การโคลนยีน การผลิตโปรตีน และการศึกษาหน้าที่ของโปรตีนเมทัลโลโปรตีเนสชนิดใหม่จากพิษงูเขียวหางไหม้ท้องเหลือง

นายอนุวัตร ภิญญะชาติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

MOLECULAR CLONING, EXPRESSION, AND FUNCTIONAL CHARACTERIZATIONS OF NOVEL SNAKE VENOM METALLOPROTEINASES FROM GREEN PIT VIPER (*Trimeresurus albolabris*)

Mr. Anuwat Pinyachat

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedical Sciences
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2010

Thesis Title	MOLECULAR CLONING, EXPRESSION, AND FUNCTIONAL CHARACTERIZATIONS OF NOVEL SNAKE VENOM METALLOPROTEINASES FROM GREEN PIT VIPER (*Trimeresurus albolabris*)
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Field of Study	Biomedical Sciences
Thesis Advisor	Professor Surang Nuchprayoon, M.D., MPH., Ph.D.
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อนุวัตร ภิญญะชาติ : การโคลนยีน การผลิตโปรตีน และการศึกษาหน้าที่ของโปรตีนเมทัลโลโปรตีเนสชนิดใหม่จากพิษงูเขียวหางไหม้ท้องเหลือง. (MOLECULAR CLONING, EXPRESSION, AND FUNCTIONAL CHARACTERIZATIONS OF NOVEL SNAKE VENOM METALLOPROTEINASES FROM GREEN PIT VIPER (*Trimeresurus albolabris*)) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ศ. พญ. ดร. สุรางค์ นุชประยูร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. นพ. ดร. พลภัทร โรจน์นครินทร์, 109 หน้า

การโคลนยีนและศึกษาหน้าที่ของเอนไซม์ snake venom metalloproteinase (SVMP) P-III ชนิดใหม่ในหลอดทดลอง โดยใช้เทคโนโลยีพันธุวิศวกรรมช่วยให้เข้าใจกลไกการทำลายเนื้อเยื่อเฉพาะที่ซึ่งไม่ตอบสนองต่อการรักษาด้วยเซรุ่มต้านพิษงูเขียวหางไหม้ท้องเหลือง (*Trimeresurus albolabris*) จากข้อมูลห้องสมุดยีนงูเขียวหางไหม้ท้องเหลือง ทำให้เราระบุและค้นหายีน SVMP P-III ชนิดใหม่โดยวิธี 5′-RACE ได้สำเร็จ เอนไซม์นี้ประกอบด้วย กรดอะมิโน 614 ตัว เรียกว่า "อัลโบคอลลาจีเนส" หากนับจากส่วนเริ่มแปลรหัสโปรตีนนี้ประกอบด้วยหลายส่วน ได้แก่ signal peptide, prodomain, metalloproteinase domain ซึ่งมีส่วนที่จับสังกะสีและมี cysteine 9 ตัว, และส่วน disintegrin-like and cysteine-rich domains ซึ่งมี DCD-motif และมี cysteine 24 ตัว ลำดับกรดอะมิโนอัลโบคอลลาจีเนสมีความคล้ายกับ SVMP P-III ของงูชนิดอื่นๆประมาณร้อยละ 70 เป็นที่สังเกตว่าส่วน prodomain มีความอนุรักษ์มากกว่าส่วนอื่นๆ คณะผู้วิจัยได้แสดงออกอัลโบคอลลาจีเนสขนาด 62 กิโลดาลตัน ซึ่งปราศจากส่วน prodomain โดยใช้ยีสต์ *Pichia pastoris* ได้สำเร็จเป็นรายแรกของโลก พบว่าเอนไซม์ดังกล่าวสามารถย่อยคอลลาเจนได้ภายใน 1 นาที แต่ไม่สามารถย่อยไฟบริโนเจนของมนุษย์ได้ อีกทั้งสามารถยับยั้งการเกาะกลุ่มของเกร็ดเลือดมนุษย์เมื่อกระตุ้นด้วยคอลลาเจน (แต่ยับยั้งไม่ได้เมื่อกระตุ้นด้วย ADP) ด้วยค่าความเข้มข้นที่ยับยั้งได้ร้อยละ 50 (IC_{50}) เท่ากับ 70 nM ผลการทดลองแนะว่าอัลโบคอลลาจีเนสมีบทบาทสำคัญต่อการทำลายเนื้อเยื่อค้ำจุน และยับยั้งการเกาะกลุ่มของเกร็ดเลือดซึ่งสัมพันธ์กับพยาธิสภาพการทำลายเนื้อเยื่อเฉพาะที่และการเกิดเลือดออกใต้ผิวหนังของผู้ที่ถูกงูเขียวหางไหม้กัด ซึ่งอาจจะนำไปสู่การค้นหาตัวยับยั้งอัลโบคอลลาจีเนสที่เหมาะสมในอนาคต

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

468 96933 20 : MAJOR BIOMEDICAL SCIENCES

KEYWORDS : GREEN PIT VIPER / ALBOCOLLAGENASE / SNAKE VENOM METALLOPROTEINASE (SVMP) / *Pichia* / COLLAGEN TYPE IV / PLATELET AGGREGATION

ANUWAT PINYACHAT : MOLECULAR CLONING, EXPRESSION, AND FUNCTIONAL CHARACTERIZATIONS OF NOVEL SNAKE VENOM METALLOPROTEINASES FROM GREEN PIT VIPER (*Trimeresurus albolabris*). THESIS ADVISOR : PROFESSOR SURANG NUCHPRAYOON, M.D., MPH., Ph.D., THESIS CO-ADVISOR : ASSOCIATE PROFESSOR PONLAPAT ROJNUCKARIN, M.D., Ph.D., 109 pp.

Molecular cloning and functional characterizations of P-III snake venom metalloproteinases (SVMPs) of the green pit viper (*Trimeresurus albolabris*) will give us deeper insights in the pathogenesis of viper bites particularly for venom-induced local tissue damages, the complication refractory to current antivenom. The aim of this study was to elucidate the *in vitro* activities of a new SVMP from the green pit viper (GPV) using recombinant DNA technology. Using the 5′-RACE method, a new P-III SVMP cDNA encoding 614 amino acid residue protein, termed "albocollagenase" was obtained. The conceptually translated protein comprised a signal peptide, pro-domain, followed by a metalloproteinase domain containing a zinc-binding motif and 9 cysteine residues, and the disintegrin-like and cysteine-rich domains possessing 24 cysteines and a DCD-motif. The albocollagenase deduced amino acid sequence alignments shown approximately 70 % identity with other P-III SVMPs. Notably, the prodomain was highly conserved, while the metalloproteinase and disintegrin-like and cysteine-rich domains contained several differences. This is the first successful report of the active 62-kDa recombinant P-III SVMP without the signal peptide and prodomain expressed in yeast *Pichia pastoris*. The recombinant albocollagenase could digest human type IV collagen from human placenta basement membrane within 1 minute, but not human fibrinogen. It also inhibited collagen-induced (but not ADP-induced) platelet aggregation with 50% inhibitory concentration (IC_{50}) of 70 nM. The results suggest the significant roles of P-III SVMP in local and systemic pathology of envenomated patients. Inhibitors of this SVMP may lead to a better treatment for viper bites in the future.

Field of Study : Biomedical Sciences

Academic Year : 2010

Student's Signature Anuwat Pinyachat

Advisor's Signature Surang Nuchprayoon

Co-Advisor's Signature

ACKNOWLEDGEMENTS

This thesis would not be possible without the supports from Thailand Research Fund (TRF) and the Commission on Higher Education. I have been supported by the Royal Golden Jubilee (RGJ) Ph.D. program scholarship from the TRF, the contract No. PHD/0246/2545. I owe my deepest gratitude to my adviser, Professor Surang Nuchprayoon, M.D., MPH., Ph.D., who made available her support in a number of ways to gain this funding. I am heartily thankful to my co-adviser, Associate Professor Ponlapat Rojnuckarin, M.D., Ph.D., who made this thesis possible. Both of them whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of this thesis project. It is an honor for me to thank Associate Professor Rachana Santiyanont, Ph.D., who inspired my vision to study in Ph.D. program. I would like to show my gratitude to Professor, Yong Poovorawan, M.D. and Professor Apiwat Mutirangura, M.D., Ph.D., the nice teachers and my scientist researcher idols. I am grateful Associate Professor Sirirat Rengpipat, Ph.D. and Dr. Allan Bollen for their valuable suggestions of vital information about the successful expression in yeast. I also would like to thank all of my committee, Assistant Professor Tewin Tencomnao, Ph.D., and Associate Professor Apinunt Udomkit, Ph.D.

I am indebted to many of my colleagues who support me including Dr. Chaunchom Maunprasitporn, Ms. Pon Singhamatr, Dr. Vivornpun Sanprasert, and all members in the laboratory for their help and support since the beginning of my laboratory practice. I would like to thank Mrs. Benjaporn Akkawat, laboratory head of division of hematology, for equipments and techniques in platelet aggregation analysis.

Last but not least, I would like to gratitude to my dear parents and, indeed, all of my older brothers/sisters for their love and understanding, which financially assisted me until today's success.

Finally, I offer my regards and blessings to all of those who supported me in every aspect during the completion of this thesis project.

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LIST OF ABBREVIATIONS

α_2M	α_2-macroglobulin is a large plasma protein found in the blood.
ADP	Adenosine 5′-diphosphate
Antipain	An oligopeptide produced by various bacteria which acts as a protease inhibitor.
ATP	adenosine 5′-triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, specific Ca^{2+} chelator
Benzamidine	Benzamidine is a reversible competitive inhibitor of trypsin, trypsin-like enzymes and serine proteases.
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CNBr	proteolysis with Achromobact*er* protease I which specifically cleaves lysyl bonds
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytosinetriphosphate
dGTP	deoxyguaninetriphosphate
DIFP	Diisopropyl fluorophosphates
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	dATP, dTTP, dGTP and dCTP
DTT	Dithiothreitol, its oxidized form is a disulfide-bonded 6-membered ring.
dTTP	deoxythyminetriphosphate
EACA	Aminocaproic acid is a derivative and analogue of the amino acid lysine, which makes it an effective inhibitor for enzymes that bind that particular residue.
ED2SC	diCalcium EDTA
EDTA	ethylenediaminetetraacetic acid

EGTA	ethylene glycol tetraacetic acid, is a polyamino carboxylic acid, a chelating agent that is related to the better known EDTA, but with a much higher affinity for calcium than for magnesium ions.
GP	glycoprotein
HMEC	Human mammary epithelial cell
IDA	Iodoacetamide, It is commonly used to bind covalently with the thiol group of cysteine so the protein cannot form disulfide bonds.
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani media
MMLV	Moloney Murine Leukemia Virus
MWCO	molecular weight cut off
OD	optical density
PACKS	platelet aggregation chromogenic kinetic system
PCMB	p-chloromercuribenzoate, a protease inhibitor
PCR	polymerase chain reaction
Pep	Pepstatin, a potent inhibitor of aspartyl proteases
Phen	O-phenanthroline, it forms strong complexes with most metal ions.
PMSF	phenylmethanesulfonylfluoride, serine protease inhibitor
PNG-F	peptide-N-glycosidase F
PVDF	Polyvinylidene Fluoride
RNA	ribodeoxyribonucleic acid
rpm	Round per minute
RT-PCR	reverse transcription polymerase chain reaction
Soybean	Soybean trypsin inhibitor
TPEN	N,N,N'N'-tetrakis(-)[2-pyridylmethyl]-ethylenediamine, specific Zn^{2+} chelator
Trasylol	(Aprotinin) The bovine version of the small protein basic pancreatic trypsin inhibitor
x g	x gravity

CHAPTER I

INTRODUCTION

1. Background and Rationale

The reported incidence of venomous snakebite in Thailand is approximately 16.6/100,000/year (Rojnuckarin, 2004) with the mortality rate around 0.02/100,000/year. The most important species are Malayan pit viper (40%), Green pit viper (34%), Cobra (12%) and Russell's viper (10%) (Jintakune and Chanhome, 1996). Therefore, the viper bites, which mainly effect hematostatic systems, are an important health problem in Thailand.

There are about 160 species of snake in Thailand and 47 of them are venomous (Jintakune and Chanhome, 1996). Two important families of the venomous snakes in Thailand are elapidae and viperidae that are neurotoxic and hematotoxic, respectively.

Family Elapidae

Cobra

Spitting cobra

King cobra

Banded krait

Malayan krait

Family Viperidae

True viper (Sub family Viperinae): Russell's viper

Pit viper (Sub family Crotalinae): Malayan pit viper and Green pit vipers

1.1 Green pit viper (GPV)

In Thailand, there are 11 species of green pit vipers. The two most common species are *Trimeresurus albolabris* and *Trimeresurus macrops* (the new names: *Cryptelytrops albolabris* and *Cryptelytrops macrops*) (Malhotra and Thorpe, 2004)). The venoms of these two species are closely related, but the venom of *T. albolabris* is more toxic than that of *T. macrops* (Rojnuckarin et al., 1999). White-lipped pit viper, *T. albolabris*, is the most commonly reported poisonous snake. *T. albolabris*, a crotaline viper, is small to medium in size and generally stays on trees. This family of snakes possesses a heat-sensitive pit organ (thermoreceptor) behind each nostril to detect a very small temperature change in warm-blooded preys (Cox et al., 1998).

Figure 1. *Trimeresurus albolabris* (Rojnuckarin, 2010)

1.2 Green pit viper bites

Green pit viper bites, an occupational hazard for farmers and rubber tappers, are still common in Southeast Asia including Thailand (Mahasandana and Jintakune, 1990; Rojnuckarin et al., 1999; Rojnuckarin et al., 2006a). They account for 40% of all bites (Viravan et al., 1992) and comprise more than 95% of venomous snakebites in Bangkok (Mahasandana and Jintakune, 1990). The victims are usually bitten as they are walking nearby a bush particularly on their hands, feet or legs. Hence, GPV bites are still prevalent and cause public health problem in our country, even in metropolitan areas, such as Bangkok. As we are invading their territory, they are adapting to live among humans in our backyards (Rojnuckarin, 2008).

1.3 Clinical manifestations

Green pit viper venom causes systemic hematotoxic effects, such as hypofibrinogenemia and thrombocytopenia, and local effects, such as limb swelling, pain, edema, blisters, ecchymosis, digital gangrene, and necrosis (Mahasandana et al., 1980). Skin blisters, resulting from dermo-epidermal separation, occur in 24.8% of victims and are significantly related to subsequent dermal necrosis and super-infection (Rojnuckarin et al., 1998). Digital gangrene, which requires prolonged daily dressing and possible surgical dermotomy, also occurs in 6.6% of cases (Rojnuckarin et al., 1998). The defibrination syndrome following a green pit viper bite may be prolonged for over a week, if no antivenom treatment is given (Visudhiphan et al., 1981).

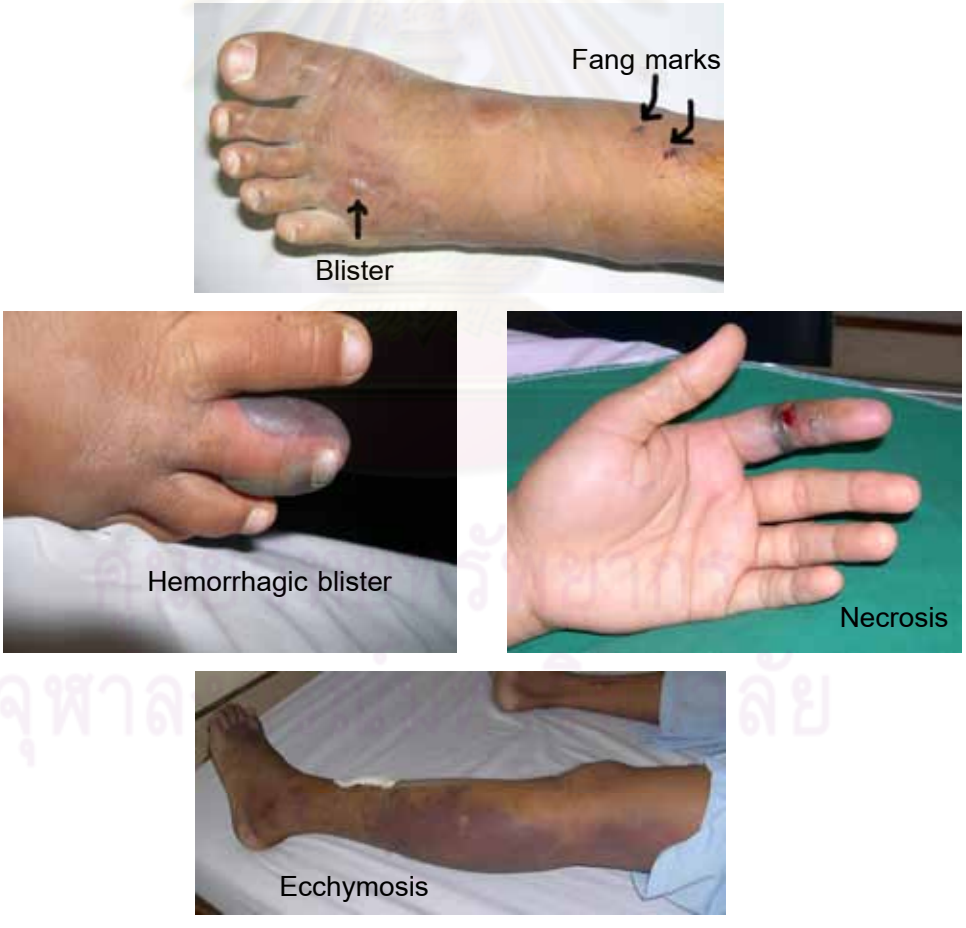


Figure 2. Clinical manifestations of GPV bites (Rojnuckarin, 2010)

1.4 Complication refractory to current antivenom

Although systemic administration of antivenom can promptly reverse coagulopathy within hours, efficacy on local edema resolution in humans is not clinically significant. As the commercially available antivenom has a risk of severe reactions, general use to treat all edema is not recommended (Rojnuckarin et al., 2006a). Furthermore, GPV-induced skin necrosis still occured, although prior systemic antivenom was administered (Chotenimitkhun and Rojnuckarin, 2008). There is, currently, no proven treatment that is effective in these cases. This is consistent with animal studies showing that venom-induced local damage was rapid and unable to be treated even by immediate antivenom (Andrews and Berndt, 2000). Therefore, production of hemorrhagic metalloproteinase protein to evaluate the functions and test for appropriate small-molecule inhibitors are required.

1.5 Snake venom metalloproteinases responsible for local effects

Snake venom metalloproteinases (SVMPs) are considered to be one of the major causes of extracellular matrix (ECM) degradation and induce both local damages and systemic bleeding in viper bite patients (Bjarnason and Fox, 1994). In addition to ECM degradation, SVMPs also affect proteins of hemostatic system. For example, the purified P-I BlaH1 from *Bothrops lanceolatus* (Stroka et al., 2005), the P-III hemorrhagin from *Trimeresurus pupureomaculatus* (Khow et al., 2002) and P-III VaH1 and VaH2 from *Vipera ammodites ammodites* (Leonardi et al., 2002) degraded fibrinogen, collagen, and elastin *in vitro* inducing hemorrhage *in vivo*. Furthermore, the purified P-III SVMP, jerdohagin, also cleaved fibrinogen and prothrombin (Chen et al., 2004).

Recent study, It has a purified and characterized alborhagin, a 60-kDa SVMP from *T. albolabris*. It was a platelet agonist and, subsequently, induced ectodomain shedding of the platelet collagen receptor glycoprotein VI (Andrews et al., 2001; Wijeyewickrema et al., 2007). However, its full sequence is not yet available.

Many structure-function studies of the SVMPs have been reported using venom purification correlating with cDNA cloning to obtain the sequences. While previous data shown that P-III SVMP was more strongly hemorrhagic than P-I and P-II SVMPs (Moura-da-Silva et al., 2008), very few reports on recombinant expression of P-III SVMPs with active protease domains were published (Moura-da-Silva et al., 1999; Silva et al., 2004; Wang et al., 2003). This may be partly due to the difficulties in protein production, purification and/or autolysis of P-III SVMP (Kamiguti et al., 2000; Moura-da-Silva et al., 2003; Oliveira et al., 2009). Recombinant P-III catrocollastatin from *Crotalus atrox* expressed using baculovirus expression system in insect cells inhibited collagen-induced platelet aggregation, but the enzymatic activity was not reported (Zhou et al., 1995). The other studies expressed the disintegrin-like domain of P-III jararhagin (Moura-da-Silva et al., 1999) and P-III DC-HF3 from *Bothrops jararaca* (Silva et al., 2004) in *E. coli* and found that they reacted with platelet.

The goal of this study was to identify a new P-III SVMP gene to investigate the direct functions related with cDNA sequences and to elucidate its *in vitro* activities that corresponded to systemic effects and local tissue damages in snakebite victims. The SVMP cDNA was cloned and expressed in the methylotrophic yeast, *P. pastoris*. The system was chosen because it enables to correct eukaryotic post-translational modifications that may be essential for enzymatic activities.

2. Research Question

What are the functions of the P-III SVMP of *T. albolabris* on human hemostatic system?

3. Hypothesis

The recombinant P-III SVMP of *T. albolabris* expressed from *Pichia pastoris* degrades basement membrane proteins and inhibits platelet aggregation contributing to local and systemic effects on envenomated human.

4. Objectives

1. To clone and characterize P-III SVMP gene of *T. albolabris* to get full-length cDNA sequence that has never been previously reported.
2. To produce recombinant P-III SVMP of *T. albolabris* expressed in *P. pastoris*.
3. To investigate the activities of recombinant P-III SVMP of *T. albolabris* on extracellular matrix proteins, the activity that probably contributes to local effects of envenomated human.

5. Keywords

Green pit viper

Albocollagenase

Snake venom metalloproteinase (SVMP)

Cloning

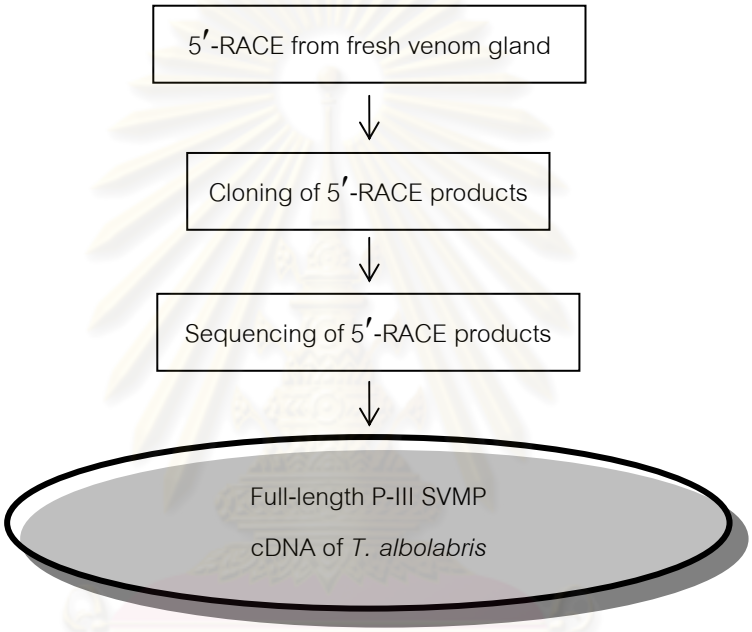
Pichia

Collagen type IV

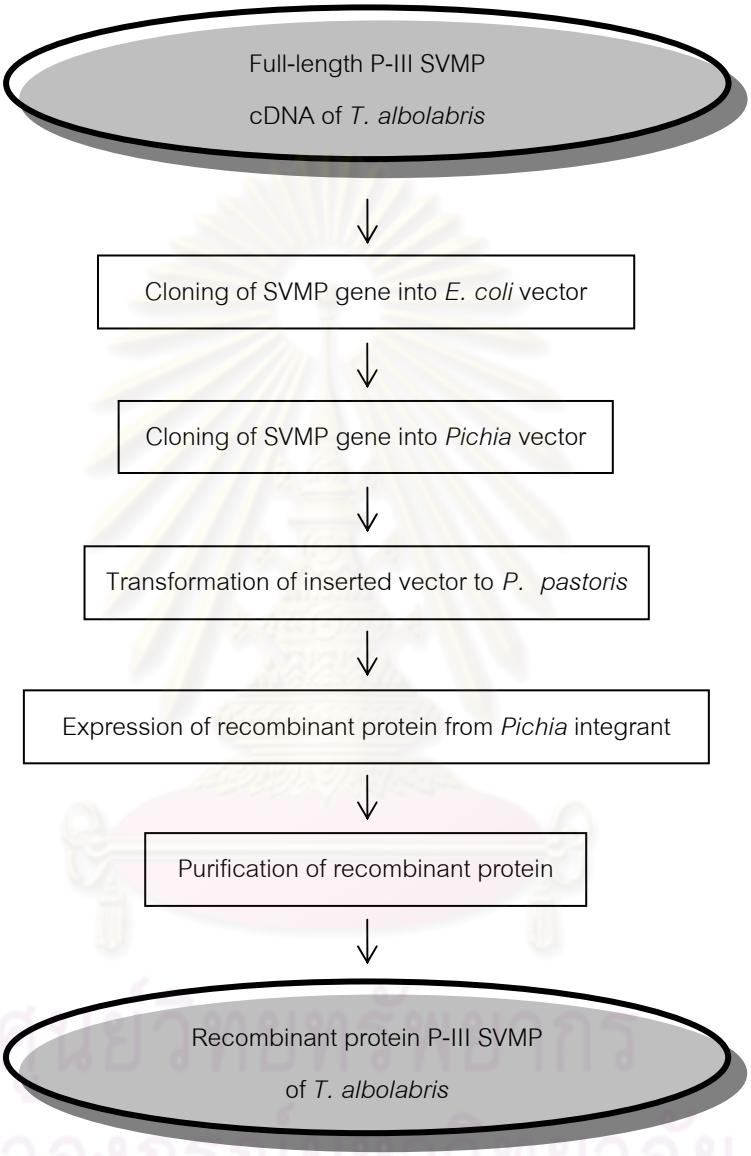
Platelet aggregation

6. Conceptual Framework

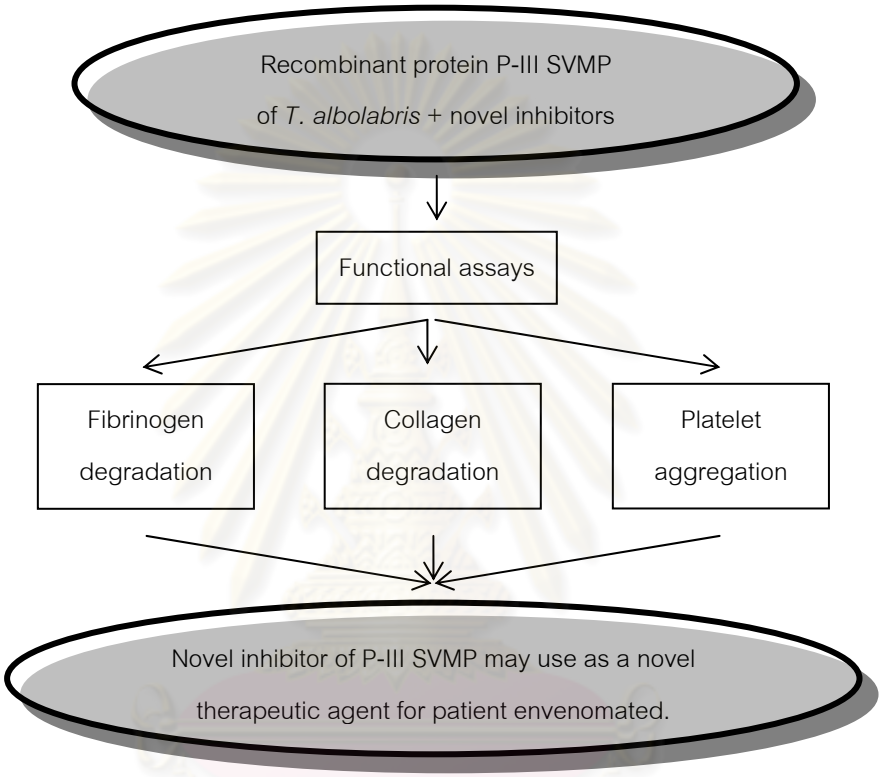
1. To clone and characterize P-III SVMP gene of *T. albolabris* to get full-length cDNA sequence that has never been previously reported. The conceptually translated protein sequence will be analyzed.



2. To express recombinant P-III SVMP of *T. albolabris* in *P. pastoris*.



3. To investigate the activities of recombinant P-III SVMP of *T. albolabris* with its putative novel inhibitors on extracellular matrix proteins, the effect that probably contribute to local tissue damages.



7. Expected benefits and applications

GPV envenomation targets the hemostatic system resulting in bleeding disorders, as well as local effects, which has no proven effective treatment at the moment. The current molecular biology permits the construction of a complementary DNA (cDNA) library of the expressed GPV gene in venom glands instead of conventional venom protein purifications. The complete and accurate genes from cDNA library drive us to select the P-III SVMP of GPV genes to study its functions correlating with the conceptual translated protein derived from 5′-RACE method. This unlimited cDNA source of P-III SVMP provides us for recombinant protein expression that can be use to investigate their functions and search for novel potentially therapeutic inhibitors. In addition, mutagenesis can be performed to study the structure–function relationship and engineer protein with desirable activities. Finally, metalloproteases may affect a wide variety of physiological and pathological processes, including angiogenesis, inflammation and cancer metastasis. Therefore, the protein, itself, also has potentials to be new diagnostic or therapeutic agents.

CHAPTER II

LITERATURE REVIEW

1. Snake venom metalloproteinases (SVMPs)

The snake venom metalloproteinases (SVMPs) are one of the major functional peptides from snake venom. SVMPs are responsible for the induction of rapid local tissue damage (Laing et al., 2003) and systemic bleeding (Rucavado et al., 2004). They interfere with the blood coagulation and hemostatic plug formation, and degrade basement membrane or extracellular matrix components of victims (Marsh, 1994; Matsui et al., 2000). All of them are zinc metalloproteinases with a Zn^{2+}-binding motif with the characteristic consensus sequence, HEXXHXXGXXH——CIM (Stocker et al. 1995), and belong to the metzincin superfamily as well as mammalian ADAMs (A Disintegrin And Metalloproteinase) (Huovila et al., 1996; Schwettmann and Tschesche, 2001), which play important roles in cell–cell fusion, cell–matrix interaction and other cellular functions (Wolfsberg et al., 1995).

SVMPs are classified into four groups by their domain structures (Hite et al., 1992). The P-I class is composed of a single metalloproteinase domain, the P-II class consists of a metalloproteinase domain and a disintegrin domain. The P-III class consists of metalloproteinase and disintegrin-like and cysteine-rich domains. Finally, the P-IV class contains an additional disulfide-linked C-type lectin-like domain compared to P-III class (Jia et al., 1996). The group P-IV of SVMPs has been recently reclassified as part of group III (Fox and Serrano, 2008). Thus, currently there are only three SVMPs groups: I, II and III.

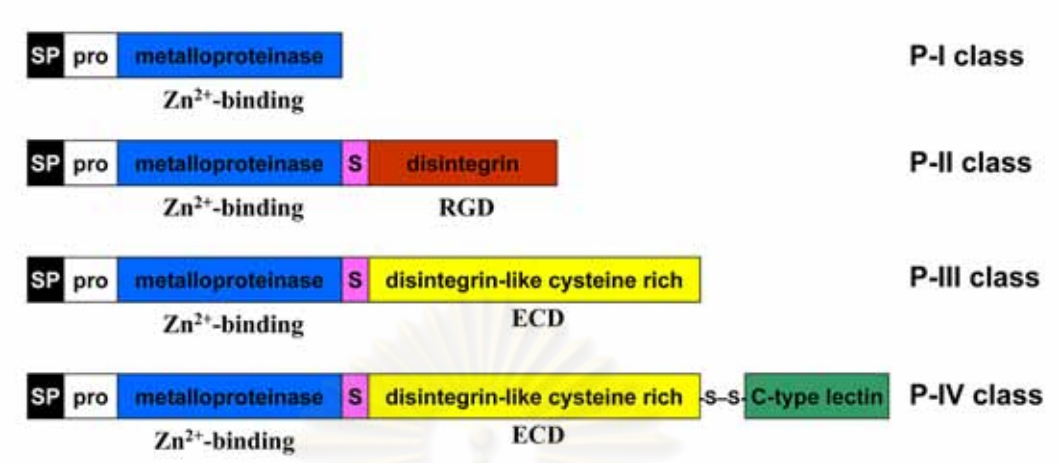


Figure 3. Schematic structures of snake venom metalloproteinases (SVMPs) were modified from Hite et al., 1994. **SP** = signal peptides, **pro** = prodomain, **S** = spacer region, **Zn^{2+}-binding** = zinc binding motif (HEXXHXXGXXH——CIM), **RGD** = conserve sequences of platelet binding glycoproteins ($\alpha_{IIb}\beta_3$ integrin), **ECD** = conserve sequences of putative collagen and/or platelet binding motif, **-S–S-** = disulfide bond

2. SVMPs induce local hemorrhage

Metalloproteinases are abundant enzymes in the venoms of *Crotalidae* and *Viperidae*. They are relevant in the pathophysiology of envenomation, being responsible for local and systemic hemorrhage frequently observed in the victims (Teixeira Cde et al., 2005). Hemorrhagic activity is determined by the rabbit skin method using one rabbit for each purification step (Kondo et al., 1960). For examining the recovery of the hemorrhagic activity during purification and for examining the effect of inhibitors, three rabbits are required (Khow et al., 2002). The minimum hemorrhagic dose (MHD) was defined as the amount (μg) of venom producing a hemorrhagic spot of 10 mm diameter in the rabbit skin. Hemorrhagic activity has been associated with enzymatic proteolytic activity, since chelation of the zinc atom by ethylenediamine tetraacetic acid (EDTA) abolishes both proteolytic and hemorrhagic effects (Bjarnason and Tu, 1978). The role

of the others domains in the toxicity of high molecular enzymes is not clear, although it has been shown that large hemorrhagic metalloproteinases contained disintegrin-like and cysteine-rich domains, are more active in inducing hemorrhage than enzymes comprising only the metalloproteinase domain (Bjarnason and Fox, 1994; Hite et al., 1994). The purified hemorrhagin from *Naja Kaouthia* treated with EDTA at a concentration of 10 mM at 37°C for 5 min and with soybean trypsin inhibitor at a concentration of 1 mg/ml at room temperature for 1 hour, hemorrhagic activity is inhibited by EDTA but not by soybean trypsin inhibitor (Khow et al., 2001). EDTA eliminates the proteolytic, as well as the hemorrhagic activity, of VaH1 while iodoacetamide, phenylmethylsulfonyl fluoride (PMSF) and pepstatin A, inhibitors of cysteine, serine and aspartic proteinases respectively, shown no effect (Leonardi et al., 2002).

3. *In vitro* assays for proteolytic activities of SVMPs

In vitro assays for proteolytic activities of snake venom have previously been described. The first method, which is the general proteolytic activity, uses casein as a substrate (Yamakawa and Omori-Satoh, 1988). The venom hydrolyzed casein in trichloroacetic acid condition and the soluble products that give the absorbance of 1.0 at 280 nm per 30 min are defined to contain one unit of activity. On the other hand, Stroka assayed in 0.1 M Tris–HCl, pH 7.8, using casein as substrate. One unit of activity was defined as an increase of 0.001 absorbance units at 280 nm/min (Stroka et al., 2005). The second method uses hide powder azure as a substrate (Chromogenic substrate for trypsin) (Omori-Satoh et al., 1995). One unit of the activity was defined as an absorbance increasing of 1.0/hour at 595 nm.

Collagenase activity was assayed using bovine Achilles tendon collagen in 0.05 M Tris–HCl, pH 7.8, as a substrate. The hydroxyproline released during a 24-h incubation was detected by a reaction with the ninhydrin reagent followed by boiling for 20 min. After cooling, 50% N-propanol was added and the resulting absorbance was read at

600 nm. Collagenolytic activity was expressed as the increasing in absorbance at 600 nm per mg (Stroka et al., 2005). To observe the band of collagen from any type, some studies use SDS-PAGE to perform the collagenase activity of snake venoms (Khow et al., 2002; Oliveira et al., 2010).

Fibrinogen degradation activity assays of snake venom were commonly performed by SDS-PAGE, since it is usually very clear to see each band of α, β and γ fibrinogen (Chen et al., 2004; Leonardi et al., 2002; Siigur et al., 1998; Willis and Tu, 1988). Some studies use the fibrin plate assay to determine the fibrinolytic activity (Siigur et al., 1991 and 1998)

4. SVMPs inhibit platelet aggregation and degrade hemostatic proteins

Generally, SVMPs can degrade ECM proteins. The proteolytic activities are thought to be resulted from metalloproteinase domain and responsible for systemic hemorrhage and local effects. In addition, they also react with platelet and hemostatic proteins. For example, the purified P-I BlaH1 from *Bothrops lanceolatus* has abilities to induce hemorrhagic activity *in vivo* and degrade fibrinogen, collagen and elastin *in vitro* (Stroka et al., 2005) similar to the purified P-III hemorrhagin from *Trimeresurus pupureomaculatus* (Khow et al., 2002) and purified P-III VaH1 and VaH2 from *Vipera ammodites ammodites* (Leonardi et al., 2002). Purified P-III jerdohagin from *Trimeresurus jerdonii* not only induces hemorrhage but also cleaves fibrinogen and fragment F1 of prothrombin (Chen et al., 2004). The P-III SVMP and its truncated form also react with platelet, inhibit collagen-induced platelet aggregation, e.g. the recombinant catrocollastatin from *Crotalus atrox* expressed in *Spodoptera frugiperda* (fall armyworm) (Zhou et al., 1995).

The *E. coli* expressed disintegrin-like and cysteine-rich domains was compared with purified P-III jararhagin from *Bothrops jararaca*. They inhibited collagen-induced platelet aggregation with IC_{50} of 140 and 40 nM, respectively (Moura-da-Silva et al.,

1999). In addition, the *Pichia pastoris* expressed disintegrin-like and cysteine-rich domains, halydin, was compared with purified P-III of halysase from *Gloydius halys*. They also inhibited platelet aggregation with IC_{50} of 178 and 87 nM, respectively (You et al., 2003; You et al., 2006). Furthermore, the *E. coli* expressed disintegrin-like and cysteine-rich domains of P-III HF3, DC-HF3, from *Bothrops jararaca* inhibits collagen-induced platelet aggregation with IC_{50} of 768 nM (Silva et al., 2004). Therefore, the disintegrin-like and cysteine-rich domains of SVMPs were hypothesized to inhibit collagen-induced platelet aggregation. The studies in P-III SVMPs revealed the specific sequences that seemed likely to react with platelet. The disintegrin-like and cysteine-rich domains were found to block $\alpha_2\beta_1$ integrin binding to collagen and apparently enhance the hemorrhagic activity of SVMPs (Jia et al., 1996). Using synthetic peptides, Kamiguti and coworkers have shown that the sequence SECDPA is involved in the inhibition of $\alpha_2\beta_1$ integrin binding to collagen (Kamiguti et al., 1997).

Interestingly, the purified P-I, which did not contain disintegrin-like and cysteine-rich domains, lebetase from *Vipera lebetina* could also inhibit ADP-induced platelet aggregation (Siigur et al., 1998) similar to the baculovirus expressed P-II agkistin from *Agkistrodon halys* (Wang et al., 2003). These recent findings suggest that the metalloproteinase domain plays also crucial roles to react with platelet that is independent with the disintegrin-like and cysteine-rich domains.

Table 1 Snake venom metalloproteinase comparative studies

Snake	SVMP (class, kDa)	Activity	Inhibitor	Reference
Agkistrodon contortrix contortrix	Purified fibrolase (I, 23)	✗ Hemorrhage ✗ Plasminogen activator ✗ p-nitroanilide chromogenic substrate ✓ Fibrinogenolysis	-	(Ahmed et al., 1990; Guan et al., 1991; Egen et al., 1987; Manning et al., 1995)
		✓ Fibrinolysis	✓ EDTA ✓ Phen ✓ Tetraethylene -pentamine ✓ DTT ✗ DIFP ✗ Soybean ✗ Trasylol ✗ PCMB	
Crotalus atrox	Purified atroxase (I, 23.5)	✗ Hemorrhage ✓ Fibrinogenolysis ✓ Fibrinolysis ✓ Insulinolysis	✓ EDTA ✓ α_2M	(Tu et al., 1996; Willis et al., 1988)
		✗ Induce platelet aggregation ✗ Inh. Plt. Aggn.	-	

Table 1 Snake venom metalloproteinase comparative studies (continue)

Snake	SVMP (class, kDa)	Activity	Inhibitor	Reference
Vipera lebetina	Purified lebetase (I, 23.7)	✓Fibrinolysis ✓Caseinolysis	✓EDTA ✓EGTA ✓Phen ✓DTT ✕PMSF ✕EACA	(Saidi et al., 1999; Siigur and Siigur, 1991; Siigur et al., 1998)
		✓Fibrinogenolysis ✓Caseinolysis	✓α2M	
		✓Hemorrhage (at high dose) ✕Plasminogen activator ✓Insulinolysis	-	
		✓Inh. Plt. Aggn. (✓ADP, ✓collagen)	-	
Agkistrodon contortrix laticinctus	Express ACLF from *E. coli* (I, 23)	✕Hemorrhage ✓Collagenolysis ✓Fibrinogenolysis ✓Lamininolysis ✓Fibronectinolysis ✓Thrombospondinolysis	✓EDTA ✕PMSF	(de Moraes et al., 2006; Ramos et al., 2003; Selistre-de-Araujo et al., 2000)
		✓Hydrolyze Abz-LVEALYQ-EDDnp	✓Phen	
		✓Decrease HeLa cell viability	-	

Table 1 Snake venom metalloproteinase comparative studies (continue)

Snake	SVMP (class, kDa)	Activity	Inhibitor	Reference
Bothrops lanceolatus	Purified BlaH1 (I,28)	✓Hemorrhage	✓Antivenom	(Stroka et al., 2005)
		✓Caseinolysis	✓EDTA ✗Trasylol ✗PMSF ✗Benzamidine	
		✓Fibrinogenolysis ✓Collagenolysis ✓Elastinolysis ✓Esterolysis ✗Phospholipase A_2	-	
Agkistrodon halys	Express agkistin and agkistin-s from baculovirus (II, D; 60, 30)	✓Inh. Plt. Aggn. (✓ADP) ✓Induce apoptosis of HMEC	-	(Wang et al., 2003)
Trimeresurus jerdonii	Purified and express jerdonitin from *P. pastoris* (II, 36)	✓Inh. Plt. Aggn. (✓ADP) ✓Induce the growth of several cell lines: (Bel7402, K562, and BGC823)	-	(Chen et al., 2003)
Trimeresurus albolabris	Purified alborhagin (60)	✓Fibrinogenolysis ✓Autodigestion ✓Induce platelet aggregation ✓Induce ectodomain shading of platelet	✓EDTA	(Andrews et al.,2001; Wijeyewic-krema et al., 2007)

Table 1 Snake venom metalloproteinase comparative studies (continue)

Snake	SVMP (class, kDa)	Activity	Inhibitor	Reference
Trimeresurus albolabris (Cryptelytrops albolabris)	Express albocollagenase from *P. pastoris* (III, 62)	✓Collagenolysis ✕Fibrinogenolysis	✓EDTA ✓BAPTA ✓TPEN ✕ED2SC ✕PMSF ✕Antivenom	This work (Pinyachat et al., 2011)
		✓Inh. Plt. Aggn. (✓collagen, ✕ADP)	-	
Crotalus atrox	Express catrocollastatin from *S. frugiperda* (III, 50)	✓Autodigestion ✓Inh. Plt. Aggn. (✓collagen, ✕ADP, ✕Thromboxane analogue U46619) ✓vWF binding ✓Collagen binding	-	(Serrano et al., 2007; Zhou et al., 1995; 1996)
Bothrops jararaca	Express jararhagin from *E. coli* (III, 52)	✓Inh. Plt. Aggn. (✓collagen)	-	(Moura-da-Silva et al., 1999)
	Purified jararhagin (III, 52)	✓Autodigestion ✓Inh. Plt. Aggn. (✓collagen, ✓ADP, ✓ristocein) ✓vWF degradation ✓vWF binding ✓$\alpha_2\beta_1$ degradation ✓Collagen binding	-	(Kamiguti et al., 1996; Serrano et al., 2007)

Table 1 Snake venom metalloproteinase comparative studies (continue)

Snake	SVMP (class, kDa)	Activity	Inhibitor	Reference
Bothrops jararaca	Purified HF3 and express DC-HF3 from *E. coli* (III, DC; 52, 28)	✓Inh. Plt. Aggn. (✓collagen) ✓Activate $\alpha_M\beta_2$ mediated phagocytosis	-	(Silva et al., 2004)
Vipera ammodites ammodites	Purified VaH1 and VaH2 (III, 70)	✓Hemorrhage ✓Caseinolysis	✓EDTA ✕IDA ✕PMSF ✕PEP	(Leonardi et al., 2002)
		✓Fibrinogenolysis ✓Insulinolysis	-	
Trimeresurus pupureoma-culatus	Purified Hemorrhagin (III,72)	✓Hemorrhage ✓Caseinolysis	✓EDTA ✓Antivenom ✕Soybean	(Khow et al., 2002)
		✓Collagenolysis ✓Gelatinolysis ✕AE-hydrolysis ✕Phospholipase A_2	-	
Trimeresurus jerdonii	Purified jerdohagin (III,96)	✓Hemorrhage ✓Fibrinogenolysis ✓Prothrombinolysis	✓EDTA ✕PMSF	(Chen et al., 2004)

Table 1 Snake venom metalloproteinase comparative studies (continue)

Snake	SVMP (class, kDa)	Activity	Inhibitor	Reference
Gloydius halys	Express halydin from *P. pastoris* (DC, 8)	✓Inh. Plt. Aggn. (✓collagen) ✓Bind&Block $\alpha_2\beta_1$	-	(You et al., 2003)
	Purified halysase (III, 66)	✓Fibrinogenolysis	✓EDTA ✓EGTA ✕IDA ✕Pep ✕Antipain ✕PMSF	(You et al., 2006)
		✓Insulinolysis	-	
		✓Inh. Plt. Aggn. (✓collagen) ✓Bind&Block $\alpha_2\beta_1$	↓EDTA	
Crotalus atrox	Purified atrolysin A (III)	✓Fibronectinolysis ✓Lamininolysis ✓Collagenolysis ✓Nidogenolysis(entactin) ✓Gelatinolysis ✓Inh. Plt. Aggn. (✓collagen, ✓ADP)	-	(Baramova et al., 1989; Jia et a., 1997; Kamiguti et al., 1996 ; Serrano et al., 2005)
		✓Bind&Block $\alpha_2\beta_1$ ✓vWF binding ✓Collagen binding	✓Reduction & alkylation	
	Express atrolysin-A from sf9 cells (DC, 22)	✓Inh. Plt. Aggn. (✓collagen, ?ADP)	-	(Jia et al., 2000)

Table 1 Snake venom metalloproteinase comparative studies (continue)

Snake	SVMP (class, kDa)	Activity	Inhibitor	Reference
Crotalus atrox	Purified acurhagin (III, 51)	✓Caseinolysis	✓EDTA	(Wang et al., 2002; 2005)
		✓Fibronectinolysis	✓EDTA ✓Phen ✕PMSF ✕Methanol	
		✓Autodigestion ✓Collagenolysis ✓Inh. Plt. Aggn. (✓collagen, ✓ADP, ✓ristocein) ✓GPVI binding ✓Collagen binding ✓vWF degradation	-	
Agkistrodon acutus	Purified AAV1 (III, 50)	✓Fibrinogenolysis ✓Caseinolysis	✓EDTA ✓Phen ✕PMSF ✕Methanol ✕DMSO	(Wang et al., 2007)
		✓Autodigestion ✕Gelatinolysis ✕Fibronectinolysis ✕Prothrombinolysis	-	
		✓Inh. Plt. Aggn. (✓collagen)	-	
		✓GPVI binding	✕Phen	
		✕GPVI degradation	✕EDTA	

Table 1 Snake venom metalloproteinase comparative studies (continue)

Snake	SVMP (class, kDa)	Activity	Inhibitor	Reference
Naja kaouthia	Purified kaouthiagin (III, 51)	✓vWF degradation	✓EDTA ✓Phen	(Hamako et al., 1998; Ito et al., 2001)
		✓vWF binding to kouthiagin	✕EDTA ✕Phen ✕PNG-F ✓CNBr	
		✓Inh. Plt. Aggn. (✓ristocetin)	✓EDTA	
		✓Inh. Plt. Aggn. (✓collagen)	✕Phen	
		✕GPIX binding ✕GPIb binding ✕GPIIbIIIa ($\alpha_{IIb}\beta_3$) binding ✕Fibrinogenolysis	-	

✓ = Yes, ✕ = No, ↓ = decrease, ? = not clear, Inh. Plt. Aggn. = Inhibit platelet aggregation, D = disintegrin, and DC = disintegrin-like and cystein-rich

CHAPTER III

MATERIALS AND METHODS

1. Materials

Table 2. Materials for DNA and RNA manipulations

Description	Name	Company
mRNA extraction and purification	TRIzol® reagent	Invitrogen™
	PolyATtract mRNA Isolation System	Promega
5′-RACE	SMART™ RACE cDNA amplification Kit ● PowerScript™ (MMLV variant) Reverse Transcriptase	CLONTECH Laboratories, Inc.
DNA extraction and purification from gel slice	Wizard® SV Gel and PCR Clean-Up System	Promega
E. coli transformation for cloning of RACE products	pGEM®-T Easy/pGEM®-T Vector system II kit ● *E. coli*, JM 109 ● T4 DNA Ligase	
Preparation of plasmid DNA from *E. coli* cells	Wizard® *Plus* SV Minipreps DNA Purification System	
DNA Sequencing	ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase	AB Applied Biosystems

Table 2. Materials for DNA and RNA manipulations (continue)

Description	Name	Company
Pichia expression system	EasySelect™ *Pichia* Expression kit Version G, 122701 • X-33 strain • KM71H strain • GS115 strain	Invitrogen™ life technologies
Proof reading PCR	BD Advantage™ 2 PCR enzyme system	BD Biosciences
Restriction enzymes	*Eco*R I *Xba* I	Promega
	Sac I	Pharmacia Biotech
Transformed *E. coli* inducer	Isopropyl-β-D-Thiogalactopyranoside (IPTG)	Sigma
β-galactosidase substrate	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)	

Table 3. Materials for Protein manipulations

Description	Name	Company
SDS-PAGE	Mini-Protean® III Electrophoresis apparatus	Bio-Rad Laboratories, Ltd.
Protein markers	SeeBlue® Plus2 Pre-Stained Standard	Invitrogen™ life technologies
	Precision Plus Protein Dual Color Standards	Bio-Rad Laboratories, Ltd.
Protein stain	Coomassie Brilliant Blue R-250	Sigma

Table 3. Materials for Protein manipulations (continue).

Description	Name	Company
Western Blot	Trans-Blot® SD semi-dry electrophoretic transfer cell	Bio-Rad Laboratories, Ltd.
	Immun-Blot™ PVDF Membrane 0.2 μm	
	Trans-Blot® Pure Nitrocellulose Membrane (0.45 μm)	
	Mouse Anti-His Antibody	GE Healthcare
	Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP	Dako Cytomation
	Amersham™ ECL Plus Western Blotting Detection System	GE Healthcare
Protein Dot blot	Bio-Dot Apparatus and Vacuum Regulator	Bio-Rad Laboratories, Ltd.
Protein Purification	The *BioLogic LP* low-pressure chromatography system	Bio-Rad Laboratories, Ltd.
	Talon™ Super-flow™ Metal Affinity Resin	BD Biosciences
	MagneHis™ Protein Purification System	Promega
Protein concentration	Vivaspin 20 (10,000 MWCO PES)	Sartorius stedim biotech
Protein Quantitative Assay	Micro BCA™ Protein Assay Reagent Kit	PIERCE Biotechnology
	Multiskan Ex	Thermo Labsystems

Table 3. Materials for Protein manipulations (continue).

Description	Name	Company
Collagen/Fibrinogen degradation	Human placenta type IV collagen powder, C7521	Sigma
	Human fibrinogen powder, F4883	
	Doxycycline, D9891	
	EDTA, E9884	
	$CaNa_2EDTA$, ED2SC	
	BAPTA, A4926	
	TPEN, P4413	
	Pepsin, P6887	
	PMSF	Roche
	Collagenase type I	Gibco BRL
	F (ab)'$_2$ antivenoms	Queen Saovabha Memorial Institute
Platelet aggregation	Platelet from two healthy human donors	-
	PACKS-4 Helena aggregometer	Helena Laboratory, TX
	Collagen solution, Cat. no. 5368	
	ADP, A2754	Sigma

Table 4. Synthetic oligonucleotides primers were purchased from Biogenomed Company, Invitrogen™ life technologies.

Primer	Sequence	Description
T7	5′–GTAATACGACTCACTATAGGGC–3′	Sequencing primer of T7 promoter
SP6	5′–ACTCAAGCTATGCATCCAAC–3′	Sequencing primer of SP6 promoter
NUP	5′–AAGCAGTGGTATCAACGCAGAGT–3′	Sequencing primer of NUP promoter
pUC/M13F	5′–GTTTTCCCAGTCACGAC–3′	Sequencing primer of pUC/M13F promoter
pUC/M13R	5′–CAGGAAACAGCTATGAC–3′	Sequencing primer of pUC/M13R promoter
SVM39.2	5′–AGAGGTTGATTAGGAGGCTCT ATTCACATCAACACA–3′	GSP1 primer of 5′-RACE PCR
SVM39.3	5′–ATGAGCTTCACTGTCGGAAAGT TCCAAGGG–3′	GSP2 primer of 5′-RACE PCR
SVM39.10	5′–CGGAATTCCATCATCATCATCATC ATGAACAACAAAGATACTTGGATGCC AAAAAATACGTTAAGTATATCTTAGTT–3′	Forward primer with N-terminal *Eco* RI and 6His tag sites

Table 4. Synthetic oligonucleotides primers were purchased from Biogenomed Company, Invitrogen™ life technologies (continue).

SVM39.9	5′–GCTCTAGATTAGGAGGCTCTATTCACA TCAACACACTGTCTGTTG–3′	Reverse primer with C-terminal *Xba* I and stop codon site
5′- *AOX1*	5′–GACTGGTTCCAATTGACAAGC–3′	*Pichia* sequencing primer
3′- *AOX1*	5′–GCAAATGGCATTCTGACATCC–3′	*Pichia* sequencing primer
α–Factor	5′–TACTATTGCCAGCATTGCTGC–3′	*Pichia* sequencing primer

2. Methods

2.1 Obtaining the full-length cDNA sequence of the P-III SVMP gene

Green pit viper venom gland cDNA library construction was previously prepared (Rojnuckarin et al., 2006b). The partial sequence of P-III SVMP was identified from cDNA library called clone 039 SVM.

2.1.1 5'-Rapid Amplification of cDNA Ends (5′-RACEs)

In principle, the objective of this experiment for obtaining full-length P-III SVMP gene using the 5'-RACE (5'-Rapid Amplification cDNA Ends) method was shown in **figure 4**. The mRNA can be prepared to cDNA using PowerScript™, a variant of MMLV reverse transcriptase exhibited terminal transferase activity by adding 3 – 5 residues (predominantly dC) to the 3′ end of the first-strand cDNA. The SMART II A™ oligonucleotide, dG residues, could anneal to the dC-rich cDNA tail and serve as an extended template for reverse transcriptase until the end. The first cDNA now contained the SMART II A™ oligonucleotide, which was complementary with the universal primer (long and short UP) and gene-specific primer (GSP) designated from *T. albolabris* cDNA library clone 039 SVM. Therefore, the fragment could be amplified by PCR.

In this method, total RNA was isolated from venom glands of *T. albolabris* using TRIzol® reagent. The mRNA was then purified using Poly ATtract mRNA Isolation System using magnetic beads coated with poly T. For preparation of 5′-RACE-Ready cDNA, we synthesized the first strand cDNA using poly T (5′-CDS) in the reaction as followed. Firstly, 500 ng of poly A^+ RNA from venom gland of *T. albolabris*, 1 μl of 5′-CDS primer, 1 μl of SMART II A™ oligonucleotide and sterile H_2O were combined to a final volume of 5 μl. It was, then, incubated at 70 °C for 2 minutes. When reach the given time, 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 in an air incubator. 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 step, we had 5′-RACE-Ready cDNA as the template for PCR. The PCR was carried out using Tm between 60 – 70 °C. PCR Master mix was prepared by combination of 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 Mix followed by adding of 10 μl of 5′-RACE-Ready cDNA, 5 μl of 10X UP primer Mix (UPM), and 10 μl of 10 pM GSP1 (SVM39.2) primer. We used Biometra®, thermal cycler, for amplifying 5′-RACE fragments using 40 cycles with 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.

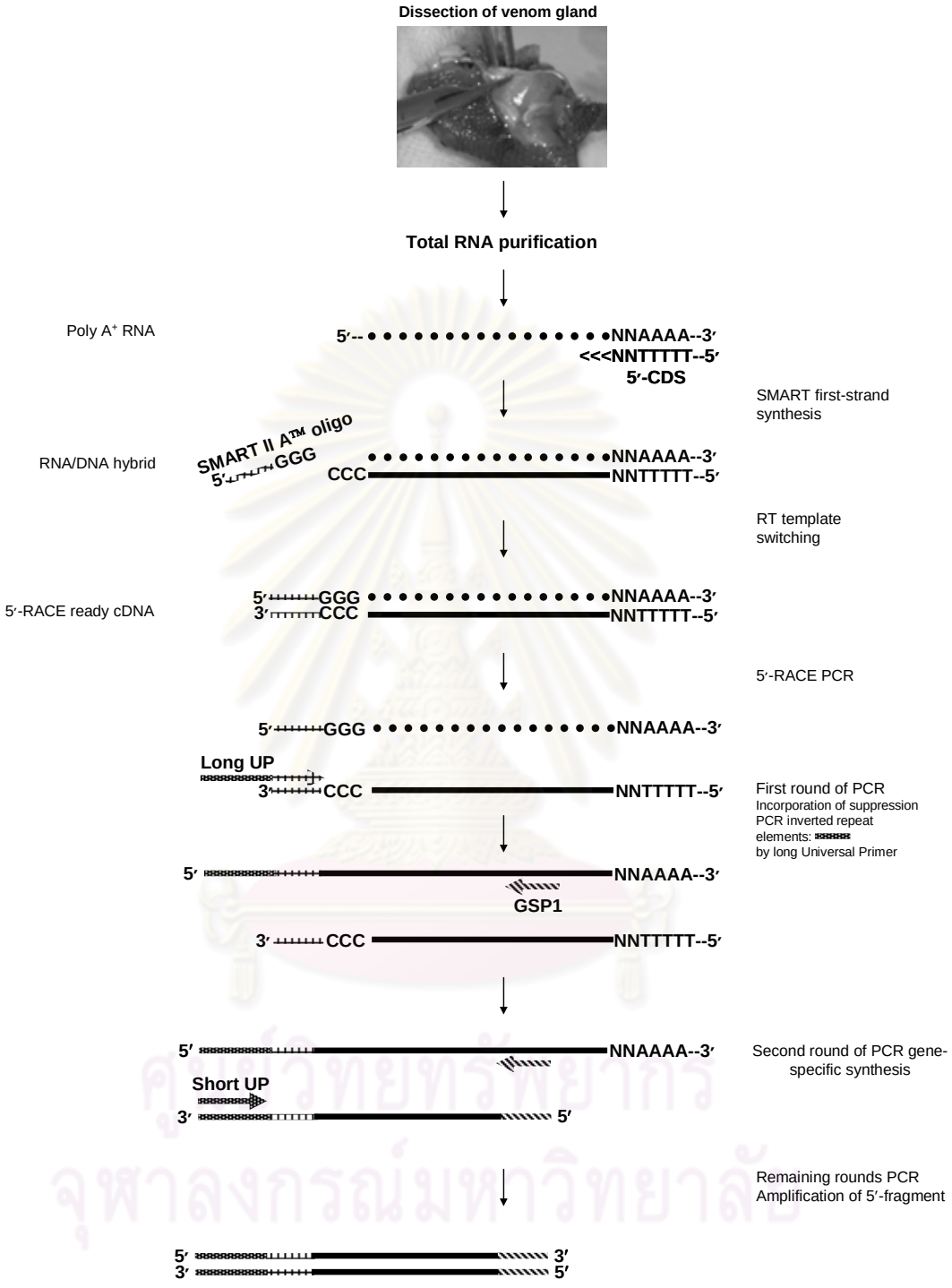


Figure 4. 5'-Rapid Amplification of cDNA Ends (5′-RACEs). This picture was modified from the SMART™ RACE cDNA amplification Kit user manual.

2.1.2 DNA Extraction and Purification from Gel Slice

The commercial kit, Gel clean system, was designed to extract and purify DNA fragments of 100 bp to 10 Kbp from standard or low-melt agarose gels in either Tris acetate (TAE) or Tris borate (TBE). After electrophoresis to separate the DNA fragments, the band(s) of interest was(were) sliced and dissolved in the presence of guanidine isothiocyanate (Membrane Binding Solution). DNA could be isolated using microcentrifugation to force the dissolved gel slice through the membrane while simultaneously binding the DNA on the surface of the silica. After washing the isolated DNA fragment, the DNA could be eluted in water.

After amplification with 5′-RACE, the products were electrophoresed on 1.2% agarose gel. A band of DNA was sliced from agarose gel with a sterile blade. The RACE products were purified by the Wizard® SV Gel and PCR Clean-Up System. Ten µL of Membrane Binding Solution per 10 mg of gel slice were mixed and incubated in 50 °C heat block for 10 minutes, or until the gel slice was completely dissolved, the tube was mixed every few minutes. The solution was transferred to SV minicolumn placed on collection tube and incubated for 1 minute at room temperature. The SV minicolumn was centrifuged at 12,000 x g for 1 minute and discarded the solution in collection tube. Then, 700 µL of Membrane Wash Solution were added to SV minicolumn assembly and centrifuged at 12,000 x g for 1 minute. The wash step was repeated with 500 µL of Membrane Wash Solution and centrifuged the SV minicolumn assembly at 12000 x g for 5 minutes and again centrifuged for 1 minute. The SV minicolumn was carefully transferred to a new 1.5 ml microcentrifuge tube, applied 50 µL of nuclease-free water directly to the center of the column without touching the membrane with the pipette tip, incubated at room temperature for 1 minute and centrifuged for 1 minute at 12,000 x g. Finally, the 50 µL of DNA were precipitated by adding 2 volumes of 0.3 M sodium acetate in 100% ethanol and incubated in -20 °C for one hour or more and centrifuged at 12,000 x g for 10 minutes. Then, the pellet was washed with 70% ethanol by inverting the tube for several times and centrifuged at 12,000 x g for 10 minutes to recover the DNA. After air dry at room temperature with turn upside down on tissue

paper to allow ethanol evaporation, the pellet was dissolved with 50 μl of TE buffer pH 8.0. The DNA solution was mixed and stored at -20 °C.

2.1.3 Ligation of RACE Products into pGEM®-T easy / pGEM®-T Vector

The commercial kit vector system was used for the cloning of PCR products. The vectors could be added a 3´terminal deoxy thymidine (dT) to both ends. These single 3´-T overhangs at the insertion site greatly improved the efficiency of ligation of a PCR product into the plasmids by preventing re-circularization of the vector by providing a compatible overhang for PCR products generated by certain thermostable polymerases. These polymerases often added a single deoxyadenosine (dA), in a template-independent fashion, to the 3´-ends of the amplified fragments. The ligation mixture was performed by adding 5′-RACE PCR product, vector and T4 DNA ligase. T4 DNA ligase catalyzed the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA.

After DNA purification, the 5′ RACE products were cloned into pGEM®-T easy Vector. The ligation procedure was carried out in a 10 μl. The ligation reaction mixture contained 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 optimized from the insert: vector ratio of 3:1 by 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.

2.1.4 Transformation of the ligation reaction into *E. coli* JM 109

E. coli JM109 that could uptake DNA was called "competent". It was made so by treatment with calcium chloride in the early log phase of growth. The bacterial cell membrane could be permeable to chloride ions but impermeable to calcium ions. As the chloride ions entered the cell, water molecules accompanied the charged particle. This influx of water caused cell swelling necessary for the uptake of DNA. The calcium chloride treatment can be followed by heat. When *E. coli* was subjected to 42 °C heat, a set of genes were expressed aiding the bacteria in surviving at such temperatures. This set of genes was called the heat shock genes. The heat shock step was necessary for the uptake of DNA. At temperatures above 42 °C, the bacterial ability to uptake DNA became reduced, and at extreme temperatures the bacteria would die (Woodcock et al., 1989). The blue-white colony selection was performed on ampicillin resistant colony plate using IPTG (galactose homolog) inducer and X-gal substrate. The white colony represented colony with an insert.

After incubation, 10 μl ligation reaction was transferred to a sterile falcon tube Cat.#2059 on ice. The *E. coli* JM 109 competent cells that were placed on ice until just thawed and mixed 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 hour with shaking at 150 rpm. Finally, 500 μl of the transformed cells were spread on LB agar plate with 100 μg/ml ampicillin 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.

2.1.5 Preparation of plasmid DNA by Alkaline Lysis: Mini-preparation

Each colony of transformed bacteria was inoculated in 3 ml of LB broth containing 100 μg/ml of ampicillin and incubated for overnight at 37 °C with shaking at 250 rpm. The culture cells were transferred to a 1.5 ml micro-centrifuge tube and centrifuged at 7,000 x g for 10 minutes. An aliquot of the original culture was stored at –70 °C in 50 % glycerol. After centrifugation, 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 again centrifuged. The bacterial pellet was lysed with 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 mixed. Subsequently, 200 μl of freshly prepared Alkaline Lysis Solution II (0.2 N NaOH, 1% w/v SDS) was added to bacterial suspension and mixed by five-time gentle inversion. The tube was incubated 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 by five-time gentle inversion and incubated on ice for 3 – 5 minutes. The bacterial lysate tube was centrifuged at 10,000 x g for 10 minutes. The supernatant was gently transferred to a new tube. Then, an equal volume of phenol: chloroform was added and vigorously mixed and then centrifuged at 10,000 x g for 10 minutes. The aqueous upper layer was transferred to a new tube. Finally, plasmid DNA was precipitated by adding of 2 volumes of 100 % ethanol and incubated in -20 °C for one hour or more and centrifuged at 12,000 x g for 10 minutes. Then, pellet was washed with 70% ethanol by inverting the tube for several times and centrifuged at 12,000 x g for 10 minutes to recover the DNA. After air dry at room temperature with turn upside down on tissue paper to allow ethanol evaporation, the pellet was dissolved with 50 μl of TE buffer pH 8.0. The DNA solution was mixed and stored at –20 °C.

Another method that we used to extract plasmid DNA from *E.coli* was Wizard® *Plus* SV Minipreps DNA Purification System. Pelleted bacterial cells were resuspended and subjected to SDS / alkaline lysis / alkaline protease to liberate the plasmid DNA. The resulting lysate was neutralized to create appropriate conditions for binding of plasmid DNA on the silica membrane in the spin column. Cell debris and SDS

precipitate were pelleted by centrifugation, and the supernatant containing the plasmid DNA was loaded onto the spin column membrane. The adsorbed DNA was washed to remove contaminants, and was then eluted with a small volume of the elution Buffer.

2.1.6 Restriction Endonuclease and Electrophoresis

The pGEM®-T Easy and pGEM®-T vector contained the *Eco* RI site and did not contain both *Eco* RI/*Xba* I sites respectively. Thus, *Eco* RI was used to screen the insertion in pGEM®-T Easy vector, and *Eco* RI/*Xba* I in pGEM®-T vector.

Approximately 500 ng of plasmid DNA were digested with 5 units of *Eco*R I according to manufacturer's protocol (Promega), 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 BSA. The digestion reaction was incubated at 37 °C for appropriate time. Then the reaction was electrophoresed on 1.5 % agarose gel. Positive clones containing the insert of interest were selected for sequencing.

2.1.7 DNA Sequencing

After the sequencing reaction steps (denature, annealing and extension) were performed. The fluorescent labeled fragments that migrate through the tube were passing a laser beam at the bottom of the tube. The laser excites the fluorescent molecules sent out light of a distinct color. That light was collected and focused by lenses into a spectrograph. Based on the wavelength, the spectrograph separates the light across a CCD camera (charge coupled device). Each base has its own color, so the sequencer can detect the order of the bases in the sequenced gene.

The sequencing was performed using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. The PCR 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, SP6, or others) 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 denaturing, 50 °C for 5 seconds of annealing, and 60 °C for 4 minutes of extension. The DNA was then precipitated by 65 % ethanol and 0.08 M sodium acetate pH 8.0. After incubation at -20 °C for one hour or more, the solution was centrifuged at 12,000 x g for 20 minutes and the supernatant was gently removed by pipetting. The pellet was then washed with 1 ml of 70 % ethanol, and centrifuged at 12,000 x g for 10 minutes. The pellet was dried in heat block 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.

2.1.8 Alignment and Computational Searching on DNA database

The DNA sequence of P-III SVMP cDNA was analyzed by comparing with nucleotide and protein database of NCBI using BLAST program. It was aligned with other identical sequences with Clustal X, GENEDOC, and Chromas program.

2.2 Expression of P-III SVMP of GPV in *Pichia pastoris*

As a eukaryote, *P. pastoris* has many of the advantages of higher eukaryotic expression systems, such as protein processing, protein folding, and post-translational modification, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. It is faster, easier, and less expensive to use compared with other eukaryotic expression systems and generally gives higher expression levels. As yeast, it shares the advantages of molecular and genetic manipulations with *Saccharomyces*. *P. pastoris* is methylotrophic, capable of metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase (*AOX* genes). In addition to formaldehyde, this reaction generates hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within a specialized cell organelle, called the peroxisome, which sequesters toxic by-products away from the rest of the cell. Alcohol oxidase has a poor affinity for O_2, and *P. pastoris* compensates by generating large

amounts of the enzyme. The promoter regulating the production of alcohol oxidase is the one used to drive heterologous protein expression in *Pichia*.

Expression of the *AOX1* gene is controlled at the level of transcription. In methanol grown cells approximately 5% of the poly A^+ RNA is from the *AOX1* gene. The regulation of the *AOX1* gene is a two step process: a repression/derepression mechanism plus an induction mechanism. Briefly, growth on glucose represses transcription, even in the presence of the inducer methanol. For this reason, growth on glycerol is recommended for optimal induction with methanol. Notably, growth on glycerol alone (derepression) is not sufficient to generate even minute levels of expression from the *AOX1* gene. The inducer, methanol, is necessary for detectable levels of *AOX1* expression (Ellis et al., 1985; Koutz et al., 1989; Tschopp et al., 1987).

2.2.1 Amplification of P-III SVMP of GPV by proof reading PCR

The BD Advantage™ 2 PCR enzyme system produced efficient, accurate, and convenient amplification of DNA from any template. It comprised Taq DNA Polymerase, a nuclease-deficient N-terminal deletion of Taq DNA polymerase plus antibody to provide automatic hot-start PCR, and a minor amount of a proof reading polymerase.

Two primers, forward primer SVM39.10 and reverse SVM39.9 were used to amplify the cDNA fragment encoding the P-III SVMP domain in pGEM®-T vector. The *Eco* RI and 6-histidine-tag residues were incorporated into the forward primer for facilitating purification and detection. The *Xba* I recognition site and UAA stop codon were incorporated into the reverse primer. The PCR reaction was carried out in a 50 μl containing 10X PCR buffer (100 mM Tris- HCl pH 8.3, 500 mM KCl, and 15 mM $MgCl_2$), 1.25 units of Tag DNA polymerase, 10 pM of each primer, 25 mM $MgCl_2$, 25 mM of each dNTPs, and 200 ng DNA template. After incubation at 94 °C for 5 minutes, amplification was carried out for 30 cycles with the following temperature cycling

parameters: 94 °C for 1 minute of denaturation, 53 °C for 1 minute of annealing, 72 °C for 1.5 minute of extension and a final extension at 72 °C for 5 minutes. The PCR products were electrophoresed in 1.2 % agarose gel. Then, the gel was sliced and DNA was purified as described above. Subsequently, the DNA was subcloned into pGEM®-T vector and transformed into *E. coli*, JM109 as described.

2.2.2 Digestion of inserted plasmid and pPICZαA vector

The inserted plasmid DNA in pGEM®-T vector and pPICZαA vector, pPICZαA were digested with *Eco*R I and *Xba* I. The digestion reaction was performed as described above and electrophoresed on 1.2 % agarose gel. Then, DNA was extracted and purified DNA from the gel as described above. After that, the DNA was precipitated by 0.3 M sodium acetate in 90 % ethanol. Then, the solution was centrifuged at 12,000 x g for 20 minutes. The pellet was washed by 1 ml of 70 % ethanol, and centrifuged at 12,000 x g for 10 minutes. The pellet was then air-dried and dissolved in sterile distilled water.

2.2.3 Ligation of P-III SVMP with pPICZαA Vector

Optimization of the appropriate amounts of inserted plasmid DNA and pPICZαA vector was described before. The ligation reaction was carried out in a 10 μl. The ligation reaction mixture contains 3 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% polyethylene glycol), 1 μl of pPICZαA vector, 5 μl of digested disintegrin construct plasmid DNA, and 3 Weiss unit of T4 DNA Ligase. The ligation reaction was incubated at 4 °C overnight.

2.2.4 Transformation of Ligation reaction into *E. coli*, JM109

Transformation was performed by heat shock method as described above. The 500 μl transformation reaction was spread onto Low Salt LB plate with 25 μg/ml Zeocin™ and incubated at 37 °C for overnight. Zeocin™-resistant colonies were picked and inoculated in 3 ml of Low Salt LB broth with 25 μg/ml Zeocin™ incubated overnight at 37 °C with shaking. The plasmid DNA was isolated using Wizard® *Plus* SV Minipreps DNA Purification System for restriction analysis and sequenced as described.

2.2.5 Linearization of the Plasmid DNA

Prior to transformation into *Pichia pastoris*, we prepared 5 – 10 μg of plasmid DNA by minipreparation and linearized with the *Sac* I, which cut one time in the 5′-*AOX1* region of pPICZαA. The 14 μl of plasmid DNA were mixed with 2 μl of 10X Buffer (300 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP), 0.1 mg/ml BSA and 1 unit of *Sac* I. The reaction was incubated at 37°C for 16-18 hours. Aliquot of reaction was electrophoresed to verify complete linearization. The reaction was then inactivated using heat at 65°C for 20 minutes. Then, plasmid DNA was precipitated by 2.5 volumes of 100 % ethanol and 1/10 volume of 3 M sodium acetate. Subsequently, the solution was centrifuged and pellet washed with 70 % ethanol, air-dried and resuspended in 5 μl sterile deionized water, and stored at –20 °C until use.

2.2.6 Preparation of competent *Pichia*

Competent *Pichia* cells were prepared by chemical method as provided with the *Pichia* EasyComp™ Kit. *Pichia pastoris*, KM71H strain, was streaked on Yeast Extract Peptone (YEPD) plate to grow as individual colonies and incubated at 30°C for 2 days. After that, a single colony was inoculated in 10 ml of YEPD and incubated overnight at 30°C in a shaking incubator (250 – 300 rpm). Yeast cells from

overnight culture was then diluted to an OD_{600} of 0.1 – 0.2 in 10 ml of YEPD and allowed to grow at 30°C in a shaking incubator until the OD_{600} reaching 0.6 – 1.0. Subsequently, the cells were centrifuged at 500 x g for 5 minutes at room temperature. The cell pellet was washed in 10 ml of Solution I (ethylene glycol and DMSO) and centrifuged at 500 x g for 5 minutes at room temperature. Finally, the pellet was resuspended in 1 ml of Solution I containing ethylene glycol and DMSO. Competent cells were aliquoted in to 50 μl in 1.5 ml sterile microcentrifuge tubes and kept in -80 °C freezer.

2.2.7 Transformation of the linearized plasmid DNA into *Pichia pastoris*, KM71H strain

The transformation was performed using the *Pichia* EasyComp™ kit from Invitrogen™ life technologies. Solutions II and III were stored at room temperature before use. The 50 μl of competent cells were thawed at room temperature for each reaction. 3 μg of the linearized plasmid DNA was mixd with competent cells. Then, 1 ml of Solution II (PEG solution) was added to the DNA/cell mixture and mixed by vortexing or flicking the tube. After that, the transformation reaction was incubated at 30°C for 1 hour in a water bath. The tube was vortexed every 15 minutes. Subsequently, the transformation reaction was subjected to heat shock at 42 °C for 10 minutes in a water bath. The transformed cells were split into 2 microcentrifuge tubes. 1 ml of YPD medium to each tube was added and incubated the transformed at 30°C for 1 hour to allow expression of Zeocin™ resistant colonies. After that, the transformed cells were centrifuged at 500 x g for 5 minutes at room temperature, resuspended in 500 μl of Solution III (Salt solution) and combined into one tube. The transformed cells were then centrifuged at 500 x g for 5 minutes at room temperature and resuspended in 100 to 150 μl of Solution III. Finally, the transformed solution was plated on YPDS plate with 100 μg/ml Zeocin™ and incubated for 3 – 10 days at 30°C.

2.2.8 PCR Analysis of *Pichia* Integrants

Genomic DNA was isolated from 6 – 10 *Pichia* clones using the protocol from the EasySelect™ *Pichia* expression kit manual. Amplification of P-III SVMP gene was carried out with 5′-*AOX1* and 3′-*AOX1* primer included in the kit. The PCR reaction was carried out in 50 μl containing 10X PCR buffer (100mM Tris- HCl pH 8.3, 500 mM KCl, and 15 mM $MgCl_2$), 25 mM $MgCl_2$, 25 mM dNTPs, 0.2 pM of both 5′-*AOX1* and 3′-*AOX1* primers, and 200 ng genomic DNA. The PCR reaction was incubated at 95°C for 5 minutes. Then, 1.25 units of Tag DNA polymerase was added to the reaction. After that, amplification was carried out for 35 cycles with the following temperature cycling parameters: 95°C for 1 minute of denaturation, 53°C for 45 seconds of annealing, 72°C for 1 minute of extension and final extension at 72°C for 7 minutes. Recombinant plasmids with and without insert were used as positive and negative controls, respectively. PCR products were analyzed on 1.5 % gel electrophoresis.

2.2.9 Small scale expression of recombinant *Pichia*

The expression was performed in 50 ml conical tubes. The *Pichia* colonies from YPDS/Zeocine™ plate were inoculated in 10 ml BMGY with shaking 250 rpm, 30 °C overnight. Then, the cells were changed into 10 ml BMMY and incubated like that for 6 days. We added the methanol every day at 0.5 % final concentration. The supernatant from each day, day 1 – 6, were pooled, concentrated with 10 MWCO of Viva spin and subject to Western blot.

2.2.10 Large scale expression of recombinant *Pichia*

A selected *Pichia* colony or glycerol stock was inoculated in YPD/Zeocin™ broth using a 50-ml Erlenmeyer flask in a shaking incubator (250 rpm) at 30°C overnight. Expression was performed in a 2-L Erlenmeyer flask. With non centrifuged, the harvested cells were resuspended in BMGY medium with starting OD_{600}

$_{nm}$ of 0.1 and grown in shaking incubator (250 rpm) at 30°C until the culture reached an $OD_{600\ nm}$ of 8 (approximately 15 hours). To measure the OD of *Pichia*, the culture media was centrifuged with 1,500 g for 5 minutes to keep supernatant to dilute the *Pichia* cells. The culture media was diluted with 1:50 before measurement of OD_{600nm} with Spectrometer. The *Pichia* cells were centrifuged with 1,500 g for 5 minutes and then were inoculated into BMMY medium with starting $OD_{600\ nm}$ of 20 and grown in shaking incubator with 250 rpm at 30°C for 3 days. In addition, methanol induction of protein expression was used and the concentration was maintained at 5% (v/v) (from preliminary results) every 24 hour.

Figure 5. Large scale expression of recombinant *Pichia* was performed in 2000 ml Erlenmeyer flask.

2.3 Protein concentration

The supernatant was concentrated using Vivaspin 20 membrane filtration with 10-kDa molecular weight cut off (MWCO). The 20 ml supernatant was poured into the Vivaspin and centrifuged at 7,000 x g (4°C).

2.4 Recombinant protein purification

2.4.1 Affinity chromatography

The recombinant protein contained a polyhistidine (6-histidine) tag that bound divalent cations like Ni^{2+} to facilitate purification. Recombinant P-III SVMP of GPV was purified according to protocol from BD TALON™ Metal Affinity Resins User Manual (BD Biosciences). All steps were carried out at 4°C. The concentrated media was separated on a Ni^{2+}-based immobilized affinity column using the main pump, BioLogic LP System. The column was manually packed, equilibrated, and washed with 50 mM sodium phosphate pH 8.0, 300 mM NaCl. The recombinant protein was eluted with 50 mM sodium phosphate pH 5.0, 300 mM NaCl into 30 fraction collector. Absorbance of each fraction was monitored at 280 nm. The appropriate fractions were pooled to re-purification with MagneHis™ Protein Purification System.

2.4.2 MagneHis™ Protein Purification System

The system containing paramagnetic pre-charged nickel particles (MagneHis™ Ni-Particles) was used to isolate polyhistidine-protein directly from a soluble protein by adding 30 μL of MagneHis™ Ni-Particles to 1 ml of protein solution. The tube was inverted (approximately 10 times) and incubated for 2 minutes at room temperature. The tube was placed in the magnetic stand for approximately 30 seconds and carefully removed the supernatant using a pipette. The washing step was done 3 times by adding 150 μl of MagneHis™ Binding/Wash Buffer using magnetic stand as described. After the supernatant was clearly removed, the elution step was done by adding 100 μl of MagneHis™ Elution Buffer.

2.5 Protein Detection

2.5.1 Sodiumdodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Coomassie Brillient Blue Staining

8% of resolving gel and 5% of stacking acrylamide gel containing 10% SDS were freshly prepared. After gel setting, the recombinant protein was mixed to 1X sample buffer (100 mM Tris-HCl pH 6.8, 4% w/v SDS, 0.2% w/v bromophenol blue, 20% v/v glycerol, 200 mM with or without β-mercaptoethanol) and then denatured at 95°C for 10 minutes and loaded into gel slots. Electrophoresis was performed at 100 v, 20 mA, and 15 w for 1 hours 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 Brillient Blue Solution for overnights in 4°C. 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 until the protein band was cleared.

2.5.2 Western Blot

After the SDS-PAGE to separate proteins according to their sizes (lengths of polypeptide chain), the protein bands were transferred from polyacrylamide gel to PVDF membrane using Trans-Blot® SD semi-dry electrophoretic transfer cell. The PVDF membrane and supporting membranes were soaked in transfer buffer for 20 minutes. All of them were sandwiched in Trans-Blot® SD semi-dry electrophoretic transfer cell as 1st-supporting membrane, PVDF membrane, polyacrylamide gel, and 2nd-supporting membrane from the bottom respectively without any air bubble. The proteins were transferred at 60 voltages, 200 mA, and 50 watts for 45 minutes. When finished, the PVDF membrane was immediately soaked with a blocking solution (10 % w/v non-fat dry milk in 1X PBS buffer, pH 7.4 and 0.1% Tween 20) for 1 hour at room temperature with gentle agitation, and then washed 3 times with 1X PBS buffer, pH 7.4, and 0.1% Tween 20, for 5 minutes each. The PVDF membrane was incubated with 1:3,000 dilution

(3.33 μL in 10 ml of milk) of monoclonal Anti-His Antibody in blocking buffer for 1 hour at room temperature with gentle agitation. The membrane was, then, washed 3 times with 1X PBS buffer, pH 7.4, and 0.1% Tween 20 for 5 minutes each. After that, the membrane was incubated with 1:1,000 dilution (10 μL in 10 ml of milk) of polyclonal Horse radish peroxidase-conjugated rabbit Anti-Mouse (IgG) in blocking buffer for 1.30 hour at room temperature with gentle agitation. The PDVF membrane was, then, washed with 1X PBS buffer, pH 7.4 for 5 times. For developing, the PDVF membrane was soaked in the Amersham™ ECL Plus Western Blotting Detection solution (2 ml Solution A, 50 μL Solution B) for 3 – 5 minutes. Then the PVDF was foiled and sandwiched with Amersham™ ECL film for appropriate durations. Finally, the film was manually developed with Kodak developer solution for a proper time (5 – 30 seconds) that the band was occurred at room temperature. The film was wash with tab water and then fixed with Kodak fixative solution for a proper time (5 – 30 seconds) that the band was cleared at room temperature, and then washed with tab water followed with drying the film in the air.

2.5.3 Protein Dot Blot

The purpose of this method is to screen the protein expression from many *Pichia* Zeocin™ resistance colonies. The supernatant of induced media was directly blotted to Nitrocellulose membrane using the Bio-Dot apparatus and vacuum regulator for enhancing the amount of recombinant protein. Like Western Blot, the recombinant protein was detected with mouse Anti-His antibody, rabbit Anti-Mouse, and Amersham™ ECL Plus Western Blotting Detection solution with the film exposure as described.

2.5.4 Quantitative Assay for Recombinant Proteins

Protein concentration was determined using the colorimetric detection, Micro BCA™ Protein Assay Reagent Kit, and quantitation of total protein.

The unique, patented method utilized bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which was formed when Cu^{+2} was reduced by protein in an alkaline environment. A purple-colored reaction product was formed by the chelating of two molecules of BCA with one cuprous ion (Cu^{+1}). This water-soluble complex exhibited a strong absorbance at 562 nm that was linearly correlated with protein concentrations. The macromolecular structure of protein, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) were reported to be responsible for color formation with BCA. Studies with di-, tri- and tetra-peptides suggested that the extent of color formation was caused by more than the mere sum of individual color-producing functional groups.

The bovine serum albumin (BSA) was diluted into 6 dilutions (0 – 60 g/l) for standard curve formation. 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 replicated were transferred 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 reader, Multiskan Ex.

2.6 Collagen degradation

Type IV collagen was selected to be the substrate according to the protocol from previous studies (Khow et al., 2002; Oliveira et al., 2010). Collagen powder was diluted to 5 mg/ml with 0.25% acetic acid. The performed reaction and all substances were incubated in a water bath at 37°C for 1 hour before used. Ten μL of the recombinant P-III SVMP (100 μg/ml) and 10 μL of soluble collagen were then mixed together for each reaction. The final concentrations were 0.8 μM and 2.5 mg/L, respectively. At every incubation time (1, 5, 10, 30 minutes; and 1, 4, 8, 24 hours), an

aliquot of each reaction was stopped using a SDS-PAGE sample buffer containing β-mercaptoethanol and 10 mM EDTA and immediately frozen at -80 °C until tested.

To determine collagen degradation, the reactions were run on an 8% reducing SDS-PAGE and then stained with Coomassie-blue R250. We used the collagenase type I as a positive control for collagen digestion.

2.7 Fibrinogen degradation

The method was previously described (Muanpasitporn and Rojnuckarin, 2007; Stroka et al., 2005). Briefly, human fibrinogen and the recombinant P-III SVMP were mixed together at 0.8 μM and 2.5 mg/L final concentrations, respectively. At every incubation time (5, 15, 30, 60, 120 minutes; and 5, 12 hours), an aliquot of each reaction was stopped and subjected to SDS-PAGE as described above. Pepsin was used as a positive control for fibrinogen degradation.

2.8 Platelet aggregation assay

Platelet aggregation assay was performed using a PACKS-4 Helenna Aggregometer, platelet aggregation chromogenic kinetic system. Venous blood (9 parts) from two healthy donors who did not receive any medication for at least 2 weeks was mixed with 3.2 % sodium citrate (1 part). The whole blood was centrifuged at 180 x g for 10 minutes to obtain platelet-rich plasma (PRP) and platelet-poor plasma (PPP) was prepared from the remaining serum by centrifuging at 2,000 x g for 10 minutes. PRP was diluted to 250 x 10^9 platelets/L with PPP. Different amount of recombinant P-III SVMP were added to PRP and incubated at 37 °C for 10 minutes before adding platelet agonist (collagen or ADP). Platelet aggregation was initiated by collagen (Helena Laboratory, TX) at the final concentration of 40 mg/L or ADP (Sigma, USA) at the concentration of 1 μM. Light transmittance reflecting percentage aggregation was measured. The maximal aggregation in the absence of the recombinant P-III SVMP was given as 100% aggregation. Bovine serum albumin was used as a negative control for platelet aggregation inhibition.

CHAPTER IV

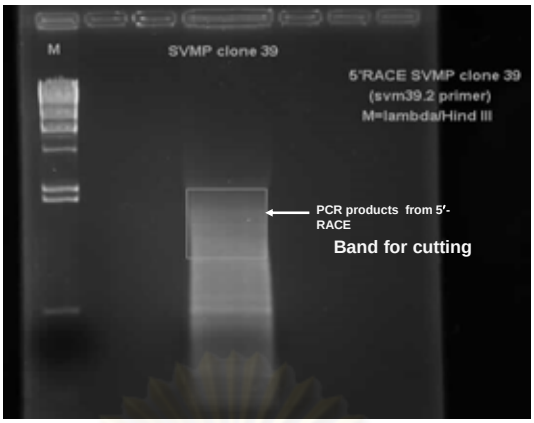
RESULTS

1. 5′-RACE products of P-III SVMP of GPV

Using a partial sequence from *T. albolabris* venom gland cDNA library, we designed a gene specific primer and performed the 5′-RACE to obtain the full-length cDNA using PCR as shown in **figure 6A and 6B**.

From the 5′-RACE, the cDNA from fresh venom glands were synthesized using poly T and reverse transcriptase. The PCR products of **figure 6A** were amplified from this cDNA as the template using the GSP1 (SVM39.2) and short UP primers. The GSP1 (SVM39.2) primer could bind across the 3′-end of translated region of albocollagenase, CVDVNRAS-… amino acid sequences as shown in **figure 10**. Then, the PCR products (smear bands) of **figure 6A** were cloned and sequenced. Although we not received the full-length sequences (from signal peptides to stop codon) from the biggest clone of the first-round 5′-RACE, we got the mature protein sequences to design the primer for mature protein expression. Thus, we re-designed the GSP2 (SVM39.3) primer to amplify the second-round of 5′-RACE. The GSP2 (SVM39.3) primer could bind the prodomain region of albocollagenase, PLELSDSEAH amino acid sequences as shown in **figure 10**. And then, the PCR product of figure 6B was cloned and sequenced.

A



B

Figure 6. 5′-RACE products of P-III SVMP of GPV. The first round of 5′-RACE products was amplified by SVM39.2 gene specific primer (A), and second round by SVM39.3 gene specific primer (B) respectively.

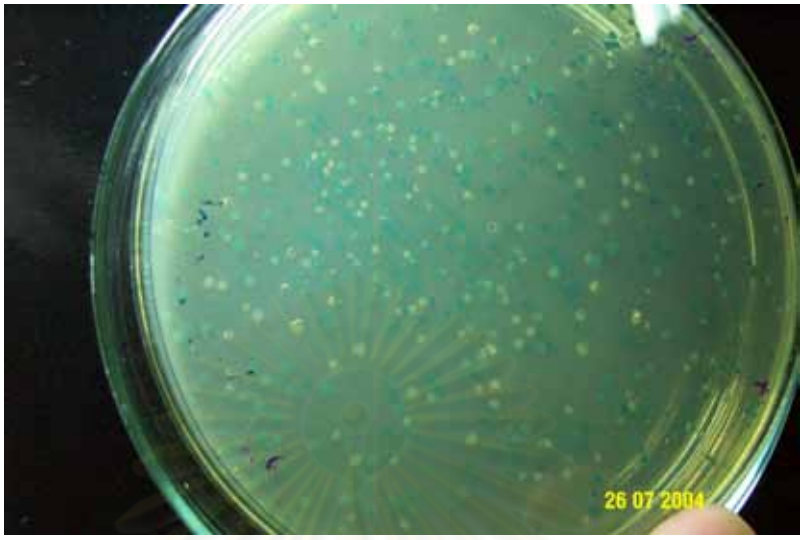


Figure 7. The *E. coli* JM109 contained pGEM®-T Easy inserted vector (white colonies) and no insert (blue colonies).

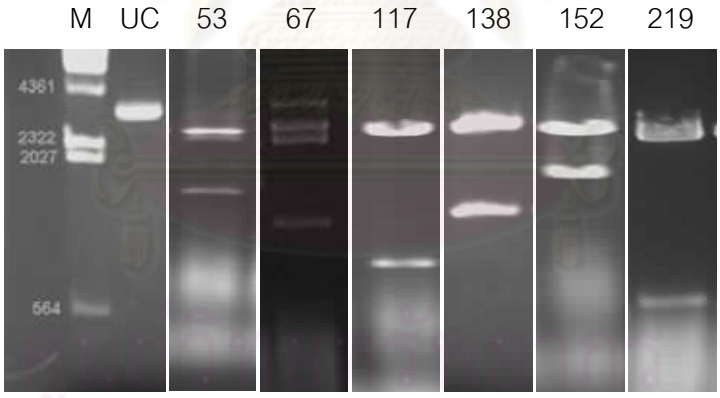


Figure 8. Six positive clones 53, 67, 117, 138 152, and 219 positive colonies of P-III SVMP in pGEM®-T Easy vector from **figure 7** were cut with *Eco* RI and run on agarose gel electrophoresis compared with the uncut vector (UC).

From the first- and second-round of 5′-RACE, we got 238 white positive colonies. We screened every clone using *Eco* RI as shown in **figure 8**. The 15 clones were selected from the different size of the cut band and were sequenced 28 times of different sequencing primers (as shown in table 4). Eight clones were shown positive results from DNA sequencing machine. The 8 DNA sequences were BLAST with NCBI website and were then analyzed to define as SVMP or not. We found that 2 clones are not SVMP and were discarded.

By homology selection, we got the six positive clones that DNA sequences from all clones were most closely with agkistin from *Agkistrodon contratruticus*. Five positive clones 53, 67, 117, 138, and 152 were got from the first-round of 5′-RACE, and a 219 positive clone was got from the second-round of 5′-RACE as shown in figure 8. The DNA sequence of each clone was compared with one another according to the agkistin from *Agkistrodon contratruticus,* which was most closely related.

```
219SP6              MIQVLLVTICLAVFPYQGSSIILESGNVNDYEVVHPRKVTALPKGAVQQKYEDAMQYEFK
Agkistin            MIQVLLVTLCLAVFPYQGSSIILESGNVNDYEVVYPRKVTVLPKGAVQPKYEDAMQYEFK

219SP6              VNGEPVVLHLEKNKGLFSEDYSETHYSPDGREITTYPSVEDHCYYHGRIQNDADSTASIS
152PUC13R                                                                   SIS
152T7                                                                       SIS
Agkistin            VNGEPVVLHLEKNKQLFSKDYSETHYSPDGREITTYPLVEDHCYYHGRIENDADSTASIS

219SP6              ACNGLKGHFK
152PUC13R           ACNGLKGHFKLQGETYLIEPLELSDSEAHAVFKYENVEKEDEAPKMCGVTQNWKSYEPIK
152T7t              ACNGLKGHFKLQGETYLIEPLELSDSEAHAVFKYENVEKEDEAPKMCGVTQNWKSYEPIK
Agkistin            ACNGLKGHFKLQGEMYLIDPLKLPDSEAHAVFKYENVEKEDEAPKMCGVTQNWESYEPIK

152PUC13R           EASQSNLTPEQQRYLDAKKYVKYILVLDRGMCTKYNTILKKMKTR
152T7               EASQSNLTPEQQRYLDAKKYVKYILVLDRGMCTKYNSDLKKMKTRMYELVNIMNEICLPL
152NUP                                  GMCTKYNSDLKKMKTRMYELVNIMNEICLPL
138SP6                               LDRGMCTKYNSDLKKMKTRMYELVNIMNEICLPL
Agkistin            KASQLNLTPEQQAYLDAKKYVEFVVVLDHGMYTKYKDNLDKIKTRIFEIVNTMNEMFIPL

152T7               NIRVALTGLIIW-LDRDRLNVTSAANVTLSYFGDWRATVLLKQKS-D
152NUP              NIRVALTGLIIW-LDRDKINVTSAANVTLSLFGDWRATVLLKQKNHDCAQLFTDTDFDGDT
138SP6              NIRVALTGLIIWVWTGDKIKLTSGSNVTVLYFGDWRATVLLNRKSMDCAQLF
53SP6                                                       DCAQLFTDTDFDGDT
Agkistin            -NIRVALICLEIWSDKDKFNMTSAANVTSISFRNWRATDLLKRKSHDNAQLLTVIDFDGPT

152NUP              VGLAYTGGICRLKHSVG
53SP6               VGLAYTGGICRLKHSVGIIQDHSTINLLMAVTMVHELGHNLGMEHDVNRDGKQCNCDACI
Agkistin            IGKAYMASMCDPKRSVGIIQDHSTINLMMAVTMAHEMGHNLGMDHDEKYCT--CGAKSCV

53SP6               MAPRLNPQPSKQFSDCSKDDYRTFLINRRPQCILNAPSKTDIVSPPVCGNELLEKGKECD
Agkistin            MAKALSRQPSKLFSNCSQEDYRKYLIKRRPKCILNEPNGTDIVSPPVCGNELLEVGEECD

53SP6               CGSPKNLSKICCNAARVTLPPGSQ
117sp6                              LPPGSQCADECGCHQCNFKRAGTEVGQAKDDCDLAESCTGRSAE
Agkistin            CGSPTNCQNPCCDAATCKLTPGSQCADGVCCDQCRFTRAGTECRQAKDDCDMADLCTGQS

117SP6              CPTDLLQRDGQPCQNNNGYCYNRTRPTMNNQCISFFGSSATVAPDGCFNFNRQSNDYSYC
67SP6                               YNRTCPTMNNQCISFFGSSATVAPDGCFNFNRQSNDYSYC
152PUC13F                    PCQNNNGYCYNRTCPTMNNQCISFLGQVQLVAPDGCFNFNRQSNDYSYC
Agkistin            CPTDRFQRNGHPCLNDNGYCYNRTCPTLKNQCIYFFGPNAAVAKDSCFKGNQKSNNHTYC

117SP6              RKENGRKIPCAPQDVKCGRLYCFPNSPGEENTCNLIYTPGREDIGMVLLGTKCADGKAC
67SP6               RKENGRKIPCAPQDVKCGRLYCFPNSPGEENTCNLIYTPGREDIGMVLLGTKCADWKACN
152PUC13F           RKENGRKIPCAPQDVKCGRLYCFPNSPGEENTCNLIYTPGREDIGMVLLGTKCADGKACN
Agkistin            RKENGKKIPCAPQDIKCGRLYCFRNLPGKKNICSVIYTPTDEDIGMVLPGTKCEDGKVCS

67SP6               SNRQCVDVNRASKSTSHFSQI
152PUC13F           SNRQCVDVNRAS
Agkistin            -NGHCVDVNIAYKSTTGFSQI
```

Figure 9. The comparison of deduced protein sequence from positive colonies 53, 67, 117, 138, 152 and 219 according to agkistin *(bold and italicized)*. The highlighted protein sequences were referred to the DNA differences between clones.

2. Sequence analysis of albocollagenase

This SVMP from *T. albolabris* was first identified in this study and termed **"albocollagenase"**. The conceptually translated sequence was analyzed. Albocollagenase was classified as a class III SVMP, P-III SVMP, since it comprised metalloproteinase domain containing the conserved Zn^{2+}-binding sequences (**Figure 10, 11**) and a following disintegrin-like and cysteine-rich domains. There were 9 and 24 cysteine residues in the metalloprotease and disintegrin-like and cysteine-rich domains, respectively. The deduced protein sequence was most closely related to the P-II SVMP agkistin from *Agkistrodon contratruticus* (Wang et al., 2003) with 73% identity using the BLAST program in NCBI database.

The full-length albocollagenase protein was compared with other P-III SVMPs including ACLD (Selistre de Araujo et al., 1997) and VMP-III (Jia and Perez, 2010) from *Agkistrodon contortrix laticinctus,* Met-isofrom 1 from *Sistrurus catenatus edwardsi* (Pahari et al., 2007), Berythractivase from *Bothrops erythromelas* (Silva et al., 2003), catrocollastatin from *Crotalus atrox* (Zhou et al., 1995) and *jararhagin* from *Bothrops jararaca* (Paine et al., 1992). The percentages of identity with albocollagenase were 74%, 74%, 74%, 70%, 65% and 63%, respectively. Notably, the identity in the prodomain ranged from 89.4% - 91.8%, while those of the mature proteins were from 54.5% - 67.7% as shown in **table 5**.

```
   1  atgatccaagttctcttggtaaccatatgcttagcagttttcccttatcaagggagctct   60
   1   M  I  Q  V  L  L  V  T  I  C  L  A  V  F  P  Y  Q  G  S  S    20

  61  ataatcctggaatctgggaacgtgaatgattatgaagtcgtgcatccacgaaaagtcact   120
  21   I  I  L  E  S  G  N  V  N  D  Y  E  V  V  H  P  R  K  V  T    40

 121  gcattacccaaaggagcagttcagcaaaagtatgaagacgccatgcaatatgaatttaag   180
  41   A  L  P  K  G  A  V  Q  Q  K  Y  E  D  A  M  Q  Y  E  F  K    60

 181  gtgaatggagagccagtagtccttcacctggaaaaaaataaaggacttttttcagaagat   240
  61   V  N  G  E  P  V  V  L  H  L  E  K  N  K  G  L  F  S  E  D    80

 241  tacagtgagactcattattcccctgatggcagagaaattacaacatacccctcggttgag   300
  81   Y  S  E  T  H  Y  S  P  D  G  R  E  I  T  T  Y  P  S  V  E   100

 301  gatcactgctattatcatggacgcatccagaatgatgctgactcaactgcaagcatcagt   360
 101   D  H  C  Y  Y  H  G  R  I  Q  N  D  A  D  S  T  A  S  I  S   120

 361  gcatgcaatggtttgaaaggacatttcaagcttcaaggggagacgtaccttattgaaccc   420
 121   A  C  N  G  L  K  G  H  F  K  L  Q  G  E  T  Y  L  I  E  P   140

 421  ttggaactttccgacagtgaagctcatgcagtattcaaatatgaaaatgtagaaaaagag   480
 141   L  E  L  S  D  S  E  A  H  A  V  F  K  Y  E  N  V  E  K  E   160

 481  gacgaggcccccaaaatgtgtggggtaacccagaattggaaatcatatgaacccatcaaa   540
 161   D  E  A  P  K  M  C  G  V  T  Q  N  W  K  S  Y  E  P  I  K   180

 541  gaggcctctcagtcgaatcttactcctgaacaacaaagatacttggatgccaaaaaatac   600
 181   E  A  S  Q  S  N  L  T  P  E  Q  Q  R  Y  L  D  A  K  K  Y   200

 601  gttaagtatatcttagttttggaccgtggaatgtgcacaaaatacaacagcgatttaaaa   660
 201   V  K  Y  I  L  V  L  D  R  G  M  C  T  K  Y  N  S  D  L  K   220

 661  aagatgaaaacaagaatgtatgaacttgtcaacattatgaatgagatctgcctccctttg   720
 221   K  M  K  T  R  M  Y  E  L  V  N  I  M  N  E  I  C  L  P  L   240

 721  aatattcgcgtagcattgactggcctaataatttggttggacagagataagattaacgtg   780
 241   N  I  R  V  A  L  T  G  L  I  I  W  L  D  R  D  K  I  N  V   260

 781  acatcagcagcaaatgttactttgtccttatttggagactggagagcgacagtcttgctg   840
 261   T  S  A  A  N  V  T  L  S  L  F  G  D  W  R  A  T  V  L  L   280

 841  aagcagaaaaatcatgattgtgctcagttattcacggacactgacttcgatggtgacact   900
 281   K  Q  K  N  H  D  C  A  Q  L  F  T  D  T  D  F  D  G  D  T   300

 901  gtaggattggcttatacgggtggcatatgccgactgaagcattctgtaggaattattcag   960
 301   V  G  L  A  Y  T  G  G  I  C  R  L  K  H  S  V  G  I  I  Q   320

 961  gatcatagcacaataaatcttctgatggcagttacaatggtccatgagctgggtcataat  1020
 321   D  H  S  T  I  N  L  L  M  A  V  T  M  V  H  E  L  G  H  N   340

1021  ctgggcatggaacatgatgtaaaccgtgatggaaagcagtgtaattgtgatgcatgcatt  1080
 341   L  G  M  E  H  D  V  N  R  D  G  K  Q  C  N  C  D  A  C  I   360

1081  atggctcccaggctaaaccctcaaccttccaaacagttcagcgattgtagtaaggatgat  1140
 361   M  A  P  R  L  N  P  Q  P  S  K  Q  F  S  D  C  S  K  D  D   380

1141  tatcggacatttcttataaatcgtagaccacaatgcattctcaatgcaccctcgaagaca  1200
 381   Y  R  T  F  L  I  N  R  R  P  Q  C  I  L  N  A  P  S  K  T   400

1201  gatattgtttcacccccagtttgtggaaatgaacttttggagaagggaaaagaatgtgac  1260
 401   D  I  V  S  P  P  V  C  G  N  E  L  L  E  K  G  K  E  C  D   420

1261  tgtggctctcctaaaaacttgtcgaaaatatgctgcaatgctgcacgtgtaactctgccc  1320
 421   C  G  S  P  K  N  L  S  K  I  C  C  N  A  A  R  V  T  L  P   440

1321  ccaggttcccaatgtgcagatgagtgtggttgtcaccagtgcaatttaagagagcagga  1380
 441   P  G  S  Q  C  A  D  E  C  G  C  H  Q  C  N  F  K  R  A  G   460

1381  acagaagtcgggcaggcaaaggatgactgtgacttggctgaaagctgcactggccgatct  1440
 461   T  E  V  G  Q  A  K  D  D  C  D  L  A  E  S  C  T  G  R  S   480

1441  gctgagtgtcccacggatctcctccaaagggatggacaaccatgccaaaacaacaatggt  1500
 481   A  E  C  P  T  D  L  L  Q  R  D  G  Q  P  C  Q  N  N  N  G   500

1501  tactgctacaataggacgcgccccaccatgaacaaccaatgtatttctttctttgggtca  1560
 501   Y  C  Y  N  R  T  R  P  T  M  N  N  Q  C  I  S  F  F  G  S   520

1561  agtgcaactgtggctccagatggatgttttaattttaaccggcaaagcaatgattatagc  1620
 521   S  A  T  V  A  P  D  G  C  F  N  F  N  R  Q  S  N  D  Y  S   540

1621  tactgcagaaaggaaaacggtagaaagattccatgtgcaccacaagatgtaaaatgtggc  1680
 541   Y  C  R  K  E  N  G  R  K  I  P  C  A  P  Q  D  V  K  C  G   560

1681  aggttatactgcttccctaattcacccggagaggagaatacttgcaatctcatatataca  1740
 561   R  L  Y  C  F  P  N  S  P  G  E  E  N  T  C  N  L  I  Y  T   580

1741  cctggtcgtgaagatattgggatggttcttctgggaacaaaatgtgcagatggaaaggcc  1800
 581   P  G  R  E  D  I  G  M  V  L  L  G  T  K  C  A  D  G  K  A   600

1801  tgcaacagcaacagacagtgtgttgatgtgaatagagcctcctaa                 1845
 601   C  N  S  N  R  Q  C  V  D  V  N  R  A  S  -                  614
```

Figure 10. (previous page) The coding nucleotide and deduced protein sequences of albocollagenase comprised 1845 base pairs and 614 amino acid residues. The protein sequence comprised the signal peptide, pro-peptide, metalloproteinase domain, and disintegrin-like and cysteine-rich domains, respectively. The putative signal peptide was *italicized and underlined*. The putative pro-peptide was *italicized*. The metalloproteinase domain was underlined. The Zn^{2+}-binding sequences followed by methionine turn (Met-turn) (HELGHNLGMEH-CIM) are underlined and highlighted. The disintegrin-like and cysteine-rich domains are **bold**. The putative collagen binding sequences, DCD, were **bold and highlighted**. The recombinant protein composed of metalloproteinase and disintegrin-like and cysteine-rich domains was expressed in this study.

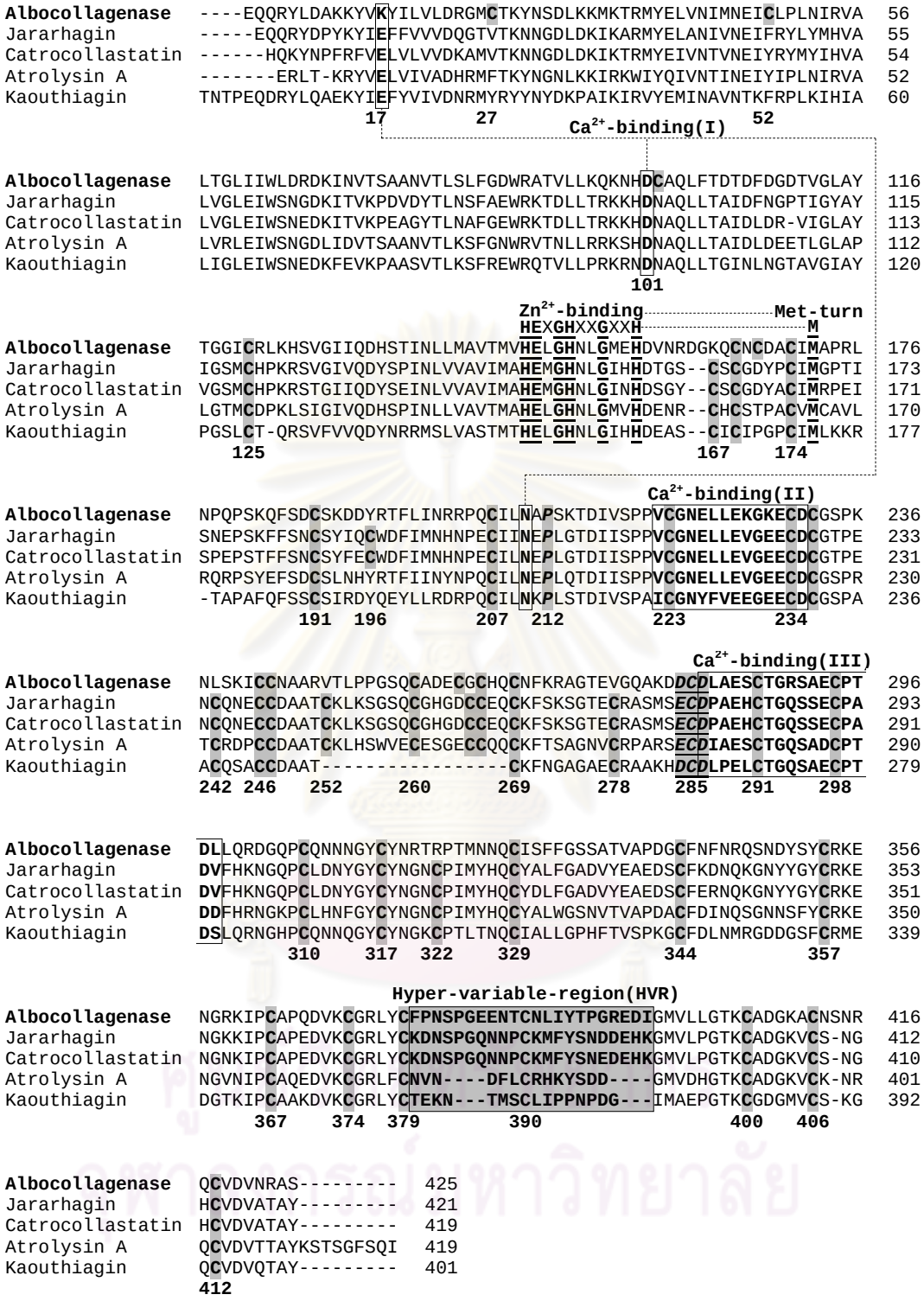
Table 5. Percentage of identity with albocollagenase compared with other SVMPs.

	%Identity compared with albocollagenase					
P-III/snake	FULL	SIG	PRO	M	DC	MDC
ACLD /*Agkistrodon contortrix laticinctus*	74.0	94.4	91.8	63.0	70.1	66.3
VMP-III /*Agkistrodon contortrix laticinctus*	74.0	94.4	91.8	63.5	69.6	66.3
Met-isofrom 1 / *Sistrurus catenatus edwardsi*	74.0	94.4	89.5	66.3	69.6	67.7
Berythractivase /*Bothrops erythromelas*	70.0	83.3	89.5	58.2	66.0	62.0
Catrocollastatin /*Crotalus atrox*	64.9	94.4	89.4	51.8	57.5	54.5
Jararhagin /*Bothrops jararaca*	63.2	75.0	90.1	51.0	57.5	54.5

FULL= full-length, SIG = signal, PRO = propeptide, M = metalloproteinase, and DC = disintegrin-like and cysteine-rich

The mature albocollagenase was then aligned with jararhagin, catrocollastatin, atrolysin A (Hite et al., 1994) from *Crotalus atrox* and kaouthiagin from *Naja kaouthia* (Ito et al., 2001) using CLUSTALW program in as shown in **Figure 11**. The conserved Zn^{2+}-binding sequences were found to be identical to other active SVMPs. However, several cysteines were different from the other P-III SVMPs. Albocollagenase disintegrin-like domain contained DCD-motif. Additionally, it contained 3 putative Ca^{2+}-binding sites as shown in **Figure 11**.

Figure 11. (next page) The multiple sequences alignment of the P-III SVMP putative mature proteins compared with other SVMPs. Cysteine residues were numbered according to kaouthiagin. The cysteine residues were highlighted. The Zn^{2+}-binding sequences were underlined. The putative self-post-translational processing position, P212, were *italicized and highlighted*. The putative collagen binding sequences, DCD, were *underlined, italicized and highlighted*. The 3 putative Ca^{2+}-binding sites were boxed. The putative hyper-variable-regions (HVR) were boxed and highlighted.


Albocollagenase ----EQQRYLDAKKYVKYILVLDRGMCTKYNSDLKKMKTRMYELVNIMNEICLPLNIRVA 56
Jararhagin -----EQQRYDPYKYIEFFVVVDQGTVTKNNGDLDKIKARMYELANIVNEIFRYLYMHVA 55
Catrocollastatin ------HQKYNPFRFVELVLVVDKAMVTKNNGDLDKIKTRMYEIVNTVNEIYRYMYIHVA 54
Atrolysin A -------ERLT-KRYVELVIVADHRMFTKYNGNLKKIRKWIYQIVNTINEIYIPLNIRVA 52
Kaouthiagin TNTPEQDRYLQAEKYIEFYVIVDNRMYRYYNYDKPAIKIRVYEMINAVNTKFRPLKIHIA 60
17 27 52
Ca2+-binding(I)
Albocollagenase LTGLIIWLDRDKINVTSAANVTLSLFGDWRATVLLKQKNHDCAQLFTDTDFDGDTVGLAY 116
Jararhagin LVGLEIWSNGDKITVKPDVDYTLNSFAEWRKTDLLTRKKHDNAQLLTAIDFNGPTIGYAY 115
Catrocollastatin LVGLEIWSNEDKITVKPEAGYTLNAFGEWRKTDLLTRKKHDNAQLLTAIDLDR-VIGLAY 113
Atrolysin A LVRLEIWSNGDLIDVTSAANVTLKSFGNWRVTNLLRRKSHDNAQLLTAIDLDEETLGLAP 112
Kaouthiagin LIGLEIWSNEDKFEVKPAASVTLKSFREWRQTVLLPRKRNDNAQLLTGINLNGTAVGIAY 120
101
Zn2+-binding
HEXGHXXGXXH
Met-turn
M
Albocollagenase TGGICRLKHSVGIIQDHSTINLLMAVTMVHELGHNLGMEHDVNRDGKQCNCDACIMAPRL 176
Jararhagin IGSMCHPKRSVGIVQDYSPINLVVAVIMAHEMGHNLGIHHDTGS--CSCGDYPCIMGPTI 173
Catrocollastatin VGSMCHPKRSTGIIQDYSEINLVVAVIMAHEMGHNLGINHDSGY--CSCGDYACIMRPEI 171
Atrolysin A LGTMCDPKLSIGIVQDHSPINLLVAVTMAHELGHNLGMVHDENR--CHCSTPACVMCAVL 170
Kaouthiagin PGSLCT-QRSVFVVQDYNRRMSLVASTMTHELGHNLGIHHDEAS--CICIPGPCIMLKKR 177
125 167 174
Ca2+-binding(II)
Albocollagenase NPQPSKQFSDCSKDDYRTFLINRRPQCILNAPSKTDIVSPPVCGNELLEKGKECDCGSPK 236
Jararhagin SNEPSKFFSNCSYIQCWDFIMNHNPECIINEPLGTDIISPPVCGNELLEVGEECDCGTPE 233
Catrocollastatin SPEPSTFFSNCSYFECWDFIMNHNPECILNEPLGTDIISPPVCGNELLEVGEECDCGTPE 231
Atrolysin A RQRPSYEFSDCSLNHYRTFIINYNPQCILNEPLQTDIISPPVCGNELLEVGEECDCGSPR 230
Kaouthiagin -TAPAFQFSSCSIRDYQEYLLRDRPQCILNKPLSTDIVSPAICGNYFVEEGEECDCGSPA 236
191 196 207 212 223 234
Ca2+-binding(III)
Albocollagenase NLSKICCNAARVTLPPGSQCADECGCHQCNFKRAGTEVGQAKDDCDLAESCTGRSAECPT 296
Jararhagin NCQNECCDAATCKLKSGSQCGHGDCCEQCKFSKSGTECRASMSECDPAEHCTGQSSECPA 293
Catrocollastatin NCQNECCDAATCKLKSGSQCGHGDCCEQCKFSKSGTECRASMSECDPAEHCTGQSSECPA 291
Atrolysin A TCRDPCCDAATCKLHSWVECESGECCQQCKFTSAGNVCRPARSECDIAESCTGQSADCPT 290
Kaouthiagin ACQSACCDAAT----------CKFNGAGAECRAAKHDCDLPELCTGQSAECPT 279
242 246 252 260 269 278 285 291 298
Albocollagenase DLLQRDGQPCQNNNGYCYNRTRPTMNNQCISFFGSSATVAPDGCFNFNRQSNDYSYCRKE 356
Jararhagin DVFHKNGQPCLDNYGYCYNGNCPIMYHQCYALFGADVYEAEDSCFKDNQKGNYYGYCRKE 353
Catrocollastatin DVFHKNGQPCLDNYGYCYNGNCPIMYHQCYDLFGADVYEAEDSCFERNQKGNYYGYCRKE 351
Atrolysin A DDFHRNGKPCLHNFGYCYNGNCPIMYHQCYALWGSNVTVAPDACFDINQSGNNSFYCRKE 350
Kaouthiagin DSLQRNGHPCQNNQGYCYNGKCPTLTNQCIALLGPHFTVSPKGCFDLNMRGDDGSFCRME 339
310 317 322 329 344 357
Hyper-variable-region(HVR)
Albocollagenase NGRKIPCAPQDVKCGRLYCFPNSPGEENTCNLIYTPGREDIGMVLLGTKCADGKACNSNR 416
Jararhagin NGKKIPCAPEDVKCGRLYCKDNSPGQNNPCKMFYSNDDEHKGMVLPGTKCADGKVCS-NG 412
Catrocollastatin NGNKIPCAPEDVKCGRLYCKDNSPGQNNPCKMFYSNEDEHKGMVLPGTKCADGKVCS-NG 410
Atrolysin A NGVNIPCAQEDVKCGRLFCNVN----DFLCRHKYSDD----GMVDHGTKCADGKVCK-NR 401
Kaouthiagin DGTKIPCAAKDVKCGRLYCTEKN---TMSCLIPPNPDG---IMAEPGTKCGDGMVCS-KG 392
367 374 379 390 400 406
Albocollagenase QCVDVNRAS--------- 425
Jararhagin HCVDVATAY--------- 421
Catrocollastatin HCVDVATAY--------- 419
Atrolysin A QCVDVTTAYKSTSGFSQI 419
Kaouthiagin QCVDVQTAY--------- 401
412

3. Construction of *Pichia* vector and *Pichia* colonies

We constructed the mature albocollagenase containing metalloproteinase and disintegrin-like and cysteine-rich domains, as well as an N terminal 6 histidine tag, in the pPICZ∝A vector. As shown in **figure 8**, we found that the *E. coli* JM109 containing inserted pGEM®-T Easy vector of clone 152 was a full-length P-III clone. Thus, we amplified the full-length P-III clone using BD Advantage™ 2 PCR enzyme system using SVM39.9 and SVM39.10 primers as shown in **figure 12**. The PCR products was ligated and cloned into pGEM®-T vector and *E. coli* JM109 respectively using 3′-T overhang. Then, the inserted vector was cut with *Eco* RI and *Xba* I as shown in **figure 13** before ligated, cloned into pPICZ∝A vector and transformed into *E. coli* JM109, which then could grow in LB/lowsalt/ Zeocin™ as shown in **figure 14**. After a frame checking of the albocollagenase insert in pPICZ∝A vector, linearization of this vector was performed using *Sac* I restriction digestion as shown in **figure 15**. *P. pastoris* KM71H was used as the host cells for transformation. The Zeocin-resistant colonies of *Pichia* (**figure 16**) were randomly selected for PCR (**figure 17**) before small-scale expression.

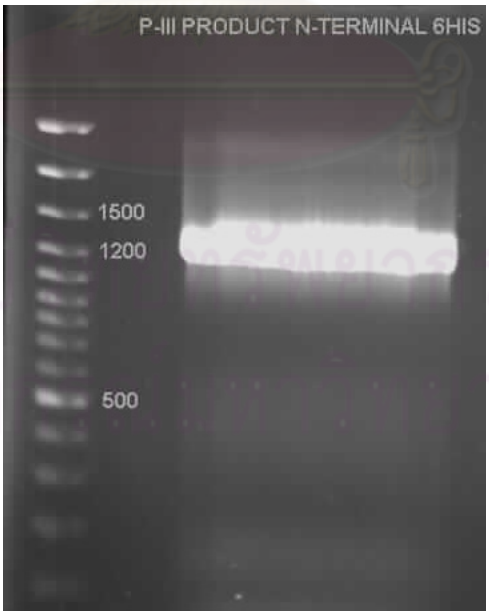


Figure 12. The PCR products of P-III SVMP of GPV gene amplified from SVM39.9 and SVM39.10 primers and inserted pGEM®-T Easy vector as a template.

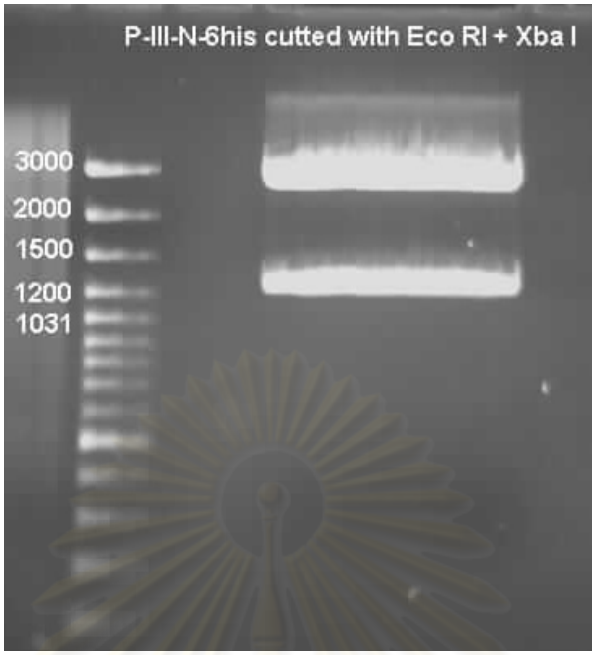


Figure 13. The pGEM®-T vector was cut with *Eco* RI and *Xba* I.

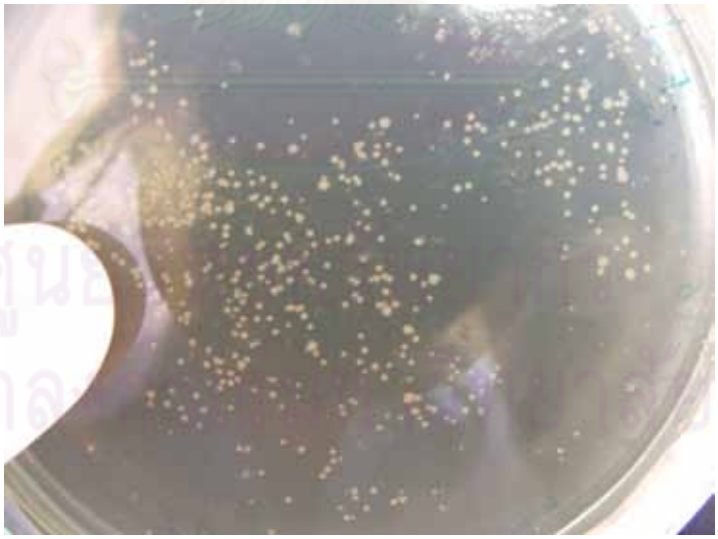


Figure 14. The *E. coli* JM109 containing inserted vector could grow in LB/lowsalt/ Zeocin™.

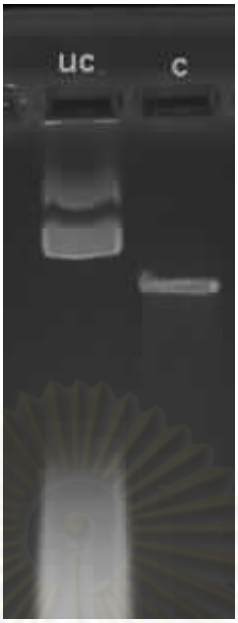


Figure 15. Linearization of the P-III insert in pPICZ∝A vector with *Sac* I restriction digestion, UC = uncut and C = cut.

Figure 16. The *P. pastoris* KM71H containing insert vector can grow in YPDS/ Zeocin™. The big colonies were selected for expression.

4. PCR checking of *Pichia* integrant

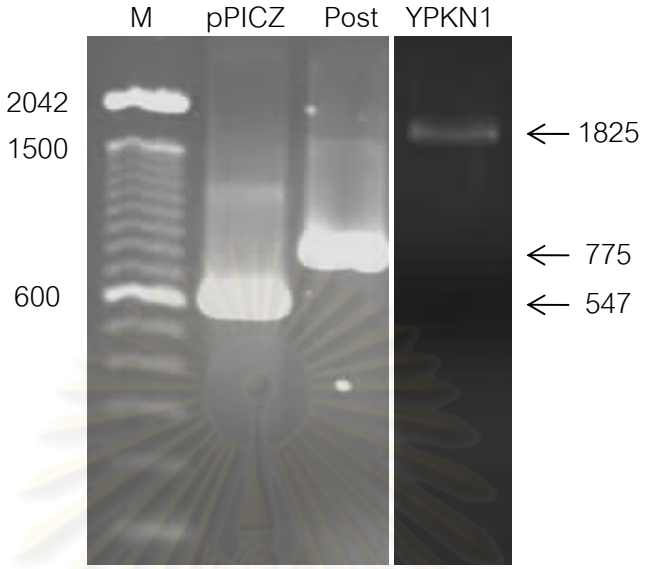


Figure 17. PCR for checking of *Pichia* integrants The YPKN1 colony of *Pichia* was selected for PCR using 3′-*AOX1* and 5′-*AOX1* primer pair. The YPKN1 shown the expected band, 1825 bp, compared with positive control, 775 bp, from genomic DNA of disintegrin clone of *Pichia* as shown in lane Post, and pPICZαA vector that did not contain DNA insert but contained the two flanking sites of 3′-*AOX1* and 5′-*AOX1* sequences, 547 bp as shown in lane pPICZ. The YPKN1 *Pichia* clone was selected for expression.

5. Expression and purification of albocollagenase

After transformation of *Pichia* integrants on YPDS/Zeocine™ agar, 16 *Pichia* colonies were inoculated in 10 ml of YPD broth in final concentration 100 μg/ml of Zeocine™ for glycerol stock with shaking 250 rpm, 30 °C, for one over night. We found that 3 *Pichia* clones were more grown than other clones. We inoculated them in YPDS agar in final concentration 100, 200, 400, and 800 μg/ml of Zeocine™. We also found that all of them could grow on YPDS agar in final concentration 100, 200, 400, and 800 μg/ml of Zeocine™. Thus, we selected them for expression.

The small scale expression was performed in 50 ml conical tube as previously described to screen the expected clone. We found that 2 of 3 clones could express the expected recombinant protein (albocollagenase) shown in Western blot (data no shown).

We randomly selected one clone to set expression conditions. **Figure 18** is the first time that we tried to express the recombinant protein without the setting conditions using random OD of *Pichia* cell mass, the method was shown in small scale expression of chapter 3. And then we tried to perform the large scale expression without setting conditions. We found that the recombinant protein had low level concentration.

The expression conditions are difficult to predict the values. “What is the suitable optical density (OD) of *Pichia* cell mass for expression” is the first question. We started the OD_{600} of 0.1 to 10 in BMGY media with 16 hours culture time in exactly 30 °C, 250 rpm shaker incubator using Erlenmeyer flask. The culture time is depended on the appropriated strain. We selected the OD_{600} of 10 by the appropriated culture time (not over 16-18 hours).This step indicate the young or old yeast cells before they were transferred to BMMY media using appropriated centrifuged force. In BMMY media, we tried to vary the OD_{600} with 10, 20, 30, 50, and 100 using 50 ml conical tube for expression. After 1st, 2nd, and 3rd-day of 0.5% every day induction with methanol, we did dot blot and found that the 3rd-day of expression with OD_{600} of 30 are the best condition for expression of albocollagenase as shown in **figure 19**. We also hypothesized that the

3rd-day of expression is the best, and from the previous Western blot results, we found that our hypothesis are true as shown in **figure 20**. “Which the percentage of methanol to maintain the induction of expression” is the 3rd hypothesis. We tried to vary the concentration of methanol with 0.5%, 1%, 5%, and 10% every day induction in BMMY media. The dot blot shown that 5% and 10% of methanol every day induction are almost the same albocollagenase expression level as shown in **figure 21** thus we selected 5% methanol for expression.

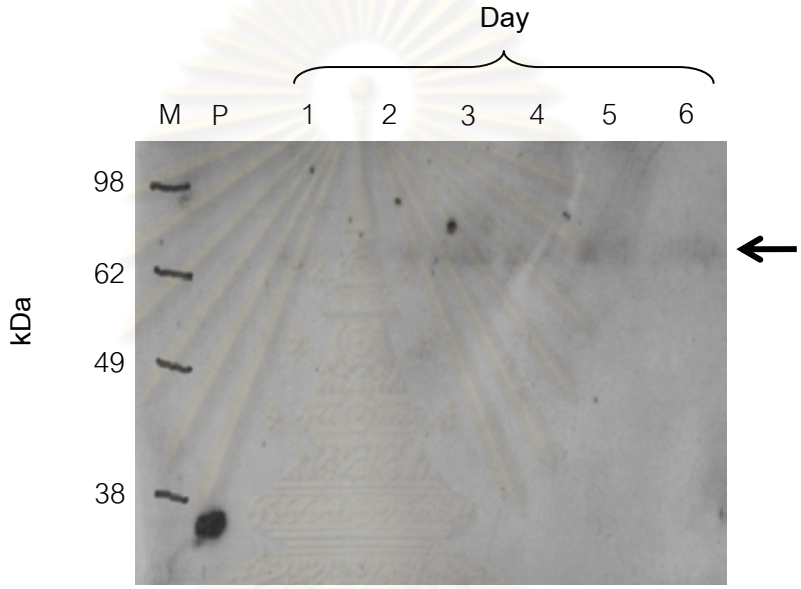


Figure 18 The Western blot of culture medium from each day of expression (Day 1 – 6), recombinant albocollagenase (Arrows). The supernatant of culture medium was concentrated using membrane filtration centrifugation with 10-kDa MWCO, electrophoresed on 8% reducing SDS-PAGE and subjected to Western blot probed with anti-histidine tag antibody. The molecular weight markers in lane M are written with a marker pen corresponding to their correct positions. Another 35-kDa 6His-protein used as a positive control for Western blot was shown in lane P.

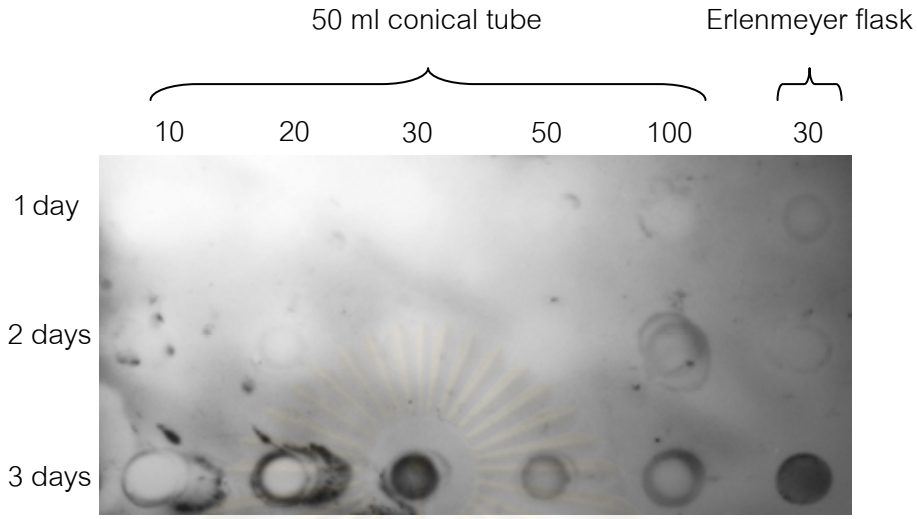


Figure 19 Dot blot of albocollagenase expression with: different OD; 10, 20, 30, 50, and 100; and different flask; 50 ml conical tube and Erlenmeyer flask. The selected clone was expressed as described in large scale expression in materials and methods of chapter 3. The supernatant with 0.5% methanol induction of each day of expression, non-concentrated, was dot blotted as previously described.

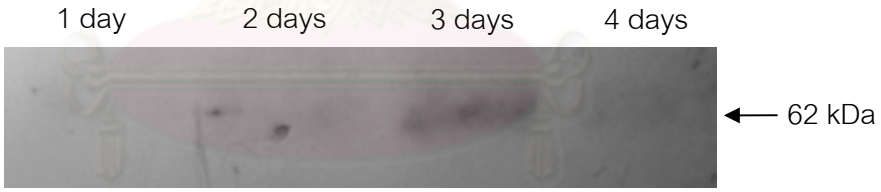


Figure 20 The Western blot of culture medium from each day of expression (Day 1 – 4), recombinant albocollagenase (Arrows). The selected clone was expressed with OD of 30 in Erlenmeyer flask as described in large scale expression in materials and methods of chapter 3. The supernatant with 0.5% methanol induction of each day of expression, non-concentrated, was Western blotted as previously described.

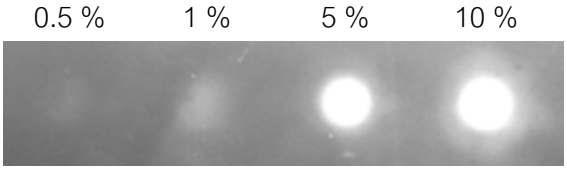


Figure 21 Dot blot of albocollagenase expression with different percent of methanol concentration; 0.5, 1, 5 and 10. The selected clone was expressed with OD of 30 in Erlenmeyer flask as described in large scale expression in materials and methods of chapter 3. The supernatant with 0.5% methanol induction of each day of expression, non-concentrated, was dot blotted as previously described.

The recombinant protein was purified using 2 tandem techniques, a Ni^{2+}-resin column (**figure 22**) and a Ni^{2+}-magnetic bead to bind recombinant 6histidine-tagged albocollagenase.

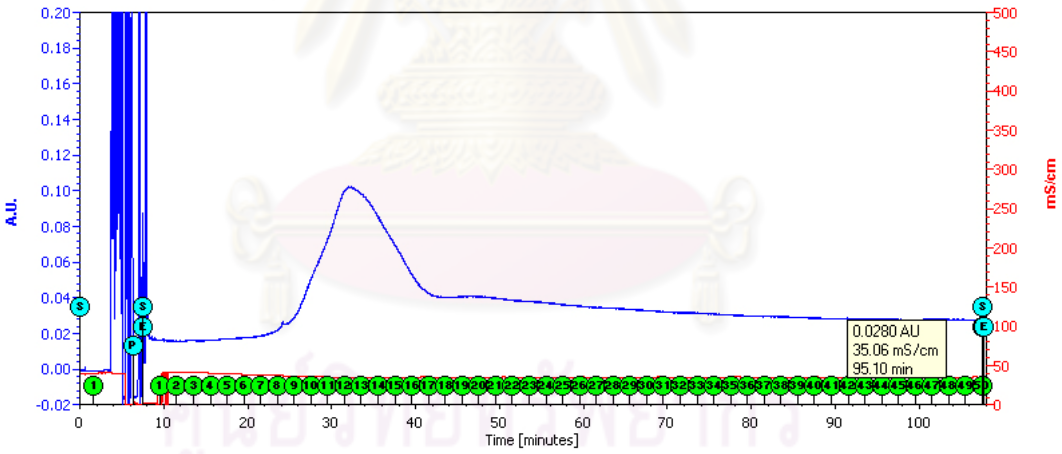


Figure 22. Purification of albocollagenase using the Ni^{2+}-resin column The 280 nm absorbance of eluted protein was high at the elution time of 24 – 42 minutes referred to the albocollagenase. It was eluted in the fractions of 8 – 17.

6. Protein concentration

The standard curve of BSA was performed as shown in **figure 23** to calculate the concentration of albocollagenase. The correlation equation of the curve is Y = 0.0156X. The albocollagenase final concentration is 100 μg/ml calculated from 0.150 of absorbance.

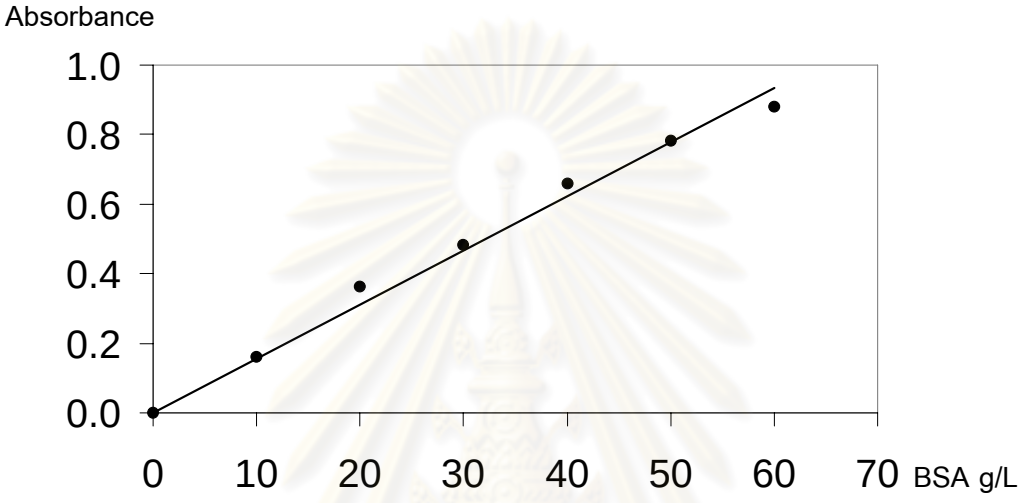


Figure 23. Standard curve of BSA using Micro BCA™ Protein Assay Reagent Kit

7. SDS-PAGE and Western Blot analysis of albocollagenase

The protein purification yielded 100 μg of recombinant albocollagenase from 400 ml of culture media. It was stored at -80°C until used. On SDS-PAGE-coomassie blue stain and Western blot, the protein bands of albocollagenase were approximately 62 kDa in both reduced and native conditions as shown in **Figure 24 and 25**, respectively.

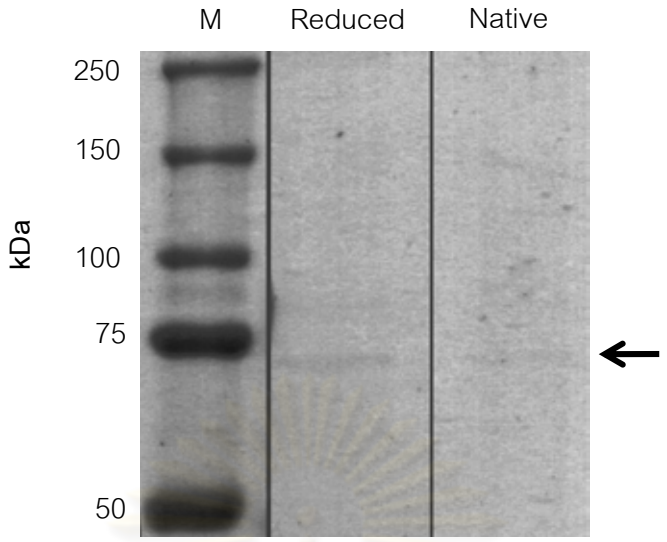


Figure 24. SDS-PAGE in reduced and native conditions of albocollagenase (arrow). The recombinant protein was purified, electrophoresed on 8% SDS-PAGE in reduced and native conditions and stained with Coomassie-blue R250. The molecular weight markers are shown in lane M.

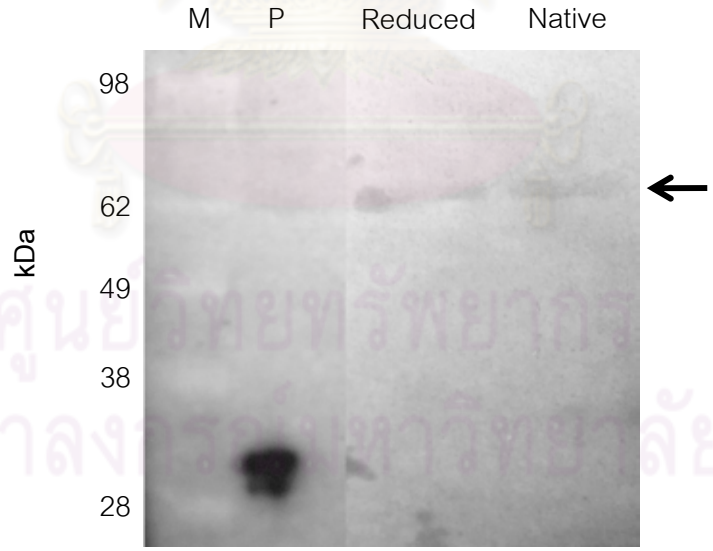


Figure 25. The Western blot of albocollagenase (arrow). The purified protein was subjected to Western blot probed with anti-histidine tag antibody. In each lane, 2 μg of protein were loaded. The 35-kDa 6His-protein was shown in lane P.

8. Albocollagenase digests type IV collagen but not fibrinogen.

The recombinant albocollagenase was incubated with type IV collagen for different periods of time as shown in **Figure 26.** Albocollagenase degraded human type IV collagen in a time-dependent manner. While the degradation of type IV collagen began at 1 minute, more digested bands were progressively more visible during the period of 24 hours.

In **Figure 27**, albocollagenase was able to digest type IV collagen like to collagenase type I, a positive control. EDTA (a metal iron chelator), but not PMSF (a serine protease inhibitor), could inhibit collagen degradation by albocollagenase suggesting that it was a metalloproteinase, not a serine protease.

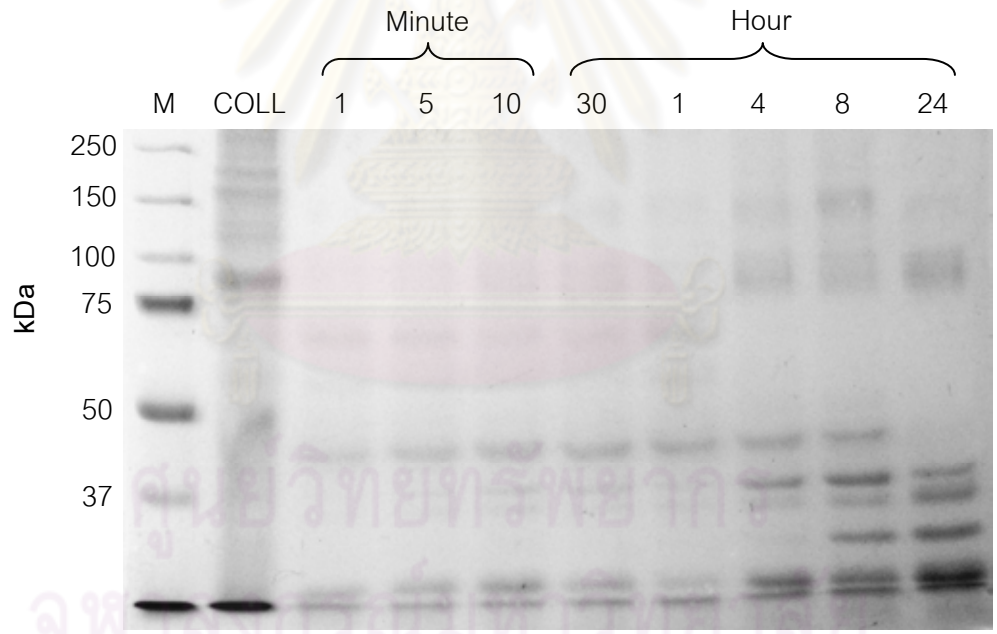


Figure 26. Time-dependent type IV collagen degradation by albocollagenase. Type IV collagen was incubated with albocollagenase at different time points as indicated in each lane. Each reaction was subjected to 8% reducing SDS-PAGE and stained with Coomassie blue. M and COLL represent the molecular weight markers and undigested collagen, respectively.

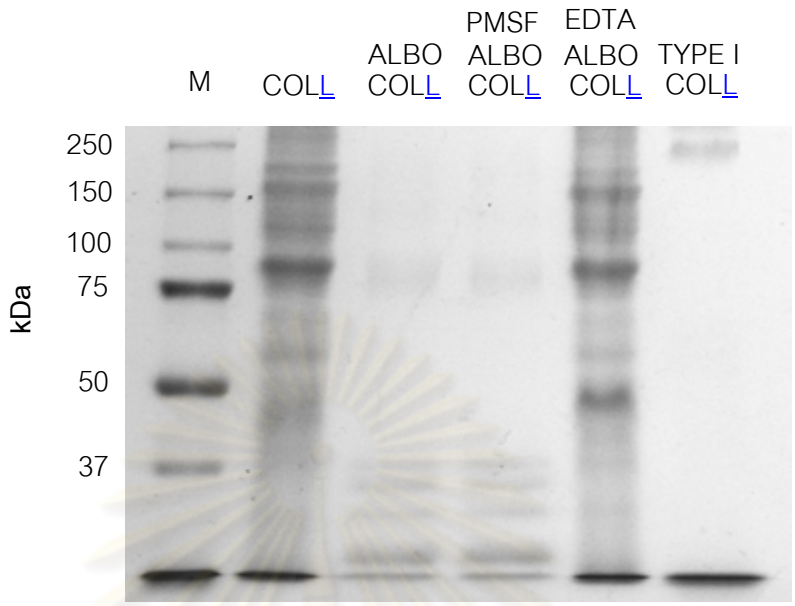


Figure 27. Albocollagenase was inhibited by EDTA but not PMSF. Type IV collagen alone and collagen plus albocollagenase incubation for 24 hours are shown in lane COLL and COLL/ALBO, respectively. In the following lanes, the albocollagenase was pre-incubated with PMSF and EDTA, respectively, at 10 mM final concentration for 1 hour before adding collagen and incubating for 24 hours. Type I collagenase (TYPE I) and collagen are shown in the last lane as a positive control.

A human fibrinogen degradation assay, **figure 28** was also performed for albocollagenase in the presence and absence of calcium ion. We found that albocollagenase could not digest human fibrinogen in either condition.

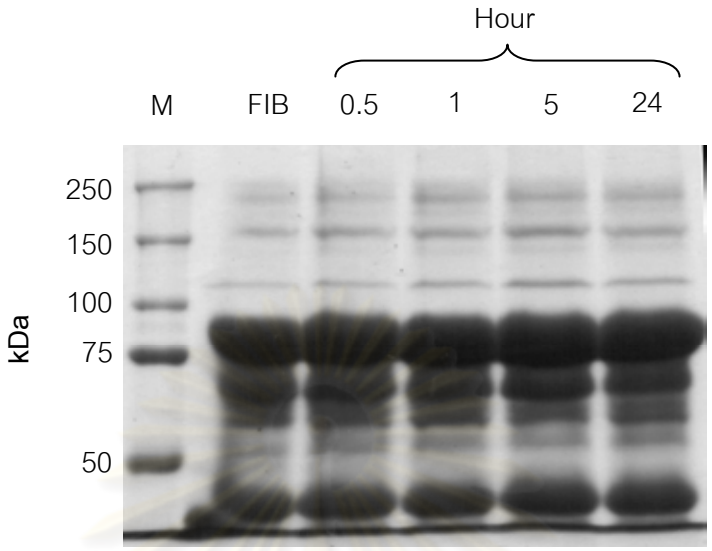


Figure 28. Time-dependent human fibrinogen degradation by albocollagenase. Human fibrinogen was incubated with albocollagenase at different time points as indicated in each lane. Each reaction was subjected to 8% reducing SDS-PAGE and stained with Coomassie blue. M and FIB represent the molecular weight markers and undigested human fibrinogen, respectively.

9. Doxycycline and GPV antivenom could not inhibit albocollagenase *in vitro*.

The metal ion chelator, EDTA, was shown to inhibit proteolytic activities of albocollagenase as shown in **figure 28**. This experiment, we tried to inhibit albocollagenase activities with edible small molecule, doxycycline, using collagen type IV degradation method compared with other specific metal ion chelators. $CaNa_2EDTA$ is also an edible form of EDTA. The other molecules, BAPTA and TPEN, were Ca^{2+} and Zn^{2+} chelators, respectively. In type IV collagen degradation activity, **figure 29**, the main collagen band remains visible to one band in lane doxycycline inhibition and to two bands in lane BAPTA and TPEN inhibition. Although we increased the concentration of doxycycline, the degradation bands still appeared **(figure 30)**. It was possible that albocollagenase was slightly inhibited by doxycycline, BAPTA and TPEN as there were

fewer degraded bands. Interestingly, $CaNa_2EDTA$ could not inhibit albocollagenase proteolytic activity.

The commercial antivenom of GPV was also performed. Current F $(ab)'_2$ antivenoms in Thailand were the products of Queen Saovabha Memorial Institute, They were pure and devoid of the Fc portion. From the instruction manual, the ratio of neutralization between 5 g/L of antivenom and the whole venom is 1 ml per 0.7 mg. Although the concentration of antivenom was increased, it was unable to inhibit proteolytic activity of albocollagenase as shown in **figure 31**.

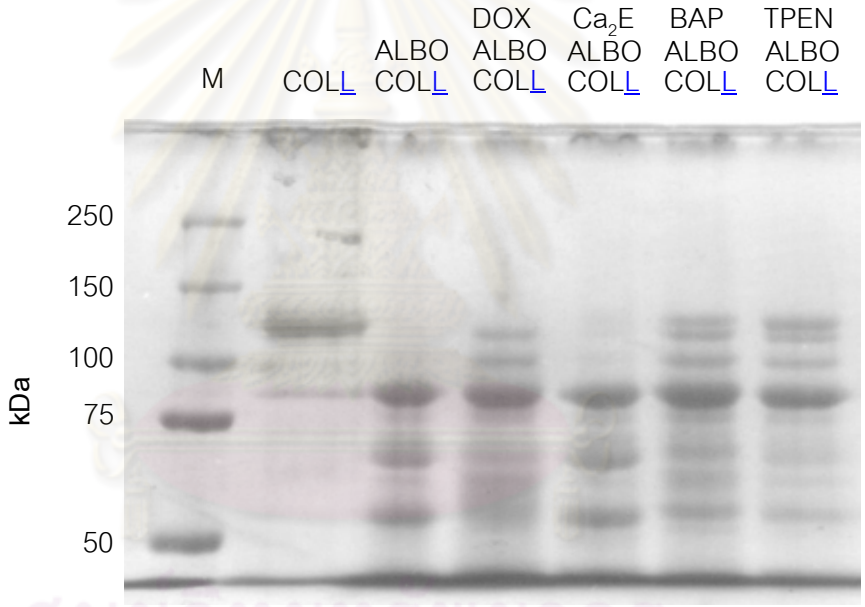


Figure 29. Albocollagenase inhibition by various specific inhibitors. Type IV collagen alone and collagen plus albocollagenase incubation for 24 hours are shown in lane COLL and COLL/ALBO, respectively. In the following lanes, the albocollagenase (ALBO) was pre-incubated with doxycycline (DOX), $CaNa_2EDTA$ (Ca_2E), BAPTA (BAP) and TPEN, respectively, at 10 mM final concentration for 1 hour before adding collagen (COLL) and incubating for 24 hours.

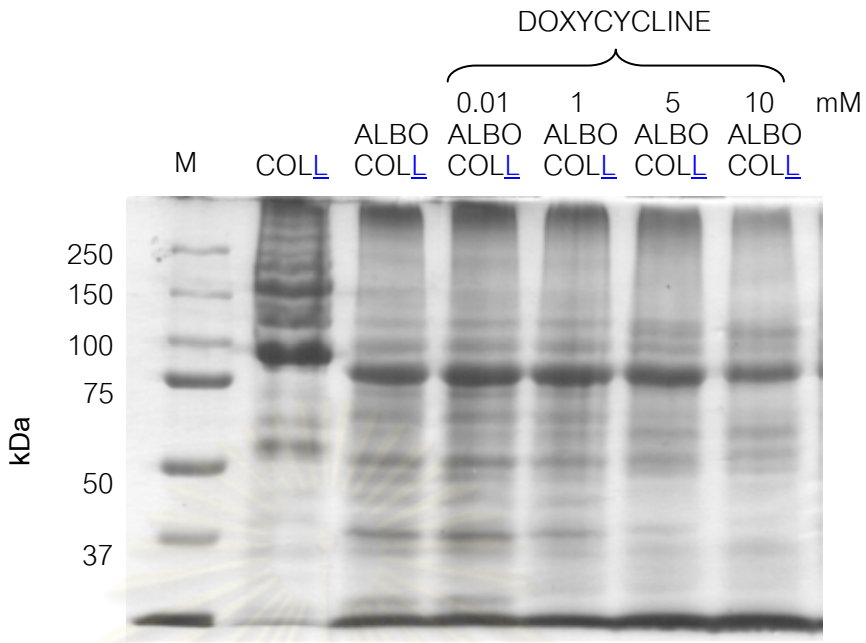


Figure 30. Doxycycline might partially inhibit albocollagenase. As described in figure 29, the albocollagenase was pre-incubated with different concentrations of doxycycline at 0.01, 1, 5 and 10 mM final concentration for 1 hour respectively before adding collagen and incubating for 24 hours.

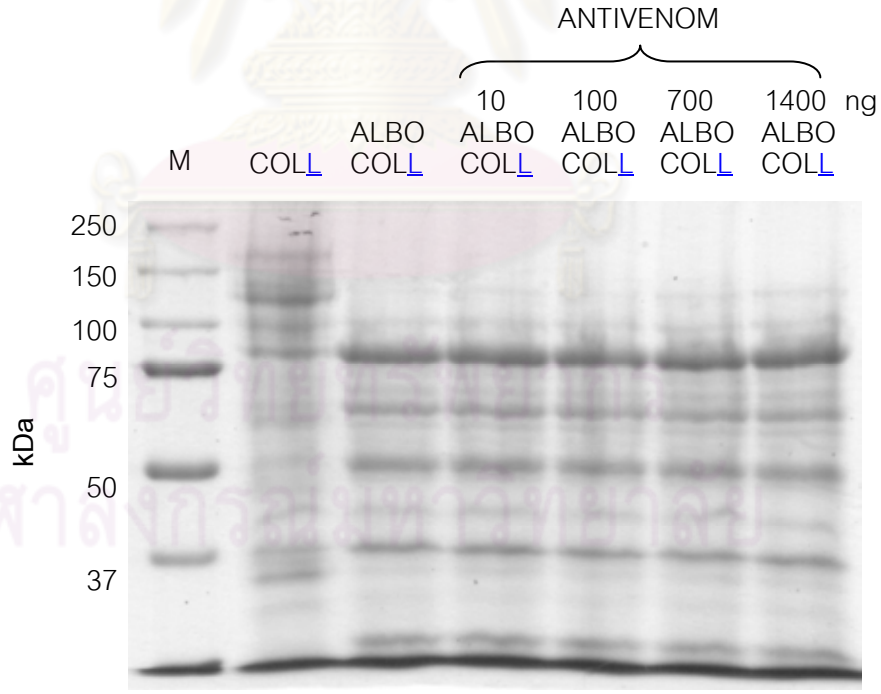


Figure 31. Antivenom could not inhibit albocollagenase. As described in figure 30, the albocollagenase was pre-incubated with different concentration of commercial GPV antivenom at 10, 100, 700 and 1400 ng final concentration for 1 hour respectively before adding collagen and incubating for 24 hours.

10. Albocollagenase inhibited collagen-induced (but not ADP-induced) platelet aggregation.

In order to test the effects of the recombinant protein and platelet, we performed platelet aggregation analysis using 10-min pre-incubation of various concentrations of albocollagenase and platelet before adding collagen or ADP as the inducer. We found that albocollagenase could inhibit collagen-induced platelet aggregation in a concentration-dependent manner as shown in **Figure 32 and 33.** The 50% of inhibitory concentration (IC_{50}) value was 70 nM.

However, there was no effect on ADP-induced platelet aggregation of albocollagenase compared with BSA as negative control inhibitor, **table 6**.

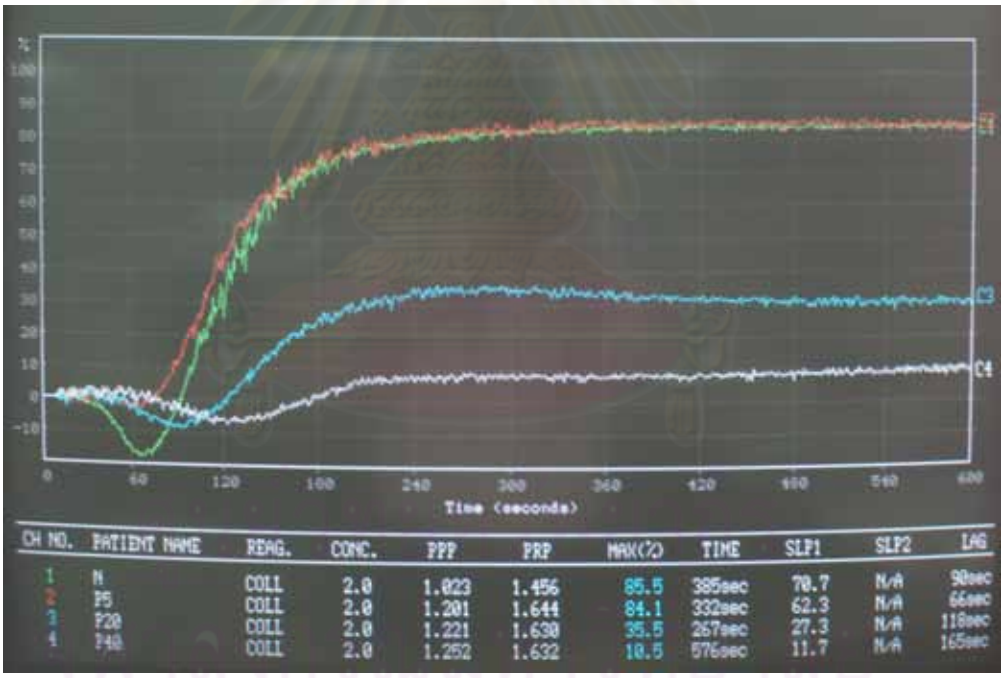


Figure 32. The PACKS 4 data (from monitor screen) showing albocollagenase inhibited collagen-induced platelet aggregation. The albocollagenase was added to reaction of PACKS 4 in a dose-dependent manner at 0, 5 20, and 40 μL which were labeled as green, red, blue and white lines respectively and correlated with the graph C1, C2, C3, and C4 that shown the percentage of maximum transmittance (Y axis) with time (X axis).

Table 6. Data of albocollagenase inhibiting collagen-induced platelet aggregation.

Inducer	100 μg/ml ALBO (μL)	ALBO in 500 μl (μg/L)	ALBO (μg/L)/62,000 (nM)	% Agg^n from PACKS 4	Adjusted % agg^n	% Inhibition
Collagen	0	0	0	85.5	100	0
	5	1000	16.1	84.1	98.3	1.6
	20	4000	64.5	35.5	41.5	58.4
	40	8000	129.0	10.5	12.2	87.7
ADP	0	0	0	100	100	0
	40	8000	129.0	91.8	91.8	8.2
BSA	-	-	-	100	100	0

ALBO = albocollagenase, Agg^n = aggregation,

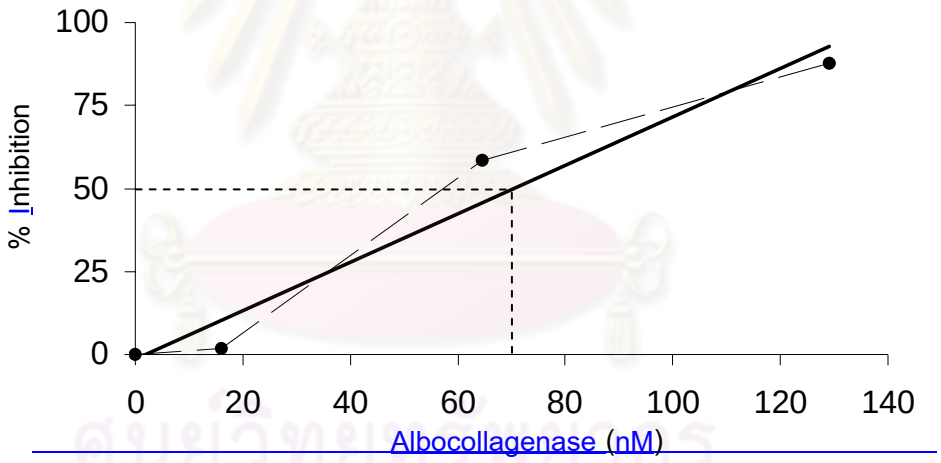


Figure 33. Albocollagenase inhibited platelet aggregation. Various concentrations of the albocollagenase (0, 0.25, 1 and 2 mg/L) were pre-incubated with platelet-rich plasma (PRP) before adding collagen. The maximal aggregation without albocollagenase was regarded as 100%. The PRP without agonist was set as 0%. The percentage of aggregation was adjusted from maximal aggregation. The percentage of inhibition was calculated from 100 minus the percentage of adjusted aggregation. The 50% of inhibitory concentration (IC_{50}) value of the albocollagenase was 70 nM by linear regression curve fitting algorithm.

CHAPTER V

DISCUSSION

Snake venom metalloproteinases (SVMPs) are the main venom components responsible for local tissue injury after viper bites. As type P-III SVMPs display more potent activities than those of P-I and P-II classes (Bjarnason and Fox, 1994; Hite et al., 1994), we focused our study on a novel P-III class SVMP, termed albocollagenase, from *T. albolabris*.

The recombinant expression in *P. pastoris* was used instead of venom purification because the structure-function relationship of the proteins could be definitely determined. Our previous studies illustrated the potentials of snake venom protein expression in *P. pastoris*, strain X-33 (Muanpasitporn and Rojnuckarin, 2007; Singhamatr and Rojnuckarin, 2007). As there were more successful data of metalloproteinase expression using *P. pastoris,* KM71H (Brouta et al., 2002; Schwettmann and Tschesche, 2001), this strain was selected for the expression in this study.

1. Sequence analysis of albocollagenase

The sequence analysis of albocollagenase in **Figure 11** revealed that several cysteine residues were conserved among P-III SVMPs. However, there were 9 cysteines in albocollagenase metalloproteinase domain compared with 5-7 residues in the other SVMPs. Although the numbers of cysteine residues vary among SVMP metalloproteinase domains, they usually contain only 3 conserved disulfide bonds (Igarashi et al., 2007; Takeda et al., 2006). The 3 'extra' cysteines in albocollagenase were all in the N-terminal portion (C27, C52, and C102) of the protease domain before reaching the highly conserved C125. These N-terminal cysteine residues may not participate in disulfide pairing and, thus, not affect the folding of SVMPs (Fox and Serrano, 2008). For example,

a crystal structure of the P-Ia SVMP, adamalysin II, shown an extra unpaired cysteine residue locating N terminal to the C125 (Gomis-Ruth et al., 1993).

From the crystal structure analysis, adamalysin II contained 2 disulfide bonds (C125-C207 and C167-C174), while the P-IIIb catrocollastatin (Igarashi et al., 2007) contained 3 disulfide pairs (C125-C207, C169-C174, and C167-C191). Interestingly, albocollagenase contained C125, C207, C169, C171, C174 and C191. The disulfide bond pattern of albocollagenase remains to be elucidated.

The disintegrin-like and cysteine-rich domains of albocollagenase contained as many as 24 cysteine residues. Data on disulfide bond pairing within this domain were conflicting between N-terminal sequencing and mass spectrometry analysis (Igarashi et al., 2007). Nevertheless, the first cysteinyl residues in disintegrin-like domain, C223, was usually lacking in disintegrin domains (Fox and Serrano, 2008). **Figure 11** shown that albocollagenase also contained this conserved C223 as all the other P-III SVMPs.

By comparison with catrocollastatin, albocollagenase contained 3 putative Ca^{2+}-binding sites as shown in **Figure 11**. Calcium ions are known to stabilize the tertiary structure of matrix metalloproteinases (MMPs) with collagenase activity (Bode et al., 1994). Alborhagin, another P-III SVMP from *T. albolabris*, could digest human fibrinogen when adding Ca^{2+} to the reaction (Andrews et al., 2001). On contrary, albocollagenase could not digest fibrinogen with or without adding Ca^{2+}.

2. Expression of albocollagenase

Albocollagenase was expressed at a low level, 0.25 mg/L of culture media, in our *Pichia* system. Snake codon usage may not be optimal for protein expression (Schmidt-Dannert et al., 1998). Another possible explanation is that the pro-domain was not included in expression construct. Pro-domains of enzymes are known to be critical for protein folding (Nagradova, 2004). Alternatively, the recombinant protein may undergo auto-proteolysis (Assakura et al., 2003; Fujimura et al., 2000). SDS-PAGE shown that the recombinant protein was larger (62 kDa) than the molecular weight calculated from the amino acid content (49 kDa). This discrepancy may be due to post-translational modifications (Oliveira et al., 2010).

3. Collagen and Fibrinogen degradation

Collagen is a structural scaffold for connective tissue and blood vessel walls comprising as many as 27 different types. Type IV collagen is an essential component of basal lamina and ocular lens. Previous reports shown that most of P-III SVMPs could degrade human type IV collagen. As shown in **Figure 26**, the degradation of type IV collagen began at 1 minute implying that the albocollagenase could rapidly degrade extracellular matrix (ECM) of envenomated patients. ECM degradation may result in vascular endothelial damages by inducing endothelial cell anoikis, a specialized form of apoptosis (Tanjoni et al., 2005). Therefore, albocollagenase probably played important roles in rapid local tissue damages in snakebite patients. Inhibition of these enzymatic activities may be helpful in clinical therapy. For example, a small molecule metalloproteinase inhibitor, doxycycline, was found to inhibit the enzyme *in vitro*. Unfortunately, it could not prevent venom hemorrhagic activities *in vivo*, if doxycycline was given after injection of crude venom (Rucavado et al., 2008).

In addition to the protease domain, the strong proteolytic activity of the P-III SVMP may result from a specific interaction with basement membrane components. Several lines of evidence suggest that the cysteine-rich domain targeted the protease to interact

with collagen fiber (Tanjoni et al., 2010) or von Willebrand factor (vWF) (Serrano et al., 2007) contributing to the hemorrhagic activity. In addition, recent crystal structure of catrocollastatin revealed the hyper-variable-region (HVR) located at the C terminal part of the cysteine-rich domain (**Figure 11**). This represented a potential exosite for substrate recognition by binding to ECM proteins (Igarashi et al., 2007). Therefore, cysteine-rich domain may function as substrate targeting to enhance metalloproteinase domain activities. Furthermore, HVR may also play a role in triggering pro-inflammatory effects by promoting leukocyte rolling (Menezes et al., 2008).

4. Platelet aggregation

Platelet aggregation contributes to hemostasis using complex mechanisms. Binding of subendothelial collagen with platelet receptor glycoprotein (GP) VI (non-integrin) stimulate the signaling pathways and up-regulate platelet integrins (inside-out signaling), such as $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$. In addition, stimulated platelets secrete the granule contents, particularly ADP, which promotes platelet activations. Like GPVI, the $\alpha_2\beta_1$ intergrin also binds collagen fibers activating platelet adhesion and spreading, as well as thrombus formation. The integrin $\alpha_{IIb}\beta_3$ plays an exclusive role in linking platelets to one another through the adhesive action of fibrinogen. Engagements of this receptor further activate platelet spreading and enhance platelet aggregation (Adam et al., 2008).

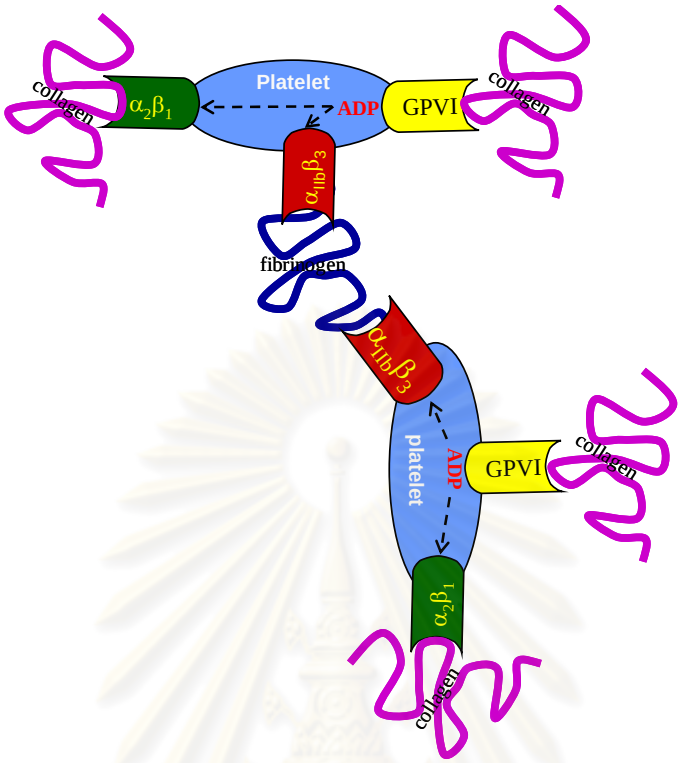
Disintegrin-like domain of SVMPs was the main part interacting with platelet. However, the purified P-I SVMP, lebetase from *Vipera lebetina*, which did not contain disintegrin or disintegrin-like domain, could also inhibit ADP-induced platelet aggregation (Siigur et al., 1998). Therefore, metalloproteinase domains may also react with platelet.

P-III SVMPs could inhibit platelet aggregation through several proposed mechanisms. First, some could degrade or interact with different platelet receptors. For example, jararhagin degraded the β subunit of integrin $\alpha_2\beta_1$ (Kamiguti et al., 1996).

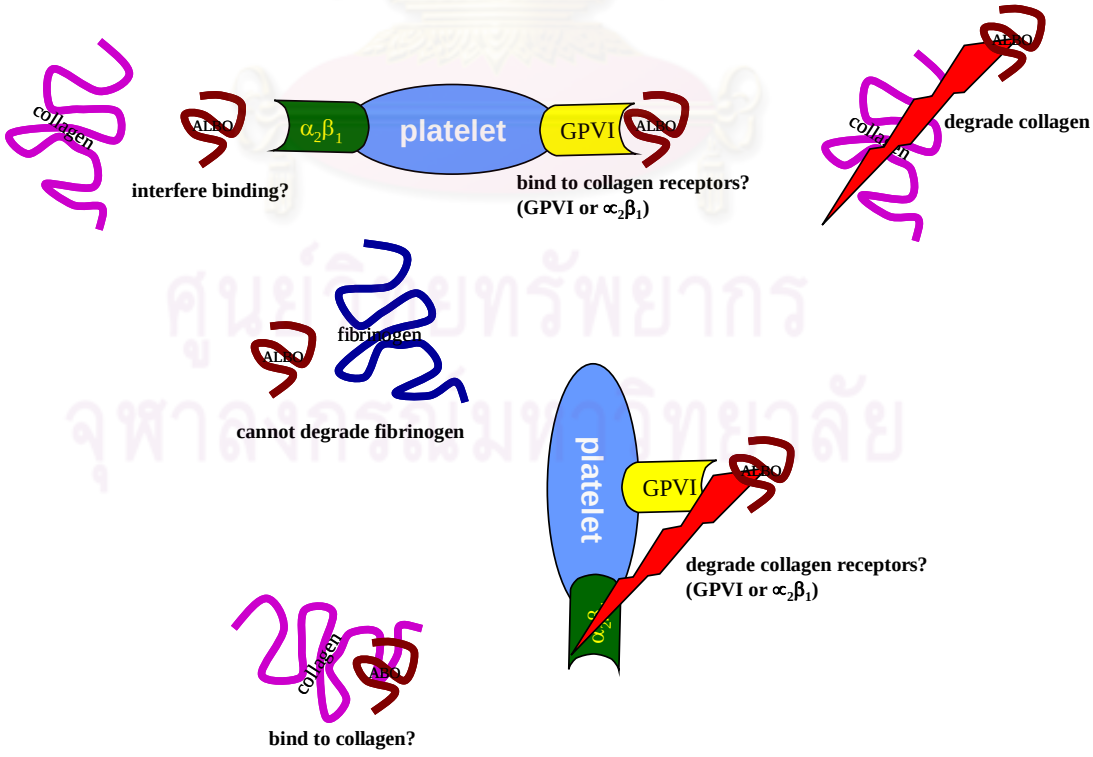
Atrolysin A bound and blocked to $\alpha_2\beta_1$ (Kamiguti et al., 2003). Acurhagin interacted with GPVI (Wang et al., 2005). Secondly, others could degrade or interact with adhesive proteins involved in haemostasis, e.g. AAV1 (Wang, 2007) and halysase degraded fibrinogen; kaouthiagin (Hamako et al., 1998) and jararhagin (Serrano et al., 2007) destroyed vWF; jararhagin, atrolysin A, and catrocollastatin interacted with vWF domain (Serrano et al., 2007); jararhagin, acurhagin, and catrocollastatin bound collagen fibers. Our results shown that albocollagenase inhibited only collagen (not ADP)-induced platelet aggregation suggesting that the venom protein specifically prevented collagen and collagen receptor (GPVI and/or $\alpha_2\beta_1$ integrin) interactions. Whether this is mediated by enzymatic degradation or non-enzymatic binding mechanisms remain to be determined.

Figure 34. (next page) The proposed mechanism of albocollagenase that inhibits platelet aggregation. A: Platelet aggregation without albocollagenase (ALBO), and B: Platelet aggregation with albocollagenase (ALBO). Albocollagenase may interfere with collagen receptor binding or directly digest collagen.

A


Platelet
ADP
GPVI
collagen
collagen
$\alpha_2\beta_1$
$\alpha_{IIb}\beta_3$
fibrinogen
$\alpha_{IIb}\beta_3$
platelet
ADP
GPVI
collagen
$\alpha_2\beta_1$
collagen

B


collagen
ALEQ
$\alpha_2\beta_1$
platelet
GPVI
ALEQ
interfere binding?
bind to collagen receptors?
(GPVI or $\alpha_2\beta_1$)
ALEQ
collagen
degrade collagen
fibrinogen
ALEQ
cannot degrade fibrinogen
platelet
GPVI
ALEQ
degrade collagen receptors?
(GPVI or $\alpha_2\beta_1$)
collagen
ALEQ
bind to collagen?

5. Doxycycline inhibition of matrix metalloproteinases

Doxycycline was shown to decreases cerebral matrix metalloproteinase (MMP)-9 activities and angiogenesis induced by vascular endothelial growth factor (VEGF) (Lee et al., 2006). At the enzymatic activity level, doxycycline started to suppress MMP-9 activity at 5 mg/kg/day, while minocycline had an effect at a lower dose, 1 mg/kg/day (Lee et al., 2006). The precise mechanism of MMP inhibition by tetracyclines remains unclear. Several mechanisms have been proposed by previous studies. The inhibition may be due to modulations including via gene transcription, scavenging reactive oxygen species or Zn^{2+} binding (Ryan et al., 2001). Previous study shown that minocycline inhibiting MMP-9 activity is also through inhibiting extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) pathways suggesting that the effects of minocycline could be through multiple pathways (Yao et al., 2004). Studies of inflammatory signaling suggested that minocycline and doxycycline might have differential effects in different species and cell types (Amin et al., 1996). A recent study in human corneal epithelial cells has shown that doxycycline markedly inhibited the tumor growth factor (TGF)-beta1- induced production of MMP-9 and activation of the Smad, c-Jun N-terminal kinase (JNK), ERK, and p38 mitogen-activated protein kinase signaling pathways (Kim et al., 2005). The SVMPs require coenzyme Zn^{2+}. Therefore, we may be able to inhibit SVMPs *in vitro* using doxycycline that binds this ion. This drug is potentially a novel therapeutic agent for snakebite patients.

However, both doxycycline and green pit viper antivenom could not completely inhibit albocollagenase *in vitro*. Other novel agents remain to be investigated.

6. Conclusion

In summary, we cloned, expressed and characterized a novel P-III SVMP, albocollagenase, from *T. albolabris* venom. Like other P-III SVMPs, it displayed a multidomain structure composed of a metalloproteinase and disintegrin-like and cysteine-rich domains. The structure-function analysis of snake venom metalloproteinases, particularly of P-III SVMPs, has been significantly limited by the lack of catalytically-active recombinant proteins. In this context, this research represents a valuable contribution and a promising alternative to obtain functional P-III SVMPs using *Pichia pastoris* and to allow site-mutagenesis studies which could pave the way for a better understanding of the structural determinants of toxicity and catalysis in P-III SVMPs. As such, this work is a relevant contribution. Recombinant albocollagenase exhibited proteolytic activities on collagen and inhibited collagen-induced platelet aggregation. Therefore, it possibly contribute to tissue necrosis and hemorrhage in snakebite patients. Future investigations to identify potent and specific inhibitors to this molecule are warranted.

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APPENDIX

1. Bacterial Media

1.1 LB Medium (per liter)

10 g Bacto®-tryptone

5 g Bacto®-yeast extract

5 g NaCl

Adjust pH to 7.0 with NaOH.

1.2 LB Plates with Ampicillin

Add 15 g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool down to 50 °C before adding ampicillin to the final concentration of 100 μg/ml. Pour 30-35 ml of medium into an 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, 100 μl 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	1 M NaCl
0.25 ml	1 M KCl
1 ml	2 M Mg^{2+} stock, filter sterilized
1 ml	2 M glucose, filter sterilized

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

2. *Pichia pastoris* Media

2.1 Low Salt LB (Luria-Bertani) Medium

1 %	Tryptone
0.5 %	Yeast Extract
0.5 %	NaCl

Adjutst to pH 7.0 with NaOH.

For 1 liter, dissolve 10 g tryphone, 5 g yeast extract and 5 g NaCl in 950 ml deionized water. Adjust the pH of the solution to 7.5 with NaOH and bring the volume up to 1 liter. Autoclave for 20 minutes at 15 lb/sq. in. Let cool to ~55 °C and add desired antibiotics at this point. Store at room temperature or at +4 °C.

2.2 **Yeast Extract Peptone Dextrose Medium - YPD or YEPD** (1 liter)

1 % Yeast Extract

2 % Peptone

2 % Dextrose (glucose)

Dissolve 10 g yeast extract and 20 g of peptone in 900 ml of water. Note: Add 20 g of agar if making YPD slants or plates. Autoclave for 20 minutes on liquid cycle. Add 100 ml of 10X D (20% Dextrose). The liquid medium is stored at room temperature. YPD slants or plates are stored at +4 °C. The shelf life is several months.

2.3 **Yeast Extract Peptone Dextrose Medium – YPDS + Zeocin™ Agar**

1 % Yeast Extract

2 % Peptone

2 % Dextrose (glucose)

1 M Sorbitol

2 % Agar

100 µg/ml Zeocin™

For 1 L, Dissolve 10 g yeast extract, 20 g peptone and 182.2 g sorbitol in 900 ml of water. Note: Add 20 g of agar and autoclave for 20 minutes on liquid cycle. Add 100 ml of 10X D (20% Dextrose). Cool solution to ~ 60 °C and add 1.0 ml of 100 mg/ml Zeocin™. Store YPDS or plates containing Zeocin™ at +4 °C in the dark. The shelf life is one to two weeks.

2.4 **Buffered Glycerol-Complex Medium and Buffered Methanol-Complex Medium – BMGY and BMMY** (1 liter)

	1 %	Yeast Extract
	2 %	Peptone
	100 mM	Potassium phosphate, pH 6.0
	1.34 %	YNB
	4×10^{-5} %	Biotin
	1 %	Glycerol (for BMGY)
Or	0.5 %	methanol (for BMMY)

Dissolve 10 g yeast extract and 20 g peptone in 700 ml of water. Autoclave for 20 minutes on liquid cycle. Cool to room temperature, then add 100 ml 1 M potassium phosphate buffer (pH 6.0), 100 ml 10X YNB, 2 ml 500X B (0.02% Biotin), and 100 ml 10X GY (10% Glycerol) and mix well. For BMMY, add 100 ml 10X M (5% Methanol) instead of glycerol. Store media at +4 °C. The shelf life of this solution is approximately two months.

3. **Buffer**

3.1 **1X Equilibration/Wash Buffer (pH 7.0)**

50 mM	Sodium Phosphate pH 7.0
300 mM	NaCl

3.2 **1X Equilibration Buffer (pH 8.0)**

50 mM	Sodium Phosphate pH 8.0
300 mM	NaCl

3.3 **1X Elution Buffer (pH 5.0)**

50 mM	Sodium Phosphate pH 5.0
300 mM	NaCl

3.4 Alkaline Lysis Solution I

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

3.5 Alkaline Lysis Solution II

0.2 N	NaOH
1 % (w/v)	SDS

3.6 Alkaline Lysis Solution III

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

3.7 STE BUFFER

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

3.8 Tris-Glycine Buffer (1X)

25 mM	Tris-Cl
250 mM	Glycine

3.9 10X Tris EDTA (TE) pH 8.0

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

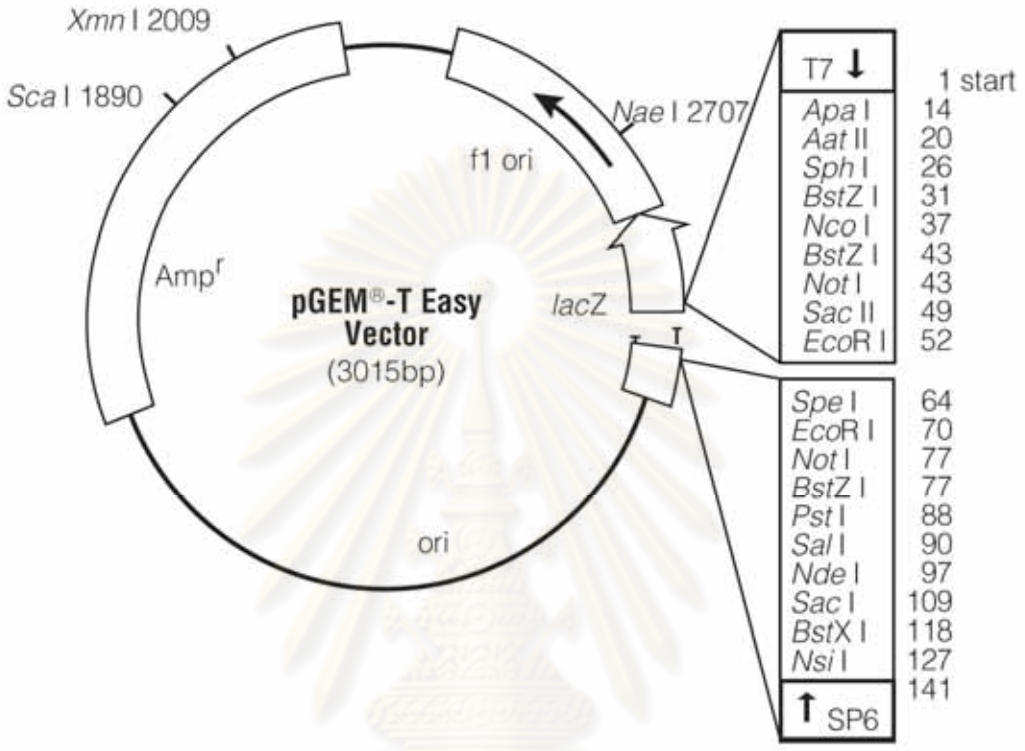
3.10 1X Phosphate-Buffered Saline (PBS)

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

Dissolve 8 g of NaCl, 0.2 g 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.

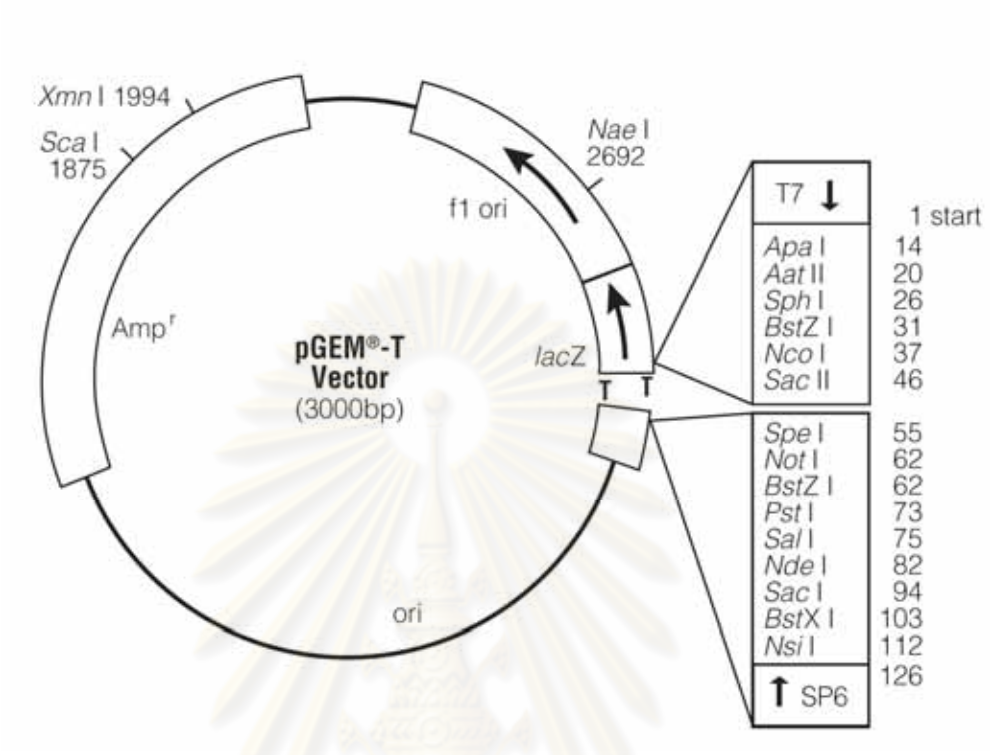
4. Vectors

4.1 **pGEM®-T Easy Vector, circle map and sequence reference points** (This figure was copied from pGEM®-T Easy/pGEM®-T Vector system II Technical Manual.)



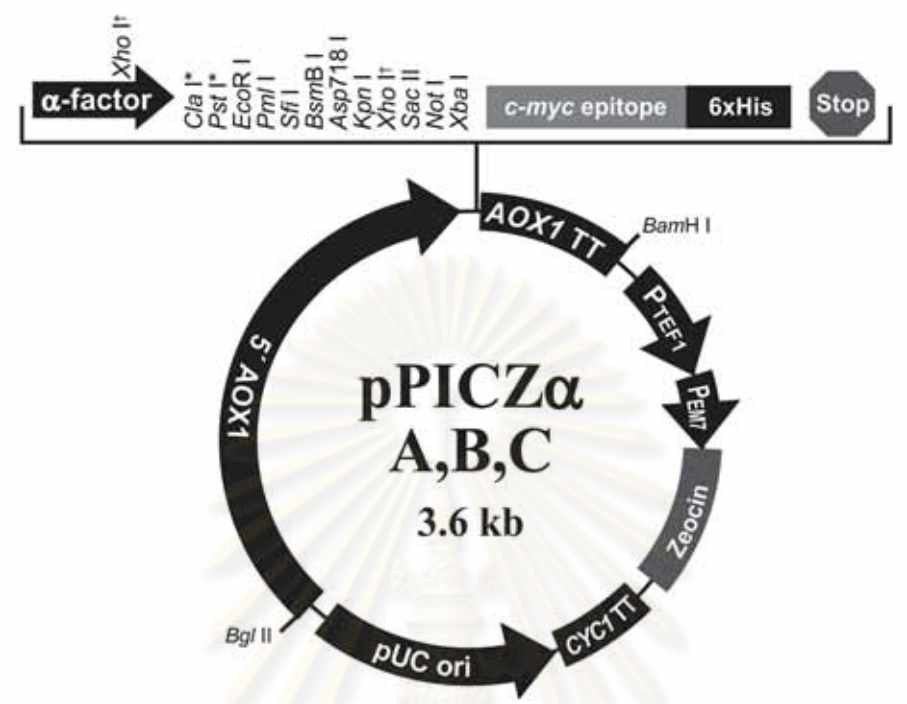
	Sequence reference point
T7 RNA polymerase transcription initiation site	1
Multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse sequencing primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward sequencing primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

4.2 pGEM®-T Vector, circle map and sequence reference points (This figure was copied from pGEM®-T Easy/pGEM®-T Vector system II Technical Manual.)



	Sequence reference point
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
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
lac operon sequences	2821–2981, 151–380
pUC/M13 Forward sequencing primer binding site	2941–2957
T7 RNA polymerase promoter (–17 to +3)	2984–3

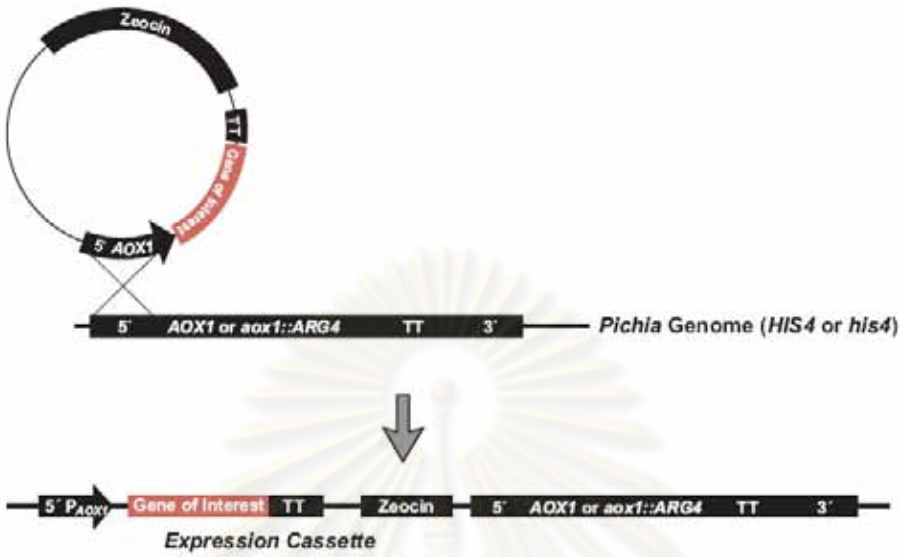
4.3 **pPICZα A, B, and C map and sequence reference points** (This figure was copied from EasySelect™ *Pichia* Expression User Manual.)



	Sequence reference point
5′ *AOX1* promoter region	1 – 1914
5′ *AOX1* priming site	855 – 875
α-factor signal sequence	941 – 1207
α-factor priming site	1144 – 1164
Multiple cloning site	1208 – 1276
c-myc epitope	1275 – 1304
Polyhistidine (6XHis) tag	1320 – 1337
3′ *AOX1* priming site	1423 – 1443
AOX1 transcription termination region	1341 – 1682
TEF1 promoter	1683 – 2093
EM7 promoter	2095 – 2162
Sh ble ORF	2163 – 2537
CYC1 transcription terminator region	2538 – 2855
pUC origin	2866 – 3539

4.4 Multiple Cloning Site of pPICZα A (This figure was copied from EasySelect™ *Pichia* Expression User Manual.)

4.5 **Gene Insertion at *AOX1* or *aox1*::ARG4** (This figure was copied from EasySelect™ *Pichia* Expression User Manual.)



5. Others

5.1 8 % Gel (5 ml) Resolving Gels for Tris-Glycine SDS-Polyacrylamide Gel Electrophoresis

2.3 ml.	H_2O
1.3 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

5.2 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

5.3 **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

5.4 **Coomassie brilliant blue, 200 ml.**

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

5.5 **Destained**

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

5.6 **Electrophoresis blotting buffer**

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

Add H_2O until 1 liter.

BIOGRAPHY

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Poster presentation

A part of this thesis was presented as a poster entitled "MOLECULAR CLONING AND CHARACTERIZATION OF SNAKE VENOM METALLOPROTEINASE GENES FROM GREEN PIT VIPER (*TRIMERESURUS ALBOLABRIS*)" by **Anuwat Pinyachat**, Ponlapat Rojnuckarin, Chuanchom Muanpasitporn, Pon Singhamatr, and Surang Nuchprayoon with **Outstanding Poster Award** at The Fifth Princess Chulabhorn International Science Congress: Evolving Genetics and Its Global Impact. August 16-20, 2004. Bangkok, Thailand

Publication

Pinyachat, A., Rojnuckarin, P., Muanpasitporn, C., Singhamatr, P., and Nuchprayoon, S. Albocollagenase, a novel recombinant P-III snake venom metalloproteinase from green pit viper (*Cryptelytrops albolabris*), digests collagen and inhibits platelet aggregation. Toxicon. 57 (2011) : 772-780.