การโคลนนิ่งและการแสดงออกของฟอสโฟไลเปสเอทูจากพิษงูแมวเซา

นางสาว สวัสดิรักษ์ พงศ์ธนานันท์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2546

ISBN 974-17-3525-1

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

CLONING AND EXPRESSION OF PHOSPHOLIPASE A_2 FROM RUSSELL'S VIPER VENOM (*Daboia russellii siamensis*)

Miss Sawatdirak Phongtananant

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Medical Science

Faculty of Medicine

Chulalongkorn University

Academic Year 2003

ISBN 974-17-3525-1

Thesis Title	CLONING AND EXPRESSION OF PHOSPHOLIPASE A_2 FROM RUSSELL'S VIPER VENOM (*Daboia russellii siamensis)*
By	Miss Sawatdirak Phongtananant
Field of study	Medical Science
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สวัสดิรักษ์ พงศ์ธนานันท์ : การโคลนนิ่งและการแสดงออกของฟอสโฟไลเปสเอทูจากพิษงูแมวเซา (CLONING AND EXPRESSION OF PHOSPHOLIPASE A_2 FROM RUSSELL'S VIPER VENOM (*Daboia russellii siamensis*)) อ. ที่ปรึกษา : รศ.นพ. อิศรางค์ นุชประยูร. 106 หน้า. ISBN 974-17-3525-1.

ฟอสโฟไลเปสเอทูในพิษงูเป็นโปรตีนที่มีขนาดเล็กประมาณ 13-15 กิโลดาลตัน มีบทบาทเกี่ยวกับความเป็นพิษหลายด้าน เช่น เป็นสารต้านการแข็งตัวของเลือด, ทำลายเม็ดเลือด, เป็นตัวชักนำทำให้เกิดการบวม, ยับยั้งการรวมตัวของเกร็ดเลือด, ทำลายระบบประสาทและกล้ามเนื้อ เป็นต้น เป็นที่ทราบกันดีว่าในพิษงูแมวเซามีฟอสโฟไลเปสเอทูหลายแบบซึ่งพบแตกต่างกันในแต่ละหน่วยย่อยของสปีชีส์ ในการศึกษานี้มีวัตถุประสงค์เพื่อตรวจหาว่างูแมวเซาของไทยมีฟอสโฟไลเปสเอทูกี่แบบโดยใช้วิธีตรวจหาจากห้องสมุดซีดีเอ็นเอ (cDNA library) ของต่อมพิษงูแมวเซาไทยและค้นหาวิธีผลิตฟอสโฟไลเปสเอทูด้วยวิธีทางพันธุวิศวกรรมและศึกษาโครงสร้างของยีนสร้างฟอสโฟไลเปสเอทู

ผลการตรวจหาฟอสโฟไลเปสเอทูจากห้องสมุดซีดีเอ็นเอพบว่ามี 2 แบบ คือ *PlaS1* และ *PlaS2* ในสัดส่วนประมาณ 1:2 มีความเหมือนกันของลำดับดีเอ็นเอในแต่ละแบบเท่ากับ 86 เปอร์เซ็นต์ ผลการเทียบกับฐานข้อมูลพบว่า *PlaS1* และ *PlaS2* เหมือนกับ *RV-4* และ *RV-7* ที่พบในงูแมวเซาไต้หวันตามลำดับ การผลิตฟอสโฟไลเปสเอทูที่เชื่อมกับฮิสติดีนจากพลาสมิดซึ่งมียีน *PlaS1* และ *PlaS2* โดยใช้แบคทีเรีย *E. coli* ได้โปรตีนขนาด 18 และ 23 กิโลดาลตันตามลำดับ เมื่อทำการละลายและปรับให้อยู่ในสภาพดั้งเดิม (refolding) ได้เอนไซม์ที่มีประสิทธิภาพในการย่อยฟอสโฟไลปิด 185.67 ไมโครโมลต่อนาทีต่อมิลลิกรัม แต่ได้โปรตีนจำนวนเพียง 2 ไมโครกรัมต่อการเลี้ยงเซลล์หนึ่งลิตร การโคลนดีเอ็นเอของฟอสโฟไลเปสเอทูจากจีโนมพบว่ามียีนสร้างฟอสโฟไลเปสเอทู 3 ยีนคือ *gPlaS1*, *gPlaS2* และ *gPlaS3* ซึ่งยีนทั้งสามมีขนาดประมาณ 2.0 กิโลเบส ประกอบด้วย 5 exon และ 4 intron นอกจากนี้ยังพบว่า *gPlaS1* และ *gPlaS2* ในช่วงบริเวณ coding และ untranslated เหมือนกับ *PlaS1* และ *PlaS2* ตามลำดับ ส่วน *gPlaS3* เมื่อลองทำนายเป็นกรดอะมิโนพบว่าแตกต่างจากฟอสโฟไลเปสเอทูของงูแมวเซาที่เคยมีรายงานมา

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สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษา.....

437 52876 30 : MAJOR MEDICAL SCIENCE
KEY WORDS: RUSSELL'S VIPER / *Daboia russellii siamensis* /
PHOSPHOLIPASE A_2 / CLONING / EXPRESSION
SAWATDIRAK PHONGTANANANT : CLONING AND EXPRESSION OF PHOSPHOLIPASE A_2 FROM RUSSELL'S VIPER VENOM (*Daboia russellii siamensis*). THESIS ADVISOR : ASSOC. PROF. ISSARANG NUCHPRAYOON. 106 pp. ISBN 974-17-3525-1.

Snake venom phospholipaseA_2 (PLA_2) enzymes are low molecular weight (13-15 kDa) proteins and exhibit a wide variety of pharmacological effects such as hemolysis, platelet aggregation inhibition, neurotoxicity and myonecrosis. Russell's viper (*Daboia russellii siamensis*, RV) venom contains several isoforms of PLA_2 different from each subspecies. The aim of this study was to search for the PLA_2 isoforms in a Thai *Daboia russellii siamensis* venom gland cDNA library, to develop a method of recombinant expression of PLA_2 by genetic engineering and study *PLA_2*s gene structure.

By using plaque-lift hybridization and random selection of cDNA library clone for DNA sequence determination, we found only 2 PLA_2 isoforms in the Thai RV venom gland cDNA library with ratio approximately 1:2. The 2 PLA_2 isoforms, designated *PlaS1* and *PlaS2*, showed 86% nucleotide sequence identity and are identical to Taiwan Russell's viper PLA_2, *RV-4* and *RV-7*, respectively. The PLA_2 isoforms were recombinantly expressed as a histidine tagged fusion protein in using an *E. coli* and yielded 18 kDa and 23 kDa proteins. Because of low level of expression, only the 18 kDa *PlaS1* was expressed in a large scale, purified by using Immobilized Metal Affinity Chromatography (IMAC) under denaturing condition, solubilized, refolded by dialysis and showed PLA_2 activity of 185.67 μmoles/min/mg. The yield of recombinant protein was about 2.0 μg/Litre of cells culture. We cloned the full-length genes encoding PLA_2 by PCR of genomic DNA from muscle tissue of an RV. We identified 3 genes about 2.0 kb in length, designated *gPlaS1*, *gPlaS2* and *gPlaS3*, including 5 exons and 4 introns in genomic PLA_2 DNA cloning. The coding and untranslated regions of *gPlaS1* and *gPlaS2* are identical to *PlaS1* and *PlaS2* cDNA, respectively. The deduced N-terminal amino acid sequences of gPlaS3, however, is different from all previous reported of *D. russellii* PLA_2s.

Field of study Medical Science Student's signature……
Academic year 2003 Advisor's signature……...

ACKNOWLEDGEMENT

I would like to express my sincere gratitude and profound appreciation to Associate Professor Dr. Issarang Nuchprayoon my advisor for his valuable advice, guidance, helpfulness, understanding and intelligential motivation throughout my study.

My grateful appreciation is extended to Associate Professor Dr. Vilai Anomasiri, Associate Professor Dr. Apiwat Mutirangura and Professor Yong Poovorawan, my thesis committees, for their valuable discussion and suggestions.

I feel profoundly indebted to Mr. Preecha Leangaramkul, Mr. Wichai Pornthanakasem and Ms. Patrawadee Pitakpolrat for their helpful, valuable suggestion and encouragement.

I am also really thankful to Mr. Arkhom Saingam, members in the Snake Venom Unit, Filariasis Unit and my friends for their encouragement, sincerity and friendship.

Finally, I will not forget to give special thanks to my parents and every members in my family for their kindness, understanding and cheerfulness.

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LIST OF ABBREVIATIONS

bp	base pairs
°C	degree Celsius
cm	centimeter
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
dNTPs	dATP, dTTP, dGTP, dCTP
EDTA	ethylenediamine tetraacetic acid
E. coli	*Escherichia coli*
g	gram (s)
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
LB	Luria-Bertani media
M	molar
MES	2-(N-Morpholino) ethanesulfonic acid
mg	milligram
min	minute
ml	millilitre
mM	millimolar
N	normal
ng	nanogram
nm	nanometer
OD	optical density
pfu	plaque forming unit
pmol	picomole
RNase	ribonuclease

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rpm	revolution per minute
SDS	sodium dodecyl sulphate
sec	second
Tris-HCl	tris-(hydroxymethyl)-aminoethane
UV	ultraviolet
µg	microgram
µl	microlitre
v/v	volume/volume
w/v	weight/volume

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CHAPTER I

INTRODUCTION

1. Background and Rationale

Approximately 15 percent of the 3,000 species of snakes found worldwide are considered to be dangerous to humans and classified into three major families: 1) Hydrophidae (sea snakes), 2) Elapidae (cobras, kraits, and coral snakes) and 3) Viperidae (vipers and pit vipers)[1]. Members of family Viperidae are the best-known venomous snakes: true viper e.g. echis species (saw-scaled viper), vipera species (vipers and Russell's viper); pit vipers e.g. crotalus and sistrurus species (rattlesnakes), agkistrodon species (cottonmouth, copperhead)[1]. Out of the 163 species of snake in Thailand, 48 species are member of Elapidae and Viperidae families[2]. These are the most danger out of all venomous snake families. The incidence of bites by venomous snake in Thailand is probably 7,000 per year[2]. Most of snakebites are due to Malayan pit viper, green pit viper, cobras, and Russell's viper, respectively[2]. However, most fatal cases of venomous snake bites in Thai victims, are cobras and Russell's viper, envenoming[2].

1.1. Russell's Viper

Russell's viper (*Daboia russellii*) is a venomous snake that widely but discontinuously distributed throughout Eastern Asia and Southeast Asia[3]. Taxonomically Russell's viper belongs to class Reptilia, subclass Lepidosauria, order Squamata, suborder Serpentes, superfamily Colubroidea, family Viperidae, subfamily Viperinae, genus *Daboia* (*Vipera*), species *russellii*[4,5]. Russell's viper can be classified to 5 subspecies based on the difference of coloration and markings, *Daboia russellii russellii* (India and Pakistan), *Daboia russellii pulchella* (Sri Lanka), *Daboia russellii*

siamensis (Thailand, Myanmar and China), *Daboia russellii formosensis* (Taiwan) and *Daboia russellii limitis* (Indonesia)[3] (**Figure 1**). In Thailand *Daboia russellii siamensis* is found in northern and central area. The snake about 90-120 cm in body length[4], portly and short shape, triangle-shape head, narrowed neck, short tail, light brown body and heavy brown oval marks[2] (**Figure 2**). They also have large retractable fangs. The average in adult is 16 mm[3]. They are a very muscular snake and can move rapidly and convulsively by lunging movements to attack the aggressor or, more commonly, to attempt escape. The natural prey includes small vertebrates, especially rodents, frogs, lizards, snakes and birds. They are nocturnal and ground dwelling but have occasionally been found swimming and climbing trees[3].

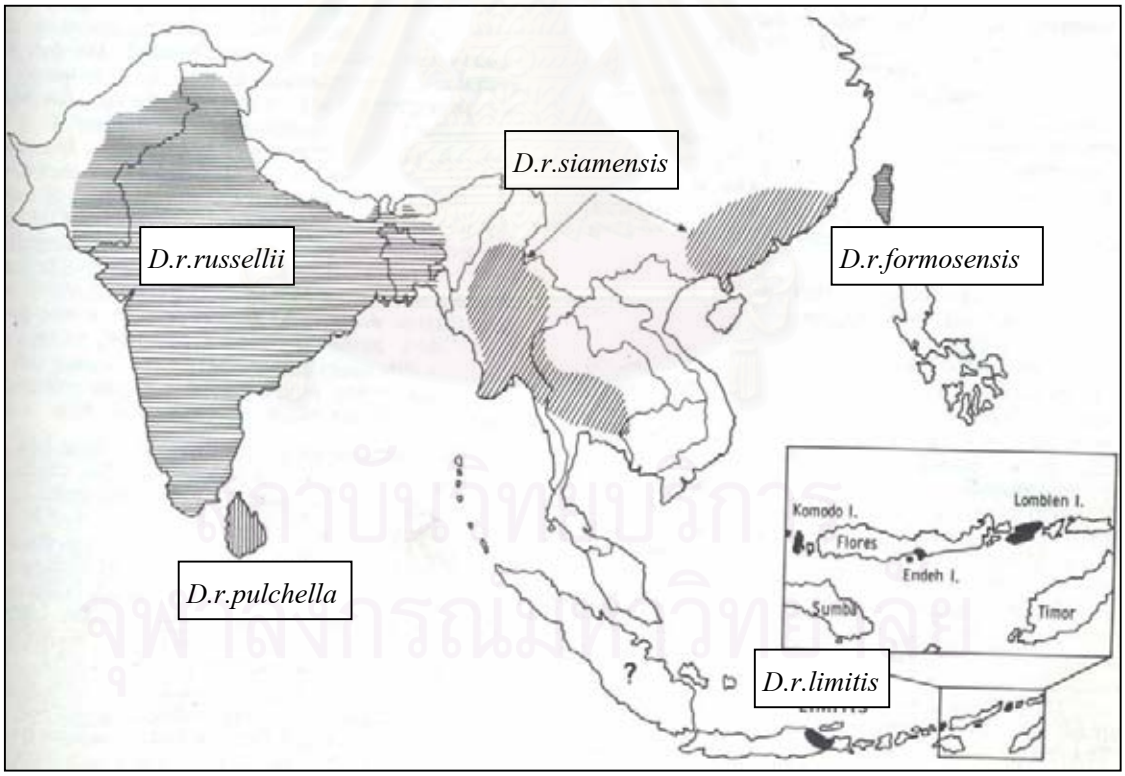


Figure 1 Distribution of *Daboia russellii* subspecies[3].

Figure 2 Morphology of *Daboia russellii siamensis*[2].

1.2. Clinical Effect of Russell's Viper Bite

The Russell's viper venom exhibits a striking geographical variation in the composition and clinical effects of venom even within same subspecies[3](**Table 1**). Haemostatic abnormalities are described from all countries but the coagulopathy was less marked in Sri Lanka than in Myanmar[6,7]. Pituitary haemorrhage has so far been described only in Myanmar and southern India[3]. Intravascular haemolysis was most marked in Sri Lanka but has been reported from India and Thailand[3]. Neuromyotoxicity is the dominant clinical feature in Sri Lanka and may well occur in India [3]. Chemosis and facial edema, as evidence of increased capillary permeability, have so far been described only in Myanmar where they are common features of severe envenoming [3]. Primary shock and hypotension are most commonly described in Myanmar but have also been mentioned in reports from the other countries [3].

Table 1 Geographical variation in the clinical manifestations of *Daboia russellii* bite[3].

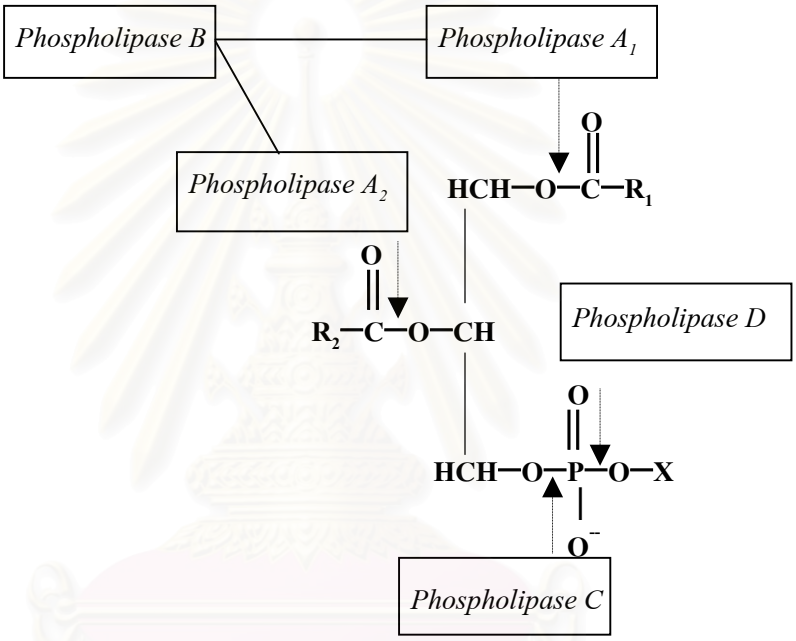
Symptoms	Sri Lanka	India	Myanmar	Thailand	Taiwan
Coagulopathy	+	++	++	++	?
Renal failure	++	+	++	+	+
Pituitary infarction	-	+	++	-	?
Intravascular haemolysis	++	+	-	+	?
Neuro-myotoxicity	++	+	-	-	?
Generalized capillary permeability	-	-	++	-	?
Primary shock/hypotension	-	+	++	-	?

1.3. Proteins in Venom

About 90% of dry weight of snake venom are proteins. Its components include neurotoxins, cytotoxins, cardiotoxins, nerve growth factors, lectins, factor IX/X-binding protein, disintegrins, various enzymes and enzyme inhibitors[8]. Several of proteins are also found in most venomous snakes including hyaluronidase, phospholipase A_2 (PLA_2), collagenase, nucleotidase, amino acid oxidase, ribonuclease, lactate dehydrogenase, deoxyribonuclease, etc.[3,8,9] The Russell's viper venom can cause the damage of vascular endothelium cause internal hemorrhage[3]. The venom induced disseminated intravascular coagulation (DIC) due to stimulation of coagulation factor X, induced edema, paralysis, wound necrosis and hypotensive effects [3,10]. Deaths which occur between 15 min and 9 days after bite and have been attributed to acute renal failure, vasodilatation, intracranial or massive intra-abdominal haemorrhage, shock caused by increased vascular permeability [3,10].

1.4. Phospholipase A_2

Phospholipase A_2 (PLA_2) enzymes are separated into several classes, namely A_1, A_2, B, C and D, depend on the site of hydrolysis on their substrate[11] (**Figure 3**).



R_1 and R_2 are hydrocarbon chains of fatty acids

X = Head groups

Figure 3 Sites of action of phospholipases[11]

PLA_2 enzymes hydrolyze phospholipids at the *sn*-2 position of the glycerol backbone, releasing lysophospholipids and fatty acids[11] (**Figure 4**). PLA_2 enzymes are found as both intracellular and extracellular form [11,12,13]. Mammalian PLA_2 enzymes have important roles in the maintenance of the cellular phospholipid pools

and a variety of physiological and pathological processes[11]. On the other hand, snake venom PLA_2 enzymes are low molecular weight proteins (13-15 kDa) and exhibit a wide diversity of pharmacological effects by interfering in normal cell functions such as anticoagulant[14,15], haemorrhage[16,17], edema[18,19,20,21], platelet aggregation inhibition[14,19], pre-postsynaptic neurotoxicity[18,19,21,22], and myonecrosis[18,21].

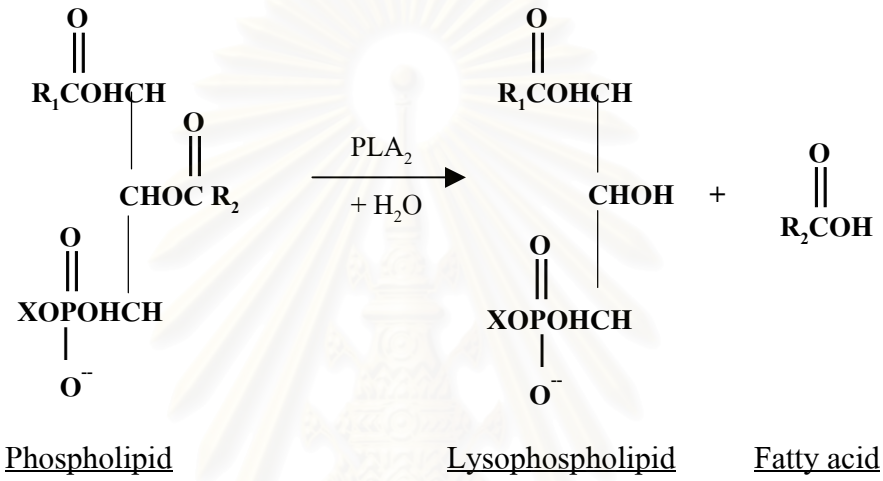


Figure 4 Reaction of catalyzed by PLA_2 [11]

Two products of PLA_2 hydrolysis, lysophospholipid and fatty acid, may act as secondary messenger molecules such as arachidonic acid (AA; 5, 8, 11, 14-eicosatetraenoic acid). Generally, these molecules are found at very low level in cells. They also function as the precursor of large family of compound eicosanoids, which includes cyclooxygenase-derived prostaglandins and lipoxygenase-derived leukotrienes. The eicosanoids possess a wide biological activities associate with inflammatory reactions[23]. The lysophospholipid may form platelet-activation factor (PAF), another potent inflammatory mediator[24].

1.5. Classification of Phospholipase A_2

PLA_2 is a large superfamily of distinct enzymes whose products are important for signal transduction processes, membrane remodeling and general lipid metabolism[13,23]. PLA_2 enzymes can be classified depending on various parameters such as their structure, localization, function, mechanism of catalysis, molecular size or nucleotide sequences[23]. In 1999, the classification of PLA_2 was updated. These PLA_2 group types are listed in **Table 2**. The secretory PLA_2s are all low molecular mass enzymes (13-15 kDa) with a very rigid tertiary structure rising from the presence of 5-8 disulfide bridges[23]. Thus, they possess both stability against proteolysis and resistance to denaturation, which allows them to maintain activity in the extracellular fluids where they are found[23].

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Table 2 Phospholipase A_2 groups [23]

Group		Sources	Location	Size (kDa)	Ca^{2+} requirement	Disulfides
I	A	Cobras, kraits	Secreted	13– 15	mM	7
	B	Porcine/ human pancreas	Secreted	13– 15	mM	7
II	A	Rattlesnakes, **vipers**, human synovial fluid/ platelets	Secreted	13– 15	mM	7
	B	Gaboon viper	Secreted	13– 15	MM	6
	C	Rat/ mouse testes	Secreted	15	MM	8
III		Bees, lizards	Secreted	16– 18	MM	5
IV	A	Raw 264.7/ rat kidney, human U937/ platelets	Cytosolic	85	< uM	
	B	Human brain	Cytosolic	100	< uM	
	C	Human heart/ skeletal muscle	Cytosolic	65	None	
V		Human/ rat/ mouse heart/ lung, P388D 1 macrophages	Secreted	14	MM	6
VI		P338D 1 macrophages, CHO cells	Cytosolic	80– 85	None	
VII	A	Human plasma	Secreted	45	None	
	B	Bovine brain	Cytosolic	42	None	
VIII		Bovine brain	Cytosolic	29	None	
IX		Marine snail	Secreted	14	MM	6
X		Human leukocytes	Secreted	14	mM	7

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1.6. Multiple Isoenzymes of PLA_2

The Russell's viper venom exhibits a geographical variation in the components of venom even within subspecies. Such as *D. russellii* venom from different regions of India was comparatively characterized[25]. The eastern region is more lethal potency than western, southern and northern, respectively. An interesting report indicated that variation of venom components from same snake species in close association with its diet[26].

Recently, venoms from some subspecies of *D. russellii* have been chromatography performed and purified. Several PLA_2 isoenzymes have been found and their biological activities have been studied. For example, purified "RV-4" of *D. r. formosensis* in Taiwan was pre-synaptic blocker of neuromuscular nerve transmission, whereas RV-7 had much lower enzymatic activity and was not toxic[22]. In addition, purified VRV-IIIb from *D. russellii* in India could inhibit platelet aggregation[19]. PLA_2 from the other species have also various effects e.g. myotoxin PLA_2 from Bothrops *asper* cause of cytolytic and myotoxic[27]. However, EC-I-PLA2 in Indian saw-scaled viper (*Echis carinatus*) was non-lethal to mice and devoid of neurotoxicity, myotoxicity, anticoagulant activity and cytotoxicity but induced mild edema in the foot of mice and inhibited platelet aggregation[28]. Difference of amino acid sequences, size of protein, number of disulfide bonds, folding of protein and conformation of enzyme loops determines the efficiency of enzyme action and presence/absence of various pharmacological functions[29].

While most of PLA_2s were isolated and purified from snake venom prior to numerous experiments such as crystallyzation, x–ray diffraction, biological activity of proteins and etc., the functional recombinant PLA_2 production was interested and attempted. Although Russell's vipers in Thailand are abundance, venom

utilizing has been limited because of the difficulty in maintain the snake in captivity. Thus, production of PLA_2, a major lethal factor in venom, is alternative choice useful to the further PLA_2 studies or advance to specific antivenom decreasing side effects.

The venom composition is an important consideration because bites by divergent subspecies may explain the variety of symptomatology in viper bite from different countries. Thus, this research is aimed at screening PLA_2 isoforms of *Daboia russellii siamensis* in Thailand and to compare with other subspecies lead to knowledge about variation of clinical effects. In addition, this research was conducted to clone and express of recombinant PLA_2 to produce *in vivo* PLA_2 protein and to study their activities.

2. Research Questions

Primary Question

How many PLA_2 isoforms of *Daboia russellii siamensis* in Thailand?

Secondary Question

What is specific enzymatic activity value of recombinant PLA_2?

3. Objective of This Research

1. To characterize cDNA, gDNA and deduced-amino acid sequences of PLA_2 of *Daboia russellii siamensis* in Thailand.

2. To produce functional recombinant PLA_2 protein useful to other studies.

4. Hypothesis

PLA_2 of *Daboia russellii siamensis* in Thailand is different from PLA_2 of other subspecies.

5. Keywords

Russell's viper

Daboia russellii siamensis

Phospholipase A_2

Cloning

Expression

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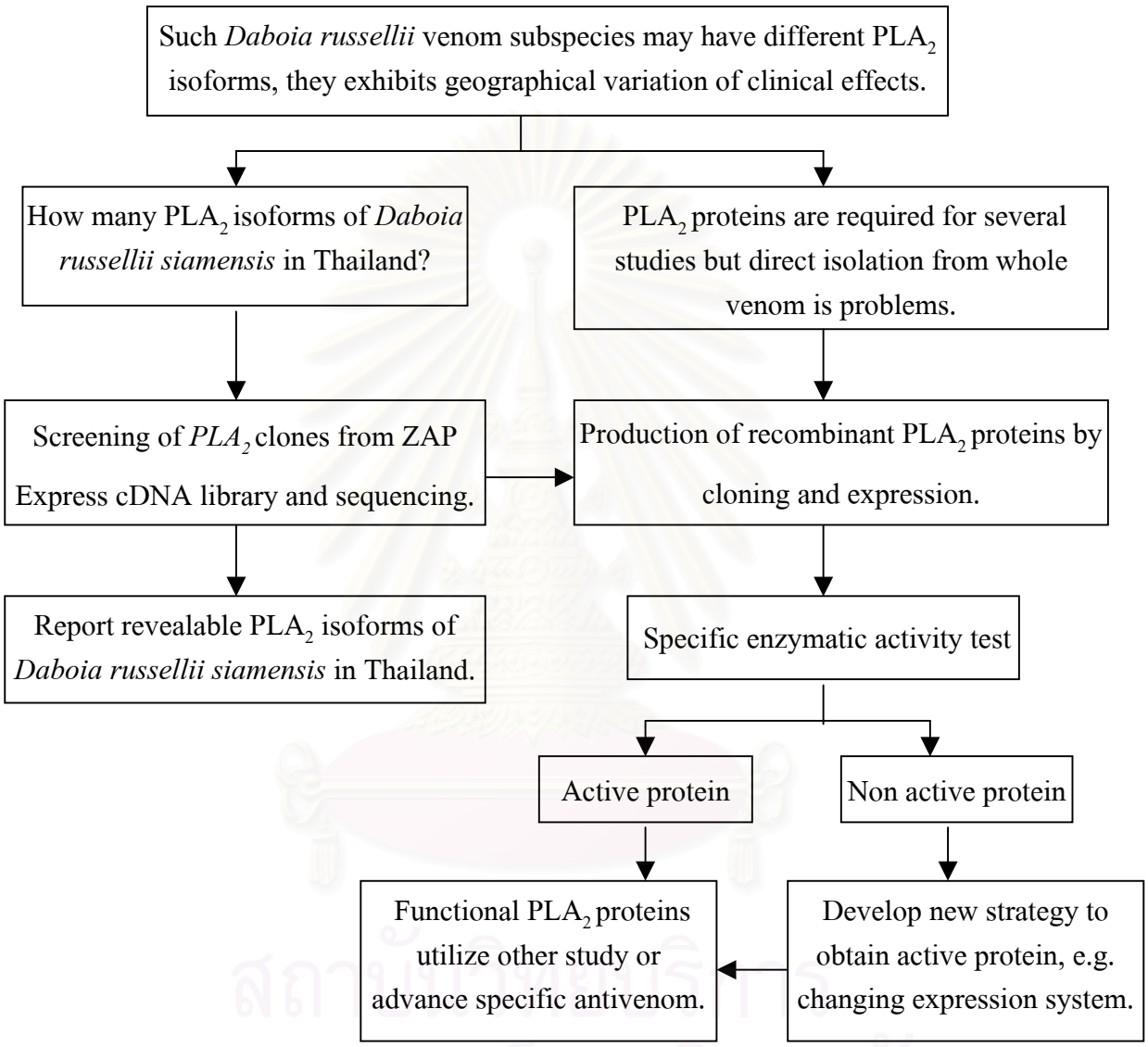
6. Conceptual Framework



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7. Expected Benefit & Application

1. Genetic information on PLA_2 of *Daboia russellii siamensis* in Thailand to PLA_2 will be useful for taxonomic classification of Russell's viper subspecies.

2. Recombinant is useful for studies of pathophysiology of Russell's viper bites, production of PLA_2-specific antibody and aid in product of diagnostic kit for snake bites.

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CHAPTER II

LITERATURE REVIEW

1. Potency of PLA_2 Isoforms

The venom of *Daboia russellii* is known to contain PLA_2, which may contribute to the toxicity of the whole venom. This enzyme was constitution 70% of the whole venom proteins[3,21]. The potent PLA_2 in venom of some subspecies was biological activity studied following purified to homogeneity by few step of chromatography as well as gel filtration.

There are several reports reveal PLA_2 functions and clinical signs in tested animals. A major PLA_2 (VRV PL-VIIIa) which constitutes 24% of the whole *D. russellii* venom in India, contributes 45% of the total PLA_2 activity of the venom[21]. It shows neurotoxic symptoms and damages vital organs such as lung, liver and kidney with LD_{50} value of 5.3 mg/kg body weight of mice[21] (the lethal potency of PLA_2s are shown in **Table 3**).

A PLA_2 from *D. russellii* venom in India (VRV PL-VIIIb) inhibiting platelet aggregation induced neurotoxic symptoms in experiment mice with an i.p. LD_{50} of 5.2 mg/kg[19]. It also induced edema in foot pads of mice but devoid of anticoagulant, myotoxic and direct haemolytic activities[19].

The isolated daboiatoxin (DbTx), a major lethal factor showing strong PLA_2 activity, was purified to homogeneity from the venom of *D. r. siamensis* (Myanma)[18]. It constitutes 12% of total venom protein and is the main lethal component of Myanmar Russell's viper venom with an i.p. LD_{50} (0.05 mg/kg) 12-fold greater than that of the whole venom (LD_{50} i.p. 0.6 mg/kg)[18]. Unlike no neurotoxic features in Myanmar victims, DbTx induces neurotoxic symptoms in mice[18]. It is

possible that neurotoxicity of PLA_2 in mice dose not predict neurotoxicity in human. DbTx also exhibits potent edema-inducing activity and a strong myonecrotic activity, but no haemorrhagic activity [18].

Furthermore, RV-4 and RV-7 from *D. r. formosensis* in Taiwan were neurotoxic and not toxic, respectively[22]. The deduced amino acid sequences of RV-4 and RV-7 were 92% identical to those of the vipoxin and vipoxin inhibitor, respectively, from *Vipera ammodytes ammodytes*[22]. Moreover, RV-7 inhibited the enzymatic activity of RV-4 *in vitro* but potentiated its neurotoxicity by reduce blocking time, and lethal potency[22]. The i.p. LD_{50} of RV-4 and RV-4/RV-7 complex were estimated to be 0.32 and 0.15 mg/kg mice, respectively[22]. It is suggested that RV-7 may facilitate the specific binding of RV-4 to its presynaptic binding sites, probably by preventing its non-specific adsorption [22].

The differences between PLA_2 isoenzymes in the same venom are not due to extent of glycosylation or any post-translational modifications[11]. Although some of the isoenzymes are closely related, recent studies of genomic sequencing have shown that these isoenzymes are derived from different genes and not by alternative splicing [11].

Table 3 Lethal potencies and specific enzymatic activities of viper PLA_2.

Protein	LD_{50} (mg/kg mouse)	Enzymatic activity[a] (μmoles/min/mg of protein)
D. r. russellii		
VRV PL-VIIIa[21]	i.p. 5.3	0.111
Whole venom[21]	i.p. 4.1	0.060
VRV PL-VIIIb[19]	i.p. 5.2	0.472
VRV PL-V[20]	i.p. 1.8	0.056
VRV PL-VI[20]	i.p. 3.5	0.084

Table 3 Lethal potencies and specific enzymatic activities of viper PLA_2. (continued)

Protein	LD_{50} (mg/kg mouse)	Enzymatic activity[a] (μmoles/min/mg of protein)
D. r. russellii		
R1 [9]	i.v. >5.00	-
R2 [9]	i.v. 2.10	-
R3 [9]	i.v. >10.00	-
R2+R3 [9]	i.v. 0.15	-
D. r. formosensis		
RV-4 [22]	i.v. 0.32	304
RV-7 [22]	i.v. >10.00	2.4
RV-4+RV-7 [22]	i.v. 0.15	192
D. r. siamensis (Myanmar)		
Daboiatoxin [18]	i.p. 0.05	91.7±4.5
Whole venom [18]	i.p. 0.6	39.2±2
V. a. ammodytes		
Vipoxin A [30]	Nontoxic	-
Vipoxin B [31]	0.05-0.15	-
Ammodytin I2 [32]	Nontoxic	-
Ammodytoxin A [33]	i.v. 0.021	280
Ammodytoxin B [34]	i.v. 0.58	520
Ammodytoxin C [35]	i.v. 0.36	-

[a] Conditions used for enzymatic activity measurements vary greatly, so comparisons of values between references must be done with caution. Substrates include egg yolk, egg lecithin, synthetic phospholipids, etc. In some cases detergents (sodium deoxycholate, Triton X-100) were added. Titrimetric, colorimetric and turbidimetric methods were used. Calcium concentration = 1-40 mM. pH = 7.3-8. Temperature = 18-50^{O}C. The common definition of 1 unit is a decrease of 0.01 in absorbance in 10 min by 1 mg of enzyme [36].

2. Enzymatic Activity

Such PLA_2 enzymes vary in their lethal and pharmacological potencies from low lethality and no specificity of action to so toxic and so specific in their actions. It is more interested in the lethal potencies and their enzymatic activity than other enzymes in snake venoms[36].

The previous report showed that there is no relationship between relative enzymatic activities of the PLA_2 enzymes and their LD_{50} values[37,38]. The enzymatic activities of some PLA_2 are shown in **Table 3**. Enzymatic activity values were reported in either as μmoles/min/mg of protein or as units. It is expressed as a decrease in absorbance per min and per μg of phospholipase[36,39]. This activity can be converted to μmoles of fatty acid released per min and per mg of phospholipase by measuring the decrease in absorbance of 0.1 which obtained by 0.01 or 0.025 μmoles of acid[39]. Because conditions used for measuring the enzymatic activities varied greatly from one study to another (see footnote to **Table 3**), the cross-comparison of values obtained by different investigators must be done with great caution[36].

3. Comparison of PLA_2 Sequences

The N-terminal amino acid sequence of some PLA_2s were shown in **Table 4** [9,18]. This report indicated that *D. r. siamensis* contains at least 5 PLA_2 isoforms[9]. The PLA_2 R3 of *D. r. russellii* and S4 of *D. r. siamensis* are probably identical to F7 (previously named RV-7) of *D. r. formosensis*[9]. The neurotoxin PLA_2 R2 and S2 are also very similar to F4 (previously named RV-4)[9]. It is noted that the neurotoxicity of R2 is dependent on the presence of R3, while F4 or S2 is moderate neurotoxic itself[9]. However, identity between S2 of *D. r. siamensis* and neurotoxic F4

of *D. r. formosensis* do not explain the lack of neurotoxicity in Thai Russell's viper bites. On the other hand, R1 and S1 are non-neurotoxic [9].

Table 4 The N-terminal amino acid sequences of viper PLA_2 toxin [9]

	* * * * *	
R1	NLFQFAEMIVKMTGKNPL-SYSDYGCYCGWGGKGKPQDATDRCCFVHDCC	
S1-2		(100%)
S1-1	Y GR FR A N	(86%)
	* * * * *	
F4	NLFQFARMINGKLGAFSVWNYISYGCYCGWGGQGTPKDATDRCCFVHDCC	
R2		(100%)
S2		(100%)
S3	DA QE F K	(88%)
VpxB	K	(98%)
	* * * * *	
F7	NLFQFGEMILEKTGKEVVHSYAIYGCYCGWGGQGRAQDATDRCCFVHDCC	
R3		(100%)
S4		(100%)
RVV012		(100%)
VpxA	D Q A R AQ	(88%)
	* * * * *	
P1	SLLEFGKMILEETGKLAIPSYSSYGCYCGWGGKGTPKDATDRCCFVHDCC	
P2-1	M V F	(94%)
P2-2	V F	(96%)
P3	M V F D	(92%)
Amdx	M G NPLT F V	(84%)
	* *	
DbTx	NFFQFAEMIVKMTGKEAVHS…	

Note R: PLA_2 isoforms from *D. r. russellii* (Pakistan) [9]

S: PLA_2 isoforms from *D. r. siamensis* (Thailand) [9]

F: PLA_2 isoforms from *D. r. formosensis* (Taiwan) [9]

P: PLA_2 isoforms from *D. r. pulchella* (Sri Lanka) [9]

RVV: PLA_2 isoform from *D. r. siamensis* (Thailand) [40]

Amdx: ammodytoxin from *Vipera ammodytes* [9]

DbTx: only 20 amino acid sequences of daboiatoxin PLA_2 from *D. r. siamensis* (Myanmar) [18]

VpxA and VpxB: vipoxin from *Vipera ammodytes ammodytes* [22]

Several amino acid sequences were deduced from nucleotide sequences. For example, cDNA cloning and sequencing of *RV-4* and *RV-7* of Taiwan Russell's viper[22]. The deduced amino acid sequences of RV-4 and RV-7 are 80% similar to each other[22](**Figure 5**).

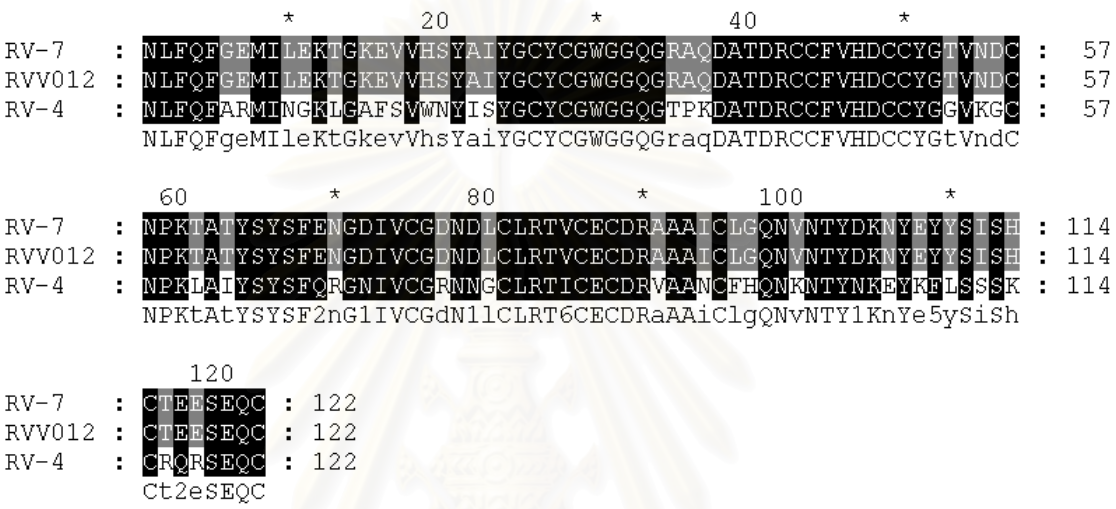

Figure 5 An alignment of deduced mature amino acid sequence PLA_2s. RVV012; PLA_2 from *D. r. siamensis*[40], RV-7 and RV-4; PLA_2 from *D. r. formosensis*[22].

4. Production of Recombinant PLA_2

The availability of the recombinant snake venom PLA_2 production system is great interest for several reasons[41]. First, snake venom contains numerous PLA_2 isoforms, among which some might constitute undesirable contaminants which could interfere with activity assays. The use of the recombinant PLA_2 should avoid such problems. Second, recombinant technology provides the possibility of producing mutants, which could be used for functional study. Finally, recombinant technique can also be utilized to achieve labeling of PLA_2s with stable isotopes prior to other studies[41].

Secreted PLA_2 have been produced in various organisms, including yeast, mammalian cells and bacteria, but snake venom PLA_2s have been produced only in *Escherichai coli*, usually a convenient host in terms of rapidity and easiness [41].

Production of recombinant PLA_2s requires a correct N-terminal residue, which is essential for enzymatic activity and proper folding of the PLA_2. Two different approaches have been used to generate appropriately cleaved PLA_2s [41]. The first one consists of producing the PLA_2 as a short or long fusion protein which is further cleaved chemically or enzymatically [41]. The second requirement for producing recombinant active PLA_2 is the correct formation of the disulfide bridges [41]. In *E. coli*, these bonds cannot be formed in the cytoplasm and hence recombinant PLA_2s produced as inclusion bodies must be refolded *in vitro* [41,42]. For example PLA_2 expression, *Vipera ammodytes ammodytes* AmmodytoxinA has been highly expressed with a long fusion protein. In addition, A-PLA_2 of *Agkistrodon halys* Pallas was expressed in *E. coli* with initiator Met[37]. This recombinant protein has been efficiently renatured with 27% activity of native enzyme [37].

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CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1. Screening of *PLA_2* Clones from ZAP Express cDNA Library

1.1.1. ZAP Express cDNA Library of Russell's Viper Venom Glands

ZAP Express cDNA library of Russell's viper venom glands used in this study was obtained from previous study in our lab[40]. ZAP Express vector map is shown in **Figure 6**.

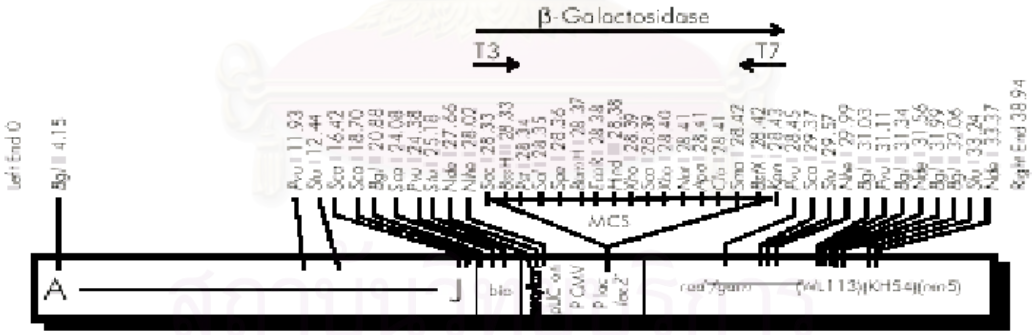


Figure 6 Map of the ZAP Express vector.

1.1.2. Plaque Lift Hybridization and Detection System

Hybond-N, Nylon membrane was purchased from Phamacia Biotech AB, USA.

North2South® Direct HRP Labeling and Detection Kit were purchased from PIERCE, USA.

1.1.3. *In Vivo* Single-clone Excision of the pBK-CMV Phagemid Vector from the ZAP Express Vector

ExAssist helper phage, provided in ZAP Express® cDNA Gigapack® III Gold Cloning Kit (Stratagene), was used for generating the pBK-CMV phagemid vector. Map of pBK-CMV phagemid vector is shown in **Figure 7**.

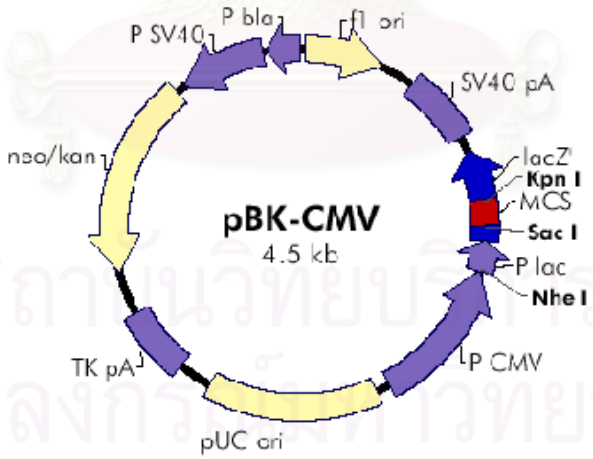


Figure 7 Map of the pBK-CMV vector.

1.1.4. Genotypes of *Escheracia coli* Strain

XL1-Blue MRF' strain; Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gryA96 relA1 lac [F' proAB $lacl^qZ\Delta M15$ $Tn10(Tet^r)$].

XLOLR strain; Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 thi-1 recA1 gryA96 relA1 lac [F' proAB $lacl^qZ\Delta M15$ $Tn10(Tet^r)$] Su^- (nonsuppressing) λ^r (lambda resistant).

1.1.5. Enzymes

Table 5 Restriction enzymes with their recognition sites, recommended buffer and manufacturer.

Enzymes	Recognition sequence	Buffer	Manufacturer
Xho I	C^TCGAG	NEBuffer 2	New England Biolabs
*Eco*R I	G^AATTC	NEBuffer 2	New England Biolabs
*Bam*H I	G^GATCC	NEBuffer 2	New England Biolabs

Note ^ represents the cleavage sites of restriction enzymes.

1.1.6. DNA Sequencing Reaction

ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit Version 2.0, was purchased from Applied Biosystems, USA.

1.2. PLA_2 Expression

1.2.1. Expression in *E. coli*

1.2.1.1. Plasmid Vector

pTrcHisA (Invitrogen), a vector containing N-terminal 6xHis tags, was used for expression of recombinant proteins in *E. coli*. Its physical map is shown in **Figure 8**.

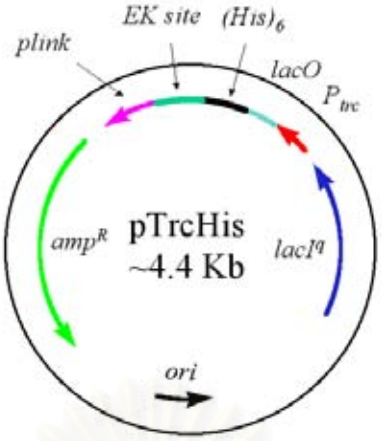


Figure 8 Map of the pTrcHisA expression vector.

1.2.1.2. Synthetic Oligonucleotides (or Primers)

Oligonucleotides were purchased from Bio Service Unit, NSTDA, Thailand.

Table 6 Oligonucleotides and their descriptions.

Name	Sequence (5'-3')	T_m (^{O}C)	Description
PLAF	CGGGATCCGGGAACCTTTTCCAGTT	50	Oligonucleotides for PCR amplification of *PLA*$_2$ gene with *Eco*R I and *Bam*H I recognition sites.
PLAR	GGAATTCCTTAGCATTGCTCTGAC	48	
5'FPLA-4F	AGCCTGGAGGTGCTTCTGG	62	Oligonucleotides for full-length amplification.
5'FPLA-7F	AGCCTGGAGGTGCTTCTGA	60	
3'FPLA-R	TTTAGTGCAGAGCTGGCACC	62	
FPLA-R	CACTCACCGCAGACGATAT	58	
T_3	AATTAACCCTCACTAAAGGG	56	Sequencing primer from T_3 or T_7 promoters.
T_7	GTAATACGACTCACTATAGGGC	64	

Note

T_m was calculated from the formula 2 ^{O}C (A+T) + 4 ^{O}C (G+C).

1.2.1.3. Enzymes

Taq DNA polymerase (Promega)

Pfu DNA polymerase (Promega)

T4 DNA ligase (USB)

*Eco*R I and *Bam*H I were listed in Table 1.

1.2.1.4. DNA Purification from Gel Slice

QIAquick® Gel Extraction Kit was purchased from QIAGEN Inc., USA.

1.2.1.5. Genotypes of *Escheracia coli* Strain

DH5α strain; supE44 ΔlacU169 (phi 80 lacZ ΔM15) hsdR17 recA1 endA gyrA96 thi-1 relA1.

TOP10 strain; mcrA Δ(mrr-hsdRMS-mcrBC) phi 80 Δlac ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara leu) 7697 galU galK λ^- rpsL endA1 mupG

1.2.2. Proteins Detection

1.2.2.1. Sodiumdodecrylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Mini-PROTEAN® 3 Electrophoresis Cell was purchased from BIO-RAD Laboratories, USA.

Low molecular weight standard was purchased from Phamacia Biotech AB, USA.

1.2.2.2. Coomassie Brillient Blue Staining

Coomassie Brilliant Blue R-250 was purchased from USB, USA.

1.2.2.3. Silver Staining

Silver Staining Kit Protein was purchased from Phamacia Biotech AB, USA.

1.2.2.4. Western Blotting Hybridization

Trans-Blot® SD semi-dry electrophoretic transfer cell (BIO-RAD Laboratories, USA) was used for transfer proteins from gel to membrane by electrophoresis.

Nitrocellulose membrane (BioTrace® NT) was purchased from Pall Gelman Science, USA.

Mouse Anti-His Antibody was purchased from Phamacia Biotech AB, USA.

Peroxidase-Conjugated Rabbit Anti-Mouse Antibody was purchased from DAKO.

3, 3'- diaminobenzidine (DAB) tetrahydrochroride was purchased from BIO BASIC, Inc., Canada.

1.2.3. Protein Purification by Using Immobilized Metal Affinity Chromatography (IMAC)

TALON® Metal Affinity Resins was purchased from CLONTECH Laboratories, Inc., USA.

1.2.4. Refolding of Protein by Dialysis

SnakeSkin® Dialysis Tubing, 10K MWCO was purchased from PIERCE, USA.

1.2.5. Concentration of Protein

Vivaspin concentrators were purchased from Vivascience Ltd., Germany.

1.2.6. Quantitative Assay of PLA_2 Protein

Bio-Rad protein assay was purchased from BIO-RAD Laboratories, USA.

1.2.7. PLA_2 Activity Test

Egg lecithin was purchased from Sigma.

1.3. Cloning of Genomic *PLA_2* DNA (Isolation of Full-length *PLA_2*)

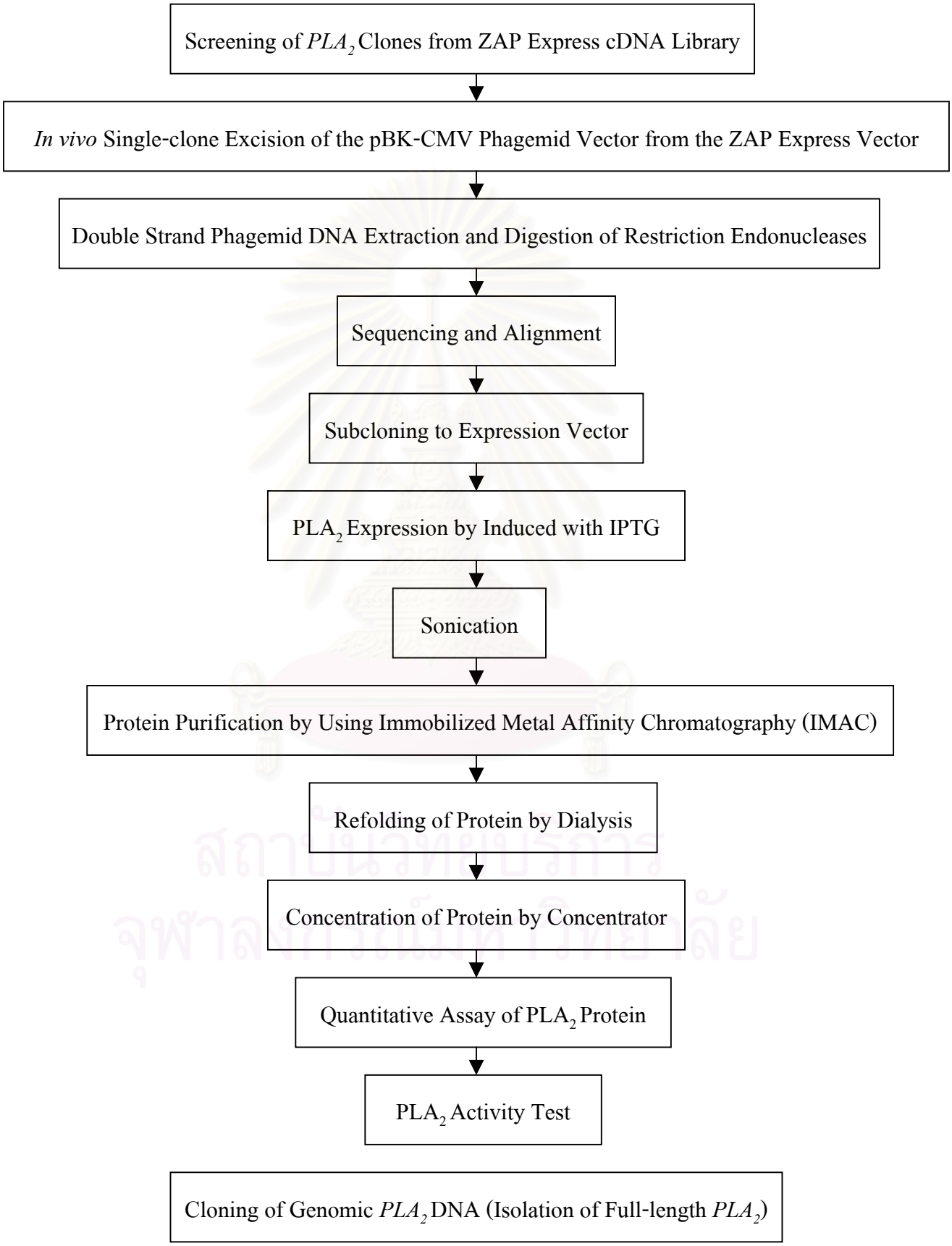
pGEM® -Teasy vector system was purchased from Promega, USA.

Bacterial strains: *Escheracia coli*, **JM109 strain**, endA1, recA1, gyrA96, thi, hsdR17 (rk –, mk +), relA1, supE44, (lac-proAB), [F, traD36, proAB, laqI q Z M15].

1.4. Chemicals

All other chemicals used in this work were either analytical or molecular biology grades purchased from many suppliers (Sigma; BIO-RAD; Phamacia Biotech AB; USB; Scharlau Chemie, S.A. and Merck).

Work Outline



2. Methods

2.1. Screening of *PLA*$_2$ Clones from ZAP Express cDNA Library

2.1.1. Plaque-lift Hybridization

2.1.1.1. Plating

The host bacteria, XL1-Blue MRF' cells, was prepared as follows. A single colony was inoculated into 3 ml of LB broth (1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract, pH 7.0) with 10 mM $MgSO_4$ and 0.2% w/v maltose, incubated with shaking at 37^{O}C overnight or to an OD_{600} of 0.5-1.0. Then, the cells were spun at 600 rpm for 2 min and supernatant was discarded. The cells were diluted to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$. The lambda phage cDNA library was diluted in SM buffer (50 mM Tris-HCl pH 7.5, -NaCl, -$MgSO_4 \cdot 7H_2O$, 0.01% gelatin) and added the equivalent of 50,000 pfu/plate to 200 µl of host cells at an OD_{600} of 0.5. The bacteria and phage mixture were then incubated at 37^{O}C for 15 min to allow the phage to attach to the cells. Three ml of NZY top agar (0.5% w/v NaCl, 0.2% w/v $MgSO_4 \cdot 7H_2O$, 0.5% w/v yeast extract, 1% NZ amine (casein hydrolysate), 0.7% w/v agarose, pH 7.5) was added into the bacteria and phage mixture and immediately pored onto an NZY agar plate (0.5% w/v NaCl, 0.2% w/v $MgSO_4 \cdot 7H_2O$, 0.5% w/v yeast extract, 1% NZ amine (casein hydrolysate), 1.5% w/v agar, pH 7.5). The plate was incubated overnight at 37^{O}C.

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2.1.1.2. Lifting

After plating, the plate was chilled for 2 hours at 4^{O}C to prevent the NZY top agar from sticking to the nitrocellulose membrane. The nitrocellulose membrane was placed onto the NZY agar plate for 2 min to allow the transfer of the phage particles to the membrane. Both of the membrane and plate were marked same position to return collect the interested clones. Following lifting step, the membrane

was denatured and neutralized by submerge into a denaturation solution (1.5 M NaCl and 0.5 M NaOH) for 2 min, and a neutralization solution (1.5 M NaCl and 0.5 M Tris-HCl, pH 8.0) for 5 min, respectively. The membrane was briefly submerged in a 0.2 M Tris-HCl, pH 7.5 and 2X SSC buffer solution (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) for 30 sec and blotted on a Whatman™ 3MM paper for 1-2 min. Finally, the membrane was baked in the oven at 80^{O}C for 1.5-2 hours to crosslink the DNA to the membrane and stored at 4^{O}C.

2.1.1.3. Probe Labeling

The *RVV012-PLA$_2$* DNA fragment[40] which isolated from previous work and shared homology to RV-7 of Taiwan PLA$_2$ isoform, was prepared from PCR amplification was labeled by North2South® Direct HRP Labeling and Detection Kit as follows. One hundred ng of *PLA$_2$* DNA fragments in 10 µl water were denatured at 95 ^{O}C for 5 min and snapped cool for 5 min. Ten µl of North2South® Direct Stabilized HRP Label and 10 µl of North2South® Direct Reaction Buffer were added. After incubation at 37^{O}C for 30 min, 30 µl of North2South® Direct Enzyme Stabilization Solution was added and mixed. The probe concentration was approximate1.67 ng/µl.

2.1.1.4. Hybridization

Hybridization of the *PLA$_2$*-cDNA on the membrane with a DNA probe was conducted by North2South® Direct HRP Labeling and Detection Kit under the following condition.

To pre-hybridization, equal volume of North2South® Direct Hybridization Buffer Component 1 and 2 were combined in a clean plastic plate at least 0.1 ml per cm^2 membrane. The hybridization solution was incubated at 55°C in a hybridization oven (HYBAID). After warming the hybridization solution at least 5 min, the blot-membrane was placed and pre-hybridized with gentle rotation for at least

15 min. Following the pre-hybridization step, about 5-10 ng of HRP-labeled DNA probe per ml of hybridization solution was added and incubated 1-4 hours with gentle rotation at 55°C. After hybridization, the membrane was washed 3 times with 0.5 ml per cm^2 membrane washing buffer 1 (2X SSC, 0.1% SDS) at 55°C for 5 min per each wash, performed same process with washing buffer 2 (2X SSC) at room temperature, and proceed to detection step.

2.1.1.5. Detection

The membrane was placed in a clean plastic bag containing equal volume of the North2South® Luminol/Enhancer Solution and North2South® Stable Peroxide Solution, incubated for 5 min at room temperature. After development, the solution was removed and the membrane was transferred and sealed in a new plastic bag. The chemiluminescent signals were detected by exposure to the X-ray film for 1 min.

2.1.2. *In vivo* Single-clone Excision of the pBK-CMV Phagemid Vector from the ZAP Express Vector

ZAP Expression vector with inserted DNA can be excised out of the phage in form of the kanamycin-resistant pBK-CMV phagemid vector by performance of the ExAssist helper phage into the host bacterial strains, XL1-Blue MRF' and XLOLR. The XL1-Blue MRF' strain can be co-infected with lambda phage cDNA library and helper phage, and secreted phagemid particles containing inserted DNA after helper phage procession. The XLOLR strain is designed to allow only the excised phagemid to replicate in the host, thus the ExAssist helper phage is unable to replicate its genome in this host cells. The XLOLR is also resistant to lambda phage infection,

so only pBK-CMV phagemid is replicated in host. *In vivo* single-clone excision was proceeded as described.

The plaques of PLA_2 clones were obtained from 2 method. The first method was plaque-lift hybridization as described in section 2.1.1. The second method was Expressed Sequence Tags (ESTs) study in our lab. The interested plaques were cored from the agar plate and transferred to a sterile microcentrifuge tube containing 500 μl SM buffer (50 mM Tris-HCl pH 7.5, 0.1 M NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, 0.01% gelatin) and 20 μl of chloroform. The content was vortexed to release the phage particles into the SM buffer, and incubated overnight at $4^{O}C$. The XL1-Blue MRF' cells and XLOLR cells were grown separately overnight in LB broth (1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract, pH 7.0) with 10 mM $MgSO_4$ and 0.2% (w/v) maltose, and in NZY broth (0.5% w/v NaCl, 0.2% w/v $MgSO_4 \cdot 7H_2O$, 0.5% w/v yeast extract, 1% NZ amine (casein hydrolysate), pH 7.5), respectively. Following inoculation, the cells were gently spun down at 6,000 rpm for 2 min and supernatant was discarded. The pellet was suspended in 10 mM $MgSO_4$ to an OD_{600} of 1.0. One μl of the ExAssist helper phage was added into the mixture containing 60 μl of XL1-Blue MRF' cells at an OD_{600} of 1.0 and 75 μl phage stock of interested plaque in SM buffer. The reaction was incubated for $37^{O}C$ for 15 min, and then 750 μl of NZY broth was add and incubated at $37^{O}C$ for 2-3 hours with shaking. After incubation, the mixture of bacteria and phage was heated at $65\text{-}70^{O}C$ for 20 min and spun down at 600 rpm for 2 min. The supernatant was decanted into a new clean tube. This stock contains the excised pBK-CMV phagemid vector packaged as filamentous phage particle. The mixture of 100 μl phage supernatant and 200 μl freshly grown XLOLR cells was incubated at $37^{O}C$ for 15 min. Three hundred μl of NZY broth was added and incubated at $45^{O}C$ for 45 min. After incubation, the XLOLR cells were spread on LB-kanamycin agar plate (50 μg/ml) and incubated overnight at $37^{O}C$.

2.1.3. Double Strand Phagemid DNA Extraction by Alkaline Lysis Method

A single colony of bacteria was inoculated in 3 ml NZY broth and incubated at 37°C with 200 rpm shaking for 16-20 hr. The cells were harvested in 1.5 ml microcentrifuge tubes by centrifugation at 5,000 rpm for 3 min and then resuspended in 100 μl of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) with 20 μg/ml RNase A. To the cell suspension, 200 μl of freshly prepared solution II (1% SDS, 0.2 N NaOH) was added and incubated on ice for 5 min. Then, 150 μl of solution Ill (3 M potassium acetate, 11.5% glacial acetic acid) was added to the mixture and incubated on ice for 5 min. The mixture was pellet by centrifugation at 10,000 rpm for 10 min. The supernatant was decanted to a new tube. The phagemid DNA was recovered from the supernatant by adding 7/10 volume of isopropanol and standing in room temperature for 10 min. The content was centrifuged at 12,000 rpm for 10 min. The pellet was washed with 500 μl of 70% ethanol, centrifuged at 12,000 rpm for 5 min and dried at room temperature. The DNA pellet was resuspended in 30 μl of TE buffer pH 7.5 (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) or sterile water.

2.1.4. Digestion of Restriction Endonucleases and Analysis

About 500 ng of phagemid DNA was double-digested with 5 units of *Eco*R I and *Xho* I according to manufacturer's protocols (New England Biolabs), 1X reaction buffer (provided), 1X BSA and sterile distilled water to a final volume of 20 μl. The digestion was incubated at 37°C for 3 hours. After digestion, digested phagemids were fractionated on 1.2% agarose gel electrophoresis. Clones which containing of about 0.6 kb insertions, the expected size of mature PLA_2 cDNA, were selected for sequencing.

2.1.5. Sequencing

The PCR sequencing was performed by using BigDye™ Terminator Cycle Sequencing Ready Reaction kit. The PCR reaction was carried out in a 10 μl reaction containing 4.0 μl of terminator ready reaction mix, 3.2 pmol of sequencing primer and 500 ng DNA template. After incubation at 95°C for 2 min, amplification was carried out for 25 cycles with the following temperature cycling parameters; 95°C for 10 sec of denaturation, 50°C for 5 sec of annealing and 60°C for 4 min of extension. The DNA was then precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of absolute ethanol and incubated at -20°C for 1 hour. After centrifugation at 12,000 rpm for 10 min, the pellet was washed with 500 μl of 70% ethanol and air dried. The DNA pellet was resuspended in 10 μl Template Suppression Reagent (Perkin-Elmer) and loaded to the ABI PRISM sequencer.

2.1.6. Alignment and Computational Searching Sequences Analysis

The nucleotide sequences obtained from the clones of interest were compared against nucleotide sequences in online database by using BLAST N (Basic Local Alignment Search Tool) program via the World Wide Web. The sequences, which have significant homology to those of PLA_2 genes, were further analyzed.

Alignments of PLA_2 sequences were made using CLUSTAL X multiple alignment program.

2.2. PLA_2 Expression

2.2.1. Expression in *E. coli*

2.2.1.1. Subcloning to Expression Vector

2.2.1.1.1. PCR Amplification

Two primers, PLAF and PLAR, were designed from two conserved sequence among the *PLA_2* isoforms (*RV-4* and *RV-7*)[22] downstream the signaling sequences. These primers also contain some restriction recognition sites at their 5' end (PLAF: *Bam*H I, PLAR: *Eco*R I) for facilitating the ligation to cloning vector.

The typical PCR reaction was carried out in a 25 μl reaction containing 1X PCR buffer, 1.25 units of Taq DNA polymerase (Promega), 1 μM of each primer, 1.5 mM MgCl2, 200 μM of each dNTPs and 200 ng DNA template. After incubation at 94°C for 2 min, amplification was carried out for 5 cycles with the following temperature cycling parameters: 94°C for 20 sec of denaturation, 48°C for 30 sec of annealing and 72°C for 30 sec of extension. Then, 30 cycles of the following temperature cycling parameters were performed: 94°C for 20 sec of denaturation, 60°C for 30 sec of annealing and 72°C for 30 sec of extension. The final amplification cycle included an addition of a 5 min extension at 72°C.

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2.2.1.1.2. DNA Purification from Gel Slice

After PCR, the amplified DNA fragments were purified by QIAquick® Gel Extraction Kit in order to remove impurities such as small RNA, proteins, unincorporated nucleotides or primers. A band of the DNA fragment of interest was excised from agarose gel with a clean, sharp razor blade. Three volumes of Buffer QG to one volume of gel (100 mg ~ 100 μl) was added and the tube was placed in a 50°C water bath incubator. After agarose gel was completely dissolved, one gel volume of

isopropanol was added, mixed and applied to the QIAquick column. After centrifugation at 10,000 xg for 1 min, the flow-through solution was discarded. The DNA fragments was washed with Buffer PE and centrifuged for 1 min. Buffer EB (10 mM Tris-HCl, pH 8.5) or distilled water was added to elute DNA and was then centrifuged for 1 min, stored at -20°C.

2.2.1.1.3. Ligation of PCR Products into Plasmid Vector

After *Bam*H I and *Eco*R I-digestion of pTrcHisA vector and PLA_2 DNA, the digested DNA was purified from gel slice by QIAquick® Gel Extraction Kit as described in section 2.3.1.1.2 and ligation was proceeded under as follows. The ligation reaction was carried out in a 10 μl reaction mixture containing pTrcHisA vector and PLA_2 DNA in the molar ratio 1:3, 2 units of T4 DNA ligase and 1X buffer. An appropriate amount of sterile water was added to make the 10 μl final volume. The pTrcHisA vector is approximate 4.4 kb and supplied at 50 ng/μl. The amount of the DNA insert was calculated from the following equation:

$$\frac{\text{ng of vector x size (kb) of insert}}{\text{size (kb) of vector}} \text{ X insert : vector molar ratio = ng of insert}$$

The ligation reaction was carried out at 16°C for 16-18 hours and the ligation products were used to transform *E. coli* competent cells prepared by $CaCl_2$ method.

2.2.1.1.4. Preparation of *E. coli* Competent Cells by $CaCl_2$ Method

A single colony of *E. coli* was inoculated into 3 ml of LB broth and incubated at 37°C with 200 rpm shaking for 16-20 hours. The overnight culture was diluted 1:100 into 100 ml of new LB broth and incubated at 37°C until an OD_{600} of 0.4-0.5. The cell culture was chilled on ice for 10 min prior to pipetting the 10 ml aliquots of the culture into 15 ml sterile polypropylene tubes. The cells were pelleted by centrifugation at 4,000 rpm for 10 min at 4°C. After that, the pellet was suspended in 5 ml of ice-cold 0.1 M $MgCl_2$, centrifuged, resuspended in 5 ml of ice-cold 0.1 M $CaCl_2$ and left on ice for 30 min to establish competency. Finally, after centrifugation, the pellet was resuspended in 750 µl of 15% v/v glycerol and 0.1 M $CaCl_2$. The cells were kept in 200 µl aliquots at -80°C until required.

2.2.1.1.5. Transformation of *E. coli* Competent Cells

Two hundred µl of *E. coli* competent cells were mixed with 2 µl of ligation products and immediately placed on ice for 30 min. The cells were subjected to heat-shock at 42°C for 45 sec and placed on ice for an additional 3 min. The transformed cells were mixed with 800 µl of LB broth and incubated at 37°C for 1 hour with shaking at 200 rpm. Finally, 100 µl of the transformed culture was spread on a LB agar plate containing 50 µg/ml ampicillin and incubated at 37°C overnight.

After transformation, the pTrcHisA vectors containing *PLA_2* DNA were extracted by alkaline lysis method as described in section 2.1.3. Then, PCR sequencing as described in section 2.1.5 was proceeded to ensure the correct *PLA_2* sequences.

2.2.1.2. PLA_2 Expression by Induced with IPTG

A single colony of *E. coli* was inoculated into 20 ml of LB broth containing 50 μg/ml ampicillin and incubated at 37°C with 200 rpm shaking for 16-20 hours. The overnight culture was diluted 1:100 into 1 L of LB broth containing 50 μg/ml ampicillin and incubated at 37°C until an OD_{600} of 0.6-0.7. Then 5 ml of cells was collected to be negative control. One mM of IPTG was added into the culture and incubated at 37°C with 180 rpm shaking for 16-20 hours. The cells were pelleted by centrifugation at 6,000 rpm for 10 min at 4°C. The pellet was washed twice with PBS buffer and proceed to sonication step.

2.2.1.3. Sonication

The pellet was suspended with 30 ml PBS buffer, pH 7.4 and vortexed. The cells were sonicated 3 times for 30 sec each time. The cell lysate was centrifuged at 12,000 rpm for 30 min, 4°C. Before the protein purification step, the lysate was washed 3 times with washing buffer 1 (0.5% v/v Triton X-100, 50 mM Tris-HCl pH 8.0, 100 mM NaCl) and centrifuged at 12,000 rpm for 20 min, 4°C and then washed twice with washing buffer 2 (50 mM Tris-HCl pH 8.0, 100 mM NaCl). The pellet was stored at -80°C.

2.3. Protein Purification by Using Immobilized Metal Affinity Chromatography (IMAC)

The pellet was suspended with 6 M Guanidine Buffer, pH 8.0 (6 M guanidine, 10 mM Tris-HCl, 50 mM NaH_2PO_4, 100 mM NaCl) and shaken at room temperature or 4°C for 1 hour. The suspension was centrifuged at 12,000 rpm for 30 min. The supernate was decanted and loaded into the column containing 6 M

guanidine buffer equilibrated-resin. The column was washed with 8 M Urea Buffer, pH 8.0 (8 M urea, 10 mM Tris-HCl, 50 mM NaH_2PO_4, 100 mM NaCl) and 8 M Urea Buffer, pH 7.0 (8 M urea, 50 mM NaH_2PO_4, 100 mM NaCl), respectively. Finally, the His-tagged protein was eluted with 50 ml Elution Buffer, pH 4.5 (8 M urea, 50 mM NaH_2PO_4, 100 mM NaCl, 20 mM MES) into 50 fractions with a flow rate of 0.5 ml/min. Fractionation was carried out at room temperature. Absorbancy of each protein elution was monitored at 280 nm.

2.4. Proteins Detection

2.4.1. Sodiumdodecrylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Twelve percentage of Resolving gel and 3.85% of Stacking gel were freshly prepared as follows:

12% of Resolving gel (1 page)

40% Acrylamide:Bisarylamide (19:1)	1.200 ml
3 M Tris-HCl, pH 8.8	0.504 ml
10% SDS	0.040 ml
Distilled water	2.223 ml
10% ammonium persulphate	0.030 ml
TEMED	0.002 ml

3.85% of Stacking gel (1 page)

40% Acrylamide:Bisarylamide (19:1)	0.144 ml
0.5 M Tris-HCl, pH 8.8	0.375 ml
10% SDS	0.015 ml
Distilled water	0.954 ml
10% ammonium persulphate	0.011 ml

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TEMED	0.001 ml

After gel setting, the protein samples were mixed with 1/4 volume of Reducing Sample Buffer (62.5 mM Tris-HCl, pH 6.8; 8% w/v SDS; 40% v/v glycerol; 0.005% Bromophenol Blue; 10% 2-mercaptoethanol), denatured at 95°C for 10 min and loaded into gel slots in submarine condition. Electrophoresis was performed at 100 volts for 80 min in 1X Running Buffer, pH 8.3 (0.25 M Tris-HCl, 1.92 M glycine, 1% w/v SDS).

2.4.2. Coomassie Brillient Blue Staining

The gel was soaked in Coomassie Brillient Blue solution (0.25% w/v Coomassie Brillient Blue R-250, 45% methanol, 10% glacial acetic acid) for 1 hour with gentle agitation. After the staining solution was removed, the destaining solution (10% glacial acetic acid, 30% methanol) was added and incubated for 2-3 hours. The destaining solution was changed 3-4 times during incubation. Lastly, the gel was wrapped in cellophane sheet and dried overnight at room temperature for preserving the gel.

2.4.3. Silver Staining

Silver Staining Kit, Protein was used to increase sensitivity of the visualization allows detection of most proteins down to the nanogram range, which is 100 times more sensitive than Coomassie Brillient Blue staining. All of solutions were freshly prepared and performed according to manufacturer's protocols at room temperature with constant gentle agitation as follows:

Fixing solution

Absolute ethanol	100.0 ml
Acetic acid glacial	25.0 ml

Make up to 250 ml with distilled water

Sensitizing solution

Sodium acetate	17.0 g
Absolute ethanol	75.0 ml
Glutardialdehyde (25% w/v)	1.25 ml
Sodium thiosulphate (5% w/v)	10.0 ml

Make up to 250 ml with distilled water

Silver solution

silver nitrate solution (2.5% w/v)	25.0 ml
Formaldehyde (37% w/v)	0.1 ml

Make up to 250 ml with distilled water

Developing solution

Sodium carbonate	6.25 g
Formaldehyde (37% w/v)	0.05 ml

Make up to 250 ml with distilled water

Stopping solution

EDTA-$Na_2 \cdot 2H_2O$	3.65 g

Make up to 250 ml with distilled water

First of all, the gel was soaked in fixing solution for 30 min, then transferred to sensitizing solution for 30 min and washed 3 times with distilled water for 5 min each time. After washing step, the gel was soaked in silver solution for 20 min, and rinsed twice in distilled water for 1 min each time. The gel was developed by soak in developing solution for 2-5 min, stopped by transfer to stopping solution for10 min, and washed in 3 times with distilled water for 5 min each time. Finally, the gel

was wrapped in cellophane sheet and dried overnight at room temperature for preserving the gel.

2.4.4. Western Blotting Hybridization

After SDS-PAGE was performed completely, the proteins were transferred from polyacrylamide gel to nitrocellulose membrane. To overcome the inefficiency of capillary transfers, Trans-Blot® SD semi-dry electrophoretic transfer cell has been adopted for eluting proteins from polyacrylamide gel as follows. The gel and membrane were soaked in transfer buffer for 15 min. Both of equilibrated gel and wetted membrane were sandwiched between sheets of transfer buffer-soaked thick filter papers, and then were placed on Trans-Blot® SD cell. The gel sandwich was transferred at 20 volt for 40 min. The transfer efficiency can be monitored by staining the transferred gel with Coomassie Brillient Blue staining or with Silver Staining Kit.

To hybridization, the marker lane was cut to stain with Coomassie Brillient Blue for 10 min and destained with destaining solution until bands of marker were visualized. The another part of membrane was incubated in blocking solution (5% w/v skim milk in PBS buffer, pH 7.4) for 1 hour with gentle agitation and then was washed 3 times with PBS buffer, pH 7.4 for 3 min each time. The membrane was transferred to a new blocking solution and 1:3,000 dilution of Mouse Anti-His Antibody was added, and incubated at room temperature with gentle agitation for 1 hour. The membrane was washed 3 times with PBS buffer, pH 7.4 for 3 min each time. Then the membrane was soaked in a new blocking solution and 1:1,000 dilution of Peroxidase-Conjugated Rabbit Anti-Mouse Antibody was added, and incubated with gentle agitation for 1 hour. After washing step, the membrane was soaked in visualizing solution (1.66 mM 3, 3'- diaminobenzidine (DAB) tetrahydrochroride, 0.04% $NiCl_2$, and 0.006% H_2O_2), and allowed to occur in dark for 5 min. Finally, the

solution was removed and the membrane was washed with PBS buffer, pH 7.4 and dried overnight.

2.5. Refolding of Protein by Dialysis

After protein purification, the SnakeSkin® Dialysis Tubing was used to eliminate denaturation buffer to refolding of protein correctly as follows. The SnakeSkin® Dialysis Tubing was presoaked in 1 mM EDTA pH 8.0 and closed the lower end of the SnakeSkin® Tubing with SnakeSkin® Dialysis Tubing Clips. The sample was poured into the SnakeSkin® Tubing and closed the open end with Clips. The SnakeSkin® Tubing containing sample was soaked in 100 volumes of the dialysate 1 (5 M urea, 50 mM NaH_2PO_4, 100 mM NaCl), 100 volumes of the dialysate 2 (2.5 M urea, 50 mM NaH_2PO_4, 125 mM NaCl), and 100 volumes of the dialysate 3 (50 mM NaH_2PO_4, 150 mM NaCl), for 3 hours each, respectively. The dialysis process was carried out at 4°C. After dialysis step, the sample was collected and stored at 4°C.

2.6. Concentration of Protein by Concentrator

Following the refolding of protein, the diluted sample was concentrated by Vivaspin concentrator as follows. The sample was poured into the concentrator and centrifuged at 3,000 xg for 1 hours. The remained sample was collected and stored at 4°C.

2.7. Quantitative Assay of PLA_2 Protein

Five dilutions of bovine serum albumin standard (1-15 μg/ml) were prepared. Eighty μl of each dilution standard or sample solution was added into 20 μl of Dye Reagent Concentrate (BIO-RAD). The reaction was incubated at room temperature for 5 min and measured at 595 nm.

2.8. PLA_2 Activity Test

The spectrophotometric method, based on the pH change due to the liberation of fatty acids, was used. This method was modified from Araujo AL and Radvanyi F[39]as follows. Four μl of the enzyme was added to the sample cuvette containing 62.5 μl of reaction medium (100mM NaCl, 10 mM $CaCl_2$, 3.5 mM lecithin solubilised with 0.5% Triton X-100 and 0.055 mM phenol red adjusted to an optical density of 1.8-2.2 at 558 nm). The same volume of solution without the enzyme was added to the reference cuvette. The change in absorbance between the reference and sample cuvettes was read at 558 nm. Enzymatic activity is expressed as a decrease in absorbance per min and per μg of phospholipase. This activity can be converted to μmoles of fatty acid released per min and per mg of phospholipase by measuring the decrease in absorbance of 0.1 which obtained by 0.01 μmoles of acid.

2.9. Cloning of Genomic *PLA_2* DNA (Isolation of Full-length *PLA_2*)

2.9.1. PCR Amplification

The primers, 5'FPLA-4F, 5'FPLA-7F and 3'FPLA-R, were designed from untranslated region of conserved sequences of the *PLA_2* isoforms (5'FPLA-4F and 5'FPLA-7F).

The PCR reaction was carried out in a 50 μl reaction containing 1X pfu buffer with 2 mM $MgSO_4$, 1.25 U of pfu DNA polymerase (Promega), 1 μM of each primer, 200 μM of each dNTPs and 1 μg genomic DNA template. After incubation at 95°C for 5 min, amplification was carried out for 30 cycles with the following temperature cycling parameters: 95°C for 40 sec of denaturation, 59°C for 30 sec of annealing and 72°C for 4 min of extension. The final amplification cycle included an addition of a 5 min extension at 72°C.

2.9.2. Ligation into pGEM-T Vector

Such PCR products were amplified without A-tail overhang from pfu DNA polymerase, A-tailing was executed before the ligation step as follows. The reaction was carried out in a 10 μl reaction mixture containing 1-7 purified PCR fragments, 1X Taq buffer, 2.5 mM $MgCl_2$, 0.2 mM dATP, 5 units of Taq DNA polymerase and sterile water to final reaction volume 10 μl. Then, the reaction was incubated at 70°C for 30 min and the ligation was proceeded.

About 3-4 μl of A-tailed PCR fragments were added into the mixture containing 50 ng of pGEM®-T Easy Vectors, 1X Rapid Ligation Buffer, 3 Weiss units of T4 DNA Ligase. An appropriate amount of sterile water was added to final reaction volume 10 μl. The ligation reaction was carried out at 4°C for 16-18 hours and the ligation products were used to transform *E. coli* competent cells prepared by $CaCl_2$ method and sequencing as described in section 2.2.1.1.5 and 2.1.5, respectively. Finally, the nucleotide sequences were compared with GENBANK database by using BLAST N program via the World Wide Web and alignments by using CLUSTAL X multiple alignment program.

CHAPTER IV

RESULTS

1. Screening of *PLA$_2$* Clones from cDNA Library

1.1. Plaque-lift Hybridization

After hybridization with *RVV012-PLA$_2$* probe and washing out at low stringency condition, 27 of about 300 plaques were positive. The hybridized membranes were exposed on an X-ray films as shown in **Figure 9**.

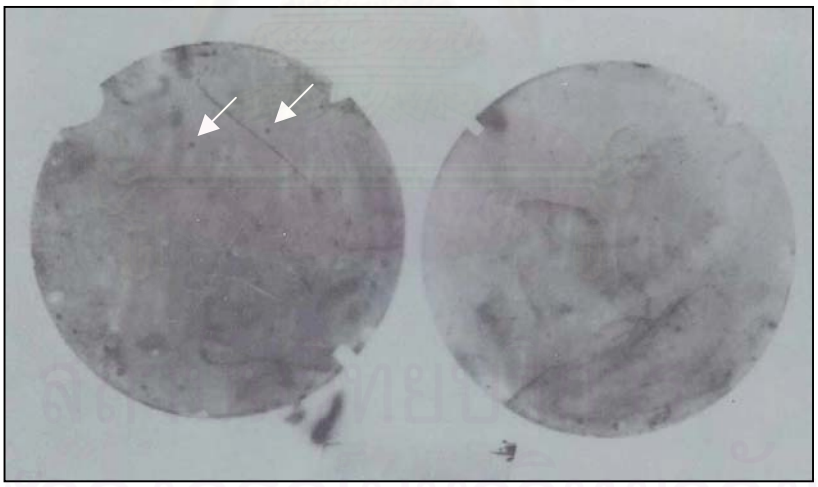

Figure 9 Positive *PLA$_2$* clones in plaque lift hybridization which were shown on X-ray film.

1.2. Digestion of Restriction Endonucleases and Analysis of the pBK-CMV Phagemid Vector

After detection of PLA_2 clones on x-ray film, the plaques of interest were cored from the agar plate. The linear DNA ZAP express with inserted clone was excised out of the phage in form of the pBK-CMV phagemid vector by performance of the ExAssist helper phage into the host bacterial strains by *in vivo* single-clone excision process. The phagemid DNA was double-digested with *Eco*R I and *Xho* I, and then fractionated on agarose gel electrophoresis to screen the clones which contain of about 0.6 kb insertions which is the expected size of mature PLA_2 cDNA (**Figure 10**).

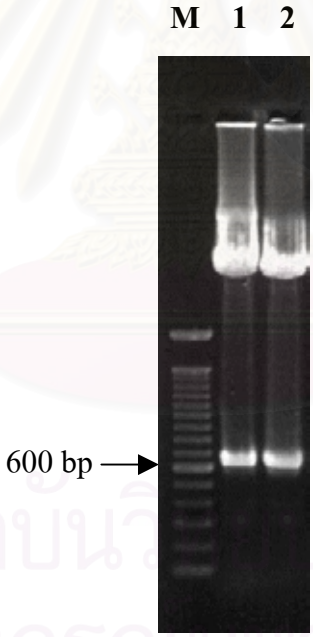

Figure 10 An ethidium bromide stained agarose gel of double strand phagemid of pBK-CMV digested by *EcoR* I and *Xho* I. Lane M; 300 ng of 100 bp DNA ladder, **lane 1** and **2**; digested-pBK-CMV containing 0.6 kb inserted cDNA.

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1.3. Sequence Alignment and Computational Searching Analysis

About 0.6 kb *PLA_2* cDNA clones were obtained from 2 methods. The first method was plaque-lift DNA hybridization using *RVV012* which was isolated from previous work as probe. Nine of *PLA_2* positive clones from hybridization were sequenced and only 2 forms of *PLA_2*, designated *PlaS1* and *PlaS2*, were found in 3 and 6 clones respectively. In the meantime, Expressed Sequence Tags (ESTs) study identified 17 *PLA_2* cDNA sequences, as same as plaque-lift DNA hybridization, only *PlaS1* and *PlaS2* were found in 5 and 12 clones respectively.

The DNA sequence analysis and comparison with GENBANK database using the BLAST N program and alignment by CLUSTAL X showed that *PlaS1* and *PlaS2* are identical to *RV-4* (GENBANK accession number S29298) and *RV-7* (GENBANK accession number S29299) which found in Taiwan Russell's viper venom. An alignment of 596 nucleotide and 122 deduced amino acid sequences of PlaS1 and PlaS2 were shown in **Figure 11** and **12**, respectively. An alignment showed 86% nucleotide sequence identity and 65% amino acid sequence identity.

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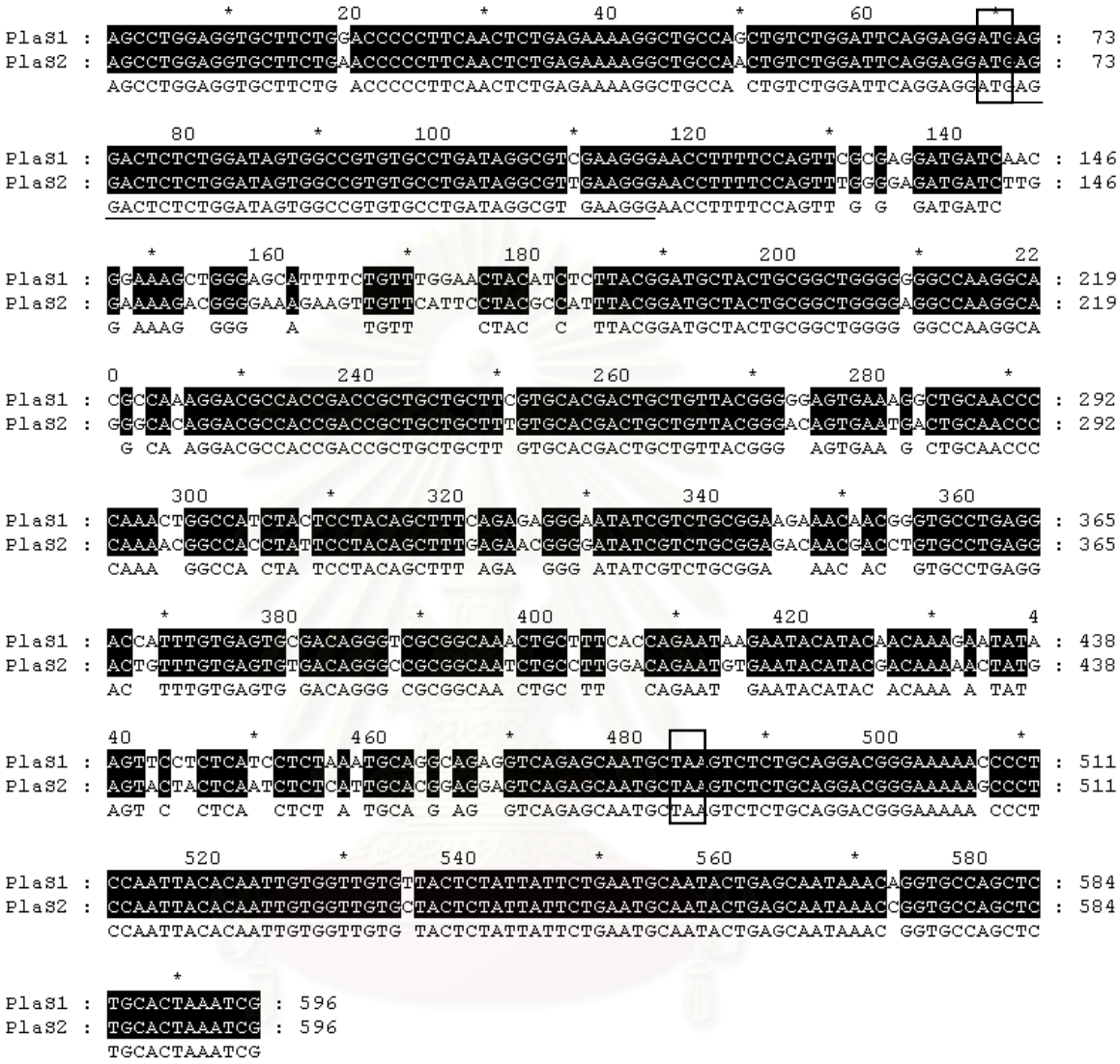

Figure 11 An alignment of nucleotide sequence of *PlaS1* and *PlaS2*. Identical nucleotides were highlighted. Start and stop codons were shown in boxes. The underlined is a signal sequence. An alignment showed 86% nucleotide sequence identity.

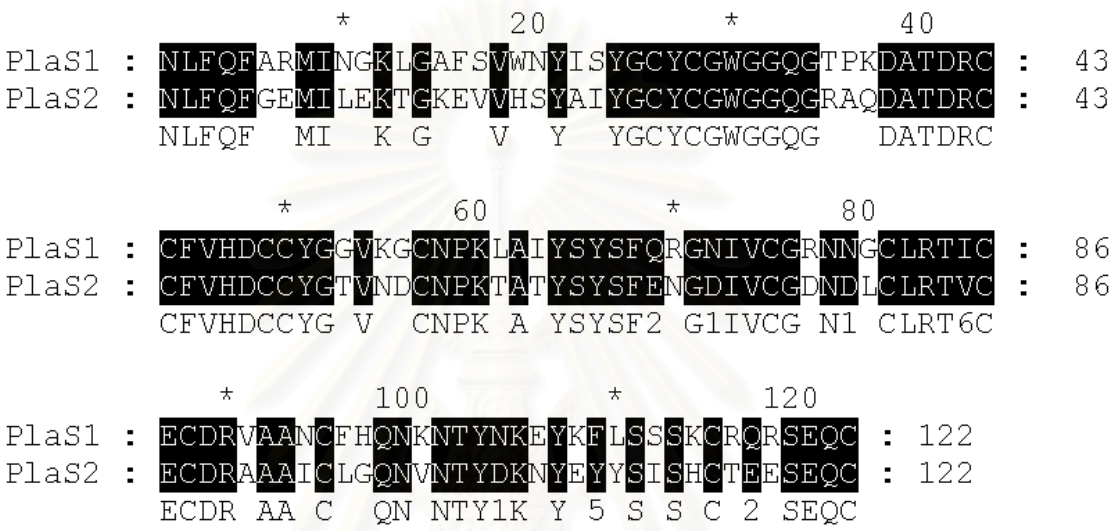

Figure 12 An alignment of deduced amino acid sequence of PlaS1 and PlaS2. Identical nucleotides were highlighted. An alignment showed 65% amino acid sequence identity.

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2. PLA_2 Expression

2.1. Expression in *E. coli*

2.1.1. Subcloning to Expression Vector

2.1.1.1. PCR Amplification

Two primers, PLAF and PLAR, were designed from two conserved sequence among the PLA_2 isoforms (*PlaS1* and *PlaS2*) downstream the signaling sequence. The PCR product size is 388 bp DNA in length (**Figure 13**). After that, the PCR products were purified and digested with restriction enzymes (*BamH* I and *EcoR* I) prior to perform the ligation.

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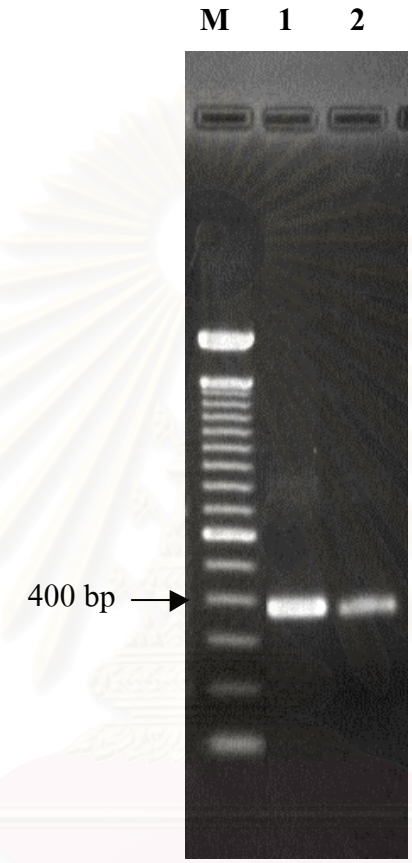

Figure 13 An ethidium bromide stained agarose gel showing the 388 bp of PCR products from pBK-CMV phagemid containing *PLA$_2$*-cDNA by using PLAF and PLAR primers. Lane M; 300 ng of 100 bp DNA ladder, lane 1; *PlaS1* PCR product, lane 2; *PlaS2* PCR product.

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2.1.1.2. Ligation of PCR Products into Plasmid Vector and Transformation of *E. coli* Competent Cells

The digested PCR product either *PlaS1* or *PlaS2* and pTrcHisA expression vector was ligated and transformed to the *E. coli* competent cells. After plasmid DNA extraction, each clone was analyzed by gel electrophoresis (**Figure 14**)

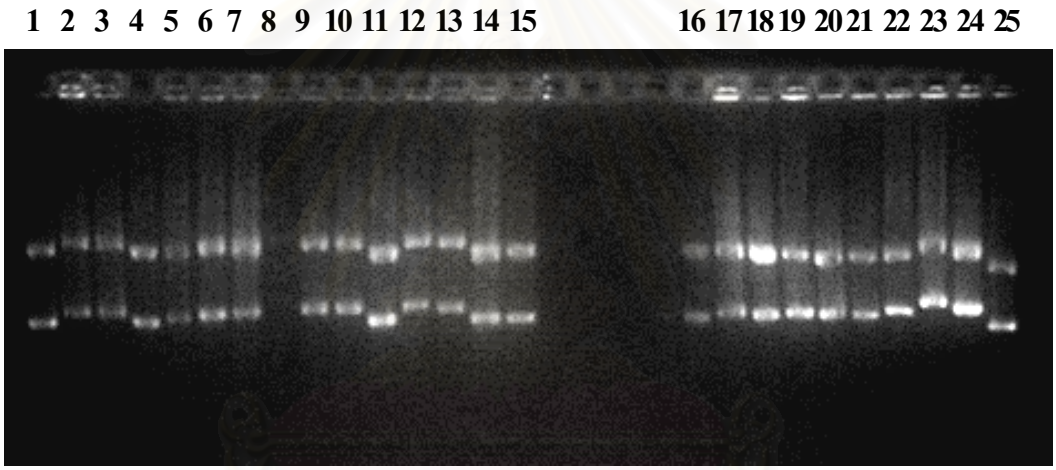

Figure 14 An ethidium bromide stained agarose gel showing ligated-pTrcHis A expression vector with PLA_2 DNA. Lane 1 and 16; 300 ng of pTrcHisA expression vector without inserted DNA, lane 2-15; 300 ng of ligated-pTrcHisA expression vector with *PlaS2* DNA, lane 17-25; 300 ng of ligated-pTrcHisA expression vector with *PlaS1* DNA.

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2.1.1.3. Digestion of Restriction Endonucleases and Analysis of the pTrcHisA Expression Vector Containing PLA_2 DNA

The selected clones from **Figure 14** which had shifted bands were double-digested with *Eco*R I and *BamH* I, and then fractionated on agarose gel electrophoresis to screen the clones which containing of 379 bp insertions (**Figure 15**). These clones were confirmed the correct sequences by sequencing to desired amino acid of protein (data not shown).

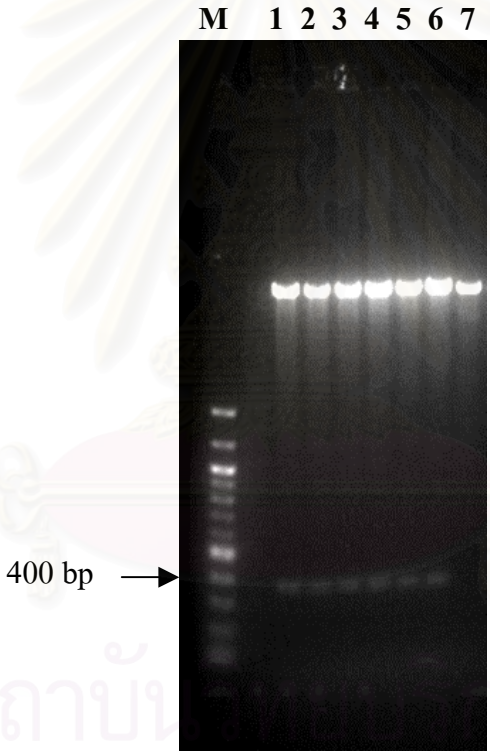

Figure 15 An ethidium bromide stained agarose gel showing the digestion of ligated-pTrcHis A. Lane M; 300 ng of 100 bp DNA ladder, lane 1-4; 400 ng digested *PlaS2* in pTrcHisA, lane 5-7; 400 ng digested *PlaS1* in pTrcHisA.

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2.1.2. Recombinant PLA_2 Expression by Induced with IPTG

The selected clones that have been confirmed by sequencing were expressed by inducing with IPTG. A single colony of *E. coli* containing recombinant *PLA_2*, was inoculated in LB broth containing 50 μg/ml ampicillin and incubated at 37°C with 200 rpm shaking for 16-20 hours. The overnight culture was subcultured into 1 Litre of LB broth containing 50 μg/ml ampicillin and incubated at 37°C until an OD_{600} of 0.6-0.7. A half mM of IPTG was added into the culture and incubated at 37°C with 180 rpm shaking for 3 hours. The cells were pelleted and heated prior to protein analysis by SDS-PAGE and Western blotting hybridization. In SDS-PAGE, Coomassie stained gel could not discriminate PLA_2 band between with or without IPTG induction. However, the Western blot showed that about 18 kDa of recombinant PlaS1 protein including extra amino acid from the vector, was expressed after inducing by IPTG. Surprisingly, in PlaS2 clone expression, about 23 kDa protein was exposed instead of 18 kDa protein (**Figure 16**). The other clones of *PlaS2* were also expressed at an equal size, 23 kDa, shown in **Figure 17**.

In addition, the optimization of IPTG concentration and incubation time after adding IPTG was performed. IPTG was added in range 0-2 mM, and incubated at 37°C, 180 rpm with shaking for 3 hours. The Western blot showed that at 1 mM concentration of IPTG is the best (**Figure 18**). The optimization of incubation time after adding IPTG in range 1 hour to overnight (16-18 hours), the highest PLA_2 expression was obtained in an overnight incubation as shown in **Figure 19**.

Furthermore, to compare the effect of host strain in PLA_2 expression, recombinant *PLA_2* plasmids were transformed into DH5α and TOP10 strain. Similar recombinant PLA_2 expression was observed as shown in **Figure 20**.

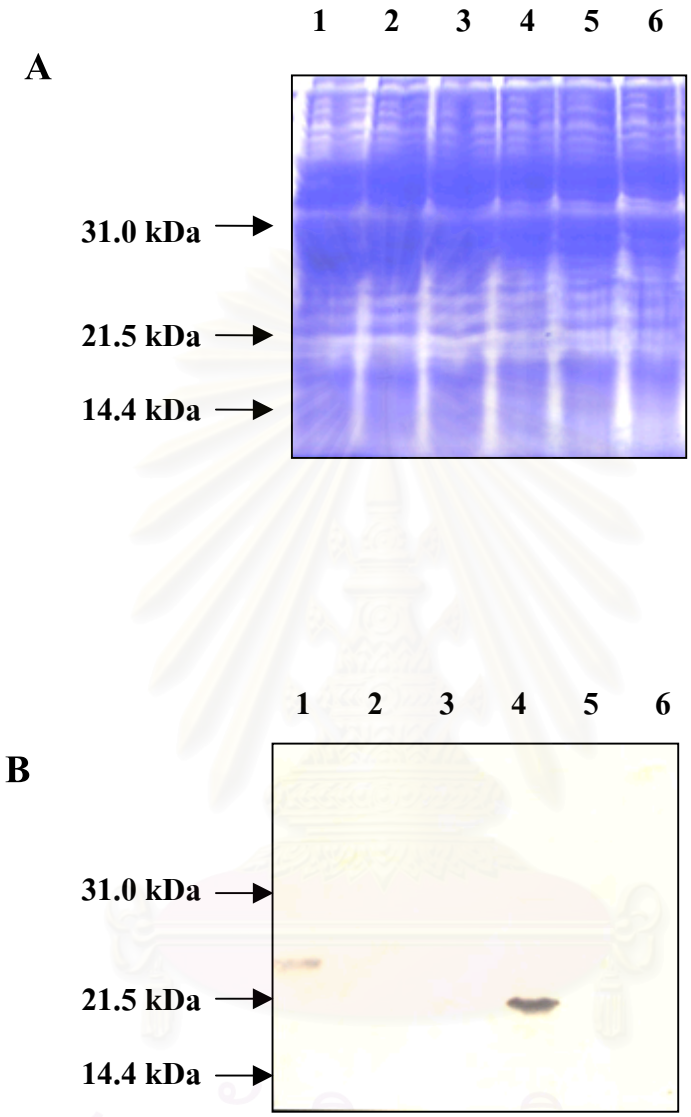

Figure 16 PlaS2 and PlaS1 expression inducing with 1 mM IPTG for 3 hours. A; Coomassie stained gel, B; Western blot. Lane 1; cells lysate of PlaS2, lane 2; cells lysate of PlaS2 without IPTG, lane 3; cells lysate of plasmid which has not *PLA*$_2$,lane 4; cells lysate of plasmid which has not *PLA*$_2$ without IPTG, lane 5; cells lysate of PlaS1, lane 6; cells lysate of PlaS1 without IPTG.

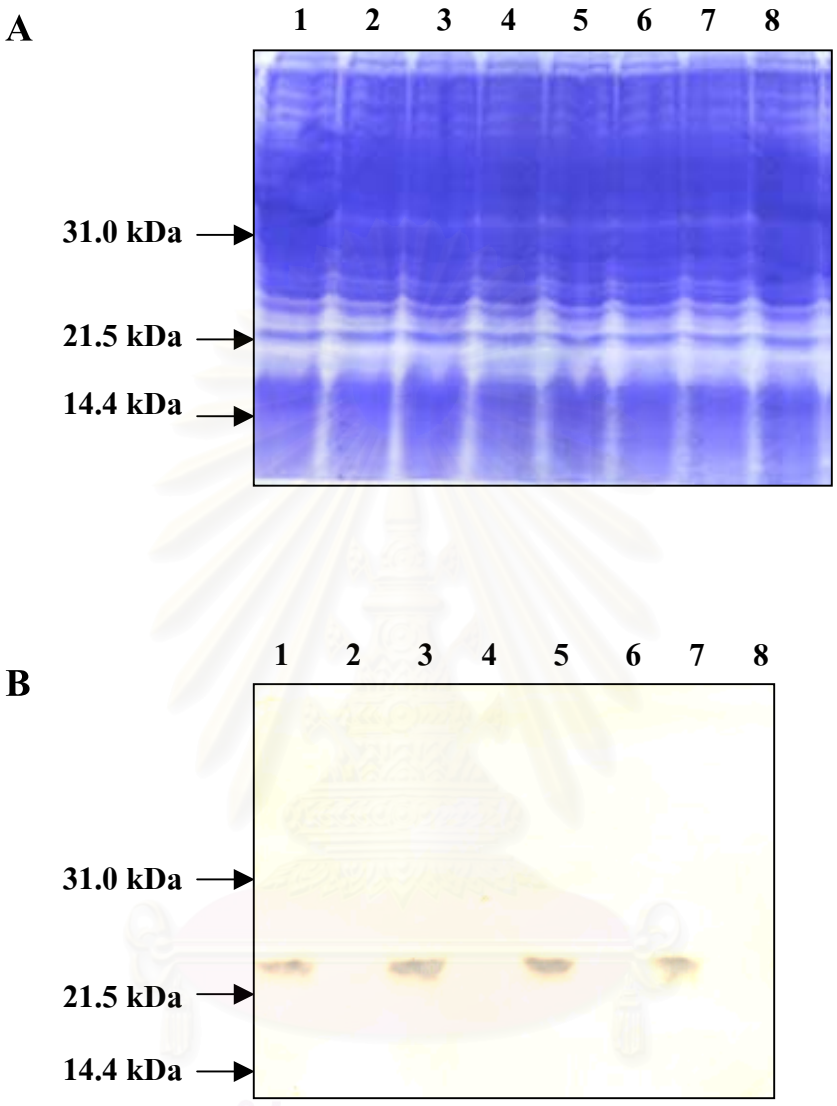

Figure 17 The other clones of PlaS2 inducing with 1 mM IPTG for 3 hours. A; Coomassie stained gel, B; Western blot. Lane 1; cells lysate of clone 2, lane 2; cells lysate of clone 2 without IPTG, lane 3; cells lysate of clone 3, lane 4; cells lysate of clone 3 without IPTG, lane 5; cells lysate of clone 4, lane 6; cells lysate of clone 4 without IPTG, lane 7; cells lysate of clone 5, lane 8; cells lysate of clone 5 without IPTG.

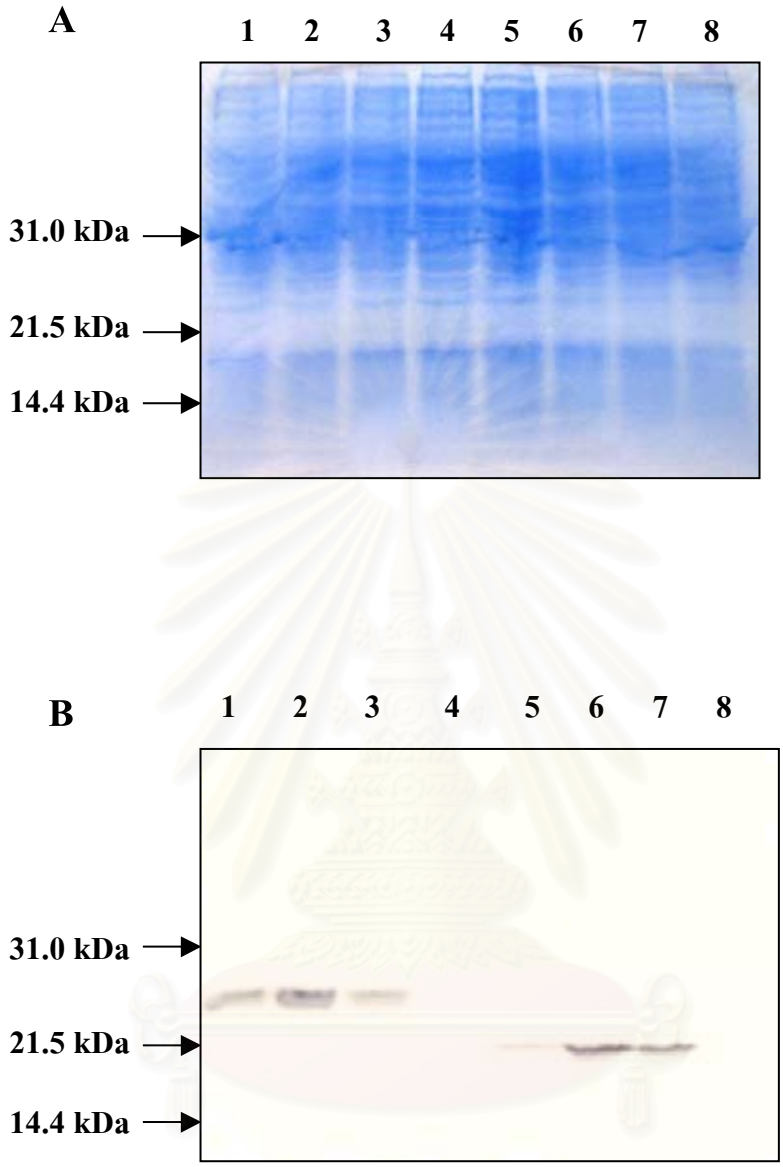

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Figure 18 Optimization of IPTG which incubation for 3 hours. A; Coomassie stained gel, B; Western blot. Lane 1; cells lysate of PlaS2with 0.5 mM of IPTG, lane 2; cells lysate of PlaS2 with 1 mM of IPTG, lane 3; cells lysate of PlaS2 with 2 mM of IPTG, lane 4; cells lysate of PlaS2 without IPTG, lane 5; cells lysate of PlaS1 with 0.5 mM of IPTG, lane 6; cells lysate of PlaS1 with 1 mM of IPTG, lane 7; cells lysate of PlaS1 with 2 mM of IPTG, lane 8; cells lysate of PlaS1 without IPTG.

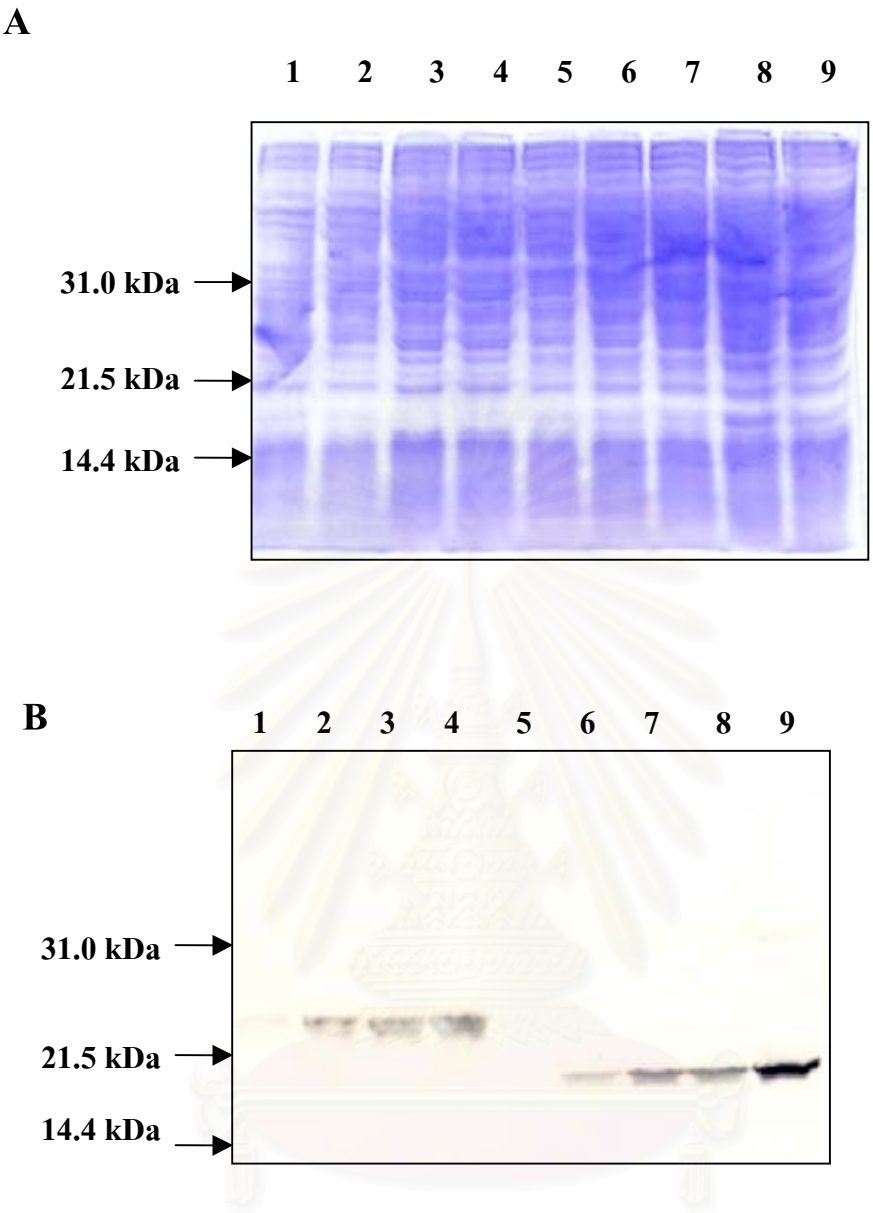

Figure 19 Optimization of incubation time after adding 1 mM of IPTG. A; Coomassie stained gel, B; Western blot. Lane 1; incubated 1 hour cells lysate of PlaS2, lane 2; incubated 3 hours cells lysate of PlaS2, lane 3; incubated 5 hours cells lysate of PlaS2, lane 4; incubated overnight cells lysate of PlaS2, lane 5; incubated overnight cells lysate of PlaS2 without IPTG, lane 6; incubated 1 hour cells lysate of PlaS1, lane 7; incubated 3 hours cells lysate of PlaS1, lane 8; incubated 5 hours cells lysate of PlaS1, lane 9; incubated overnight cells lysate of PlaS1.

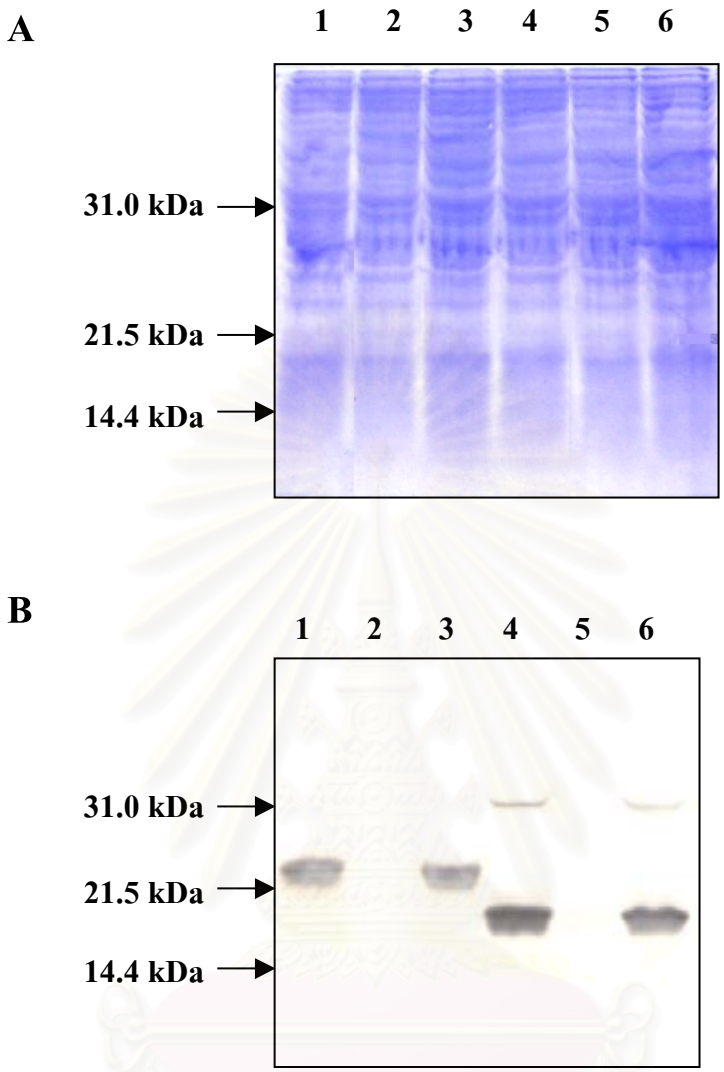

Figure 20 Comparison of PLA_2 expression between DH5α and TOP10 strain that were induced with 1 mM of IPTG for overnight. A; Coomassie stained gel, B; Western blot. Lane 1; cells lysate of PlaS2 in TOP10, lane 2; cells lysate of PlaS2 in TOP10 without IPTG, lane 3; cells lysate of PlaS2 in DH5α, lane 4; cells lysate of PlaS1 in TOP10, lane 5; cells lysate of PlaS1 in TOP10 without IPTG, lane 6; cells lysate of PlaS1 in DH5α.

2.1.3. Sonication

After cells culture, the cells in PBS buffer were sonicated 3 times for 30 sec. The cell lysate was centrifuged at 12,000 rpm for 30 min, 4°C. The supernate and pellet parts were analyzed by SDS-PAGE. Majority of recombinant PLA_2 protein was observed in the pellet part as inclusion bodies (**Figure 21** and **22**).

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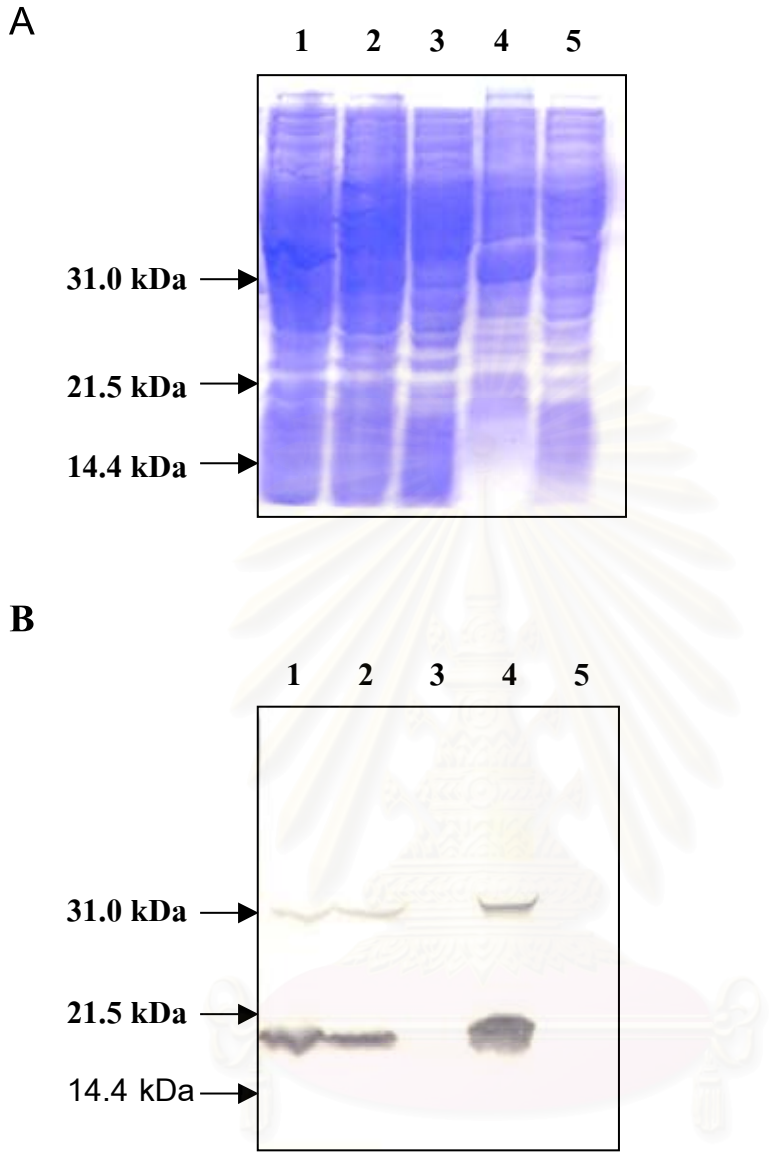

Figure 21 Recombinant PlaS1 expression after sonication. A; Coomassie stained gel, B; Western blot. Lane 1; before sonication, lane 2; after sonication, lane 3; supernate after sonication, lane 4; pellet after sonication, lane 5; cells lysate without IPTG.

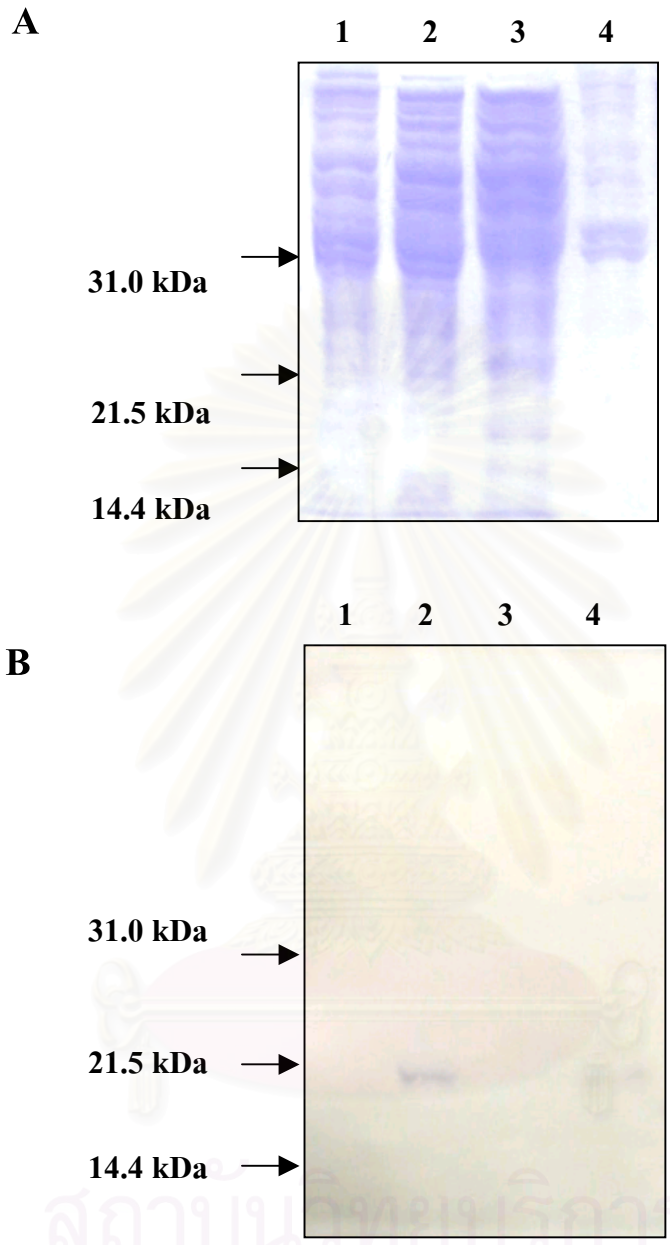

Figure 22 Recombinant PlaS2 expression after sonication. A; Coomassie stained gel, B; Western blot. Lane 1; cells lysate without IPTG, lane 2; before sonication, lane 3; supernate after sonication, lane 4; pellet after sonication.

3. Protein Purification by Using Immobilized Metal Affinity Chromatography (IMAC)

The pellet was suspended with 6 M Guanidine Buffer, pH 8.0. After centrifuged at 12,000 rpm for 30 min, the supernate was loaded into the column containing 6 M guanidine buffer equilibrated-resin. The column was washed with 8 M Urea Buffer, pH 8.0 and 8 M Urea Buffer, pH 7.0, respectively. The His-tagged protein was eluted with 50 ml Elution Buffer, pH 4.5, into 50 fractions with a flow rate of 0.5 ml/min. Absorbancy of each protein elution was monitored at 280 nm. No major peak was obtained in absorbant chromatogram (**Figure 23**). However, recombinant PLA_2 protein bands was observed in some fractions by using Western blot (**Figure 24)**.

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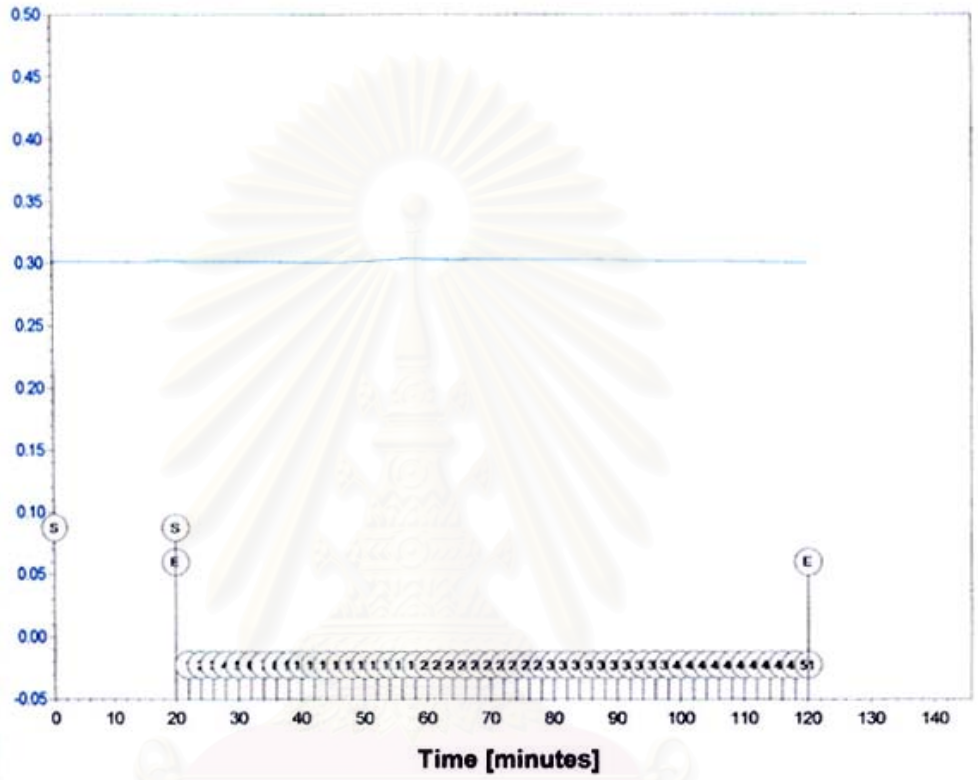

Figure 23 Absorbant chromatogram of purified recombinant PlaS1 fractions.

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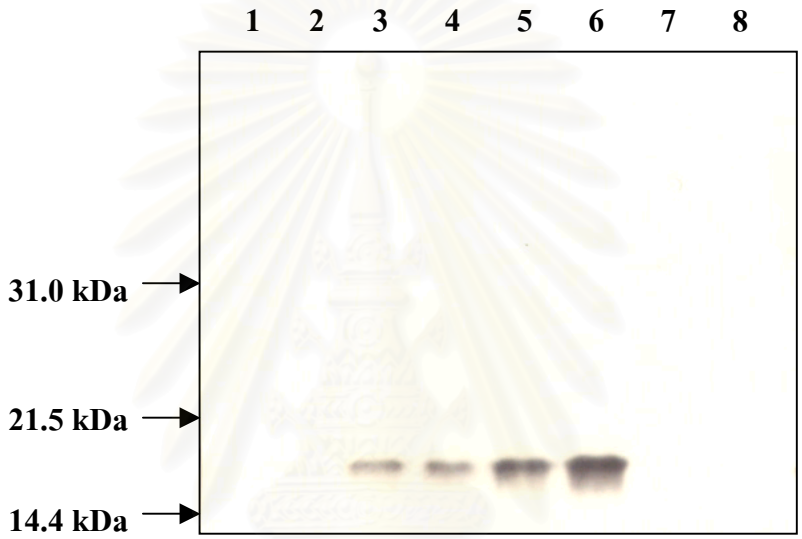

Figure 24 Western blot of purified recombinant PlaS1 fractions. Lane 1; fraction 7, lane 2; fraction 8, lane 3; fraction 11, lane 4; fraction 12, lane 5; fraction 13, lane 6; fraction 15, lane 7; fraction 27, lane 8; fraction 28.

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4. Refolding of Protein by Dialysis

The SnakeSkin® Dialysis Tubing was used to eliminate denaturation buffer to refolding of protein. About 37 ml of purified recombinant PLA_2 fractions from 1.75 Litre of cell culture, proved contain PLA_2 protein by Western blot, were dialysis. Final volume after dialysis was increased to 45 ml.

5. Concentration of Protein by Concentrator

The diluted protein sample was concentrated by Vivaspin concentrator. After centrifugation, the volume of remaining protein sample was reduced to about 230 μl. A silver stained gel showed before and after concentrated sample (**Figure 25).** PlaS1 protein of the concentrated sample was present while it was absent in the passed-through. Thus, almost refolded PlaS1 was recovered.

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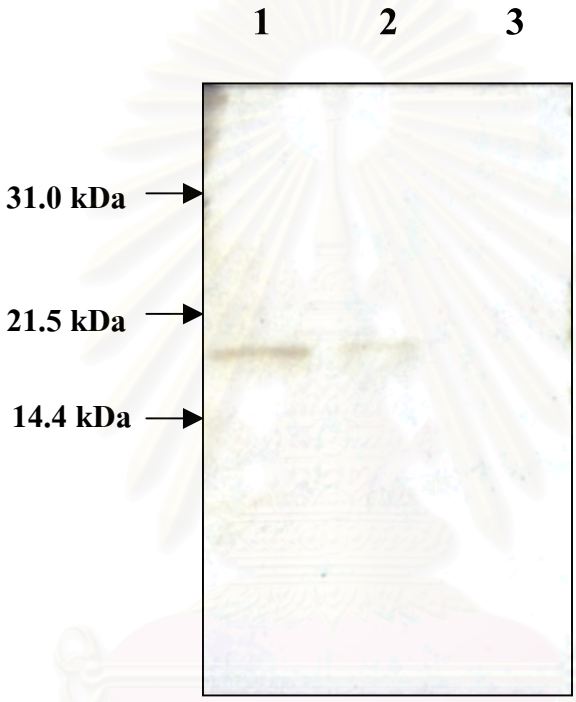

Figure 25 A silver stained gel of PlaS1. Lane 1; 1:400 volume of pooled PlaS1 before concentration, lane 2; 1:62 volume of concentrated PlaS1, lane 3; 1:400 volume of passed through the filter-solution after concentration.

6. Quantitative Assay of Recombinant PlaS1 Protein

Five dilutions of bovine serum albumin standard (0.8-12 μg/ml) were plotted a standard curve (**Figure 26**). Absorbency average 0.098 at 595 nm of diluted 2.8 times protein sample was calculated concentration of protein equal to 4.29 μg/ml. Therefore, undiluted PlaS1 protein was 15.01 μg/ml or 3.45 μg/230 μl of concentrated total volume. The yield of purified protein at this step was about 2.0 μg/Litre culture.

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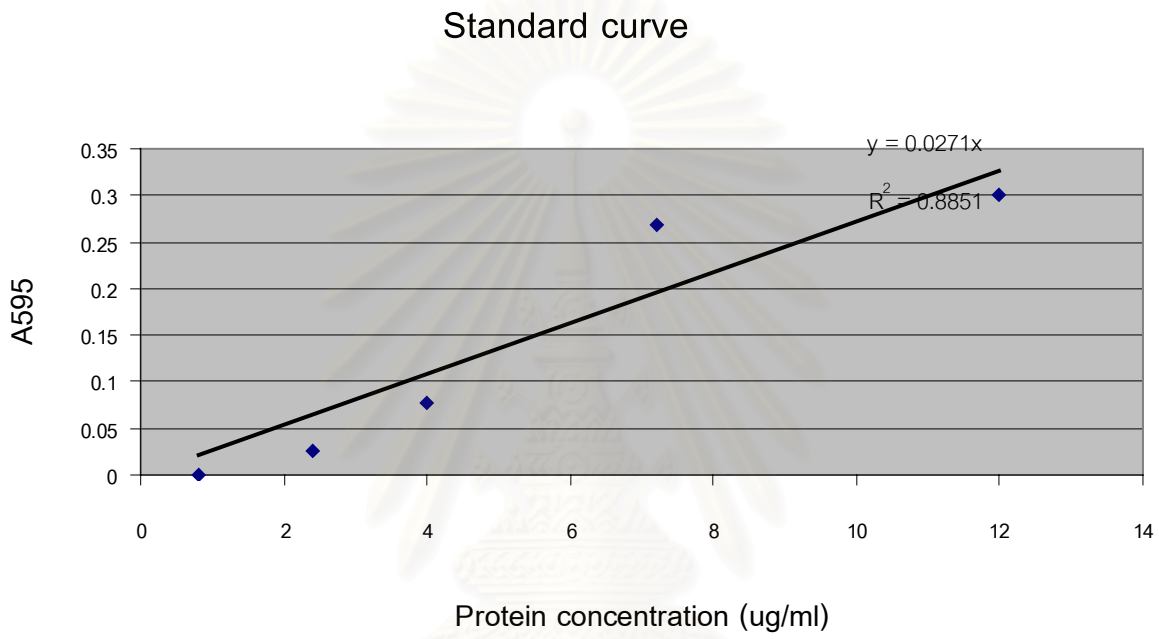

Figure 26 Standard curve of protein concentration.

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7. PLA_2 Activity Test

The spectrophotometric method, based on the pH change due to the liberation of fatty acids, was used. Sixty ng of PLA_2 enzyme was added to the sample cuvette containing 62.5 µl of reaction medium (100mM NaCl, 10 mM $CaCl_2$, 3.5 mM lecithin solubilised with 0.5% Triton X-100 and 0.055 mM phenol red adjusted to an optical density of 1.8-2.2 at 558 nm). The same volume of buffer without the enzyme or 60 ng BSA negative control protein was added to the reference cuvette. While the buffer without and BSA protein can not change absorbancy at 558 nm, an absorbancy of PLA_2 was dramatically decreased (**Table 7** and **Figure 27**).

Table 7 Absorbancy values of PLA_2 activity test.

Min / Sample	0	10 sec	1	2	3	4	5	6	7	8	9	10
Buffer	2.096	2.124	2.110	2.121	2.183	2.186	2.070	2.096	2.124	2.185	2.056	2.131
PLA_2	2.124	1.160	1.077	1.058	1.015	1.110	1.097	1.052	1.121	1.088	1.038	1.010
BSA	2.124	2.045	2.124	2.086	1.997	2.030	2.070	1.978	1.978	2.010	2.032	1.992

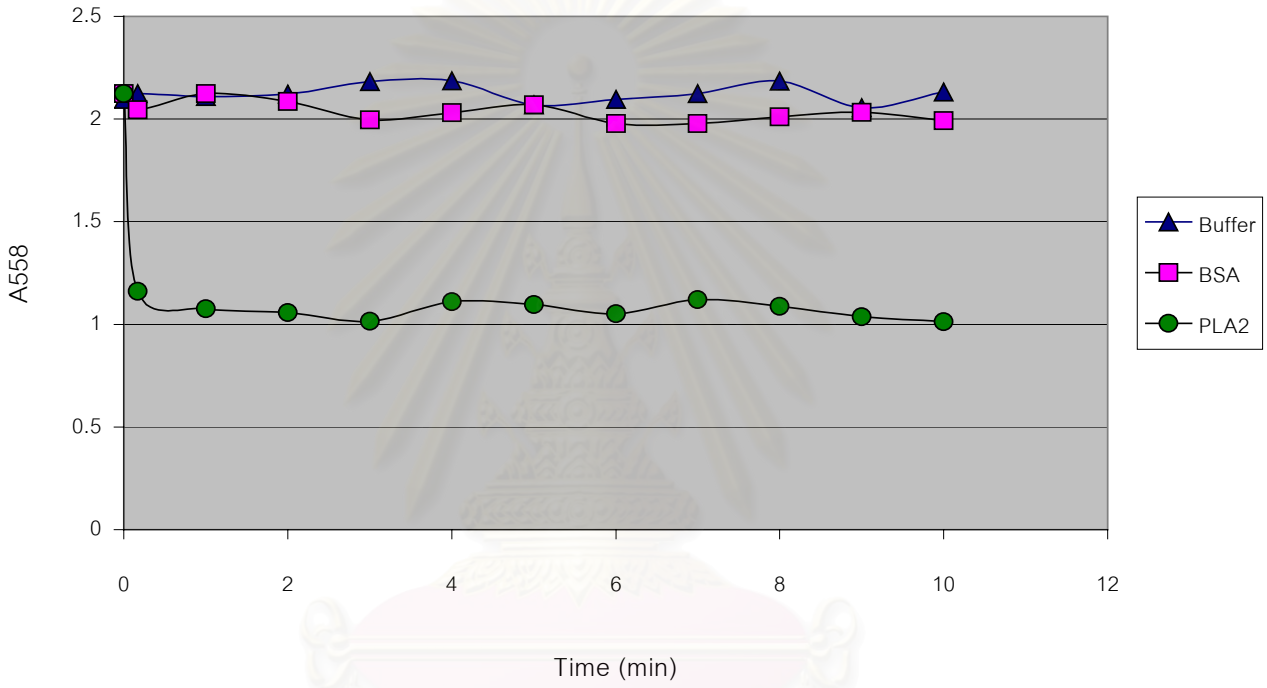

Figure 27 Absorbance of PLA_2 activity test by spectrophotometric method.

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The PLA_2 activity was calculated from a decrease in absorbance per min and per μg of phospholipase. The enzymatic activity of expressed PLA_2 was 1.86 units at 10 min.

A_{558} at 0 min = 2.124

A_{558} at 10 min = 1.010

Enzymatic activity

ΔA_{558}/min x μg = (2.124 -1.010)/ 10 min x 0.06 μg

= 1.86 Units

The enzymatic activity was converted to 185.67 μmoles of fatty acid released per min and per mg of phospholipase by measuring the decrease in absorbance of 0.1 that obtained by 0.01 μmoles of acid.

μmoles/min x mg = 0.01 x (2.124 - 1.010)/0.1(10 min x $6x10^{-5}$mg)

= 185.67

8. Cloning of Genomic *PLA_2* DNA (Isolation of Full-length *PLA_2*)

8.1. PCR Amplification

Two primer sets, 5'FPLA-4F or 5'FPLA-7F and 3'FPLA-R, were designed from untranslated region of two conserved sequence among the *PLA_2* isoforms (5'FPLA-4F and 5'FPLA-7F). The PCR product size is about 2.0 kb DNA in length (**Figure 28**). After that, the PCR products were purified and digested with restriction enzymes (*EcoR* I) prior to perform the ligation.

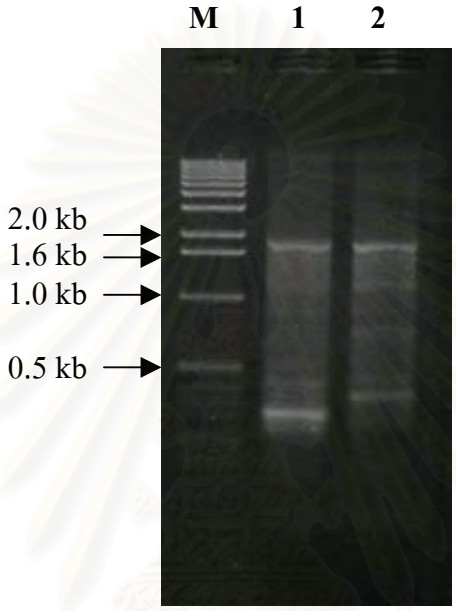

Figure 28 An ethidium bromide stained agarose gel showing the PCR products from genomic DNA using 5'FPLA-4F or 5'FPLA-7F and 3'FPLA-R primers. Lane M; 300 ng of 1 kb DNA ladder, lane 1; 5'FPLA-4F and 3'FPLA-R primers, lane 2; 5'FPLA-7F and 3'FPLA-R primers.

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8.2. Digestion of Recombinant pGEM-T with Restriction Endonuclease

After ligation and transformation, the recombinant pGEM-T plasmids were digested with *Eco*R I. On agarose gel showed that pGEM-T plasmids contain size variation of insert DNA (**Figure 29**).

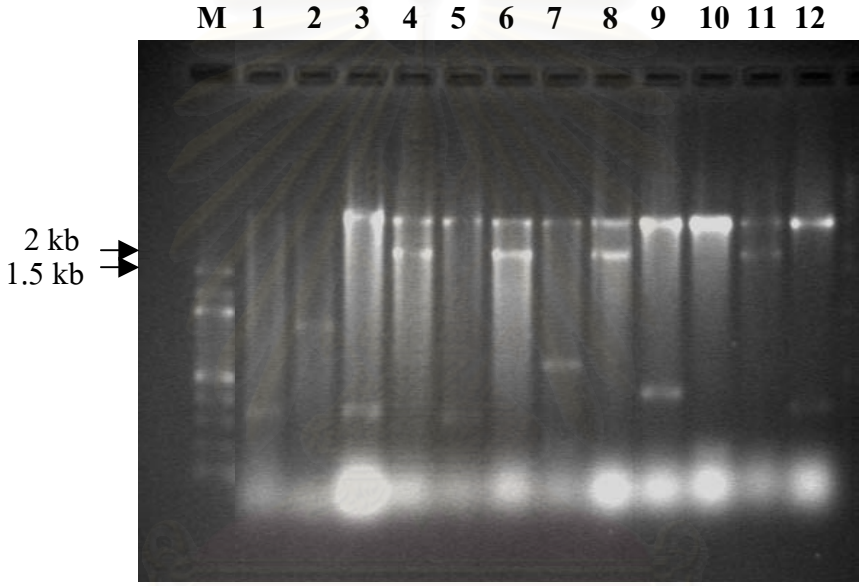

Figure 29 An ethidium bromide stained agarose gel showing the digested pGEM-T plasmids. Lane M; 300 ng of 100 bp DNA ladder, lane 1-10; digested pGEM-T plasmid clone 1-10.

8.3. Sequence Alignment and Computational Searching Analysis

The DNA sequence were analyzed and compared to the entries in database using the BLAST N and CLUSTAL X program. From 7 selected clones, there were 3 clones of full-length PLA_2 with length of about 2 kb and 4 clones of non specific genes with length less than 2 kb. Three clones of full-length PLA_2 were designated *gPlaS1*, *gPlaS2* and *gPlaS3*. The highest BLAST score of *PlaS1* showed homologous to *Vipera aspis zinnikeri* vaspin B, accession number AY158635 (**Figure 30)**. Whereas the highest BLAST score of *gPlaS2* and *gPlaS3* showed homologous to *Vipera aspis aspis* ammodytin I2, accession number AY158637 (**Figure 31-32)**. An alignment between nucleotide sequences of *gPlaS1*, *gPlaS2* and *gPlaS3* showed 87% (*PlaS1*:*PlaS2*), 88% (*PlaS1*:*PlaS3*) and 98% (*PlaS2*:*PlaS3*) nucleotide sequence identity (**Figure 33)**. Exon 1 encode 5' UTR, exon 2 encodes most of the signal peptide, and exon 3-5 encode regions of protein residues 1-43, 43-76, 76-122 and 3' UTR, respectively. The comparison between genomic DNA sequence of *gPlaS1* and its cDNA sequence, *PlaS1*, indicated that the coding and untranslated regions are identical (**Figure 34**). This observation was also found for *gPlaS2* and *PlaS2*. However, cDNA sequence encoded by *gPlaS3* could not be obtained, therefore, no comparison were shown. An alignment of deduced amino acid sequences of gPlaS1, gPlaS2 and gPlaS3 showed 69%(*gPlaS1*:*gPlaS2*), 70%(*gPlaS1*:*gPlaS3*) and 92%(*gPlaS2*:*gPlaS3*) amino acid sequence identity **(Figure 35)**.

By using the BLAST P program, deduced amino acid sequence of PlaS3 was highest homologous to Phospholipase A2 RV-7 precursor, accession number P31100 (**Figure 36**). Comparison of deduced amino acid sequences of gPlaS1, gPlaS2, gPlaS3 with other N-terminal amino acid sequences *D. russellii* PLA_2 reports showed that gPlaS3 showed some amino acid differences from the other (**Figure 37).**

Comparison between cDNA and gDNA showed that PLA_2 gene has 5 exons and 4 introns (**Figure 38**).

Sequences producing significant alignments:		Score (bits)	E Value
gi|33187117|gb|AY158635.1|	Vipera aspis zinnikeri vaspin B ...	1285	0.0
gi|33187115|gb|AY158634.1|	Vipera aspis vaspin B (VaspB) ge...	1285	0.0
gi|33187139|gb|AY243575.1|	Vipera aspis aspis vaspin B isof...	1285	0.0
gi|33187137|gb|AY243574.1|	Vipera aspis zinnikeri vaspin B ...	1285	0.0
gi|33187143|gb|AY243577.1|	Vipera aspis zinnikeri vaspin B ...	1277	0.0
gi|33187141|gb|AY243576.1|	Vipera aspis aspis vaspin B isof...	1269	0.0
gi|33187113|gb|AY152843.1|	Vipera aspis zinnikeri vaspin A ...	815	0.0
gi|33187751|gb|AF548351.1|	Vipera aspis vaspin A (vaspA) ge...	815	0.0
gi|33187119|gb|AY158636.1|	Vipera berus berus phospholipase...	682	0.0
gi|33187133|gb|AY159810.1|	Vipera aspis zinnikeri ammodytin...	678	0.0
gi|33187129|gb|AY159808.1|	Vipera aspis ammodytin I1 isofor...	678	0.0
gi|33187127|gb|AY159807.1|	Vipera aspis aspis ammodytin I1 ...	678	0.0
gi|33187131|gb|AY159809.1|	Vipera aspis ammodytin I1 isofor...	662	0.0

Figure 30 Homology searching via Internet result of *gPlaS1* by using BLAST N program at www.ncbi.nlm.nih.

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Sequences producing significant alignments:	Score (bits)	E Value
gi|33187121|gb|AY158637.1| Vipera aspis aspis ammodytin I2 ...	1144	0.0
gi|6967297|emb|X84018.1|VAMMI2 V.ammodytes ammodytin I2 gene	1082	0.0
gi|33187113|gb|AY152843.1| Vipera aspis zinnikeri vaspin A ...	1049	0.0
gi|33187751|gb|AF548351.1| Vipera aspis vaspin A (vaspA) ge...	1049	0.0
gi|33187125|gb|AY158639.1| Vipera berus berus ammodytin I2 ...	825	0.0
gi|13936540|gb|.1|AF253048 Vipip8 Vipera ammodytes ammodyt...	783	0.0
gi|33187135|gb|AY159811.1| Vipera berus berus ammodytin I1 ...	775	0.0
gi|33187123|gb|AY158638.1| Vipera aspis ammodytin I2 (AmtI2...	769	0.0
gi|33187131|gb|AY159809.1| Vipera aspis ammodytin I1 isofor...	767	0.0
gi|3885849|gb|AF091855.1|AF091855 Vipera palaestinae VP7 ph...	747	0.0
gi|3885847|gb|AF091854.1|AF091854 Vipera palaestinae VP8 ph...	686	0.0
gi|33187119|gb|AY158636.1| Vipera berus berus phospholipase...	674	0.0
gi|33187129|gb|AY159808.1| Vipera aspis ammodytin I1 isofor...	642	0.0
gi|33187127|gb|AY159807.1| Vipera aspis aspis ammodytin I1 ...	642	0.0

Figure 31 Homology searching via Internet result of *gPlaS2* by using BLAST N program at www.ncbi.nlm.nih.

Sequences producing significant alignments:	Score (bits)	E Value
gi|33187121|gb|AY158637.1| Vipera aspis aspis ammodytin I2 ...	1136	0.0
gi|6967297|emb|X84018.1|VAMMI2 V.ammodytes ammodytin I2 gene	1074	0.0
gi|33187113|gb|AY152843.1| Vipera aspis zinnikeri vaspin A ...	997	0.0
gi|33187751|gb|AF548351.1| Vipera aspis vaspin A (vaspA) ge...	997	0.0
gi|33187125|gb|AY158639.1| Vipera berus berus ammodytin I2 ...	825	0.0
gi|13936540|gb|AF253048.1|AF253048 Vipera ammodytes ammodyt...	783	0.0
gi|33187135|gb|AY159811.1| Vipera berus berus ammodytin I1 ...	775	0.0
gi|33187123|gb|AY158638.1| Vipera aspis ammodytin I2 (AmtI2...	769	0.0
gi|33187131|gb|AY159809.1| Vipera aspis ammodytin I1 isofor...	767	0.0
gi|3885849|gb|AF091855.1|AF091855 Vipera palaestinae VP7 ph...	747	0.0
gi|3885847|gb|AF091854.1|AF091854 Vipera palaestinae VP8 ph...	686	0.0
gi|33187119|gb|AY158636.1| Vipera berus berus phospholipase...	674	0.0
gi|33187129|gb|AY159808.1| Vipera aspis ammodytin I1 isofor...	642	0.0
gi|33187127|gb|AY159807.1| Vipera aspis aspis ammodytin I1 ...	642	0.0

Figure 32 Homology searching via Internet result of *gPlaS3* by using BLAST N program at www.ncbi.nlm.nih.

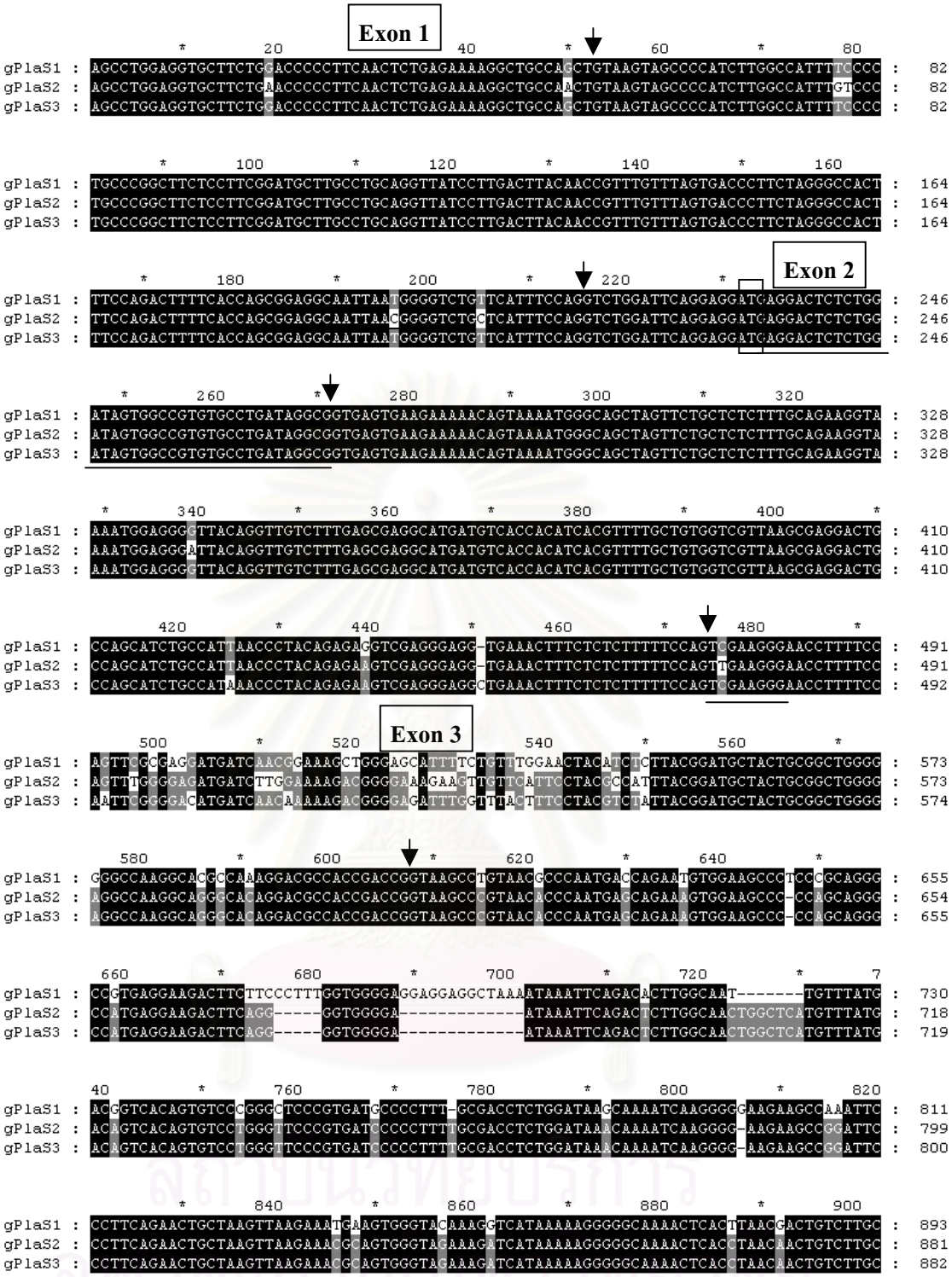

Figure 33 An alignment of nucleotide sequences of *gPlaS1*, *gPlaS2* and *gPlaS3*. Identical nucleotides were highlighted. Gaps have been introduced to optimize the alignment. Arrows indicated exon separation. Start and stop codons were shown in boxes. The underlined is a signal sequence.

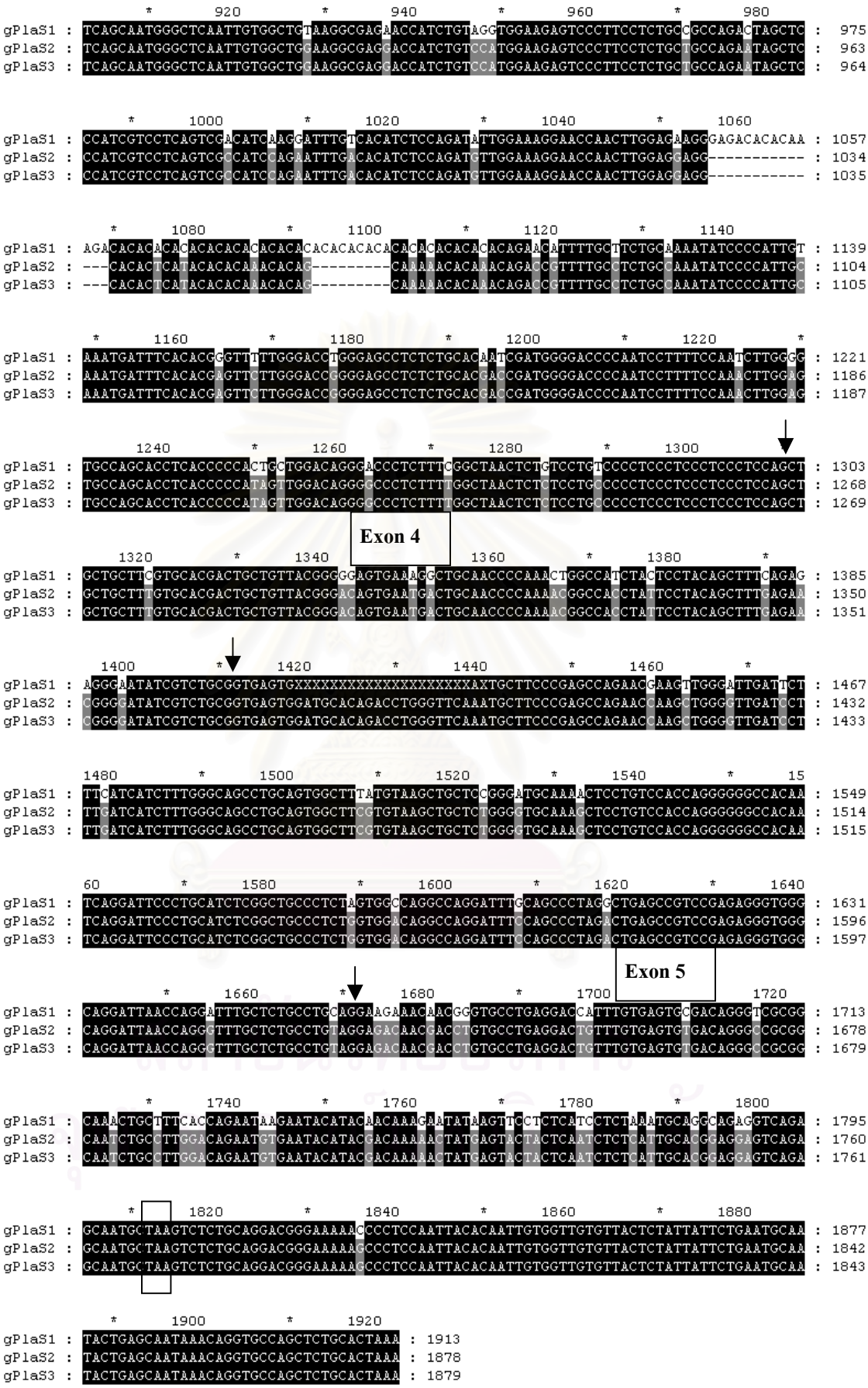

Figure 33 (continued)

```
PlaS1-cDNA AGCCTGGAGGTGCTTCTGGACCCCCTTCAACTCTGAGAAAAGGCTGCCAGCT----------------------
PlaS2-cDNA AGCCTGGAGGTGCTTCTGAACCCCCTTCAACTCTGAGAAAAGGCTGCCAACT----------------------
PlaS1-DNA  AGCCTGGAGGTGCTTCTGGACCCCCTTCAACTCTGAGAAAAGGCTGCCAGCTGTAAGTAGCCCCATCTTGGCCAT
PlaS2-DNA  AGCCTGGAGGTGCTTCTGAACCCCCTTCAACTCTGAGAAAAGGCTGCCAACTGTAAGTAGCCCCATCTTGGCCAT
PlaS3-DNA  AGCCTGGAGGTGCTTCTGGACCCCCTTCAACTCTGAGAAAAGGCTGCCAGCTGTAAGTAGCCCCATCTTGGCCAT

PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  TTTCCCCTGCCCGGCTTCTCCTTCGGATGCTTGCCTGCAGGTTATCCTTGACTTACAACCGTTTGTTTAGTGACC
PlaS2-DNA  TTGTCCCTGCCCGGCTTCTCCTTCGGATGCTTGCCTGCAGGTTATCCTTGACTTACAACCGTTTGTTTAGTGACC
PlaS3-DNA  TTTCCCCTGCCCGGCTTCTCCTTCGGATGCTTGCCTGCAGGTTATCCTTGACTTACAACCGTTTGTTTAGTGACC

PlaS1-cDNA ----------------------------------------------------------------GTCTGGATTC
PlaS2-cDNA ----------------------------------------------------------------GTCTGGATTC
PlaS1-DNA  CTTCTAGGGCCACTTTCCAGACTTTTCACCAGCGGAGGCAATTAATGGGGTCTGTTCATTTCCAGGTCTGGATTC
PlaS2-DNA  CTTCTAGGGCCACTTTCCAGACTTTTCACCAGCGGAGGCAATTAACGGGGTCTGCTCATTTCCAGGTCTGGATTC
PlaS3-DNA  CTTCTAGGGCCACTTTCCAGACTTTTCACCAGCGGAGGCAATTAATGGGGTCTGTTCATTTCCAGGTCTGGATTC

PlaS1-cDNA AGGAGGATGAGGACTCTCTGGATAGTGGCCGTGTGCCTGATAGGCG----------------------------
PlaS2-cDNA AGGAGGATGAGGACTCTCTGGATAGTGGCCGTGTGCCTGATAGGCG----------------------------
PlaS1-DNA  AGGAGGATGAGGACTCTCTGGATAGTGGCCGTGTGCCTGATAGGCGGTGAGTGAAGAAAAACAGTAAAATGGGCA
PlaS2-DNA  AGGAGGATGAGGACTCTCTGGATAGTGGCCGTGTGCCTGATAGGCGGTGAGTGAAGAAAAACAGTAAAATGGGCA
PlaS3-DNA  AGGAGGATGAGGACTCTCTGGATAGTGGCCGTGTGCCTGATAGGCGGTGAGTGAAGAAAAACAGTAAAATGGGCA

PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  GCTAGTTCTGCTCTCTTTGCAGAAGGTAAAATGGAGGGGTTACAGGTTGTCTTTGAGCGAGGCATGATGTCACCA
PlaS2-DNA  GCTAGTTCTGCTCTCTTTGCAGAAGGTAAAATGGAGGGATTACAGGTTGTCTTTGAGCGAGGCATGATGTCACCA
PlaS3-DNA  GCTAGTTCTGCTCTCTTTGCAGAAGGTAAAATGGAGGGGTTACAGGTTGTCTTTGAGCGAGGCATGATGTCACCA

PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  CATCACGTTTTGCTGTGGTCGTTAAGCGAGGACTGCCAGCATCTGCCATTAACCCTACAGAGAGGTCGAGGGAGG
PlaS2-DNA  CATCACGTTTTGCTGTGGTCGTTAAGCGAGGACTGCCAGCATCTGCCATTAACCCTACAGAGAAGTCGAGGGAGG
PlaS3-DNA  CATCACGTTTTGCTGTGGTCGTTAAGCGAGGACTGCCAGCATCTGCCATAAACCCTACAGAGAAGTCGAGGGAGG

PlaS1-cDNA ------------------------TCGAAGGGAACCTTTTCCAGTTCGCGAGGATGATCAACGGAAAGCTGGGAG
PlaS2-cDNA ------------------------TTGAAGGGAACCTTTTCCAGTTTGGGGAGATGATCTTGGAAAAGACGGGGA
PlaS1-DNA  -TGAAACTTTCTCTCTTTTTCCAGTCGAAGGGAACCTTTTCCAGTTCGCGAGGATGATCAACGGAAAGCTGGGAG
PlaS2-DNA  -TGAAACTTTCTCTCTTTTTCCAGTTGAAGGGAACCTTTTCCAGTTTGGGGAGATGATCTTGGAAAAGACGGGGA
PlaS3-DNA  CTGAAACTTTCTCTCTTTTTCCAGTCGAAGGGAACCTTTTCCAATTCGGGGACATGATCAACAAAAAGACGGGGA

PlaS1-cDNA CATTTTCTGTTTGGAACTACATCTCTTACGGATGCTACTGCGGCTGGGGGGGCCAAGGCACGCCAAAGGACGCCA
PlaS2-cDNA AAGAAGTTGTTCATTCCTACGCCATTTACGGATGCTACTGCGGCTGGGGAGGCCAAGGCAGGGCACAGGACGCCA
PlaS1-DNA  CATTTTCTGTTTGGAACTACATCTCTTACGGATGCTACTGCGGCTGGGGGGGCCAAGGCACGCCAAAGGACGCCA
PlaS2-DNA  AAGAAGTTGTTCATTCCTACGCCATTTACGGATGCTACTGCGGCTGGGGAGGCCAAGGCAGGGCACAGGACGCCA
PlaS3-DNA  GATTTGGTTTACTTTCCTACGTCTATTACGGATGCTACTGCGGCTGGGGAGGCCAAGGCAGGGCACAGGACGCCA

PlaS1-cDNA CCGACCG--------------------------------------------------------------------
PlaS2-cDNA CCGACCG--------------------------------------------------------------------
PlaS1-DNA  CCGACCGGTAAGCCTGTAACGCCCAATGACCAGAATGTGGAAGCCCTCCCGCAGGGCCGTGAGGAAGACTTCTTC
PlaS2-DNA  CCGACCGGTAAGCCCGTAACACCCAATGAGCAGAAAGTGGAAGCCC-CCAGCAGGGCCATGAGGAAGACTTCAGG
PlaS3-DNA  CCGACCGGTAAGCCCGTAACACCCAATGAGCAGAAAGTGGAAGCCC-CCAGCAGGGCCATGAGGAAGACTTCAGG

PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  CCTTTGGTGGGGAGGAGGAGGCTAAAATAAATTCAGACACTTGGCAAT-------TGTTTATGACGGTCACAGTG
PlaS2-DNA  -----GGTGGGGA-------------ATAAATTCAGACTCTTGGCAACTGGCTCATGTTTATGACAGTCACAGTG
PlaS3-DNA  -----GGTGGGGA-------------ATAAATTCAGACTCTTGGCAACTGGCTCATGTTTATGACAGTCACAGTG
```

Figure 34 Comparison between cDNA (*PlaS1*, *PlaS2*) and gDNA (*gPlaS1*, *gPlaS2* and *gPlaS3*). Bold letters indicated start and stop codons. The underlined is a signal sequence.

```
PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  TCCCGGGCTCCCGTGATGCCCCTTT-GCGACCTCTGGATAAGCAAAATCAAGGGGGAAGAAGCCAAATTCCCTTC
PlaS2-DNA  TCCTGGGTTCCCGTGATCCCCCTTTTGCGACCTCTGGATAAACAAAATCAAGGGG-AAGAAGCCGGATTCCCTTC
PlaS3-DNA  TCCTGGGTTCCCGTGATCCCCCTTTTGCGACCTCTGGATAAACAAAATCAAGGGG-AAGAAGCCGGATTCCCTTC

PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  AGAACTGCTAAGTTAAGAAATGAAGTGGGTACAAAGGTCATAAAAAGGGGGCAAAACTCACTTAACGACTGTCTT
PlaS2-DNA  AGAACTGCTAAGTTAAGAAACGCAGTGGGTAGAAAGATCATAAAAAGGGGGCAAAACTCACCTAACAACTGTCTT
PlaS3-DNA  AGAACTGCTAAGTTAAGAAACGCAGTGGGTAGAAAGATCATAAAAAGGGGGCAAAACTCACCTAACAACTGTCTT

PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  GCTCAGCAATGGGCTCAATTGTGGCTGTAAGGCGAGAACCATCTGTAGGTGGAAGAGTCCCTTCCTCTGCCGCCA
PlaS2-DNA  GCTCAGCAATGGGCTCAATTGTGGCTGGAAGGCGAGGACCATCTGTCCATGGAAGAGTCCCTTCCTCTGCTGCCA
PlaS3-DNA  GCTCAGCAATGGGCTCAATTGTGGCTGGAAGGCGAGGACCATCTGTCCATGGAAGAGTCCCTTCCTCTGCTGCCA

PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  GACTAGCTCCCATCGTCCTCAGTCGACATCAAGGATTTGTCACATCTCCAGATATTGGAAAGGAACCAACTTGGA
PlaS2-DNA  GAATAGCTCCCATCGTCCTCAGTCGCCATCCAGAATTTGACACATCTCCAGATGTTGGAAAGGAACCAACTTGGA
PlaS3-DNA  GAATAGCTCCCATCGTCCTCAGTCGCCATCCAGAATTTGACACATCTCCAGATGTTGGAAAGGAACCAACTTGGA

PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  GAAGGGAGACACACAAAGACACACACACACACACACACACACACACACACACACACACACACAGAACATTTTG
PlaS2-DNA  GGAGG--------------CACACTCATACACACAAACACAG---------CAAAAACACAAACAGACCGTTTTG
PlaS3-DNA  GGAGG--------------CACACTCATACACACAAACACAG---------CAAAAACACAAACAGACCGTTTTG

PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  CTTCTGCAAAATATCCCCATTGTAAATGATTTCACACGGGTTTTTGGGACCTGGGAGCCTCTCTGCACAATCGAT
PlaS2-DNA  CCTCTGCCAAATATCCCCATTGCAAATGATTTCACACGAGTTCTTGGGACCGGGGAGCCTCTCTGCACGACCGAT
PlaS3-DNA  CCTCTGCCAAATATCCCCATTGCAAATGATTTCACACGAGTTCTTGGGACCGGGGAGCCTCTCTGCACGACCGAT

PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  GGGGACCCCAATCCTTTTCCAATCTTGGGGTGCCAGCACCTCACCCCCACTGCTGGACAGGGACCCTCTTTCGGC
PlaS2-DNA  GGGGACCCCAATCCTTTTCCAAACTTGGAGTGCCAGCACCTCACCCCCATAGTTGGACAGGGGCCCTCTTTTGGC
PlaS3-DNA  GGGGACCCCAATCCTTTTCCAAACTTGGAGTGCCAGCACCTCACCCCCATAGTTGGACAGGGGCCCTCTTTTGGC

PlaS1-cDNA ----------------------------------CTGCTGCTTCGTGCACGACTGCTGTTACGGGGGAGTGAAA
PlaS2-cDNA ----------------------------------CTGCTGCTTTGTGCACGACTGCTGTTACGGGACAGTGAAT
PlaS1-DNA  TAACTCTGTCCTGTCCCCTCCCTCCCTCCCTCCAGCTGCTGCTTCGTGCACGACTGCTGTTACGGGGGAGTGAAA
PlaS2-DNA  TAACTCTCTCCTGCCCCCTCCCTCCCTCCCTCCAGCTGCTGCTTTGTGCACGACTGCTGTTACGGGACAGTGAAT
PlaS3-DNA  TAACTCTCTCCTGCCCCCTCCCTCCCTCCCTCCAGCTGCTGCTTTGTGCACGACTGCTGTTACGGGACAGTGAAT

PlaS1-cDNA GGCTGCAACCCCAAACTGGCCATCTACTCCTACAGCTTTCAGAGAGGGAATATCGTCTGCG--------------
PlaS2-cDNA GACTGCAACCCCAAAACGGCCACCTATTCCTACAGCTTTGAGAACGGGGATATCGTCTGCG--------------
PlaS1-DNA  GGCTGCAACCCCAAACTGGCCATCTACTCCTACAGCTTTCAGAGAGGGAATATCGTCTGCGGTGAGTGXXXXXXX
PlaS2-DNA  GACTGCAACCCCAAAACGGCCACCTATTCCTACAGCTTTGAGAACGGGGATATCGTCTGCGGTGAGTGGATGCAC
PlaS3-DNA  GACTGCAACCCCAAAACGGCCACCTATTCCTACAGCTTTGAGAACGGGGATATCGTCTGCGGTGAGTGGATGCAC

PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  XXXXXXXXXXXXXXAXTGCTTCCCGAGCCAGAACGAAGTTGGGATTGATTCTTTCATCATCTTTGGGCAGCCTGCA
PlaS2-DNA  AGACCTGGGTTCAAATGCTTCCCGAGCCAGAACCAAGCTGGGGTTGATCCTTTGATCATCTTTGGGCAGCCTGCA
PlaS3-DNA  AGACCTGGGTTCAAATGCTTCCCGAGCCAGAACCAAGCTGGGGTTGATCCTTTGATCATCTTTGGGCAGCCTGCA

PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  GTGGCTTTATGTAAGCTGCTCCGGGATGCAAAACTCCTGTCCACCAGGGGGGCCACAATCAGGATTCCCTGCATC
PlaS2-DNA  GTGGCTTCGTGTAAGCTGCTCTGGGGTGCAAAGCTCCTGTCCACCAGGGGGGCCACAATCAGGATTCCCTGCATC
PlaS3-DNA  GTGGCTTCGTGTAAGCTGCTCTGGGGTGCAAAGCTCCTGTCCACCAGGGGGGCCACAATCAGGATTCCCTGCATC
```

Figure 34 (continued)

```
PlaS1-cDNA ------------------------------------------------------------------------
PlaS2-cDNA ------------------------------------------------------------------------
PlaS1-DNA  TCGGCTGCCCTCTAGTGGCCAGGCCAGGATTTGCAGCCCTAGGCTGAGCCGTCCGAGAGGGTGGGCAGGATTAAC
PlaS2-DNA  TCGGCTGCCCTCTGGTGGACAGGCCAGGATTTCCAGCCCTAGACTGAGCCGTCCGAGAGGGTGGGCAGGATTAAC
PlaS3-DNA  TCGGCTGCCCTCTGGTGGACAGGCCAGGATTTCCAGCCCTAGACTGAGCCGTCCGAGAGGGTGGGCAGGATTAAC

PlaS1-cDNA ----------------------GAAGAAACAACGGGTGCCTGAGGACCATTTGTGAGTGCGACAGGGTCGCGGCAA
PlaS2-cDNA ----------------------GAGACAACGACCTGTGCCTGAGGACTGTTTGTGAGTGTGACAGGGCCGCGGCAA
PlaS1-DNA  CAGGATTTGCTCTGCCTGCAGGAAGAAACAACGGGTGCCTGAGGACCATTTGTGAGTGCGACAGGGTCGCGGCAA
PlaS2-DNA  CAGGGTTTGCTCTGCCTGTAGGAGACAACGACCTGTGCCTGAGGACTGTTTGTGAGTGTGACAGGGCCGCGGCAA
PlaS3-DNA  CAGGGTTTGCTCTGCCTGTAGGAGACAACGACCTGTGCCTGAGGACTGTTTGTGAGTGTGACAGGGCCGCGGCAA

PlaS1-cDNA ACTGCTTTCACCAGAATAAGAATACATACAACAAAGAATATAAGTTCCTCTCATCCTCTAAATGCAGGCAGAGGT
PlaS2-cDNA TCTGCCTTGGACAGAATGTGAATACATACGACAAAAACTATGAGTACTACTCAATCTCTCATTGCACGGAGGAGT
PlaS1-DNA  ACTGCTTTCACCAGAATAAGAATACATACAACAAAGAATATAAGTTCCTCTCATCCTCTAAATGCAGGCAGAGGT
PlaS2-DNA  TCTGCCTTGGACAGAATGTGAATACATACGACAAAAACTATGAGTACTACTCAATCTCTCATTGCACGGAGGAGT
PlaS3-DNA  TCTGCCTTGGACAGAATGTGAATACATACGACAAAAACTATGAGTACTACTCAATCTCTCATTGCACGGAGGAGT

PlaS1-cDNA CAGAGCAATGCTAAGTCTCTGCAGGACGGGAAAAACCCCTCCAATTACACAATTGTGGTTGTGTTACTCTATTAT
PlaS2-cDNA CAGAGCAATGCTAAGTCTCTGCAGGACGGGAAAAAGCCCTCCAATTACACAATTGTGGTTGTGTTACTCTATTAT
PlaS1-DNA  CAGAGCAATGCTAAGTCTCTGCAGGACGGGAAAAACCCCTCCAATTACACAATTGTGGTTGTGTTACTCTATTAT
PlaS2-DNA  CAGAGCAATGCTAAGTCTCTGCAGGACGGGAAAAAGCCCTCCAATTACACAATTGTGGTTGTGTTACTCTATTAT
PlaS3-DNA  CAGAGCAATGCTAAGTCTCTGCAGGACGGGAAAAAGCCCTCCAATTACACAATTGTGGTTGTGTTACTCTATTAT

PlaS1-cDNA TCTGAATGCAATACTGAGCAATAAACAGGTGCCAGCTCTGCACTAAA
PlaS2-cDNA TCTGAATGCAATACTGAGCAATAAACAGGTGCCAGCTCTGCACTAAA
PlaS1-DNA  TCTGAATGCAATACTGAGCAATAAACAGGTGCCAGCTCTGCACTAAA
PlaS2-DNA  TCTGAATGCAATACTGAGCAATAAACAGGTGCCAGCTCTGCACTAAA
PlaS3-DNA  TCTGAATGCAATACTGAGCAATAAACAGGTGCCAGCTCTGCACTAAA
```

Figure 34 (continued)

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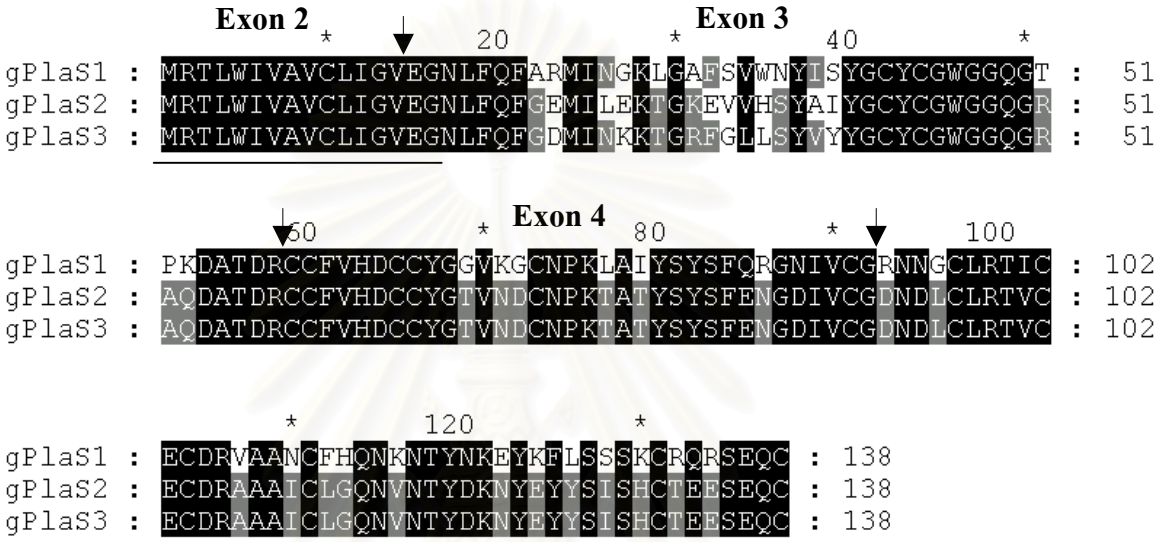

Figure 35 An alignment of deduced amino acid sequences of gPlaS1, gPlaS2 and gPlaS3. Identical nucleotides were highlighted. Signal peptide sequences were underlined. Arrows indicated exon separation. An alignment showed 69% (*gPlaS1*:*gPlaS2*), 70%(*gPlaS1*:*gPlaS3*) and 92%(*gPlaS2*:*gPlaS3*) amino acid sequence identity.

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Sequences producing significant alignments:	Score (bits)	E Value
gi|400714|sp|P31100|PA27_DABRU Phospholipase A2 RV-7 precur...	202	1e-51
gi|30142140|gb|AAP13805.1| phospholipase A2-I [Vipera russe...	201	1e-51
gi|26006833|sp|Q8JFG1|PA2I_VIPAP Phospholipase A2 inhibitor...	194	2e-49
gi|31790290|gb|AAP58959.1| phospholipase A2-III [Vipera rus...	189	6e-48
gi|6647690|sp|Q98996|PA2A_VIPPA Phospholipase A2 isozyme ac...	178	2e-44
gi|28201853|sp|Q9PWR6|PA27_VIPPA Phospholipase A2 isozyme V...	175	1e-43
gi|33187136|gb|AAN59990.1| ammodytin I1 [Vipera berus berus]	172	8e-43
gi|33187128|gb|AAN59986.1| ammodytin I1 [Vipera aspis aspis...	172	8e-43
gi|25453141|sp|Q910A1|PA2_VIPAA Phospholipase A2, ammodytin...	171	2e-42
gi|2851544|sp|P04084|PA2I_VIPAE Phospholipase A2 inhibitor ...	165	1e-40
gi|2144447|pir||PSVII phospholipase A2 inhibitor - western ...	165	1e-40
gi|16974940|pdb|1JLT|A Chain A, Vipoxin Complex	165	1e-40

Figure 36 Homology searching via Internet result of deduced amino acid sequence gPlaS3 by using BLAST P program.

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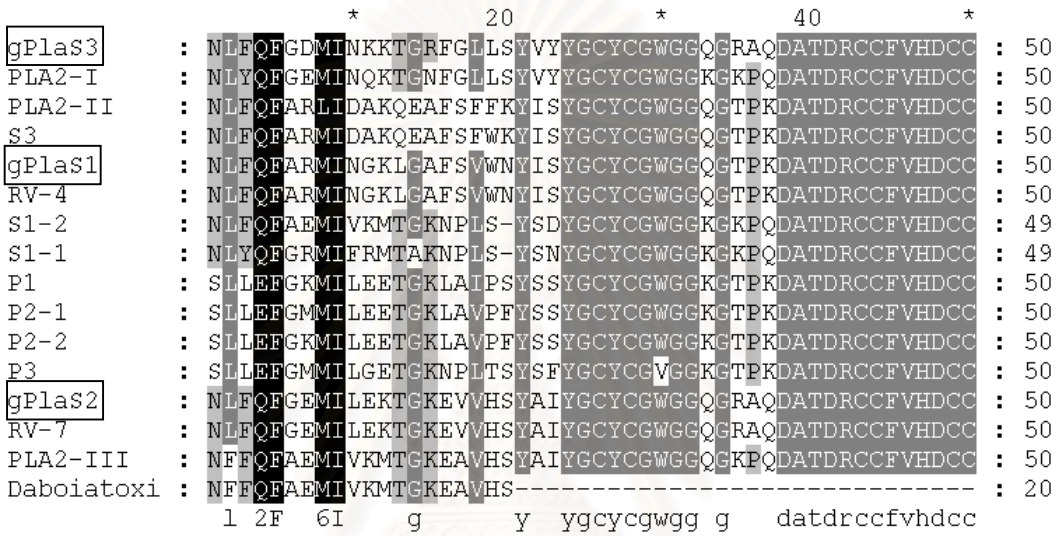

Figure 37 **An alignment of N-terminal amino acid sequence PLA_2s. gPlaS1, gPlaS2 and gPlaS3 were deduced from DNA. PLA2-I; *D. r. siamensis* (#AY256974), PLA2-II; *D. r. siamensis* (#AY286006), PLA2-III; *D. r. siamensis* (#AY303800), RV-4; *D. r. formosensis* (#S29298), RV-7; *D. r. formosensis* (#S29299), Daboiatoxin; *D. r. siamensis*[18], S1-1; *D. r. siamensis*[9], S1-2; *D. r. siamensis*[9], S3; *D. r. siamensis*[9], P1; *D. r. pulchella*[9], P2-1; *D. r. pulchella*[9], P2-2; *D. r. pulchella*[9], P3; *D. r. pulchella*[9].**

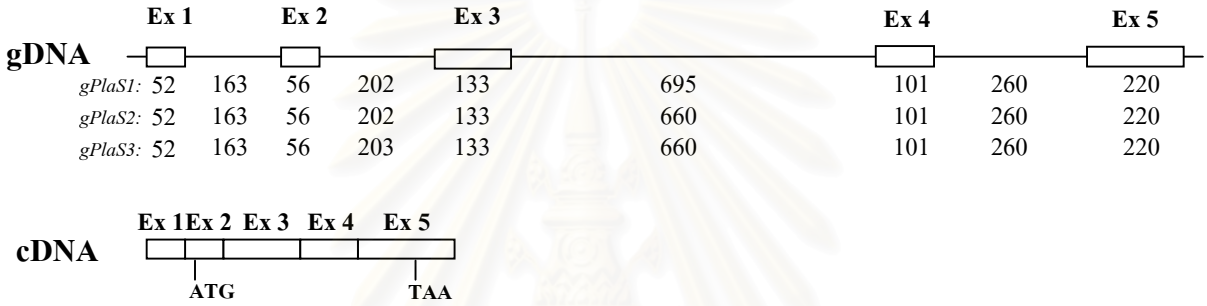

Figure 38 Comparison between cDNA and gDNA of PLA_2s.

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CHAPTER V

CONCLUSION AND DISCUSSION

The objective of this study is to screen PLA_2 isoforms of Thai Russell's viper venom and *in vivo* express of revealed PLA_2. The PLA_2 expression is performed in *E. coli* and its enzymatic activity is determined. The previous reports[9,18,19,22] suggested that several PLA_2 isoforms are found in Russell's viper venom subspecies. However, there is little information both PLA_2 isoforms of Thai Russell's viper venom and their nucleotide/amino acid sequences.

1. Two Types of *PLA_2* Account for Most of the Expressed PLA_2 in Thai Russell's Viper

We used 2 methods to clone *PLA_2* cDNA. The first is by plaque-lift DNA hybridization from cDNA library using *RVV012* probe. The second is by random sequencing for *PLA_2* of cDNA library obtained from ESTs study. In total of 26 clones, we found only 2 forms of *PLA_2* that designated *PlaS1* and *PlaS2*. The ratio of *PlaS1* and *PlaS2* cDNA found in the venom is 8:18.

Previously, Russell's viper PLA_2 studies relied on PLA_2 proteins, which were isolated and purified directly from snake venom by column chromatography technique. Most of PLA_2 protein sequences were reported as their short N-terminal sequences usually less than 50 amino acid residues[9,18]. Therefore, limited data of nucleotide sequences and full-length protein sequences of PLA_2 is hinder in identification and characterization clones of interest. The nucleotide sequences of Taiwan Russell's viper were available for comparison. We found that Thai Russell's viper *PlaS1* is identical to Taiwan Russell's viper PLA_2, *RV-4*, and *PlaS2* is identical to

RV-7. Although Thai Russell's viper (*D. r. siamensis*) and Taiwan Russell's viper (*D. r. formosensis*) are classified in different subspecies, the identical in PLA_2 gene sequence suggest they have closely relationship. In contrast, Daboiatoxin purified from Myanmar Russell's viper (*D. r. siamensis*) show less homology to Thai Russell's viper PLA_2. Thus, classification of snake subspecies by using different in coloration and marking may insufficient.

Despite 2 PLA_2 isoforms were found in cDNA library, the previous study which isolation PLA_2 from whole venom[9] showed that at least 5 isoforms of PLA_2 were found in *D. r. siamensis*. However, the isoforms classification from previous report discriminated by using only short N-terminal amino acid sequences of isolated PLA_2. The absent of other PLA_2 isoforms probably causes by relative low amount of their mRNA compare to *PlaS1* and *PlaS2* or using snake from different geographic region.

The genomic *PLA_2* gene organization is also one of interested. There are no full-length genomic *PLA_2* of *Daboia russellii spp.* reported to date. We found 3 forms of *PLA_2* gene, which designated *gPlaS1*, *gPlaS2* and *gPlaS3*. Sequence analysis of *gPlaS1* and *gPlaS2* are shown that their encode for *PlaS1* and *PlaS2* cDNA, respectively. Interestingly, *gPlaS3* was resemble to *gPlaS2* (98% nucleotide sequence identity). The isoelectric point (pI) predictation of deduced amino acid gPlaS1, gPlaS2 and gPlaS3 by using Compute pI/Mw Tool[43], is 8.96, 4.46, and 4.56, respectively. The predictation result indicated that gPlaS1 is a basic PLA_2 protein, while gPlaS2 and PlaS3 are acidic PLA_2 proteins. Surprisingly, an alignment of N-terminal amino acid sequences indicated that deduced amino acid sequences of gPlaS3 is different from previous reported *D. russellii* PLA_2s suggest that it is a new isoform reported todate. Therefore, this is the first report of genomic DNA sequences of *D. russellii* PLA_2. However, we can not clone cDNA of *gPlaS3* from cDNA library. Probably, cDNA of

gPlaS3 may express at very low level compare to *PlaS1* and *PlaS2*. Because predicted protein encoded from *gPlaS3* is not identical to any reported snake venom PLA_2, it is possible that *gPlaS3* may express in another tissue rather than venom gland.

The *gPlaS1*, *gPlaS2* and *gPlaS3* DNAs are about 2.0 kb in length. These *PLA_2*s gene compose of 5 exons and 4 introns similar to previously reported Viper *$gPLA_2$* [41,44,45,46] such as *Vipera ammodytes* ammodytin I1 (accession number AF253048) which are 2142 bp and 5 exons. The initiation codon (ATG) of all 3 forms of *$gPLA_2$* is located in exon 2 and the termination codon is located in exon 5. The signal peptides of these genes are 100% identical indicate the conservation of this region. All introns in these genes start by GT and end with AG as found in any eukaryotes. All the disulfide bridges, the active-site residues (His48, Asp49, Tyr52, Tys73 and Asp99) and the calcium-binding site (25-33, 49) of PLA_2 family[22,47,48] are all conserved (**Figure 39**).

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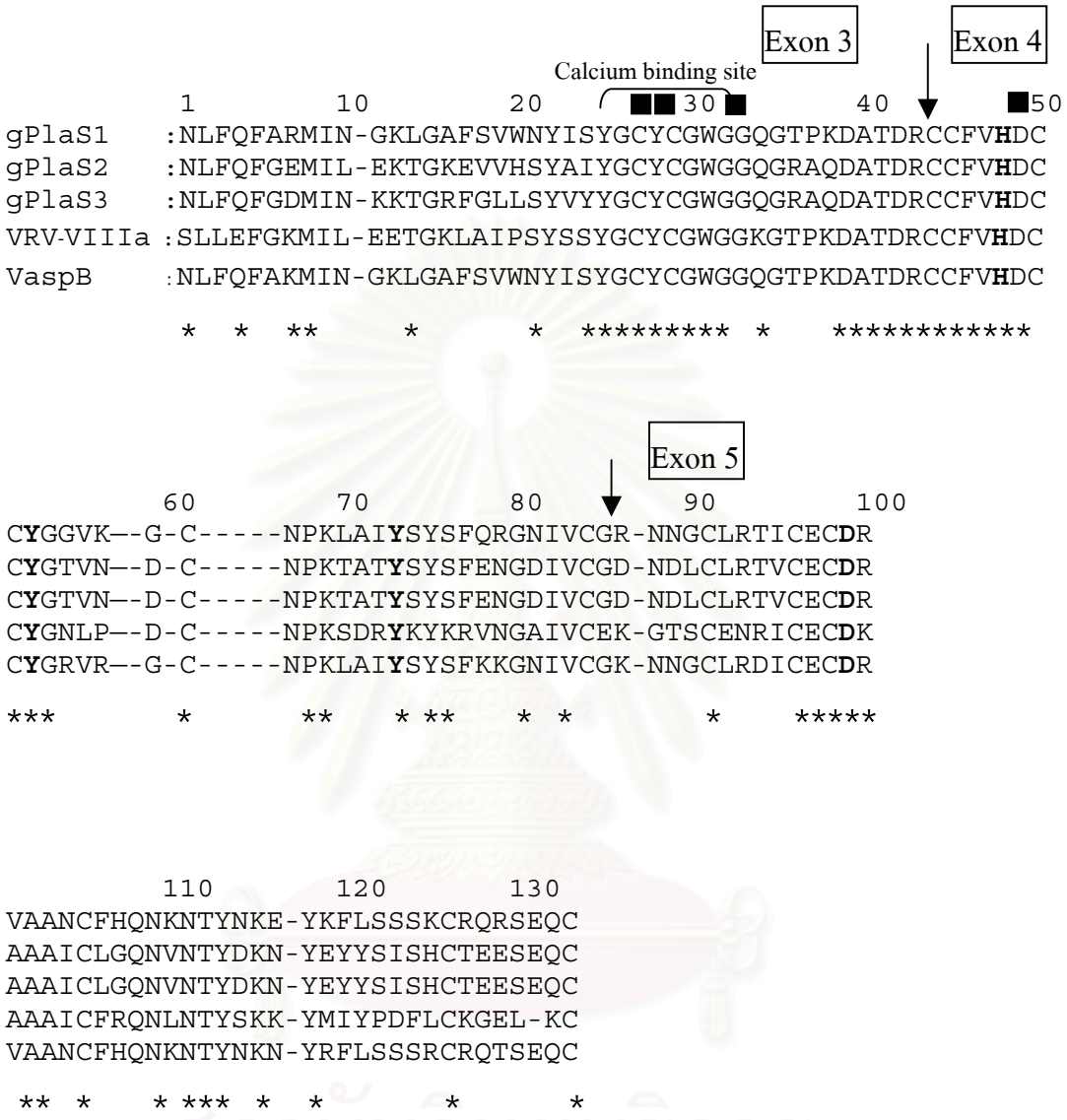

Figure 39 Comparison of amino acid sequences of PLA_2s. gPlaS3 were deduced from nucleotide sequence. **VRV-VIIIa;** VRV-PL-VIIIa from D. r. pulchella[49],**VaspB;** accession number AY158635. The sequences were aligned and spaced by the consensus numbering system for PLA_2[50]. Arrows indicated exon separation.Conserved residues were denoted by asterisk. Bold letter indicated residues involved in catalytic and supporting catalytic. Residues involved in coordination of the primary Ca^{2+} were indicated by closed boxes.

2. Expression of Recombinant Russell's Viper PLA_2

The successful expression of fully active recombinant PLA_2 of *Agkistrodon halys* Pallas in *E. coli* was reported[51]. High level of recombinant RVV-X expression by using vector pTrcHisA in *E. coli* was also obtained in our lab. Hence, we try to produce recombinant PlaS1 and PlaS2 in the *E. coli* using the expression vector pTrcHisA.

To optimize the expression level, IPTG concentration, incubation time after adding IPTG, and bacterial host strain were compared. As the result, 1 mM of IPTG, overnight incubation, and TOP10 host strain were selected. However, low production of PLA_2 was still observed (2.0 ug protein/Litre of cell culture). Generally, the average recombinant protein expression using pTrcHisA vector in *E. coli* was about 6-7 mg protein/Litre of cell culture[52].

Because of a low level of PLA_2 expression, SDS-PAGE analysis following IPTG induction did not discrimination PLA_2 band between with and without IPTG induction. The Western blot analysis revealed the expected band of about 18 kDa of recombinant PlaS1. However, the expected band of recombinant PlaS2 was also 18 kDa but the 23 kDa band was obtained instead. The shifted band of recombinant PlaS2 may be caused by the unused of stop codon in its sequence. Nevertheless, amino acid prediction of PlaS2 after stop codon revealed many stop codons in all 3 frames and none of them give an expected 23 kDa protein. Moreover, recombinant PlaS2 protein also showed the lower expression level than PlaS1.

The low level of recombinant PLA_2 expression is probably due to mRNA secondary structure[38]. In previous report of PLA_2 expression, a double mutant ammodytoxin A was expressed in *E. coli* at a low level not exceeding 0.5% of total cell proteins[38]. They also found that ammodytoxin A mRNA form strong secondary

structure using RNA secondary structure prediction program. Consequently, after the replacement of strong bases that involved hindering effective translation at the ribosome with weaker bases, the new mutant was successfully produced at a level of 15% of total proteins[38]. Thus, we try to produce recombinant PlaS1 and PlaS2 by using Rapid Translation System (RTS) which is *In vitro* protein synthesis system based on *E.coli* lysate. The silent mutational variants calculation of our DNA with ProteoExpert RTS E. coli HY Program was used to avoid the effect of mRNA secondary structure on the level of expression. However, recombinant PLA_2 was not expressed in this system while recombinant GFP control was detected.

The other reasons that may cause the low level of PLA_2 expression are the low stability of *PLA_2* mRNA or PLA_2 protein itself. Depletion of 5' UTR or signal peptide may cause instability of mRNA or PLA_2 protein as described elsewhere[41]. Moreover, expression as inclusion body in cells consequently loss of protein after solubilisation and refolding steps. An alternative strategy of soluble protein production is to direct the protein to the periplasm of *E.coli* cells, where disulfide bridges are spontaneously formed, by using signal peptide tag such as moltose-binding protein[53].

3. PLA_2 Activity

PLA_2 is tested for their *in vitro* enzymatic activity by using spectrophotometric method[39]. This method is easy and rapid. The result showed that recombinant PlaS1 had enzymatic activity of 185.67 μmoles/min/mg. We attempted to a used the chromatographic fraction containing PLA_2 proteins separated from Russell's viper crude venom as a control in activity assay. However, after incubation of control with its substrate, the mixture was precipitated in a few minutes. Although the enzymatic activity of PLA_2 control could not be assessed, the mixture reaction shown

the changing in color after enzyme adding, indicating that it possesses enzymatic activity.

There was a paper reported that the enzymatic activity of PLA_2 is not correlate with their LD_{50} [36]. However, the amount of recombinant pLA_2 was not enough to assess the biological role of PLA_2 that was previously planned, for example platelet aggregation inhibition and LD_{50}.

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APPENDIX

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APPENDIX

CHEMICAL AGENTS AND INSTRUMENTS

A. Research Instruments

Automatic adjustable micropipette (Eppendorf, Germany)

Balance (Precisa, Switzerland)

Beaker (Pyrex)

Combs (BIO-RAD, USA)

Chromatography and fraction collection system (BioLogig LP™ System from BIO-RAD, USA)

DNA Thermal cycler 2400 (Perkin Elmer, Cetus USA)

Electrophoresis Chamber set (BIO-RAD, USA)

Flask (Pyrex)

Chemi Doc (BIO-RAD, USA)

Glass Pipette (Witeg, Germany)

Heat block (Bockel)

Parafilm (American National Can, USA)

Pipette boy (Tecnomara, Switzerland)

Pipette rack (Autopack,USA)

Pipette tip (Axygen, USA)

Plastic wrap

Polypropylene conical tube (Elkay, USA)

Power supply model

pH meter (Eutech Cybernataics)

Microcentrifuge (Eppendorf, USA)

Microcentrifuge tube (BIO-RAD, Elkay, USA)

Reagent bottle (Duran)

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Spectrophotometer (BIO-RAD, USA)

Thermometer (Precision, Germany)

Vortex (scientific Industry, USA)

Water bath

B. General Reagents

Absolute ethanol (Merck)

Acetic acid (Merck)

Acrylamide:Bisarylamide (Phamacia Amersham)

Agar (Scharlau)

Agarose (USB)

Ammonium persulphate (Phamacia Amersham)

Ampicillin (M&H manufacturing)

Bromophenol blue (USB)

Calcium chloride (Merck)

Chloroform (Merck)

EDTA (Merck)

Ethidium bromide (Sigma)

Guanidine (USB)

Glucose (Merck)

Glycerol (Phamacia Amersham)

Glycine (USB)

Hydrochloric acid (Merck)

Hydrogen peroxide (Sigma)

IPTG (USB)

Isoamyl alcohol (Merck)

Maltose (Sigma)

Magnesium chloride (Fluka)

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Magnesium sulphate (Sigma)

2-Mercaptoethanol (Phamacia Amersham)

MES (USB)

Methanol (Merck)

Nickel chloride (Sigma)

NZY (Gibco)

Phenol (Sigma)

Phenol red (Sigma)

Potassium acetate (BDH)

Proteinase K (Phamacia Amersham)

RNase A

Sodium acetate (Merck)

Sodium chloride (Scharlau)

Sodium citrate (Sigma)

SDS (Sigma)

Sodium dihydrogenphosphate (Fisher Scientific)

Sodium hydroxide (Merck)

Sucrose (Sigma)

TEMED (Gibco)

Tris base (USB)

Triton X-100 (Sigma)

Tryptone (Scharlau)

Urea (USB)

Yeast extract (Scharlau)

100 bp DNA ladder (Biolabs)

1 kb DNA ladder (Gibco)

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C. Buffer of enzymes

1. *Taq* DNA Polymerase 10X buffer (50 mM KCl, 10 mM Tris-HCl (pH9.0 at 25^{O}C), 1.5 mM $MgCl_2$ and 0.1% TritonX-100 when diluted 1:10)
2. *Pfu* DNA Polymerase 10X buffer (100 mM KCl, 200 mM Tris-HCl (pH8.8 at 25^{O}C), 20 mM $MgSO_4$, 100 mM $(NH_4)_2SO_4$, 1 mg/ml nuclease-free BSA and 1% TritonX-100)
3. T4 DNA Ligase 10 X buffer (660 mM Tris-HCl (pH 7.6), 66 mM $MgCl_2$, 100 mM DTT, 660 μM ATP)

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