence. Two glutamic acid residues were added to flank this sequence at next to linker in order to adjust pI to 5. The modified consensus sequence was placed at aa 78 of truncated WHcAg (chimeric VLP; WHcAg-FL-DIII) and a construct which was different from chimeric VLP (chimeric VLP without FL; WHcAg-DIII). As truncated WHcAg was a construct without insertion and was under a positive control in comparison between shapes and characteristics of wild-type and chimeric VLP.

3.4 Synthesis of Three Constructs

As two constructs are chimeric (WHcAg + DENV fragment protein) and as the fusion loop is naturally not close to the domain III in E protein from DENV-2. Three genes were synthesize to facilitate the cloning (Genscript, USA and GeneArt, Germany) which were inserted in the cloning vector pUC57 by (WHcAg-FL-DIII), pMA-T (WHcAg) and pET15b (WHcAg-DIII) (Fig. 5) which changed the nucleotide sequences into *E. coli* codon usage, and were added 2 stop codons (TGATAA) at the end 3' to stop the protein translation apart from the target protein, and then added restriction

sites of *Nco* I and *Xho* I at the end 5' and 3', orderly, for serving as double digestion and cloning site to investigate and select a specific target sequences.

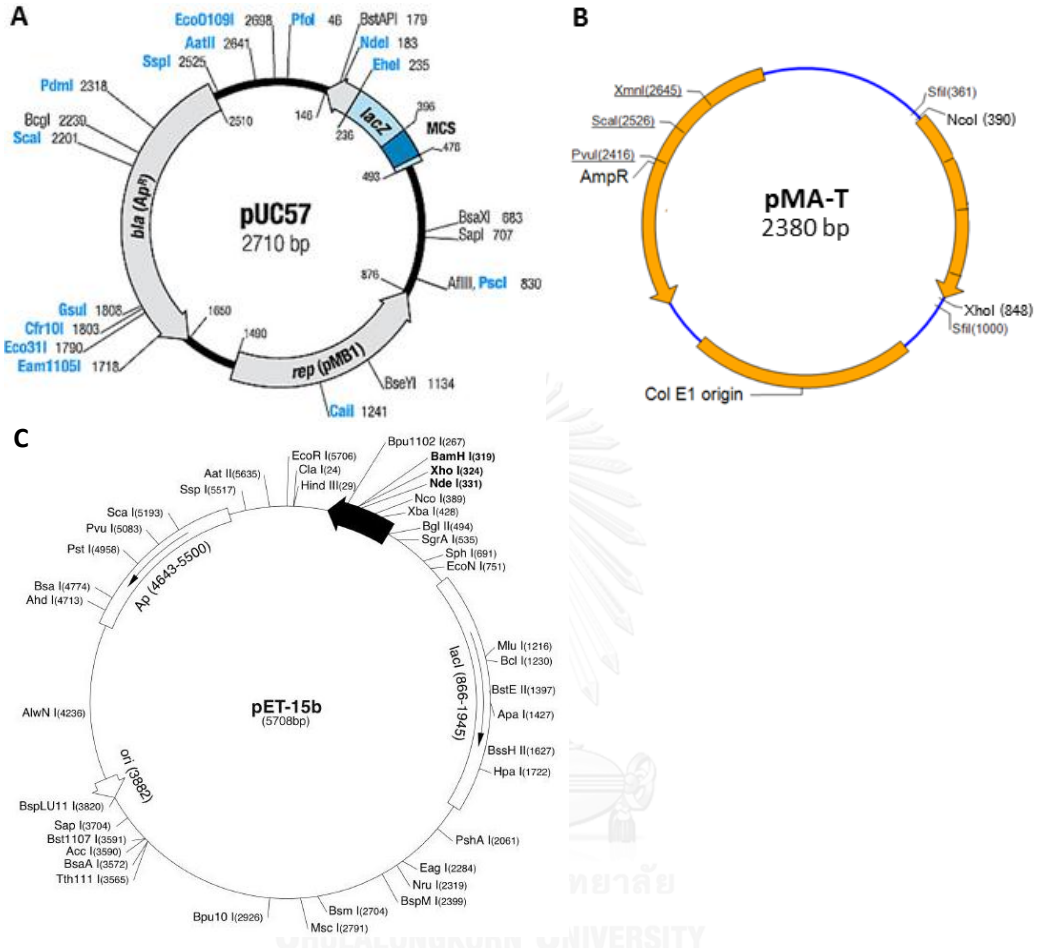

Figure 5: Empty plasmid DNA for three synthetic constructs including pUC57 (A), pMA-T (B) and pET15b (C) (data from (133-135))

3.5 Preparation of Competent Cells

DH5α E. coli strain (Invitrogen) was prepared to be a competent cell through the process of gently thawing glycerol *E. coli* stocks on ice and streaked LB-agar plate before incubating at 37ºC, overnight. Picked colonies (only large size, approximately 2 mm, 10 - 12 colonies) and cultured in 250 ml of SOB medium in flask by using a shaking incubator, 200 rpm for a night at 18ºC until the OD_{600} which measurement with spectrophotometer (Bio-Rad, USA) was 0.6, for 2 - 3 days. After that, bacterial

cells were incubated on ice for 10 min, divided 30 ml into each tube and centrifuged at the speed of 3,000 rpm for 7 min at 4ºC and discarded supernatant, and made the pellet re-suspension with ice/cold transformation buffer (TB) 10 ml by gently rotating. Put it on ice for 10 min and discarded supernatant after centrifugation as before, then added TB 2.5 ml to re-suspend pellet. Pooled all tubes together, becoming the total volume 20 ml, and gradually added DMSO 1.4 ml and gently mixed with the final concentration, 7%, then left on ice for 10 min. Ultimately, divided into tubes, each 250 µl, and instantly dipped in liquid nitrogen and then stored at -80ºC.

3.6 Plasmid Transformation

The competent cells were thawed gently on ice and plasmids DNA were added into the cells. In this step, the competent cells were left on ice for 30 min. After that the ingredients were quickly dipped in water of 42ºC for 90 seconds (this step is called heat-shock), then they were quickly dipped in ice for 10 min. Next, 900 µl SOC medium was added and shaken at 37ºC with the speed of 200 rpm for an hour. Lastly, they were placed on LB-antibiotic plates (Ampicillin 100 µg/ml) to observe colonies after being grown overnight at 37ºC. One colony from each construct was picked and grown in LB medium containing (Amp 100 µg/ml) to prepare stock, and plasmids DNA were extracted and then were digested with *Nco* I and *Xho* I to check the presence of the appropriate inserts, as described in the protocol below.

3.7 DNA Extraction

Recombinant plasmids DNA from 15% glycerol stock were thawed from -80°C and inoculated in LB-Amp medium (bacterial cell: LB; 1: 300). Cultured 5 ml of bacterial cells with 200 rpm at 37°C in shaking incubator, and after 16 h, had it centrifuged at a speed of 4,000 x g for 10 min at 4°C to be bacterial pellet. The High-speed kit (Geneaid Biotech, Taiwan) was used for extracting recombinant plasmids DNA following the protocol of manufacturer by making the cell pellet into re-suspension with PD1 buffer 200 µl and filled with PD2 buffer 200 µl as well, then

mixed them by inverting 10 times gently, and left it at least two min at room temperature. Put PD3 buffer 300 µl until it became homogeneous, and inverted the same. After that centrifuged the mixture for 3 min at 10,000 x g, kept the supernatant that was isolated into PD column, and then centrifuged for 30 seconds. After adding wash buffer 600 µl, centrifuged with the same speed and time and dried membrane by centrifuging for 3 min. DNA that bound to the membrane would be eluted by elution buffer 30 µl, measured the concentration of DNA and stored at -20°C.

3.8 Double Digestion

Purified plasmids DNA were double restricted by *Nco* I and *Xho* I restriction endonuclease (NEB, USA). Each reaction (20 µl) consisting of 1/10 volume of 10X buffer, 1/10 volume of 10X BSA, 20 U/µl of *Xho* I, 10 U/µl of *Nco* I, 1 µg of DNA and sterile deionized water was filled to final volume, then had the mixture to be incubated for 1 h, at 37ºC and can be measured by running 1% gel electrophoresis in TAE buffer to isolate DNA by size.

3.9 Plasmid Preparation

The lyophilized recombinant pUC57, pET15b and pMA-T plasmids DNA were re-suspended in 20 µl of sterilized DNase-RNase free water at a final concentration of 200 µg/ml. Then transformed the plasmids DNA (100 ng) into competent DH5**α** *E. coli* cells to increase the amount of recombinant plasmids DNA. Picked one colony from each construct by using a sterile loop and then grew at 37°C (shaking incubator at the speed 200 rpm) in fresh LB-Amp medium for 16 h. Next, checked size and selected the insert piece by running 1.5% gel electrophoresis.

3.10 Expression Vector Preparation (pET15b)

Nco I and *Xho* I sites in empty pET-15b are so close that make the size after cutting one and two enzymes equal. Thus, it is therefore difficult to confirm if the double digestion occurs. Start by double restriction (*Xho* I/*Nco* I) the recombinant

pET-15b contained derp2 gene, to get ready to be inserted by DNA piece of each construct (excluding WHcAg-DIII construct), and ran 1.5% agarose gel electrophoresis to isolate pET15b vector.

3.11 DNA Ligation

Made the gel cut, only DNA piece and pET15b expression vector part, that were isolated by the expected size and then purified it to extract the agarose gel by using QIAquick Gel extraction kit (Qiagen, Australia) according to the manufacturer; that incubated a piece of the gel (300 mg as 300 µl) to dissolve in buffer QG (ratio 1 mg: 3 µl) at 50ºC for 10 min, after that put isopropanol by the same volume of gel and homogenized them by vortexing. Brought the mixture into QIAquick column and centrifuged at 13,000 rpm, 1 min then washed with buffer PE 700 µl. Besides, dried the column for another 1 min, eluted with elution buffer 30 µl and measured the concentration of DNA by NanoDrop (Thermo Fisher Scientific, USA) before storing it at -20ºC. To create pET15b-WHcAg-DENV, T4 DNA ligase was used (Roche, Germany) to ligate the purified insert and pET15b expression vector together, according to the formula was shown below.

$$\text{Insert quantity (ng)} = \frac{\text{Insert (ratio)}}{\text{Vector (ratio)}} \times \left[\frac{\text{Vector (50 to 100 ng) x insert length (ng)}}{\text{Vector length (kb)}} \right]$$

All were in the mixture of 20 µl per reaction consisting of sterile water, 5X DNA dilution buffer (1/10 volume), 2X T4 ligation buffer (10 µl), T4 ligase (1 µl), vector 100 ng and its ratio of insert to vector is 3 to 1 ng. Then ligated by incubating at 16ºC, overnight and transformed the mixture of ligation into DH5**α** *E. coli* strain. After that, chose the colony that resists ampicillin. To determine the colony could do the same way-extracted plasmid DNA, used *Nco* I/*Xho* I double restrictions and ran colony PCR which took colony as a template by using two synthesized specific primers, for preparation of PCR reaction was described in the protocol below, and

then sent to process DNA sequencing as well. The positive colonies were stored in 15% glycerol at a -80°C refrigerator.

3.12 Colony PCR Screening

Colony PCR screening was prepared by a mixture per 25 µl reaction (contains 5X PCR buffer (1/5 volume), 25 mM $MgCl_2$ (1.5 mM), 10 mM dNTPs (8 mM), 10 pmole forward and reverse primer (0.2 pmole), Tag polymerase (1.5 Unit), DNase-RNase free water at a final volume of 25 µl), put the DNA template and then ran PCR in a thermal cycler (Appiled biosystems, USA) by setting 3 steps following; step 1 is a step before template denaturation at 95ºC for 4 min. Step 2 is the PCR process consisting of template denaturation 95ºC for 30 seconds, primer annealing which reduced the temperature to 54ºC for 30 seconds, and extension by addition of nucleotides in DNA synthesis at 72ºC, 1 min. All the three sub-stages is a cycle which repeated continuously all along 35 cycles. The last step is about more extension at 72ºC for 7 min, and kept the temperature constant at 4ºC. The PCR product was determined the size by 1.5% agarose gel electrophoresis.

The used pair of primer was designed from nucleotide sequence, around the end of two sides of the fusion loop EDII plus EDIII DENV-2, by using Oligo Primer Analysis Software (Molecular Biology Insights, Inc., USA) which a forward primer designed from the 5' and a reverse primer from the 3'end. Furthermore, T7 promoter and terminator had been cooperated with, that were specific to the area of pET15 vector, which flanked the DENV E gene. The two pairs of primer could be alternated matching between forward and reverse primers for more accuracy of PCR product-size detection.

3.13 Protein Expression Assay

Preparing *Origami* (Novagen), *SHuffle* and *Lemo 21 E. coli* strains (NEB, UK) competent cells followed the same instruction as used with DH5α *E. coli* cells. When it was transformed by recombinant pET15b plasmids, colony of each construct should be investigated with colony PCR and *Xho* I/*Nco* I double digestion pattern. After that, positive recombinant clones were cultured overnight at 37ºC (*Origami*) and

30ºC (*SHuffle* and *Lemo 21*) in LB-Amp medium to become a starter. Then it was grown in fresh LB medium 500 ml until the OD_{600} reached 0.4 to 0.6 then added Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Vivantis, USA) which is an inducer. Every construct was induced under several conditions such as final concentration of IPTG from 0.04 to 0.5 mM, inducing period from 1 to 17 h and temperature during inducing between 16 and 37°C. For harvesting, the bacterial cells were centrifuged at 4,000 rpm for 10 min and then resuspended in 50 ml of Tris-HCl pH 7.5. After that the cells were dissociated by using high pressure homogenizer (TS series benchtop 0.75 kW model, UK). The lysates were centrifuged at 4,000 rpm for 15 min (Beckman, USA) to separate inclusion bodies which found in pellet fraction. Then supernatant fraction was ultracentrifuged at 40,000 rpm for 1 h (SW41 Ti rotor, Beckman) to separate soluble (cytoplasmic proteins) from insoluble (membrane associated) proteins. Lastly, the three fractions were kept at -20ºC for protein expression analysis.

3.13.1 SDS-PAGE and immunoblot analysis

Investigating protein expression level and recognizing proteins by using specific antibodies for the proteins included three fractions (inclusion bodies, cytoplasmic and membrane associated fractions) relying on SDS polyacrylamide gel electrophoresis (SDS-PAGE), in which stacking gel was added with 6% of polyacrylamide concentration including 40% Acrylamide/Bis acrylamide, 0.5 M Tris-HCl pH 6.8, 10% SDS, 10% ammonium persulfate (APS), distilled water and TEMED. For 12% of polyacrylamide concentration in separating gel including 40% Acrylamide/Bis acrylamide, 1.5 M Tris-HCl pH 8.8, 10% SDS, 10% APS, distilled water and TEMED. The next step was Western Blotting by transferring proteins which were differentiated based on molecular weight from SDS-PAGE. After that it was transferred to nitrocellulose membrane by using electricity with semi-dry blotting (Bio-Rad, USA) method. Then the membrane was blocked at 4ºC, overnight with 5% (w/v) of non-fat dried milk in TBS-Tween buffer. Next, the membrane was incubated for 1 h at room temperature in order to detect WHc and EDIII of DENV-2 by using a primary antibody which is a mouse monoclonal antibody (mAb) 14E11 (Millipore, USA) at 1/5000 dilution in blocking buffer and mAb 3H5 (kindly provided by Dr.Chunya Puttikhunt,

National Center for Genetic Engineering and Biotechnology) at dilution 1/1000, respectively. Then it was washed with TBS-Tween buffer 5 min at a time for 3 times. Lastly, incubated the membrane with secondary antibody which is anti-mouse AP-conjugated antibody (Millipore, USA) at dilution 1/5000 for 1 h and washed 10 min at a time for 3 times with TBS-Tween buffer, in which a position of secondary antibody was found by AP substrate as a chromogenic detection (Bio-Rad, USA).

3.14 Purification of Cytoplasmic Fraction

3.14.1 Sucrose Density Gradient

Cytoplasmic fraction 0.5 ml (three constructs) was loaded into each tube containing 10% to 60% (w/v) sucrose gradient with 1.6 ml per concentration. Then the tube was first loaded by 60% (w/v) sucrose at the bottom (including 10%, 20%, 30%, 40%, 50% and 60% in TNE buffer. After that it was ultracentrifuged for 2 h with 41,000 rpm at 4°C. When the time set was due, the 14 fractions were collected gently from top to bottom of the tube. Then analysis was applied to find VLP zone with SDS-PAGE (Coomassie blue staining) and Western blotting in the form of chemiluminescent detection. Tris-HCl pH 8 was placed by phosphate buffer saline (PBS) (Vivantis, USA) in this detection and the secondary antibody which is different from chromogenic detection by being incubated with anti-mouse HPP-conjugated antibody (KPL, USA) at dilution 1/5000 for 1 h, washed 10 min at a time for 3 times with PBS-Tween buffer, added with HRP substrate (Millipore, USA) and exposed to X-ray film.

3.15 Characterization of VLP by Electron Microscopy (EM)

The protein derived from fractions of VLP zone 20 µl were shaped with 2% gluteraldehyde in 0.1 M PBS. Then it was absorbed for 5 min with copper grid which coated with Formvar and carbon (Becthai, Thailand). Next, distilled water was dropped through the grid and negatively stained with 2% uranyl acetate for 1 min.

After that it was left to dry and observed by using transmission electron microscope, 100 kV (Hitachi HT7700 model, Japan).

3.16 Inclusion Body Solubilization

The re-suspended inclusion bodies (IB) was centrifuged and the acquired pellet was washed then, by 30 ml of 0.5% Triton-X100 (Calbiochem) in 50 mM Tris-HCl pH 8, and the optimized one that was mixed with 0.5% Triton-X100 and various buffers containing DL-Dithiothreitol (DTT) (Bio Basic, Canada) and Urea (Millipore, USA) was shown in table 4 and then stirred it with a rotator at 4ºC overnight. After that, centrifuged it at 15,000 rpm for 15 min to remove sticky materials and re-suspended pellet with denaturing buffer, which was optimized for proper condition to extract IBs by adding 6 M Urea, DTT, NaCl (Millipore, USA), Ethylenediaminetetraacetic acid (EDTA) (Bio-Rad, USA).

Table 4: The condition for washing and solubilizing of inclusion body

IB wash buffer (in 50 mM Tris-HCl pH 8)	**IB solubilization (in 50 mM Tris-HCl pH 8)**
0.5% Triton-X100	6 M Urea + 500 mM NaCl + 1 mM DTT
0.5% Triton-X100 + 1 M Urea	6 M Urea
0.5% Triton-X100 + 1 M Urea + 5 mM DTT	6 M Urea + 500 mM NaCl + 5 mM DTT
0.5% Triton-X100 + 2 M Urea	6 M Urea + 500 mM NaCl + 5 mM DTT + 2 mM EDTA

3.18 Gel Filtration Chromatography

soluble fraction 500 µl was applied onto the Superose 6 and Superdex 75 columns and used AKTA Prime system (GE Healthcare Lifesciences, UK), which the pore size of matrix in the range of 5 - 5,000 kDa and 3 - 70 kDa, respectively, by equilibrating in filtrated PBS pH 7.2 (at flow rate 0.5 ml/min) and checked the purity

of each fraction selected from the range of high molecular weight in chromatogram and ran SDS-PAGE (Coomassie blue staining and Western blotting).

3.17 Protein Refolding

There are two strategies for refolding by dialysis method. The first strategy is two dialysis steps from 6 M to 3 M Urea and 3 M Urea to 0 M Urea which contained 100 mM L-Arginine (Sigma, Singapore) with optimization condition as shown in table 5 and then was applied onto Superpose 6 GF column to purify in native condition by using PBS (see gel filtration protocol) and performed SDS-PAGE analysis. Another strategy is purification of the monomeric proteins in denaturing conditions and followed by protein refolding. Before purification, the denatured proteins were precipitated by addition of 4 M saturated ammonium sulfate to make protein higher in concentration which some remaining volume was removed by gel filtration. For refolding the proteins after purification step begin with evaluated the positive peaks of chromatogram by SDS-PAGE and pooled targeted bands. Then gradually reduced the concentration of Urea (3 M to 0 M Urea) in 50 mM Tris-HCl pH 8 or added reduced/oxidized (GSH/GSSG) glutathione (5/0.5 mM and 2/0.2 mM) in 50 mM Tris-HCl pH 9 by dialysis method (Table 5). The dialysis buffer volume was 100 times of the denatured protein (5 ml), which was contained in a dialysis tubing (Thermo Fisher Scientific, USA). By then stirred buffer at 4ºC in each step for around one night, or at least 8 h, after that, centrifuged at 15,000 rpm for 15 min to separate the soluble and aggregated protein. Finally, determined the reassembly by sucrose gradient ultracentrifugation and ran SDS-PAGE.

Table 5: Dialysis in native and denaturing conditions

Dialysis step 1	Dialysis step 2
Native conditions	
150 mM NaCl	50 mM Tris-HCl pH 8
150 mM NaCl + 3 M Urea	50 mM Tris-HCl pH 8
150 mM NaCl + 100 mM Arginine	50 mM Tris-HCl pH 8
Denaturing conditions	
3 M Urea	50 mM Tris-HCl pH 8
3 M Urea + 50 mM NaCl + 5 mM GSH + 0.5 mM GSSG in 50 mM Tris-HCl pH 9	50 mM NaCl + 2 mM GSH + 0.2 mM GSSG in 50 mM Tris-HCl pH 9

CHAPTER 4

RESULTS

4.1 Design of a Synthetic Gene Encoding WHcAg-FL-DIII

In order to produce a chimeric WHcAg-based VLP, the DNA sequence encoding the fusion loop + DIII of the DENV-2 E protein was inserted into the truncated WHcAg cDNA (deleted RNA/DNA binding motifs at codon 150 - 188) at position corresponding to codon 78. E protein fragments were flanked with linkers encoding the $(Gly_4Ser)_3$ amino acid sequences. The goal of this linker addition is to avoid destabilization of VLP formation by the presence of the inserted E-based sequences. Moreover, the WHcAg-FL-DIII DNA sequence was modified through addition of extra codons for two glutamic acid residues between the truncated WHcAg and linker as it was previously demonstrated that such acidic residues facilitate the VLP formation (120). The resulting chimeric WHcAg structure is summarized in figure 6. Together with this chimeric construction, the corresponding truncated WHcAg cDNA sequence without any insertion was constructed. These two cDNA molecules were cloned into the prokaryotic expression vector pET-15b. Following transformation in *E. coli* cells, recombinant clones were selected through Ampicillin resistance. The two recombinant DNA constructs were subsequently isolated from large scale culture of DH5**α** *E. coli* strain corresponding clones. Competent *Origami E. coli* cells, an appropriate strain for T7-based protein expression were subsequently transformed with the two purified plasmids. Ampicillin resistant colonies were also screened by colony PCR for the detection of target DNA (data not shown) as well as by a *Xho* I/*Nco* I double restriction of recombinant pET-15b vectors to demonstrate the presence of the corresponding inserted sequences (truncated WHcAg and chimeric WHcAg) (Fig. 7).

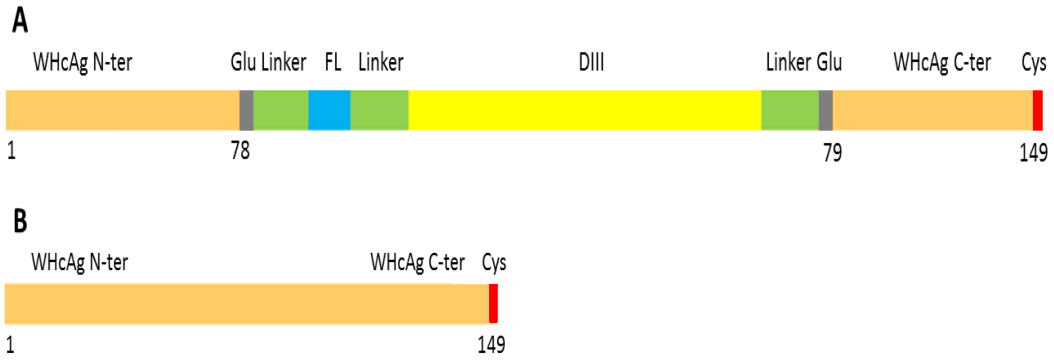

Figure 6: Scheme of the WHcAg-FL-DIII (Panel A) and truncated WHcAg (Panel B). The WHcAg N-terminus and C-terminus domains, the extra glutamic acid residues, the $(Gly_4Ser)_3$ linkers, the fusion loop of EDII, the full-length EDIII and a cysteine residue are highlighted in beige, grey, green, blue, yellow and red, respectively.

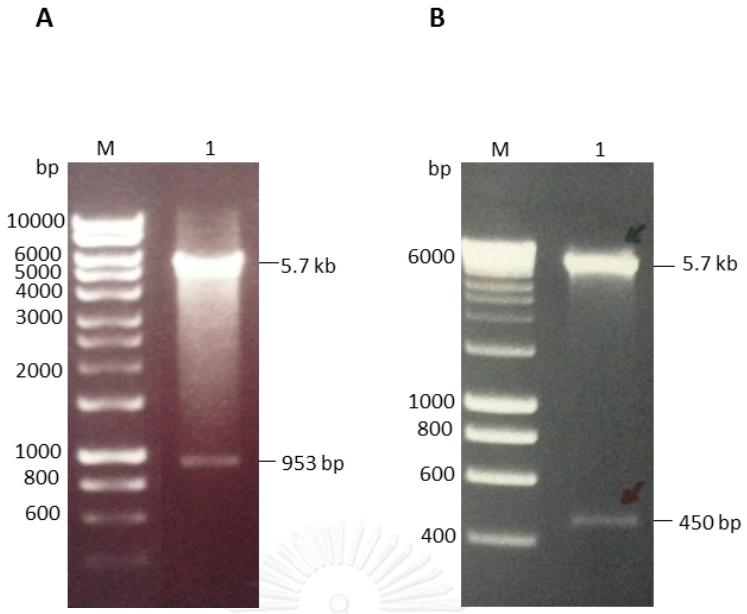

Figure 7: Digestion of the recombinant pET15b-WHcAg-FL-DIII (Panel A, lane 1) and pET-15b-truncated WHcAg (Panel B, lane 1) with Nco I and Xho I. Lane M is hyperladder 1 kb DNA marker. The 5.7 kb band corresponds to the linearized pET-15b vector, the 953 bp and 450 bp bands correspond respectively to cDNA encoding WHcAg-FL-DIII and truncated WHcAg.

4.2 Protein Expression in *Origami E. coli* Strain

For the self-assembly of chimeric WHcAg into VLP, two important issues need to be addressed: the protein expression level as well as the solubility of the expressed proteins into the bacteria. For those reasons, the *Origami E. coli* strain was selected, which carries DE3 gene encoding T7 RNA polymerase for high expression level and is deleted at the level of thioredoxin reductase (*trxB*) and glutaredoxin reductase (*gor*) genes. These two deletions would improve the production of soluble proteins through correct disulfide bond formation (136). When recombinant *Origami E. coli* clones were cultured in the presence of 0.5 mM IPTG at 37ºC for 3 h, both constructs successfully expressed the corresponding proteins with the expected size (Fig. 8).

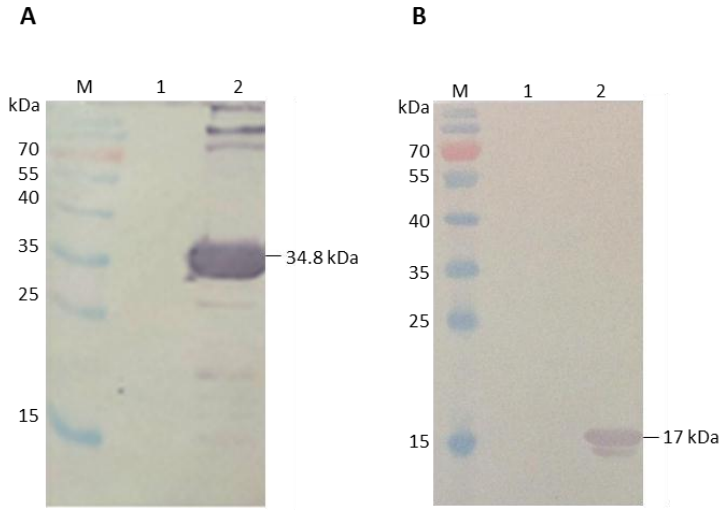

Figure 8: Expression of WHcAg derivatives in Origami E. coli cells. Intracellular expression of WHcAg-FL-DIII (Panel A, 34.8 kDa), and truncated WHcAg (Panel B, 17 kDa). Protein expression was assayed by western blot analysis (WB) using anti-DENV-2 EDIII mAb; 3H5 (Panel A, lane 2) and anti-WHcAg mAb; 14E11 (Panel B, lane 2). Lane 1: non induced, Lane M: pre-stained protein ladder.

To evaluate whether the expressed proteins in *E. coli* were soluble, preparative *E. coli* cell lysates were obtained through cell disruption under high-pressure. Putative inclusion bodies were isolated following a low speed centrifugation step (4,000 rpm, 15 min) and the fully soluble cytoplasmic proteins were separated from the residual membranes through a second centrifugation step at high speed (40,000 rpm, 1 h) as shown in figure 9. The presence of truncated WHcAg, as well as, WHcAg-FL-DIII in these three different protein fractions, were evidenced by WB. This result clearly indicated that the WHcAg-FL-DIII was strictly expressed as insoluble inclusion bodies, (Fig. 10 A). In contrast, the truncated WHcAg was highly soluble but present in inclusion bodies fractions as well (Fig. 10 B).

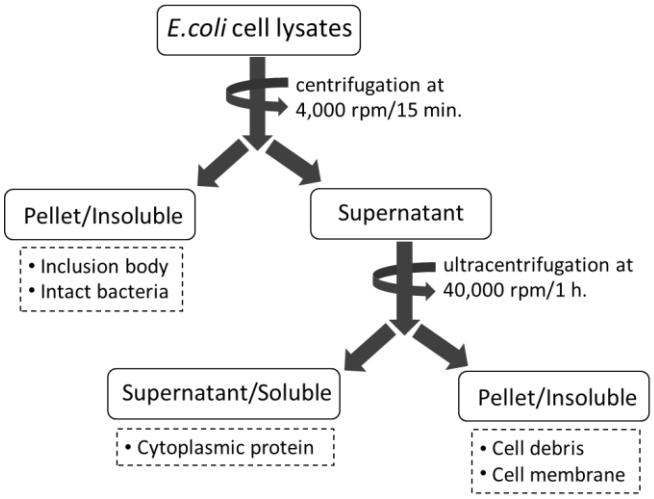

Figure 9: Inclusion body and soluble cytoplasmic protein isolation by successive centrifugation steps

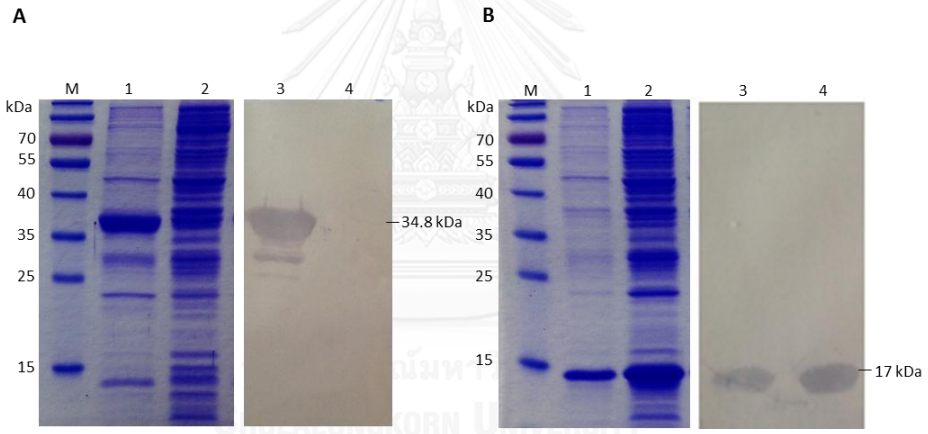

Figure 10: Solubility of expressed WHcAg-FL-DIII (Panel A) and truncated WHcAg (Panel B). SDS-PAGE and WB analysis using coomassie blue staining, anti-DENV-2 EDIII mAb (Panel A) and anti-WHcAg mAb (Panel B). Lanes 1 and 3: inclusion bodies, lanes 2 and 4: cytoplasmic fraction following ultracentrifugation.

4.3 Optimization of Soluble Protein Production

In order to optimize the yield of soluble WHcAg-FL-DIII, controlling protein expression by keeping IPTG at low concentration (control T7 promoter), under low temperature during inducing long period supports low protein expression which

enhances protein folding into a correct structure. This avoids protein over expression which is insoluble and found in inclusion bodies. The solubility of WHcAg-FL-DIII was evaluated whether the different intermediate temperatures (4, 16, 22 and 37ºC), the different intermediate IPTG concentrations (0.1, 0.5, 1 and 10 mM) and the different intermediate times (30 min, 1, 3, 7, 17, 34 and 51 h) during the induction step could influence. The table 6 represents typical optimization assays to improve the yield of soluble WHcAg-FL-DIII. Unfortunately, whatever the different experimental conditions, WHcAg-FL-DIII remained insoluble (data not shown)

Table 6: Optimization of the induction parameters for the production of soluble WHcAg-FL-DIII in Origami E. coli strain

Temperature	IPTG conc. (mM)	Duration of induction
37ºC	0.1	1 h, 3 h and 7 h
	0.5	1 h, 3 h and 17 h
	1	30 min, 1 h, 3 h and 7 h
	10	30 min, 1 h and 3 h
22ºC	0.5	1 h, 3 h and 17 h
16ºC	0.5	17 h and 34 h
4ºC	0.1	34 h and 51 h
	0.5	3 h, 17 h, 34 h and 51 h
	1	34 h and 51 h
	10	34 h and 51 h

4.4 Expression of WHcAg-FL-DIII in the *SHuffle E. coli* Strain

The *SHuffle E. coli* strain was used to optimize the folding of WHcAg-FL-DIII protein and consequently the yield of soluble protein. Indeed, the *SHuffle E. coli* strain notably is considered to improve the disulfide bond formation through deletions of thioredoxin reductase (*trxB*) and glutaredoxin reductase (*gor*) genes and expression of disulfide isomerase (DsbC). DsbC enzyme acts as chaperone to promote proper folding by shuffling the mis-oxidized cysteine pairs (137). Through

several expression assays using *SHuffle E. coli* strain, the duration of induction (1, 3, 7 and 17 h), IPTG concentration (0.4 and 0.04 mM) and temperature of induction (16, 22, 30 and 37°C) were varied (Table 7), in the hope to increase the percentage of soluble protein (data not shown). According to the following induction conditions (0.04 mM IPTG for 17 h, at 16°C), a small percentage of fully soluble WHcAg-FL-DIII protein was detected in the cytoplasmic fraction (Fig. 11), the large majority of the WHcAg-FL-DIII always being expressed as inclusion bodies.

Table 7: Optimization of the induction parameters for the production of soluble WHcAg-FL-DIII in SHuffle E. coli strain

Temperature	IPTG conc. (mM)	Duration of induction
37ºC	0.4	3 h
30ºC	0.4	3 h
	0.04	1, 3, 7 and 17 h
22ºC	0.4	17 h
	0.04	1, 3, 7 and 17 h
16ºC	0.4	17 h
	0.04	1, 3, 7 and 17 h

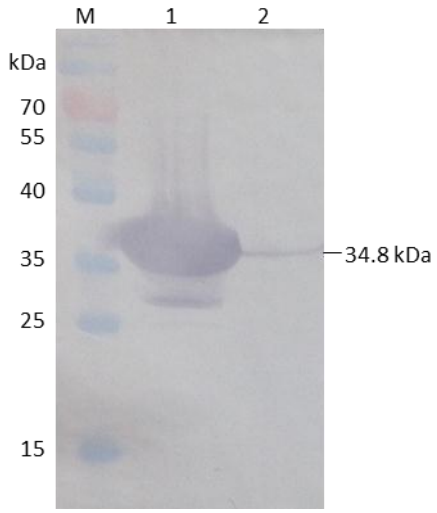

Figure 11: Expression of WHcAg-FL-DIII in the *SHuffle E. coli* strain. WB analysis using anti-DENV-2 EDIII mAb, lane 1: inclusion bodies, lane 2: cytoplasmic fraction following ultracentrifugation.

4.5 Characterization of Two Soluble WHcAg Protein Derivatives by Sucrose Density Gradient Ultracentrifugation

To demonstrate the VLP formation through self-assembly of truncated WHcAg and recombinant WHcAg-FL-DIII, the corresponding soluble forms of these proteins were applied onto a sucrose density gradient (10 - 60%) and ultracentrifuged at 41,000 rpm for 2 h. This semi-purification method is typically used to evidence VLP particle formation. Following the sample migrations, the sucrose gradients were subsequently fractioned into 14 tubes and the presence of truncated and chimeric WHcAg into these fractions was analyzed by SDS-PAGE and WB. In agreement with the literature (138) together with, the distribution of truncated WHcAg along the density gradient (Fig. 12 C and D can be delimited into three main zones. The fractions 1 to 6, 7 to 10 and 11 to 14 (fractions 1 and 14 corresponding to the zone of the gradient with the lowest and the highest density respectively) correspond to density values for monomers/non-VLP oligomers, VLP and aggregates respectively. These results clearly showed that truncated WHcAg produced as a soluble protein in *Origami E. coli* strain self-assembled appropriately as VLP (fractions 7 - 10) but some

protein aggregates were also evidenced (fractions 11 - 14) (Fig. 12 C and D). The soluble chimeric WHcAg migrated also into two density zones, VLP particles were detected in fractions 7 - 10 together with their presence into the monomeric/non-VLP oligomeric zone (fractions 2 - 6) (Fig. 12 A and B). These results indicated that the chimeric WHcAg produced as a soluble protein in *SHuffle E. coli* strain self-assembled partially into VLPs. Moreover, such VLP particles co-migrated into the density gradient with numerous host-cell proteins contaminants, demonstrating that the sucrose density gradient ultracentrifugation needs to be combined with other purification steps to obtain pure VLP.

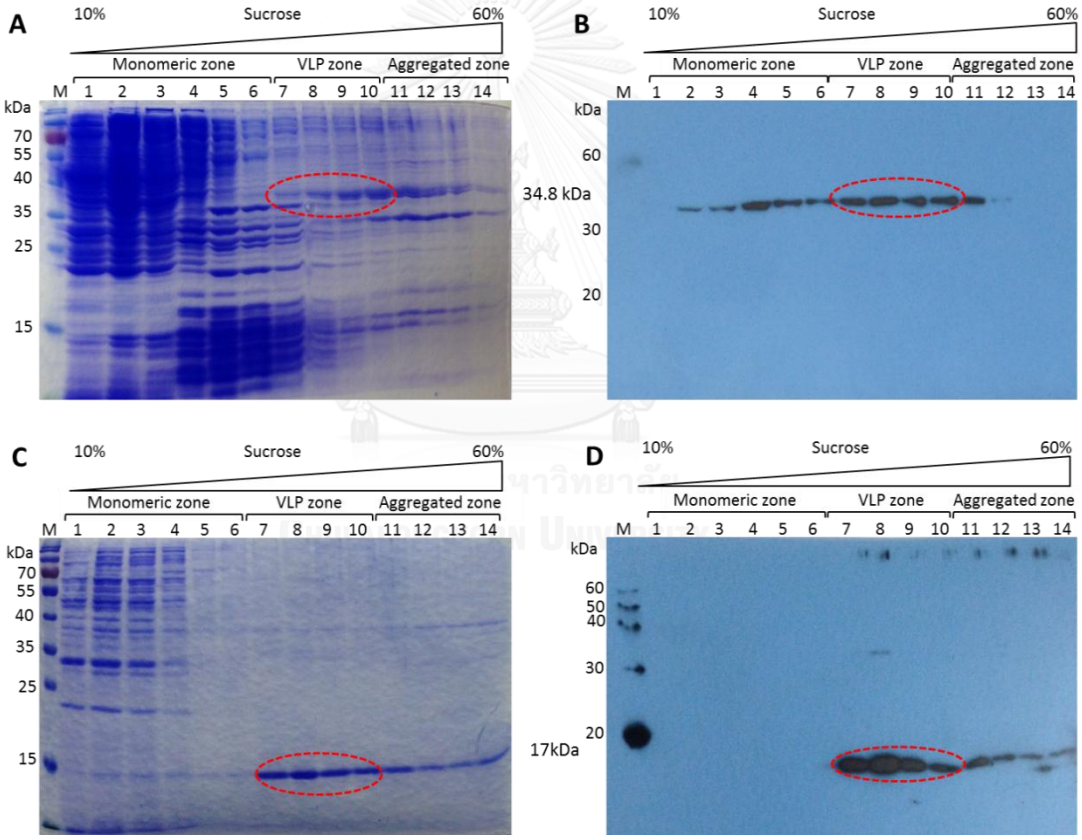

Figure 12: Distribution of truncated and chimeric WHcAg into a 10 – 60% (w/v) sucrose density gradient. The different fractions from the gradients were analyzed by SDS-PAGE (Panels A and C) and WB (using anti-DENV-2 EDIII mAb in Panel B, anti-WHcAg mAb in Panel D). Migration of soluble WHcAg-FL-DIII (Panels A and B) and truncated WHcAg (Panels C and D). The red ovals indicate in the VLP zone.

4.6 Analysis of VLP by Transmission Electron Microscopy

The VLP structure from sucrose density gradient (fractions 7 – 10) was also characterized by transmission electron microscope. These results from the fraction 8 of sucrose gradient showed that truncated WHcAg-based VLP displayed homogenous particle size (average diameter 35 nm which corresponded to the expected size for HBcAg or WHcAg particles (102) (Fig. 13 A). In contrast, the VLP structures based on WHcAg-FL-DIII from the fraction 8 of sucrose density gradient were more heterogeneous (the measured diameter values ranged from 20 to 35 nm Fig. 13 B).

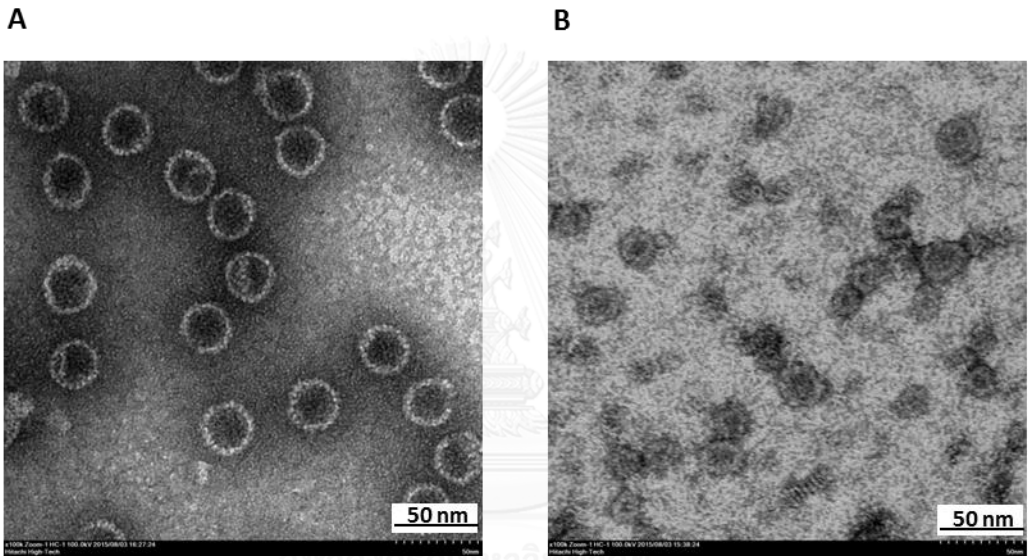

Figure 13: VLP structure formation based on WHcAg-FL-DIII (Panel A) and truncated WHcAg (Panel B) from the fraction 8 were negatively stained (2% uranyl acetate). Scale bar (50 nm) is indicated on the right bottom. Magnification: x 100,000

4.7 Production of WHcAg-DIII

The poor solubility of the expressed WHcAg-FL-DIII in bacteria could likely be explained by the hydrophobicity of the fusion loop (DRGWGNGCGLF). To demonstrate this hypothesis, a new chimeric WHcAg containing only the DIII (WHcAg-DIII) was expressed (Fig. 14), using the same expression vector and induction conditions in the *SHuffle E. coli* strain as WHcAg-FL-DIII (see table 7).

Unfortunately, under the experimental conditions, similar results were observed with a WHcAg-FL-DIII which is highly expressed protein as inclusion bodies, (Fig. 15).

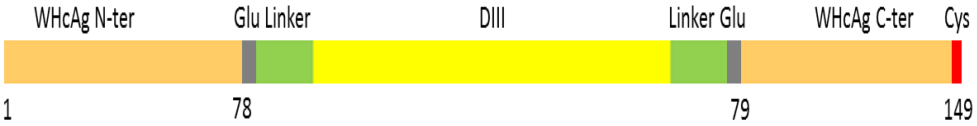

Figure 14: Scheme of the WHcAg-DIII. The WHcAg N-terminus and C-terminus domains, the $(Gly_4Ser)_3$ linkers, the extra glutamic acid residues, the full-length EDIII and a cysteine residue are highlighted in beige, grey, green, yellow and red, respectively.

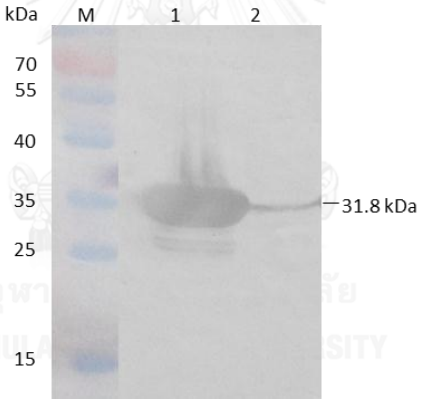

Figure 15: Expression of WHcAg-DIII in *SHuffle E. coli* strain. WB detected by anti-DENV-2 EDIII mAb. Lane 1: inclusion bodies, lane 2: cytoplasmic fraction following ultracentrifugation.

4.8 Expression of WHcAg-FL-DIII in *Lemo 21 E. coli* Strain

Finally, WHcAg-FL-DIII was expressed as a soluble form by using *Lemo 21* (DE3) *E. coli* strain. Through the tight control of T7 RNA polymerase activity by the rhamnose-dependent T7 lysozyme expression, it is possible to reduce the

recombinant protein expression level to limit the inclusion bodies formation (Fig. 16). Several induction conditions were tested: IPTG concentration (0.04 and 0.4 mM), L-rhamnose concentration (0, 100, 250, 500, 1,500 and 3,000 µM), temperatures (16, 20, 25 and 30°C) and duration of expression (4, 7 and 17 h) (Table 2). Unfortunately, whatever the conditions of induction, WHcAg-FL-DIII was always expressed as inclusion bodies in this *E. coli* strain (Fig. 17).

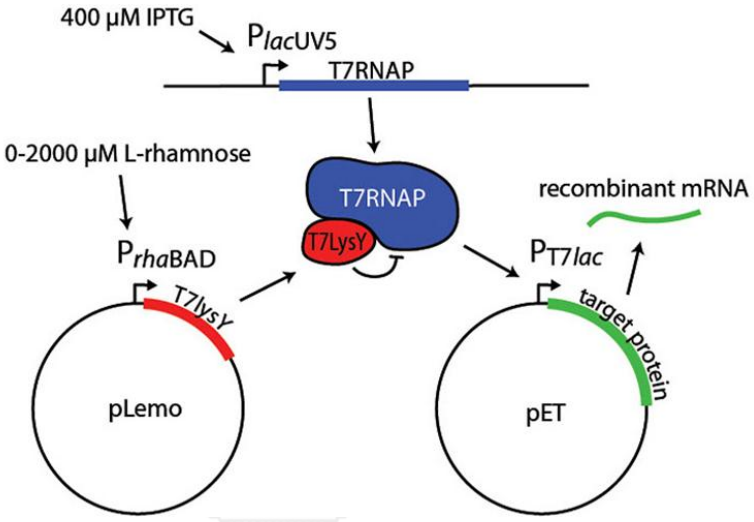

Figure 16: Regulation of heterologous protein expression in Lemo 21 (DE3) E. coli strain. The T7 promoter (PT7 Lac; the small arrow) in pET vector is activated by the expression of T7 RNA polymerase (dark blue) following IPTG induction .The optimal concentration of L-rhamnose (0 - 2,000 µM) can tightly control the expression of T7 lysozyme, at the level of the rhamnose promoter (PrhaBAD). T7 lysozyme regulates the activity of T7 RNA polymerase.

Table 8: Optimization of the induction parameters for the production of soluble WHcAg-FL-DIII in Lemo 21 E. coli strain

Temperature	IPTG conc. (mM)	Duration of induction	L-rhamnose conc. (µM)
30°C	0.4	4 and 17 h	0, 100, 250, 500, 1500 and 3000
	0.04	4 h	100
		7 h	0, 100 and 250
25°C	0.4	4 and 17 h	0, 100 and 250
20°C	0.04	4 h	100
		7 h	0, 100 and 250
16°C	0.4	4 and 17 h	0, 100 and 250

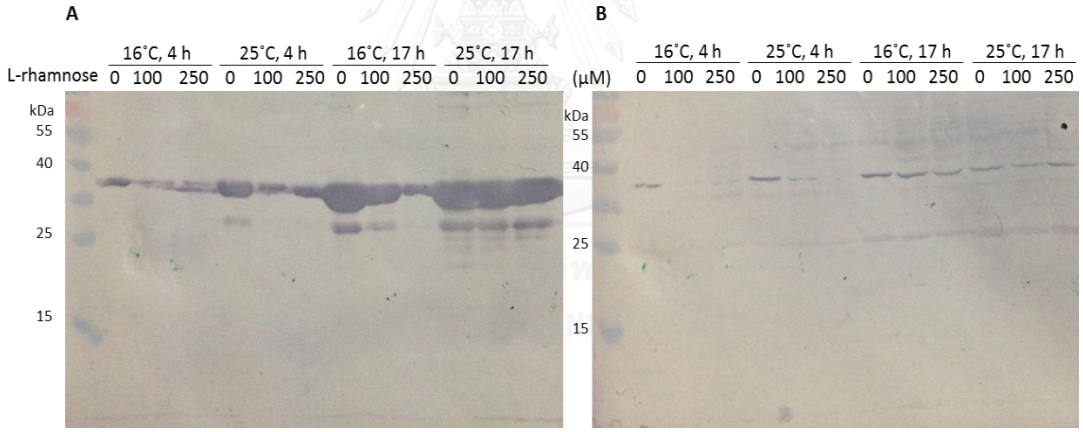

Figure 17: Optimization of WHcAg-FL-DIII expression in *Lemo 21 E. coli* strain. Protein was detected in inclusion bodies (Panel A) and ultracentrifuged cytoplasmic soluble proteins (Panel B) by WB anti-DENV-2 EDIII mAb. The protein was expressed at different temperatures (16 and 25°C), using different periods of induction (4 and 17 h) and 0.4 mM IPTG with L-rhamnose concentration (0, 100 and 250 µM).

4.9 Refolding of WHcAg-FL-DIII from Inclusion Bodies

As the yield of the expressed soluble WHcAg-FL-DIII is small amount of yield in the tested experimental conditions, the possibility to obtain VLP particles from the self-assembly of extracted and refolded WHcAg-FL-DIII from the inclusion bodies were evaluated. Although such insoluble form of WHcAg-FL-DIII is abundantly produced, the refolding and the subsequent VLP formation represent a challenge. However, through previously published studies, such strategy met some success in the production of soluble VLP particles (26, 117, 126, 127, 129, 139). In brief, WHcAg-FL-DIII expressed as inclusion bodies needs to be solubilized under denaturing conditions and subsequently refolded through denaturing buffer replacement with a native one in order to maintain the protein solubility but, this time, under native conditions. The first optimization under denaturing conditions can solubilize the WHcAg-FL-DIII. It was found that the inclusion bodies could be fully solubilized using 6 M Urea + 500 mM NaCl + 1 mM DTT in 50 mM Tris-HCl pH 8. The inclusion bodies extract was subsequently refolded by two successive dialysis steps: a first one using L-Arginine at 100 mM or 3 M Urea was added in the 150 mM NaCl and 50 mM Tris-HCl buffer, followed by the second step, in 50 mM Tris-HCl pH 8. Although the proteins were successfully refolded by such strategy, as evidenced by the absence of precipitation (Fig. 18 A), the VLP particle formation was failed to evidence by electron microscopy (Fig. 18 C), even if the refolded chimeric WHcAg partly migrated in the VLP from a sucrose density gradient (Fig. 18 B).

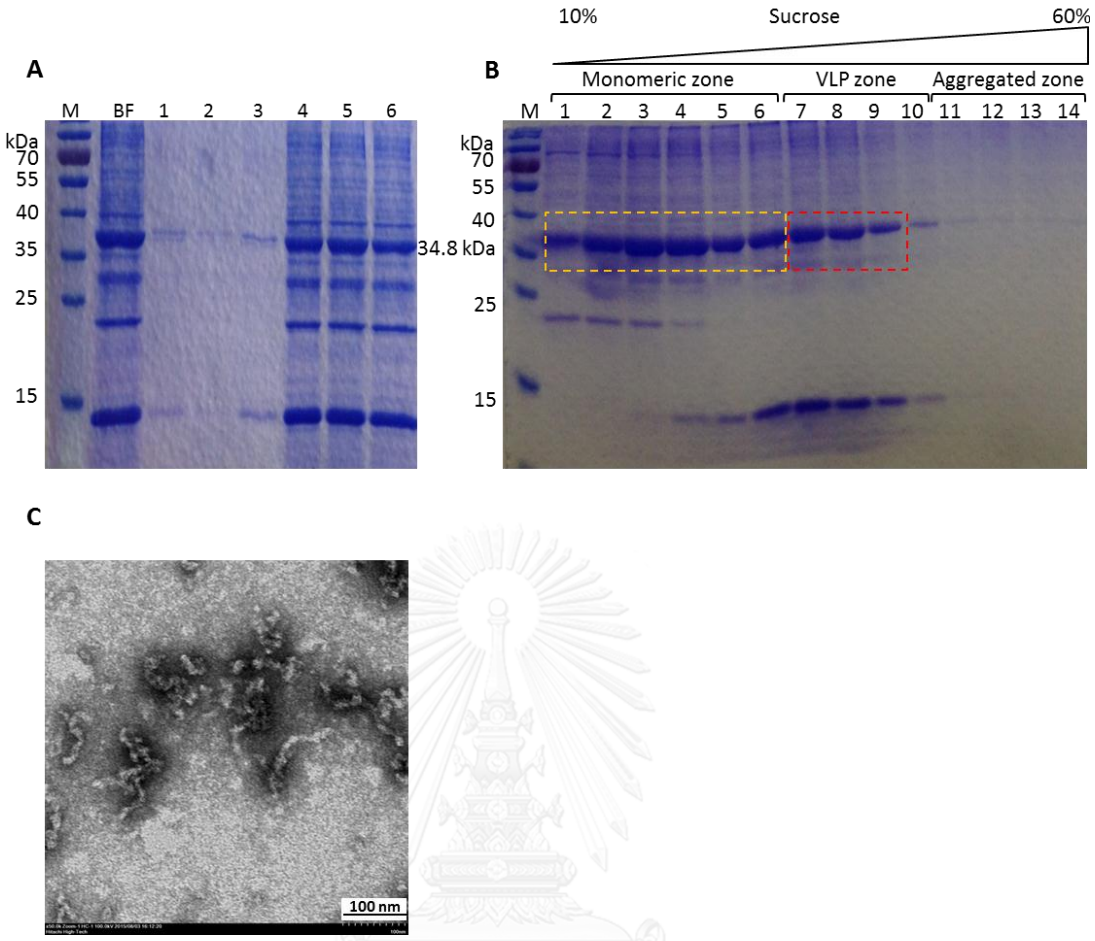

Figure 18: Refolding of WHcAg-FL-DIII from inclusion bodies denaturation. SDS-PAGE profile of centrifuged soluble (Panel A, lanes 4-6) and insoluble (Panel A lanes 1-3) proteins, BF: before refolding by dialysis first step in three different conditions: 150 mM NaCl in Tris-HCl (lanes 1 and 4), Tris-HCl with 3 M Urea (lanes 2 and 5) and Tris-HCl with 100 mM Arginine (lanes 3 and 6). Distribution of refolded WHcAg-FL-DIII was in 10 - 60% (w/v) sucrose density gradient (Panel B). The different fractions from the gradient were analyzed by SDS-PAGE (Fig. 18 A), the orange and red boxes indicate the identified proteins by using coomassie blue staining monomeric/non-VLP oligomeric and VLP zone, respectively. The fraction 8 from the sucrose gradient from panel B was analyzed by electron microscopic analysis (Panel C). Scale bar, 50 nm and magnification, x 50,000

The absence of VLP formation during the refolding could be explained by the low purity level of the extracted WHcAg-FL-DIII, the presence of host cell protein contaminants (notably a 14 kDa protein) could interfere with the self-assembly. Consequently, once the inclusion bodies were solubilized under denaturing conditions, the solubilized inclusion bodies were applied onto a Superdex 75 gel filtration column equilibrated under the same denaturing conditions. This step was performed to purify denatured target protein, ideally under a monomeric form. As shown in figure 19 A, WHcAg-FL-DIII can be further purified by this method. However, WHcAg-FL-DIII was detected in a broad number of fractions: from at least fractions 17 to 31, indicating that, in this extract, the size of denatured WHcAg-FL-DIII is largely heterogenous. This result suggested that WHcAg-FL-DIII is not extracted under a unique homogenous monomeric population. Moreover, the purity of the protein is variable: the fractions corresponding to the exclusion peak (peaks 16 - 18) being much more pure than the following ones. These results suggested that the tested denaturing extraction conditions are not efficient enough to solubilize WHcAg-FL-DIII molecule into monomeric form. The fractions 17 – 21, with the highest amount of WHcAg-FL-DIII, (Fig. 19 B) were pooled to initiate the refolding by the same two steps dialysis strategy as previously evaluated (3 M Urea in Tris-HCl buffer and Tris-HCl buffer only). SDS-PAGE analysis of the refolded fractions showed the presence of some residual contaminants (Fig. 19 C). However, the purity was shown to be drastically improved compared with the one reached following a protocol without gel filtration (Fig. 18 A).

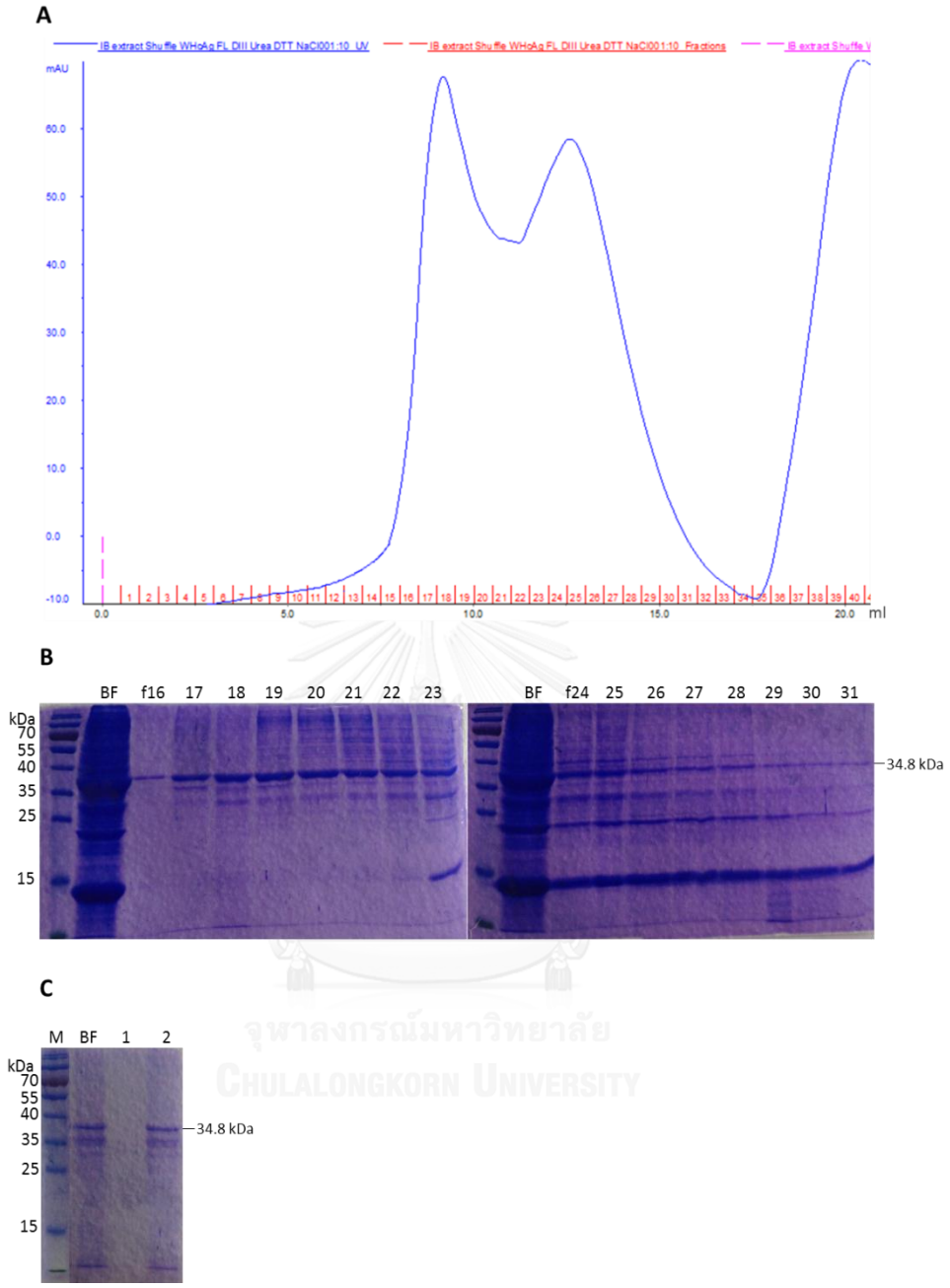

Figure 19: Purification of the extracted WHcAg-FL-DIII from SHuffle E. coli strain onto Superdex 75 column under denaturing conditions. The elution fractions as seen in chromatogram (Panel A) and SDS-PAGE (Panel B), BF: before a gel filtration step. The solubility of WHcAg-FL-DIII was showed after the refolding step (Panel C). The refolded proteins were submitted to centrifugation, BF: before refolding, lane 1: insoluble material, lane 2: soluble fractions.

In another experiment, the extracted inclusion bodies were purified under the same denaturing conditions (6 M Urea + 0.5 mM NaCl + 5 mM DTT + 2 mM EDTA in 50 mM Tris-HCl pH 8) by a gel filtration step using a Superose 6. Such gel filtration matrix is particularly dedicated to the separation of large proteins with a Mw ranging from 5 - 5,000 kDa. The Superose 6 chromatographic profile showed that, similarly to the one generated with the Superdex 75 column, the denatured WHcAg-FL-DIII is broadly distributed in a large numbers of fractions, indicating the presence of multiple populations (Fig. 20 A). In addition, the purity level of the target protein was variable. However, WHcAg-FL-DIII in fractions corresponding to the exclusion volume (fractions 16 - 21 and 23 - 32, elution volume for proteins with Mw>5000 kDa) was pure. These fractions were pooled and refolded by dialysis. Although the refolded WHcAg-FL-DIII was fully soluble in native conditions (Fig. 20 B), neither the sucrose density gradient nor the EM evidenced VLP particles (data not shown).

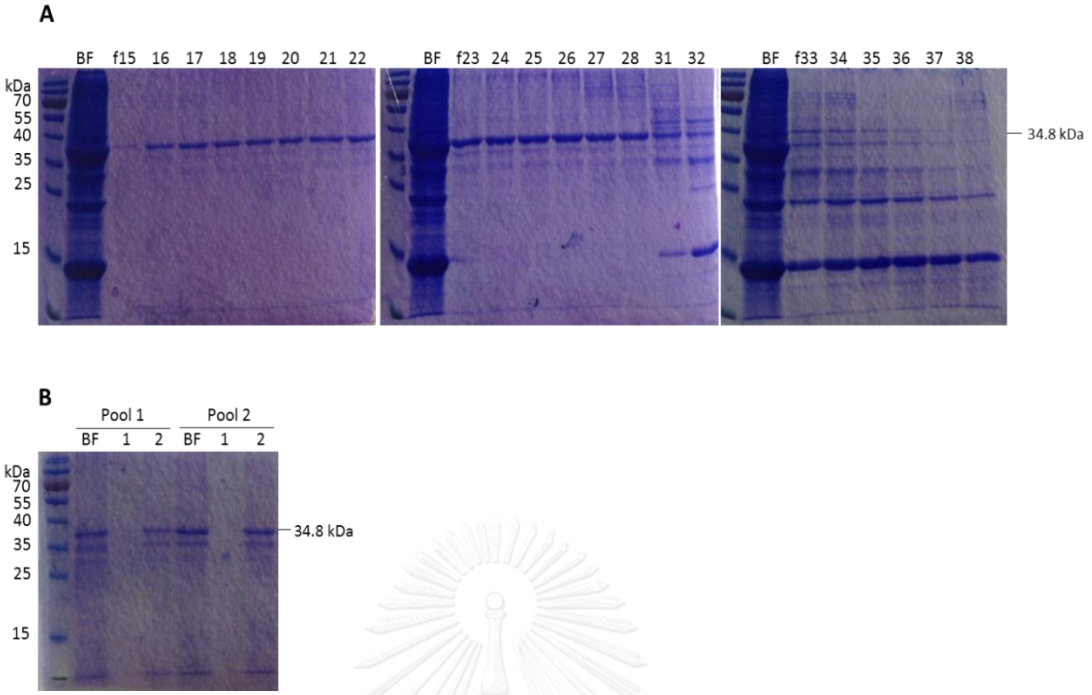

Figure 20: Purification of extracted WHcAg-FL-DIII from SHuffle E. coli strain onto Superose 6 column in denaturing conditions. SDS-PAGE profile (Panel A), BF: before a gel filtration step. From this profile, two pools were constituted for the subsequent refolding by dialysis fractions 16 - 21 (pool 1), fractions 22 - 26 (pool 2). Refolding of pools 1 and 2 (Panel B). The refolded proteins were submitted to centrifugation, BF: before refolding, lane 1: insoluble material, lane 2: soluble fractions.

A comparison of the SDS-PAGE profile of extracted WHcAg-FL-DIII from inclusion bodies obtained in *SHuffle* and *Origami E. coli* strains suggested that the level of host cell protein contaminants are lower using the *Origami E. coli* strain. Consequently, inclusion bodies from WHcAg-FL-DIII which produced in *Origami E. coli* strain was solubilized under the same experimental denaturing conditions (6 M Urea + 500 mM NaCl + 5 mM DTT + 2 mM EDTA). Such buffer fully solubilized the inclusion bodies (data not shown). The IB extracts from *Origami E. coli* strain were applied onto the Superose 6 column equilibrated in the same denaturing condition as described above. A similar broad distribution of the target protein in chromatographic fractions was observed, suggesting that the extracted WHcAg-FL-DIII from *Origami E. coli* strain is present under multimers (Fig. 21 A - C). However, the

WHcAg-FL-DIII purity was higher compared with that obtained with the inclusion bodies from *SHuffle E. coli* strain (see Fig. 20). The fractions 16 - 21 and 22 - 26, according to the purity were separately pooled. WHcAg-FL-DIII was, once again, refolded by the same dialysis-based protocol (PBS + 3 M Urea and PBS only). The refolded chimeric WHcAg was subsequently submitted to the sucrose density gradient ultracentrifugation to demonstrate the VLP formation. These results clearly evidenced the absence of VLP formation during the refolding of WHcAg-FL-DIII as the protein was detected in the monomeric/non-VLP multimeric zone of the density gradient (Fig. 21 D and E).

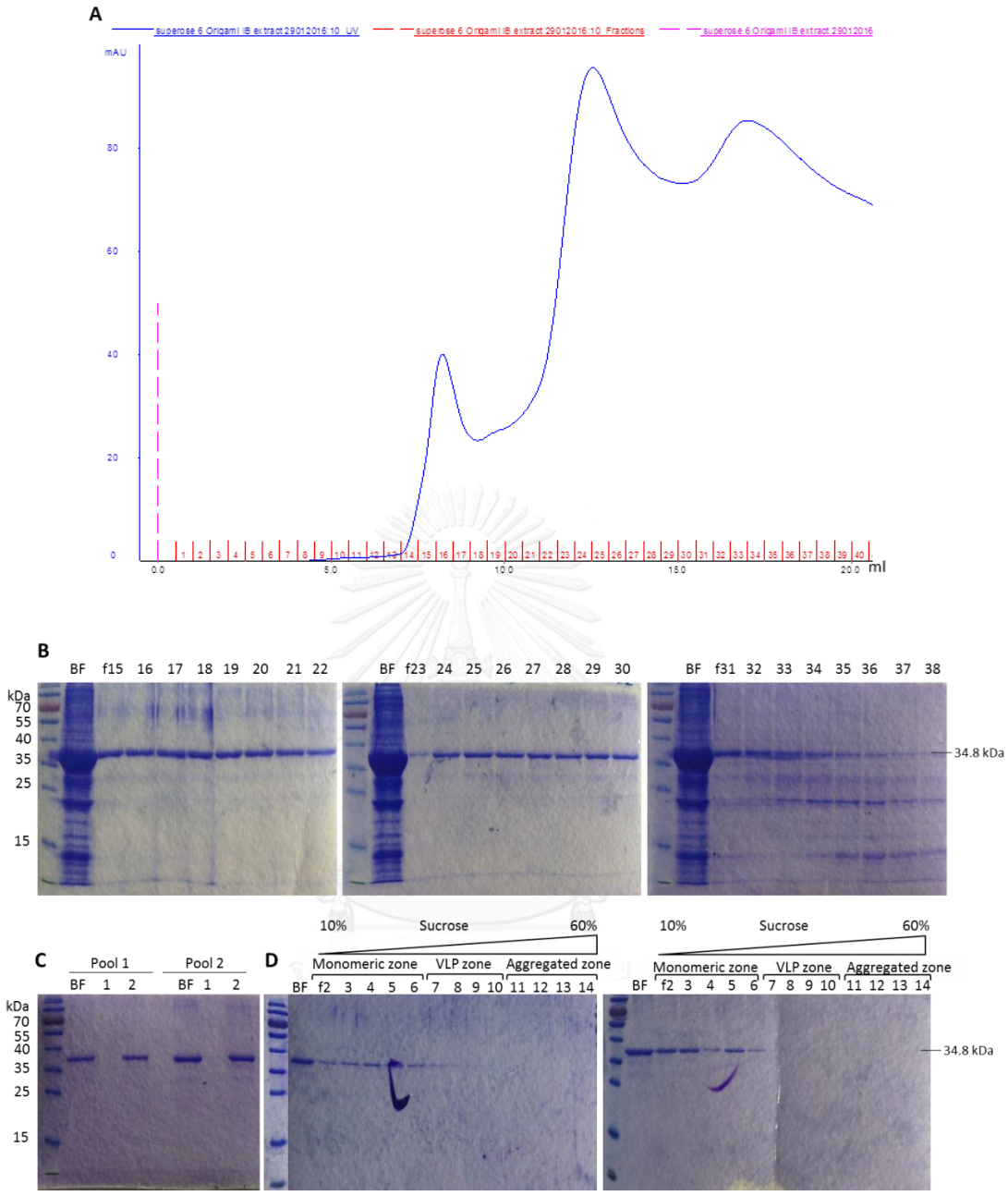

Figure 21: Purification of extracted WHcAg-FL-DIII from Origami E. coli strain onto Superose 6 column under denaturing conditions. The elution (Panel A) and SDS-PAGE profile (Panel B), BF: before a gel filtration step. The solubility of WHcAg-FL-DIII was showed after the refolding step (Panel C). The refolded proteins were submitted to centrifugation; BF: before the refolding, lane 1: insoluble material, lane 2: soluble fractions. Distribution of refolded proteins from the two pools was in sucrose density gradient (Panels D and E)

Finally, the effect of the addition of reduced of oxidized glutathione was evaluated during the refolding step on the VLP formation. Indeed, the absence of VLP particle formation during the refolding could be explained by mismatches at the level of the disulfide bonds of the chimeric WHcAg (7 cysteine residues in WHcAg-FL-DIII can form 3 disulfide bridges), using the two selected pools from the Superose 6 gel filtration (see Fig. 21), the proteins were refolded by dialysis using buffer containing reduced glutathione (5 or 2 mM) and oxidized glutathione (0.5 or 0.2 mM). The refolded proteins, under these conditions were submitted to sucrose density gradient ultracentrifugation. The SDS-PAGE analysis showed that the refolded proteins of each pooled was distributed into the monomeric/non-VLP oligomeric zone, indicating the absence of VLP particle formation during the refolding (Fig. 22B and C).

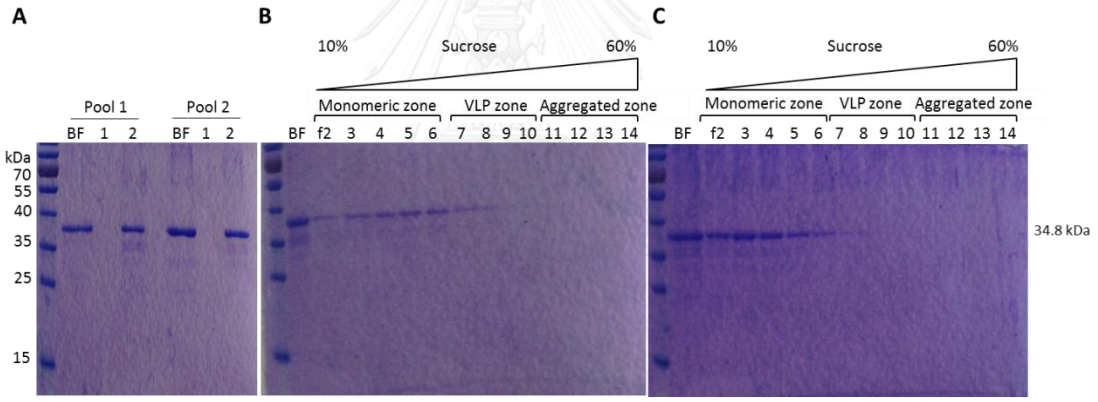

Figure 22: Refolding of purified WHcAg-FL-DIII from the two pools. The solubility of WHcAg-FL-DIII was showed after the refolding step (Panel A). The refolded proteins were submitted to centrifugation; BF: before refolding, lane 1: insoluble material, lane 2: soluble fractions. Distribution of refolded proteins from the two pools was in sucrose density gradient (Panels B and C).

CHAPTER 5

DISCUSSION AND CONCLUSION

The development of efficient vaccine candidates against the four DENV serotypes is still under way. Although the first live-attenuated dengue vaccine of Sanofi Pasteur group (Dengvaxia) was registered for use in the Philippines, Mexico, Brazil and El Salvador (140), the effectiveness of prevention in phase III clinical trials reached roughly around 60%. More importantly, its effectiveness against dengue virus serotype 2 infection was limited to 42.3% (75, 76). Consequently, an improved vaccine candidate against DENV-2 (which is a serotype had been largely causing outbreaks in Thailand from 2009 to 2013) is needed. As a proof of concept, the DENV-2 was used through the combination of two protein fragments of the E protein: the full-length Domain III (DIII), which is responsible of the neutralizing antibody response (89), and the fusion loop, which is a cross-reactive epitope (17). Both fragments were inserted into the loop of the woodchuck hepatitis core antigen (WHcAg) which spontaneously self-assembles to create virus-like particles (VLPs). Such vaccine candidate consequently represents a chimeric VLP of DENV-2 DIII and fusion loop.

This study inserted the DIII and the fusion loop between residue 78 and 79. This position was chosen because the previous study by Billaud *et al.* indicated that it is located at the tip of the immunodominant B-cell epitope of WHcAg and also found that inserting in this position is suitable for stimulating the antibody and still remains self-assemble without aggregation during purification (120). The selection of WHcAg instead of HBcAg was justified by the large seropositivity to human hepatitis B virus in Asian population (141). Consequently, the use of WHcAg-based VLP could avoid pre-existing immunity issues which would drastically reduce the vaccine efficacy.

As it was previously reported that HBcAg/WHcAg antigens can be successfully expressed as VLP in *E. coli*, the WHcAg (wild type VLP) could be also produced in *Origami* and *SHuffle E. coli* strains. Whereas, the chimeric VLP, although various experimental conditions were tested, the chimeric protein was produced exclusively as inclusion bodies in *Origami E. coli* strain but obtained 5 - 10% soluble protein through the use of the *SHuffle E. coli* strain. To increase the yield of soluble protein, various parameters were optimized including concentration of IPTG, duration of induction and temperature. Unfortunately, the expression level was so high in any cases that inclusion bodies were formed and, consequently, the 5 - 10% solubility yield was not improved, similar results were obtained with a chimeric WHcAg containing only DIII, suggesting that the hydrophobic fusion loop was not responsible for that poor solubility.

The previous result showed that DIII alone can be produced as a soluble protein in *E. coli* (93, 142, 143) but insoluble protein was observed when inserting DIII into the HBcAg (23). The insertion of DIII or FL-DIII induced the aggregation of the protein, it could be hypothesized that the insolubility of chimeric protein probably due to improper folding of chimeric protein.

Beside the insertion effect, protein insolubility may cause by too high expression of the target proteins and form inclusion body (IB). IB formation, theoretically, depends on the kinetic competition between specific protein folding and the rate of generating aggregation which relates to synthesis rate (144). As a result of protein overexpression and formed IB, may need to control the synthesis rate of protein starting from de-optimization of codon as all three constructs are codons optimized for *E. coli*, which may be the main cause of high levels of protein expression. Furthermore, the expression vector (pET15b), which is a medium copy number (40 copies/cell) may be changed to be a lower copy number of expression vector (145). This suggests that a vector with lower copy number leads to the lower amount of vectors in *E. coli* and thus reduces the level of protein expression and accumulation/aggregation.

In order to decrease protein synthesis rate, there were various strategies which studied on promoter controlling. This study is working with a promoter from the bacteriophage T7, a strong promoter. It commonly uses to produce recombinant proteins, employing T7 RNA polymerase. This enzyme synthesizes mRNA much higher than usual *E. coli* RNA polymerase (146); so protein synthesis rate is too high and causes inclusion bodies formation. Thus, using of the weaker promoters would reduce the aggregation (145). An interesting promoter is CspA promoter which found in pCold vector. This vector expresses protein effectively when culturing at low temperature, 15ºC. This because of the structure of downstream 5 'UTR is stable only at low temperature (147). Moreover, co-expression with chaperones are widely used for preventing protein misfolding which cause aggregation and help such proteins fold correctly as seen in native form (148). This suggested the necessary to use *E. coli* strain that contains chaperones to increase correctly folded soluble protein (149).

In addition, inserting any improper size of foreign antigens may lead to improper protein folding of each monomer, or it may cause some problems during dimerization and result in aggregation, dimers and oligomers eventually. This new technique is called SplitCore, which splitted c/e1 loop of HBcAg into two fragments called core N and core C and select one core to make a fusion with foreign antigen, as shown in figure 23 A and B. Previous report showed that this can increase the soluble VLP formation (150).

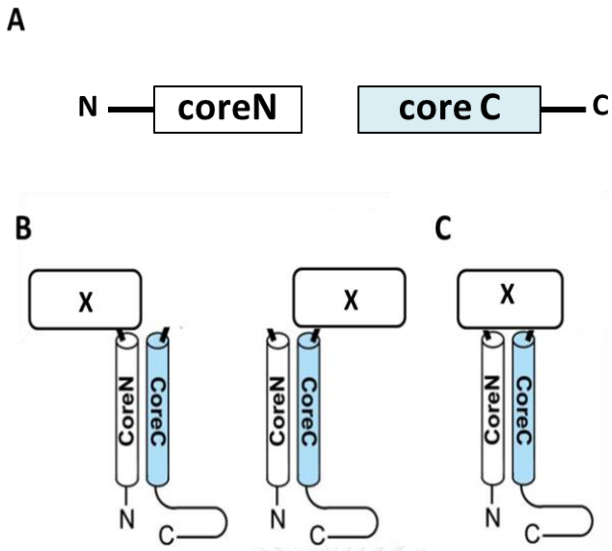

Figure 23: The schemes of splitted c/e 1 loop of Hepatitis B core gene (A). Foreign antigen (X) exposing on the surface of particles using different strategies, SplitCore N (B, left), SplitCore C (B, right) and unsplitted c/e 1 loop (C) (modified from (150)).

The results showed that at this insertion site, when comparing the pattern of distribution with wild-type VLP (truncated WHcAg); the soluble chimeric WHcAg can self-assemble into VLP although all of the soluble proteins do not migrate into the VLP zone but at least some. As the amount of the soluble chimeric WHcAg produced in *SHuffle E. coli* strain was not appropriate for future vaccine *in vivo* experiments, the inclusion bodies were used to evaluate the possibility to generate VLP. The strategy was to solubilize and refold the IBs in order to reassemble proteins in native conditions. Under experimental conditions, the VLP formation from inclusion body urea extracts was unsuccessful. The gel filtration profile could explain the failure of self-assembly into VLP: the urea extraction solubilized WHcAg-FL-DIII under different forms with broad range of molecular weight and, consequently, a homogenous monomeric population was not evidenced. By considering about purity, it was found that the contaminant was very low after being purified. It would be interesting to evaluate stronger denaturing conditions to obtain monomers, using more potent dissociating agents such as guanidium hydrochloride at high concentrations (which is a strong denaturant and better to completely destroy hydrogen bond more than

6 M urea) or detergents such as Sarkosyl (N-lauroylsarcosine). Sarkosyl is commonly used to extract inclusion bodies which preserve the protein structure and it is not necessary to refold (149). The demonstration of homogenous monomeric chimeric WHcAg will be further evidenced by gel filtration. The refolding could be performed only using these fractions.

In conclusion, according to the VLP detection in sucrose gradient of soluble WHcAg-FL-DIII fraction, it could be confirmed that this construction strategy is succeeded. However, it would be interested to evaluate whether the solubility issue is also related to the insertion position (150). Unfortunately, under these experimental conditions cannot create enough VLP with a good purity to perform *in-vivo* experiments. Future experiments will be attempted to solubilize the IB to obtain the first monomeric unfolded WHcAg under denaturing conditions in order to increase the chance to trigger self-assembly (VLP formation) during the refolding.

In addition, a new construction strategy is interesting. For example, expressing FL-linker-DIII and WHcAg independently then further conjugate the target protein on the surface of the WHcAg-based VLP (21). Alternatively, expression of WHcAg-FL-DIII in *P. pastoris* might be able to solve the folding issue. For further experiments, with the purified VLP (by any methods), experiments in mice will be conducted to compare the immunogenicity of vaccine candidates in various aspects: 1) WHcAg-FL-DIII with/without adjuvant, 2) truncated FL-DIII protein with/without WHcAg, with/without adjuvant, 3) WHcAg alone (control). Binding and neutralizing antibodies titers will be determined to demonstrate the potential of particulate antigens in the amplification of the antigen-specific immune responses.

Once when serotype 2 is successful in, the remaining 3 serotypes (serotype 1, 3 and 4) will be constructed. Furthermore, multiple strategies that used to increase dengue vaccine coverage or cost-effectiveness such as by making multi-serotypes (eg. tetravalent) within a single construct, creating DNA shuffling, single DNA fragment which stimulate neutralizing antibodies against all the 4 serotypes. DNA shuffling could be constructed by randomly select E gene fragment from each serotype and

recombine to be a single the new chimeric (151). This technology is also used for HPV, allergies and malaria vaccine candidates. Therefore, it is interesting to explore Shuffled DNA to insert into the truncated WHcAg.

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APPENDIX

Reagent/Buffers

6X DNA loading dye

60% (w/v) glycerol

60 mM EDTA, pH 8

0.01 % (w/v) Bromophenol blue

50X Tris-acetate buffer (TAE buffer)

2 M Tris-acetate

100 mM Na_2EDTA, pH 8

Distilled water up to 1L

SOB medium

2% (w/v) tryptone

0.5% (w/v) yeast extract

10 mM NaCl

10 mM $MgCl_2$

2.5 mM KCl

10 mM $MgSO_4$

SOC medium

20 mM glucose in SOB medium

Transformation buffer (TB), pH 6.7

10 mM Pipes

15 mM $CaCl_2$

250 mM KCl

55 mM $MnCl_2$

Luria Broth (LB) media (1000 ml)

10 g tryptone

5 g yeast extract

10 g NaCl

LB-agar plate

1.5% (w/v) Degranulated agar in LB medium

4x protein loading dye

200 mM Tris pH 6.8

40% (v/v) Glycerol

8% (w/v) SDS

0.4% (w/v) Bromphenol blue

10% (v/v) β-mercaptoethanol

Running buffer, pH 8.3

25 mM Tris

192 mM Glycine

0.1% (w/v) SDS

Transfer buffer, pH 8.3

25 mM Tris

192 mM Glycine

20% (v/v) Methanol

TBS buffer, pH 7.5

50 mM Tris

150 mM NaCl

TBS-Tween buffer

0.05% (v/v) Tween 20 in TBS buffer

Destaining solution

50% (v/v) DW

40% (v/v) Methanol

10% (v/v) acetic acid

TNE buffer

10 mM Tris HCl pH 7.8

100 mM NaCl

1 mM EDTA

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