การทำให้บริสุทธิ์และการโคลนยีนของโปรตีนที่กระตุ้นเกล็ดเลือดจากพิษงูเขียวหางไหม้ (คริบเทไลทรอปส์ อัลโบลาบริส)

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PURIFICATION AND CLONING OF PLATELET ACTIVATING PROTEINS FROM GREEN PIT VIPER VENOM (*CRYPTELYTROPS ALBOLABRIS)*

Miss Ponthip Mekchay

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Medical Science

Faculty of Medicine

Chulalongkorn University

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By	Miss Ponthip Mekchay
Field of Study	Medical Science
Thesis Advisor	Associate Professor Ponlapat Rojnuckarin, M.D., Ph.D.

Accepted by the Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of the Faculty of Medicine
(Professor Adisorn Patradul, M.D.)

THESIS COMMITTEE

…….. Chairman
(Professor Apiwat Mutirangura, M.D., Ph.D.)

…….. Thesis Advisor
(Associate Professor Ponlapat Rojnuckarin, M.D., Ph.D.)

…….. Examiner
(Assistant Professor Tewin Tencomnao,Ph.D.)

…….. External Examiner
(Assistant Professor Yuttana Mundee,Ph.D.)

พรทิพย์ เมฆฉาย : การทำให้บริสุทธิ์ และการโคลนยีนของโปรตีนที่กระตุ้นเกล็ดเลือดจากพิษงูเขียวหางไหม้ (คริบเทไลทรอปส์ อัลโบลาบรีส). (PURIFICATION AND CLONING OF PLATELET ACTIVATING PROTEINS FROM GREEN PIT VIPER VENOM (*CRYPTELYTROPS ALBOLABRIS*)) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รองศาสตราจารย์ นพ.ดร. พลภัทร โรจน์นครินทร์ 64 หน้า.

งูเขียวหางไหม้เป็นงูพิษที่พบมากและกัดคนบ่อยในกรุงเทพ ซีไทป์เลคตินไลค์โปรตีนเป็นโปรตีนชนิดหนึ่งที่พบในพิษงูซึ่งออกฤทธิ์ต่อระบบโลหิต งานวิจัยนี้มีวัตถุประสงค์เพื่อโคลนนิ่ง แยกบริสุทธิ์ซีไทป์เลคตินไลค์โปรตีนตามขนาดและตามประจุและศึกษาผลกระทบของโปรตีนนี้ต่อเกล็ดเลือดมนุษย์โดยวิธีการหาปริมาณการเกาะกลุ่มของเกล็ดเลือดและการกระตุ้นเกล็ดเลือด

จากข้อมูลลำดับเบสจากห้องสมุดซีดีเอ็นเอของต่อมพิษงูเขียวหางไหม้เราได้ทำการโคลนยีนซีไทป์เลคตินโดยวิธี 5'-RACE และ 3'RACE จนสามารถโคลนยีนซีไทป์เลคตินไลค์โปรตีนได้ทั้งยีนของส่วนอัลฟาและยีนของส่วนบีต้า การวิเคราะห์ลำดับนิวคลีโอไทด์ พบว่าซีดีเอ็นเอของส่วนอัลฟาลและบีต้าประกอบด้วย 477 และ 447 เบสตามลำดับ ทำให้ได้โปรตีนที่มีกรดอะมิโน135 และ 125 ตัวตามลำดับ และมีส่วนซิกแนลเปปไทด์ 23 ตัว ผลที่ได้จากการแยกบริสุทธิ์พบว่าซีไทป์เลคตินไลค์โปรตีนมีขนาด 120 กิโลดาลตันในภาวะที่ไม่มีการทำลายพันธะไดซัลไฟด์ และมีขนาด 14, 17 กิโลดาลตันในภาวะที่มีการทำลายพันธะไดซัลไฟด์แสดงว่าน่าจะมีการเรียงตัวแบบ $(\alpha\beta)_4$ นอกจากนี้ยังใช้วิธีลิควิดโครมาโตรกราฟีแทนเดมแมสสเปคโตรสะโคปปีในการยืนยันว่าโปรตีนตัวนี้โคลนได้จากต่อมพิษงูจริง การวิเคราะห์ลำดับกรดอะมิโนพบซิสเทอีนที่น่าจะเชื่อมสายโปรตีนเข้าด้วยกันจนมีขนาดใหญ่ ในการทดสอบกับเกล็ดเลือดมนุษย์พบว่าโปรตีนนี้มีฤทธิ์เกาะกลุ่มเกล็ดเลือดโดยไม่ต้องอาศัยสารร่วมด้วยค่าอีซี 50 เท่ากับ 0.25 นาโนโมลาร์ แอนติบอดี้ต่อไกลโคโปรตีนวันบีและไกลโคโปรตีนซิกสามารถยับยั้งการเกาะกลุ่มของเกร็ดเลือดที่ถูกกระตุ้นด้วยซีไทป์เลคตินไลค์โปรตีนและยังพบว่าโปรตีนนี้กระตุ้นการเกิดไทโรซีนฟอสโฟรีเรชั่นซึ่งมีผลทำให้เกิดการส่งสัญญาณภายในเกล็ดเลือดมนุษย์ จากการศึกษาจนถึงปัจจุบันพบว่ารายงานนี้เป็นรายงานแรกของซีไทป์เลคตินไลค์โปรตีนตัวใหม่และซีดีเอ็นเอของซีไทป์เลคตินไลค์โปรตีนนี้ที่ได้จากพิษงูเขียวหางไหม้

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORDS : *CRYPTELYTROPS ALBOLABRIS* / C-TYPE LECTIN PROTEINS / PLATELET AGGREGATION / PLATELET ACTIVATION

PONTHIP MEKCHAY: PURIFICATION AND CLONING OF PLATELET ACTIVATING PROTEINS FROM GREEN PIT VIPER VENOM (*CRYPTELYTROPS ALBOLABRIS*.). THESIS ADVISOR: ASSOCIATE PROFESSOR PONLAPAT ROJNUCKARIN, MD., PhD., 64 pp.

Green pit viper (GPV) is the most common cause of snakebites in Bangkok. C-type lectin-like protein (CLP) obtained from the venom acts on haemostatic system. This study is aimed to clone, purifying a high-molecular weight CLP and characterize its effects on human platelets.

Based on the partial cDNA library of *Cryptelytrops albolabris*, we had cloned full-length cDNA encoding the CLP subunit by 5'-RACE and 3'RACE method. The cDNA sequence of α subunit was 477 bp that was translated into 23 amino acid residue signal peptide and a 135 amino acid residue mature protein. The cDNA sequence of β subunit was 447 bp and translated into 23 amino acid residue signal peptide and a 125 amino acid residue mature protein. The novel *C. albolabris* CLP was isolated from crude venom using gel filtration column and followed by ion-exchange chromatography. The purified *C. albolabris* CLP was electrophoresed on SDS-PAGE showing the apparent molecular mass of 120 kDa (native condition) and displayed 2 bands of 14 and 17 kDa, (reduced condition) suggesting a tetramer of heterodimers $(\alpha\beta)_4$. Liquid chromatography-tandem mass spectrometry analysis of the peptides found a perfect match with the conceptually-translated sequence in the green pit viper venom gland cDNA library. The peptide contained extra cysteine residues that probably formed inter-chain disulfide bonds. CLP induced human platelet aggregation in the absence of any cofactor with EC_{50} of 0.25 nM. Antibodies against both GPIb and GPVI inhibited platelet aggregation induced by *C. albolabris* CLP. Furthermore, *C. albolabris* CLP was found to activate tyrosine phosphorylation in human platelet. Up to now, this is the first report of the novel CLP and its cDNA cloning from *C. albolabris*.

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LIST OF ABBREVIATIONS

3D	three-dimension
ACN	Acetonitrile
ADP	adenosine diphosphate
Amu	atom mass unit
ATP	adenosine triphosphate
bp	base pair
BCA	bicinchoninic acid
^{o}C	degree Celsius
cDNA	complementary deoxyribonucleic acid
CCA	alpha cyano cinnamic acid
CD	cluster of differentiation
CID	collision-induced dissociation energy
CLPs	C-type lectin like proteins
cm	centimeter
CP/CPK	creatine phosphate/creatine phosphokinase
CRDs	carbohydrate recognition domain
Cys	cysteine
dNTPs	dATP, dTTP, dGTP and dCTP
DNA	deoxyribonucleic acid

DTT	dithiothreitol
E. coli	*Escherichia coli*
EC_{50}	the concentration of an agonist required to induce 50% aggregation
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FcR	Fc receptor
g	gram
Gly	glycine
GP	glycoprotein
IC_{50}	the concentration of an inhibitor required to inhibit 50 % aggregation
IgE	immunoglobulin E
IgM	immunoglobulin M
IPTG	isopropyl-β-D-thiogalactopyranoside
ITAM	immunoreceptor tyrosine-based activation motif
Kb	kilobase
kDa	kiloDalton
L	Liter
LB	Luria-Bertani media
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
mg	milligram

ml	milliliter
mM	millimolar
nM	nanomolar
M	molar
MMLV	Moloney Murine Leukemia Virus
MWCO	moleculare weight cut off
ng	nanogram
nm	nanometer
OD	optical density
PACKS	platelet aggregation chromogenic kinetic system
pmol	picomole
PCR	polymerase chain reaction
PMFs	peptide mass fingerprints
PT	plain tyrode
RACE	rapid amplification of cDNA end
rpm	round per minute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodiumdodecylsulphate
SDS-PAGE	sodiumdodesylsulphate polyacrylamide gel electrophoresis
TFA	trifluoroacetic Acid

Tris-HCl	tris-(hydroxymethyl)-aminoethane
UTR	untranslated region
vWF	von Willebrand factor
v/v	volume/volume
w/v	weight/volume
µg	microgram
µl	microliter
x g	gravity

CHAPTER I
INTRODUCTION

1.1 Background and Rationale

The wide range of topographical and climatic conditions in Thailand supports a variety of snakes of more than 160 species, but the most common venomous snake that is widely distributed and causes an important snakebite problem is the green pit viper. There are two important species, *Cryptelytrops albolabris* and *Cryptelytrops macrops*. They account for 40% of all bites (Viravan *et al*., 1992) in Thailand and comprise more than 95% of venomous snakebites in Bangkok (Mahasandana and Jintakune, 1990). More than 400 snakebite patients came to Chulalongkorn hospital each year (P. Rojnuckarin *et al*., 2005), therefore viper snakebites still cause both public health and economic problems.

Cryptelytrops albolabris, a crotaline viper, is small to medium in size and generally stays on trees. These snakes possess a heat-sensitive pit organ (thermoreceptor) behind a nostril to detect a very small temperature change in warm-blooded preys. The venom of *C. albolabris* is more toxic than that of *C. macrops.* It can produce severe local effects. Swelling occurs very rapidly and may involve the whole limb or adjacent trunk. Bruising, blistering and necrosis occur within few days. Necrosis is common in tight fascial compartments, such as the digits. Hematotoxic effects are hypofribrinogenemia and thrombocytopenia (Mahasandana *et al*., 1980). The venom has been shown to contain a large number of toxins that exert powerful both inhibitory and stimulatory effects on platelets. Platelet aggregating agents in venom were proposed to activate platelets *in vivo* resulting in platelet consumption and thrombocytopenia. These platelet activators in viper venoms are usually in the family of C-type lectin-like proteins.

Recently, a number of C-type lectin-like proteins affecting the platelet functions have been isolated and characterized. Some of them inhibit platelet aggregation by binding to glycoprotein (GP) Ib-IX-V and blocking vWF binding, whereas the others promote platelet aggregation by targeting against von Willebrand factor (vWF), GPIb-IX-V, GPIV and possibly

other platelet receptors. In this study, we will purify and clone a novel C-type lectin-like protein and identify some of the involved signaling molecules.

1.1.1 Green Pit vipers

In Thailand, there are 11 types of green pit vipers. White-lipped pit viper or *Cryptelytrops albolabris* is the most commonly found poisonous snake. Its head and body are green with a red tail and yellowish or whitish abdomen. There are 21 rows of keeled scales in the middle of body. Scales under abdomen are smooth. The top of a head contains 11-12 fine scales. The prominent feature of green pit vipers is the lip scales that connect with nostril scales. Males have a white stripe on the first body scale row which is absent in females. The length of males and females are about 50 and 70 centimeters, respectively. The heat-sensitive pit organ (thermoreceptor) is behind the nostril in order to detect a very small temperature change of warm-blooded preys. It often stays on trees hanging by tail, spends daytime resting and always hunts at night on the ground. It feeds on mice, birds, lizards and frogs. At the time it is disturbed, green pit vipers usually escape in the opposite direction and crawl high up. It rarely strikes unless it is in an urgent situation. *Cryptelytrops albolabris* is ovoviviparous, i.e. embryos develop inside eggs that are retained within the mother's body until hatching. It always gives birth in rainy season yielding 7-15 offsprings per litter.

1.1.2 Clinical signs and symptoms of green pit viper bites

The venom is mainly hematotoxic. It often produces no serious systemic symptom except pain, swelling of the affected part with extension to the limb, and abnormal blood coagulolation. Some patients have purpura and ecchymoses at the affected extremities. In cases of severe envenoming, patients are hypotensive effects immediately after the bites and recover later on. This is followed by gum bleeding, hematemesis, hematuria, diarrhea, anuria or oliguria and acute renal failure. Other systemic bleeding such as purpura, ecchymoses, subconjunctival hemorrhage, bleeding from puncture wounds and abdominal pain are observed. Uremic symptoms, including nausea, vomiting, hiccough, edema and

signs of pulmonary edema, are possible. Hypofibrinogenemia, thrombocytopenia, anemia and an abnormal renal function test can be found. With prompt green pit viper antivenom serum, symptomatic and supportive treatments all patients should survive with complete recovery.

1.1.3 Components of green pit viper venom

The snakes use venom to kill and help to digest preys, as well as for self defense. In snakebites, the venom is injected into a victim via the sharp fangs causing damages in several ways. Green pit viper venom is a complex mixture of biologically active proteins and peptides. Many of them affect hemostasis by activating or inhibiting coagulant factors or platelets. Green pit viper venom components have been classified into various families, such as serine proteases, metalloproteinases, phospholipases A_2, disintegrins and C-type lectins.

Serine protease is a protein family that contains histidine, aspartate and serine residues on the catalytic sites. They selectively affect many steps in the blood coagulation cascade by activation or inhibition of specific blood factors involved in platelet aggregation, coagulation or fibrinolysis. In *Cryptelytrops albolabris,* serine proteases show effects on thrombin clotting, plasminogen activation, fibrinogenolysis and anticoagulation. The effects can be neutralized *in vitro* using the specific antivenom

Snake venom metalloproteinases (SVMPs) are 20 to 100 kDa, multi-domain proteins that compose of a catalytic domain and one or several non-catalytic domain. SVMPs are classified into four major groups (Grams F. *et al*., 1993). The P-I class has a single metalloproteinase domain. The P-II class has a metalloproteinase and a disintegrin domain that are separated post-translationally. The P-III class has metalloproteinase, disintegrin-like (containing a disulfide-linked XXCD, mostly SECD, in place of RGD), and cysteine-rich domains. The P-IV class contains an additional disulfide-linked C-type lectin-like domain, compared with the P-III class. The hemorrhagic metalloproteinases are stored as inactive zymogens and activated by a cysteine switch-like mechanism. Metalloproteinase - disintegrin, alborrhagin from the viper *Cryptelytrops albolabris*, selectively targets GPVI on

platelets or transfected cells, and induces GPVI-dependent signaling and platelet aggregation. (Robert K. *et al*., 2001)

Phospholipase A_2 (PL A_2), a non-glycosylated protein, belongs to the superfamily of enzymes that hydrolyze the sn-2 acyl groups of membrane phospholipids to release arachidonic acid (AA) and lysophospholipids. Made up of 120 to 135 amino acids with about 7 disulfide bonds, snake PLA_2s can be monomeric, homodimeric or heterodimeric. It has a conserved protein folding consists of three major α-helices and β-strand structure. Their enzymatic activity ranges from strong to undetectable. They affect not only nerves and muscles, but also to platelets, leukocytes, and erythrocytes (Xiao-Yan Du *et al*., 2006).

Disintegrin is a low molecular weight protein from viper venom that binds to the transmembrane integrins selectively. Disintegrin belong to a class of non-enzymatic cysteine-rich peptides. The conserved Arg-Gly-Asp (RGD) sequence together with certain non-conserved amino acids of disintegrins determines their binding specificity and their ability to interact with platelet $\alpha_{IIb}\beta_3$ (Xiao-Yan Du *et al*., 2006).

C-type lectins are also non-enzymatic proteins that Ca^{2+}-dependently bind to mono- and oligosaccharides. They are multi-domain proteins and contain one or more copies of a highly conserved region consisting of 115 to 130 amino acid residues, called the carbohydrate recognition domain (CRD) which has a uniquely mixed αβ topology. C-type lectin can be classified into seven groups based on structural characteristics.

C-type lectin-like proteins (CLPs) are found only in snake venoms and belong to group VII of C-type lectin proteins. Although CLPs are highly homologous to C-type lectins, there are some differences as they lack carbohydrate binding activity and do not depend on Calcium. CLPs show diverse pharmacological activities against coagulation factors, platelets and affect hemostasis and thrombosis. A prominent feature of CLPs is that they are heterodimers or oligomeric complexes of heterodimers comprising two homologous subunits: subunit a (α chain) of 14–15 kDa and subunit b (β chain) of 13–14 kDa linked by interchain disulfide bond. CLPs occur in a variety of oligomeric forms, including αβ, $(\alpha\beta)_2$, and $(\alpha\beta)_4$(Atoda *et al*., 1991; Fukuda *et al*., 2000; Wang and Huang, 2001; Wang *et al*.,

2003). Snake venom C-type lectins are Ca^{2+}-dependent proteins that are exclusively homodimers or homo-oligomers.

From what has been mentioned, it seems clear that CLP is an important protein and should be thoroughly investigated in order to understand pathogenesis of snake venom-induced platelet abnormalities in order to find effective treatments for snakebite patients. Furthermore, studying the molecular mechanisms of platelet activation by CLPs will give us deeper insights in platelet signaling pathways. Finally, these proteins display great potentials to be diagnostic agents for platelet function tests in patients with bleeding or thrombotic disorders.

1.2 Research Questions

1. What are the full-length cDNA and deduced amino acid sequences of platelet-activating C-type lectin-like protein from *C. albolabris*?

2. What are the effects of this protein on platelet aggregation, receptors and signal transduction?

1.3 Objectives of the study

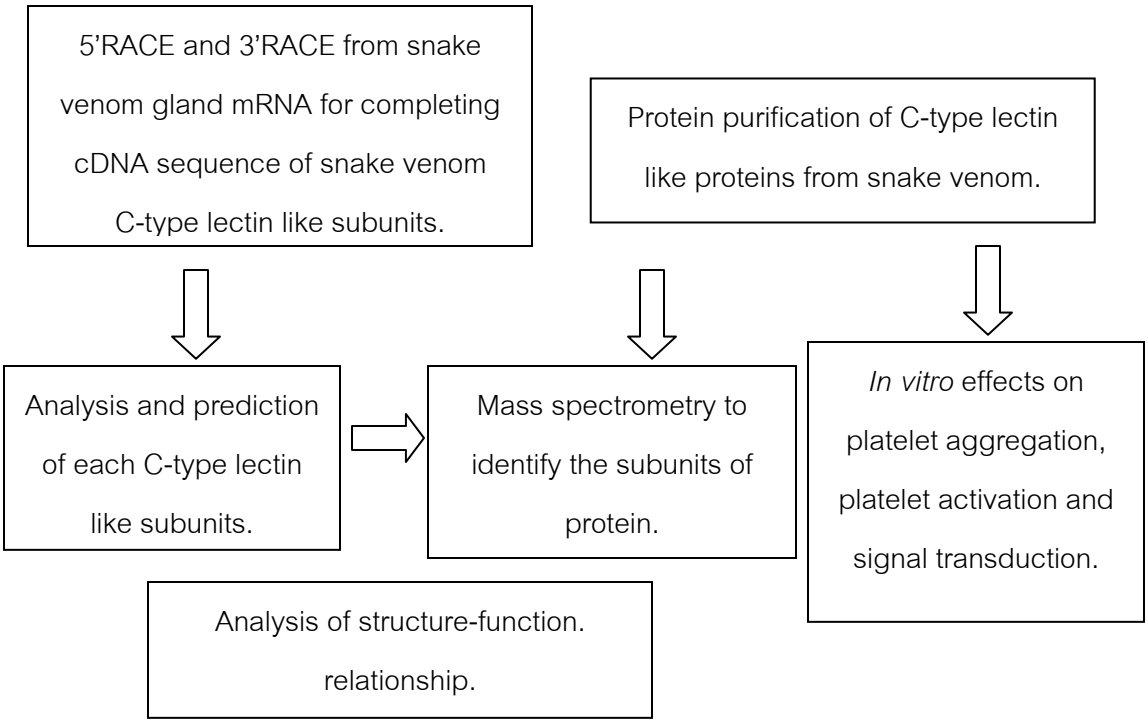
1. Purification of CLP and study its effects on human platelet aggregation and signal transduction.

2. Molecular cloning of the platelet activating protein from green pit viper (*C. albolabris*.) venom and analysis of the sequence correlating with its functions.

1.4 Keywords

C-type lectin proteins, Platelet aggregation, Platelet activation, *Cryptelytrops albolabris*.

1.5 Conceptual Framework



1.6 Benefits and Applications

1. This protein is potentially useful as a diagnosis agents or reagents to dissect mechanisms of platelet activation.
2. This proteins may be useful as an agent to diagnosis the abnormality of platelet function in patients.
3. This protein has the potential to be used for diagnostic tests and the development of new medical compounds.
4. This study will give deeper insights of structure-function relationship of snake venom C-type lectin like proteins and molecular pathogenesis of green pit viper envenomation.

CHAPTER II

LITERATURE REVIEW

2.1 The C-type lectin proteins from snake venom

C-type lectins are non-enzymatic proteins that are found in many animals and bind mono- and oligosaccharides in a Ca^{2+}-dependent manner. C-type lectins contain one or more copies of a highly conserved region, called the carbohydrate recognition domain (CRD), consisting of 115-130 amino acid residues. C-type lectins are typically multi-domain proteins. They can be divided into seven groups (I-VII) based on their structural characteristics (Weis *et al*., 1992; Drickamer, 1999).

The C-type lectin-like proteins (CLPs) belong to the group VII of the C-type lectin family. CLPs are found only in snake venoms. A prominent feature of CLPs is that they are heterodimers or oligomeric complexes of heterodimers comprising a basic structure of two homologous subunits: subunit a (α chain) of 14–15 kDa and subunit b (β chain) of 13–14 kDa linked by an interchain disulfide bond. CLPs show a variety of oligomeric forms, including αβ, $(\alpha\beta)_2$, and $(\alpha\beta)_4$ (Atoda *et al*., 1991; Fukuda *et al*., 2000; Wang and Huang, 2001; Wang *et al*., 2003). The basic homologous dimerization (αβ) of these CLPs is formed by three-dimensional (3D) domain swapping. Despite the similar name, C-type lectin-like proteins (CLPs) of snake venom differ from the C-type lectins. C-type lectins display Ca^{2+}-dependent (C type) binding and they are exclusively homodimers or homo-oligomers.In contrast to C-type lectins, CLPs have no lectin, or carbohydrate-binding, activity (Drickamer, 1999; Morita, 2004b). They possess various biological activities, including anticoagulant- and platelet-modulating activities.

2.2 Snake venom C-type lectin-like proteins (CLPs): functions

CLPs can be classified into 4 subgroups on the basis of their functions, namely anticoagulant lectins, coagulant proteins, platelet aggregation agonists, and platelet aggregation antagonists (Table 1).

Table1: C-type lectin-like proteins (CLPs) of snake venom that modulate hemostatic system (Morita, 2005).

Function	CLP	Structure	Ligands or substrates
Anticoagulant	IX/X-bp, IX-bp IX/X-bp, X-bp Bothrojaracin	αβ αβ αβ	Factor IX Factor X α-Thrombin
Coagulant	RVV-X Carinactivase-1(CA-1)	αβ/metallaprotease αβ/metallaprotease	Factor X Prothrombin
Platelet agonist	Botrocetin,bilitiscetin Alboaggregin-B Agglucetin Aggregin/rhodocytin Convulxin	αβ αβ Tetrameric structure αβ $(\alpha\beta)_4$	vWF GPIbα GPIbα GPIa/IIa GPVI
Platelet antagonist	Echicetin Flavocetin-A(FL-A) EMS16	αβ $(\alpha\beta)_4$ αβ	GPIbα GPIbα GPIa/IIa

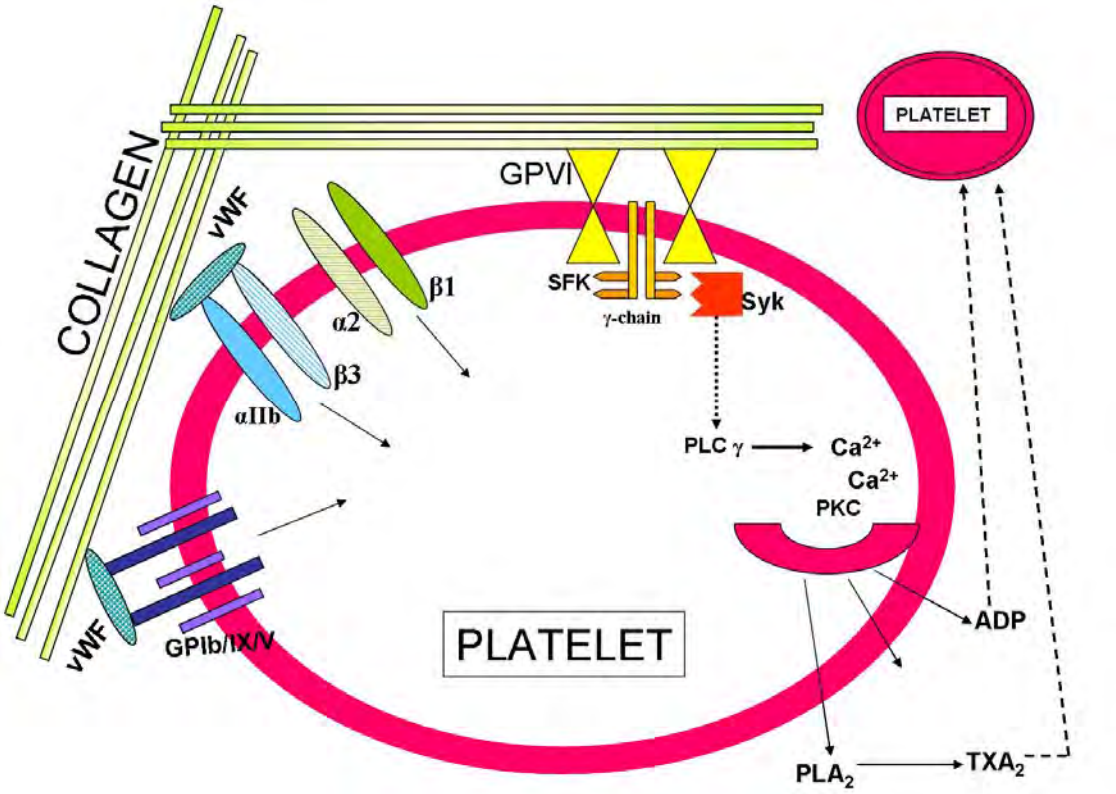
Abbreviations: IX/X-bp = factor IX/X binding protein, IX-bp = factor IX binding protein, X-bp = factor X binding protein, RVV-X = Russell's viper venom factor X activator, vWF = von willebrand factor, GP = Glycoprotein

Platelet adhesion and aggregation are essential processes to control blood loss at sites of vascular injury. The first platelet response to vascular injury is adhesion to exposed subendothelial matrix. Circulating platelets arrest on and are activated by exposed collagen and collagen-bound von Willebrand factor in the subendothelial matrix using platelet receptors that can bind to von Willebrand factor (the glycoprotein (GP) Ib/IX/V complex and integrin $\alpha_{IIb}\beta_3$ or GPIIb/IIIa) and collagen (the Integrin $\alpha_2\beta_1$ or GPIa/IIa and GPVI). The initial intracellular signaling then allows the accumulation of a platelet monolayer that will subsequently support thrombin generation and the formation of larger platelet aggregates. This adhesion process and platelet agonists activate platelet GPIIb/IIIa structure leading to expression of fibrinogen-binding sites which are not available on inactive platelets. Binding of fibrinogen results in the linkage of activated platelets through fibrinogen bridges, thereby mediating aggregation. When the

additional platelets accumulate on the initial monolayer, there is the stimulation of the receptors presence on platelet by soluble agonists, such as thrombin, TxA_2 and ADP amplifying the processes. The late events of platelet plug formation are characterized by direct interactions between platelets making contact-dependent signaling possible for example the outside-in signaling through integrins and the signaling which generated by non-receptor tyrosine kinases. These late events stabilize the platelet plug, prevent premature disaggregation and regulate clot retraction (Woulfe *et al*., 2004).

The GPIb complex on and platelets and von Willebrand factor (VWF) are involved in platelet adhesion by linking with collagen on the subendothelium. Subsequently, platelets are either torn away by the shear force of the blood stream and continue to circulate or are stopped and adhere firmly to the subendothelium mediated by integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$. (Qiumin Lu *et al*., 2004). Another critical receptor is GPVI, which is one of the major platelet receptors for collagen. GPVI has been established as the central platelet collagen receptor that is essential for platelet adhesion and aggregation on immobilized collagen *in vitro,* as it mediates the activation of different adhesive receptors, including integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$. GPVI is a 60–65- kDa type I transmembrane glycoprotein belonging to the immunoglobulin superfamily that forms a complex with the Fc receptor (FcR) γ chain on the cell surface in human and mouse platelets. Signaling through GPVI occurs via a pathway similar to that is used by immunoreceptors as revealed by the tyrosine phosphorylation of the FcR γ chain immunoreceptor tyrosine-based activation (ITAM) motif by a Src-like kinase. (Massberg *et al*., 2003)

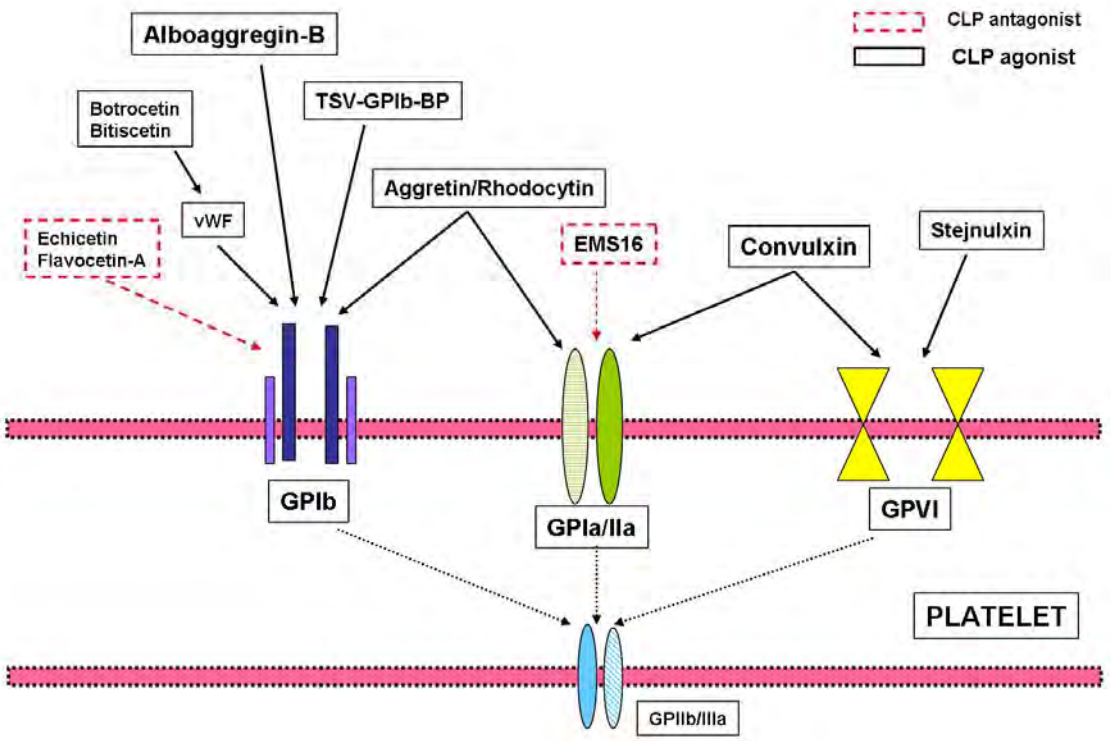
Figure1. Platelet activation (Modified from Woulfe *et al.*, 2004).



Snake venom CLPs affecting platelet functions

Glycoprotein receptors on platelets are also targets for snake venom C-type lectin-like protein as shown in figure 2.

Figure2. CLPs from snake venom that modulate the platelet function. (Modified from Morita., 2005)



1. vWF/GPIb-binding CLPs

Botrocetin: The heterdimeric (αβ) CLP was isolated from the venom of *Bothrops Jararaca*. It is the first identified CLP that induces platelet agglutination (Brinkhous *et al*., 1981, 1983) and can promote the vWF-dependent agglutination of platelets. Botrocetin first binds to the A1 domain of vWF to form an active complex, which then induces platelet agglutination.

Bitiscetin: The protein was isolated from the venom of *Bitis arietans*. It is another heterdimeric (αβ) CLP that induces platelet agglutination (Hamako *et al*., 1996). Several biochemical studies of bitiscetin indicate that the vWF A1 domain, which binds GPIb, is essential for the biological activity (Matsui *et al*., 2002).

2. GPIb-binding CLPs

Mucrocetin: Mucrocetin purified from the venom of *Trimeresurus mucrosquamatus*. A tetrameric $(\alpha\beta)_4$ platelet-agglutinating factor, molecular mass 121.1 kDa, which bind specific site of glycoprotein Ibα. Monoclonal antibody to CD42b Ab-1, raised against the platelet GPIb, dose-dependently blocked the agglutination induced by mucrocetin. The α- and β -subunits of mucrocetin bear higher structural similarity to the β- and γ-chains of alboaggregin A 60.7% and 71.2%respectively. (HUANG *et al*., 2004)

Alboaggregin-B (AL-B): The heterodimeric (αβ) CLP was isolated from *Cryptelytrops albolabris* venom by ion-exchange chromatography. It agglutinates platelets without the need for any cofactors or platelet activation. (Peng., 1991) Platelet agglutination induced by AL-B is completely blocked by the anti-GpIbα antibody indicating that GpIbα is the specific target of AL-B (J. Arpijuntarangkoon *et al*, 2007).

TSV-GPIb-BP: The dimeric protein was isolated from *Viridovipera stejnegeri* venom. The α-subunit of TSV-GPIb-BP is identical to that alboaggregin-B and the sequence identity of their β-subunitis is 94.3%. Similar to AL-B, TSV-GPIb-BP agglutinates platelets without platelet activation. This platelet aggregation activity was inhibited by specific GPIbα antibodies but not by antibodies against platelet GPIa, GPIIa, GPIIb and GPIIIa (Lee *et al*. 2003).

Flavocetin-A: The CLP has been isolated from the habu snake, *Trimeresurus flavoviridis* (Taniuchi *et al*., 1995). This protein has a molecular mass of 149 kDa and binds with highest affinity to platelet GPIb. This protein strongly inhibits vWF-dependent aggregation of fixed platelets and inhibits shear-induced platelet aggregation at high shear stress. The 3D structure of this high molecular mass CLP was determined by X-ray crystallography to understand its functional specificity and to identify the molecular recognition sites within its heterodimeric structure (Fukuda *et al*., 2000). The X-ray structure of flavocetin-A revealed that it is composed of a cyclic tetramer made up from four identical αβ-heterodimers arranging in head-to-tail orientation.

3. Platelet collagen receptor-binding CLPs

Both platelet GPVI and GPIa/IIa are collagen receptors that participate in platelet activation.

Aggretin: A heterodimeric $(\alpha\beta)_2$ CLPs, potent platelet activator was isolated from *Calloselasma rhodostoma* venom. It activates platelets by binding to platelet GPIa/IIa. Aggretin-induced platelet activation was inhibited by a monoclonal antibody to GPIb as well as by the antibody to GPIa/IIa. No binding of other major platelet membrane glycoproteins, in particular GPVI was detectable (Navdaev *et al*., 2001).

EMS16: This proein was isolated from the venom of *Echis multisquamatus*. Its structure was $\alpha_2\beta_1$. It was reported as the first selective GPIa/IIa antagonist from snake venom (Marcinkiewicz *et al*., 2000). It binds to the GPIa/IIa domain responsible for collagen-binding inhibiting collagen-induced platelet aggregation.

Convulxin: The CLP was isolated from venom of *Crotalus durissus terrificus*. It is a platelet agonist that acts on GPVI. Convulxin is a high molecular mass CLP that binds the collagen receptor GPVI with high affinity (Kd = 30 pM) and is a very potent inducer of platelet aggregation. The crystal structure revealed that it is a disulfide-linked cyclic $(\alpha\beta)_4$ heterotetramer (Batuwangala *et al*., 2004; Murakami *et al*., 2003). There is an extra intermolecular disulfide bond, Cys135 in the C-terminus of the α chain and Cys3 in the N-terminal region of the β chain that are required for the cyclic structure of convulxin.

Stejnulxin: a hexameric $(\alpha\beta)_3$ CLP, was isolated from *Viridovipera stejnegeri*. The high-molecular mass CLPs (120 kDa) contains potent platelet activating activity that acts on GPVI. Stejnulxin induces human platelet aggregation in a dose dependent manner. Antibodies against $\alpha_{IIb}\beta_3$ inhibited the aggregation response to stejnulxin, indicating that activation of $\alpha_{IIb}\beta_3$ is involved in stejnuxin-induced platelet aggregation. Antibodies against GPIb had no significant effect on stejnuxin-induced platelet aggregation. In addition stejnulxin-induced platelet activation was blocked by anti-GPVI

antibodies, suggested that stejnulxin bound specifically to platelet membrane GPVI. (Lee *et al*.., 2003)

C-type lectin-like protein from green pit viper (*Cryptelytrops albolabris*)

Previous studies have purified 4 green pit viper platelet-agglutinating proteins, alboaggregins A, B, and C, that were C-type lectin like-proteins from *C. albolabris* and shown to bind to platelet GPIb.

Alboaggregin B, a 25-kDa protein, has a simple heterodimeric structure and agglutinates platelets by binding to GPIb but does not cause a major activation of platelet signaling pathways. Alboaggregin B cDNA has been cloned and identified a conserved motif (SRTY) in β-subunit that may responsible for platelet aggregation (J. Arpijuntarangkoon *et al*., 2007). On the other hand, alboaggregin A, 50 kDa proteins, showed 3 bands at 14, 15, and 16 kDa in the reduced condition. It is tetrameric and activates platelets strongly, presumably, by means of receptor clustering mechanism. Both alboaggregins agglutinate fixed platelets by cross-linking receptors between platelets. Studies of the signaling in platelets induced by alboaggregin A have suggested that it generated signaling by means of GPIb. Because alboaggregin A gives much stronger signals than other GPIb ligands, it was proposed to cross-link the receptor to a higher degree than the other agonists and could, therefore, be used to explore GPIb signaling pathways of platelet activation. However, because the pattern of tyrosine phosphorylation induced in platelets by alboaggregin A differs considerably from that caused by other ligands of GPIb, an alternative explanation for its strong agonist effects on platelets could be that it binds not only to GPIb but also to another platelet receptor. Along with binding to GPIb, alboaggregin A binds to platelet GPVI, a major receptor involved in platelet activation by this snake CLPs (Do¨rmann *et al*., 2001). Alboaggregin C, a 121 kDa protein, is still poorly defined. Recently, alboluxin, a potent platelet activator with a mass of 120 kDa, contains, after reduction, subunits of 17 and 24 kDa. Alboluxin is a hexamer $(\alpha\beta)_3$, which activates platelets via both GPIb and GPVI (Du *et al*., 2002).

Although partial protein sequence of alboaggregin A and B from *C. albolabris* have been published, molecular cloning of cDNA will give us more complete and accurate protein sequences for the studies of structure-function relationship and for future expression of the recombinant proteins. Due to the availability of 3D structures and sequence of C-type lectin-like protein, structure and function analysis is possible for our cloned genes by comparison among homologous viper venom CLPs. This will give us insights in molecular mechanisms of platelet activation and also introductory data for future recombinant protein expression by designing C-type lectin-like with desirable functions for research uses and platelet function tests in patients with bleeding of thrombotic disorders.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Obtaining Full Length cDNA

3.1.1.1 Rapid Amplification of cDNA Ends (RACE)

We used SMART™ RACE cDNA Amplification Kit purchased from CLONTECH Laboratories Inc, CA, USA.

3.1.1.2 Gene Specific Primers (GSP)

Synthetic oligonucleotides were purchased from BGM, Bangkok, Thailand.

3.1.1.3 DNA Extraction and Purification from gel slice

QIAquick® Gel Extraction Kit was purchased from QIAGEN Inc., Valencia, U.S.A.

Wizard® SV Gel and PCR Clean-Up System were purchased from Promega, WI, U.S.A.

3.1.1.4 Cloning of RACE Products

pGEM®-T Easy Vector System II was purchased from Promega, WI, U.S.A. It contains *Eschericia coli*, JM 109 strain, pGEM®-T Easy Vector, T4 DNA Ligase and 2x Rapid Ligation Buffer.

Isopropyl-β-D-Thiogalactopyranoside (IPTG), Dioxane-Free, Formula weight 238.3, was purchased from Promega, WI, U.S.A.

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 100 mg was purchased from Promega, WI, U.S.A.

3.1.1.5 Enzymes

Tag DNA polymerase (Invitrogen™ life technologies, CA, USA)

T4 DNA Ligase (Promega, WI, USA)

*Eco*R I (Sigma, MO, USA)

3.1.1.6 DNA Sequencing

We use ABI PRISM® BigDye® Terminator V.3.1 Cycle Sequencing Kit purchased from AB Applied Biosystems, CA, USA

3.1.2 Proteins Detection

3.1.2.1 Sodium Dodesyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE.)

Mini-Protein 3 Electrophoresis apparatus was purchased from Bio-Rad Laboratories, Ltd., CA, U.S.A.

Protein marker See Blue Plus 2 was purchased from Invitrogen™ life technologies, CA, U.S.A.

Coomassie Brilliant Blue R-250 was purchased from USB, Ohio, U.S.A.

3.1.2.2 Western Blotting Hybridization

Trans-Blot® SD semi-dry electrophoretic transfer cell was purchased from Bio-Rad Laboratories,Ltd.

Immun-Blot™ PVDF membrane 0.2 μl was purchased from Bio-Rad Laboratories,Ltd.

Monoclonal antibody to phosphotyrosine clone BDI820 from Biodesign international, A Division of meridian life science, Inc, U.S.A.

Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP was purchased from Dako Cytomation, Denmark.

3.1.3 Protein Purification

Protein purification using BIORAD Biological LP system, gel filtration sephacryl S200 and RESOURSE Q ion-exchange chromatography were purchased from Amesharm Biosciences, Uppsala, Sweden.

3.1.4 Concentration of Protein

Vivasspin ultra filtration was purchased from Vivascience Sartorius Group., Göttingen, Germany.

3.1.5 Protein Quantitative Assay

Micro BCA^{TM} Protein Assay Reagent Kit was purchased from PIERCE Biotechnology., IL, U.S.A.

3.1.6 Activity Assay

Monoclonal antibody to phosphotyrosine clone BDI820 from Biodesign international, A Division of meridian life science, Inc, U.S.A.

Collagen reagent (calf skin) was purchased from AMAX trinity Biotech plc, Bray, Co. Wicklow, Ireland.

Monoclonal antibody CD42b (GPIbα) clone SZ2 from Immunotech, a Beckman Coulter company, France.

Rabbit polyclonal to glycoprotein VI was purchased from abcam plc, UK.

3.2 Methods

3.2.1 Obtaining full length C-type lectin subunit cDNA

Green pit viper venom gland library has been previously prepared. Two partial C-type lectin cDNA have cloned from green pit viper, called clone 041 and 051 Lectin.

3.2.1.1 5'and 3' RACE

5'-RACE or anchored PCR is the technique that facilitates the isolation, characterization of 5' ends from low-copy messages for generating full length cDNA. The templates of 5'-RACE-PCR were prepared by reverse transcriptase polymerase chain reaction (RT-PCR) using the joint action of the SMART IIA™ oligonucleotide and PowerScript™, a variant of MMLV reverse transcriptase, reverse transcription. For preparation of 5'-RACE-Ready cDNA, we synthesized the first strand cDNA using poly A^+ RNA in the reaction as followed. Firstly, 500 ng of poly A^+ RNA from venom gland of *Cryptelytrops albolabris*, 1 μl of 5'-CDS primer, 1 μl of SMART IIA oligonucleotide and sterile H_2O are combined to a final volume of 5 μl. It was, then, incubated at 70°C for 2 minutes. Subsequently, the reaction tube was kept on ice for 2 minutes. After that, the following reagents were added to the reaction; 2 μl of 5X first-strand buffer (250 mM Tris-HCl pH 8.3,375 mM KCl,30 mM $MgCl_2$), 1 μl of DTT (20 mM), 1 μl of dNTP Mix (10 mM each), and 1 μl of PowerScript reverse transcriptase. The tube was then incubated at 42°C for 1.5 hours. Finally, the first-strand reaction solution was diluted with Tris-EDTA buffer (10 mM Tris-KOH pH 8.5, 1 mM EDTA) and heated at 72°C for 7 minutes.

At this point, we have 5'-RACE-Ready cDNA templates with the SMART IIA™ oligonucleotide incorporated in its 5'end. 5'-RACE was carried out using the SMART RACE cDNA amplification Kit with gene specific primers based on nucleotide sequences derived from the primary library. The calculate Tm should be between 60 – 70°C. Firstly, PCR Master mix was prepared by combining 27 μl of PCR-Grade Water, 5 μl of 10X Advantage 2 PCR Buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 1 μl of dNTP Mix (10 mM each), and 1 μl of 50X Advantage 2 Polymerase. Secondly, 10 μl of 5′-RACE-Ready cDNA, 5 μl of 10X Universal Primer A Mix (UPM) that was complementary to the SMART IIA oligonucleotide, and 10 μl of 10 pM Gene specific primer for clone 041 or 051 were then added to 34 μl of PCR Master mix as described. We used PE GeneAmp Systems 2400 thermal cycle for amplifying 5'-RACE fragments using 40 cycles with following the temperature cycling parameters: 94°C for 5 seconds of denaturation, 68°C for 10 seconds of annealing and 72°C for 3 minutes of extension.

3.2.1.2 DNA Extraction and Purification from Gel Slice

After amplification of 5'-RACE, the RACE products was electrophoresed on 1.2 % agarose gel. A band of DNA was excised from an agarose gel using a sterile blade. The RACE products were purified by the NucleoTrap™ Gel Extraction Kit. Two volumes of NT 1 Buffer to one volume of gel are added and the tube was placed in 50°C water bath. After agarose gel was completely dissolved, the tube was centrifuged at 20,000x g for 30 seconds and supernatant was discarded. Subsequently, 500 µl of the NT 2 Buffer was added, mixed, and centrifuged at 20,000 x g for 30 seconds. The supernatant was discarded. This step was repeated once. The pellet was air-dried. Finally, EB buffer was added to elute DNA before centrifugation at 20,000 x g for 10 minutes. After that, DNA was precipitated using 0.3 M sodium acetate in 100% ethanol before centrifugation at 20,000 x g for 10 minutes. The supernatant was discarded. The pellet was washed with 1 ml of 70 % ethanol, and centrifuged at 20,000 x g for 10 minutes. The supernatant was discarded. The dry pellet was dissolved in TE buffer pH 8.0 (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0) or distilled water and stored at –20°C until use.

3.2.1.3 Cloning of RACE Products

3.2.1.3.1 Ligation of RACE Products into pGEM®-T easy Vector.

After purified by the NucleoTrap gel extraction kit, the 5'-RACE products were cloned into pGEM®-T easy vector. The ligation procedure was carried out in a 10 µl reaction containing 5 µl of 2X Rapid Ligation Buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% PEG), 50 ng of pGEM®-T easy vector, 3 Weiss units of T4 DNA Ligase and an appropriate amount of A-tailing PCR products that optimized from the insert: vector ratio of 3:1 using the following equation.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \frac{3}{1} = \text{ng of insert}$$

Subsequently, deionized water was added to a final volume of 10 µl. Finally, the ligation reaction was mixed by pipetting and incubated at 4°C for 16 – 18 hours.

3.2.1.3.2 Transformation to E. Coli, JM 109

The 10 μl of ligation reaction was added to a sterile falcon tube Cat. no. 2059 on ice. JM 109 competent cells that were placed on ice until just thawed were then mixed with DNA by gently flicking. Subsequently, 50 μl of competent cells were carefully transferred into falcon tube and gently mixed and placed on ice for 20 minutes. The reaction tube was then subjected to heat-shock for 40 – 50 seconds in a water bath at exactly 42°C and immediately returned to ice for 2 minutes. The transformed cells were mixed with 450 μl of SOC medium and incubated at 37°C for 1.5 hours with shaking at 150 rpm. Finally, 500 μl of the transformed cells were plated on LB agar plate with 100 μg/ml amplicillin supplemented with 100 mM IPTG and 50 μg/ml of X-gal for blue/white screening. The plate was incubated at 37°C for 16 – 24 hours.

3.2.1.3.3 Preparation of plasmid DNA by Alkaline Lysis Minipreparation

Each colony of transformed bacteria was inoculated in 3 ml of LB broth containing 100 μg/ml of ampicillin. The culture was incubated overnight at 37 °C with shaking at 250 rpm. The culture cells were poured into 1.5 ml microcentrifuge tube and centrifuged at 20,000 x g for 10 minutes. An aliquot of the original culture was stored at –70 °C in 50 % glycerol. After centrifugation, supernatant was removed by aspiration and the pellet was resuspended in 500 μl of cold STE buffer (10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1 mM EDTA pH 8.0) and repeat centrifugation. The bacterial pellet was lysed in 100 μl of ice-cold Alkaline lysis Solution I (50 mM Glucose, 25mM Tris-HCL pH 8.0, and 10 mM EDTA pH 8.0) and vigorously vortexed. Subsequently, 200 μl of freshly prepared Alkaline lysis Solution II (0.2 N NaOH, 1% w/v SDS) was added to bacterial suspension. The tube was closed and mixed by gently inversion five times. The tube was stored on ice for 10 – 30 minutes. After that, 150 μl of ice-cold Alkaline lysis Solution III (5 M Potassium acetate, glacial acetic acid, and H_2O) was added and mixed. The tube was stored on ice for 3 – 5 minutes. The bacterial lysate tube was centrifuged at 20,000 x g for 10 minutes. The supernatant was transferred to a fresh tube. Then, an equal volume of phenol: chloroform was added. The tube was mixed by vortexing and then centrifuged at 20,000 x g for 10 minutes. The aqueous upper layer was transferred to a fresh tube. Finally, plasmid DNA was recovered by precipitation from the

supernatant by adding 2 volumes of 100 % ethanol. The solution was mixed by vortexing and centrifuged at 20,000 x g for 10 minutes. The supernatant was removed by gentle aspiration. The tube was stood in an inverted position on a paper to allow all of the fluid to drain away. Then, the pellet was washed with 70 % ethanol and the tube was inverted several times. The tube was centrifuged at 20,000 x g for 10 minutes to recover the DNA. The supernatant was removed from the tube and open the tube at room temperature to allow ethanol evaporation. Finally, the pellet was dissolved with 50 μl of TE buffer pH 8.0. The DNA solution was mixed and stored at –20°C.

3.2.1.3.4 Restriction Endonuclease and Electrophoresis

Approximately 500 ng of plasmid DNA was digested with 5 units of *Eco*R I according to manufacturer's protocol (Sigma) using 1 μl of 10X Buffer (300 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP), and 0.1 mg/ml bovine serum albumin. The digestion reaction was incubated overnight at 37°C. After digestion, the reaction was electrophoresed on 1.5 % gel. Clones containing the insert of interest were selected for sequencing.

3.2.1.3.5 DNA purification

Plasmid DNA from Alkaline Lysis Minipreparation method was cleaned up using QIAquick PCR purification kit before sequencing.

3.2.1.3.6 DNA Sequencing

The sequencing was performed using BigDye™ Terminator reaction Sequencing Ready Reaction Kit. The primer extension reaction was carried out in a 10 μl containing 4 μl of terminator ready reaction mix (AmpliTag DNA polymerase and FS with thermostable pyrophosphatase), 1 pM sequencing primer (T7 or SP6) and 1 μg DNA template. After incubation at 95 °C for 30 seconds, amplification was carried out for 25 cycles of the following thermal cycling parameters: 95°C for 10 seconds of denaturation, 50°C for 5 seconds of annealing, and 60°C for 4 minutes of extension. The DNA was then precipitated by 95 % ethanol and 0.08 M sodium acetate pH 8.0. The solution was centrifuge at 25,000 x g for 20 minutes and the supernatant was removed

by pipetting. The pellet was then washed with 1 ml of 70 % ethanol, and tube centrifuged at 25,000 x g for 8 minutes. Subsequently, the supernatant was removed. The pellet was dried in heated incubator at 95°C for 2 minutes. Finally, the DNA pellet was resuspended in 10 μl Template Suppression Reagent (Perkin-Elmer) and loaded to the ABI PRISM sequencer.

3.2.1.3.7 Alignment and Computational Searching Sequences Analysis

The nucleotide sequences and their conceptual translation obtained from the clones of interest were compared against nucleotide or protein sequences in online databases using BLAST N (Basic Local Alignment Search Tool) program via the World Wide Web. Alignments of sequence are made using CLUSTALW multiple sequence alignment program.

3.2.2 Purification of Proteins

3.2.2.1 Gel filtration Chromatography

Five hundred mg of lyophilized *C.albolabris* venom from Queen Savabha institute was dissolved in 0.05M Tris buffer, pH 8.0 and precipitate was removed by filtration through a 0.22μm filter. The sample was clear after filtration and free form visible lipid contamination. The venom was first purified using gel filtration chromatography from Automate Biorad Biologic LP system with LP Data view software. Sephacryl S-200(16/60) was equilibrated with one-half column volume of distilled water at flow rate of 0.5ml/min and two column volume of 0.05M Tris-HCl, 0.15M NaCl, PH 7.2 (start buffer) at 1ml/min. Subsequently, samples were loaded on a sephacryl column and eluted using 150 ml of start buffer. Protein concentration of each 1 ml per collecting fraction was detected by LP Data view software using the absorbance at 280 nm. Each peak of proteins that can aggregate wash or formalin-fixed platelets was collected for further purification.

3.2.2.2 Ion-Exchange Chromatography

Proteins fraction 37-40 that showed strong platelet aggregation were pooled and concentrated by ultrafiltration using Vivaspin concentrator, which contained MWCO of 5,000 Da, to the final volume of 1 ml. Samples were loaded on a RESOURCE Q column, pre-equilibrate with two column volumes of 20mM Tris-HCl, pH8.0 (start buffer), two column volumes of 20mM Tris-HCl, PH 8.0 with 0.5M NaCl and five column volumes of start buffer. Elution was performed with a linear 0-0.5M NaCl gradient. Platelet aggregation inducing activity was concentrated in the fraction 34-36. Purified proteins was analyzed by Coomassie-stained SDS-PAGE

3.2.2.3 Concentration of Proteins

The peak of proteins, which aggregated human washed platelets, was concentrated using Vivaspin concentrator ultracentrifugation, which contains MWCO of 5,000 Da. The supernatant was poured into the concentrator at the maximum volume, and then the concentrator was placed in a 50 ml centrifuge tube. Subsequently, the assembled concentrator was centrifuged at 25,000 x g for 40 minutes. The remaining samples from the bottom of the concentrated pocket were recovered using a pipette.

3.2.3 Protein Detection

3.2.3.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue Staining

12 % of resolving gel and 5 % of stacking acrylamide gel containing 10% SDS were freshly prepared. After gel setting, the recombinant protein was mixed with ¼ volume of 2X sample buffer (100 mM Tris-HCl pH 6.8, 4% w/v SDS, 0.2% w/v bromophenol blue, 20% v/v glycerol, 200 mM with or without β-mercaptoethanol) and then denatured at 95°C for 10 minutes and loaded into gel slots. Electrophoresis was performed at 125 volts for 90 minutes in 1X running buffer, pH 8.3 (0.25 M Tris-HCl, 1.92 M glycine, 1 % w/v SDS). After electrophoresis, the gel was soaked in Coomassie Brilliant Blue Solution for 30 minutes 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 to 4 times during incubation

3.2.3.2 Quantitative Assay for Purified Proteins

Protein concentration was determined using Micro BCA™ Protein assay reagent kit (Pierce). The method utilizes bicinchoninic acid (BCA) as the detection reagent for Cu^{+} that is formed when Cu^{2+} is reduced by protein in an alkaline environment. The bovine serum albumin standards (BSA) were diluted into 6 dilutions (0.025 – 0.1 mg/ml). Then fresh working reagent was prepared by mixing 25 parts of Micro BCA™ Reagent MA containing sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH and 24 parts Reagent MB containing 4% bicinchoninic acid in water with 1 part of Reagent MC containing 4% cupric sulfate, pentahydrate in water. 150 μl of each standard or the sample solution replicates were pipetted into microplate wells and 150 μl of the working reagent was added to each well and mixed. The plate was covered and incubated at 37°C for 2 hours. The reaction was then measured the absorbance at 570 nm on an ELISA plate reader.

3.2.4 Protein Identification Methods

3.2.4.1 LC/MS/MS

The tryptic peptides were resuspensed with 0.1% formic acid and then analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Peptides were elute for each analysis during a gradient from7% to 65% buffer B in 65 min (buffer A: 0.1 % formic acid /water, buffer B: 0.1% formic/ Acetonitrite, flow rate: 100 μl /min). Peptide ions were detected in a survey scan from 400 to 1600 atom mass unit (amu) followed by one data-dependent MS/MS scan (isolation width 3 amu, 25% collision-induced dissociation energy (CID), dynamic exclusion for 180 seconds).

3.2.4.2 Protein idenitfication

All MS/MS spectra were searched using Biowork™ 3.3 software (Sequest algorithm) to comparing between experiment product ion spectra and theory product

ion spectra in the nr database. All MS/MS spectra were searched against the database using the following criteria: enzyme trypsin, static modification of cysteine (+57.05130 Da), differential modification of methionine (+15.99940). The result of searching were filters by Xcorr versus charge state (+1≥1.5, +2≥2.0, +3≥2.5) and protein probability (minimum 1.00E-3).

3.2.4.3 Activity Assay

3.2.4.3.1 Platelet Aggregation Assay

Platelet rich plasma and the platelet pellet were isolated by successive centrifugation steps. Platelets were resuspended in 113mM NaCl, 4.3 mM K_2HPO_4, 24.4 mM NaH_2PO_4, 5.5 mM glucose, pH6.5 (buffer B) and centrifuged at 180 x g for 10 min. The platelet-rich supernatant was centrifuged at 1942xg for 10 min, and the platelets were washed once more with buffer B. Washed platelets were resuspended in 20 mM Hepes, 140 mM NaCl, 4 mM KCl, 5.5 mM glucose, pH 7.4 (buffer C), and the platelet count was adjusted to 350 x 10^9 platelet/ml by dilution with buffer C. The platelets were incubated at 37°C for 2 min before analysis. Different amount of purified C-type lectin-like proteins was added to washed platelets and incubated with continuous magnetic stirring at 37°C for 10 minutes. Platelet aggregation was measured by determining the change in light transmission with platelet aggregation chromogenic kinetic system (PACKS-4, Helena Laboratories, USA).

3.2.4.3.2 Preparation of washed platelets.

Platelet rich plasma and the platelet pellet were isolated by successive centrifugation steps. Platelets were resuspended in 113mM NaCl, 4.3 mM K_2HPO_4, 24.4 mM NaH_2PO_4, 5.5 mM glucose, pH6.5 (buffer B) and centrifuged at 180 x g for 10 min. The platelet-rich supernatant was centrifuged at 1942 x g for 10 min, and the platelets were washed once more with buffer B. Washed platelets were resuspended in 20 mM Hepes, 140 mM NaCl, 4 mM KCl, 5.5 mM glucose, pH 7.4 (buffer C), and the platelet count was adjusted to 3 x 10^8 platelet/ml by dilution with buffer C. The platelets were incubated at 37°C for 2 min before analysis.

3.2.4.3.3 Platelet Activation Assay

Fifty microliters of Purified C-type lectin-like proteins were added to 450 μl of washed normal platelets (350,000 platelets/μl). Aliquots were taken at fixed times (5 minutes) and the platelet suspension was lysed by adding the aliquots to 5 μl of HEPES containing 10% SDS, 10 mM NEM, 20 mM Na_3VO_4 and 20 mM EDTA. After centrifugation, the supernatant were electrophoresed on an 8 %SDS-polyacrylamide gel and then electro blotted onto PVDF membrane for 1 hour. After that, blotted-PVDF membrane was washed with distilled water 3 times for 5 minutes each. Subsequently, the blotted-PVDF membrane was blocked by 3% non-fat dry milk in TBS for approximately an hour. The monocloclonal antibody to phosphotyrosine, 4G10, was applied to detect tyrosine phosphorylated protein as primary antibody. After an overnight incubation at 4°C, the membrane was washed with TBS-tween solution 5 times for 5 minutes each, followed by water washing 2 times for 5 minutes each, in order to remove non-specific binding. Blotted-PVDF membrane was, then, incubated with peroxides-coupled rabbit-anti-mouse secondary antibody for 1 hour at room temperature, washed again by TBS-tween solution and followed by water as stated above. Bound antibody was detected using ECL-chemiluminescence reaction and exposure to hypersensitivity film about 15 minutes.

CHAPTER IV

RESULTS

4.1 Molecular cloning of Full Length C-Type Lectin-Like proteins from Green Pit Viper

Green pit viper (*C. albolabris*) venom gland library has been previously prepared. Two partial clones of C-type lectin cDNA have been discovered, called clone 041 and clone 051.

4.1.1 5'-RACE

From primary cDNA library of *Cryptelytrops albolabris* venom gland (Rojnuckarin et al, 2006), we derived two partial cDNA sequences of C-type lectin like protein (CLPs) clones (Figure 3) which were used to design the CLP-specific primers (041 and 051) in order to obtain the complete cDNAs encoding CLPs by using 5'-RACE technique (Figure 4). The sequences primers of clone 041 and 051 are shown in Table 2. The products showed smear patterns on gels indicated that they were consisted of several different genes. The 5'-RACE products, which were approximately 600-700 base pairs in sizes, were eluted and sub-cloned into pGEM®-T easy vector and transformed in to *Escherichia coli*, JM109. The positive clones were identified by using blue-white color selection system (Figure 5). More than 100 positive clones were identified and isolated by alkaline lysis minipreparation method and digested with *Eco*R I to screen for clones that contained correct-sized inserts (Figure 6). The inserts were varying in sizes indicating that there were different products. Next, the inserted plasmids were sequenced using ABI PRISM (Perkin-Elmer) system. The sequencing was performed in both orientations using NUP and SP6 sequencing primers. The sequences were analyzed in comparison with GENBANK database using the BLAST N and CLUSTAL W program. The first 5'-RACE results indicated that all these clones contained full-length cDNA inserts encoding C-type lectins that can be divided in to 2 major groups. Clone 041 derivatives were homologous to factor IX binding proteins and those of clone 051 were similar to platelet-binding proteins, alboaggregins. Full-length cDNA of the β

subunits of alboaggregin B (AL-B) was obtained from 5'RACE using the 051 lectin primer (Table2).

4.1.2 3'-RACE

The cDNA sequences around the ATG start site and 5'UTR sequences of various lectin proteins in the same species are relatively conserved (personal observation). The 5'UTR sequences of clone051 were analyzed and used for designing a primer (common lectin primer; Table 2) to obtain *C. albolabris* platelet-active CLPs. Subsequently 3'-RACE technique was used in order to obtain other CLP subunits. More than forty positive clones were identified and isolated. In the 3'-RACE results, we found 3 additional major groups apart from 5'-RACE. Three clones were homologous to α subunit of alboaggregin B (AL-B). The sequences of α and β subunits AL-B were previously reported by Arpijuntarangkoon et al, 2007. Furthermore, 8 clones were related to α subunit and 8 clones were homologous to β subunit of the other platelet-binding CLP, which will be focused in this study.

Table 2: Oligonucleotides primers and their descriptions.

Name	Sequence	Description
T7	5'- GTAATACGACTCACTATAGGGC -3'	Sequencing primer
SP6	5'-ACTCAAGCTATGCATCCAAC –3'	Sequencing primer
Lectin41	5'-CTCCAGACTTCACTCAGCTGGACCTTC-3'	5'-RACE PCR for clone 041
Lectin51	5'-CCAGACTTCAGACAGCTGGATCTT-3'	5'-RACE PCR for clone 051
Common lectin	5'-CCAGACTTCAGACAGCTGGATCTT-3'	3'-RACE PCR

Figure 3: Partial cDNA sequences of 041 and 051 CLP clones from the primary library of *C. albolabris* venom gland

Partial cDNA of 041

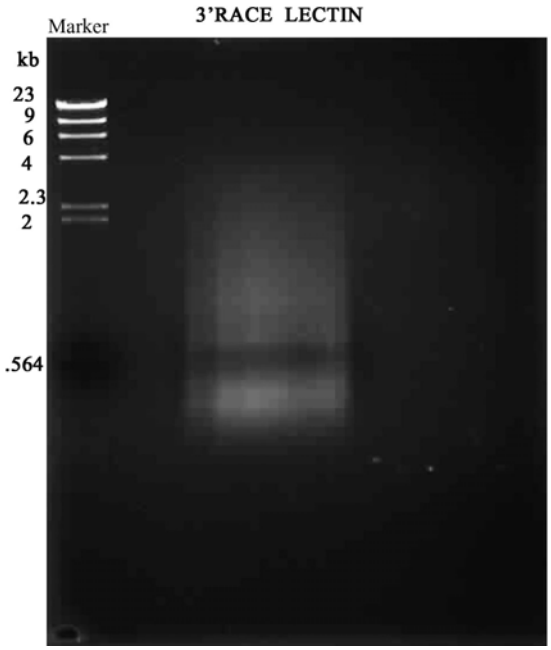
CATGAGCAGCGAATAGCGGCATCTGTCTTATCGAAGCGCCGAGAAGCAGCTTCGG
GCCCAGTGTTTCTGAGACATACAGAACACCAAAGCTATGTTGATCGGCTGAGGTT
CAAGGCAAGAAACAATGCACCTCGGAGTGGAGCGATGGCTCCAGCGCAGTTATGA
GACCTGATTGATGCAGAATCCAAAACGTGTCTTGGCTGGACCAAGAGACAGAGTT
TCGTAAGTGGGTCAATATTTACTGGGACTACAAAATCCATTCGTTTGCGAGGCAT
AGTCCCGAAGGTCCAGCTGAGTGAAGTCTGGAGAAGCAAGGAAGACCCCCACCCA
CCCCCACCCCCCACCTGCCGCAATCTCTGCTCTGCCCCCTTCGCTCAAGGGATGC
TCTCTGTAGCTGGATCTGGTTTTGCTGCTCCTGATGGGCCAGAAGGTCCAATAAA
TTCTGCCTAGCAAAAAAAAAAAAAAAAA

Partial cDNA of 051

GAATTCGGCACGAGGCCTCGTGCCGAATTCGGCACGAGGCAGCCACCTGCTCTCC
TTCCACAGCAGTGAAGAAGTAGATTTTGTGGTCTCCAAGACCCTCCCAATTCTGA
AAGCGGATTTTGTCTGGATCGGACTCACCGATGTCTGGAGTGCATGCAGGTTGCA
GTGGAGCGATGGCACCGAGCTCAAGTACAACGCCTGGACTGCAGAATCGGAGTGC
ATCGCATCCAAGACAACTGATAACCAATGGTGGACTAGATCCTGCAGCAGGACTT
ACCCTTTCGTCTGCAAGTTGGAGGTATAGTCTGAAGATCCAGCTGTCTGAAGTCT
GGAGAACAAAGAAGACCCCAACTTTACACTCTCCATCCCACACCCCACCCCGCCT
GGCCCAATTTCTGCCCCCTTCGCTCACGGATGCTCTCTGGAACTGG

Figure 4: 3'-RACE and 5'-RACE products of CLPs electrophoresed on 1.2% agarose gel. Lane 1: molecular weight marker (M) λ *Hin*d III; Lane 2:3'-RACE (A) and 5'-RACE (B) cDNA product

A



B

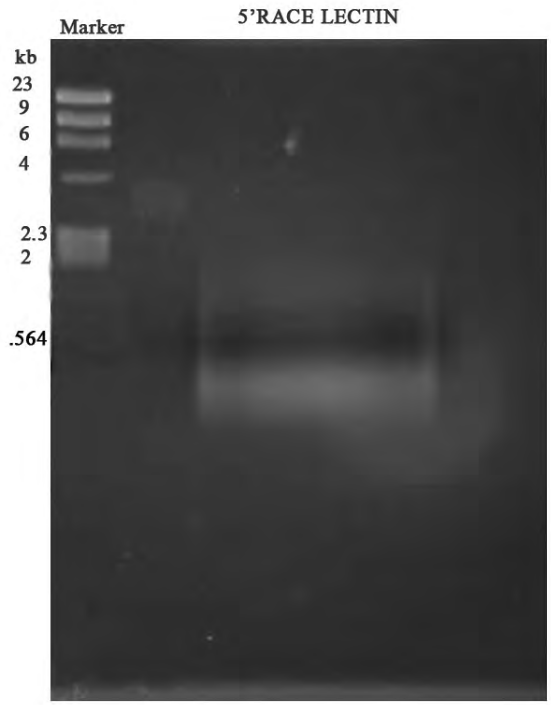


Figure 5: *E. coli*, JM 109, transformed with C-type lectin-like protein cDNA. The positive transformants were white colonies as a blue-white selection system was used.

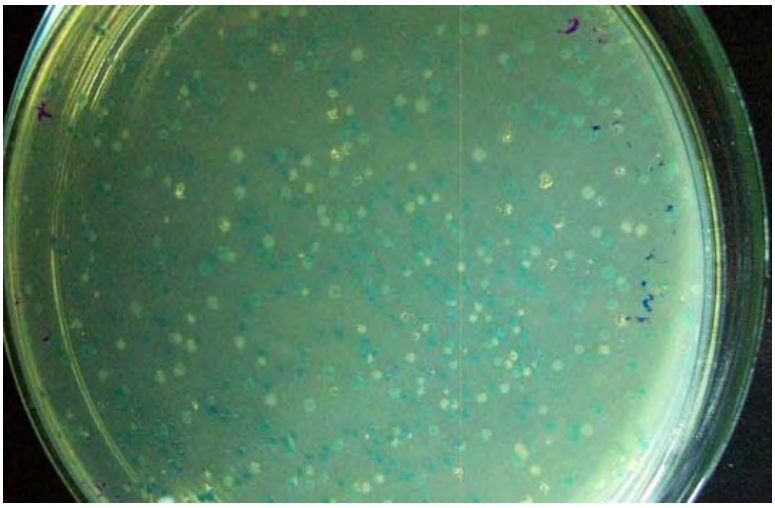
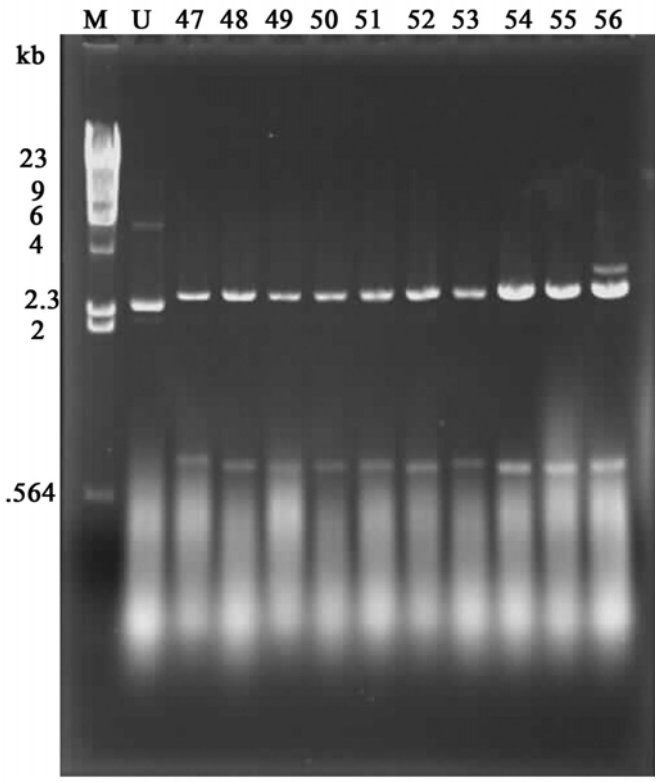


Figure 6: EcoRI digestion of recombinant plasmids clones 47-56 of CLP cDNA from 3'RACE. The inserts were varying in sizes. Clone 47- 56 showed the presence of inserts. U = recombinant plasmid that did not contain insert. M = molecular weight marker λ *Hin*d III.



4.1.3 Sequence Alignment and Computational Searching Analysis

The insert-positive clones from 3'-RACE were sequenced and compared with the GENBANK database. The sequences of α and β subunits of alboaggregin B were previously published by our group (Arpijuntarangkoon et al, 2007). This study focused on the other novel alboaggregin.

Eight cDNA clones (6-Lec, 21-Lec, 22-Lec, 23-Lec, 36-Lec, 41-Lec, 71-Lec and 81-Lec) encoded for α subunits and eight cDNA clones (68-Lec, 75-Lec, 79-Lec, 81-Lec, 87-Lec, 96-Lec, 99-Lec and 103-Lec) encoded for β subunits. The full length sequence α subunit CLP was 477 bp conceptually translated into 23 amino acid residue signal peptide and a 135 amino acid residue mature protein (Figure 7). On the other hand, the β subunit contained 447 bp translating into 23 amino acid residue signal peptide and a 125 amino acid residue mature protein (Figure 8). The identity of the deduced mature amino acid sequences of CLP α and β was 41.77% (Figure 9).

Using BLASTN, the nucleotide sequence of α subunit showed the highest homology to *Protobothrops mucrosquamatus* mucrocetin alpha chain mRNA (Figure 10) and β subunits showed the highest identity to *Crotalus durissus* convulxin beta mRNA (Figure 11). An alignment between translated sequences of CLP-α and *Protobothrops mucrosquamatus* alpha chain protein showed 86.7% amino acid sequences identity (figure 12), while translated sequences of CLP-β and *Crotalus durissus* convulxin beta showed 77.02% amino acid sequences identity (Figure 13).

Subsequently, the amino acid sequence of CLP α and β were compared with other snake venom CPLs using Clustal W multiple sequence alignment. The results revealed that the identity scores of CLP α with other known platelet collagen receptor-binding CLPs snake venom were as follows: Stejnulxin 66.45%; Convulxin 58.86%; EMS16 56.32% and Aggretin 48.10% (Figure 14). The identity scores of CLP β subunit with other platelet collagen receptor-binding CLPs snake venom β subunits were as follows Stejnulxin 71.60%; Convulxin 77.70%; EMS16 54.72% and Aggretin 60.14% (Figure 15).

Figure 7: The cDNA and the deduced amino acid sequence of CLP α subunit from *C. albolabris.* The translation stop codon is indicated by the asterisk. The poly adenylation signal (aataa) is underlined.

```
 1   ttctctctgcattaaggaaggaagaccATGGGGCGATTCATCTTCGGGAGCTTCGGCTTG  60
                               M  G  R  F  I  F  G  S  F  G  L
 61  CTGGTGGTGTTCCTCTCCTTAAGTGGTACTGGAGCTGATTTTGATTGTCCCCCTGGTTGG 120
      L  V  V  F  L  S  L  S  G  T  G  A  D  F  D  C  P  P  G  W
121  TCTGCCTATGATCGGTATTGCTACCAGGCCTTCAGTGAACCGAAAACCTGGGAAGATGCA 180
      S  A  Y  D  R  Y  C  Y  Q  A  F  S  E  P  K  T  W  E  D  A
181  GAGAGTTTCTGCATGGAGGGGGTGAAGGACTCGCATCTGGTCTCTATCGAAAGCTCCGGA 240
      E  S  F  C  M  E  G  V  K  D  S  H  L  V  S  I  E  S  S  G
241  GAAGCCGACTTCGTGGCCCAGCTGATCAACGAGAAAATAAAGACATCCTTTCGCTATGTC 300
      E  A  D  F  V  A  Q  L  I  N  E  K  I  K  T  S  F  R  Y  V
301  TGGATTGGACTGAGGATTCAAAACAAAGAACAGCAATGCAGGTCGGAGTGGAGCGATGCC 360
      W  I  G  L  R  I  Q  N  K  E  Q  Q  C  R  S  E  W  S  D  A
361  TCCAGTGTCAGTTATGAGAACTTGATTAAAAAAGTTTCAAAAAAATGTTATGGGCTGAAA 420
      S  S  V  S  Y  E  N  L  I  K  K  V  S  K  K  C  Y  G  L  K
421  AAAGGGACAGAGCTTCGCACGTGGTTCAATGTTTACTGTGCAGAACTAAATCCTTTCATC 480
      K  G  T  E  L  R  T  W  F  N  V  Y  C  A  E  L  N  P  F  I
481  TGCAAGTTCCCGCCAGAGGTGTAAgatccagctgagtgaagtctggagaagcaaggaaga 540
      C  K  F  P  P  E  C  *
541  cccccacccacccccaacccttcgctcaacggatgctctcggtagcgggatctgctttgc 600
601  tgctcctgatgggccagaaggtccaataaa-poly A
```

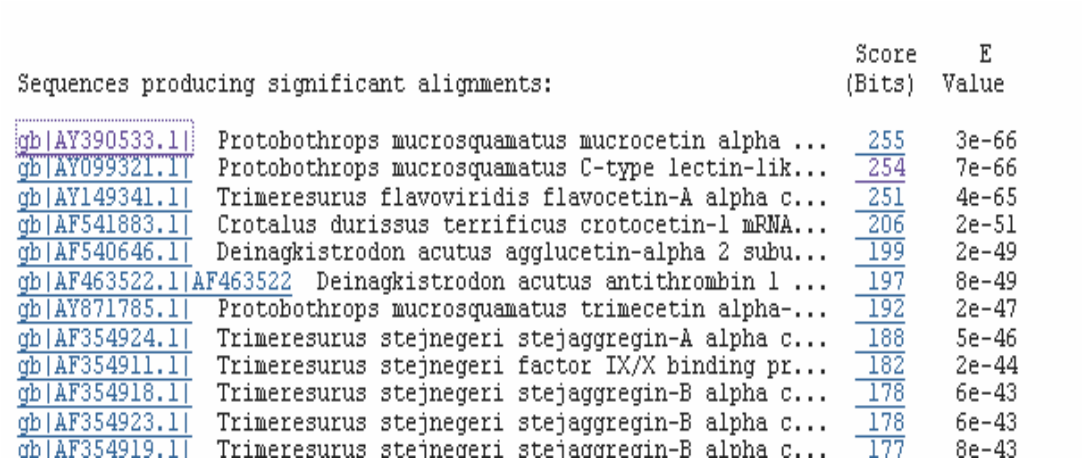
Figure 8: The cDNA and the deduced amino acid sequence of CLP β subunit from *C. albolabris.* The translation stop codon is indicated by the asterisk. The poly adenylation signal (aataa) is underlined.

```
  1  aagaccATGGGGCGATTCATCTCCGTGAGCTTCGGCTTGCTGGTTGTGTTCCTCTCCCTG   60
           M  G  R  F  I  S  V  S  F  G  L  L  V  V  F  L  S  L
 61  AGTGGAGCTGGAGCTGGTTTGTGTTGTCCCTTGGATTGGTCTTCCTATGATCTGTATTGC  120
      S  G  A  G  A  G  L  C  C  P  L  D  W  S  S  Y  D  L  Y  C
121  TACAAGGTCTTCAAACAACAGATGAACTGGACGGATGCAGAGCAATTCTGCACACAACAG  180
      Y  K  V  F  K  Q  Q  M  N  W  T  D  A  E  Q  F  C  T  Q  Q
181  CACACAGGCAGCCACCTGGTCTCCTTTCACAGCACTGAAGAAGTAGATTTTGTGGTCCAG  240
      H  T  G  S  H  L  V  S  F  H  S  T  E  E  V  D  F  V  V  Q
241  ATGAGCTACAAGAGTTTGGACACCACTTTTTTCTGGATCGGAGTAAACAACATCTGGAAT  300
      M  S  Y  K  S  L  D  T  T  F  F  W  I  G  V  N  N  I  W  N
301  GGATGCAACTGGCAGTGGAGCGATGGCACCGGGCTCGACTACAAAGAATGGCGTGAACAA  360
      G  C  N  W  Q  W  S  D  G  T  G  L  D  Y  K  E  W  R  E  Q
361  TTTGAATGTCTCGTAGCCAAGACATTTGATAACCAGTGGTGGAGTATGGACTGCAACAGT  420
      F  E  C  L  V  A  K  T  F  D  N  Q  W  W  S  M  D  C  N  S
421  ACTTACTCTTTCGTCTGCAAGTTCCAGGCATAGtctgaagatccagctgtgtgaagtctg  480
      T  Y  S  F  V  C  K  F  Q  A  *
481  gagaagcaaggaagccccccccccaccccccaccttcgctcagtggatgctctctgtagc  540
541  tggatctggttttgctgctcctgatgggccagaaggtccaataaattctgcctagc-Poly A 600
```

Figure 9: An alignment of the deduced amino acid sequences of *C. albolabris* CLP α and β subunit using CLUSTAL W multiple sequences alignment program showed 41.8% identity. Residues (***** = the residues or nucleotides in that column are identical in all sequences in the alignment, ":" = conserved substitutions are observed and "." = semi-conserved substitutions are observed). Dark highlight represent the signal peptide of α and β subunit.

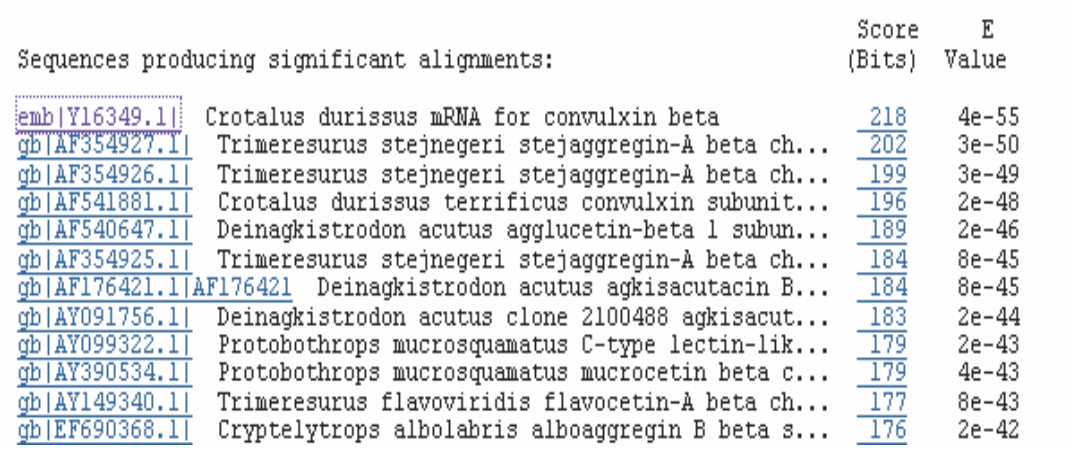
```
CPL alpha      MGRFIFGSFGLLVVFLSLSGTGADFDCPPGWSAYDRYCYQAFSEPKTWEDAESFCMEGVK
CPL beta       MGRFISVSFGLLVVFLSLSGAGAGLCCPLDWSSYDLYCYKVFKQQMNWTDAEQFCTQQHT
               *****  *************:**.: ** .**:** ***:.*.:  .* ***.** :  .

CPL alpha      DSHLVSVESSGEADFVAQLVNENIKTSFRYVWIGLRIQNKEQQCRSEWSDASSVSYENLI
CPL beta       GSHLVSFHSTEEVDFVVQMSYKSLDTTF--FWIG--VNNIWNGCNWQWSDGTGLDYK---
               .*****..*: *.***.*:  :.:.*:*  .***  ::*  : *. :***.:.:.*:

CPL alpha      KKVSKKCYGLKKGTELRTWFNVYCAELNPFICKFPPEC 100%
CPL beta       -EWREQFECLVAKTFDNQWWSMDCNSTYSFVCKFQA--   41.77%
                :  ::   *   *  . *:.: * .  .*:*** .
```

Figure 10: Homology searching of *C. albolabris* CLP α subunit using BLAST N program



Sequences producing significant alignments:		Score (Bits)	E Value
gb|AY390533.1|	Protobothrops mucrosquamatus mucrocetin alpha ...	255	3e-66
gb|AY099321.1|	Protobothrops mucrosquamatus C-type lectin-lik...	254	7e-66
gb|AY149341.1|	Trimeresurus flavoviridis flavocetin-A alpha c...	251	4e-65
gb|AF541883.1|	Crotalus durissus terrificus crotocetin-1 mRNA...	206	2e-51
gb|AF540646.1|	Deinagkistrodon acutus agglucetin-alpha 2 subu...	199	2e-49
gb|AF463522.1|AF463522	Deinagkistrodon acutus antithrombin 1 ...	197	8e-49
gb|AY871785.1|	Protobothrops mucrosquamatus trimecetin alpha-...	192	2e-47
gb|AF354924.1|	Trimeresurus stejnegeri stejaggregin-A alpha c...	188	5e-46
gb|AF354911.1|	Trimeresurus stejnegeri factor IX/X binding pr...	182	2e-44
gb|AF354918.1|	Trimeresurus stejnegeri stejaggregin-B alpha c...	178	6e-43
gb|AF354923.1|	Trimeresurus stejnegeri stejaggregin-B alpha c...	178	6e-43
gb|AF354919.1|	Trimeresurus stejnegeri stejaggregin-B alpha c...	177	8e-43

Figure11: Homology searching of *C. albolabris* CLP β subunit using BLAST N program.



Sequences producing significant alignments:		Score (Bits)	E Value
emb|Y16349.1|	Crotalus durissus mRNA for convulxin beta	218	4e-55
gb|AF354927.1|	Trimeresurus stejnegeri stejaggregin-A beta ch...	202	3e-50
gb|AF354926.1|	Trimeresurus stejnegeri stejaggregin-A beta ch...	199	3e-49
gb|AF541881.1|	Crotalus durissus terrificus convulxin subunit...	196	2e-48
gb|AF540647.1|	Deinagkistrodon acutus agglucetin-beta 1 subun...	189	2e-46
gb|AF354925.1|	Trimeresurus stejnegeri stejaggregin-A beta ch...	184	8e-45
gb|AF176421.1|AF176421	Deinagkistrodon acutus agkisacutacin B...	184	8e-45
gb|AY091756.1|	Deinagkistrodon acutus clone 2100488 agkisacut...	183	2e-44
gb|AY099322.1|	Protobothrops mucrosquamatus C-type lectin-lik...	179	2e-43
gb|AY390534.1|	Protobothrops mucrosquamatus mucrocetin beta c...	179	4e-43
gb|AY149340.1|	Trimeresurus flavoviridis flavocetin-A beta ch...	177	8e-43
gb|EF690368.1|	Cryptelytrops albolabris alboaggregin B beta s...	176	2e-42

Figure 12: An alignment of deduced amino acid sequence of *C. albolabris* CLP α subunit and *Protobothrops mucrosquamatus* mucrocetin alpha chain. An alignment showed 86.7 % amino acid sequence identity. (***** = the residues or nucleotides in that column are identical in all sequences in the alignment, ":" = conserved substitutions are observed and "." = semi-conserved substitutions are observed.)

```
CLP         MGRFIFGSFGLLVVFLSLSGTGADFDCPPGWSAYDRYCYQAFSEPKTWEDAESFCMEGVK 60
Mucrocetin  MGRFIFVSFGLLVVFLSLSGTGADFDCIPGWSAYDRYCYQAFSEPKNWEDAESFCEEGVK 60
            ****** ******************** ******************.******** ****

CLP         DSHLVSVESSGEADFVAQLVNENIKTSFRYVWIGLRIQNKEQQCRSEWSDASSVSYENLI 120
Mucrocetin  TSHLVSIESSGEGDFVAQLVAEKIKTSFQYVWIGLRIQNKEQQCRSEWSDASSVNYENLY 120
             *****:*****.******* *:*****:************************.****

CLP         KKVSKKCYGLKKGTELRTWFNVYCAELNPFICKFPPEC 158
Mucrocetin  KQSSKKCYALKKGTELRTWFNVYCGRENPFVCKYTPEC 158
            *: *****.***************.. ***:**:.***
```

Figure 13: An alignment of deduced amino acid sequence of *C. albolabris* CLP β subunit and *Crotalus durissus* convulxin beta. An alignment showed 77.0 % amino acid sequence identity. (***** = the residues or nucleotides in that column are identical in all sequences in the alignment, ":" = conserved substitutions are observed, "." = semi-conserved substitutions are observed.)

```
CLP         MGRFISVSFGLLVVFLSLSGAGAGLCCPLDWSSYDLYCYKVFKQQMNWTDAEQFCTQQHT 60
convulxin   MGRFIFVSFGLLVVFLSLSGSEAGFCCPSHWSSYDRYCYKVFKQEMTWADAEKFCTQQHT 60
            ***** **************: **:*** .***** ********:*.*:***:*******

CLP         GSHLVSFHSTEEVDFVVQMSYKSLDTTFFWIGVNNIWNGCNWQWSDGTGLDYKEWREQFE 120
convulxin   GSHLVSFHSTEEVDFVVKMTHQSLKSTFFWIGANNIWNKCNWQWSDGTKPEYKEWHEEFE 120
            *****************:*:::**.:******.***** *********  :****:*:**

CLP         CLVAKTFDNQWWSMDCNSTYSFVCKFQA 148
convulxin   CLISRTFDNQWLSAPCSDTYSFVCKFEA 148
            **:::****** *  *..********:*
```

Figure 14: Comparison of the deduced amino acid sequence of *C. albolabris* CLP α subunit with those of other known platelet collagen receptor-binding snake venom CLP α subunits.

```
CLP           MGRFIFGSFGLLVVFLSLSGTGADFDCPPGWSAYDRYCYQAFSEPKTWEDAESFCMEGVK 60
Stejnulxin    MGRFISVSFGLLVVFLSLSGTGADFDCPSGWSAYDWYCYKPFNEPQTWDDAERFCTEQAK 60
convulxin     MGRFIFVSFGLLVLFLSLSGTGAGLHCPSDWYYYDQHCYRIFNEEMNWEDAEWFCTKQAK 60
EMS16         MGRFISVSFGLLVVFLSLSGTGADFDCPSDWTAYDQHCYLAIGEPQNWYEAERFCTEQAK 60
aggretin      ----------------GLE------DCDFGWSPYDQHCYQAFNEQKTWDEAEKFCRAQEN 38
                             .*.      .*  .*  ** :**  :.*  .* :** **    :

CLP           DSHLVSVESSGEADFVAQLVNENIKTS-FRYVWIGLRIQNKEQQCRSEWSDASSVSYENL 119
Stejnulxin    GGHLVSIESSGEADFVGQLVSENIQRP-EIYVWIGLRDRRKEQQCSSEWSDGTSIIYVNW 119
convulxin     GAHLVSIKSAKEADFVAWMVTQNIEES-FSHVSIGLRVQNKEKQCSTKWSDGSSVSYDNL 119
EMS16         DGHLVSIQSREEGNFVAQLVSGFMHRS-EIYVWIGLRDRREEQQCNPEWNDGSKIIYVNW 119
aggretin      GAHLASIESNGEADFVSWLISQKDELADEDYVWIGLRAQNKEQQCSSEWSDGSSVSYENL 98
              ..**.*::*  *.:**. ::.   . .   :* **** :.:*:** .:*.*.:.: * *

CLP           IKKVSKKCYGLKKGTELRTWFNVYCAELNPFICKFPPEC 158       100%
Stejnulxin    NKGESQMCQGLSKWTNFLKWDNTDCQAKNPFVCKFPPQC 158        66.45%
convulxin     LDLYITKCSLLKKETGFRKWFVASCIGKIPFVCKFPPQC 158        58.86%
EMS16         KEGESKMCQGLTKWTNFHDWNNINCEDLYPFVCKFSAV- 157        56.32%
aggretin      IDLHTKKCGALEKLTGFRKWVNYYCEQMHAFVCKLLPY- 136        48.10%
               .     *  * * * :  *    *    .*:**: .
```

Figure 15: Comparison of the deduced amino acid sequence of *C. albolabris* CLP β subunit with those of other known platelet collagen receptor-binding snake venom CLP β subunits.

```
CLP           MGRFISVSFGLLVVFLSLSGAGAGLCCPLDWSSYDLYCYKVFKQQMNWTDAEQFCTQQHT 60
Stejnulxin    MGQFIFVSFGLLVVLLSLSGAGAGFCCPLGWSSYDLYCYKVFKQQMNWTDAEKFCTEQHT 60
convulxin     MGRFIFVSFGLLVVFLSLSGSEAGFCCPSHWSSYDRYCYKVFKQEMTWADAEKFCTQQHT 60
EMS16         MGRLISVRFSLLVVFLSLSGIGAGLCCPLGWSSFDQHCYKVFEPVKNWTEAEEICMQQHK 60
aggretin      MGRFIFVSFGLLVVFLSLSGTGAD--CPSGWSSYEGHCYKPFNEPKNWADAERFCKLQPK 58
              **::* * *.****:*****  *.  **  ***:: :*** *:   .*::**.:*  * .

CLP           GSHLVSFHSTEEVDFVVQMSYKSLDTTFFWIGVNNIWNGCNWQWSDGTGLDYKEWREQFE 120
Stejnulxin    GSHLVSFHSSEEADFVVNMTYPILKLDFVWIGLSNVWNQCNSEWSDGTKLDYKDWSGESE 120
convulxin     GSHLVSFHSTEEVDFVVKMTHQSLKSTFFWIGANNIWNKCNWQWSDGTKPEYKEWHEEFE 120
EMS16         GSRLASIHGSEEEAFVSKLASKALKFTSMWIGLNNPWKDCKWEWSDNARFDYKAWKRRPY 120
aggretin      HSHLVSFQSAEEADFVVKLTRPRLKANLVWMGLSNIWHGCNWQWSDGARLNYKDWQEQSE 118
               *:*.*::.:**  ** :::   *.   .*:* .* *: *: :***.:  :** *  .

CLP           C--LVAKTFDNQWWSMDCNSTYSFVCKFQA---- 148  100%
Stejnulxin    C--IASKTVENQWWTKSCSRTHYVVCKFQA---- 148   71.60%
convulxin     C--LISRTFDNQWLSAPCSDTYSFVCKFEA---- 148   77.70%
EMS16         CTVMVVKPDRIFWFTRGCEKSVSFVCKFLTDPAV 154   54.72%
aggretin      C--LAFRGVHTEWLNMDCSSTCSFVCKFKA---- 146   60.14%
              *  :  :     * .  *. :  .**** :
```

4.2 Proteins purification and SDS-PAGE analysis

Gel filtration chromatography of 0.5 g of crude *C. albolabris* venom was performed on Sephacyl G-200 column resulting in seven protein peaks. Peak 1 and 4 showed strong platelet aggregation-inducing activity. They were separately pooled and concentrated for further purification. The low molecular weighted peak 4 was identified as alboaggregin B and previously reported (Arpijuntarangkoon et al, 2007). The high molecular weight CLP was further investigated in this study. The pooled peak 1 was fractionated on Resourse Q ion-exchange column using a linear gradient of NaCl from 0 to 0.5 M. This CLP was eluted at approximately 0.38 M NaCl as determined by platelet aggregatin activity (Figure 16 and 17).

Purified high molecular weighted *C. albolabris* CLP was analyzed by SDS-PAGE under reducing and non-reducing condition. It had an apparent molecular mass on SDS-PAGE of ~120 kDa under non-reducing condition. Upon reduction, it separated into two bands with apparent molecular weights of 14 kDa and 17 kDa consistent with α and β subunit, respectively. (Figure 18)

Figure 16: Gel filtration chromatogram of *C. albolabris* venom (0.5g) on a Sephacyl G-200 column. Protein concentrations were estimated from the absorbance at 280 nm. Highlights represent the platelet aggregation-induced activity proteins, peak I is the novel *C. albolabris* CLP in this study and peak IV is alboaggregin B.

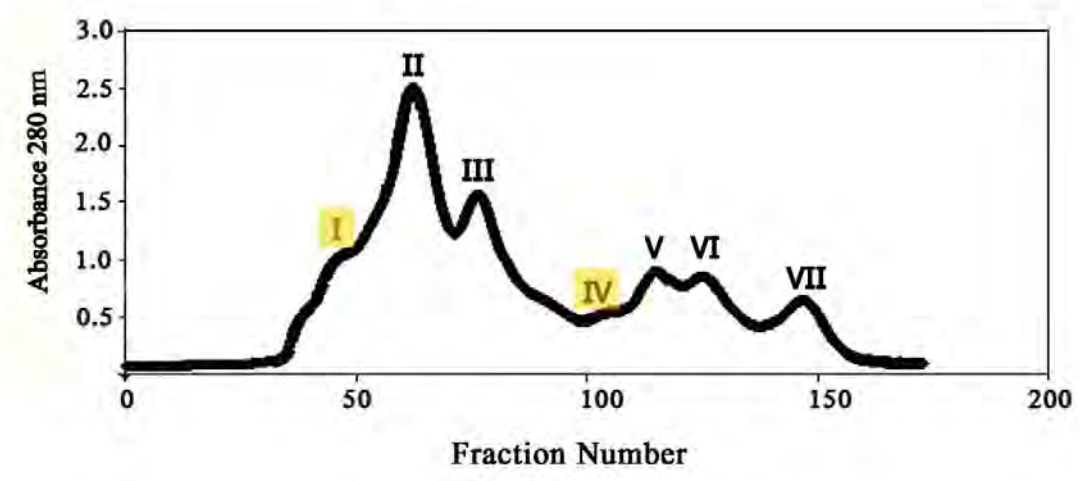


Figure 17: Ion-exchange chromatogram of partially purified *C. albolabris* CLP on Resourse Q column. Protein concentrations were estimated from the absorbance at 280 nm. The CLP containing peak determined by platelet-aggregating activity was indicated by an arrow.

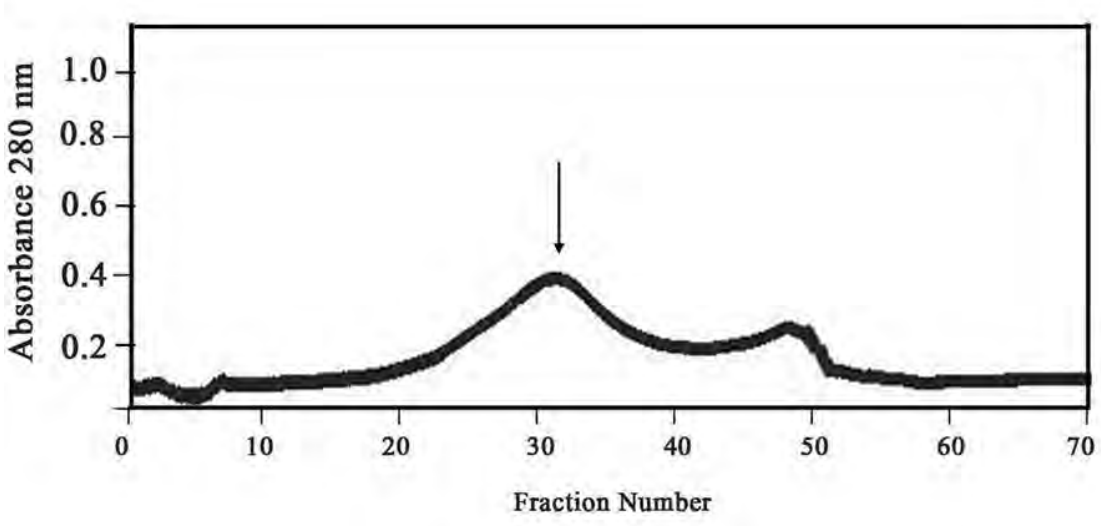
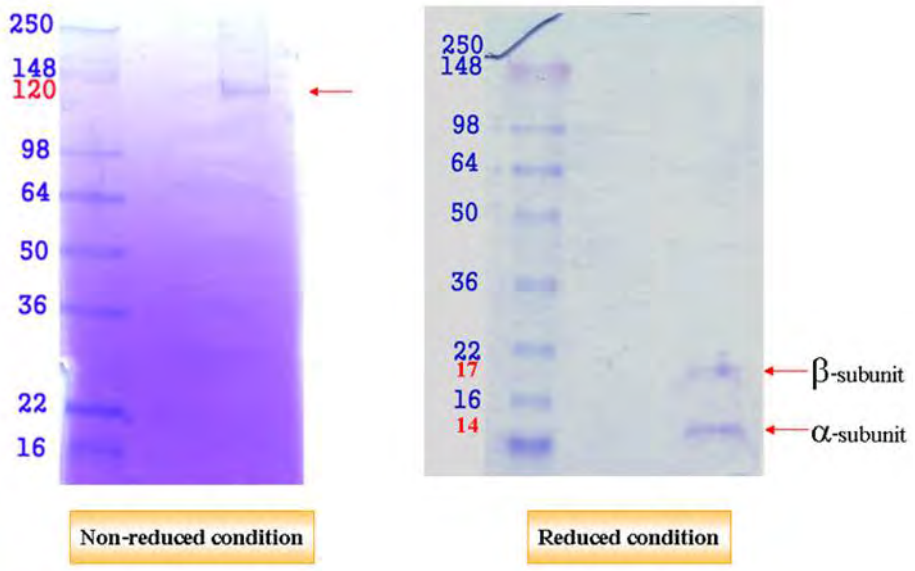


Figure 18: SDS-PAGE of purified *C. albolabris* CLP under non-reducing and reducing conditions, respectively.



4.3 Proteins Identification by LC/MS/MS

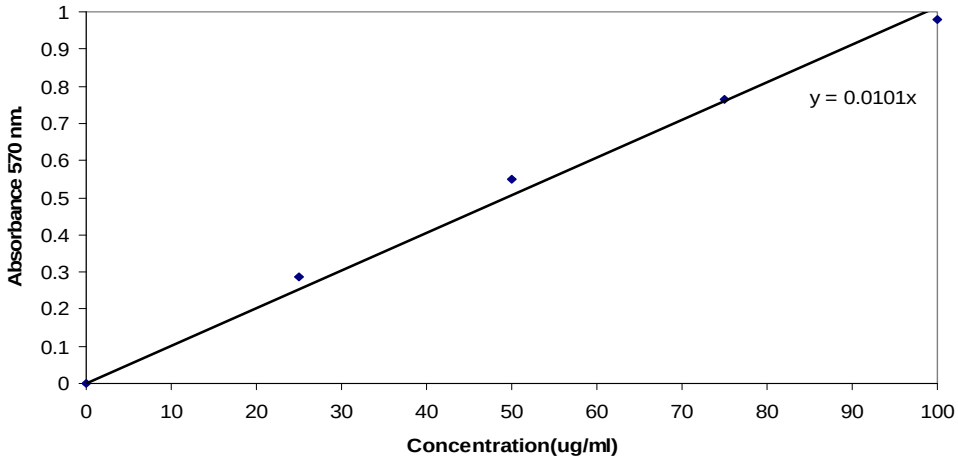
After obtaining the results from SDS-PAGE, the identities of purified *C. albolabris* CLP α and β subunits were determined by liquid chromatography coupled with tandem mass spectrometry. Each band of α and β subunit was eluted by in-gel digestion method (chapter III) and prepared for trypsin-digested peptide mass fingerprinting. The LC/MS/MS results of the fragments from α and β subunits sequences were R.YCYQAFSEPK.N and R.EQFECLVA.K respectively. They were matched with deduced amino acid sequences of α and β subunits obtained from cDNA library as previously described. Moreover the molecular mass of α and β subunits acquired from LC/MS/MS were 15728.4 Da and 16963.1Da, respectively.

4.4 Quantitative Assay of Purified *C. albolabris* CLP.

To determine the quantity of purified CLP, the Micro BCA™ Protein Assay was used for measuring the protein concentration. Absorbance average of 0.79 at 570 nm at 1:10 dilution of protein sample indicating that the calculated undiluted purified protein concentration was 647.5 µg/ml. The total yield of protein was 1.64 mg from the initial 500 mg of crude venom. Therefore, this *C. albolabris* CLP comprised approximately 0.33 % of the total venom proteins.

Figure 19: The standard curve of protein concentrations fitted by linear regression

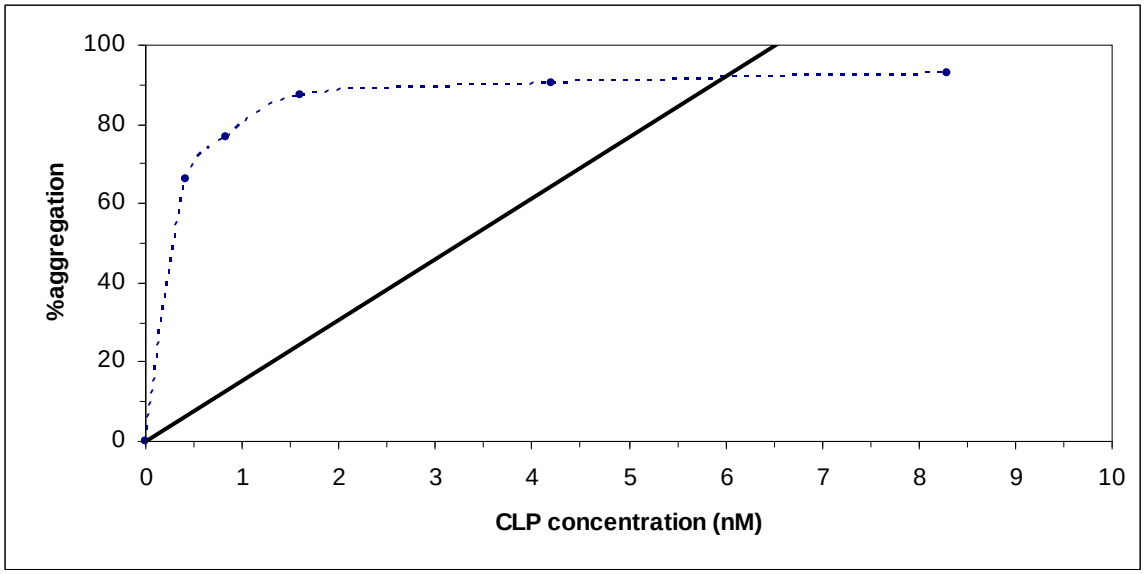
(y = mx + b, m = 0.0122, b = 0)



4.5 The Effects of the novel CLP on washed human platelets

From previous studies, high-molecular-weighted CLPs from snake venom, such as convulxin (from *Crotalus durissus terrificus*), strejnulxin (from *Cryptyletrops strejnegeri* venom) and alboluxin (from *Cryptyletrops albolabris* venom) were found to aggregate washed platelets without any cofactor. Similarly, a high molecular CLP purified in our laboratory induced aggregation of washed platelets. The maximum platelet aggregation dose toward washed human platelets in our study was 4.2 nM. Interestingly, the dose-response curve is hyperbolic. The concentration that induced 50% aggregation of washed platelets was 0.25 nM. (Figure20)

Figure 20: The effect of *C. albolabris* CLP on washed platelets aggregation. The EC_{50} (the concentration that induced 50% aggregation) was approximately 0.25 nM.



The direct human platelet aggregation activity of *C. albolabris* CLP was dose-dependently inhibited by monoclonal anti-GPIbα antibodies SZ2 and polyclonal GPVI antibody. Different concentrations of monoclonal anti-GPIbα antibodies SZ2 and polyclonal GPVI antibody were incubated at 37 °C for 5 minutes with 450 µl washed human platelets (3 x 10^8/ml), and then a fixed concentration of *C. albolabris* CLP (10 µg/ml, 8.3nM final concentration) was added. The maximum aggregation response

obtained from addition of *C. albolabris* CLP without antibody was set as 100 % aggregation. The anti-GPIbα antibody was found to inhibit CLP induced platelet aggregation in a dose dependent manner with the IC_{50} of 87.4 nM. In addition, anti-GPVI antibody was found to inhibit CLP-induced platelet aggregation more strongly than monoclonal anti-GPIb antibody with the lower IC_{50} of 44.8 nM. Either of the antibodies did not completely inhibit platelet aggregation. Therefore, we incubated anti-GPIbα and anti-GPVI antibody together with the highest concentration of each antibody (20 µg/ml of anti-GPIbα antibody and 5 µg/ml of anti-GPVI antibody). The combination of antibodies completely inhibited platelet aggregation induced by *C. albolabris* CLP.

Figure 21: Inhibitory effect of monoclonal anti-GPIb antibody on *C. albolabris* CLP-induced platelet aggregation. IC_{50} was determined by using curve fitting with linear regression

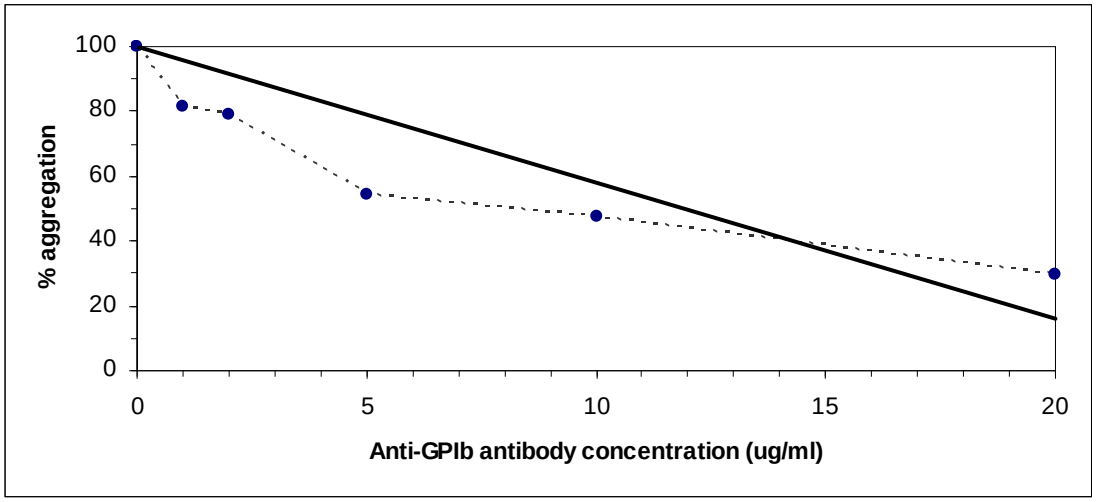
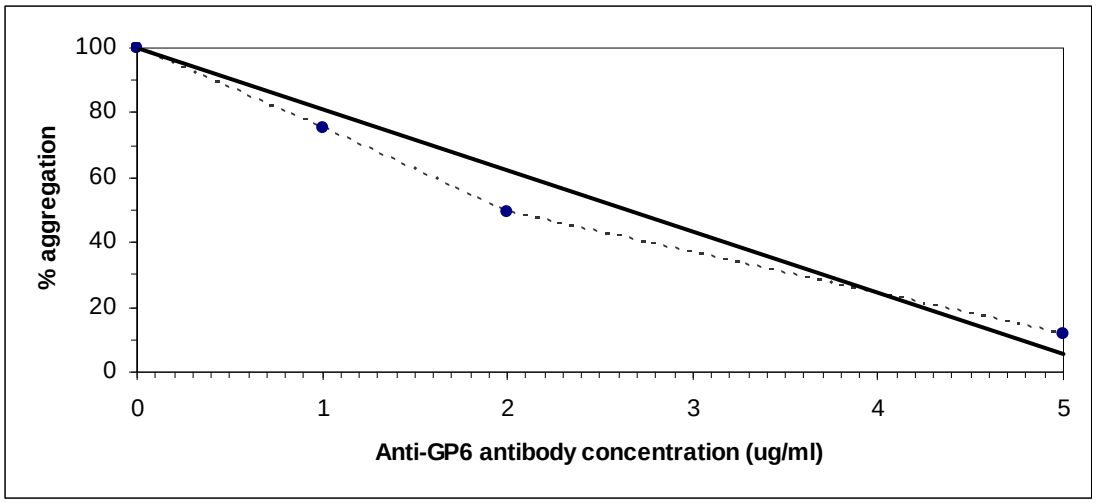


Figure 22: Inhibitory effect of polyclonal GPVI antibody on *C. albolabris* CLP-induced platelet aggregation. IC_{50} was determined by using curve fitting with linear regression

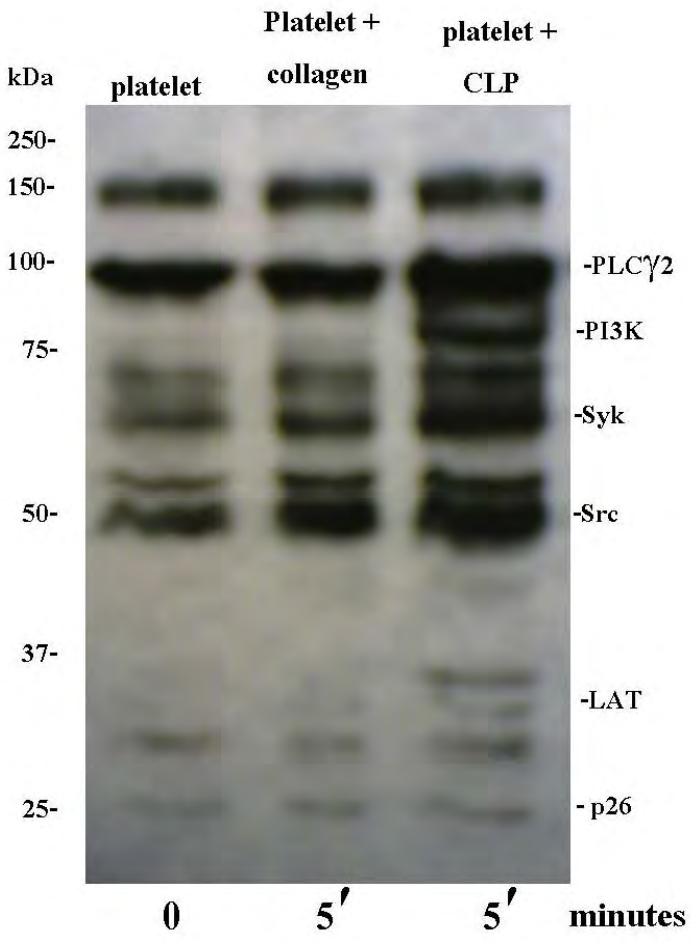


4.6 Platelet Signal Transduction Induced by *C. albolabris* CLP

C. albolabris CLP is a potent platelet agonist, similar to collagen, based on the aggregation slopes of washed platelet suspensions when treated with CLP or collagen. The final concentrations of 10 µg/ml of collagen and 5 µg/ml of the novel *C. albolabris* CLP gave comparable platelet aggregation rate as estimated by the slopes of light transmission aggregometer curves.

Subsequently, tyrosine phosphorylation of platelet signaling proteins after stimulation by the equivalent doses of CLP and collagen was determined. After 5-min incubation, platelet lysates were subjected to Western blotting probed by anti-phosphotyrosine antibody. Compared with platelets activated by collagen, CLP-stimulated platelets yielded stronger tyrosine phosphorylation of a range of proteins, including PLCγ2, PI3K, Syk, Src, LAT, and p26 (Figure 23).

Figure 23: Tyrosine phosphorylation of human platelets after activation by 5 µg/ml of *C. albolabris* CLP compared with those of 10 µg/ml of collagen.



CHAPTER V

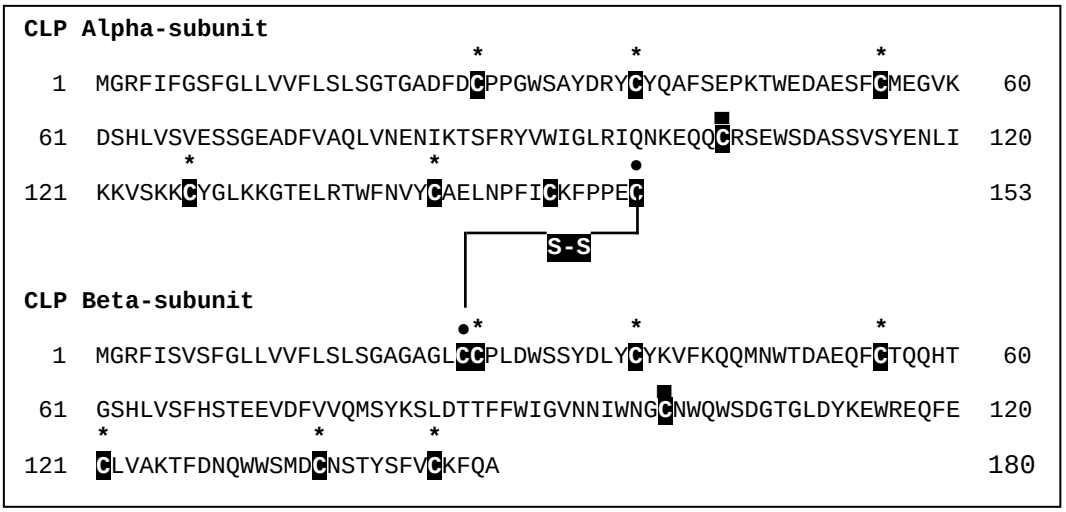
DISCUSSION AND CONCLUSION

From previous studies, our laboratory has constructed a cDNA library of green pit viper, *Cryptelytrops albolabris*'s venom glands and cloned venom genes from it. In this study, we have characterized and purified a novel 120-kDa C-type lectin like-protein (CLP) from *C. albolabris* crude venom It has ability to induce platelet aggregation via both GPIbα and GPVI. From the sequences and molecular weights of its subunits, this CLP has not been previously reported as it is not alboaggregin A, alboaggregin C or alboluxin (See detail below). The molecular masses of the non-reduced and reduced forms suggest that *C. albolabris* CLP is an octameric heterodimers composed of $(\alpha\beta)_4$. The pattern of disulfide bridges of this C-type lectin protein was then compared with those of other homologous snake venoms, There is an additional cysteine residue in each subunit in high molecular weight CLPs compared with low molecular weight CLPs to form extra links, resulting in a large multimeric structure. There are seven conserved cysteine residues in each subunit of *C. albolabris* CLP. The multimeric inter-chain disulfide bonds pattern between α and β subunit of *C. albolabris* CLP are predicted to be cross-linked by a disulfide bond between Cys 158 of α subunit and Cys 26 of β subunit. This characteristic disulfide bond is required for the cyclic structure. The dimeric inter-chain disulfide bond is predicted to cross-link between Cys 104 of α subunit and Cys 100 of β subunit. Moreover, there are three intra-chain disulfide bonds in each subunit (Figure 24). The cDNA sequence of α subunit composed of 477 bp in length that is translated into 23 amino acid residue signal peptide and a 135 amino acid residue mature protein. The cDNA sequence of βsubunit composed of 447 bp in length and translated into 23 amino acid residue signal peptide and a 125 amino acid residue mature protein. Excluding signal peptides, the theoretical molecular masses of mature α and β subunits were 15658.61 and 14922.59 Da, respectively.

Five hundred mg of crude venom was purified by gel filtration and ion-exchange chromatography. They separated proteins by size and charge respectively. The purified CLP was analyzed by SDS-PAGE under non-reducing and reducing conditions. It

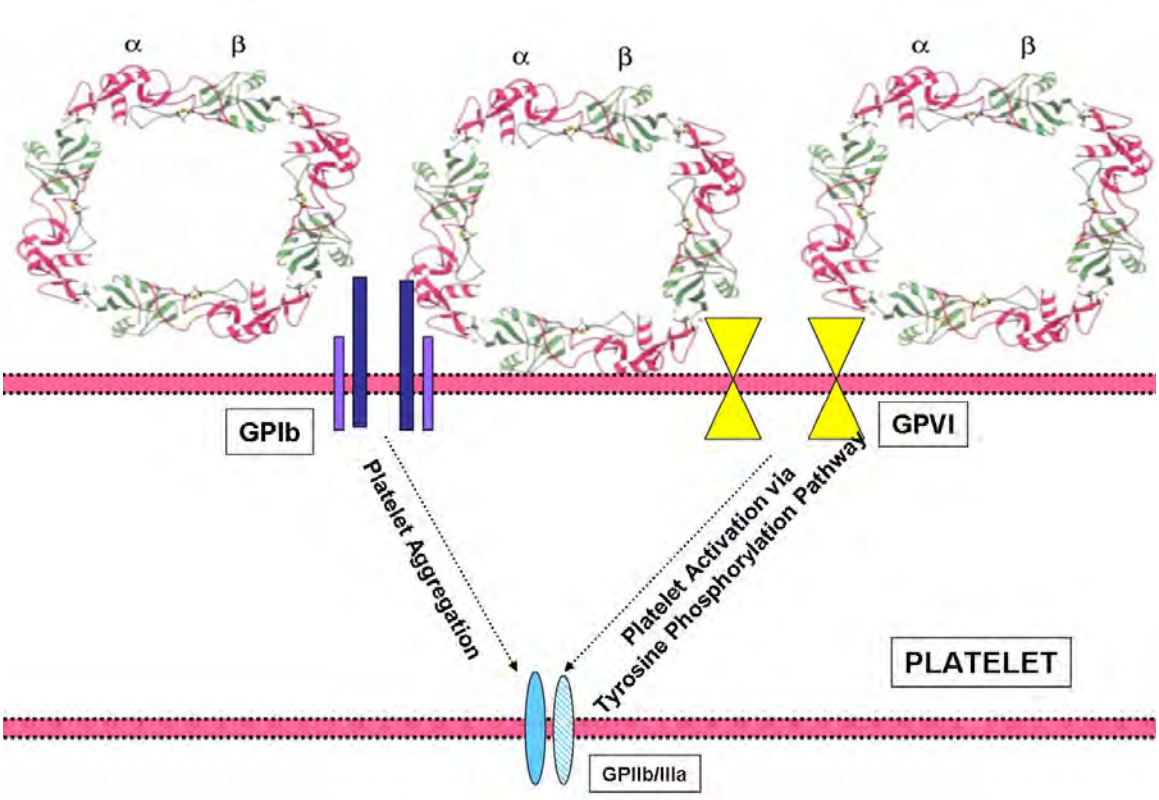
exhibited a single band with molecular weight of 120,000 Da (non-reduced condition) and two bands with apparent molecular weights of 14,000 and 17,000 Da (reduced condition) for α and β subunits, respectively. Moreover, we used liquid chromatography-tandem mass spectrometry (LC/MS/MS) to confirm the identity amino acid sequence of purified CLP. Each subunit of CLP were separated by SDS-PAGE analysis and digested with trypsin. The fragments of α and β subunits showed peptide mass fingerprints as R.YCYQAFSEPK.N and R.EQFECLVA.K. These amino acid sequences were identical to those respective subunits of snake venom C-type lectins. The measured peptide ions in LC/MS/MS were detected in a survey scan ranging from 400 to 1600 amu. Therefore, it was possible that protein fragments smaller than 400 amu or larger than 1600 amu could not be observed. There were 9 fragments of α subunit and 2 fragments of β subunit that predicted to be in this range. However, fragments of protein might have been lost during the process. Nevertheless, the 2 derived peptide sequences' results were completely matched with amino acid sequences of α and β subunits obtained from cDNA library suggesting that they were the same protein. The theoretical molecular masses of α and β subunits were 15658.61 and 14922.59 Da, while the molecular masses obtained from LC/MS/MS of α and β subunits were 15728.4 and 16963.1Da. The actual molecular masses from LC/MS/MS were higher than theoretical masses, possibly due to the post translation modifications.

Figure 24: Conceptually-translated protein sequence of α- and β- subunits from cDNA library, *, ■ and ● represent intra-chain, dimeric inter-chain and multimeric inter-chain disulfide bonds, respectively.



Each snake venom contains more than one C-type lectin like-protein that acts via platelet membrane receptors, either inducing or inhibiting platelet activation. Many snakes C-type lectin like-proteins that activate platelet have been reported. They may either have different receptors or different affinities for the same receptors. *C. albolabris* CLP is a powerful platelet activator. It showed a distinct, dose-dependent platelet aggregating activity. Furthermore, it displayed a lag phase before the start of aggregation similar to collagen. This lag time is probably the period of platelet signal transduction. This high potency that causes agglutination is thought to come from cooperative action of multiple binding sites of its octameric structure similar to convulxin, stejnulxin and alboluxin (Ja´nos Polga´ *et al*, 1997; Lee *et al*., 2003; Du *et al*., 2002) (Figure25). This large polyvalent structure may cross-link and cluster several molecules of platelet glycoprotein resulting in strong activation.

Figure 25: Mechanism of action of *C. albolabris* CLP: binding both GpIb and Gp VI.



There are 4 snake venom C-type lectins, which have already been isolated from *C. albolabris* and characterized.

1. Alboaggregin A is a tetrameric $(\alpha\beta)_2$ CLP with the molecular mass of 50 kDa. It is a powerful activator of platelets through GPVI and GPIb. Alboaggregin A induces platelet tyrosine phosphorylation pattern similar to that is caused by collagen. (Do¨rmann *et al*, 2001).

2. Alboaggregin B is a $\alpha\beta$ heterodimers with the molecular weight of 25 kDa. It agglutinates platelets by binding solely to GPIb. However, it does not activate or only weakly activate platelet signal transduction pathway (J. Arpijuntarangkoon *et al*., 2007).

3. Alboaggregin C is a 120 kDa CLP under a non-reduced condition. Upon reduction, the 17 and 19 kDa subunits are revealed. Alboaggregin C only agglutinates platelets without signal transduction stimulation (Du *et al*., 2002).

4. Alboluxin is another tetrameric $(\alpha\beta)_2$ CLP with the molecular mass of 120 kDa. The 17 and 24 kDa subunits are found upon reduction. Alboluxin is a powerful platelet agonist which activates platelets through both platelet GPVI and GPIb. Moreover, the tyrosine phosphorylation pattern of alboluxin is the same as that induced by collagen (Du *et al*., 2002).

From platelet aggregation inhibition studies, anti-GPVI polyclonal antibodies more strongly inhibited *C. albolabris* CLP-induced platelet aggregation than anti-GPIbα antibodies. The concentration of monoclonal GPIb antibody and polyclonal GPVI that inhibited 50% aggregation (IC50) induced by *C. albolabris* CLP (8.3 nM final concentration) were 87.4 nM and 44.8 nM, respectively. This provides the evidence that *C. albolabris* CLP activates platelets through both GPIbα and GPVI. In another study (Xiao-Yan Du *et al*., 2002) has shown that another snake venom C-type lectin-like protein, alboluxin, also induced platelet aggregation via both GPVI and GPIbα. The IC_{50} of monoclonal GPIb antibody and polyclonal GPVI that effects on alboluxin were 296 nM (final concentration of alboluxin = 0.25 nM) and 31000 nM (final concentration of alboluxin = 0.33 nM). Therefore, alboluxin appears to be a stronger platelet agonist

because it requires larger quantities of antibodies in order to inhibit platelet aggregation. Furthermore this experimental data demonstrate that *C. albolabris* CLP bind specifically to both GPIbα and GPVI resulting in activation platelets and inducing phosphorylation of tyrosine residues in signaling proteins. These 2 platelet receptors are likely to function synergistically because one of the antibodies almost completely inhibited the activity of CLP. However, only one of the receptor is sufficient for platelet activation by this CLP because both antibodies were required to completely inhibit CLP-induced platelet aggregation. Examining the *C. albolabris* CLP-induced tyrosine phosphorylation profile in platelets, we found that it was fairly similar to those produced by collagen and alboluxin, except that platelets activated by albolulxin showed particularly strong Fcγ phosphorylation.

From the data above, Our *C. albolabris* CLP are similar in molecular mass under non-reduced condition to alboaggregin C and alboluxin. However, compared with alboaggregin C, the *C. albolabris* CLP contains different properties in activating platelet signal transduction. Therefore, our C. albolabris CLP is unlikely to be the alboaggregin C that only agglutinated platelets in the previous report.

Comparing between alboluxin and *C. albolabris* CLP, we found that both were not only similar in non-reduced molecular mass but also showed similar ability in activate platelets. Therefore, the possibility that *C. albolabris* CLP is alboluxin needs to be considered. According to the previous study, alboluxin α and β subunit had shown the molecular masses under reduced condition of 17 and 24 kDa, respectively. On the other hand, *C. albolabris* CLP α and β subunit displayed the reduced masses of 14 and 17 kDa. Moreover, the reported N-terminal sequence of β subunit of alboluxin was NFSPPDWYAYD, which was not matched with either subunit sequence of *C.albolabris* CLP. The N-terminal sequence of alboluxin's α subunit is still unclear. For these reasons, we do not think that alboluxin and our *C. albolabris* CLP are the same protein. Therefore, *C. albolabris* CLP is probably a novel C-type lectin like-protein.

Platelet activating CLPs are useful tools to investigate GPIb and GPVI functions, signal transduction and interactions. Genetic mutations of GPIb cause a bleeding

disorder termed Bernard-Soulier syndrome. Furthermore, genetic polymorphisms of GPVI have been reported to be associated with thrombotic and/or bleeding tendency. Our CLP may be a useful agent to test platelet functions in these patients. Moreover, several cases of undefined platelet dysfunctions have been reported as platelet signal transduction defects. This CLP may be helpful in elucidating the molecular defects in these patients.

We hypothesize that the *C. albolabris* CLP draws GPIb and GPVI into close proximity resulting in synergistic signal transduction between these 2 receptors (Figure 25). Therefore, one CLP molecule should contain separate GPIb and GPVI binding sites. These sites on the CLP should be identified. Further studies on expression, characterization and mutagenesis of recombinant *C. albolabris* CLP are essential to elucidate this structure-function relationship. These experiments will give us deeper insights in molecular mechanisms of platelet aggregation induced by these 2 receptors.

References

[1] Arpijuntarangkoon J. *et al*. Molecular cloning and sequence analysis of alboaggregin B. Platelets. 18(4) (2007) : 266–272.

[2] Atoda, H., Hyuga, M., Morita, T. The primary structure of coagulation factor IX/factor X-binding protein isolated from the venom of *Trimeresurus flavoviridis*. Homology with asialoglycoprotein receptors, proteoglycan core protein, tetranectin and lymphocyte Fc epsilon receptor for immunoglobulin. E. J. Biol. Chem. 266 (1991) : 14903–14911.

[3] Batuwangala, T., Leduc, M., Gibbins, J.M., Bon, C., Jones, E.Y., 2004. Structure of the snake-venom toxin convulxin Acta Crystallogr. D Biol. Crystallogr. 60 (Pt 1) (2004) : 46–53.

[4] Brinkhous, K.M., Barnes, D.S., Potter, J.Y., Read, M.S. Von Willebrand syndrome induced by a Bothrops venom factor: bioassay for venom coagglutinin. Proc. Natl Acad. Sci. USA. 78(5) (1981) : 3230–3234.

[5] Brinkhous, K.M., Read, M.S., Fricke, W.A., Wagner, R.H. Botrocetin (venom coagglutinin) reaction with a broad spectrum of multimeric forms of factor VIII macromolecular complex. Proc. Natl Acad. Sci. USA. 80 (5) (1983) : 1463–1466.

[6] Dagmar Do¨rmann *et al*. Alboaggregin A activates platelets by a mechanism involving glycoprotein VI as well as glycoprotein Ib. BLOOD (2001): 929-936.

[7] Drickamer, K. C-type lectin-like domains. Curr. Opin. Struct. Biol. (1999) : 585-590.

[8] Fukuda, K., Mizuno, H., Atoda, H., Morita, T. Crystal structure of flavocetin-A, a platelet glycoprotein Ib-binding protein, reveals a novel cyclic tetramer of C-type lectin-like heterodimers. Biochemistry. 39 (8) (2000) : 1915–1923.

[9] Grams F, Huber R, Kress LF, Moroder L, Bode W. Activation of snake venom metalloproteinases by a cysteine switch-like mechanism. FEBS Lett. 335 (1993) : 76–80.

[10] Hamako, J., Matsui, T., Suzuki, M., Ito, M., Makita, K., Fujimura, Y., Ozeki, Y., Titani, K. Purification and characterization of bitiscetin, a novel von Willebrand factor modulator protein from Bitis arietans snake venom. Biochem. Biophys. Res. Commun. 226(1) (1996) : 273–279.

[11] Hirabayashi, J., Kusunoki, T., Kasai, K. Complete primary structure of a galactose-specific lectin from the venom of the rattlesnake Crotalus atrox. Homologies with Ca^{2+} dependenttype lectins. J. Biol. Chem. 266(4) (1991) : 2320–2326.

[12] Ja'nos Polga'r, Jeannine M. Clemetson, Beate E. Kehrel, Markus Wiedemann, Edith M. Magnenat, Timothy N. C. Wells, and Kenneth J. Clemetson. the journal of biological chemistry. 272 (1997) : 13576–13583.

[13] Kai-Fa HUANG, Tzu-Ping KO, Chin-Chun HUNG, John CHU, Andrew H.-J. WANG and Shyh-Horng CHIOU. Crystal structure of a platelet-agglutinating factor isolated from the venom of Taiwan habu (Trimeresurus mucrosquamatus). Biochem. J. 378 (2004) : 399–407

[14] Mahasandana S. *et al*. Clinical manifestrations of bleeding following Russell's viper and green pit viper bites in adults. Southeast Asian Trop Med Pub Health. 11 (1980) : 285-93.

[15] Mahasandana S., Jintakune P. The species of green pit viper in Bangkok. Southeast Asian J. Trop Med Pub Health. 21 (1990) : 225-255.

[16] Matsui, T., Hamako, J., Matsushita, T., Nakayama, T., Fujimura, Y., Titani, K. Binding site on human von Willebrand factor of bitiscetin, a snake venom-derived platelet aggregation inducer. Biochemistry. 41(25) (2002) : 7939–7946.

[17] Marcinkiewicz, C., Lobb, R.R., Marcinkiewicz, M.M., Daniel, J.L., Smith, J.B., Dangelmaier, C., Weinreb, P.H., Beacham, D.A., Niewiarowski, S. Isolation and characterization of EMS16, a C-lectin type protein from Echis multisquamatus venom, a potent and selective inhibitor of the $\alpha_2\beta_1$ integrin. Biochemistry. 39(32) (2002) : 9859–9867.

[18] Morita, T. C-type lectin-related proteins from snake venoms. Current Drug Targets Cardiovascular & Haematological Disorders. 4(4) (2004b) : 357–373.

[19] Morita, T. Structures and functions of snake venom CLPs (C-type lectin-like proteins) with anticoagulant-, procoagulant-, and platelet-modulating activities. Toxicon. 45 (2005) : 1099–1114.

[20] Murakami, M.T., Zela, S.P., Gava, L.M., Michelan-Duarte, S., Cintra, A.C., Arni, R.K. Crystal structure of the platelet activator convulxin, a disulfide-linked $\alpha_4\beta_4$ cyclic tetramer from the venom of *Crotalus durissus terrificus*. Biochem. Biophys. Res. Commun. 310(2) (2003) : 478–482.

[21] Navdaev, A., Clemetson, J.M., Polgar, J., Kehrel, B.E., Glauner, M., Magnenat, E., Wells, T.N., Clemetson, K.J. Aggretin, a heterodimeric C-type lectin from Calloselasma rhodostoma (malayan pit viper), stimulates platelets by binding to $\alpha_2\beta_1$ integrin and glycoprotein Ib, activating Syk and phospholipase Cγ 2, but does not involve the glycoprotein VI/Fc receptor γ chain collagen receptor. J. Biol. Chem. 276(24) (2001) : 20882–20889.

[22] Peng, M., Lu, W., Kirby, E.P. Alboaggregin-B: a new platelet agonist that binds to platelet membrane glycoprotein Ib. Biochemistry. 30(49) (1991) : 11529–11536.

[23] Qiumin Lu *et al*. GPIb is involved in platelet aggregation induced by mucetin, a snake C-type lectin protein from Chinese habu (*Trimeresurus mucrosquamatus*) venom. Thromb Haemost. 91 (2004) : 1168–76.

[24] Robert K. Andrewse, Aura S. Kamigutib, Oscar Berlangaa, Mireille Leducc, R. David G. Theakstond, Steve P. Watsona. The use of snake venom toxins as tools to study platelet receptors for collagen and von Willebrand Factor. Haemostasis. 31 (2001) : 155–172

[25] Rojnuckarin P. *et al*. A randomized, double-blind, placebo-controlled trial of antivenom for local effects of green pit viper bites. Transactions of the Royal Society of Tropical Medicine and Hygiene. 100 (2005) : 879-884.

[26] Steffen Massberg *et al*. A Crucial Role of Glycoprotein VI for Platelet Recruitment to the Injured Arterial Wall in Vivo. J. Exp. Med. 197 (2003) : 41–49.

[27] Taniuchi, Y., Kawasaki, T., Fujimura, Y., Suzuki, M., Titani, K., Sakai, Y., Kaku, S., Hisamichi, N., Satoh, N., Takenaka, T.,et al. Flavocetin-A and -B, two high molecular mass glycoprotein Ib binding proteins with high affinity purified from Trimeresurus flavoviridis venom, inhibit platelet aggregation at high shear stress. Biochim. Biophys. Acta 1244(2–3) (1995) : 331–338.

[28] Viravan *et al*. A national hospital-based survey of snakes responsible for bites in Thailand. Trans Royal Soc Trop Med Hyg. 86 (1992) : 100-106.

[29] Wang, W.J., Huang, T.F. A novel tetrameric venom protein, agglucetin from *Agkistrodon acutus*, acts as a glycoprotein Ib agonist. Thromb. Haemost. 86(4) (2001) : 1077–1086.

[30] Wang, W.J., Ling, Q.D., Liau, M.Y., Huang, T.F. A tetrameric glycoprotein Ib-binding protein, agglucetin, from Formosan pit viper: structure and interaction with human platelets. Thromb. Haemost. 90(3) (2003) : 465–475.

[31] Weis, W.I., Kahn, R., Fourme, R., Drickamer, K.,Hendrickson, W.A. Structure of the calcium-dependent lectin domain from a rat mannose-binding protein determined by MAD phasing. Science. 254(5038) (1991) : 1608–1615.

[32] Weis, W.I., Drickamer, K., Hendrickson, W.A. Structure of a C-type mannose-binding protein complexed with an oligosaccharide. Nature. 360(6400) (1992) : 127–134.

[33] Wen-Hui Lee *et al*. Molecular cloning and characterization of a platelet glycoprotein Ib-binding protein from the venom of *Trimeresurus stejnegri*. Toxicon 41 (2003) : 885-892.

[34] Wen-Hui Lee *et al*. Stejnulxin, a novel snake C-type lectin-like protein from *Trimeresurus stejnegeri* venom is a potent platelet agonist acting specifically via GPVI. Thromb Haemost. 90 (2003) : 662-71.

[35] Woulfe *et al*. Signaling receptors on platelets and megakaryocytes. Methods in Molecular biology 273 (2004).

[36] Xiao-Yan Du *et al*. Alboluxin, a Snake C-type Lectin from *Trimeresurus albolabris* Venom is a potent Platelet Agonist acting via GPIb and GPVI. Thromb Haemost 87 (2002) : 692–8.

[37] Xiao-Yan Du, Derek S. Sim , Wen-Hui Lee , Yun Zhang. Blood cells as targets of snake toxins. Blood Cells Molecules and Diseases 36 (2006) : 414–421 415.

APPENDIX

APPENDIX

1. Bacterial Media

1.1 LB Medium (per liter)

10g	Bacto®-tryptone
5g	Bacto®-yeast extract
5g	NaCl

Adjust pH to 7.0 with NaOH.

1.2 LB Plates with Ampicillin

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50 °C before adding ampicillin to a final concentration of 100 µg/ml. Pour 30-35 ml of medium into 85 mm petri dishes. Let the agar harden. Store at 4 °C for up to 1 month or at room temperature for up to 1 week.

1.3 LB Plates with Ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above; then supplement with 0.5 mM IPTG and 80 µg/ml X-Gal and pour the plates. Alternatively, 100µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal may be spreaded over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37 °C prior to use.

1.4 SOC Medium (100ml)

2.0 g	Bacto®-tryptone
0.5 g	Bacto®-yeast extract
1 ml	1M NaCl
0.25 ml	1M KCl
1 ml	2M Mg^{2+} stock, filter sterilized
1 ml	2M glucose, filter sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97 ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2 M Mg^{2+} stock and 2 M glucose, each to a final concentration of 20 mM. Bring to 100 ml with sterile, distilled water. The final pH should be 7.0.

2. Buffer

2.1 1X Equilibration/Wash Buffer (pH 7.0)

50 mM	Sodium Phosphate pH 7.0
300 mM	NaCl

2.2 1X Equilibration Buffer (pH 8.0)

50 mM	Sodium Phosphate pH 8.0
300 mM	NaCl

2.3 1X Elution Buffer (pH 5.0)

50 mM	Sodium Phosphate pH 5.0
300 mM	NaCl

2.4 Alkaline Lysis Solution I

50 mM	Glycine
25 mM	Tris-Chloride, pH 8.0
10 mM	EDTA, pH 8.0

2.5 Alkaline Lysis Solution II

0.2 N	NaOH
1 % (w/v)	SDS

2.6 Alkaline Lysis Solution III

60 ml.	5 M Potassium Acetate
11.5 ml.	Glacial Acetic Acid
28.5 ml.	dH_2O

2.7 STE BUFFER

10 mM	Tris-Cl pH 8.0
0.1 M	NaCl
1 mM	EDTA pH 8.0

2.8 Tris-Glycine Buffer (1X)

25 mM	Tris-Cl
250 mM	Glycine

2.9 10X Tris EDTA (TE) pH 8.0

100 mM	Tris-Cl, pH 8.0
10 mM	EDTA, pH 8.0

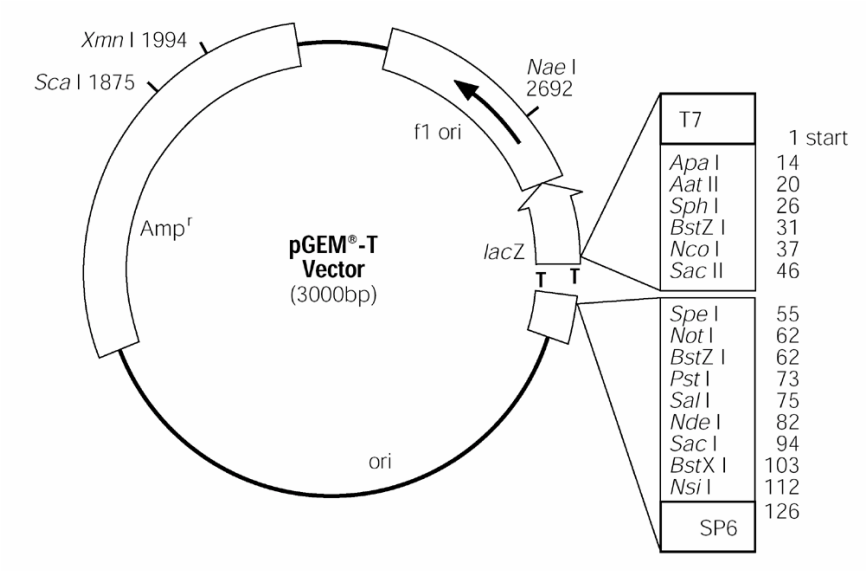
2.10 1X Phosphate-Buffered Salive (PBS)

137 mM	NaCl
2.7 mM	KCl
10 mM	Na_2HPO_4
2 mM	KH_2PO_4

Dissolve 8 g of NaCl, 0.2 of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 800 ml of dH_2O. Adjust pH to 7.4 with HCl. Add dH_2O to 1 liter and sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm^2) on liquid cycle. Store the buffer at room temperature.

3. Vector

3.1 pGEM®-T Vector Circle Map and Sequence Reference Points.

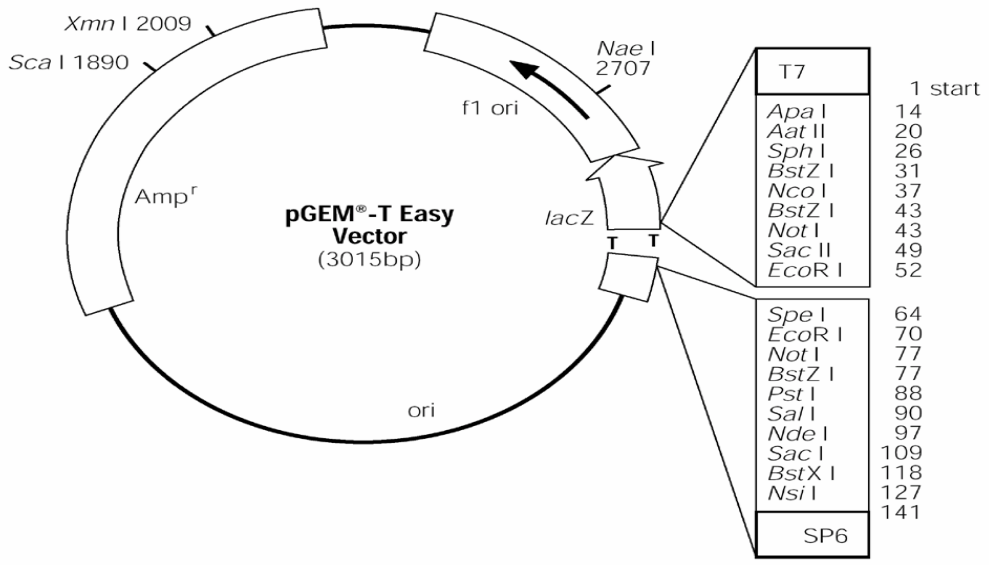


pGEM®-T Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–113
SP6 RNA polymerase promoter (–17 to +3)	124–143
SP6 RNA polymerase transcription initiation site	126
pUC/M13 Reverse Sequencing Primer binding site	161–177
lacZ start codon	165

lacoperator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
lacoperon sequences	2821–2981, 151–380
pUC/M13 Forward Sequencing Primer binding site	2941–2957
T7 RNA polymerase promoter (–17 to +3)	2984–3

3.2 pGEM®-T Easy Vector Circle Map and Sequence Reference Points.



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180

lacoperator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lacoperon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

4. Others

4.1 12 % Gel (5 ml) Resolving Gels for Tris-Glycine SDS-Polyacrylamide Gel Electrophoresis

1.6 ml.	H_2O
2.0 ml.	30 % acrylamide mix
1.3 ml.	1.5 M Tris, pH 8.8
0.05 ml.	10 % SDS
0.05 ml.	10 % ammonium persulfate
0.002 ml.	TEMED

4.2 5 % Stacking Gel (1 ml)

0.068 ml.	H_2O
0.17 ml.	30 % acrylamide mix
0.13 ml.	1.0 M Tris, pH 6.8
0.01 ml.	10 % SDS
0.01 ml.	10 % ammonium persulfate
0.001 ml.	TEMED
0.1 %	SDS

4.3 2X SDS Gel-Loading Buffer

100 mM	Tris-Cl, pH 8.8
4 % w/v	SDS
0.2 % w/v	bromphenol blue
20 % v/v	glycerol
200 mM	dithiothreitol or β-mercaptoethanol

4.4 Coomassie brilliant blue, 200 ml.

Coomassie blue	0.5 g.
H_2O	90 ml.
CH_3OH	90 ml.
CH_3COOH	20 ml.

4.5 Washing buffer

Stock I	2.5 ml.
Stock II	0.5 ml.
Stock III	1 ml.

Glucose anhydrous 0.05 g., 17.5 % bovimealbumin 1 ml. pH 7.35

4.5.1 Stock I

$NaHCO_3$	1 g.
KCl	0.2 g.
NaH_2PO_4	0.056 g.
NaCl	8 g.
H_2O (DW)	50 ml.

4.5.2 Stock II

$MgCl_2$ $6H_2O$ 203 g. in 10 ml. DW

4.5.3 Stock III

$CaCl_2$ $2H_2O$ 147 g. in 10 ml DW

4.6 buffer I

30 ml. washing buffer, 300 µl. heparin 5,000 U/ml., 300 µl CP/CPK

4.7 buffer II

20 ml. washing buffer, 200 µl CP/CPK

4.8 Destained

4.8.1 Destained I

H_2O	880 ml.
CH_3OH	50 ml.
CH_3COOH	70 ml.

4.8.2 Destained II

H_2O	160 ml.
CH_3OH	200 ml.
CH_3COOH	40 ml.

4.9 Electrophoresis blotting buffer

25mM Tris	3.03 g.
192M Glycine	14.04 g.
20% CH_3OH	200 ml.

Add H_2O until 1 liter.

BIOGRAPHY

Personal Data

Name	Miss Ponthip Mekchay
Date of Birth	July 12, 1983
Address	77/71 Ratchathewi tower, Payathai Road, Ratchathewi, Bangkok, Thailand 10400

Education

1999-2001	Sacred Heart College, Chiangmai, Thailand
2002-2005	Faculty of Associated Medical Sciences, Chiangmai Unversity, Chiangmai, Thailand

Experience

2004	Training in Medical Laboratory, McCormick Hospital, Chiangmai, Thailand
2005	Training in Medical Laboratory and Blood Bank Unit, Maharaj Nakorn Chiangmai Hospital, Chiangmai, Thailand