การโคลนนิ่งและศึกษาโปรตีนที่กระตุ้นให้เกิดการเกาะกลุ่มของเกร็ดเลือด จากพิษงูเขียวหางไหม้ท้องเหลือง (ไตรเมอรีซูรัส อัลโบลาบรีส)

นายจรัสพงษ์ อภิจันทรางกูร

สถาบันวิทยบริการ
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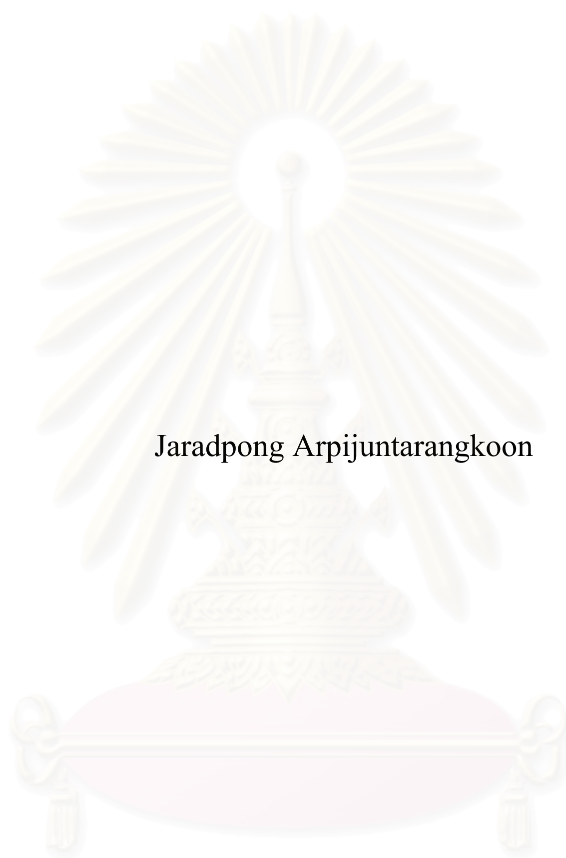
CLONING AND CHARACTERIZATION OF PLATELET-AGGREGATING PROTEINS FROM *TRIMERESURUS ALBOLABRIS* VENOM



Jaradpong Arpijuntarangkoon

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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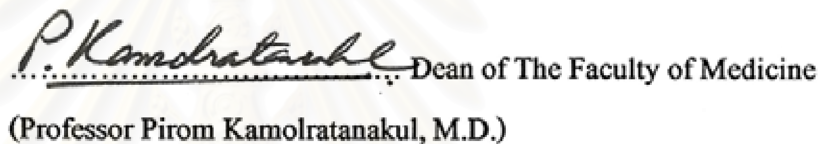
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จรัสพงษ์ อภิจันทรางกูร : การโคลนนิ่งและศึกษาโปรตีนที่กระตุ้นให้เกิดการเกาะกลุ่มของเกร็ดเลือดจากพิษงูเขียวหางไหม้ท้องเหลือง (ไตรเมอรีซูรัส อัลโบลาบรีส) (CLONING AND CHARACTERIZATION OF PLATELET-AGGREGATING PROTEINS FROM TRIMERESURUS ALBOLABRIS VENOM) อ. ที่ปรึกษา รศ.นพ.พลภัทร โรจน์นครินทร์, จำนวน 84.หน้า, ISBN 974-17-3575-8

ในพิษงูกลุ่มโครทาไลน์ซึ่งมีผลต่อระบบเลือดพบมีโปรตีนชนิดซีไทป์เลคตินหลายชนิด โปรตีนเหล่านี้ประกอบด้วยส่วนย่อยสองสายขึ้นไปเช่นส่วนอัลฟ่าและเบต้าเชื่อมต่อกันด้วยพันธะไดซัลไฟด์ บางตัวมีฤทธิ์กระตุ้นแต่บางตัวมีฤทธิ์ยับยั้งการเกาะกลุ่มของเกร็ดเลือด และบางชนิดสามารถยับยั้งการทำงานของปัจจัยการแข็งตัวของเลือดที่9และ10

การศึกษาก่อนหน้านี้ได้แยกโปรตีนอัลโบแอคกริกินบี (AL-B) ซึ่งเป็นซีไทป์เลคตินจากพิษงูเขียวหางไหม้ท้องเหลือง พบว่าสามารถจับไกลโคโปรตีนวันบีของเกร็ดเลือดและรายงานลำดับกรดอะมิโน แต่ยังไม่มีรายงานถึงลำดับนิวคลีโอไทด์ของซีดีเอ็นเอของ AL-B มาก่อน จากข้อมูลลำดับเบสจากห้องสมุดซีดีเอ็นเอของต่อมพิษงูเขียวหางไหม้ท้องเหลือง เราได้ออกแบบโอลิโกนิวคลีโอไทด์ไพรเมอร์เพื่อโคลนยีนซีไทป์เลคตินโดยวิธี 5′-RACE และจากข้อมูลนี้ได้ออกแบบไพรเมอร์เพื่อทำ 3′-RACE จนสามารถโคลนยีนของ AL-B ได้ทั้งยีนของส่วนอัลฟ่าและยีนของส่วนเบต้า การวิเคราะห์ลำดับนิวคลีโอไทด์ พบว่าซีดีเอ็นเอของส่วนอัลฟ่าและเบต้าประกอบด้วย 468 และ 438 เบสตามลำดับ ทำให้ได้โปรตีนที่มีกรดอะมิโน133 และ 123 ตัวตามลำดับ และมีส่วนซิกแนลเปปไทด์ 23 ตัว การวิเคราะห์ลำดับกรดอะมิโนพบว่าส่วนอัลฟ่าและเบต้ามีส่วนเหมือนกับซีไทป์เลคตินของงูเขียวไผ่ 85 และ 79 เปอร์เซนต์ตามลำดับ นอกจากนี้ จากการวิเคราะห์ลำดับกรดอะมิโนเทียบซีไทป์เลคตินชนิดต่างๆเรายังพบส่วนของโปรตีน (เซอรีน,อาร์จินีน,ทรีโอนีน,ไทโรซีน) ที่น่าจะทำหน้าที่เกาะกลุ่มเกร็ดเลือด การศึกษาโปรตีน AL-B พบว่าสามารถเกาะกลุ่มเกร็ดเลือดโดยอาศัยไกลโคโปรตีนวันบีบนผิวเกร็ดเลือดด้วยค่าอีซี50 เท่ากับ 180 นาโนโมลาร์ นอกจากนี้ยังใช้วิธีแมสสเปคโตรเมทที่ในการยืนยันโปรตีนตัวนี้ว่าเหมือนกับยีนที่โคลนได้จริง จากการค้นคว้าวารสารต่างๆพบว่ารายงานนี้เป็นรายงานแรกของซีดีเอ็นเอของโปรตีนกลุ่มซีไทป์เลคตินจากพิษงูเขียวหางไหม้ท้องเหลือง

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JARADPONG ARPIJUNTARANGKOON: CLONING AND CHARACTERIZATION OF PLATELET-AGGREGATING PROTEINS FROM TRIMERESURUS ALBOLABRIS VENOM. THESIS ADVISOR: ASSOC. PROF. PONLAPAT ROJNUCKARIN, 84 pp. ISBN 974-17-3575-8

Crotaline venoms contain a variety of C-type lectin-like proteins that participate in hematotoxicity. Previously characterized lectins show conserved folds with several disulfide bridges. The structures are composed of $\alpha\beta$ hetero-oligomers linked by interchain disulfide bonds. They may enhance or inhibit platelet-ligand bindings, as well as inhibition of clotting factor IX and X.

In previous studies, the platelet glycoproteinIb-binding protein from *Trimeresurus albolabris* named alboaggregin B (AL-B), has been purified and sequenced. However, there is no report of the cDNA sequences of AL-B. The aim of this thesis is to analyze the full-length sequence of AL-B α and β subunits from the cDNA library of *T. albolabris* venom gland. Moreover the protein has been purified and characterized. Based on partial sequence of the primary library, oligonucleotide primers were designed and used to clone full-length cDNA encoding the C-type lectin subunit by 5′-RACE method. Additional homologous subunit was obtained using 3'-RACE. Analysis of the full-length nucleotide sequence found that the coding region of AL-B α subunit was 468 bp and βsubunit was 438 bp with the deduced protein sequence of 133 and 123 amino acid residues respectively, including 23 residues signal peptides. The nucleotide sequence of AL-B α and β subunit showed 85.25 % and 79.45 % amino acid sequence identity with stejaggregin from *T. stejnegeri*, respectively. In addition, by sequence comparison a conserved motif in AL-B β (SRTY) that may be responsible for platelet aggregation activity was found. Furthermore, AL-B protein was purified from the snake venom and confirmed to be identical to the cloned protein using the MALDI TOF Mass spectroscopy. AL-B agglutinated fixed human platelets with the EC50 of 180 nM and was completely inhibited by anti GPIb antibody. To our knowledge, this is the first report of cDNA cloning of C-type lectin-like protein from *T. albolabris*.

Field of study ___Medical Science___ Student's signature ...Jaradpong Arpijuntarangkoon...

Academic year ______2005________ Advisor's signature...Ponlapat Rojnuckarin...

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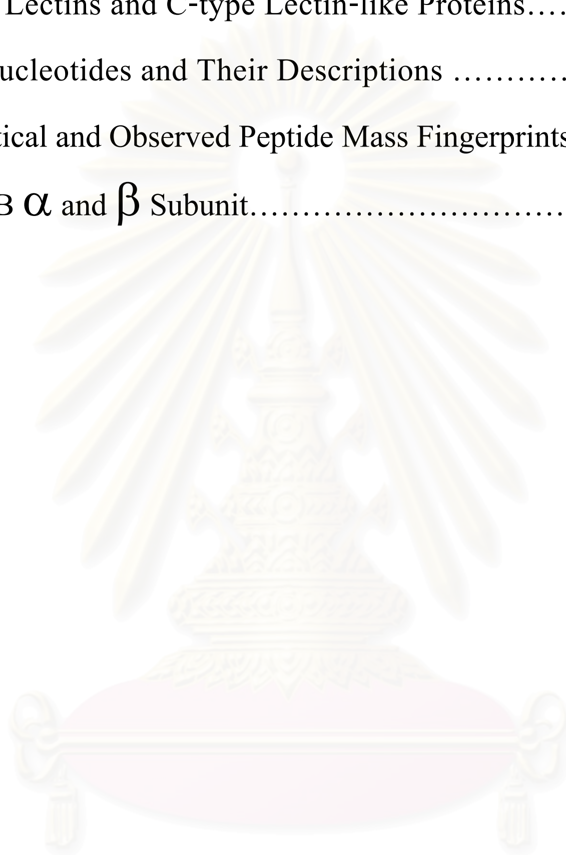
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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ADP	adenosine diphosphate
ATP	adenosine triphosphate
bp	base pair
BCA	bicinchoninic acid
$^{\circ}C$	degree Celsius
cDNA	complementary deoxyribonucleic acid
CCA	alpha cyano cinnamic acid
CD	cluster of differentiation
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
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	isopropy1-β-D-thiogalactopyranoside
Kb	kilobase
kDa	kiloDalton
L	Liter
LB	Luria-Bertani media
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
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

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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

Viper snakebites still pose public health problems worldwide. In Thailand, green pit viper (*Trimeresurus albolabris* or *Trimeresurus macrops*), a crotaline viper, accounts for 40% of all bites (Viravan et.al, 1992). In addition, green pit viper is most widely distributed and comprises more than 95% of venomous snakebites in Bangkok (Meemano et.al, 1987 Mahasandana and Jintakune, 1990). As we invaded their habitat, green pit viper adapted to live with humans. Consequently, more than 400 new cases of bites came to Chulalongkorn hospital each year. Most of them were bitten in Bangkok or nearby regions, despite extensive urbanization.

Green pit viper venom causes hypofibrinogenemia and thrombocytopenia that may result in systemic bleeding (Mitrakul and Impun, 1973 Mitrakul, 1973 1982 Mahasandana et.al, 1980 Rojnakarin et.al, 1999). In some patients, severe thrombocytopenia occurs with relatively normal clotting time suggesting separate destructive mechanisms of fibrinogen and platelets. While the cause of hypofibrinogenemia was found to be the thrombin-like and fibrinolytic effects of venom, the pathogenesis of thrombocytopenia remains to be determined. Platelet-aggregating agents in the 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 proteins. Although these proteins have been purified from crude venom and partially sequenced at the protein level, cDNA sequences and complete protein sequences have not been reported.

Obtaining the complete cDNA sequences of these proteins will give us more accurate amino acid sequences. *In vitro* activity of lectin proteins purified from Thai green pit viper venom will also determined. These sequence and activity data of platelet activators will be correlated with each other, as well as with data from this and other species. This will give us deeper insights in structure-function relationship of the C-type lectin proteins.

1.1.1 Green pit vipers

Venomous snake in family Viperidae can be divided in to 2 subfamilies, viperinae and crotalinae. Crotalinae has pit organs but viperinae does not. Green pit vipers (GPV) are venomous snake in the family viperidae, subfamily crotalinae. A pit organ is a sensory cavity located between an eye and a nostril. It consists of numerous nerve ending that are sensitive to temperature changes of only 0.002 °C. This property helps GPV to precisely locate the position of preys, such as mice and birds that have high body temperature. GPVs are members of genus Trimeresurus. The two most common species of Trimeresurus in Thailand, especially in the middle part including Bangkok, are *Trimeresurus albolabris* and *T. macrops* (Mahasandana and Jitakune, 1990).

T. albolabris or white-lipped pit viper is a medium sized snake. The average length of male is approximately 50 centimeters, while female is 70 centimeters, It has a triangular head that looks different from other nonvenomous species. The head is distinguishly larger than the neck. The head and body are light green. Ventrum is yellow or white. Some snakes show white lateral lines along the bodies. Notably, they have brilliantly red tail. Most have yellow eyes but some have red. It can hunt on trees. The preys are rats, birds, lizards or small amphibians. They are oovivorous with approximately 7-15 offsprings per litter.

T. albolarbris is the most important and dangerous of the GPV group. Although the death rate of patients who were bitten is low, the number of victims as compared with that of other vipers is relatively high (approximately 34.8%).

1.1.2 Clinical signs and symptoms of green pit viper bites

Snake venoms can be classified by their effects into three categories: neurotoxin, myotoxin and hematotoxin. Green pit viper venom affects hemostatic system. Similar to those of other venomous snakes, the toxic symptoms can be divided into two categories: local and systemic effects. The local effects start to appear within 30 to 60 minutes after pit viper envenomation. These effects include pain, edema, blister, hemorrhage and ecchymosis at the bite sites (Trishnanaada, 1979). The systemic effects include coagulopathy, hypofibrinogenemia and thrombocytopenia (Rojnuckarin et al., 1996, 1999, Ferrer, 2001). Green pit viper bites may also result in hypotension, respiratory distress, or severe tachycardia.

1.1.3 Components of green pit viper venom

GPV venom contains a variety of proteins with biological activities and many of these proteins belong to a few proteins families, such as serine proteinases that contain the catalytic triad (histidine, aspatate and serine) and affect coagulation factors and fibrinolytic system; phospholipases A_2 that induce a wide variety of pharmacological effects by interfering with several physiological functions, including endothelial injury, myotoxicity, thrombosis and hemostasis; metalloproteinases-disintegrin that is also termed catalytic hemorrhagic proteins, and C-type lectins that affect platelets and coagulation factors.

The C-type animal lectin superfamily is defined by a sequence motif, Carbohydrate- Recognition Domain (CRD) that binds carbohydrates (*i.e.* lectin) in a Calcium-dependent manner (*i.e.* C-type). They can be classified in to seven groups (I to VII) base on their structural characteristics. (Weis et al., 1998 Drickamer, 1999)

Group I (a proteoglycan core peptide and a single C-type lectin domain in the vicinity of the C-terminus), **group III** (collectins, which participate in the host defense mechanism through complement activation), and **group VII** (found in pancreas and snake venoms) are soluble proteins, containing one isolated CRD. (Tomohisa et al, 2005)

Group IV (selectins, which mediate the adhesive interaction between leukocytes and vascular endothelial cells) and **group VI** (macrophage cell surface mannose receptors and DEC-205, a dendritic cell surface molecule) are type I membrane proteins (an extra cellular region consisting of a cystein-rich domain, a fibronectin type II domain, and tandem CRDs followed by a transmembrane domain and a carboxyl terminal cytoplasmic domain) (Tomohisa et al, 2005)

Group II (asialoglycoprotein receptor of hepatocytes) and **group V** (natural killer cell receptors and the low affinity IgE receptor CD23) are type II membrane proteins (an extracllular carboxyl terminus, a single transmembrane domain and a cytoplasmic amino terminus) (Stockert, 1995 Lanier, 1998 Kijimoto-Ochiai, 2002 Tomohisa et al, 2005)

Snake venom C-type lectin-like proteins are in the group VII animal lectins containing conserved folds and several disulfide bridges. The simplest structure of lectins is composed of $\alpha\beta$ heterodimers linked by an interchain disulfide bond (Fig. 1). Higher multimers or $(\alpha\beta)_n$ are present in some proteins (Fig. 2). In addition, structures may be more complex, e.g. heterotetrameric $(\alpha_1\alpha_2\beta_1\beta_2)$. Each subunit is translated from one gene. Therefore, one lectin protein derives from multiple genes.

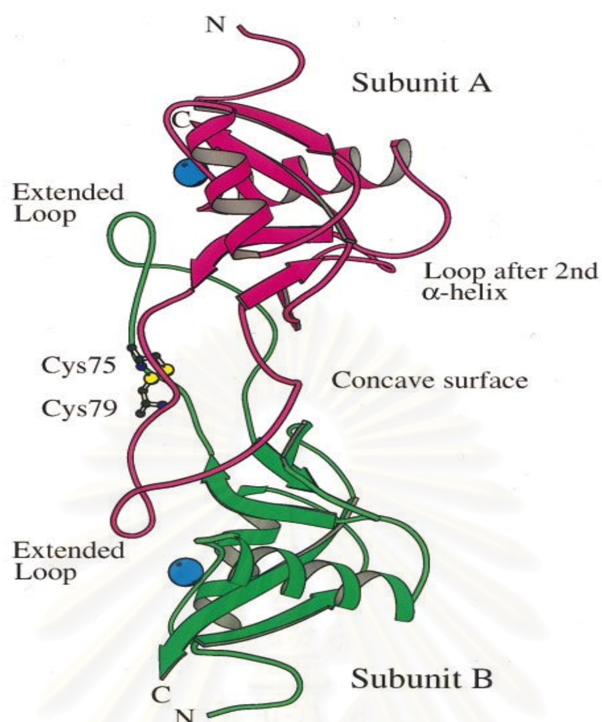

Figure 1 A 3-D structure of a heterodimeric $\alpha\beta$ C-type lectin

(Factor IX Binding Protein from Habu snake: Mizuno, et al 1999)

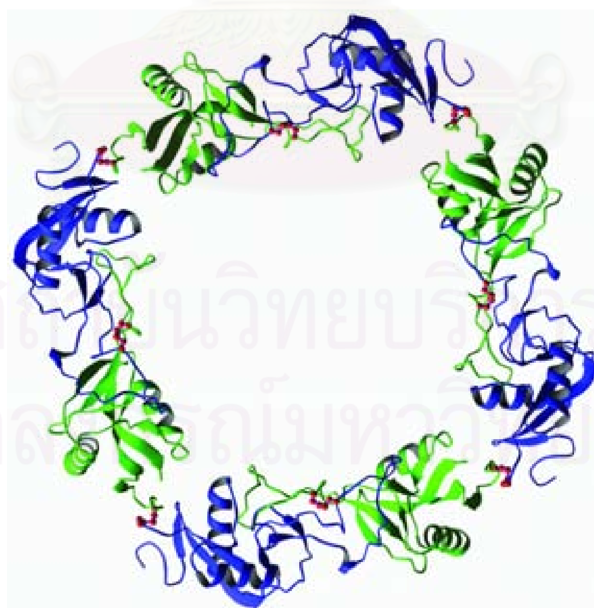

Figure 2 A 3-D structure of a higher multimeric $(\alpha\beta)_4$ C-type lectin

(Convulxin from *Crotalus durissus terrificus*: Muramaki et al., 2003)

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1.2 Research Questions

What are the complete cDNA sequences and characteristics of platelet-aggregating C-type lectin-like proteins from *T. albolabris*?

Is there any conserved motif that may contribute to their function?

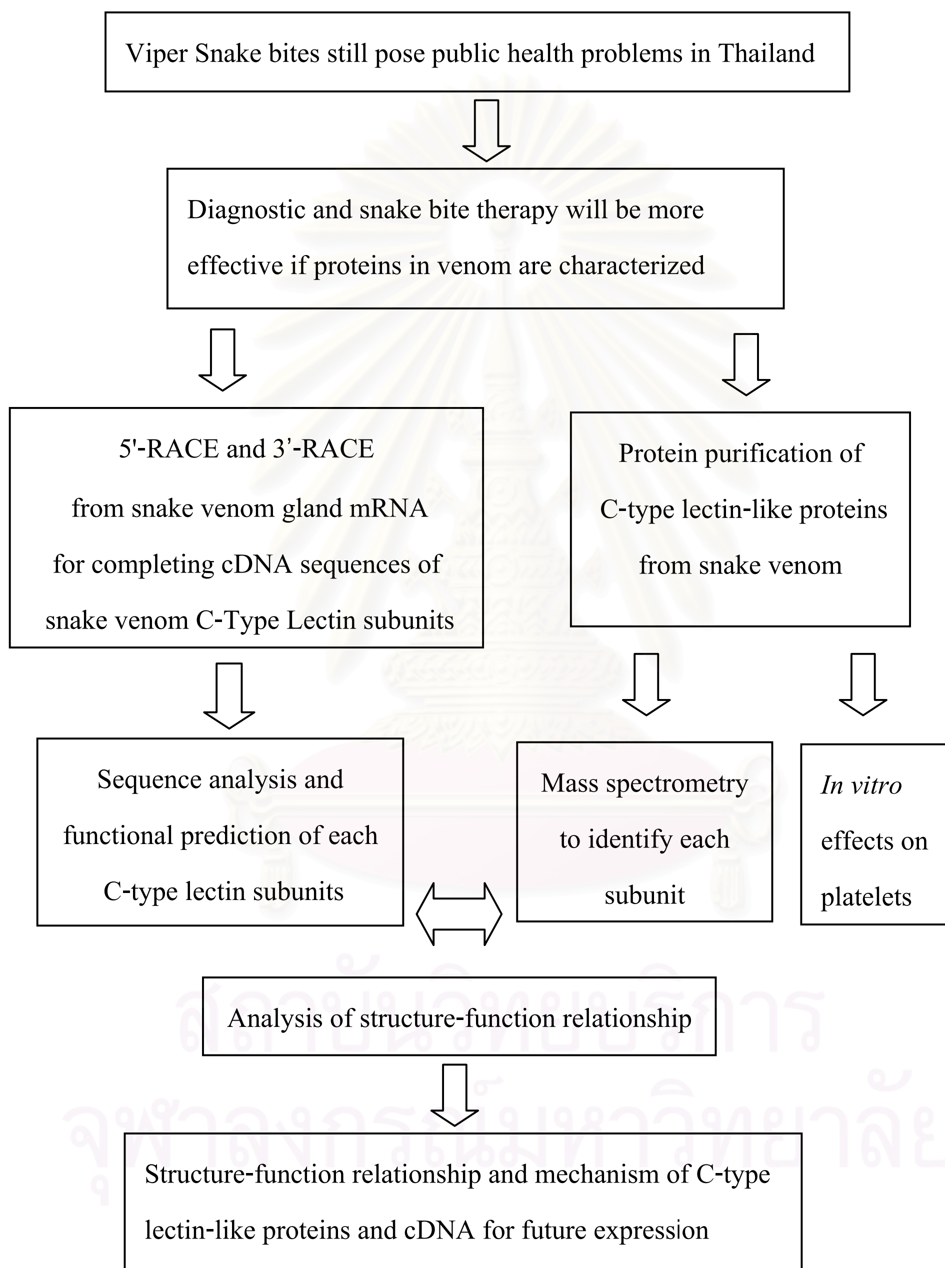
1.3 Objectives of the Study

1. Molecular cloning and sequence analysis of the platelet aggregation C-type lectin-like proteins from green pit viper (*T. albolabris*) venom
2. Purification and characterization of their effects on human platelets.

1.4 Keywords

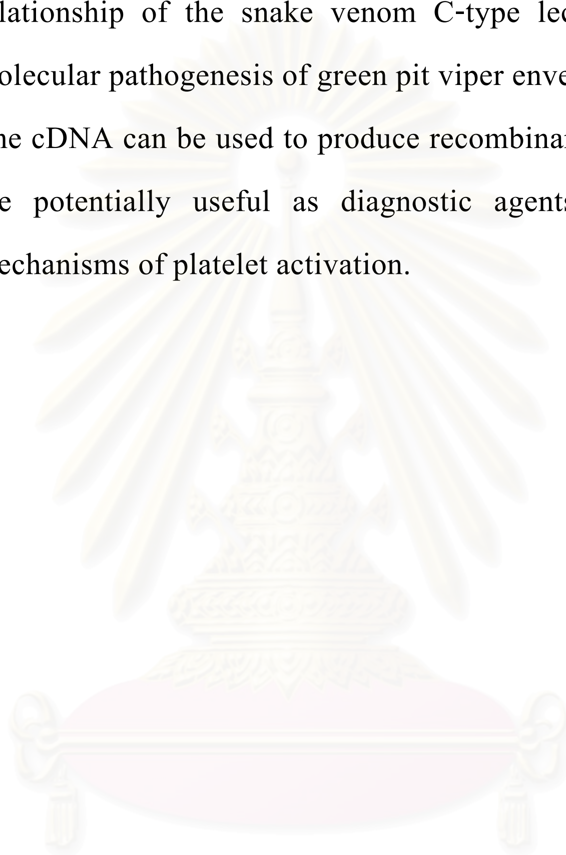
C-type lectin-like proteins, Platelet aggregation, *T. albolabris*

1.5 Conceptual Framework



1.6 Benefits and Applications

1. The study will give us deeper insights in the structure-function relationship of the snake venom C-type lectin-like proteins and the molecular pathogenesis of green pit viper envenomation.
2. The cDNA can be used to produce recombinant proteins. These proteins are potentially useful as diagnostic agents or reagents to dissect mechanisms of platelet activation.



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CHAPTER II

LITERATURE REVIEW

2.1 The C-type lectin proteins from snake venom.

Snake venoms C-type lectin proteins containing carbohydrate recognition domain (CRDs), Ca^{2+} dependent, sugar (galactose) binding domains are generally found in homodimeric or homooligomeric forms, such as galactose-specific C-type lectin from the venom of the rattlesnake (*Crotarus atrox*) (Hirabayashi et al., 1991), B. jararaca lectin from *Bothrops jararaca* (Ozeki et al., 1994), BjcuL from *Bothrops jararacussu* (de Carvalho et al., 2002), PAL from *Bitis arietans* (Nikai et al., 1995), LmsL from *Lachesismuta stenophyrs* (Aragon-Ortiz et al., 1996), HHL from *T. okinavensis* (Nikai et al., 2000), and TSL from *T. stejnegeri* (Xu et al., 1999). Although highly homologous to C-type lectin proteins, the C-type lectin like proteins (CLPs) differs in their physiological activities. CLPs show typical folds similar to classic C-type lectins and display a variety of biological properties, including anticoagulant, procoagulant, and agonist or antagonist of platelet activation. However they lack carbohydrate-binding activity (Tomohisa et.al, 2005).

2.2 Snake CLPs: Characteristics and Relationships

CLP activities on hemostatic system are diverse. They may enhance or inhibit platelet-ligand bindings or platelet aggregation, as well as inhibition of clotting factor IX, X or thrombin. The main target proteins of snake venom lectins are the von Willebrand factor (vWF) receptor and collagen receptors on platelet surface. These receptors not only mediate platelet adhesion to subendothelium, but also co-operatively activate signal transduction in platelets upon ligand binding, resulting in platelet aggregation. For example, stejnulxin from *T. stejnegeri* (Chinese green viper)

(Wen-Hui Lee et al., 2003), convulxin, a hetero-octamer lectin, $(\alpha\beta)_4$, from *Crotalus durissus terrificus* (Tropical rattlesnake) (Polgar et al., 1997 Leduc et al., 1998 Kanaji et al., 2003 Muramaki et al., 2003) and ophioluxin from *Ophiophagus hannah* (King cobra) (Du et al., 2002) induce platelet aggregation by acting via glycoprotein VI (GpVI), a collagen receptor on platelet surface resulting in activation of Src kinases (Cicmil et al., 2000), phosphorylation of the Fc receptor γ chain, and activation of $p72^{SYK}$ (Polgar et al., 1997). Alboluxin and alboaggregin A from *T. albolabris* (Green pit viper) (Peng et al., 1992 Andrews et al., 1996 Kowalska et al., 1998 Dormann et al., 2001) induce platelet aggregation via both Gp VI and glycoprotein Ib (GpIb), a part of the Gp Ib-IX-V complex that is the platelet vWF receptor. Mamushigin from *Agkistrodon halys blomhoffii* (Mamushisnake) (Sakurai et al., 1998), alboaggregin B from *T. albolabris* (Peng et al., 1991 Kawasaki et al., 1998) and TSV-GPIb-bp from *T. stejnegeri* (Lee and Zhang, 2003) can bind to platelet Gp Ib and directly aggregate platelets. On the other hand, flavocetin A and B, high-molecular-weight lectins from *T. flavovirides* (Habu snake) (Taniuchi et al., 1995 Fukada et al., 2000), agkicetin C and agglucetin from *A. acutus* (Hundred-pace pit viper) (Chen and Tsai, 1995 Chen et al., 2000 Wang et al., 2003), CHH-A and B from *Crotalus horridus horridus* (Andrews et al., 1996), mucetin and mucrocetin from *T. mucrosquamatus* (Wei et al., 2002 Lu et al., 2004 Huang et al., 2004), lebecetin from *Macrovipera lebetina* (Sarray et al., 2001, 2003, 2004), tokaracetin from *T. tokarensis* (Kawasaki et al., 1995), purpureotin from *T. purpureomaculatus* (Li et al., 2004) and echicetin from *Echis carinatus* (Saw-scaled viper) (Polgar et al., 1997b Navdaev et al., 2001b) can bind platelet Gp Ib and directly block vWF binding and, hence, inhibit platelet aggregation. Interestingly, when echicetin is multimerized by IgM, platelet will be activated suggesting the higher multimer structure may crosslink receptors together resulting in activation instead of inhibition. Bothrocetin from *Brothrops*

jararaca (Jararaca snake) (Usami et al., 1993 Sen et al., 2001 Fukada et al., 2002) binds vWF and activates its function in agglutinating platelets via Gp Ib. This has a potential role as a diagnostic reagent for von Willebrand disease, a common disease characterized by lacking vWF. Bitiscetin from *Bitis arientans* (Puff adder) (Hamako et al., 1996 Hirotsu et al., 2001 Maita et al., 2003) binds both Gp Ib and vWF and enhances their interaction, resulting in platelet activation. Bilinexin from *Agkistrodon bilineatus* (Mexican moccasin) (Du et al., 2001) agglutinates platelets using both Gp Ib and integrin $\alpha_2\beta_1$, another collagen receptor on platelet. Rhodocetin from *Calloselasma rhodostoma* (Malayan pit viper) (Wang et al., 1999 Eble and Tuckwell, 2003) and EMS16 from *Echis multisquamatus* (Central Asian sand viper) (Marcinkiewicz et al., 2000 Horii et al., 2003) inhibit collagen- induced platelet aggregation by blocking collagen binding to integrin $\alpha_2\beta_1$.

On the other hand, several lectins can inhibit coagulation factors. TSV-factor IX binding protein (FIX-BP) from *T. stejnegeri* (Lee et al., 2003b), AHP IX-BP from *A. halys pallas* (Zang et al., 2003), and Habu FIX-BP isolated from *T. flavovirides* (Morita et al., 1996) inhibited clotting factor IX. Jararaca IX/X-BP from *B. jararaca* (Sekiya et al., 1993), Habu FIX/X-BP isolated from *T. flavovirides* (Atoda et al., 1995 Mizuno et al., 1999), halyxin from *A. halys brevicaudus* (Koo et al., 2002),and ECLV from *Echis carinatus leucogaster* (Chen and Tsai, 1996) can bind and inhibited the activity of blood coagulation factor IX/X. Furthermore, Bothrojaracin from *Bothrops jararaca* (Zingali et al., 1993 Monteiro et al., 2001 Monteiro and Zingali., 2002) binds to both positively-charged sites of thrombin, the exositeI and exositeII, and, thus, inhibits thrombin-fibrinogen recognition. These proteins may become novel classes of anticoagulants in the future.

Table1. Functional classification of snake venom C-type lectins and C-type lectin-like proteins.

Type	Target Molecules	Examples: Snake species	Examples: Protein	Subunit structure	PDB Code	References
1. Lectins	Sugar (galactose)	Bj	B.jararaca lectin	Homodimer	-	Ozeki et al. (1994)
		Bs	BjcuL	Homodimer	-	De Carvalho et al. (2002)
		Bia	PAL	-	-	Nikai et al. (1995)
		Ca	RSL	Homodimer	-	Hirabayashi et al. (1991)
			-	-	1MUQ	Walker et al. (2004)
		Lm	LmsL	Homodimer	-	Argon-Ortiz et al. (1996)
		To	HHL	-	-	Nikai et al. (2000)
		Ts	TSL	Homodimer	-	Xu et al. (1999)
2. Platelet aggregation agonists	GPIaIIa	Cr	Rhodocytin	$\alpha\beta$	-	Shin and Morita (1998)
	GPVI/ GPIaIIa	Oh	Ophioluxin	$\alpha\beta$	-	Du et al. (2002a,b)
	GPVI/GPIb	Ts	Stejnulxin	$\alpha\beta_3$	-	Lee et al. (2003a)
		Cd	Convulxin	-	-	Polgar et al. (1997)
			-	-	-	Jandrot-Perrus et al. (1997)
			-	$\alpha_4\beta_4$	-	Leduc and Bon (1998)
			-	-	1UMR	Muramaki et al. (2003)
		Ta	Alboagregin A	$\alpha_1\alpha_2\beta_1\beta_2$	-	Dormann et al. (2001)
	vWF(GPIb)	Bj	Botrocetin	$\alpha\beta$	-	Read et al. (1989)
			-	-	-	Andrew et al. (1989)
			-	-	-	Fujimura et al. (1991)
			-	-	1FVU	Sen et al. (2001)
			-	+vWF-A1	1IJK	Fukuda et al. (2002)
		Bs	Bitiscetin	$\alpha\beta$	-	Hamako et al. (1996)
			-	-	1JWI	Hirotsu et al. (2001)
			-	+vWF-A1	1UEX	Maita et al. (2003)
	GPIb	Ab	Mamushigin	$\alpha\beta$	-	Sakurai et al. (1998)
		Ta	Alboaggregin B	$\alpha\beta$	-	Peng et al. (1991)
			-	-	-	Usami et al. (1996)
		Ts	TSV-GPIb-BP	$\alpha\beta$	-	Lee and Zhang (2003)

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3. Platelet	GPIb	Aa	Agkicetin	$\alpha\beta$	-	Chen and Tsai (1995)
aggregation		Ch	CHH-A/B	$\alpha\beta$	-	Andrews et al. (1996)
antagonists		Ec	Echicetin	$\alpha\beta$	-	Peng et al. (1993)
			-	-	-	Peng et al. (1995)
			-	-	-	Navdaev et al. (2001)
		Tf	Flavocetin A	$\alpha_4\beta_4$	-	Shin et al. (2000)
			-	$\alpha_4\beta_4$cyclic	-	Fukuda et al. (2000)
		Aa	Agglucetin	$\alpha\beta$	-	Wang et al. (2003)
		Tt	Tokaracetin	$\alpha\beta$	1C3A	Kawasaki et al. (1995)
		Tp	Purpureotin	$\alpha\beta$	-	Li et al. (2004)
	GPIaIIa	Cr	Rhodocetin	$\alpha\beta$	-	Wang et al. (1999)
		Em	EMS16	$\alpha\beta$	-	Marcinkiewicz et al. (2000)
			-	-	-	Okuda et al. (2003)
			-	-	-	Horii et al. (2003)
		Ba	Bothroalternin	$\alpha\beta$	-	Castro et al. (1998)
		Bj	Bothrojaracin a/b	$\alpha\beta$	-	Zingali et al. (1993)
			-	-	1UKM	Arocas et al. (1997)
4. Anti	Factor IX/X	Ah	Halyxin	$\alpha\beta$	-	Koo et al. (2002)
coagulant		Bj	jararacaIX/X-BP	$\alpha\beta$	-	Sekiya et al. (1993)
proteins		Ec	ECLV IX/X-BP	$\alpha\beta$	-	Chen and Tsai (1996)
		Tf	habuIX/X-BP	$\alpha\beta$	1IXX	Atoda and Morita (1993)
	Factor IX	Ap	AHP-IX-BP	$\alpha\beta$	-	Zang et al. (2003)
		Tf	Habu IX-BP	$\alpha\beta$	1BJ3	Atoda et al. (1998)
		Ts	TSV-FIX-Bp	$\alpha\beta$	-	Lee et al. (2003)
	Factor X	Aa	ACP	$\alpha\beta$	-	Tani et al. (2002)
		Da	X-BP	-	1IDO	Atoda et al. (1998)

Abbreviations: BP, binding protein; Ah, *Agkistrodon halys brevicaudus*; Ap, *A. halys Pallas*; Ab, *A.halys blomohoffi*; Aa, *A. acutus*; Bj, *Bothrops jararaca*; Bs, *B. jararacussu*; Ba, *B. alternatus*; Bia, *Bitis arietans*; Ca, *Crotalus atrox*; Ch, *C. horridus*; Cd, *C. durissus terifficus*; Cr, *Calloselasma rhodostoma*; Da, *Deinagkistrodon acutus*; Ec, *Echis carinutus leucogaster*; Em, *E. multisquamatus*; Lm, *Lachesismuta stenophyrs*; Oh, *Ophiophagus hannah*; Ta, *Trimeresurus albolabris*; Tf, *T. flvoviridis*; Tm, *T. mucrosquamatus*; To, *T. okinavensis*;Tp, *T. purpureomaculatus*; Ts, *T. stejnegeri*; Tt, *T. tokarensis*.

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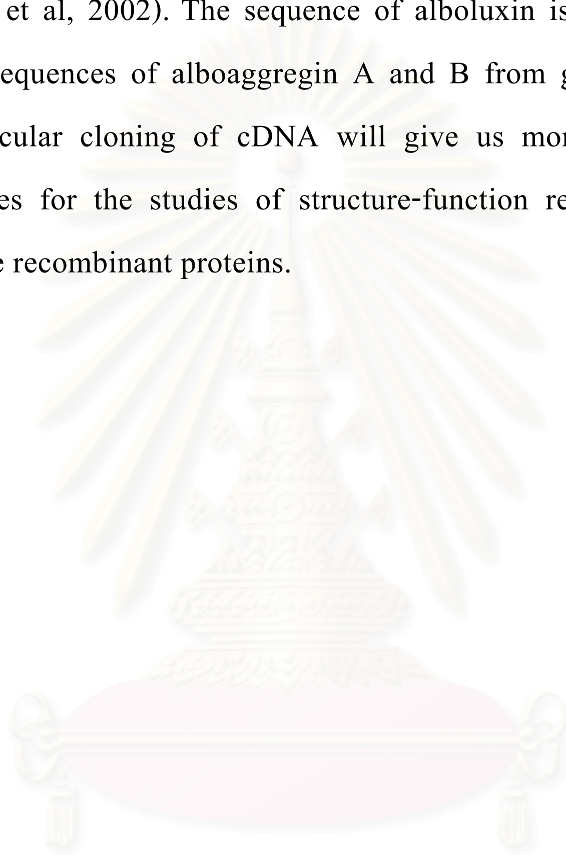
Three dimensional (3-D) structures of several lectins have been solved using X-ray crystallography. The basic structure is a dimer providing two convex surfaces, intervened by the interface between the dimer providing one concave surface (Figure1). The interface between two subunits is formed by a domain swap, *i.e.* a loop of one subunit protrudes into the other and *vice versa*. This concave interface serves as a binding site for various lectin target proteins including platelet Gp Ib, vWF and clotting factor (Morita T et al, 1996 Batuwangala T et al, 2003). Due to the availability of 3-D structures and sequence of various lectins, structure-function analysis is possible for our cloned genes by comparison among homologous viper venom proteins. This will give us not only insights in the molecular mechanisms of platelet activation, but also preliminary data for future recombinant protein expression by designing lectins with desirable functions for clinical or research uses.

In previous studies, platelet-aggregating proteins, alboaggregin A, B and C, have been purified from *T. albolabris* venom. They are oligomeric protein of the C-type lectin family. Both alboaggregin A, a 50 kDa heterotetramer and alboaggregin B, a 25 kDa heterodimer, bind to platelet membrane glycoprotein Ib-IX-V causing platelet agglutination (Peng et al., 1992). Alboaggregin A has been demonstrated to be a stronger platelet activator of the two with EC_{50} of 10 nM, compared with 200 nM of alboaggregin B. The mechanisms of action of alboaggregin A have recently been shown to activate the collagen receptor, glycoprotein VI in addition to Gp Ib binding. This contributes to tyrosine phosphorylation of signaling molecules in platelets leading to platelet shape change and aggregation mediated by glycoprotein IIb/IIIa and fibrinogen (Kowalska MA et al, 1998 Dormann D et al, 2001 Asazuma N et al, 2001).

The *in vitro* activity, as well as the protein sequence, of alboaggregin C, a 121 kDa protein, is still poorly defined. Recently, alboluxin, a novel 120 kDa multimeric protein, $(\alpha\beta)_3$, was purified from *T. albolabris* venom. It was found to be potent platelet activator acting via both Gp Ib and Gp VI on platelet, similar to alboaggregin A (Du Xiao-Yan et al, 2002). The sequence of alboluxin is still unknown. Although partial protein sequences of alboaggregin A and B from green pit viper 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.



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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, BKK, Thailand

Table 2 Oligonucleotides 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

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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

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3.1.2 Proteins Detection

Sodiumdodesylsulphate 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.

3.1.3 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

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

3.1.5 Protein Quantitative Assay

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

3.1.6 Activity Assay

Creatine phosphokinase was purchased from Sigma, MO, U.S.A.

Monoclonal antibody GPIb clone SZ2 from Immunotech, Radiová 1, France

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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 was used for generating full length cDNA. The templates of 5′-RACE-PCR are 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 *Trimeresurus 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 incubator. After agarose gel was completely dissolved, the tube was centrifuged at 20,000 x 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

10 μl ligation reaction was added to a sterile falcon tube Cat. #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 are 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 *T.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-fix platelets was collected for further purification.

3.2.2.2 Ion-Exchange Chromatography

Proteins fraction 68-72 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 washed formalin-fix 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 Sodiumdodecylsulphate 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 $^{\circ}$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 In-Gel Digestion

The preparation of trypsin-digested proteins for mass spectrometric sequencing is according to the book of Protein Sequencing and Identification Using Tandem Mass Spectrometry (Kinter M. and Sherman E.N.). The gel was rinsed with water. Bands of interest are excised using clean scalpel cutting as close to the edge of the spot or band as possible. It was important to reduce the volume of "background" gel. The excised bands were chopped into very small pieces (ca. 1x1 mm.). Gel particles were transferred into a microcentrifuge tube (0.5 ml or 1.5 ml eppendorf). The gel particle was washed with 100-500 μl of water (5 min, ca. 2-3 times) and spinned down to remove the liquid. Acetonitrile (ACN)/0.1M NH_4HCO_3 (1:1, ca. 3-4 times equal the volume of gel pieces) was added. The gel pieces will be shrunk,

become white and stick together. The gel particles were spinned down to remove all liquid. The gel particle was dried in a vacuum centrifuge.

The gel pieces were swelled in 10 mM dithiotriol/0.1M NH_4HCO_3/1mM EDTA by adding the liquid enough to cover gel and incubating for 45 min at 60°C to reduce the proteins. In-gel reduction was recommended even if proteins were previously reduced. After incubation with iodoacetamide/0.1M NH_4HCO_3 for 30 min at room temperature in the dark, iodoacetamide solution was removed and the gel particles were washed with 150-200 µl of 0.05 M Tris-HCl pH 8.5/50% acetonitrile (ca. 3-4 times). Gel particles were rehydrated in the 180 µl digestion buffers (containing 100 µl of 0.1 M Tris-HCl, pH 8.5, 2 µl of 100 mM $CaCl_2$, 20 µl of ACN and 78 µl of distilled water) and 20 µl of trypsin solution. Then, incubate overnight at 37°C. After that, 100 µl of supernatant was kept in a microcentrifuge tube and 100 µl of 2% trifluroacetic acid (TFA) was added and incubated for 30 min at 60°C. After incubation, supernatant was combined and dried in a vacuum centrifuge. 30 µl of digestion buffer was added to the tube containing the gel pieces, incubated for 10 min at 30°C and sonicated for 5 min. Supernatant was removed and combined. 5% formic acid/ACN was added to gel, incubated for 10 min at 30°C and sonicated for 5 min. All supernatant was combined in a tube and dry in a vacuum centrifuge.

3.2.4.2 Sample preparation for Mass spectrometry

Peptide and proteolytic digests from the In-gel digestion were desalted and purified by a microscale reversed phase cartridge. The first step was to clean the cartridge with acetonitrile (ACN) 500 µl and then equilibrate with 0.1% formic acid 300 µl. Each sample was dissolved in an aqueous acidic solution (0.1 %(v/v) formic acid) and loaded on the column. The salt was eluted with 100 to 300 µl of ACN: water

(20:80). The peptide was eluted with 0.1% (v/v) formic acid in ACN: water (80:20, 75μl).

3.2.4.3 MALDI-ToF Mass spectrometry

Mass measurements were carried out on a Biflex (Bruker, Germany) matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-ToF). The dried samples were dissolved in 0.1% TFA and vortexed. The dried droplet method was used for MALDI-MS sample preparation. A saturated solution of α-cyano-4-hydroxycinnamic acid (CCA) in 50% ACN/0.1%TFA was used as a matrix. For peptide sample, 1 μl of sample solution was mixed with 5 μl of a matrix solution. Then, 1 μl of the mixture solution was spotted on target and allowed to dry at room temperature. MALDI peptide spectra were performed in reflectron mode and internally calibrated with CCA ($\{2M+H\}^+$, 379.09) and ubiquitin ($\{M+2H\}^+$, 4283.45) in oxidized form. The resulting peptide mass fingerprints (PMFs) were compared with calculated peptide fragment (digested by trypsin) of proteins conceptually translated from molecular cloning method.

3.2.4.4 Activity Assay

3.2.4.4.1 Preparation of formalin-fixed platelets.

Two bags of platelet concentrate from the blood bank department of Chulalongkorn hospital were used to prepare washed formalin-fixed platelets. The platelet-rich supernant was centrifuged at 180 x g for 1 minute, 1942 x g for 15 minutes. Platelets were resuspended in 30 ml washing buffer, 300μl heparin 500 U, 300μl CP/CPK, pH 7.35 (buffer I), incubate 37°C for 10 minutes, centrifuged at 650 x g for 1 minute, 1942 x g for 15 minutes. Platelets were resuspended in 20 ml washing buffer, contain 200μl CP/CPK (buffer II) and incubated at 37°C for 10 minutes, add

2% formaldehyde 20ml and incubated for 1 hour, or overnight at 4°C. Platelets were then washed three time in plain tyrode (PT), centrifuged at 1942 x g 15 minutes, and resuspended in 20ml PT followed by adding 2% sodium azide 350µl/ml, and storing at 4°C.

3.2.4.4.2 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 supernant was centrifuged at 1942 x g for 10 min, and the platelets were washed once more with buffer B. Washed platelets were resuspend 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.

3.2.4.4.3 Platelet Aggregation Assay

1 ml of stored fixed platelets are resuspended in 10ml plain tyrode (PT), centrifuged at 1942 x g for 15minutes to remove sodium azide. The platelet count was adjusted to 350,000 platelet/µl. Purified C-type lectin-like proteins was added to the platelets and incubated with continuous magnetic stirring at 37 °C. Platelet aggregation was measured by determining the change in light transmission with platelet aggregation chromogenic kinetic system (PACKS-4, Helena Laboratories, USA).

3.2.4.4.4 Effects of monoclonal anti GPIbα antibody.

Washed human platelets 450 μl (350,000 platelet/ml) were incubated with different concentration of monoclonal anti-GPIbα antibody clone SZ2 at 37°C for 2 min, then fixed concentration of AL-B was add (10 μg/ml, final concentration). Platelet aggregation was measured by determining the change in light transmission with platelet aggregation chromogenic kinetic system (PACKS-4).

CHAPTER IV

RESULTS

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

4.1.1 5′-RACE

Two partial cDNA sequences of C-type lectin like protein (CLPs) clones derived from primary cDNA library (unpublished) of *Trimeresurus albolabris* venom gland (Fig. 3), were used to design primers (041 and 051) in order to obtain complete cDNAs encoding CLPs using 5′-RACE technique (Fig. 4). The products showed smear patterns on gels suggesting that they were consisted of several different genes. The 5′-RACE products, which were about 600-700 base pairs in sizes, were eluted and subcloned into pGEM®-T easy vector and transformed in to *Escherichia coli*, JM109. The positive clones were identified by white colonies using blue-white color selection system (Fig. 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 (Fig. 6). The inserts wre varying in sizes indicating that there were different products. Subsequently, the plasmids containing inserts 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 CIUSTAL 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-lengh cDNA of β

subunit of alboaggregin B (AL-B) was obtained from 5'RACE using 051 lectin primer. All sequences are not been previously reported.

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 clone041 and clone051 were analyzed and used for designing a primer (common lectin primer). Subsequently 3′-RACE technique was used in order to obtain other CLPs. 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). Eight clones were related to α subunit and nine clones were homologous to β subunit of another alboaggregin. Moreover we found that in the group β subunit of AL-B, there were 2 polymorphisms; which were different at 3 base pairs positions. Two were silent, but the other was a difference in one conceptual protein translation (K instead of Q, Figure 12).

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Partial cDNA of 041

```
CATGAGCAGCGAATAGCGGCATCTGTCTTATCGAAGCGCCGAGAAGCAGCTTCGG
GCCCAGTGTTTCTGAGACATACAGAACACCAAAGCTATGTTGATCGGCTGAGGTT
CAAGGCAAGAAACAATGCACCTCGGAGTGGAGCGATGGCTCCAGCGCAGTTATGA
GACCTGATTGATGCAGAATCCAAAACGTGTCTTGGCTGGACCAAGAGACAGAGTT
TCGTAAGTGGGTCAATATTTACTGGGACTACAAAATCCATTCGTTTGCGAGGCAT
AGTCCCGAAGGTCCAGCTGAGTGAAGTCTGGAGAAGCAAGGAAGACCCCCACCCA
CCCCCACCCCCCACCTGCCGCAATCTCTGCTCTGCCCCCTTCGCTCAAGGGATGC
TCTCTGTAGCTGGATCTGGTTTTGCTGCTCCTGATGGGCCAGAAGGTCCAATAAA
TTCTGCCTAGCAAAAAAAAAAAAAAAAAA
```

Partial cDNA of 051

```
GAATTCGGCACGAGGCCTCGTGCCGAATTCGGCACGAGGCAGCCACCTGCTCTCC
TTCCACAGCAGTGAAGAAGTAGATTTTGTGGTCTCCAAGACCCTCCCAATTCTGA
AAGCGGATTTTGTCTGGATCGGACTCACCGATGTCTGGAGTGCATGCAGGTTGCA
GTGGAGCGATGGCACCGAGCTCAAGTACAACGCCTGGACTGCAGAATCGGAGTGC
ATCGCATCCAAGACAACTGATAACCAATGGTGGACTAGATCCTGCAGCAGGACTT
ACCCTTTCGTCTGCAAGTTGGAGGTATAGTCTGAAGATCCAGCTGTCTGAAGTCT
GGAGAACAAAGAAGACCCCAACTTTACACTCTCCATCCCACACCCCACCCCGCCT
GGCCCAATTTCTGCCCCCTTCGCTCACGGATGCTCTCTGGAACTGG
```

Figure 3 Partial cDNA sequences of 041 and 051 CLPs from the primary library of *T.albolabris* venom gland.

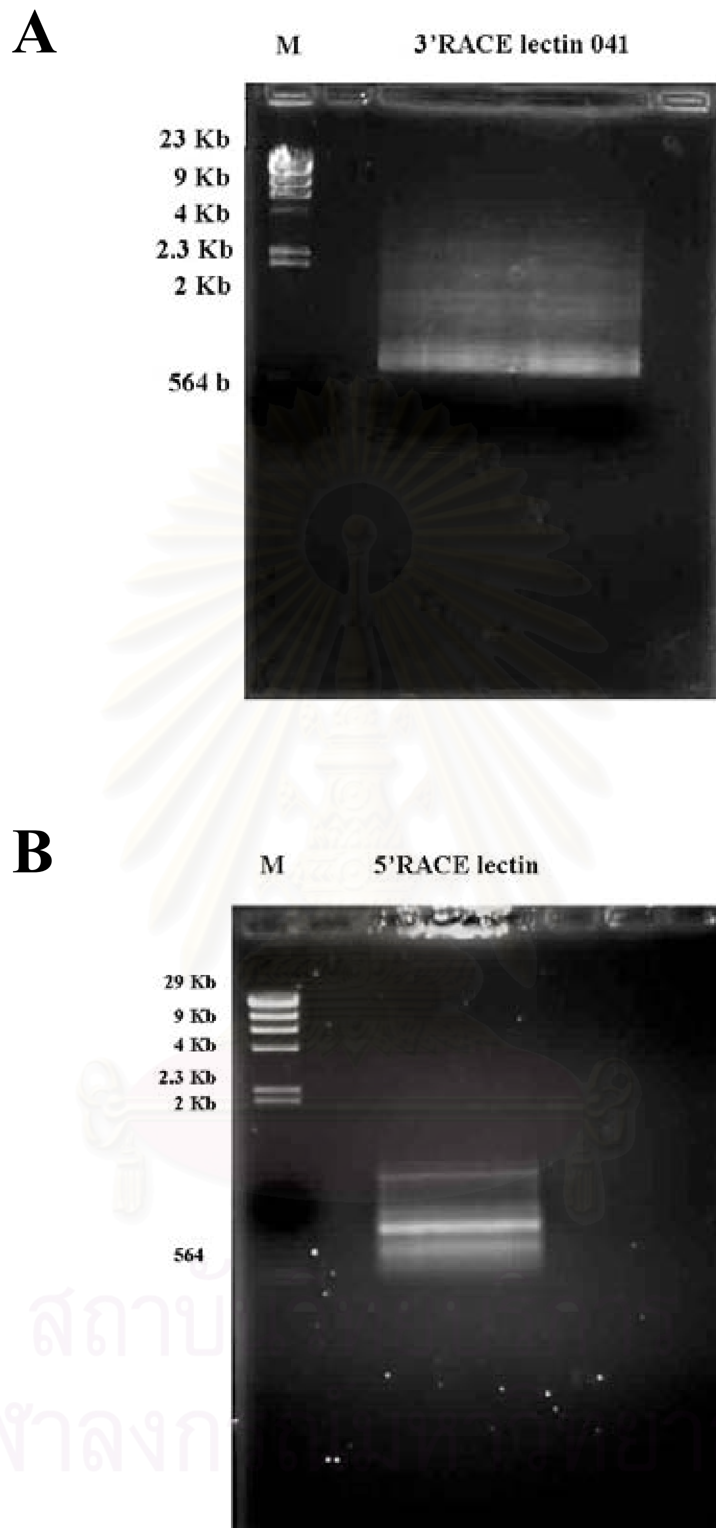

Figure 4 3'-RACE and 5′-RACE products of CLPs electrophoresed on 1.2% agarose gel . Lane 1: molecular weight marker (M) λ *Hin*d III; Lane 2: 3'-RACE (A) and 5′-RACE (B) cDNA product.

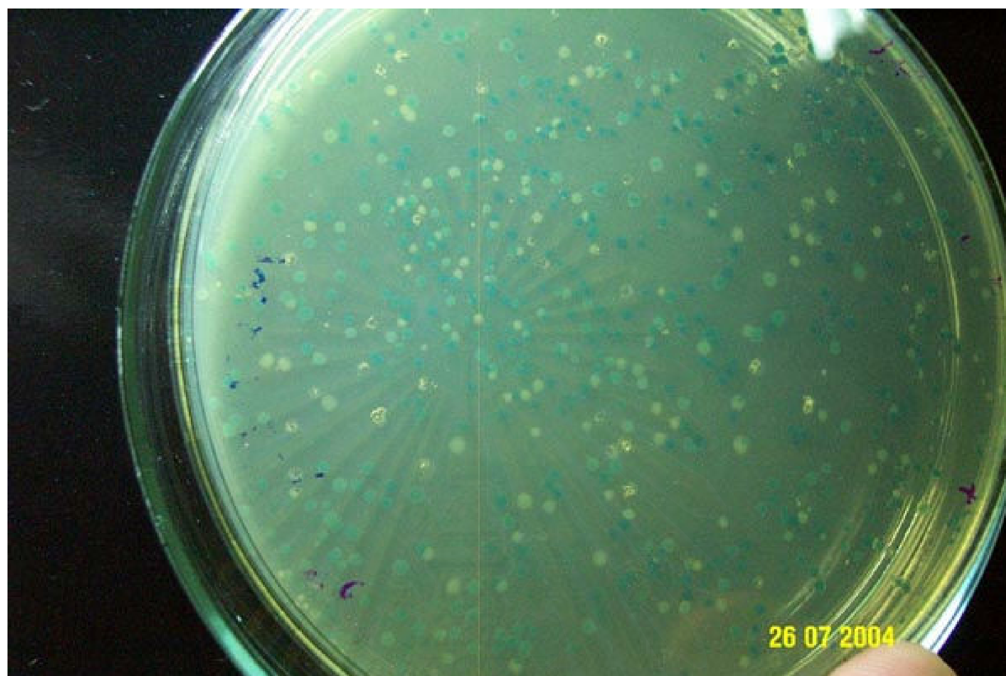

Figure 5 *E. coli*, JM 109, transformed with C-type lectin-like protein cDNA. The positive transformants were white colonies as using a blue-white selection system.

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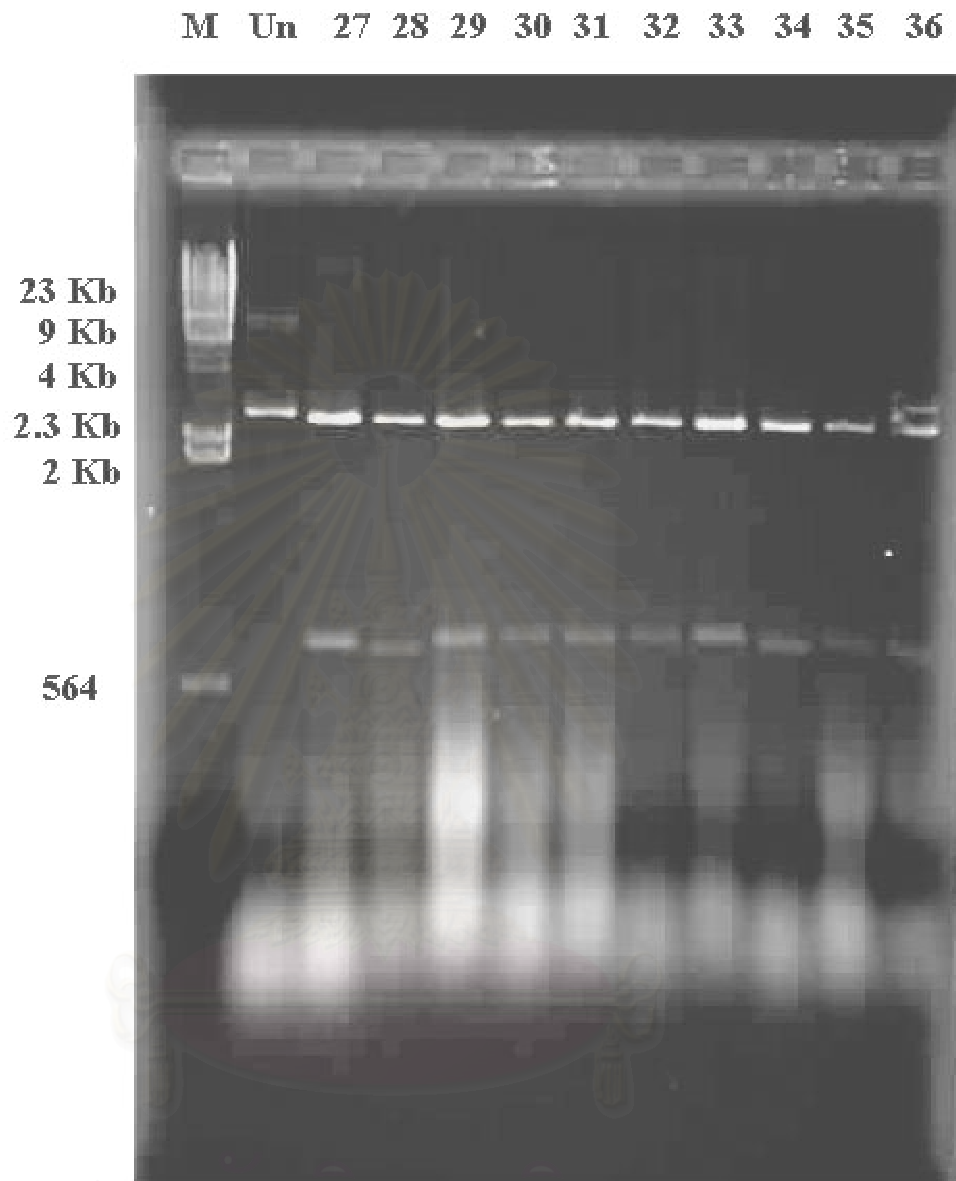

Figure 6 EcoRI digestion of recombinant plasmids clone 27-36 of CLP cDNA. The inserts were varying in sizes. Clone 27- 36 showed the presence of inserts. Un = recombinant plasmid that did not contain insert. M = molecular weight marker λ *Hin*d III.

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4.1.3 Sequence Alignment and Computational Searching Analysis

The insert-positive clones from 3'-RACE and 5′-RACE were sequenced and compared with the GENBANK database. Three cDNA clones (20-Lec, 74-Lec and 84-Lec) encoded for AL-B α subunits and twelve clones (14-Lec, 27-Lec, 47-Lec, 54-Lec, 55-Lec, 69-Lec, 70-Lec, 71-Lec, 84-Lec, 92-Lec, 93-Lec and 97-Lec) encoded for AL-B β subunits. Using BLASTN the nucleotide sequence of AL-B α subunit showed the highest homology to *T .stejnegeri* stejaggregin α (Fig. 7) and AL-B β subunits showed highest identity to *T .stejnegeri* stejaggregin β (Fig. 8). An alignment between translated DNA sequence of AL-B α and stejagregin B α showed 85.25 % amino acids sequence identity (Figure 9), translated DNA sequence of AL-B β and stejagregin B β showed 79.45% amino acids sequence identity (Figure 10). The full-length of AL-B α subunit was 468 bp containing a 23 amino acid residue signal peptide and 133 amino acid residue mature proteins (Fig. 11). AL-B β subunit was 438 bp. The deduced protein sequence of AL-B β comprised 123 residue mature protein and 23 amino acid residue signal peptide (Fig. 12). All seven half-cystinyl residues in each subunit of AL-B are well conserved. The deduced mature protein amino acid sequence of AL-B α and β subunits showed 33.3 % identical to each other (Fig. 13). The amino acid sequence of AL-B α and β were compared with other snake venom CPLs using Clustal W multiple sequence alignment. The results revealed that the identity scores of AL-B α with other known snake venom GPIb-BP α subunit were as follows: TSV-GPIb-BP, 83.45%; mamushigin, 60.15%; jararaca GPIb-BP, 51.87%; FL-A, 54.88%; CHH-B, 42.10% and echicetin, 45.86% (Fig. 14). The identity scores of AL-B β subunit with that of other snake venom GPIb-BP β subunits were as follows: TSV-GPIb-BP, 78.76%; mamushigin, 74.65%; jararaca GPIb-BP, 60.97%; FL-A, 65.75%; CHH-B, 59.34% and echicetin, 54.47% (Fig 15).

Sequences producing significant alignments:	Score (Bits)	E Value
gi|33341207|gb|AF354923.1| Trimeresurus stejnegeri stejaggreg...	656	0.0
gi|33341205|gb|AF354922.1| Trimeresurus stejnegeri clone 3 st...	648	0.0
gi|33341203|gb|AF354921.1| Trimeresurus stejnegeri clone 2 st...	648	0.0
gi|33341201|gb|AF354920.1| Trimeresurus stejnegeri clone 1 st...	648	0.0
gi|33341199|gb|AF354919.1| Trimeresurus stejnegeri stejaggreg...	632	2e-178
gi|33341197|gb|AF354918.1| Trimeresurus stejnegeri stejaggreg...	632	2e-178
gi|58293943|gb|AY871785.1| Protobothrops mucrosquamatus trime...	482	5e-133
gi|11967284|gb|AF102901.1|AF102901 Deinagkistrodon acutus agk...	385	8e-104
gi|20562944|gb|AY091762.1| Deinagkistrodon acutus clone 3019 ...	377	2e-101
gi|33332304|gb|AF541883.1| Crotalus durissus terrificus crotocet	361	1e-96
gi|20273043|gb|AF176420.2| Deinagkistrodon acutus clone 21004...	244	2e-61

Figure 7 Homology searching of AL-B α subunit using BLAST N program.

Sequences producing significant alignments:	Score (Bits)	E Value
gi|33341193|gb|AF354916.1| Trimeresurus stejnegeri stejaggreg...	787	0.0
gi|33341195|gb|AF354917.1| Trimeresurus stejnegeri stejaggreg...	749	0.0
gi|33341211|gb|AF354925.1| Trimeresurus stejnegeri stejaggreg...	472	7e-130
gi|33341215|gb|AF354927.1| Trimeresurus stejnegeri stejaggreg...	454	2e-124
gi|21260583|gb|AY099322.1| Deinagkistrodon acutus clone 21002...	448	1e-122
gi|3882118|dbj|AB019616.1| Agkistrodon blomhoffi mRNA for mamush	448	1e-122
gi|20562932|gb|AY091755.1| Trimeresurus stejnegeri stejaggreg...	351	2e-93
gi|58293945|gb|AY871786.1| Protobothrops mucrosquamatus trime...	412	5e-112
gi|11967286|gb|AF102902.1|AF102902 Deinagkistrodon acutus agk...	379	8e-102
gi|37575444|gb|AY390534.1| Protobothrops mucrosquamatus mucro...	377	3e-101
gi|33341213|gb|AF354926.1| Protobothrops mucrosquamatus C-typ...	414	1e-112

Figure 8 Homology searching of AL-B β subunit using BLAST N program.

```
AL-B    1   MGRFIFVSFGLLVVFLSLSGTGADCPSDWSSFKQYCYQIVKELKTWEDAEKFCSEQANDG
Stej    1   MGRFIFVSFGLLVVFLSLSGTGADCPSDWSSFKQYCYQIIKQLKTWEDAERFCMDQVKGA

AL-B   61   HLVSIESYREAVFVAELLSENVKTTKYNVWIGLSVQNKGQQCSSEWSDGSSVSYENLVKP
Stej   61   HLVSIESYREAVFVAQQLSENVKTTKYDVWIGLSVVNKGQQCSSEWSDGSSVSYENLVKP

AL-B  121   NPKKCFVLKKESEFRTWSNVYCEQKHIFMCKFLGSR      100%
Stej  121   LSKKCFVLKKGTEFRKWFNVACEQKHLFMCKFLRPR      85.25%
```

Figure 9 An alignment of deduced amino acid sequence of AL-B α subunit and stejaggregin. An alignment showed 85.25 % amino acid sequence identity. Dark highlights represent the identical residues and light ones for the homologous residues.

```
AL-B   1   MGRFIFGSFGLLVLFLSLSGTGADCPSDWSSYDLYCYKVFQQRMNWEDAEQFCRQQHTGS
Stej   1   MGRFIFVSFGLLVVFLSLSGTGADCPSDWSSYDLYCYRVFQEKKNWEDAEKFCRQQHTDS

AL-B   61  HLLSFHSSEEVDFVVSKTLPILKADFVWIGLTDVWSACRLQWSDGTELKYNAWTAESECI
Stej   61  HLVSFDSSEEADFVASKTFPVLNYDLVWIGLGSVWNACKLQWSDGTELKYNAWSAESECI

AL-B   121 ASKTTDNQWWTRSCSRTYPFVCKLEV 100%
Stej   121 TSKSIDNQWFTRSCSQTYPFVCKFQA 79.45%
```

Figure 10 An alignment of deduced amino acid sequence of AL-B β subunit and stejaggregin. An alignment showed 79.45 % amino acid sequence identity. Dark highlights represent the identical residues and light ones for the homologous residues.

```
1      gaaggaaggaagaccATGGGGCGATTCATCTTCGTGAGCTTCGGCTTGCTGGTCGTGTTC   60
                      M  G  R  F  I  F  V  S  F  G  L  L  V  V  F
61     CTCTCCCTGAGTGGTACTGGAGCTGACTGTCCTTCTGATTGGTCCTCCTTTAAACAATAT  120
        L  S  L  S  G  T  G  A  D  C  P  S  D  W  S  S  F  K  Q  Y
121    TGCTACCAGATCGTCAAAGAACTCAAGACCTGGGAAGATGCAGAGAAGTTCTGCTCGGAG  180
        C  Y  Q  I  V  K  E  L  K  T  W  E  D  A  E  K  F  C  S  E
181    CAGGCGAACGACGGGCATCTGGTCTCTATCGAAAGCTACAGAGAAGCCGTCTTCGTGGCC  240
        Q  A  N  D  G  H  L  V  S  I  E  S  Y  R  E  A  V  F  V  A
241    GAGCTGCTCTCTGAGAACGTAAAGACAACCAAATACAATGTCTGGATTGGACTGAGTGTT  300
        E  L  L  S  E  N  V  K  T  T  K  Y  N  V  W  I  G  L  S  V
301    CAAAACAAAGGACAGCAATGCAGCTCGGAGTGGAGCGATGGCTCCAGCGTCAGTTATGAG  360
        Q  N  K  G  Q  Q  C  S  S  E  W  S  D  G  S  S  V  S  Y  E
361    AACTTGGTTAAACCAAATCCCAAAAAGTGTTTTGTGCTGAAAAAAGAGTCAGAGTTTCGC  420
        N  L  V  K  P  N  P  K  K  C  F  V  L  K  K  E  S  E  F  R
421    ACGTGGTCCAATGTTTACTGTGAACAAAAACATATTTTCATGTGCAAATTCCTGGGATCA  480
        T  W  S  N  V  Y  C  E  Q  K  H  I  F  M  C  K  F  L  G  S
481    CGTTAAgatccggctgtgtgaagtctggagacgcaaggaagccccccaccccccaccc  540
        R  *
541    ttcgctcaacggatgctctctgtagctggatctggttttgctgctcctgatgggccagaa  600
601    ggtccaataaattctgcctagcat-Poly A
```

Figure 11 The nucleotide sequence and the deduced amino acid sequence of AL-B α subunit from *Trimeresurus albolabris.* The translation stop codon is indicated by the asterisk. The poly adenylation signal (aataa) is underlined.

```
1      ggcggcgcgaattcactagtgattgaaggaaggaagaccATGGGGCGATTCATCTTCGGG   60
                                               M  G  R  F  I  F  G
61     AGCTTCGGCTTGCTGGTCCTGTTCCTCTCCCTGAGTGGTACTGGAGCTGATTGTCCCTCT   120
        S  F  G  L  L  V  L  F  L  S  L  S  G  T  G  A  D  C  P  S
121    GATTGGTCCTCCTATGATCTGTATTGCTACAAGGTCTTCCAACAAAGGATGAACTGGGAA   180
        D  W  S  S  Y  D  L  Y  C  Y  K  V  F  Q  Q  R  M  N  W  E
181    GATGCAGAGCAATTCTGCAGACAACAGCACACAGGCAGCCACCTGCTCTCCTTCCACAGC   240
        D  A  E  Q  F  C  R  Q  Q  H  T  G  S  H  L  L  S  F  H  S
241    AGTGAAGAAGTAGATTTTGTGGTCTCCAAGACCCTCCCAATTCTGAAAGCGGATTTTGTC   300
        S  E  E  V  D  F  V  V  S  K  T  L  P  I  L  K  A  D  F  V
301    TGGATCGGACTCACCGATGTCTGGAGTGCATGCAGGTTGCAGTGGAGCGATGGCACCGAG   360
        W  I  G  L  T  D  V  W  S  A  C  R  L  Q  W  S  D  G  T  E
361    CTCAAGTACAACGCCTGGACTGCAGAATCGGAGTGTATCGCATCCAAGACAACTGATAAC   420
        L  K  Y  N  A  W  T  A  E  S  E  C  I  A  S  K  T  T  D  N
421    CAATGGTGGACTAGATCCTGCAGCAGGACTTACCCTTTCGTCTGCAAGTTGGAGGTATAG   480
        Q  W  W  T  R  S  C  S  R  T  Y  P  F  V  C  K  L  E  V  *
481    tctgaagatccagctgtctgaagtctggagaagcaaagaagaccccaccttgacactctc   540
541    catccccacacccccaccccgcctgccccaatctctgctctgccccccttcgctcaacgga   600
601    tgctctcgtgtagctggatctggttttgctgcttcctgatgggccagaaggtgcccaata   660
661    aatttcggcctaggcatc-Poly A
```

Figure 12 The nucleotide sequence and the deduced amino acid sequence of AL-B β subunit from *Trimeresurus albolabris*. The translation stop codon is indicated by the asterisk. The poly adenylation signal (aataa) is underlined. The highlight nucleotide can be found in another clone of AL-B β subunit (A instead of C changes the translated amino acid from Q to K. At the other 2 sites, T instead of C does not change the translated amino acids).

```
AL-B alpha    MGRFIFVSFGLLVVFLSLSGTGADCPSDWSSFKQYCYQIVKELKTWEDAEKFCSEQANDG
AL-B beta     MGRFIFGSFGLLVLFLSLSGTGADCPSDWSSYDLYCYKVFQQRMNWEDAEQFCRQQHTGS

AL-B alpha    HLVSIESYREAVFVAELLSENVKTTKYNVWIGLSVQNKGQQCSSEWSDGSSVSYENLVKP
AL-B beta     HLLSFHSSEEVDFVVSKTLP--ILKADFVWIGLTDVWS--ACRLQWSDGTELKYN--AWT

AL-B alpha    NPKKCFVLKKESEFRTWSNVYCEQKHIFMCKFLGSR   100%
AL-B beta     AESEC--IASKTTDNQWWTRSCSRTYPFVCKLEV--   33.3%
```

Figure 13 An alignment of the deduced amino acid sequences of AL-B α and β. An alignment showed 33.3 % amino acid sequence identity. Dark highlights represent the identical residues and light ones for the homologous residues.

```
AL-B         1 MGRFIFVSFGLLVVFLSLSGTG--ADCPSDWSSFKQYCYQIVKELKTWED-----AEKFC
TSV-GPIb     1 MGRFISVSFGLLVVFLSLSGTG--ADCPSDWSSFKQYCYQIIKQLKTWED-----AERFC
Mamushigin   1 MGRFIFVSFGLLVVFLSLSGAEDDSDCPSDWSSNGRFCYKLFQQKMKWAD-----AERFC
jararaca       ---------------------DTPFECPSDWSTHRQYCYKFFQQKESWDDRSEYDAERFC
flavocetin   1 MERLIFVSFGLLVVILSLSGTGADFDCIPGWSAYDRYCYQAFSKPKNWED-----AESFC
CHH-B          -----------------------DLECPSGWSSYDRYCYKPFKQEMTWAD-----AERFC
Echicetin1     -----------------------DQDCLSGWSFYEGHCYQLFR-LKTWDE-----AEKYC

AL-B        54 SEQANDGHLVSIESYREAVFVAELLSENVKT--TKYNVWIGLSVQNKGQQCSSEWSDGSS
TSV-GPIb    54 MDQVKGAHLVSIESYREAVFVAQQLSENVKT--TKYDVWIGLSVVNKGQQCSSEWSDGSS
Mamushigin  56 TEQRTGAHLVSIESNTEAAFVNQMISENIKK--TDY-VWIGLTVQNEEQQCKSRWSDRSS
jararaca    40 SEQAKGGHLVSIESDEEADFVAQLVAPNIGK--SKYYVWIGLRIENKKQQCSSKWSDYSS
flavocetin  56 EEGVKTSHLVSIESSGEGDFVAQLVAEKIKT--SFQYVWIGLRIQNKEQQCRSEWSDASS
CHH-B       33 SEQAKGRHLLSVETALEASFVDNVLYANKEY--LTRYIWIGLRVQNKGQPCSSIYSENLV
Echicetin   32 NQWDG-GHLVSIESNAKAEFVAQLISRKLPKSAIEDRVWIGLRDRSKREQCGHLWTDNSF

AL-B          112 VSYENLVKPNPKKCFVLKKESEFRTWSNVYCEQKHIFMCKFLGSR-   100%
TSV-GPIb      112 VSYENLVKPLSKKCFVLKKGTEFRKWFNVACEQKHLFMCKFLRPR- 83.45%
Mamushigin    113 VSYENLVKPNSKKCFVLKEYEGSRKWFNVYCGQKYAFMCKFLRPR- 60.15%
jararaca      98  VSYENLVRGNVKKCFALEKKQGFRKWVNIDCVEGNPFVCKFIRPR- 51.87%
flavocetin    114 VNYENLVKQFSKKCYALKKGTELRTWFNVYCGTENPFVCKYTPEC- 54.88%
CHH-B         91  DPFD---------CFMVSRDTRLREWFKVDCEQQHSFICKFTRPRR 42.10%
Echicetin     91  VHYEHVVPP--TKCFVLERQTEFRKWIAVNCEFKFPFVCKAKIPR- 45.86%
```

Figure 14 Comparison of the deduced amino acid sequence of AL-B α subunit with those of other known snake venom GPIb-BP α subunits. The sequence are: TSV-GPIb-BP (Lee et al., 2003); mamushigin (Sakurai et al., 1998); jararaca GPIB-BP (Kawasaki et al., 1996); flavocetin-A (Shin et al., 2000); CHH-B (Andrew et al., 1996); echicetin α chain (Polgar et al., 1997). Gaps are inserted to maximize similarity. Identical and homologous residues are shaded in black and gray, respectively.

```
AL-B        1  MGRFIFGSFGLLVLFLSLSGTGA--DCPSDWSSYDLYCYKVFQQRMNWEDAEQFCRQQHT
TSV-GPIb    1  MGRFIFVSFGLLVVFLSLSGSGA--DCPSDWSSYDLYCYRVFQEKKNWEDAEKFCTQQHT
Mamushigin  1  MGRFIFLSFGLLVVFVSLSGTGA--DCPSDWSSYEGHCYRVFQKEMTWEDAEKFCTQQRK
jararaca1   1  -------------------------DCPSDWSPYGGHCYKLFKQRMNWADAENLCAQQRK
flavocetin  1  MGQFIFVSFGFLVVATSLSGTEAGFCCPLGWSSYDEHCYQVFQQKMNWEDAEKFCTQQHK
CHH-B       1  -------------------------DCPSDWSSYEGHCYRVFQQEMTWDDAEKFCTQQHT
Echicetin   1  -------------------------NCLPDWSVYEGYCYKVFKERMNWADAEKFCMKQVK

AL-B       59 GSHLLSFHSSEEVDFVVSKTLPILKADFVWIGLTDVWSACRLQWSDGTELKYNAWTAESE
TSV-GPIb   59 DSHLVSFDSSEEADFVASKTFPVLNYDLVWIGLGSVWNACKLQWSDGTELKYNAWSAESE
Mamushigin 59 ESHLVSFHSSEEVDFVVSMTWPILKYDFVWIGLNNIWNECMVEWTDGTRLSHNAWITESE
jararaca   36 ESHLVSFHSSEEVDFLVLLTFPILGPDLYWTGLSNIWNGCSFEWSDGTKVNYNAWASESE
flavocetin 61 GSHLVSFHSSEEVDFVTSKTFPILKYDFVWIGLSNVWNECTKEWSDGTKLDYKAWSGGSD
CHH-B      36 GGHLVSFRSSEEVDFLVS----ILKFDLFWMGWRDIWNERRLQWSDGTKVNYKAWSAEPE
Echicetin  36 DGHLVSFRNSKEVDFMISLAFPMLKMELVWIGLSDYWRDCYWEWSDGAQLDYKAWDNERH

AL-B         119 CIASKTTDNQWWTRSCSRTYPFVCKLEV         100%
TSV-GPIb     119 CITSKSIDNQWFTRSCSQTYPFVCKFQA       78.76%
Mamushigin   119 CIAAKTTDNQWLSRPCSRTYNVVCKFQE       74.65%
jararaca      96 CVASKTTDNQWWSFPCTRLQYFVCEFQA       60.97%
flavocetin   121 CIVSKTTDNQWLSMDCSSKRYVVCKFQA       65.75%
CHH-B         92 CIVCRATDNQWLSTSCSKTHNVVCKF-        59.34%
Echicetin     96 CFAAKTTDNQWMRRKCSGEFYFVCKCPA       54.47%
```

Figure 15 Comparison of the deduced amino acid sequence of AL-B β subunit with those of other known snake venom GPIb-BP β subunits. The sequence are: TSV-GPIb-BP (Lee et al., 2003); mamushigin (Sakurai et al., 1998); jararaca GPIB-BP (Kawasaki et al., 1996); flavocetin-A (Shin et al., 2000); CHH-B (Andrew et al., 1996); echicetin β chain (Peng et al., 1994). Gaps are inserted to maximize similarity. Identical and homologous residues are shaded in black and gray, respectively.

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4.2 Proteins purification and SDS-PAGE analysis

Not only the cDNA cloning but also protein purification and characterization of platelet aggregating activity of CLPs have been studied in this thesis. The purification of AL-B was followed by determining the aggregation-inducing activity of fixed platelets in each fraction. Gel filtration chromatography of 0.5 g of crude *T .albolabris* venom was performed on Sephacyl G-200 column resulting in seven protein peaks (Fig 16). Peak1 and 4, which showed strong platelet aggregation-inducing activity, were separately pooled and concentrated for further purification. Pooled peak 4 were fractionated on Resourse Q ion-exchange column using a linear gradient of NaCl from 0-0.5 M. AL-B was eluted at approximately 0.38 M NaCl (Fig 17).

Purified AL-B was analyzed by SDS-PAGE under reducing and non-reducing condition. AL-B gave a 25 kDa band on SDS-PAGE under native condition. Upon reduction, it separated into two bands with apparent molecular weights of 14 kDa and 12 kDa consistent with α and β subunit, respectively (Fig. 18).

4.3 Proteins Identification by MALDI-ToF Mass spectrometry

After obtaining the results from SDS-PAGE, The identities of AL-B α and β subunits were confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry (Fig. 19, 20). 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 resulting observed peptide mass fingerprints (PMFs) were compared with theoretical peptide mass calculated based on cloned sequence using Protein Calculation Program (Table. 2). The 2 polymorphisms of AL-B β could not be differented due to the equal molecular masses of lysine and glutamine residues. In addition, no glycosylation was detectable in either subunit using mass spectrometry.

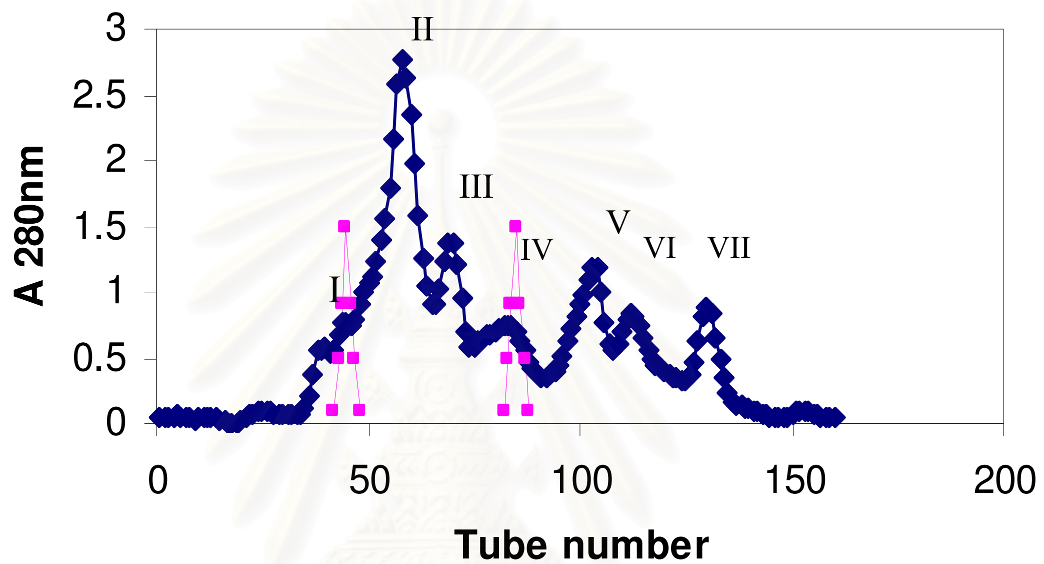

Figure 16 Gel filtration chromatogram of *T. albolabris* venom (0.5g) on a Sephacyl G-200 column. Protein concentrations (◆) were estimated from the absorbance at 280 nm. Fixed platelet aggregation-inducing activity (■) was assayed as described in Chapter III.

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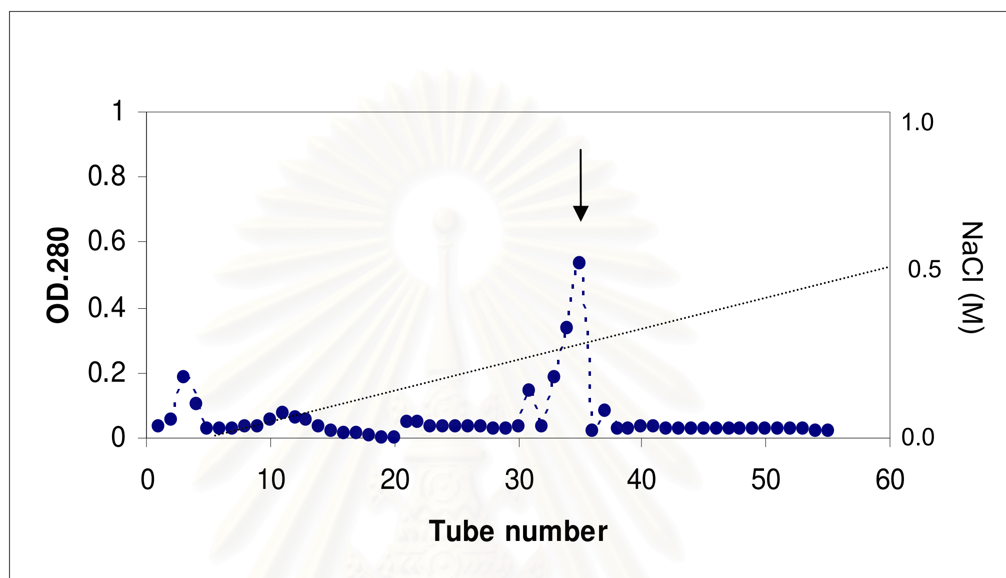

Figure 17 Ion-exchange chromatogram of partially purified AL-B on Resourse Q column. Protein concentrations (●) were estimated from the absorbance at 280 nm. The elution was performed with a NaCl gradient represented by a straight line (---). The AL-B containing peak determined by platelet-aggregating activity was indicated by an arrow.

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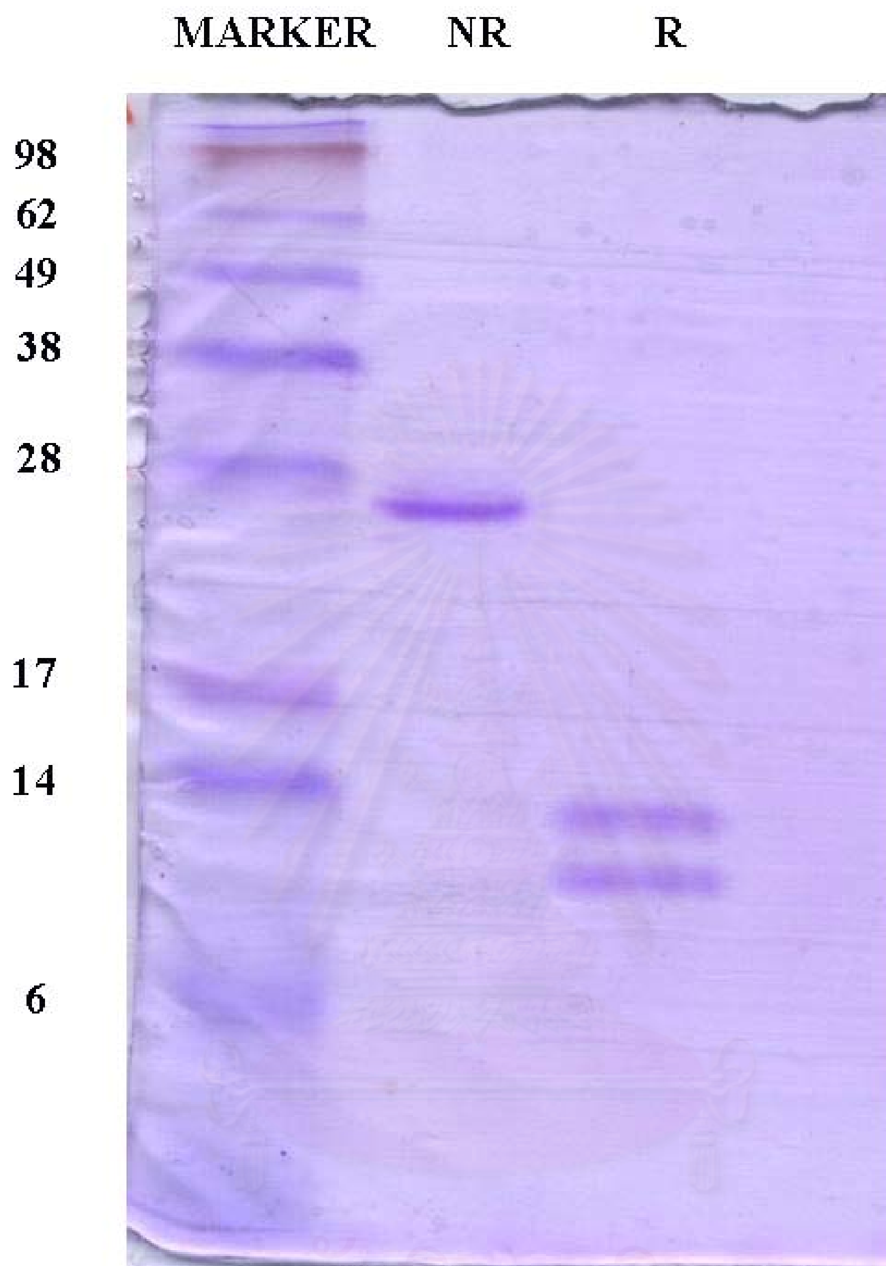

Figure 18 SDS-PAGE of AL-B. Lane 1: pre-stained protein marker; Lanes 2 and 3: AL-B under non-reducing (NR) and reducing conditions (R), respectively.

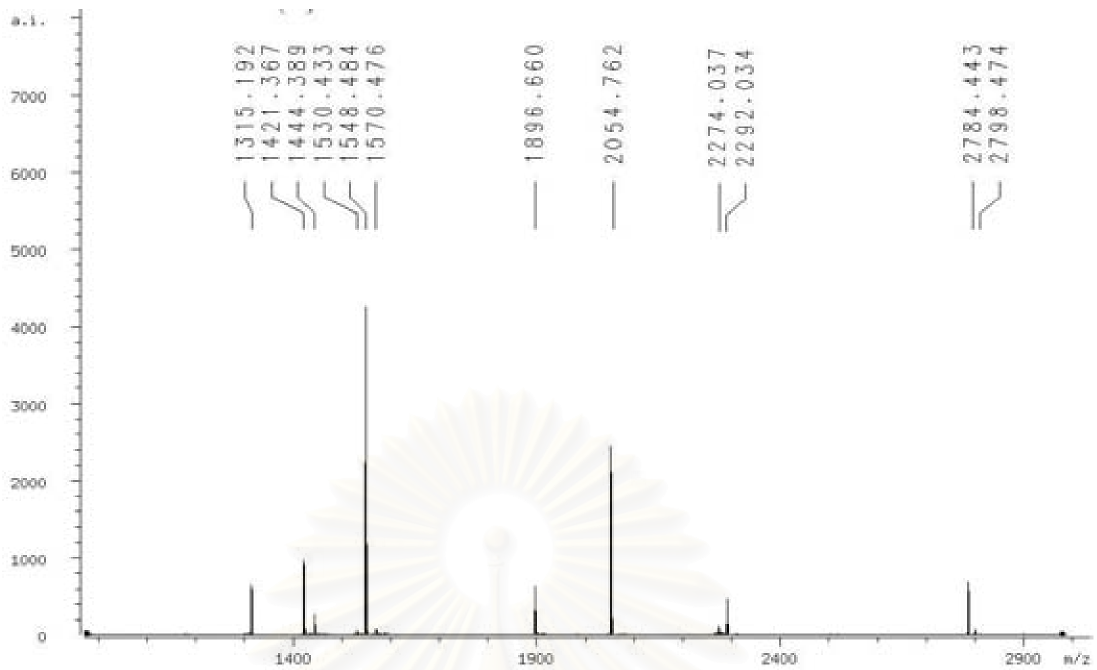

Figure 19 The peptide mass fingerprint of trypsin-digested AL-B α subunit as determined by MALDI-ToF mass spectrometry.

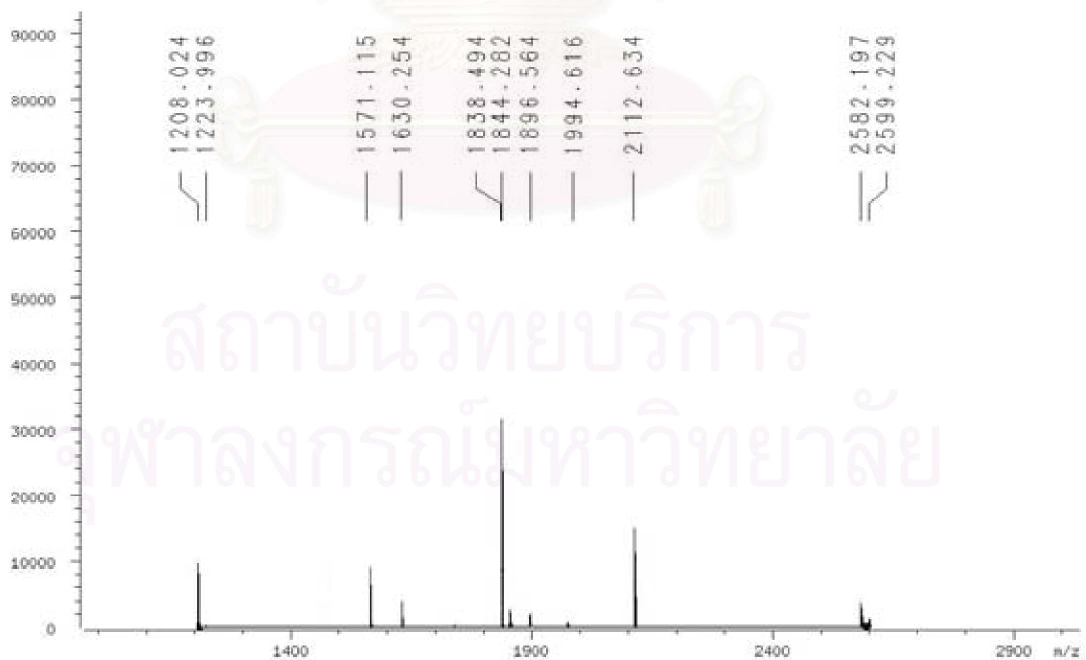

Figure 20 The peptide mass fingerprint of trypsin-digested AL-B α subunit as determined by MALDI-ToF mass spectrometry.

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Subunit	Sequence	Theoretical masses (Da)	Observed masses (Da)
α	1-10 DCPSDWSSFK	1171.24	-
	11-28 QYCYQIVKELKTWEDAEK	2274.57	2274.037
	29-46 FCSEQANDGHLVSIESYR	2055.21	2054.762
	47-60 EAVFVAELLSENVK	1547.77	1548.484
	47-63 EAVFVAELLSENVKTTK	1878.14	1896.660
	64-75 YNVWIGLSVQNK	1420.63	1421.367
	76-96 GQQCSSEWSDGSSVSYENLVK	2290.40	2292.034
	101-112 KCFVLKKESEFR	1513.82	1530.433
	102-122CFVLKKESEFRTWSNVYCEQK	2794.21	2798.474
	123-133 HIFMCKFLGSR	1338.64	1315.192
β	1-15 DCPSDWSSYDLYCYK	1844.98	1844.282
	16-31 VFQQRMNWEDAEQFCR	2087.30	2112.634
	32-54QQHTGSHLLSFHSSEEVDFVVSK	2598.81	2599.229
	61-76 ADFVWIGLTDVWSACR	1839.1	1838.494
	77-86 LQWSDGTELK	1176.29	-
	87-100 YNAWTAESECIASK	1572.71	1571.115
	101-109 TTDNQWWTR	1207.27	1208.024
	110-123 SCSRTYPFVCKLEV	1631.92	1630.254

Table 3 Theoretical and observed peptide mass fingerprints of AL-B α and β subunits.

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4.4 Quantitative Assay of Purified Alboaggregin B.

To determine the quantity of purified alboagregin B (AL-B), the Micro BCA™ Protein Assay was used for measuring the protein concentration. Absorbance average of 0.9 at 570 nm at 1:10 dilution of protein sample indicating that the calculated undiluted purified protein concentration was 95.7μg/ml (Figure 21). The total yield of protein was 2.4 mg from the initial 500 mg of crude venom. Therefore, AL-B comprised approximately 0.5 % of the total venom weight.

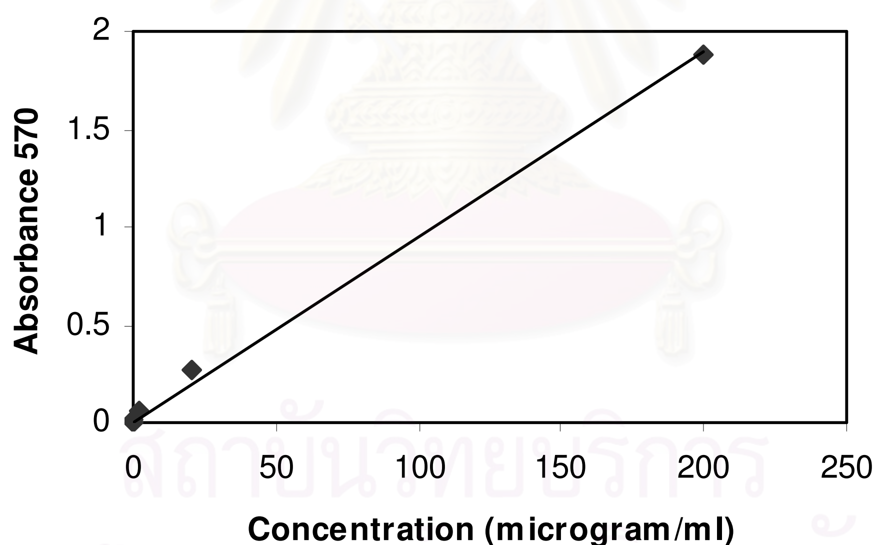

Figure 21 The standard curve of protein concentrations fitted by linear regression ($y = mx + b$, $m = 0.0095$, $b = 0$, $r^2 = 0.9966$)

4.5 The Effects of Alboaggregin B on Washed platelets and Formalin-fixed platelets

In previous studies, AL-B was found to agglutinate washed platelets and formalin-fixed platelets without any cofactor. Similarly, AL-B purified in our laboratory induced aggregation of both washed and fixed platelets but AL-B purified in our laboratory was less active than that of the previous report. The maximum platelet aggregation dose toward washed human platelets in our study was 10μg/ml, while the reported AL-B concentration was 0.48μg/ml. Moreover, AL-B at the dose of 10μg/ml could only induce approximately 35 % aggregation of formalin-fixed platelets. (Figure22). The concentrations that induced 50% aggregation of washed and fixed platelets were 180 nM and 184 nM, respectively, similar to the previous reported value of AL-B of 200 nM (Kowalska et al., 1998). The direct human platelet aggregation activity of AL-B was dose-dependently inhibited by monoclonal anti-GPIbα antibodies SZ2. Different concentrations of monoclonal anti-GPIbα antibodies SZ2 were incubated at 37 °C for 2 minute with 450 μl washed human platelets (3 x 10^8/ml), and then, a fixed concentration of AL-B was added (10 μg/ml, final concentration). The maximum aggregation response obtained from addition of AL-B without antibody was set as 100 % aggregation. (Figure23). The anti-GPIbα antibody was found to inhibit AL-B induced platelet aggregation in a dose dependent manner with IC_{50} = 19.4 nM. Furthermore, the complete inhibition occurred at the final antibody concentration of 20μg/ml (117.6 nM).

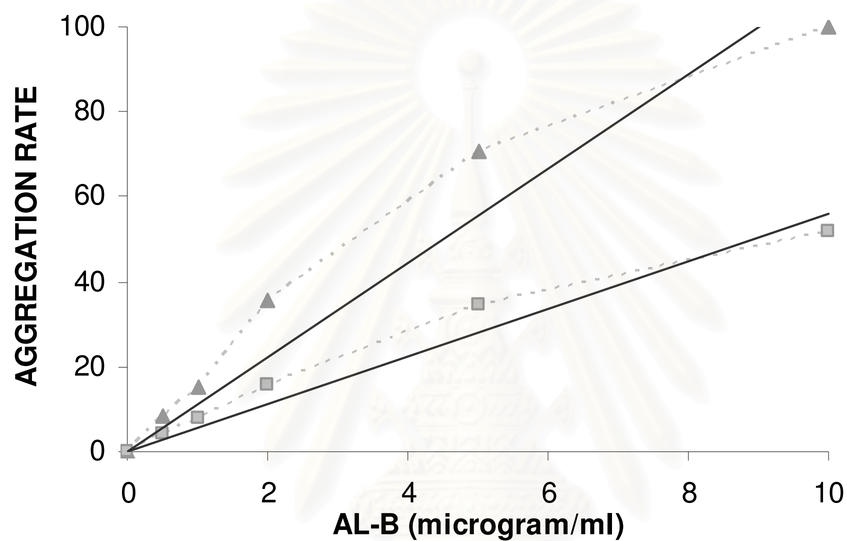

Figure 22 Effects of AL-B on washed (▲)and formalin-fixed platelets (■) (final concentration). EC50 (the concentration that induced 50% aggregation) was determined using curve fitting with linear regression (——).

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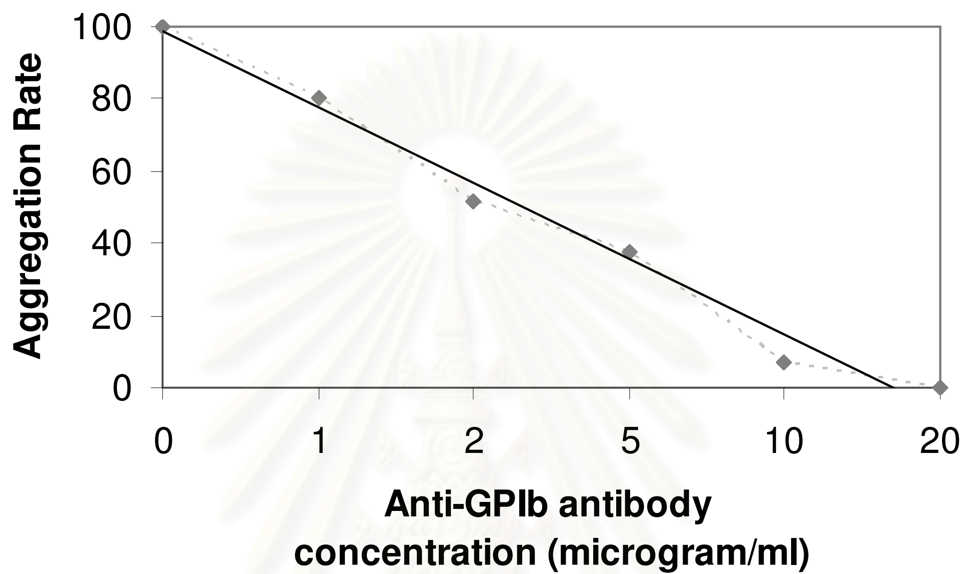

Figure 23 Inhibitory effects of monoclonal anti-GPIb antibody on AL-B-induced platelets aggregation. IC50 (the concentration that inhibited 50% aggregation) was determined using curve fitting with linear regression (——).

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CHAPTER V

DISCUSSION AND CONCLUSION

Although Alboaggregin B (AL-B), a 25 kDa heterodimeric C-type lectin-like protein that induced platelet aggregation via GPIbα from *T. albolabris*, has been purified and characterized, there is no report of the complete nucleotide sequence of its cDNA. This is the first report of the complete cDNA sequences. AL-B is a heterodimer composed of α and β subunits. The cDNA sequence of α subunit is 471 bp in length and translated in to 133 amino acid residue mature protein. The cDNA sequence of β subunit is 441 bp in length and translated in to 123 amino acid residues. All seven cysteine residues in each subunit of AL-B are well conserved. The overall 3-D structures of all the platelet-aggregating lectins are closely similar. Therefore, the disulfide bond pattern of AL-B is that α and β chains are predicted to be cross-linked by a disulfide bond between Cys 79 of α chain and Cys 75 of β chains and each chain contains three intra-chain disulfide bonds between Cys 2-13, 30-126 and 110-118 (Kowalska et al., 1998). Comparing the amino acid sequences of α and β chain of AL-B determined by Usami et al. and Kowalska et al. with the amino acid sequences of α and β chain of AL-B deduced from nucleotide sequences from our laboratory revealed 81.20% and 78.86% amino acid identity, respectively (Fig 24).The reason for these differences may be caused by the variations of proteins isolated from different subspecies or different geographical locations. In addition, nucleotide sequencing technique is likely to be more accurate than direct protein sequencing.

Using the combination of gel filtration and ion-exchange chromatography, *T. albolabris* venom proteins were purified by molecular size and charges, respectively. The result of gel filtration, shows 7 protein peaks. Formalin-fixed platelets were used to identify the specific activity, which is direct platelet agglutination. The fixed platelets were used in obtaining AL-B to verify that platelet activation and plasma factors were not required. Peak 1 and 4 showed fixed-platelet aggregating activity. The fractions in both peaks were separately pooled and further purified by ion-exchange chromatography. The latter peak (peak4) contains smaller proteins due to gel filtration separation. This peak, which contains platelet-aggregating activity contained AL-B. Purified AL-B was analyzed by SDS-PAGE under non-reducing and reducing conditions. It appeared as a single band with molecular weight of 25,000 and two bands with apparent molecular weights of 14,000 and 13,000 for α and β subunits, respectively. Moreover, the identity of purified AL-B was confirmed by MALDI ToF Mass spectrometry. Each subunit of AL-B separated by SDS-PAGE was digested with trypsin and protein fragments were detected. The results of α subunit, showed 9 protein fragments with molecular weights of 1315.129, 1421.367, 1530.433, 1548.484, 1896.660, 2054.762, 2274.037, 2798.474 and 2292.037 those of β subunits displayed 7 protein fragments with molecular weights of 1208.024, 1571.115, 1630.254, 1838.494, 1844.282, 2112.634 and 2599.229. When comparing between observed and theoretical masses, it can be demonstrated that all the observed mass of 9 protein fragments were matched with the theoretical mass of the α subunit. However, protein fragment of amino acid 1-11 did not match with any observed mass. Similarly, all 7 observed masses of protein fragments were identical to theoretical mass of β subunits. However, the protein fragments position at 77-86 did not corresponded with any observed masses. This was because the range of measured mass was 1200-3000 Da. Therefore, it is possible that protein fragments lower than 1200 or

higher than 3000 can not be observed or the protein fragments were lost during the process.

In this study, AL-B was isolated from the venom of *T. albolabris*. It comprised approximately 0.5 % of the total dry weight. Like the previous study (Peng et al., 1991), AL-B directly aggregated washed and fixed platelet in a dose-dependent manner and the aggregation activity was completely blocked by specific monoclonal anti-GPIbα antibodies SZ2. Therefore, GPIbα is likely the site specific platelet receptor for AL-B. The degree of aggregation is higher with the living (washed) than dead (fixed) platelets, suggesting that there is platelet activation induced by the AL-B. However, some GPIb loss during formalin fixation is also possible.

```
AL-B        1    MGRFIFVSFGLLVVFLSLSGTGADCPSDWSSFKQYCYQIVKELKTWEDAEKFCSEQANDG
Usami       1    -----------------------DCPSDWSSFKQYCYQIIKELKTWEDAEXFCMDQVKGA
Kowalska    1    -----------------------DCPSDWSSFKQYCYQIVKELKTWEDAEXFCSEQANDG

AL-B        61   HLVSIESYREAVFVAELLSENVKTTKYNVWIGLSVQNKGQQCSSEWSDGSSVSYENLVKP
Usami       38   HLVSIESYREAVFAQQLXSENVKTTKYDVWIGLSVVNKXQQCSSEWSGDSSVSYENLVKP
Kowalska    38   HLVSIESYREAVFVAELLSENVXXXKYHXWIGLSVQNKXQQCSSEWSDGSSVTYENLIXX

AL-B        121    NPKKCFVLKKESEFRTWSNVYCEQKHIFMCKFLGSR     100%
Usami       98     LSKKCFVLKKGTEFRTWSNVACEQKHLFMCKFLRPR    81.20%
Kowalska    98     XXXKCFVLKKESEFRTWSNVYCEQKHIFMCKFLGS-    89.47% (98.34%)
```

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Figure 24 AL-B α subunits sequence compared with previous protein sequence reported by Usami and Kowalska. Percentages of identical residues are also shown. The value in the parenthesis is percentage identity when Xs are excluded.

```
AL-B      1   MGRFIFGSFGLLVLFLSLSGTGADCPSDWSSYDLYCYKVFQQRMNWEDAEQFCRQQHTGS
Usami     1   -----------------------DCPSDWSSYDLYCYKVFQEKKNWEDAEFKCTQQHTDS
Kowalska 1    -----------------------DCPSDWSSYDLYCYKVFQQRMNWEDAEQFCRQQHTGS


AL-B     61   HLLSFHSSEEVDFVVSKTLPILKADFVWIGLTDVWSACRLQWSDGTELKYNAWTAESECI
Usami    38   HLVSFDSSEEVDFVASKTEPVLKHDLVWIGLGSVWNACKLQWSDGTELKYNAWSAESECI
Kowalska 38   HLLSFXXXXXADFVXXXXXXXXXXXXXWIGLTDVWSACRLQWSDGTTLSKNAWTAESECI


AL-B     121  ASKTTDNQWWTRSCSRTYPFVCKLEV       100%
Usami     98  TSKSTDNEWLTRSCSRTYPFVCKFQA       78.86%
Kowalska 98   ASKTTDNQWWTRSCSRTYPFV-----       78.04% (95.8%)
```

Figure 25 AL-B β subunits sequence compared with previous protein sequence reported by Usami and Kowalska. . Percentages of identical residues are also shown. The value in the parenthesis is percentage identity when Xs are excluded.

The relationship between biological functions and the binding sites of GPIb-binding venom proteins is still poorly understood. GPIbα extracellular domain contains several distinguishable domains: residues 1-35 (N-terminal flanking sequence), residues 36-200 (seven leucine-rich repeats), residues 201-268 (double-loop or C-terminal flanking sequence) and residues 269-282 (anionic region). Both the monoclonal anti-GPIbα antibody, SZ2 which is specific for GPIbα, and the monoclonal antibody AK2, which binds to N-terminal peptide domain of GPIbα, can inhibit platelet aggregation induced by AL-B (Andrews et al, 1996.). The GPIbα binding domain for AL-B should be investigated by 3-D crystallography or competitive studies using epitope-mapping antibodies in the future.

Interestingly, known snake GPIb binding proteins (GPIb-BPs) that contain direct platelet-aggregating activity include only AL-B, mamushigin and TSV GPIb-BP. When comparing amino acid sequence of AL-B with other snake venom GPIb-BPs (Fig 26-27), we found that the amino acid motif 109-114 of AL-B, mamushigin and TSV-GPIb-BP is well-conserved, while the same motif from others were more variable. The crystal structure of factor X binding protein (X-BP) complexed with the factor X Gla domain peptide has been determined at 2.3 Å resolution (Mizuno et al., 2001). The Gla domain of factor X contains three hydrophobic amino residues, Phe4, Leu5 and Val8, in the N-terminal loop that contribute to membrane binding. These hydrophobic residues interact with the hydrophobic amino acids in a loop of X-BP β subunit. The aromatic ring of Phe4 in the Gla domain stacks over the guanidine group of Arg112 from the X-BP β subunit. Leu5 of the Gla domain participates in a hydrophobic interaction with Ile114 of subunit β. In addition, Val8 of the Gla domain interacts with Met113 and Ile 114 of subunit β. Therefore, the amino acid sequence Arg 112-Met 113-Ile 114 in the C-terminal region of X-BP β subunit is critical for the formation of complex between the Gla domain and X-BP. This RMM/RMI motif in a β subunit of anticoagulant CLPs contributes to their specificity for the Gla domain of factor IX or factor X (Morita, 2005). In jararaca GPIb-BP, the reduced β chain separated by electrophoresis significantly blocked the biotin-labeled jararaca GPIb-BP binding to platelets, but the similarly treated α chain did not, underlying the significance of the β subunit (Sakurai et al., 1998). Notably, RMM/RMI sequence is well-conserved in IX, IX/X and X binding proteins suggesting that this area in β subunit is the site for target protein binding. The platelet GPIb binding proteins which induced platelet aggregation (AL-B, mamushigin and TSV-GPIb-BP) also contain a conserved motif of Ser (S-112), Arg (R-113), Thr (T-114), Tyr (Y-115): (Q was present instead of R in TSV-GPIb-BP) at this homologous site in β subunit. This suggests that this motif may be critical for activating GPIb binding.

On the other hand, AL-B is a strong inhibitor of the binding of von Willebrand factor (vWF) to platelets (Peng et al., 1991). Furthermore, TSV-GPIb-BP dose-dependently inhibits ristocetin-induced platelet aggregation (Lee W.H. et al, 2003) and mamushigin obviously blocks shear stress-induced platelet aggregation (SIPA) at high-shear stress, the condition that induces GPIb-vWF interaction (Sakurai K. et al, 1998). It is interesting to note that at low-shear stress these proteins enhance platelet aggregation, while platelet aggregation was inhibited at high-shear stress indicating that AL-B is a less potent GPIb ligand than the natural GPIb ligand, vWF.

Most platelet-aggregating C-type lectin-like proteins (CLPs) contain complex multimeric structures that can bind several GPIb per CLP molecule and activate platelets via GPVI. However, the majority of dimeric snake venom GPIb-BPs inhibits platelet aggregation, only AL-B, mamushigin and TSV-GPIb-BP are reported to be bi-functional *ie*. activation when present alone and inhibition with vWF or at high shear. It is possible that these proteins contain more than one GPIb-binding site (bivalent) but difference binding affinity, thereby under low-shear stress the molecule can agglutinate the adjacent platelets together. However, under high-shear stress, the molecule may functional as a monomer (preventing vWF binding) (Sakurai et al, 1998). Moreover, based on the determined crystal structure of flavocetin-A (FL-A), a platelet aggregation antagonist, the platelet GPIb-binding site of FL-A is mainly attributed to two hydrophilic patched in β subunit. The hydrophilic patch I is formed by Ser-46, Ser-47, Glu-48 and Asp-52 and the hydrophilic patch II is composed of Lys-102, Thr-104, Asp-105, Asn-106 and Gln-107 of the β subunit (Fukuda et al, 2000). TSV-GPIb-BP, AL-B and mamushigin show variable amino acid residues in hydrophilic patch II. Inaddition, other dimeric snake venom GPIb-BPs that do not have direct platelet-activating activity including jararaca GPIb-BP, CHH-B, echicetin and FL-A, retain a relatively concerved hydrophilic patch II (except CHH-B, in which Lys was replaced by Arg in hydrophilic patch II) (Lee et al., 2003). These data

suggest that the presence of both SRTY motif and the conserved hydrophilic patches contribute to the bi-functional GPIb binding of AL-B.

The importance of SRTY motif and hydrophilic patches in direct platelet aggregation inducing activity by snake venom GPIb-BPs needs further investigation. In addition, further studies on the expression and characterization of recombinant AL-B protein and mutagenesis of AL-B are essential to elucidate the structure-function relationships and molecular mechanisms of platelet aggregating-activity.

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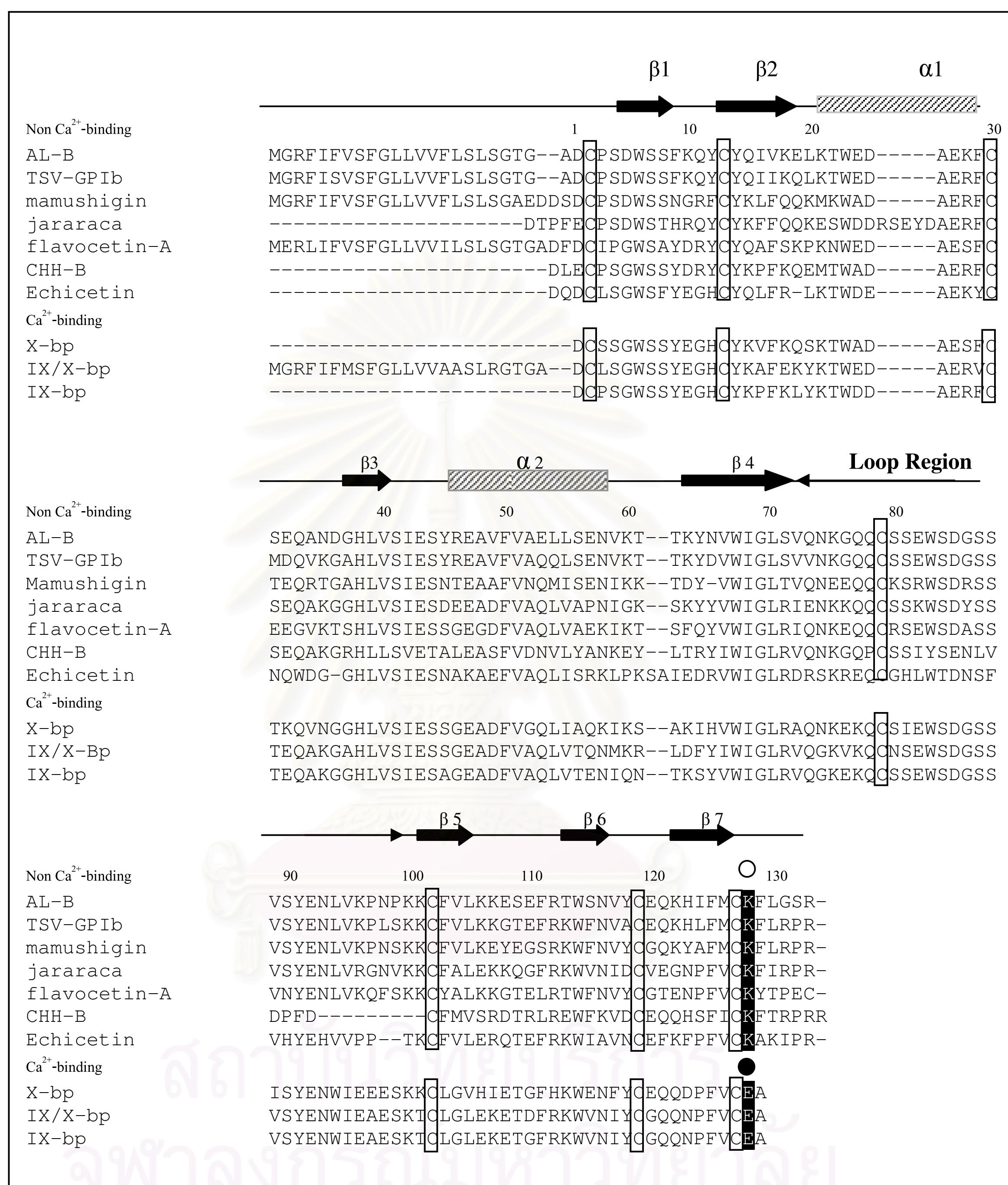

Figure 26 Comparison between amino acid sequence of AL-B α subunit and structurally related CLPs. Ca^{2+}-binding site are shaded and marked with closed circle (●), and the corresponding residues on non-Ca^{2+}-binding lectins are shaded and shown in white circle (○). Secondary structures are shown as hatched boxes (α helix, α1-α2) and black arrows (β strand, β1- β7), above the sequence. Half-cystine residues are in square boxes (Modified from Tomohisa et al., 2005).

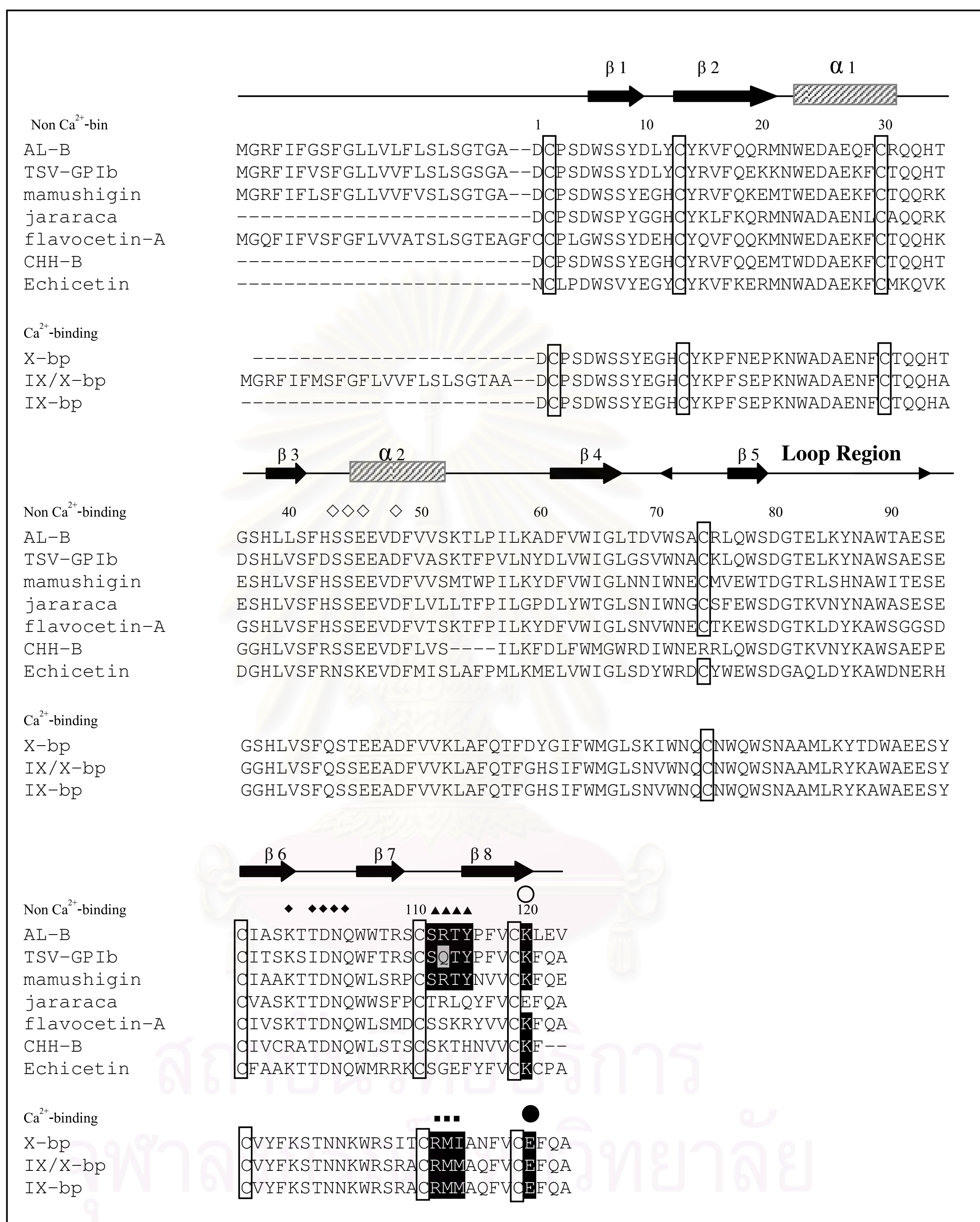

Figure 27 Comparison between amino acid sequence of AL-B β subunit and structurally related CLPs. Ca^{2+}-binding site are shaded and marked with closed circle (●), and the corresponding residues on non-Ca^{2+}-binding lectins are shaded and shown in white circle (○). Secondary structures are shown as hatch boxes (α helix, α1-α2) and black arrows (β strand, β1- β7), above the sequence. Half-cystine

residues are in square boxes (Modified from Tomohisa et al., 2005). The black squares (■) mark the position of the critically conserved three amino acid sequence RMM/RMI in coagulation factor IX and X-binding CLPs. The black triangle (▲) denote the putative conserved motif in GPIb-binding CLPs motif that cause direct platelet aggregation. Hydrophobic patch I and II are labeled with (◇) and (◆), respectively.

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REFERENCES

Andrews, R.K., Booth, W.J., Gormann, J.J., Castaldi, P.A., Berndt, M.C. 1989. Purification of botrocetin from *Bothrops jararaca* venom. Analysis of the botrocetin-mediated interaction between von Wellebrand factor and the human platelet membrane glycoprotein Ib-IX complex. Biochemistry 28, 8317-8326.

Andrews, R.K., Kroll, M.H., Ward, C.M., Rose, J.W., Scarborough, R.M., Smith, A.I., Lopez, J.A., Berndt, M.C. 1996. Binding of a novel 50-kilodalton alboaggregin from *Trimeresurus albolabris* and related viper venom proteins to the platelet membrane glycoprotein Ib-IX-V complex. Effect on platelet aggregation and glycoprotein Ib-mediated platelet activation. Biochemistry 35, 12629-12639.

Aragon-Ortiz., F., Mentele, R., Auerswald, E.A. 1996. Amino acid sequence of a lectin-like protein from *Lachesis muta stenophyrs* venom. Toxicon 34, 763-769.

Arocas, V., Castro, H.C., Zingali, R.B., Guillin, M.C., Jandrot-Perrus, M., Bon, C., Wisner, A. 1997. Molecular cloning and expression of bothrojaracin, a potent thrombin inhibitor from snake venom. Eur. J. Biochem. 248, 550-557.

Asazuma, N., Marshall, S.J., Berlanga, O., Snell, D., Poole, A.W., Berndt, M.C., Andrews, R.K., Watson, S.P. 2001. The snake venom toxin alboaggregin-A activates glycoprotein VI. Blood 97. 39, 89-91.

Atoda, H., Morita, T. 1993. Arrangement of the disulfide bridges in a blood coagulation factor IX/factorX-binding protein from the venom of *Trimeresurus flavoviridis*. J. Biochem. 113, 159-163.

Atoda, H., Ishikawa, M., Yoshida, N., Sekiya, F., Morita, T. 1995. Blood coagulation factor IX-binding protein from the venom of *Trimeresurus flavoviridis*: purification and characterization. J. Biochem. 118, 965-973.

Atoda, H., Ishikawa, M., Mizuno, H., Morita, T. 1998. Coagulation factor IX-binding protein from *Deinagkistrodon acutus* venom is a Gla domain-binding protein. Biochemistry 37, 17361-17370.

Batuwangala, T., Leduc, M., Gibbson, G.M., Bon, C., Jones, E.Y. 2004. Structure of the snake-venom toxin convulxin. Acta Crystallogr. D Biol. Crystallogr. 60, 46-53.

Castro, H.C., Dutra, D.L., Oliveira-Carvalho, A.L., Zingali, R.B. 1998. Bothroalternin, a thrombin inhibitor from the venom of *Bothrops alternatus*. Toxicon 36, 1903-1912.

Chen, Y.L., Tsai, I.H. 1995. Function and sequence characterization of agkicetin, a new glycoprotein Ib antagonist isolated from *Agkistrodon acutus* venom. Biochem. Biophys. Res. Common. 210, 472-477.

Chen, Y.L., Tsai, I.H. 1996. Functional and sequence characterization of coagulation factor IX/factor X-binding protein from the venom of *Echis carinatus leucogaster*. Biochemistry 35, 5264-5271.

Chen, Y.L., Tsai, K.W., Chang, T., Hong, T.M., Tsai, I.M. 2000. Glycoprotein Ib-binding protein from the venom of *Deinagkistrodon acutus*-cDNA sequence, functional characterization, and three-dimentional modeling. Thromb. Heamost. 83, 119-126.

Cicmil, M., Thomas, J.M., Sage, T., Baryy, F.A., Leduc, M., Bon, C., Gibbins, J.M. 2000. Collagen, convulxin, and thrombin stimulate aggregation-independent tyrosine phosphorylation of CD31 in platelet. Evidence for the involvement of Src family kinases. J. Biol. Chem. 275, 27339-27347.

de Carvalho, D.D., Marangoni, S., Novello, J.C. 2002. Primary structure characterization of *Bothrops jararacussu* snake venom lectin. J. Protein Chem. 21, 43-50.

Dormann, D., Clemetson, J.M., Navdaev, A., Kehrel, B.E., Clemetson, K.J. 2001. Alboaggregin A activates platelets by a mechanism involving glycoprotein VI as well as glycoprotein Ib. Blood. 97, 929-936.

Drickamer, K. 1999. C-type lectin-like domains. Curr. Opin. Struct. Biol.,.9, 585-590

Du, X.Y., Navdaev, A., Clemetson J.M., Magnenat, E., Wells T.N., Clemetson, K.J. 2001. Bilinexin, a snake C-type lectin from *Agkistrodon bilineatus* venom agglutinates platelets via GPIb and $\alpha_2\beta_1$. Thromb. Haemost. 86, 1277-1283.

Du, X.-Y., Clemetson, J.M., Navdaev,, A., Magnenat, E.M., Wells, T.N., Clemetson, K.J. 2002a. Opioluxin, a convulxin-like C-type lectin from *Ophiophagus hannah* (king cobra) is a powerful platelet activator via glycoprotein VI. J. Biol. Chem. 277, 35124-35132.

Du, X,-Y., Magnenat, E., Wells, T.N., Clemetson, K.J. 2002b. Alboluxin, a snake C-type lectin from *Trimeresurus albolabris* venom is a potent platelet agonist acting via GPIb and GPVI. Thromb Haemost. 87, 692-698.

Eble, J.A., Tuckwell, D.S. 2003. The $\alpha_2\beta_1$ inhibitor rhodocetin bind to the A-domain of the integrin α_2 subunit proximal to the collagen binding site. Biochem. J. 15, 77-85.

Ferrer, E. 2001. Snake venom: the pain and potential of poison. Available from http://www.annieappleseedproject.org

Fujimura, Y., Titani, K., Usami, Y., Suzuki, M., Oyama, R., Matsui, T., Fukai, H., Sugimoto, M., Ruggeri, Z.M. 1991. Isolation and chemical characterization of two structurally and functionally distinct forms of botrocetin, the platelet coagglutinin isolated from the venom of *Bothrops jararaca*. Biochemistry 30, 1957-1964.

Fukada, K., Mizuno, H., Atoda, H., Morita, T. 2000. Crystal atructure of flavocetin-A, a platelet glycoprotein Ib-binding protein, reveals a novel cyclic tetramer of C-type lectin-like heterodimers. Biochemistry 39, 1915-1923.

Fukada, K., Doggett, T.A., Bankston, L.A., Cruz, M.A., Diacovo, T.G., Liddington, R.C. 2002. Structure basic of von Willebrand factor activation by the snake toxin botrocetin. Structure 10, 943-950.

Hamako, J., Matsui, T., Suzuki, M., Ito, M., Makita, K., Fujimura, Y., Ozeki, Y., Titani, K. 1996. Purification and characterization of bitiscetin, a novel von Willebrand factor modulator protein from *Bitis arietans* snake venom. Biochem. Biophys. Res. Common. 226, 273-279.

Hirabayashi , J., Kusunoki, T., Kasai, K. 1991. Complete primary structure of a galactose-specific lectin from the venom of the rattle snake Crotalus atrox. Homologies with $Ca^{2(+)}$-dependent-type lectins. J. Biol. Chem. 266, 2320-2326.

Hirotsu, S., Mizuno, H., Fukuda, K., Qi, M.C., Matsuio, T., Hanako, J., Morita, T., Titani, K. 2001. Crystal structure of bitiscetin, a von Willebrand factor-dependent platelet aggregation inducer. Biochemistry 40, 13592-13597.

Horii, K., Okuda, D., Morita, T., Mizuno, H. 2003. Structure characterization of EMS16, an antagonist of collagen receptor (GPIa/IIa) from the venom of *Echis multisquamatus*. Biochemistry 42, 12497-13502.

Huang, K.F., Ko, T.P., Hung C.C., Chu, J., Wang, A.H., Chiou, S.H. 2004. Crystal structure of a platelet-agglutinating factor isolated from the venom of Taiwan habu (*Trimeresurus mucrosquamatus*). Biochem. J. 378(Pt 2), 399-407.

Jandrot-Perrus, M., Lagrue, A.H., Okuma, M., Bon, C. 1997. Adhesion and activation of human platelets induced by convulxin involve glycoprotein VI and integrin alpha2beta1. J. Biol. Chem. 272, 27035-27041.

Kanaji, S., Kanaji, T., Furihata, K., Kato, K., Ware, J.L., Kuicki, T.J. 2003. Convulxin binds to native, human glycoprotein Ib alpha. J. Biol. Chem. 278, 39452-39460.

Kawasaki, T., Taniuchi, Y., Hisamichi, N., Fujimura, Y., Suzuki, M., Titani, K., Sakai, Y., Kaku, S., Satoh, N., Takenaka, T., et al. 1995. Tokaracetin, a new platelet antagonist that bind to platelet glycoprotein Ib and inhibits von Willebrand factor-dependent shear-induced platelet aggregation. Biochem. J. 308(Pt 3), 947-953.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Kijimoto-Ochiai, S. 2002. CD23 (the low-affiity IgE receptor) as a C-type lectin: a multidomain and multifunctional molecul. Cell Mol. Life Sci. 59, 648-664.

Kinter M. and Sherman E.N., Protein Sequencing and Identification Using Tandem Mass Spectrometry (CANADA: John Wiley & Sons, 2000c), pp. 147-160.

Koo, B.H., Sohn, Y.D., Hwang, K.C., Jang, Y., Kim, D.S., Chung, K.H. 2002. Characterization and cDNA cloning of halyxin, a heterogeneous three-chain anticoagulant protein from the venom of *Agkistrodon halys brevicaudus*. Toxicon 40, 947-957.

Kowalska, M.A., Tan, .L, Holt, J.C., Peng, M., Karczewski, J., Calvete, J.J., Niewiarowski, S. 1998. Alboaggregins A and B. Structure and interaction with human platelets. Thromb Haemost. 79, 609-613.

Laneir, L.L. 1998. NK cell receptors. Annu. Rwv. Immunol. 16., 359-393.

Leduc, M., Bon, C. 1998. Cloning of subunit of convulxin, a collagen-like platelet-aggregation protein from *Crotalus durissus terrificus* venom. Biochem. J. 333, 389-393.

Lee, W.H., Zhang, Y. 2003. Molecular cloning and characterization of a platelet glycoprotein Ib-binding protein from the venom of *Trimeresurus stejnegeri*. Toxicon 41, 885-892.

Lee, W.H., Du, X.Y., Lu, Q.M., Clemetson, K.J., Zhang, Y. 2003a. 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, 662-671.

Lee, W.H., Zhuang, Q.Y., Zhang ,Y. 2003b Cloning and characterization of a blood coagulation factor IX-binding protein from the venom of *Trimeresurus stejnegeri*. Toxicon 41, 765-772.

Li, X., Zheng, L., Kong, C., Kolatkar, P.R., Chung, M.C. 2004. Purpureotin: a novel di-dimeric C-type lectin-like protein from *Trimeresurus purpureomaculatus* venom is stabilized by noncovalent interaction. Arch. Biochem. Biophys. 424, 53-62.

Lu, Q.M., Navdaev, A., Clemetson, J.M., Clemetson K.J. 2004. GPIb is involved in platelet aggregation induce by mucetin, a snake C-type lectin protein from Chinese habu (*Trimeresurus mucrosquamatus*) venom. Thromb. Haemost. 91(6), 1168-1176.

Mahasandana , S., Rungruxsirivorn, Y., Chantarangkul, V. 1980. Clinical manifestations of bleeding following Russell's viper and green pit vier bites in adults. Southeast Asian Trop Med Pub Health 11, 285-293.

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

Maita, N., Nishio, K., Nishimoto, E., Matsui, T., Shikamoto, Y., Morita, T., Sadler, J.E., Mizuno, H. 2003. Crystal structure of von Willebrand factor A1 domain complexed with snake venom, bitiscetin: insight into glycoprotein Ibalpha binding mechanism induced by snake venom proteins. J. Biol. Chem. 278, 37777-37781.

Marcinkiewicz, C., Lobb, R.R., Marcinkiewicz, M.M., Daniel, J.L., Smith, J.B., Dangelmaier, C., Weinreb, P.H., Beacham, D.A., Niewiarowski, S. 2000. Isolation and characterization of EMS16, a C-lectin type protein from *Echis multisquamatus* venom, a potant and selective inhibitor of the $\alpha_2\beta_1$ integrin. Biochemistry 39, 9859-9867.

Meemano, K., Pochanugool C., Limthongkul, S. 1987. Incidence of snakebites at Chulalongkorn hospital. In: Gopalakrishnakone P, Tan CK. Progress in venom and toxin research. Singapore: National University of Singapore, 36-40

Mitrakul, C., Impun, C. 1973. The hemorrhagic phenomena associated with green pit viper (*Trimeresurs erythrurus* and *T.popeorum*) bites in children. A report of studies to elucidate their pathogenesis. Clin Pediatr 12, 215-218.

Mitrakul , C. 1973. Effects of green pit viper (*Trimeresurs erythrurus* and *T. popeorum*) venoms on blood coagulation, platelet and fubrinolytic enzyme systems: Studies in vivo and in vitro. Am J Clin Pathol 60, 654-662.

Mitrakul , C. 1982. Clinical features of viper bites in 72 thai children. Southeast Asian J. Trop. Med Pub. Hlth.13(4), 628-636.

Mizuno, H., Fujimoto, Z., Koizumi, M., Kano, H., Atoda, H., Morita, T. 1999. Crystal structure of coagulation factor IX-binding protein from habu snake venom at 2.6 Å: implication of central loop swapping based on deletion in the linker region. J. Mol. Biol. 289, 103-112.

Monteiro, R.Q., Bock, P.B., Bianconi, M.L., Zingali, R.B. 2001. Characterization of bothrojaracin interaction with human prothrombin. Protein Sci. 10, 1897-1904.

Monteiro, R.Q., Zingali. 2002. Bothrojaracin, a proexosite I ligand, inhibits factor V_a-accelerated prothrombin activation. Thromb. Haemost. 87, 288-293.

Morita, T., Atoda, H., Sekiya, F. 1996. Structure and functions of coagulation factor IX/factor X-binding protein isolated from the venom of *Trimeresurus flavoviridis*. Adv Exp Med Biol. 391,187-196.

Muramaki, M.T., Zela, S.P., Gava, L.M., Michelan-Duarte, S., Cintra, A.C., Arni, R.K. 2003. Crystal structure of the platelet activator convulxin, a disulfide-linked alpha4beta4 cyclic tetramer from the venom of *Crotalus durissus terrificus*. Biochem. Biophys. Res. Common. 310, 478-482.

Navdaev, A., Dormann, D., Clemetson, J.M., Clemetson, K.J. 2001b. Echicetin, a GPIb-binding snake C-type lectin from *Echis carinatus*, also contains a binding site for IgMkappa responsible for platelet agglutination in plasma and inducing signal transduction. Blood 97, 2333-2341.

Nikai, T., Suzuki, J., Komori, Y., Ohkura, M., Ohizumi, Y., Sugihara, H. 1995. Primary structure of the lectin from the venom of *Bitis arietans* (puff-adder)/ Biol. Pharm. Bull. 18, 1620-1622.

Nikai, Kato S, Komori Y, Sugihara H. 2000. Amino acid sequence and biological properties of the lectin from the venom of Trimeresurus okinavensis (Himehabu). Toxicon 38, 707-711.

Okuda, D., Horii, K., Mizuno, H., Morita, T. 2003. Characterization and preliminary crystallographic studies of EMS16, an antagonist of collagen receptor (GPIa/IIa) from the venom of *Echis multisquamatus*. J. Biochem. (Tokyo) 134 (1), 19-23.

Ozeki, Y., Matsu, T., Hamako, J., Suzuki, M., Fujimura, Y., Yoshida, E., Nishida, S., Titani, K. 1994. C-type galactoside-binding lectin from *Bothrops jararaca* venom: comparison of its structure and function with those of botrocetin. Arch. Biochem. Biophys. 308, 306-310.

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

Peng, M., Lu, W., Kirby, E.P. 1992. Characterization of three alboaggregins purified from *Trimeresurus albolabris* venom. Thromb Haemost. 67, 702-707.

Peng, M., Lu, W., Beviglia, L., Niewiarowski, S., Kirby, E.P. 1993. Echicetin, a snake venom protein that inhibits binding of von Willebrand factor and alboaggregins to platelet glycoprotein Ib. Blood 81, 2321-2328.

Peng, M., Emig, F.A., Mao, A., Lu, W., Kirby, E.P., Niewiarowski, S., Kowalska, M.A. 1995. Interaction of echicetin with high affinity thrombin binding site on platelet glycoprotein GPIb. Thromb. Haemost. 74, 954-957.

Polgar, J., Chemetson, J.M., Kehrel, B.E., Wiedemann, M., Magnenat, E.M., Wells, T.N., Clementson, K.J. 1997a. Platelet activation and signal transduction by convulxin, a C-type lectin from *Crotalus durissus terrificus* (tropical rattlesnake) venom via the p62/GPVI collagen receptor . J. Biol. Chem. 272, 13576-13583.

Polgar, j., Magnenat, E.M., Peitsch, M.C., Wells, T.N., Saqi, M.S., Clemetson, K.J. 1997b. Amono acid sequence of the alpha subunit and computer modelling of the alpha and beta subunits of echicetin from the venom of *Echis carinatus* (saw-scaled viper). Biochem. J. 323, 533-537.

Read, M.S., Smith, S.V., Lamb, M.A., Brinkhous, K.M. 1989. Role of botrocetin in platelet agglutination: formation of an activated complex of botrocetin and von Willebrand factor. Blood 74, 1031-1035.

Rojnuckarin P, Mahasandana S., Intragumtornchi T., Swasdikul D., Sutcharitchan P. 1996. Moderate to severe cases of green pit viper bites in Chulalongkorn hospital. Thai journal of hematology and transfusion medicine 6, 199-205.

Rojnuckarin P, Intragumtornchi T, Sattapiboon R, Maunpasitporn C, Pakmanee N, Khow O., Swasdikul D. 1999. The effects of green pit viper (*Trimeresurus albolabris* and *Trimeresurus macrops*) venom on the fibrinolytic system in human. Toxicon 37, 743-55.

Sakurai, Y., Fujimura, Y., Kokubo, T., Imamura, K., Kawasaki, T., Handa, M., Suzuki, M., Matsui, T., Titani, K., Yoshioka, A. 1998. The cDNA cloning and molecular characterization of a snake venom platelet glycoprotein Ib-binding protein, mamushigin,from *Akistrodon halys blomhoffii* venom. Thromb. Haemost. 79, 1199-1207.

Sarray, S., Srairi, N., Luis, J., Marvaldi, J., El Ayeb, M., Marrakchi, N. 2001. Lebecetin, a C-lectin protein from the venom of Macrovipera lebetina that inhibits platelet aggregation and adhesion of cancerous cells. Haemostasis 31, 173-176.

Sarray, S., Srairi, N., Hatmi, M., Luis, J., Louzir, H., Regaya, I., Slema, H., Marvaldi, J., El Ayeb, M., Marrakchi, N. 2003. Lebecetin, a potent antiplatelet C-type lectin from *Macrovipera lebetina* venom. Biochem. Biophys. Acta. 1651, 30-40.

Sekiya, F., Atoda, H., Morita, T. 1993. Isolation and characterization of an anticoagulant protein homologous to botrocetin from the venom of *Bothrops jararaca*. Biochemistry 32, 6892-6897.

Sen, U., Vasudevan, S., Subbarao, G., McClintock, R.A., Celikel, R., Ruggeri, Z.M., Varughese, K.I. 2001. Crystal structure of the von Willebrand factor modulator bothrocetin. Biochemistry 40, 345-352.

Shin, Y., Morita, T. 1998. Rhodocytin, a functional novel platelet agonist belonging to the heterodimeric C-type lectin familly, induces platelet aggregation independent of glycoprotein Ib. Biochem. Biophys. Res. Commun. 245, 741-745.

Shin, Y., Okuyama, I., Hasegawa, J., Morita, T. 2000. Molecular cloning of glycoprotein Ib-binding protein, flavocetin-A, which inhibits platelet aggregation. Thromb. Res. 99, 239-247.

Stockert. R.J. 1995. The asialoglycoprotein receptor: Relationships between structure, function, and expression Physiol. Rev. 75: 591-609.

Tani, A., Ogawa, T., Nose, T., Nikandrov, N.N., Deshimaru, M., Chijiwa, T., Chang, C.C., Fukumaki, Y., Ohno, M. 2002. Characterization, primary structure and molecular evolution of anticoagulant protein from *Agkistrodon acutus* venom. Toxicon 40, 803-813.

Taniuchi, Y., Kawasaki, T., Fujimura, Y., Suzuki, M., Titani, K., Sakai, Y., Kabu, S., Hisamichi, N., Satoh, N., Takenaka, T., etal. 1995. Flavocetin-A and –B two hight molecular mass glycoprotein Ib binding proteins with high affinity purified from *Trimeresurus flavoviridis* venom, inhibit platelet aggregation at high shear stress. Biochem. Biophys. Acta 1244, 331-338.

Tomohisa O., Tankahito C., Naoko Oda-Ueda., Motonori O. 2005. Molecular diversity and accelerated evolution of C-type lectin-like proteins from snake venom. Toxicon. 45, 1-14.

Trishnanaada, M. 1979 Incidence, clinical manifestations and general management of snake bites. Southeast Asian J Trop Med Public Health 10, 248-250.

Usami, Y., Fujimura, Y., Suzuki, M., Ozeki, Y., Nishio, K., Fukui, H., Titani, K. 1993. Primary structure of two-chain botrocetin; a von Willebrand factor modulator purified from the venom of *Bothrops jararaca*. Proc. Natl. Acad. Sci. USA 90, 928-932.

Usami, Y., Suzuki, M., Yoshida, E., Sakurai, Y., Hirano, K., Kawasaki, T., Fujimura, Y., Titani, K. 1996. Primary structure of alboaggregin-B purified from the venom of *Trimeresurus albolabris*. Biochem. Biophys. Res. Commun. 219, 727-733.

Viravan C, Looareesuwan S, Kosakarn W, et al. 1992 A national hospital-based survey of snakes responsible for bites in Thailand. Trans Royal Soc Trop Med Hyg 86, 100-106

Wang, R., Kini, R.M., Chung, M.C. 1999. Rhodocetin, a novel platelet aggregation inhibitor from the venom of *Calloselasma rhodostoma* (Malayan pit viper): synergistic and noncovalent interaction between its subunits. Biochemistry 38, 7584-7593.

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

Weis WI, Taylor ME, Drickamer K. 1998. The C-type lectin superfamily in the immune system. Immunol Rev. 163, 19-34

Wei, Q., Lu, Q.M., Jin, Y., Li, R., Wei, J.F., Wang, W.Y., Xiong, Y.L. 2002. Purification and cloning of a novel C-type lectin-like protein with platelet aggregation activity from *Trimeresurus mucrosquamatus* venom. Toxicon 40, 1331-1338.

Xu, Q., Wu, X.F., Xia, Q.C., Wang, K.Y. 1999. Cloning of a galactose-binding lectin from the venom of *Trimeresurus stejnegeri*. Biochem. J. 341, 733-737.

Zang, J., Teng, M., Niu, L. 2003. Purification, crystallization and preliminary crystallographic analysis of AHP IX-bp, a zinc ion and pH-dependent coagulation factor IX binding protein from *Agkistrodon halys Pallas* venom. Acta Crystallogr. D Biol. Crystallogr. 59 (Pt 4). 730-737.

Zingali, R.B., Jandrot-Perrus, M., Guillin, M.-C., Bon, C. 1993. Bothrojaracin, a new thrombin inhibitor isolated from *Bothrops jararaca* venom: Characterization and mechanism of thrombin inhibitor. Biochemistry 32, 10794-10802.

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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

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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

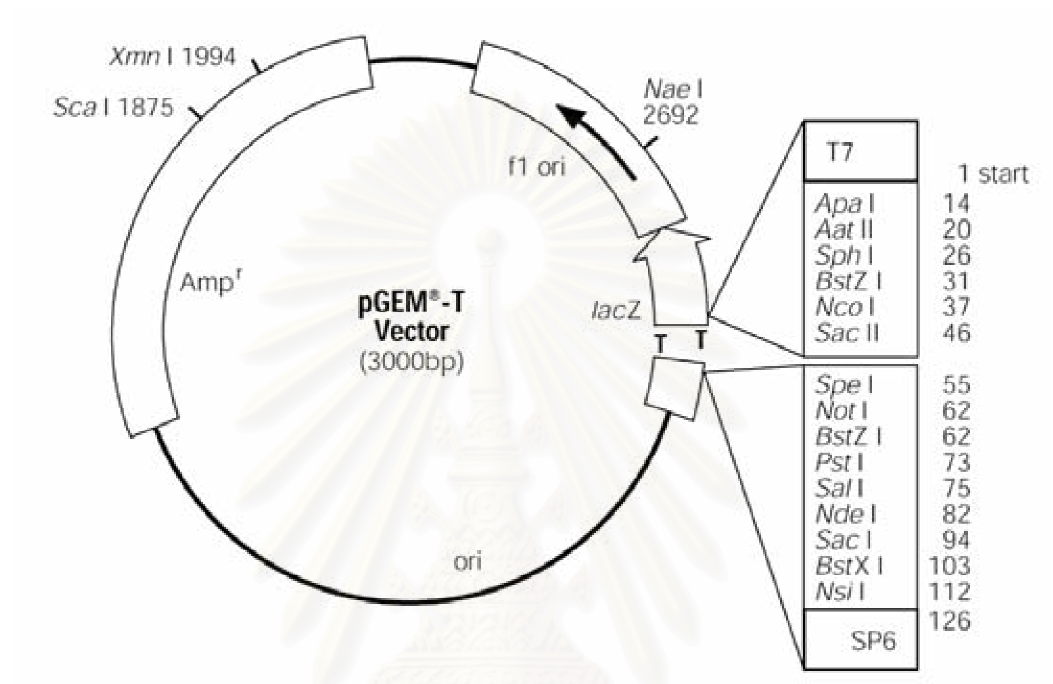
2.10 1X Phosphate-Buffered Salive (PBS)

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

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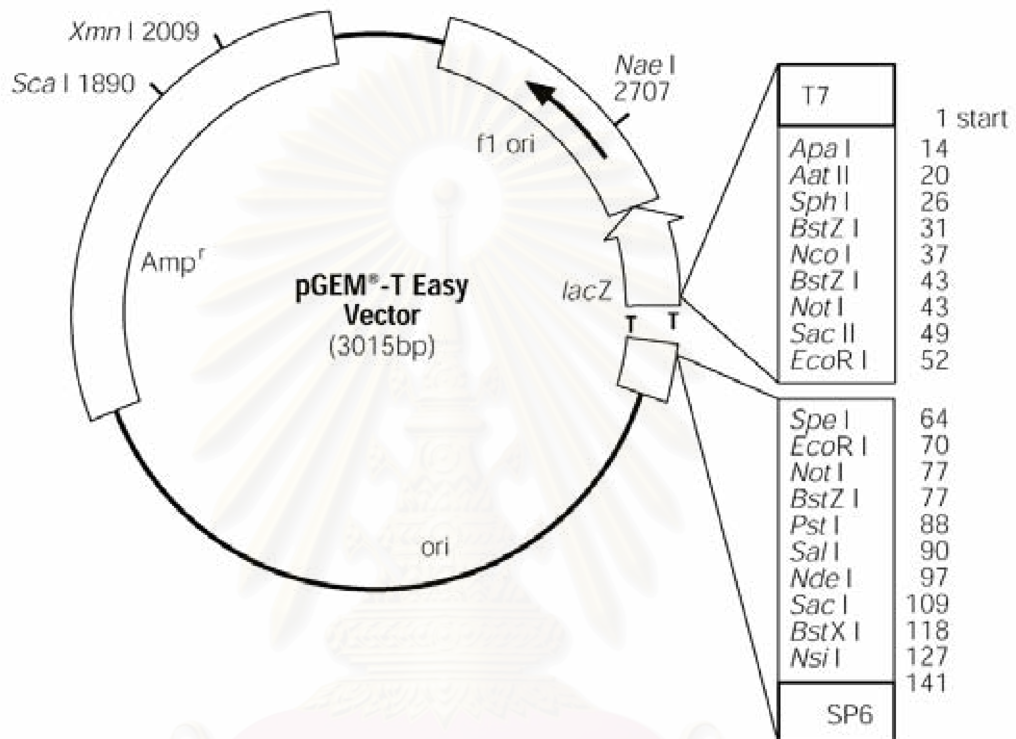
pUC/M13 Forward Sequencing Primer binding site 2941–2957

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

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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 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

4.4.1 Stock I

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

4.4.2 Stock II

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

4.4.3 Stock III

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

4.5 buffer I

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

4.6 buffer II

20 ml. washing buffer, 200 µl CP/CPK

BIOGRAPHY

Mister Jaradpong Arpijuntarangkoon was born in 1981. He obtained the Bachelor’s Degree from the Faculty of Allied Health Science, Chulalongkorn University in 1999. He continued his study for Master’s Degree in Medical Science, Faculty of Medicine, Chulalongkorn University in 2003.

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