การโคลน และ การแสดงออกของโปรตีนแฟคเตอร์ไฟฟ์แอคติเวเตอร์ของพิษงูแมวเซา

นายพัทธดนย์ สุขพันธุ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

MOLECULAR CLONING AND EXPRESSION OF FACTOR V ACTIVATOR (RVV-V) FROM RUSSELL'S VIPER VENOM

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

Thesis Title	MOLECULAR CLONING AND EXPRESSION OF FACTOR V ACTIVATOR (RVV-V) FROM RUSSELL'S VIPER VENOM.
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Field of Study	Biomedical Science
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พัทธดนย์ สุขพันธุ์ : การโคลน และแสดงออกของโปรตีนแฟคเตอร์ไฟฟ์แอคติเวเตอร์ของพิษงูแมวเซา. (MOLECULAR CLONING AND EXPRESSION OF FACTOR V ACTIVATOR (RVV-V) FROM RUSSELL'S VIPER VENOM) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รองศาสตราจารย์ ดร. นายแพทย์ อิศรางค์ นุชประยูร, 99 หน้า.

พิษงูประกอบไปด้วยเอ็นไซม์หลายชนิด เช่น ฟอสโฟไลเปสเอทู, เมทัลโลโปรตีเอส, เซอรีนโปรตีเอส และ แอล-อะมิโน เอซิด อ๊อกซิเดส ขณะที่โปรตีนที่ไม่ใช่เอนไซม์เช่น ซี-ไทป์ เล็คติน, ดิสอินทีกริน ก็มีการค้นพบ งูแมวเซา (*Daboia russelli siamensis*) มีความสำคัญทางการแพทย์ในพื้นที่เกษตรกรรมภาคกลางของประเทศไทย เมื่อถูกงูกัดผู้ป่วยจะมีอาการได้หลากหลายซึ่งสัมพันธ์กับการที่ระดับของปัจจัยการแข็งตัวของเลือด จุดประสงค์ของการศึกษานี้คือ (1) เพื่อโคลนและศึกษาลักษณะของซีดีเอ็นเอของโปรตีนแฟคเตอร์ไฟฟ์แอ็คติเวเตอร์ (RVV-V) จากห้องสมุดซีดีเอ็นเอของต่อมพิษงูแมวเซา และ (2) เพื่อผลิต recombinant RVV-V ในจุลชีพโดยวิธีพันธุวิศวกรรม RVV-V ถูกจัดในกลุ่มของเซอรีนโปรตีเอส ประกอบด้วย catalytic triad ที่เป็น active site, กรดอะมิโนซิสเทอีน สิบสองหน่วยที่จะสร้างพันธะ disulfide และ glycosylation site หนึ่งจุด ส่วน 5' ของ RVV-V cDNA ได้ห้องสมุดยีนโดยการทำ 5' - RACE ลำดับเบสและกรดอะมิโนของ RVV-V ได้ถูกนำมาวิเคราะห์โดยโปรแกรมทางชีววิทยาระดับโมเลกุล การศึกษานี้ยังค้นพบ cDNA ของ serine proteinases ชนิดใหม่ที่ยังไม่เคยถูกค้นพบ คือ RVAF และ RVBF โดยลำดับเบสของ RVV-V, RVAF และ RVBF ได้ถูกรายงานใน GenBank เป็นที่เรียบร้อยแล้ว ทั้งนี้ เพื่อปรับปรุงความเข้าใจของความสัมพันธ์ทาง phylogenetic ของ serine proteinases ลำดับกรดอะมิโนของโปรตีนของ RVV–V และเซอรีนโปรตีเอสจากพิษงูอื่น ๆได้ถูกมาใช้เพื่อสร้าง phylogenetic tree ซึ่งเป็นเครื่องมือที่มีประโยชน์สำหรับการจำแนกโครงสร้างและหน้าที่ของโปรตีนและดีเอ็นเอ ดีเอ็นที่เข้ารหัสของ RVV-V ได้ถูกโคลนเพื่อผลิตโปรตีนลูกผสมในแบคทีเรียและยีสต์ (rRVV–V) เพื่อที่จะสร้าง rRVV-V ในเชื้อ *E. coli* ชิ้นส่วนดีเอ็นเอได้ถูก subclone เพื่อเข้า pET32a ซึ่งเป็นเวคเตอร์ที่ช่วยเพิ่มการละลายของโปรตีนลูกผสมโดยรวม thioredoxin tag กับลูกผสมนั้น นอกจากนี้ rRVV-V ยังถูกสร้างกับ molecular chaperone ทั้งนี้สามารถพบ rRVV-V ใน *E. coli* lysate อย่างไรก็ตาม rRVV-V ไม่มีการทำงานกับสาร chromogenic ในความพยายามในการปรับปรุงระบบการแสดงออกจึงย้ายไปที่ระบบการแสดงออกของยีสต์ *Picia pastoris* ซึ่งเป็นยีสต์ชนิด methylotrophic ที่ใช้ในการผลิตโปรตีนลูกผสมที่ผ่านขั้นตอน post-translational modification โดยใช้เวคเตอร์สองชนิดคือ pPIC-Zα และ pPink อย่างไรก็ตามการแสดงออกของ rRVV-V ไม่ประสบความสำเร็จเนื่องจากไม่พบ rRVV-V ตามที่พบจากการทำ SDS-PAGE และไม่พบการทำงานเมื่อทดลองร่วมกับสาร chromogenic ดังนั้น ในการศึกษาต่อไปจึงจำเป็นต้องมีการพัฒนาระบบของการสร้างโปรตีนลูกผสมเพื่อที่จะสามารถผลิตโปรตีน rRVV-V ที่สามารถทำงานได้จริงเพื่อประยุกต์ใช้ในอนาคต

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

4689675020 : MAJOR BIOMEDICAL SCIENCE
KEYWORDS : RUSSEL'S VIPER/FACTOR V ACTIVATOR/CLONING/ CDNA/EXPRESSION

PATTADON SUKKAPAN : MOLECULAR CLONING AND EXPRESSION OF FACTOR V ACTIVATOR (RVV-V) FROM RUSSELL'S VIPER VENOM. THESIS ADVISOR: ASSOC. PROF. ISSARANG NUCHPRAYOON, M.D., Ph.D., 99 pp.

Snake venoms contain various types of enzyme such as phospholipase A2, metalloproteinases, serine proteases and L-amino acid oxidases, while non enzymatic proteins including C-types lectins and disintegrins are also found. *Daboia russelli siamensis* or Russell's viper (RV) is a medically important snake in the central rice-growing area of Thailand. Bitten by such snake causes variety of signs and symptoms associated with severe reduction of blood coagulation factor. The aim of this study is (1) to clone and characterize the factor V activator (RVV-V) cDNA from Russell's viper gland cDNA library, and (2) to produce the recombinant RVV-V in microorganisms through genetic engineering approach. RVV-V is a member of serine protienase family, consisting of the catalytic triad containing active site, 12 cysteins performing 6 disulfide bonds and one N-glycosylation site. The 5' segment of the RVV-V cDNA was obtained from the cDNA library by 5'-RACE. The nucleotide amino acid sequences of RVV-V were analyzed by molecular biology softwares. In addition, two novel serine proteinases cDNA, designed RVAF and RVBF, were also cloned. Since only amino acid sequence of RVV-Vγ was reported, cDNAs of the RVV-V, RVAF anf RVBF from this study have been submitted in GenBank. In addition, to improve our understanding of the phylogenetic relationship, the amino acid sequences of mature proteins of the RVV-V, RVAF, RVBF and other snake venom serine proteinases were used to construct of phylogenetic tree, a useful tool for classification of structure and function of proteins and DNAs. The mature protein-encoding sequence of RVV-V was cloned to produce the recombinant protein (rRVV-V) in bacteria and yeast. To express rRVV-V in *E. coli*, the DNA fragment was subcloned in to the pET32a vector, the expression vector that enhances solubility of the recombinant protein, by including the thioredoxin tag with the expressed recombinant protein. In addition, the rRVV-V was also co-expressed with molecular chaperone. The solubilized rRVV-V was observed in the *E. coli* cell lysate. However, the rRVV-V had no activity on the chromogenic substrate. The effort to improve the expression system had moved to the yeast expression system. *Picia pastoris*, a methylotrophic yeast that allows production of post-translational modified recombinant. Two expression vectors for P. pastoris were used, pPIC-Zα and pPink. However, expression of rRVV-V was unsuccessful in both vectors. The rRVV-V could not be observed as shown by SDS-PAGE analysis, and protease activity on chromogenic substrate was not found. Therefore, improvement of recombinant protein expression system is required in further study to produce the functional rRVV-V for further applications.

Field of Study : Biomedical Science Student's Signature .....

Academic Year : 2010 Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and profound appreciation to my advisor, Professor Dr. Issarang Nuchprayoon for his valuable supervision, guidance, encouragement, discussions, and criticism which have inspired me to accomplish my study. I am very grateful to my thesis committees, Professor Dr. Apiwat Mutirangura, Associate Professor Dr. Ponlapat Rojnuckarin, Associate Professor Dr. Wilai Anomasiri, Assistant Professor Dr. Tewin Tencomnao, and Professor John C. Perez for their suggestions and criticisms.

I also appreciate Dr. Vivornpan Sanprasert, Dr. Montamas Suntravat, Dr. Alisa Junpee, Dr. Arkhom Sai-ngam, and all members in Snake Bite and Venom Research Unit and Lymphatic Filariasis Research Unit, as well as all staffs in Biomedical science program, Chulalongkorn University, for their technical assistance and help.

I am particularly indebted to the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program, the National Research Council of Thailand, as well as the Graduate Thesis Grant from Graduate School, Chulalongkorn University for supporting this study.

Finally, much appreciation is special expressed to my parents, and my family for their loves, kindness, cheerfulness, and moral support throughout this study.

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LIST OF ABBREVIATIONS

A	=	Absoebance
A. p. leucostoma	=	*Agkistrodon piscivorus leucostoma*
ABI	=	Applied Biosystems
APL-C	=	protein C activator from *A. p. leucostoma*
APL-PA	=	plasminogen activator from *A. p. leucostoma*
BCA	=	bicinchoninic acid
bp	=	base pair
BSA	=	bovine serum albumin
°C	=	degree Celsius
cDNA	=	complementary deoxyribonucleic acid
cm	=	centimeter
CO_2	=	carbon dioxide
Da	=	dalton
DNA	=	deoxyribonucleic acid
EDTA	=	ethylenediamine tetraacetic acid
g	=	gram (s)
g	=	gravitational constant
IPTG	=	isopropyl-β-D-thiogalactopyranoside
K_A	=	Numbers of non-synonymous substitutions per non-synonymous site
kDa	=	kilodalton
kg	=	kilogram
K_N	=	Numbers of nucleotide substitutions per site

K_S	=	Number of nucleotide substitutions per synonymous site
LB	=	Luria-Bertani media
mg	=	milligram
ng	=	nanogram
OD	=	optical density
ORF	=	open reading frame
PBS	=	phosphate buffered saline
PCR	=	polymerase chain reaction
pI	=	isoelectric point
M	=	molar
MCS	=	multiple cloning site
mg	=	milligram
mM	=	millimolar
mRNA	=	messenger RNA
MW	=	molecular weight
NCBI	=	National Center for Biotechnology Information
ng	=	nanogram
NH_4HCO_3	=	ammonium bicarbonate
NO	=	nitric oxide
PCR	=	polymerase chain reaction
RNA	=	ribonucleic acid
RNase	=	ribonuclease
RT	=	reverse transcriptase
RVAF	=	α-fibrinogenase homologue from RVV

=

RVBF	=	β- fibrinogenase homologue from RVV
RVV	=	Russell's viper venom
RVV-V	=	factor V activator from RVV
SDS-PAGE	=	sodium dodecyl sulfate-polyacylamide gel electrophoresis
S.N.A.P. column	=	small nucleic acid purification column
Tris	=	tris-(hydroxymethyl)-aminomethane
μg	=	microgram
μl	=	microliter
UV	=	ultraviolet
V	=	volts

CHAPTER I

INTRODUCTION

1. Background and Rationale

Snake venoms are complex mixtures molecules with various biological activities, which are used for capturing digesting prey; however, these same molecules could have therapeutic value once characterized and cloned. Snake venoms contain various enzymes such as phospholipase A2, metalloproteinases, serine proteases and L-amino acid oxidases, and non enzymatic peptides including C-types lectins and disintegrins (Cidade et al., 2006; Jia et al., 2008; Nuchprayoon et al., 2001; Sai-Ngam et al., 2008).

Venomous snake bite is an important medical problem in Southeast Asia, including Thailand. *Daboia russelli* or Russell's viper (RV) is a medically important snake in many Southeast Asian countries including Thailand, Myanmar, India, Sri Lanka, China, Taiwan and Indonesia (Chanhome et al., 1998; Warrell, 1989). It is the major cause of snake bite morbidity and mortality in the central rice-growing area of Thailand. Bitten by the *Daboia Russelli siamensis*, the subspecies found in Thailand, is an occupational hazard of rice farmers. RV envenomation various symptoms including edema, pain, thrombocytopenia with increased risk of systemic bleeding from disseminated intravascular coagulation (DIC) and severe reduction of coagulation factor V, X and XIII (Mitrakul, 1979; Warrell, 1989).

As observed in other snake venoms, the RV venom is a mixture of several poisonous components, including phospholipase A_2, which could be at least seven isoenzymes, L-amino oxidase, endonuclease, phosphodiesterase, 5'-nucleotidase, phosphomonoesterase, paraoxonase, hyaluronidase, and a variety of proteinases (Dennis, 1994; Pukrittayakamee et al., 1988). Recent studies of Russell's viper venom gland cDNA indicates that phospholipase A_2 isoforms were predominantly expressed (Nuchprayoon et al., 2001; Sai-Ngam et al., 2008). Two well-known procoagulant enzymes found in the Russell's viper venom have been characterized: factor X activator (RVV-X), a type IV metalloproteinase, and factor V activator (RVV-V), a single chain serine proteinase, (Furie and Furie, 1976; Kisiel, 1979; Kisiel et al., 1976; Tokunaga et al., 1988). According to its biochemical properties and amino acid sequence, RVV-V has been grouped in a serine proteinase family (Kisiel, 1979; Tokunaga et al., 1988).

Serine protease is a family of proteases in which one of the "catalytic triad" active site is serine, histidine and aspartic acid (Hedstrom, 2002b). They are widely found in eukaryotes, prokaryotes, archae, and viruses (Hedstrom, 2002b). In snake, they are present in venoms of the families Viperidae, Crotalidae, Elapidae and Colubridae. Snake venom serine proteinases show stringent macromolecular substrate specificity that contrasts with the less specific activity of trypsin (Serrano and Maroun, 2005). Although the amino acid sequences of snake venom serine proteases share high similarity, these venom proteases exhibit different substrate specificity and function (Serrano and Maroun, 2005). A number of hemostasis-affecting snake venom serine proteases have been reported, including procoagulant, anticoagulant, platelet

aggregating- and fibrinolytic proteases (Kini, 2005; Matsui et al., 2000; Serrano and Maroun, 2005).

RVV-V is a 29 kDa single chain serine proteinase, consisting of 236 amino acid residues and 6% carbohydrate (Kisiel, 1979; Tokunaga et al., 1988). RVV-V specifically cleaves the single peptide bond between Arg1545 and Ser1546, resulting in activation of factor V, a key component of the haemostatic system which acts as a co-factor in prothrombinase complex (Kalafatis et al., 2003; Keller et al., 1995; Rosing et al., 2001). Factor Va accelerates factor X-catalyzed prothrombin conversion by 300,000-fold (Mann and Kalafatis, 2003). The amino acid sequence of RVV-V has been reported (Tokunaga et al., 1988) and Crystallized (Nakayama et al., 2009). RVV-V was also observed in a proteomic study of Russell's viper venom (Risch et al., 2009).

To understand pathogenesis and improve the therapy of RV bite, characterization in both biochemistry and molecular levels of the venom components is required. The aims of this study were to clone the RVV-V cDNA and analyze its nucleotide and amino acid sequences compared with other snake venom serine proteinases. The effort to produce functional recombinant RVV-V also performed on *E. coli* and yeast expression systems.

2. Objectives

1) To obtain and characterize the RVV-V cDNA which has not been reported
2) To obtain novel cDNA encoding serine proteinases from RVV gland transcripts
3) To produce recombinant RVV-V by genetic engineering approaches using *E. coli* and yeast (*Pichia pastoris*)

3. Keywords

Snake venom

Russell's viper

Factor V activator

Phylogenetic analysis

4. Expected benefits and applications

Obtaining of RVV-V cDNA provide more information of the RVV instead of knowing only the amino acid sequence. In addition, acquiring the novel RVV serine proteinases cDNA can improve knowledge about pathogenesis of RVV bite. Analysis of the nucleotide/amino acid sequences of the RVV-V and other snake venom serine proteinases would provide more information of molecular biology of snake venom. Study of snake venom toxins, by bioinformatics, molecular biology and biochemistry approaches, may lead to the better treatment of RV bite, as well as the discovery of novel medical useful agents. Active rRVV-V may be applied in medical used, e.g. in laboratory diagnostic in hematology. Further developed strategies are required for production of the functional rRVV-V.

CHARTER II

LITERATURE REVIEWS

1. Snake venom and hemostasis

1.1 Snake venom

Snake venoms are complex mixtures of proteins, including enzymes and other biologically active components, which cause variety of symptoms after snake bite. Many venom enzymes have been found, such as phospholipase A2, metalloproteinases, serine proteases and L-amino acid oxidases, as well as non enzymatic proteins including neurotoxins, C-types lectins, disintegrins and bradykinin-potentiating peptides (Cidade et al., 2006; Jia et al., 2008; Junqueira-de-Azevedo and Ho, 2002; Nuchprayoon et al., 2001). Because not all of these toxins are presented in each snake, various pathogenesis were found depend on type of the snake such as paralysis, systemic myolysis, coagulopathy and haemorrhage, renal damage and failure, cardiotoxicity and local tissue injury at the bite site. The symptoms suggest that snake venoms affect various systems, particularly the central nervous system (CNS), cardiovascular system, muscular and haemostatic system (Koh et al., 2006). Some toxins have multiple effects. However, the function of some components is still unclear. For example, "nerve growth factor", found in cobra venom, plays a major role in the growth of nerve tissue (Kostiza and Meier, 1996). Why this

molecule is present at high concentration in venom in the first place remains an open question. It has been proposed that this toxin promotes the absorption of venom by releasing various mediators from mastocytes. The common toxins found in snake venoms and their actions were shown in Table 1.

Table 1. Common toxins in snake venom (Ernst and Zug, 1996; Koh et al., 2006)

Compounds	Action	Snakes genera
Hyaluronidases	Catalyze reactions that break mucopolysaccharide links in connective tissues, thereby enhancing diffusion of venom	Several genera
Proteolytic enzymes	Catalyze the breakdown of structural components of tissues.	All venomous species
Phospholipases	Catalyze reactions that harm musculature and nerves	Almost all venomous species
Thrombin-like enzymes	Inhibit blood clotting	Vipers, pit vipers, and a few rare elapids
Neurotoxins	Disrupts nerve-impulse transmission, causing heart or respiratory failure	Mambas, *Vipera, Crotalus, Bungarus, Naja, Laticaua, Hydrophis* all with different types of toxin.
Myotoxins	bind specifically to the sarcoplasmic reticulum of muscles, leading to swelling and disintegration of both the sarcoplasmic reticulum and muscle fibrils	Rattlesnakes and other pit vipers
Nerve growth factors	Stimulates the growth of nerve cells	*Agkistrodon, Crotalus*
Disintegrins	Inhibit various integrins by recognition motifs. The well-known RGD motif binds the GPIIb/IIIa receptor and other integrins on platelet, blocking platelet functions.	Vipers and pit vipers
C-type lectin-like proteins	Bind to a wide range of coagulation factors and to platelet receptors, display both anti-coagulant-and platelet-modulating activities	Elapidae and Viperidae

1.2 Blood coagulation

Blood coagulation acts as an important defense mechanism against bleeding, classified into intrinsic and extrinsic pathways depend on initiating factors (Dahlbäck, 2000). Reactions of blood coagulation cascade are progressed by many coagulation factors comprised of enzymes and cofactors those are transcribed as zymogens and procofactor respectively, required proteolyses to have activities (Butenas and Mann, 2002). After initiated, both pathways similarly activate prothrombinase, a complex enzyme involved in common coagulation pathway. This complex contains factor Xa (active factor X), factor Va, calcium ion and membrane phospholipids, and functions as an activator of prothrombin to promote formation of thrombin, a key enzyme of coagulation system that convert fibrinogen to fibrin network. Many snake venoms have been found to have procoagulant activities because they can activate many coagulation factors in haemostasis system (Koh et al., 2006; Markland, 1998; Matsui et al., 2000) (Fig 1). Consequently, consumption of coagulation factors by these venoms results in clinical anticoagulation and bleeding (White, 2005).

1.3 Snake venom proteins affecting hemostatic system

The venoms of *Viperidae* and *Crotalidae* snakes contain a large variety of proteins and peptides affecting the hemostatic system. The major symptoms from snake bite affecting the haemostatic system are (i) reduced coagulability of blood, resulting in an increased tendency to bleed, (ii) bleeding due to the damage to blood vessels, (iii) secondary effects of increased bleeding, ranging from hypovolaemic shock to secondary-organ damage, such as intracerebral haemorrhage, anterior

pituitary haemorrhage or renal damage, and (iv) direct pathologic thrombosis and its sequelae, particularly pulmonary embolism (Koh et al., 2006; Numeric et al., 2002). Venoms from vipers and some Australian snakes are rich sources of proteases that strongly affect the haemostatic mechanism (White, 2005). These proteins may be classified as coagulant, anticoagulant or fibrinolytic factors (Braud et al., 2000; Markland, 1998). Coagulant enzymes include the thrombin-like enzymes and the activators of the blood coagulation factors II (prothrombin), V and X. Anti-coagulants include protein C activators, inhibitors of prothrombin complex formation and phospholipase A_2s. Fibrinolytic factors include fibrin(ogen) degradation enzymes and plasminogen activators. However, the thrombin-like enzymes are intermediates between the true coagulants and true anticoagulants because their activity is clotting *in vitro* but defibrination (anticoagulation) *in vivo*. Moreover, snake venom proteins, including the disintegrins and C-type lectin like proteins, also affect platelets by inducing or inhibiting platelet aggregation (McLane et al., 1998; Wijeyewickrema et al., 2005). Snake venom proteins that affect the blood coagulation cascade are summarized in Figure 1. Various toxin families involved in the hemostatic system have been reported (Braud et al., 2000; Koh et al., 2006; Markland, 1998). These activators or inhibitors include serine proteases, metalloproteinases, C-type lectins, disintegrins, and phospholipases.

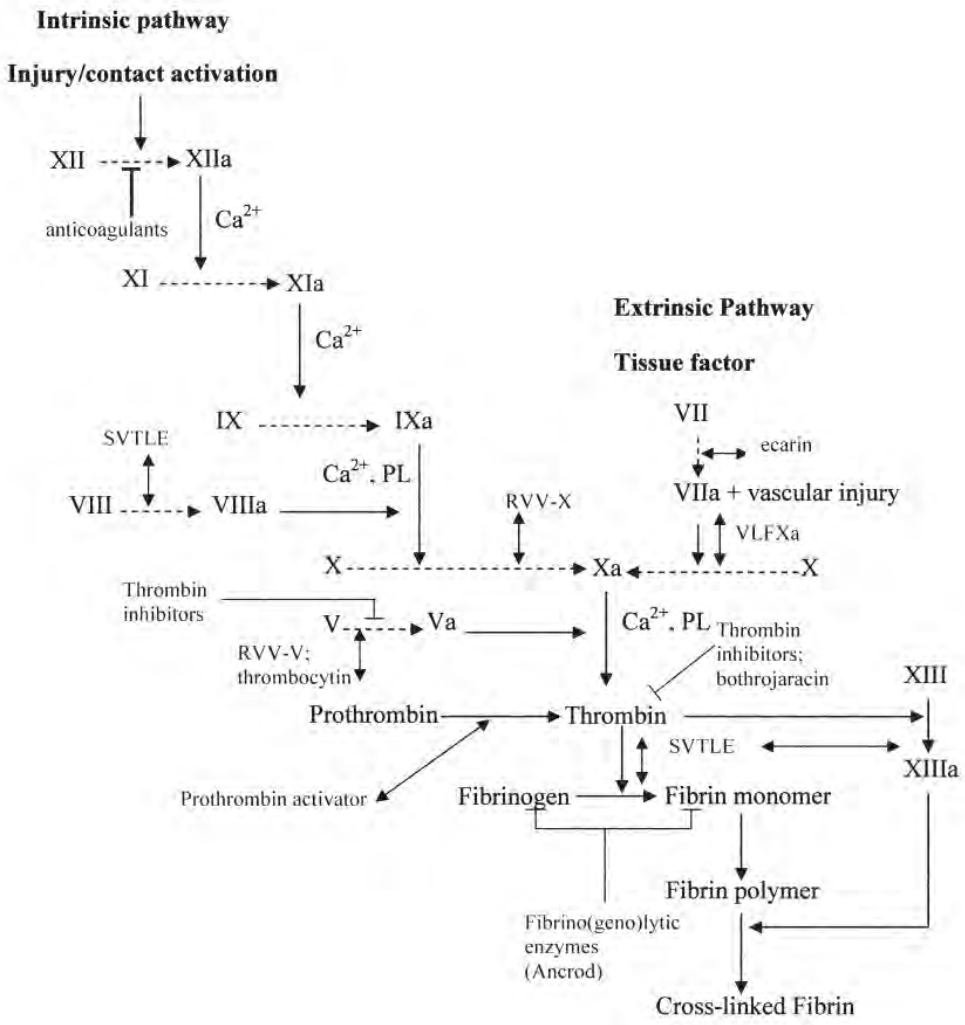


Figure 1. Blood coagulation pathways and the steps in which snake venom proteins interfere. SVTLE, snake venom thrombin-like enzymes; RVV-V, Russell's viper venom factor V activator; RVV-X, Russell's viper venom factor X activator; VLFXa, Vipera lebetina factor X activator. Activation by venom protein is denoted by ↔ , inhibition by |−−−−− (Koh et al., 2006).

1.4 Snake venom serine proteinases

Serine protease is a family of proteases which contains the "catalytic triad" active site: highly reactive serine, histidine and aspartic acid (Hedstrom, 2002b). They belong to the trypsin family S1 of clan SA, the largest family of peptidases (Halfon and Craik, 1998). They are widely found in eukaryotes, prokaryotes, archaea, and viruses (Hedstrom, 2002b). In snake, they are present in venoms of the families Viperidae, Crotalidae, Elapidae and Colubridae.

Snake venom serine proteinases (SVSPs) are defined by a common catalytic mechanism that includes a highly reactive serine residue that plays a key role of the active site with the presence of histidine and aspartic acid residues within (Hedstrom, 2002b; Serrano and Maroun, 2005). Therefore, they are sensitive to the serine-modifying reagents phenylmethylsulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP). SVSPs are glycoproteins. A variable number of N- or O-glycosylation sites in amino acid sequence that differ from one SVSP to the other are observed (Serrano and Maroun, 2005; Yamazaki and Morita, 2007). They contain twelve conserved cysteine residues which form six disulfide bonds, in which one disulfide bridge is unique among SVSPs.

Snake venom serine proteinases show stringent macromolecular substrate specificity that contrasts with the less specific activity of trypsin (Serrano and Maroun, 2005). Although the amino acid sequences of snake venom serine proteases share high similarity, these venom proteases exhibit different substrate specificity and function (Serrano and Maroun, 2005). A number of hemostasis-affecting snake venom serine proteases have been reported, including procoagulant, anticoagulant, platelet

aggregating- and fibrinolytic proteases (Kini, 2005; Matsui et al., 2000; Serrano and Maroun, 2005). They are not lethal by themselves, but they contribute to the toxic effect of the venom when associated with other venom proteins (Braud et al., 2000).

2. Russell's viper venom

Russell's viper (RV), *Daboia russelli*, is a medically important venomous snake widely distributed in East and Southeast Asia. Its body is stout, light brown background with a series of multicolor blotches. These blotches vary in shape and size, are dark brown with black inner and white outer edges. Head is distinctly triangular covered with small-keeled scales. At least 5 subspecies of RV have been classified according to minor difference in color and markings: *D. r. russelli* in India, *D. r. pulchella* in Sri Lanka, *D. r. siamensis* in Myanmar, Thailand, and China, D. *r. formosensis* in Taiwan, and *D. r. limitis* in Indonesia (Warrell, 1989) (Fig. 2). Some of the most important symptoms of Daboia russelli bites are ascribed to the phospolipase A_2 which induces neurotoxicity, myotoxicity, edema, and hemorrhage (Carredano et al., 1998; Uma and Veerabasappa Gowda, 2000). In Thailand, Russell's viper (*Daboia russelli siamensis*) (Fig. 3) is found throughout the central rice-growing area. Bitten by these snakes is an occupational hazard of rice farmers responsibly for morbidity in the area (Chanhome et al., 1998; Warrell, 1989).

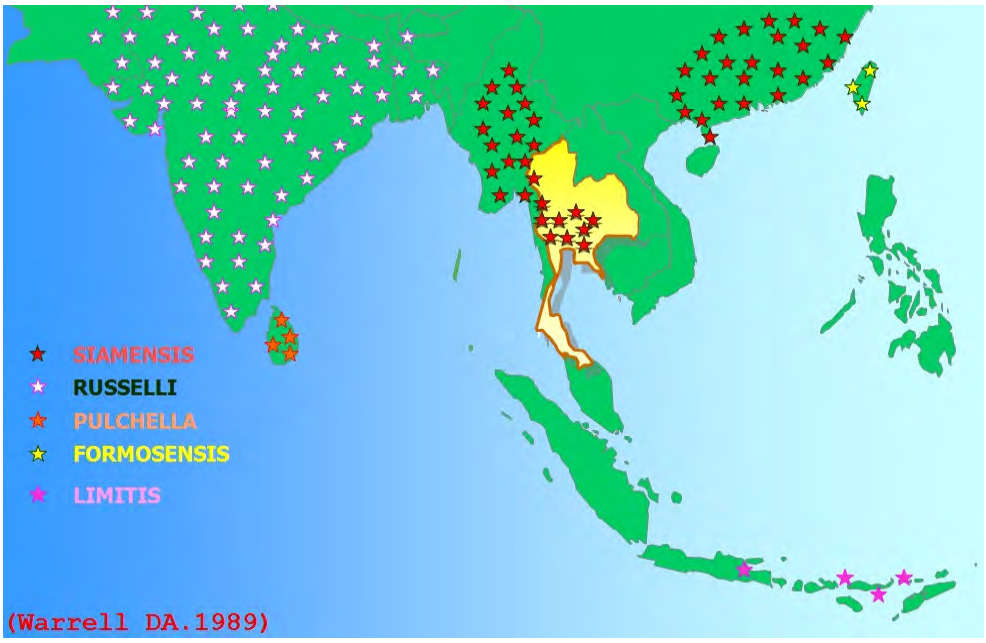


Figure 2. Distribution of Russell's vipers (modified from Warrell, 1989)

Figure 3. Morphology of Russell's viper in Thailand.

Signs and symptoms of RV bite include disseminated intravascular coagulation (DIC), haemolysis, rhabdomyolysis, neurotoxicity, platelet damage, oedema formation, vasodilatation causing hypotention and release of autacoids such as histamine, etc (Kini and Iwanaga, 1986; Slater et al., 1988). One of the common clinical manifestations is incoagulable blood associated with severe reduction of coagulation factor V, X and XIIIA (Mitrakul, 1979; Warrell, 1989). However, There are some genetic variations among RVs from different geographical locations, lead to the differences in venom composition and cause broad symptoms or effects to prey (Warrell, 1989) (Table 2).

Table 2. Geographical variation in the clinical manifestations of Russell's viper bite (Warrell, 1989)

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

"+" represents detectable symptom.

"–" indicates the absence of detectable symptom.

"?" indicates no clinical data.

2.1 Enzymes and peptides in Russell's viper venom

RV venom is a mixture of enzymes and peptides. A variety of phospholipase A_2s, L-amino oxidase, endonuclease, phosphodiesterase, 5'-nucleotidase, phosphomonoesterase, paraoxonase, hyaluronidase, and a number of proteinases were observed (Dennis, 1994; Pukrittayakamee et al., 1988; Warrell, 1989). However, RV venoms from different geographical regions and subspecies often exhibit different pharmacological or pathological and antigenic properties. This is because of the difference in the compositions between subspecies. For example, a potent heterodimeric PLA2 neurotoxin, Russtoxin, was found in the venoms of almost Russell's vipers except that of *D. r. pulchella* from Sri Lanka and South India (Tsai et al., 1996). Furthermore, RV venoms from different regions of India also exhibited variation in ion-exchange chromatography profile and SDS-PAGE pattern (Prasad et al., 1999).

Recent studies of Russell's viper (*D. r. siamensis*) venom gland transcriptome indicated that phospholipase A_2 isoforms were predominantly expressed (Nuchprayoon et al., 2001; Sai-Ngam et al., 2008). As shown in a proteomic study of *D. r. siamensis* from Myanmar, RV venom comprised toxins from six protein families: serine proteinases, metalloproteinases, phospholipases A_2, L-amino acid oxidases, vascular endothelial growth factors and C-type lectin-like proteins (Risch et al., 2009). Two potent procoagulant enzymes in RV venom have been characterized: factor X activator (RVV-X) and factor V activator (RVV-V) (Furie and Furie, 1976). RVV-X is a well-known 93 kDa P-IV snake venom metalloproteinase comprising of a heavy chain and two light chains (Gowda et al., 1994; Kisiel et al., 1976).

2.2 Russell's viper factor V activator (RVV-V)

RVV-V is a 29 kDa single chain serine proteinase, consisting of 236 amino acid residues and 6% carbohydrate (Kisiel, 1979; Tokunaga et al., 1988). Similarly to the other snake venom serine proteinases, the venom protein exhibits three highly conserved amino acids residues- His44, Asp88 and Ser182, the "catalytic triad" relied on the active site of the enzyme, and twelve cysteins which form six disulfide bonds to stabilize tertiary structure of the enzyme. It also contains the putative site for N-glycosylation (Tokunaga et al., 1988). Three RVV-V isoproteins, namely RVV-Vα, RVV-Vβ and RVV-Vγ, were purified by HPLC in a weight ratio 2:1:6 and sequenced. Unfortunately, the difference in their activity is unknown because they are separable only under denaturing condition (Tokunaga et al., 1988). Crystallization of RVV-V were reported (Nakayama et al., 2009). RVV-V was also observed in a proteomic study of Russell's viper venom (Risch et al., 2009).

Activation of coagulation factor V by RVV-V is different from that by thrombin. In normal physiological process, the 330 kDa coagulation factor V is activated by thrombin-cleaved at Arg709, Arg1018 and Arg1545 to remove internal activation domain and generate the active factor V (Va) comprised of 105 kDa heavy chain and 71/74 kDa light chain that accelerates factor X-catalyzed prothrombin conversion by 300,000-fold (Mann and Kalafatis, 2003). On the other hand, RVV-V specifically activates coagulation factor V by cleavage only at Arg1018 and Arg1545, producing factor Va consisting of two fragments of 150 kDa and 71/74 kDa (Fig 4) (Keller et al., 1995; Segers et al., 2006). This RVV-activated factor Va has coagulant activity similarly to that activated by thrombin (Kalafatis et al., 2003; Keller et al.,

1995). Therefore, activation of coagulation factor V by RVV-V plays important roles in pathogenesis of Russell's viper bite because of enhancing activity of RVV-X-activated factor Xa and meanwhile reducing the level of factor V. RVV-V activity is inhibited by diisopropylfluorophosphate (DFP), similarly to the other serine proteinases, but not by antithrombin III (Kisiel, 1979; Segers et al., 2006).

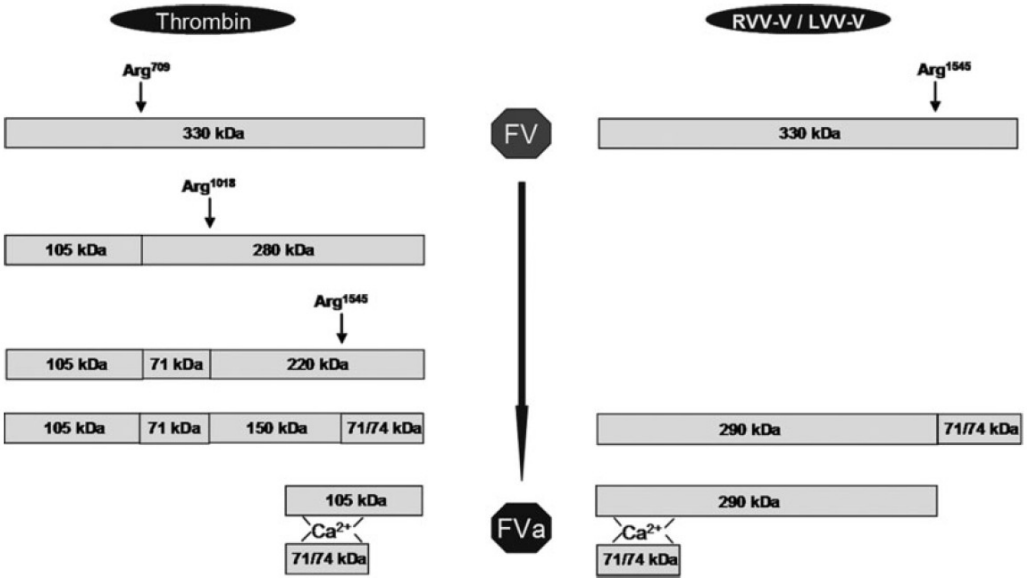


Figure 4. Activation of human FV by thrombin and the snake venom FV activators RVV-V and LVV-V (Segers et al., 2006). Thrombin-catalyzed FV activation proceeds via three sequential cleavages at Arg709, Arg1018, and Arg1545. The resulting activated FV molecule (FVa) is a heterodimeric protein consisting of a light chain doublet (71/74 kDa) and a heavy chain (105 kDa). The snake venom FV activators from *Daboia russelli* and *Daboia lebetina* cleave FV at Arg1545, giving rise to a FVa molecule with a light chain of 71/74 kDa and a heavy chain of around 290 kDa.

In addition to RVV-V, another serine proteinase factor V activator from *Vipera lebetina* (VLFVA), was isolated and characterized (Siigur et al., 1999; Siigur et al., 1998). *Vipera lebetina* is classified in the same subfamily with *D. russelli*. Complementary DNA (cDNA) sequence indicated that mature protein-encoding region of VLFVA is 84% homology with the amino acid sequence of RVV-V and showed the particular features such as the catalytic triad, 12 conserved cysteins and N-glycosylation site. Furthermore, the pre- and proprotein-encoding regions were represented, suggesting that this protein is a zymogen that requires proteolytic activation to become the functional enzyme. Owing to high degree of conservation between snake venom serine proteinases in spite of variation among their substrate specificity (Serrano and Maroun, 2005), the polyclonal antibody against from VLFVA-immunized mouse have cross-reacted with venom components of several snakes including Russell's viper (Siigur et al., 2000).

3. Production of recombinant proteins in microorganisms

3.1 *Escherichia coli* expression system

Prokaryotic expression systems, particularly *E. coli*, have been exploited for the production of a variety of therapeutic proteins, on an industrial scale. Prokaryotic cells (*E. coli*) are normally the preferred host for the expression of heterologous proteins because they offer (a) inexpensive carbon source requirements for growth, (b) rapid biomass accumulation, (c) capability for high-cell density fermentation, and (d) simple process scale up (Berrow et al., 2006; Sahdev et al., 2008). However, lack of post-translational machinery and production of inactive protein due to the

formation of inclusion bodies, offer a significant challenge in these expression systems. Strategies to improve productivity of functional recombinant protein have been developed, including co-expression of molecular chaperones (Haacke et al., 2009; Sahdev et al., 2008). Molecular chaperones are proteins adapted to assist *de novo* protein folding and facilitate expressed polypeptide's proper conformation attainment without becoming a part of the final structure (Hartl, 1996). It has been shown that chaperones helped in recombinant protein refolding and involved in preventing protein aggregation (Haacke et al., 2009; Nishihara et al., 2000; Xu et al., 2005).

3.2 *Pichia pastoris* expression system

Pichia pastoris is a species of methylotrophic yeast. Pichia yeast is widely used for protein expression using recombinant DNA techniques. It is used in biochemical and genetic research in academia and the biotechnical industry (Cereghino and Cregg, 2000). *P. pastoris* expression systems offer significant advantages over E. coli expression systems for the production of many heterologous eukaryotic proteins that require post-translational modifications including disulfide bond and glycosylation (Daly and Hearn, 2005). *P. pastoris* also has a high growth rate, and it is able to grow on a simple and inexpensive medium. In addition, Pichia is suitable for both small and large scale production in fermentor.

P. pastoris has two alcohol oxidase genes, AOX1 and AOX2, which have a strongly inducible promoter. These genes allow *P. pastoris* to use methanol as a carbon and energy source. Most *P. pastoris* expression systems use the methanol-

induced alcohol oxidase (AOX1) promoter, since the fraction of total soluble protein that is composed of alcohol oxidase can typically rise to 30% upon induction by methanol (Li et al., 2007). Some expression vectors also include the secretion signal of the α-mating factor from *Saccharomyces cerevisiae*, resulting in secretion of the recombinant protein into the growth medium. This greatly facilitates subsequent protein purification. Numerous strains of *P. pastoris* with a wide range of expression vectors have been used to express many heterologous snake toxins (Raveendra Anangi et al., 2007).

CHAPTER III

MATERIALS AND METHODS

1 Materials

1.1 cDNA library of Russell's viper venom glands

The Russell's viper venom gland mRNA and cDNA library were obtained from previous study (Nuchprayoon et al., 2001).

1.2 5' Rapid Amplification of cDNA Ends (5' RACE)

5' RACE System kit was purchased from Invitrogen, CA, USA. This kit requires three gene specific primers (GSP).

1.3 Synthetic oligonucleotides

Oligonucleotides were synthesized from Bio Service Unit, NSTDA, Thailand. The DNA sequences were shown in Table 3.

1.4 DNA sequencing reaction

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

1.5 Restriction endonuclease and modified enzymes

All Restriction endonucleases (EcoRI, XbaI, XhoI, etc.) were purchased from NEB Biolabs. *Taq* DNA polymerase (Promega), T4 DNA ligase (USB)

Table 3. Synthetic oligonucleotide

	Primer name	Sequence (5'–3')	Bases
5′ RACE			
	GSP1	CAT TAC AGA TGA GCG GTC C	19
	GSP2	CCG TGA CAT GTA TCT CTG CCT CC	23
	GSP3	CCA TGG ATA AAG TGG TTC ACA CC	23
Consensus PCR			
	VSP–F	CCG CTT GGG TTA TCT GAT TAG	21
	VSP–R	GCA CCT CAC CCT AAA ACA G	19
pET–32a (E. coli)			
	pET–VF	CCA TGG AGT CGT TGG AGG TGA TG	23
	pET–VR	CTC GAG TCA CGG GGG GCA AG	20
pPicZαA			
	pPic–VF	GAA TTC GTC GTT GGA GGT GAT G	22
	pPic–VR	TCT AGA TCA CGG GGG GCA AG	20
pPinkα–HC			
	pPink–SF	CGG GAG AGT CGT GGA GTC GTT GGA GGT GAT G	31
	pPink–SR	TAT GGC CGG CCT CAC GGG GGG CA	23

1.6 DNA Purification from gel slice

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

1.7 Protein detection

Mini-PROTEAN Electrophoresis Cell was purchased from BIO-RAD Laboratories, USA. Low molecular weight standard was purchased from Phamacia Biotech AB, USA.

1.8 Coomassie Brillient Blue staining

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

1.9 Western blotting

Trans-Blot SD semi-dry electrophoretic transfer cell (BIO-RAD Laboratories, USA) was used for transfer proteins from gel to membrane by electrophoresis. Nitrocellulose membrane (BioTrace NT) was purchased from Pall Gelman Science, USA.

Anti-His (C-terminal) Antibody was purchased from Invitrogen, USA. Peroxidase-Conjugated Rabbit Anti-Mouse Antibody was purchased from DAKO.
3, 3'- diaminobenzidine (DAB) tetrahydrochroride was purchased from BIO BASIC, Inc., Canada.

1.10 Concentration of protein

Amicon® Ultra-15 concentrators were purchased from Millipore, Billerica, MA, USA.

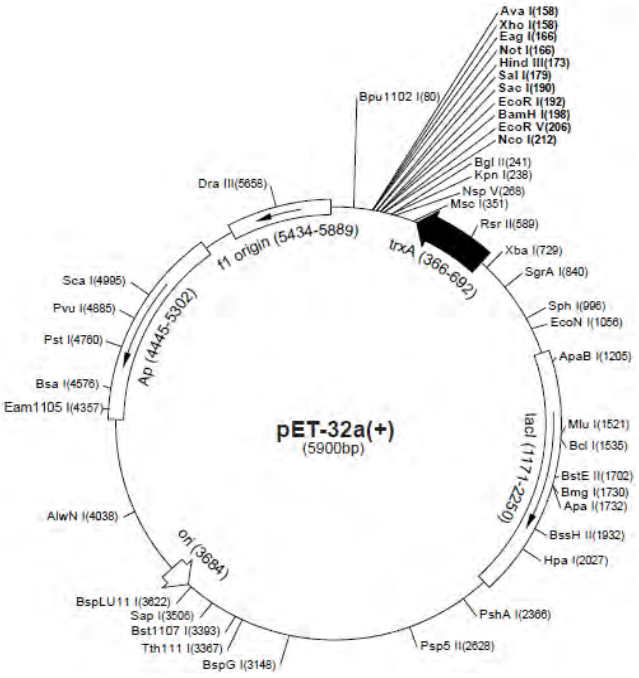
1.11 Purification of His-Tag protein

Purification of the soluble rRVV-V was performed by chromatography with TALON® Metal Affinity Resins (CLONTECH Laboratories, Inc., Mountain View, CA).

1.12 pET-32a

pET32a, an expression vector for *E. coli*, was purchased from Novagen, USA. This vector provides for fusion of the desired protein to thioredoxin (Trx) at N-terminal. Trx enhances the solubility of many target proteinsand catalyzes the formation of disulfide bonds. Its physical feature and restriction map were shown in Figure 5.

A



B

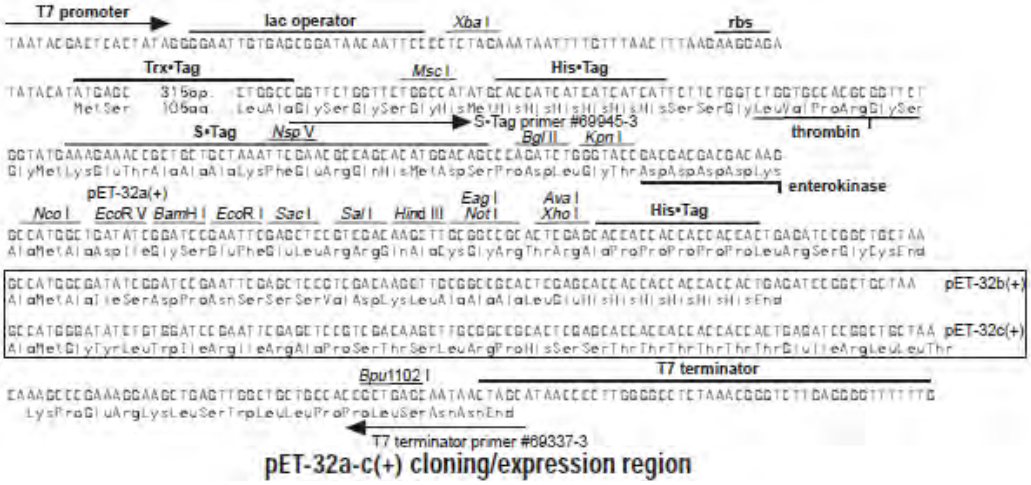
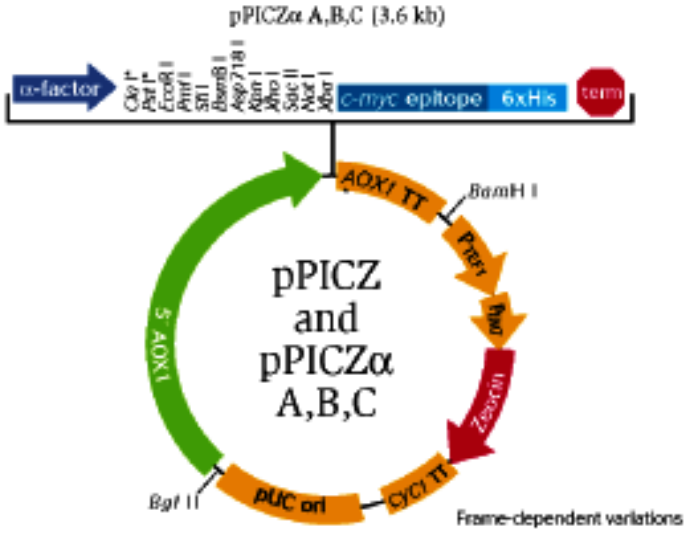


Figure 5. Map and Features of the pET-32a expression vector. (A) pET-32a map, (B) Multiple cloning sites of pPicZα-A. The pictures were obtained from the manual of pET-32a, published by Novagen.

1.13 pPicZα A

pPicZα A, an expression vector for *P. pastoris*, was purchase from Invitrogen, USA (Figure 6). This vector provides α-factor signal sequence for secretion of the recombinant protein. The selectable marker for this vector is Zeocin.

A



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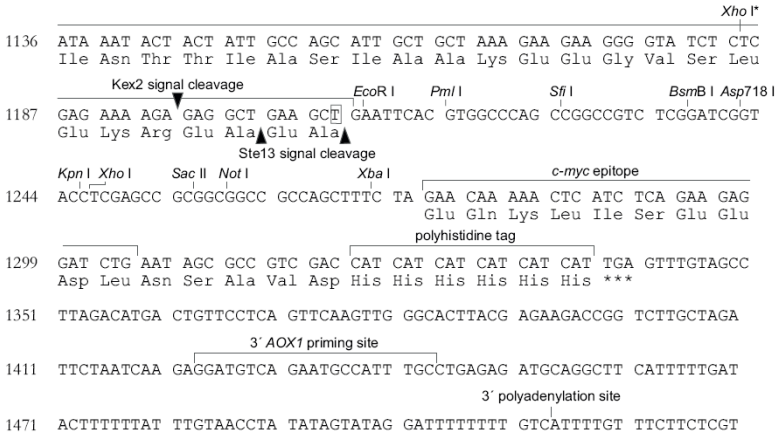
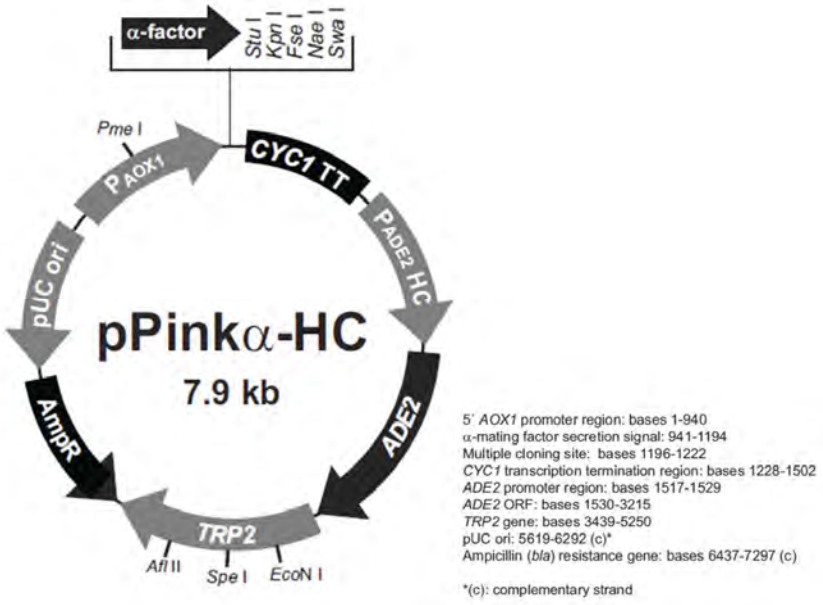


Figure 6. Map and Features of the pPicZα-A expression vector. (A) pPicZα-A map. (B) Multiple cloning sites of pPicZα-A. The pictures were obtained from the manual of pPicZα A, published by Invitrogen.

1.14 pPinkα-HC

pPinkα-HC, an expression vector for *P. pastoris*, was purchased from Invitrogen, USA. Its physical feature and restriction map were shown in Figure 7.

A



B

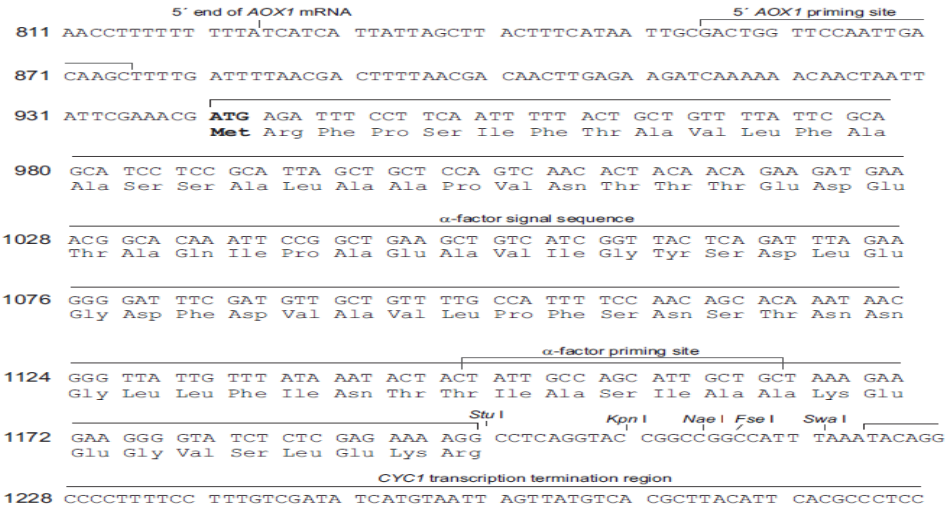


Figure 7. Map and Features of the pPinkα-HC expression vector. (A) pPinkα-HC map. (B) Multiple cloning sites of pPinkα-HC. The pictures were obtained from the manual of pPinkα-HC, published by Invitrogen.

pPinkα-HC provides *Phosphoribosylaminoimidazole carboxylase* gene (*ADE2*) for selection in PichiaPink™ Strains *Pichia pastoris*, whick is a mutant strain that cannot synthesize adenine. Only the pPinkα-HC-transformed PichiaPink™ cells on adenine dropout medium. More than one copy of the DNA can be integrated and the yeast harboring high copy of the DNA can express the *ADE2* gene at high level. Accumulation of the intermediate from adenine synthesis pathway causes pink color while the recombinant clones expressing high *ADE2* become white. Therefore, the yeast clones with high dosage of integrated DNA become white but those with low dosage become pink or red.

1.15 pGEM-T Easy vector

The pGEM-T Easy vectors, purchased from Promega, are linearized vectors with the single 3'-terminal thymidine at both ends, which greatly improve the efficiency of ligation of a PCR product into the plasmids. In addition, the vector contains multiple restriction sites within the MCS. These restriction sites allow for the release of the insert by single-enzyme digestion. Its physical feature and restriction map were shown in Figure 8.

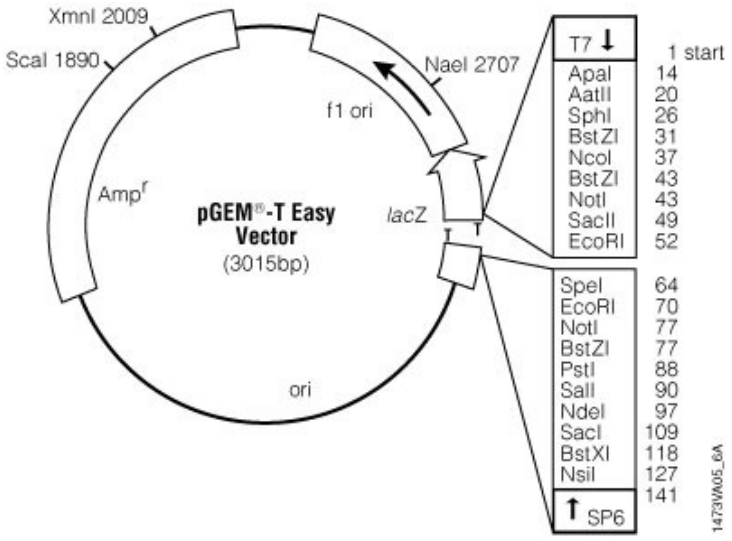


Figure 8. Map of the pGEM®-T vector. The picture was obtained from the manual of pGEM-T easy vector, published by Promega.

1.16 *E. coli* strains

DH5α strain was used for cloning and BL21 strain was used for recombinant protein expression.

1.17 Pichia yeast strain

X-33 was come along with EasySelect Pichia Expression Kit.

1.18 Chemicals

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

1.19 Nucleotide and amino acid sequence of serine proteinase from other snakes

The cDNA of the APL-PA and APL-C were obtained from the cDNA library of *A. p. leucostoma* (Jia et al., 2008). cDNA and amino acid sequences of snake venom serine proteinases included in this study were obtained from GenBank database (Table 4).

Table 4. Snake venom serine proteinases from GenBank database used in this study.

Serine proteinases	GenBank accession number		Species
	Protein	DNA	
VLFVA (factor V activator)	Q9PT41	AF163973.1	*Macrovipera lebetina*
TLG2A (serine proteinase 2A)	O13060	D67082.1	*Trimeresurus gramineus*
Serine protease KN4 homolog	Q71QJ4	AF395763.1	*Viridovipera stejnegeri*
TjsvSPH	B0ZT25	EU400543.1	*Trimeresurus jerdonii*
TLF2	O13057	D67079.1	*Trimeresurus flavoviridis*
Flavoxobin	P05620	D67078.1	*Trimeresurus flavoviridis*
Catroxase II	Q8QHK2	AF227154.1	*Crotalus atrox*
Haly-PA[a]	Q9YGJ8	AF017737.1	*Gloydius blomhoffi brevicaudus*
Serine beta-fibrinogenase (VLBF)	Q8JH62	-	*Macrovipera lebetina*
TLG3 (Venom serine proteinase 3)	O13063	D67085.1	*Trimeresurus gramineus*
Salmobin	O73800	AF056033.1	*Gloydius halys*
TLF3	O13058	D67080.1	*Trimeresurus flavoviridis*
LV-PA [a]	Q27J47	DQ396477.1	*Lachesis muta muta*
TSV-PA [a]	Q91516	U21903.1	*Viridovipera stejnegeri*
Acutobin	Q9I8X2	AF159057.1	*Deinagkistrodon acutus*
TLG2C	O13062	D67084.1	*Trimeresurus gramineus*
PTLE1	Q802F0	AY225505.1	*Gloydius halys*
CPI-enzyme 2	O42207	AF018568.1	*Gloydius ussuriensis*
KN-BJ 2	O13069	AB004067.1	*Bothrops jararaca*
Dav-KN	Q9I8X0	AF159059.1	*Deinagkistrodon acutus*
PA-BJ [a]	P81824	-	*Bothrops jararaca*
Serine alpha-fibrinogenase (VLAF)	Q8JH85	AF528193.1	*Macrovipera lebetina*
ACC-C (protein C activator)	P09872	-	*Agkistrodon contortrix contortrix*
Dav-X	Q9I8W9	AF159060.1	*Deinagkistrodon acutus*
Elegaxobin I	P84788	-	*Protobothrops elegans*
Ancrod	P47797	L07308.1	*Calloselasma rhodostoma*
Contortrixobin	P82981	-	*Agkistrodon contortrix contortrix*
LM-TL	P33589	S35689	*Lachesis muta muta*
Acutin	Q9YGS1	AF089847.1	*Deinagkistrodon acutus*
Ohs1	ABN72544	EF080837	*Ophiophagus hannah*
Nasp	ABN72541.1	EF080834	*Naja atra*
BmSP	ABN72545	EF080838	*Bungarus multicinctus*

[a]PA: plasminogen activator

2 Methods

2.1 5'-Rapid Amplification of cDNA Ends (5'-RACE)

To obtain the 5' end nucleotide sequence of RVV-V, the 5'-end amplification of cDNA was performed using the 5'-RACE System kit (Invitrogen, CA, USA) according to the manufacturer's manual (Fig 9). Three gene specific primers (GSP), as shown in Table 3, were designed from RVV cDNA library.

2.1.1 First Strand cDNA Synthesis from Total RNA

The single strand cDNAs were reverse transcribed from 5 µg of Russell's viper gland mRNA using GSP1. mRNA was mixed with 2.5 pmoles of GSP1 and DEPC-treated water then in the mixture was incubated at 10 min at 70°C. Subsequently, the mixture of 10X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP mix, and 0.1 M DTT was added. After Incubation for 1 min at 42°C, SuperScript™ II RT was added and incubation for 50 min at 42°C was performed. The mixture was then incubateed at 70°C for 15 min to terminate the reaction. RNase mix was added and incubation was done for for 30 min at 37°C. Final composition of the reaction is 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 100 nM cDNA primer (GSP1), 400 µM each dATP, dCTP, dGTP, dTTP, and 200 units SuperScript™ II RT.

2.1.2 S.N.A.P. Column Purification of cDNA

Binding solution (6 M NaI) was added to the first strand reaction product from 2.1.1. The cDNA/NaI solution was then transfered to the Small Nucleic Acid

Purification column (S.N.A.P. column) and centrifuged at 13,000 x g for 20 s. After washed by 1X washing buffer (4 times) and 70% ethanol, the cDNA was eluted by sterilized distilled water.

2.1.3 TdT Tailing of cDNA

After RNA digestion and purification, the cDNAs were tailed with poly-cytosine (poly-C) at the 5' terminal by in 10 mM Tris-HCl (pH 8.4) containing 25 mM KCl, 1.5 mM $MgCl_2$ and 200 μM dCTP.

2.1.4 PCR of dC-tailed cDNA

Tailed cDNA obtained from the preceding protocol was amplified directly by PCR. Final composition of the reaction includes 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 400 nM GSP2, 400 nM Abridged Anchor Primer, 200 μM each dATP, dCTP, dGTP, dTTP, tailed cDNA, and 2.5 units Taq DNA polymerase. A 35 cycles of PCR was performed: 94°C for 2 min, 30 cycles of 94°C for 1 min, 55°C for 0.5-1 min and 72°C for 1-2 min, followed by Final extension of 72°C, 7 min.

2.1.5 Nested Amplification

PCR product from previous step was 100-fold diluted in TE buffer [10 mM Tris-HCl, (pH 8.0), 1 mM EDTA]. The diluted 1st PCR product then was used in 2nd PCR reaction. Final composition of the 2nd PCR is 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 nM nested GSP, 200 nM universal amplification primer (UAP), 200 μM each dATP, dCTP, dGTP, dTTP and 2.5 units Taq DNA polymerase. A 35 cycles of PCR was performed: 94°C for 2 min, 30 cycles of 94°C for 1 min,

55°C for 0.5-1 min and 72°C for 1-2 min, followed by Final extension of 72°C, 7 min. PCR product was analyzed by agarose gel electrophoresis.

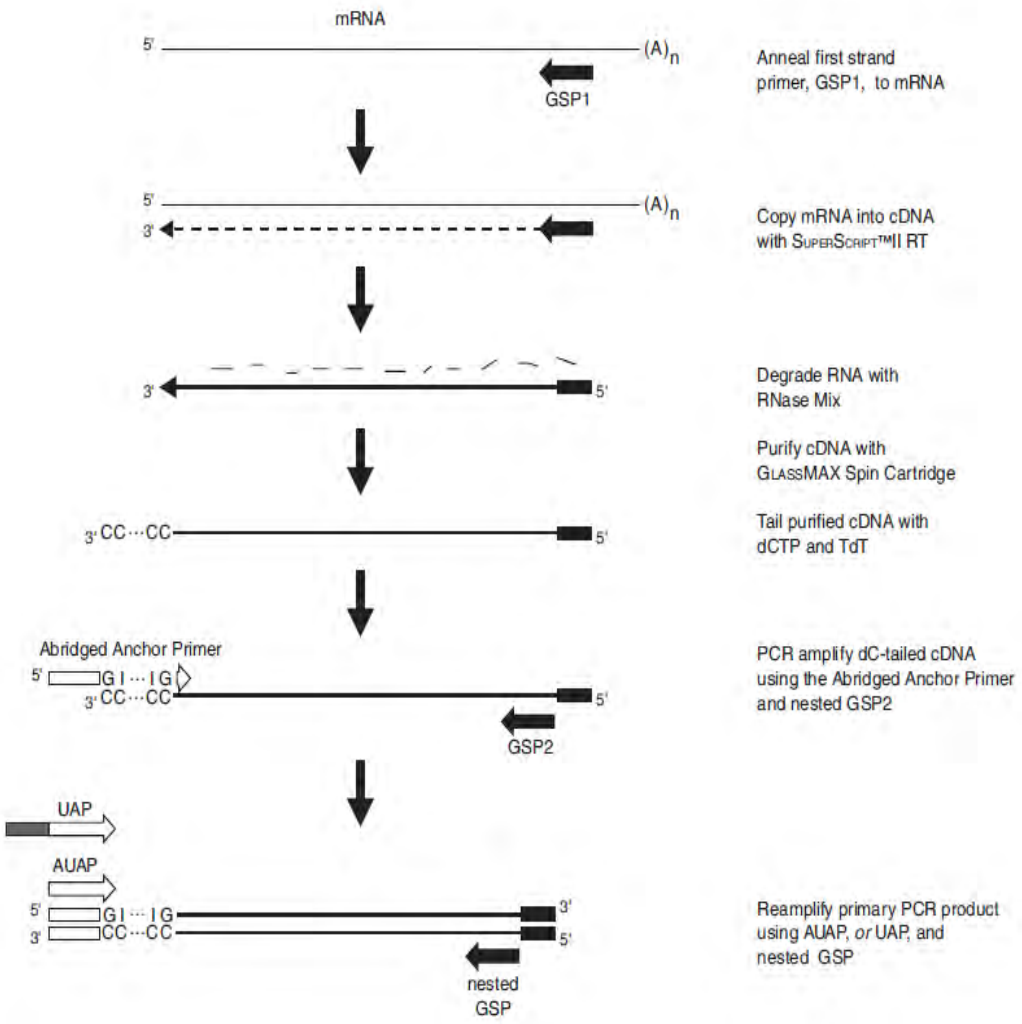


Figure 9. Overview of the 5' RACE Procedure. The picture was obtained from the manual of 5' RACE system kit, published by Invitrogen.

2.2 Ligation of PCR Products into Plasmid Vector

The amplified cDNA fragments were purified from gel slice by QIAquick® Gel Extraction Kit, ligation was proceeded under as follows. The ligation reaction was carried out in a 10 µl reaction mixture containing pGEM-T easy vector and DNA insert in the molar ratio 1:3, 2 units of T4 DNA ligase and 1X buffer. An appropriate amount of sterile water was added to make the 10 µl final volume. The amount of the DNA insert was calculated from the following equation:

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

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

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

A single colony of E. coli was inoculated into 3 ml of LB broth and incubated at 37°C with 200 rpm shaking for 16-20 hrs. The overnight culture was diluted 1:100 into 100 ml of new LB broth and incubated at 37°C until an OD600 of 0.4-0.5. The cell culture was chilled on ice for 10 min prior to pipetting the 10 ml aliquots of the culture into 15 ml sterile polypropylene tubes. The cells were pelleted by centrifugation at 4,000 rpm for 10 min at 4°C. After that, the pellet was suspended in

5 ml of ice-cold 0.1 M $MgCl_2$, centrifuged, resuspended in 5 ml of ice-cold 0.1 M CaCl2 and left on ice for 30 min to establish competency. Finally, after centrifugation, the pellet was resuspended in 750 µl of 15% v/v glycerol and 0.1 M $CaCl_2$. The cells were kept in 200 µl aliquots at -80 µC until required.

2.4 Transformation of *E. coli* Competent Cells

E. coli DH5-α competent cells were mixed with 2 µl of ligation products and immediately placed on ice for 30 min. The cells were subjected to heat-shock at 42°C for 45 seconds and placed on ice for an additional 3 minutes. The transformed cells were mixed with 800 µl of LB broth and incubated at 37°C for 1 hour with shaking at 200 rpm. Finally, 100 µl of the transformed culture was spread on a LB agar plate containing 50 µg/ml ampicillin, pre-spreaded with 10 µl of 1M IPTG, and 20 µl of 5mg/ml X-Gal. The plate was incubated at 37°C overnight. White colonies containing the inserts were selected and grown in LB broth. The recombinant plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen) and cut with *EcoR*I to confirm the presence of recombinant plasmids. The plasmids were sequenced in both directions to confirm that the *wsp* gene represented correct orientation.

2.5 Plasmid preparation and DNA sequencing

Individual colonies were randomly picked from the Luria-Bertani (LB) with ampicilin plates and inoculated for overnight at 37 ^{o}C. Plasmid DNAs were purified from the overnight cultures by minipreparation or the Sigma Plasmid Miniprep Kit (Sigma, CA, USA) according to the manufacture's instruction manual. Extracted plasmid DNAs were subjected for sequencing for both directions using BigDye3.1 on an Applied Biosystems 9700 thermal cycler. The PCR reaction was carried out in a 10 µl reaction containing 4.0 µl of terminator ready reaction mix, 3.2 pmol of sequencing primer and 500 ng DNA template. After incubation at 95^{o}C for 2 min, amplification was carried out for 25 cycles with the following temperature cycling parameters; 95^{o}C for 10 sec of denaturation, 50^{o}C for 5 sec of annealing and 60^{o}C for 4 min of extension. The DNA was then precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of absolute ethanol and incubated at -20^{o}C for 1 hr. After centrifugation at 12,000 rpm for 10 min, the pellet was washed with 500 µl l of 70% ethanol and air dried. The DNA pellet was resuspended in 10 µl l Template Suppression Reagent (Perkin-Elmer) and loaded to the ABI PRISM sequencer.

2.6 Sequence analysis

The open reading frame (ORF) in cDNA sequences was analyzed using BioEdit Sequence Alignment Editor version 7.0.9. Nucleotide and amino acid sequences of snake venom serine proteinase, as shown in Table 1, were obtained from NCBI databases for phylogenetic analysis. Putative N-linked and O-linked glycosylation sites were predicted by NetNGlyc 1.0 and NetOGlyc 3.1 Server,

respectively. Multiple sequence alignment of amino acid sequences was performed using Clustal X version 2.0.11 and GeneDoc version 2.7. The aligned amino acid sequences were edited by BioEdit . The nucleotide sequences alignment was buffered according to the amino acid sequence alignment using DAMBE version 5.1.5. Phylogenetic analysis of the aligned amino acid or nucleotide sequences was performed with PHYLIP package version 3.69. Genetic distances were determined with F84 model for the aligned nucleotide sequences or JTT model for the aligned amino acid sequences. The distance matrixes were subsequently used to construct the phylogenetic trees by the neighbour-joining method. Bootstrap estimates analysis was conducted in 1000 replicates. Numbers of non-synonymous substitutions per non-synonymous site (K_A) and numbers of synonymous substitutions per synonymous site (K_S) in protein coding regions, and numbers of nucleotide substitutions per site (K_N) for the 3' UTR of snake venom serine proteinase cDNAs were calculated by MEGA4 program using Nei and Gojobori (1986) method (Tamura et al., 2007). The one-tailed test was used to determine the significance of differences between K_A and K_S for test of positive selection by MEGA4 program.

K_N values were calculated from the following equation

$$K_N = \log(1/(1-2P-Q)) + \log(1/(1-2Q))$$

P: Observed number of transitional differences
Q: Observed number of transversion differences
K_N: Numbers of nucleotide substitutions per site

K_A and K_S values were calculated from the following equations

$$\hat{P}_S = \frac{\text{Synonymous differences}}{\text{Synonymous sites}} \qquad \hat{P}_A = \frac{\text{Nonsynonymous differences}}{\text{Nonsynonymous sites}}$$

$$K_S = -\frac{3}{4} \ln\left(1 - \frac{4}{3}\hat{P}_S\right) \qquad K_A = -\frac{3}{4} \ln\left(1 - \frac{4}{3}\hat{P}_A\right)$$

2.7 Amplification of mature protein-encoding cDNA

2.7.1 Consensus PCR

A pair of primers, VSP-F and VSP-R (Table3) was designed from the conserved sequences of the 5'- and 3'-untranslated region (UTR) from the cDNAs encoding RVV-V and snake venom serine preteinases in Genbank database. A 5 µg of the Russell's viper gland mRNA was used in RT- PCR using SuperScript™ One-Step RT-PCR kit (Invitrogen, CA, USA). cDNA was synthesized by reverse transcription at 45°C for 45 min. PCR amplification consisting of 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min followed by final extension at 72 °C for 7 min was performed. RT-PCR products were electrophoresed by 1% agarose gel and purified with QIAquick Gel Extraction Kit (Qiagen, CA, USA). The purified RT-PCR products were subsequently cloned into the pGEM-T easy plasmid (Promega, MA, USA). Individual colonies were randomly picked for plasmid extraction and DNA sequencing.

2.7.2 PCR for cloning into expression vectors

DNA encoding mature RVV-V was amplified by PCR with the primer pairs designed for each expression vector (Table 3). The typical PCR reaction was carried out in a 50 µl reaction containing 1X PCR buffer, 1.25 units of Taq DNA polymerase (Promega), 1 µM of each primer, 1.5 mM $MgCl_2$, 200 µM of each dNTPs and 1 ng of plasmid DNA template. After incubation at 94°C for 3 min, amplification was carried out for 30 cycles of the following temperature cycling parameters were performed: 94°C for 20 sec of denaturation, 55°C for 30 sec of annealing and 72°C for 30 sec of extension. The final amplification cycle included an addition of a 5 min extension at 72°C. The amplified DNA fragments were purified by QIAquick® Gel Extraction Kit (QIAGEN, Valencia, CA) in order to remove impurities such as small RNA, proteins, unincorporated nucleotides or primers. The purified PCR products were then cloned into pGEM-T east vector prior to subsequently be subcloned into the expression vectors.

2.8 Subcloning to Expression Vector

The RVV-V cDNA fragment in pGEM-T easy vector was restriction digested by the same enzymes used to digest the desired expression vector (Table 3). *Nco*I/*Xho*I or *Eco*RI/*Xba*I were used for cloning into pET-32a or pPicZα-A, respectively. For the pPink system, the RVV-V cDNA was digested *SmaI* and *FseI* while digestion of the pPinkα-HC was performed with *StuI* and *FseI*. After restriction digestion, the digested DNA fragments were purified from gel slice by QIAquick Gel

Extraction Kit. Ligation and transformation were preceded as described in 2.2 and 2.4. Plasmid extraction and DNA sequencing performed as described in 2.5.

2.9 Recombinant RVV-V expression in *E. coli*

2.9.1 Transformation

pET32a-RVV-V plasmid was transformed into the *E. coli* strain BL21, a strain suitable for recombinant protein expression, according to protocol described in 2.4. To co-express the rRVV-V with molecular chaperone, transformation of pG-KJE8 was also performed. pG-KJE8 is an expression vector that express molecular chaperones under control by L-arabinose and tetracyclin.

2.9.2 Protein expression

The *E. coli* strain BL21 containing pET32a-RVV-V plasmid was cultured in shaking flasks containing Luria–Bertani (LB) medium overnight. A 10 ml of the overnight culture was inoculated at 37^{o}C in 1000 ml of fresh LB medium. When OD_{600} reach 0.4, final concentration of 0.5 mg/ml of L-arabinose and 5 ng/ml of tetracycline were added. A final concentration of 0.1 mM isopropyl b-D-thiogalactoside (IPTG) was added when OD_{600} reach 0.5, and the culture was induced at various temperatures. Cells were collected by centrifugation and resuspended in Binding buffer for affinity purification (10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 7.0). Sonication was performed on ice. After centrifugation of the cell lysate, the supernatant was collected for purification.

2.9.3 Protein purification using Immobilized Metal Affinity Chromatography (IMAC)

Purification of the soluble rRVV-V was performed by chromatography with TALON® Metal Affinity Resins (CLONTECH Laboratories, Inc., Mountain View, CA) under naturing condition. The rRVV-V was solubilized in denaturing binding buffer (10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 7.0). After sonication, the suspension was centrifuged at 12,000 rpm for 30 minutes at 4°C. Prior to loading onto the column containing the resin, the supernatant was clarified by filtration through a 0.45-μm nylon membrane (Millipore, Billerica, MA), and the purification column was prepared by washing with 5-column volume of the binding buffer. Then the sample was loaded onto the column, and wash with 10-column volume of the binding buffer. The column was washed with another 10-colume volume of naturing washing buffer (20 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 7.0). The rRVV-V was eluted with naturing elution buffer (200 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 7.0). Fractionation was performed at 4°C. Absorbance of each protein elusion was monitored at 280 nm.

2.10 Recombinant RVV-V expression in *P. pastoris*

2.10.1 Transformation

i) pPicZα-A – X33

Recombinant pPicZα-A was chemically transformed into X33 *Pichia pastoris* by using EasyComp transformation kit. Briefly, 3-5 ug of linearized recombinant

pPicZα-A were mixed to the Pichia competent cells. Finally, the competent cells were spread onto zeocin-YPD plates and incubated at 28-30 ^{o}C for 3-7 days. The growing yeast colonies were picked to test for the recombinant pPicZα-A integration by PCR.

ii) pPinkα-HC - PichiaPink™

Recombinant pPinkα-HC was transformed into PichiaPink™ Strains *Pichia pastoris* by electrophoration. A 5 ug of linearized recombinant pPinkα-HC was mixed to the PichiaPink™ cells and electrophorated with Bio-Rad electroporation system. The competent cells were then spread onto adenine dropout medium and incubated at 28-30 ^{o}C for 3-7 days. The growing white colonies were picked to confirm the presence of recombinant pPicZα-A integration by PCR.

2.10.2 Protein expression

A single colony was inoculated in 100 ml of Buffered Glycerol-complex Medium (BMGY) in a 1 liter baffled flask at 28–30°C in a shaking incubator (250–300 rpm) until the culture reaches an OD600 = 2–6. The cells were harvested by centrifuging at 1,500–3,000 xg for 5 minutes at room temperature. The supernatant was then discarded and cell pellet was resuspended in Buffered Methanol-complex Medium (BMMY). The yeast was grown at 28–30°C with shaking. A final concentration of 0.5% methanol was added every 24 hours. Supernatant was collected by centrifuging at 1,500–3,000 xg for 5 minutes at room temperature.

2.11 Protein concentration by concentrator

Protein samples were concentrated by centrifugal concentrator with a nominal molecular weight limit of 10 kDa (Amicon® Ultra-15; Millipore, Billerica, MA). The column was pre-washed with 20 mM Tris-HCl (pH 8.5). The sample was poured into the concentrator and centrifuged at 3,000 *xg* for 20-30 minutes. The remained sample was collected. Protein concentration was measured by a BCA protein assay (Pierce) and stored in aliquots at -70°C

2.12 Sodiumdodecrylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Twelve percentages of Resolving gel and 4% of Stacking gel were freshly prepared as follows:

12% of Resolving gel (for 2 mini-gels)

30% Acrylamide:Bisarylamide (37.5:1)	3,200 µl
1.5 M Tris-HCl, pH 8.8	2,000 µl
10% SDS	80 µl
Distilled water	2,680 µl
10% ammonium persulphate (APS)	40 µl
TEMED	4 µl

4% of Stacking gel (for 2 mini-gels)

30% Acrylamide:Bisarylamide (37.5:1)	462 µl
0.5 M Tris-HCl, pH 6.8	882 µl
10% SDS	35 µl
Distilled water	2,100 µl
10% ammonium persulphate (APS)	17.5 µl
TEMED	3.5 µl

After gel setting, the protein samples were mixed with 1/4 volume of 4X Reducing Sample Buffer (62.5 mM Tris-HCl, pH 6.8; 8% w/v SDS; 40% v/v glycerol; 0.005% bromophenol Blue; 10% 2-mercaptoethanol), denatured at 95°C for 10 minutes and loaded into gel slots in submarine condition. Electrophoresis was performed in 1X Running Buffer, pH 8.3 (0.25 M Tris-HCl, 1.92 M glycine, 1% w/v SDS) at 80 volts for 15 minutes followed by 100 volts for 90 minutes.

2.13 Coomassie Brillient Blue staining

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

2.14 Western blot analysis using anti-His antibodies

After SDS-PAGE was performed completely, the separated protein was transferred from polyacrylamide gel to nitrocellulose membrane using Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-Rad Laboratories). The gel and membrane were soaked in transfer buffer (20 mM Tris, 150 mM Glycine, 20% methanol) for 15 minutes. Both of equilibrated gel and wetted membrane were sandwiched between sheets of transfer buffer-soaked thick filter papers, and then were placed on Trans-Blot® SD cell. The gel sandwich was transferred at 20 volts for 35 minutes. The transfer efficiency can be monitored by staining the transferred gel with Coomassie Brillient Blue staining.

The nitrocellulose membrane blotted with rRVV-V proteins was blocked with 5% non-fat dry milk (Carnation) in PBS (pH 7.4) at 4°C overnight. The blot was then incubated with mouse anti-His antibody diluted in the blocking buffer (1:3,000) at room temperature for 1 hour. After washing in 3 changes of PBS (pH 7.4) for 5 minutes, the secondary antibody, rabbit anti-mouse antibody conjugated with HRP (Zymed) diluted in the blocking buffer (1:10,000), was applied, and followed by incubation for 1 hour at room temperature. The blot was then washed in 5 changes of PBS (pH 7.4) for 10 minutes. After washing, the blot was soaked in the substrate solution (1.66 mM DAB tetrahydrochroride, 0.04% $NiCl_2$, and 0.006% H_2O_2) at room temperature in dark for 5 minutes. Finally, the solution was removed and the blot was washed with distilled water and dried at room temperature overnight.

2.15 Arginine esterase activity

Benzoylarginine ethyl ester (BAEE), a low molecular wieght arginine ester substrate, was used as a substrate to measure arginine esterase activity of rRVV-V. A 200 µl of rRVV-V was mixed with 3 ml of 0.25 mM BAEE in 67 mM Sodium Phosphate Buffer, pH 7.6 at 25°C. The increase in A_{253} nm was recorded for approximately 5 minutes. Trypsin was used as positive control.

CHAPTER IV

RESULTS

1. Molecular cloning and sequence analysis of rRVV-V

1.1. 5’-RACE

5'-RACE using RVV-V specific primers amplified 700 bp DNA fragments, which represented the 5'-untranslated region (UTR), pre- and proprotein- encoding regions and N-terminal-encoding region of RVV-V cDNA. The complete sequence of the RVV-V cDNA was obtained by combination of the overlapping sequences between the 5'-RACE product and the cDNA library. The RVV-V cDNA contained a start codon for methionine (ATG), stop codon (TGA), polyadenylation signal (AATAAA) and poly (A) tail (Fig. 10). The 5'- and the 3'-UTR were 192 and 606 base pairs, respectively. The 780 bp open reading frame (ORF) encoded signal peptides (54 base pairs, 18 amino acids), activation peptides (18 basepairs, 6 amino acids) and mature enzyme coding region (708 base pairs, 236 amino acids). The translated signal peptide sequence of the RVV-V was rich in hydrophobic amino acid residues which were highly conserved among snake venom serine proteinases (Fig. 11). Using Blastx and BioEdit program revealed that the deduced amino acid sequence of the mature RVV-V in this study was 99% identical with the RVV-Vγ since there was one amino acid substitution (H203K) (Fig. 11) (Tokunaga et al., 1988). The catalytic triad (His67-Asp112-Ser206), 12 conserved cysteins and one putative N-glycosylation site were presented (Fig. 10, 11). Therefore, the RVV-V

cDNA revealed that RVV-V is expressed as a zymogen, which requires proteolytic cleavage for activation. Since only amino acid sequence of RVV-Vγ was reported, the novel RVV-V cDNA from this study has been submitted in GenBank (Accession no. HQ270463).

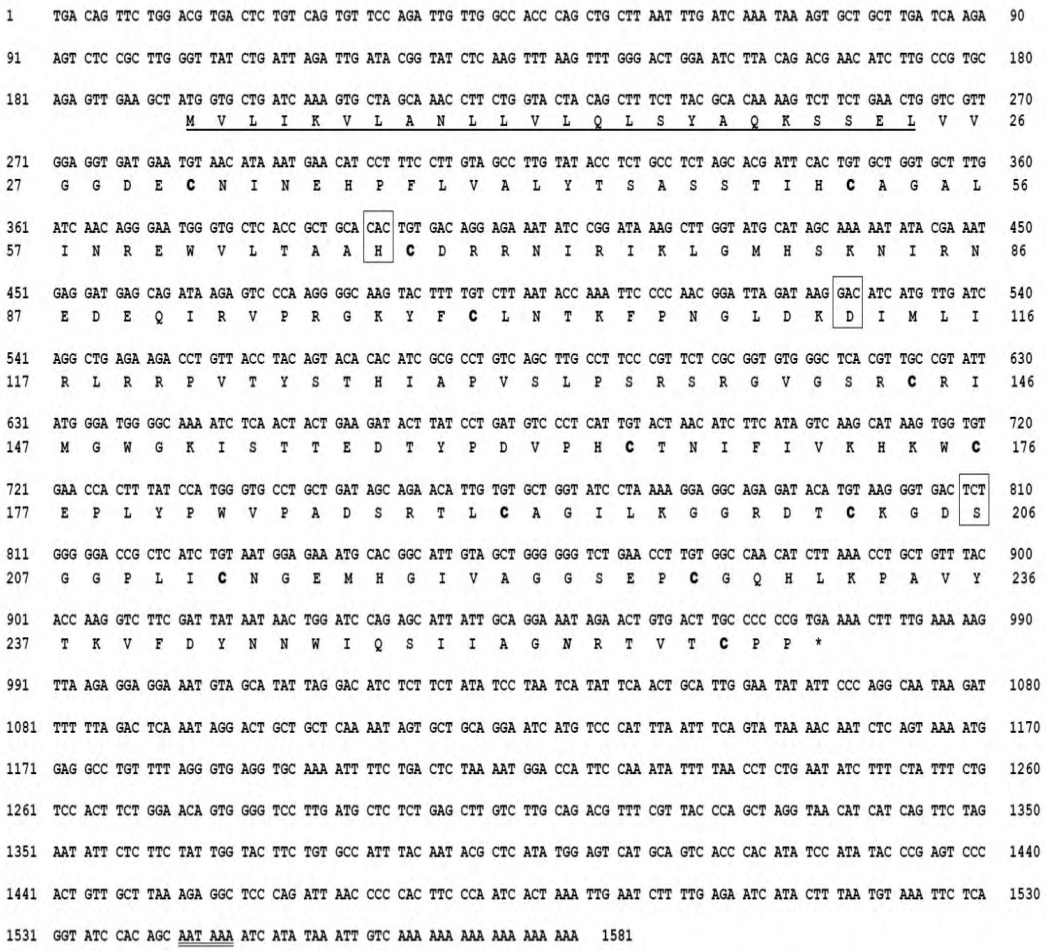


Fig. 10. Complete cDNA sequence of factor V activator (RVV-V) from Russell's viper cDNA. His-Asp-Ser residues of catalytic triad were boxed. Twelve conserved cysteines were bolded. The single underlined sequences indicated the signal and activation peptides. Polyadenylation signal was double underlined.

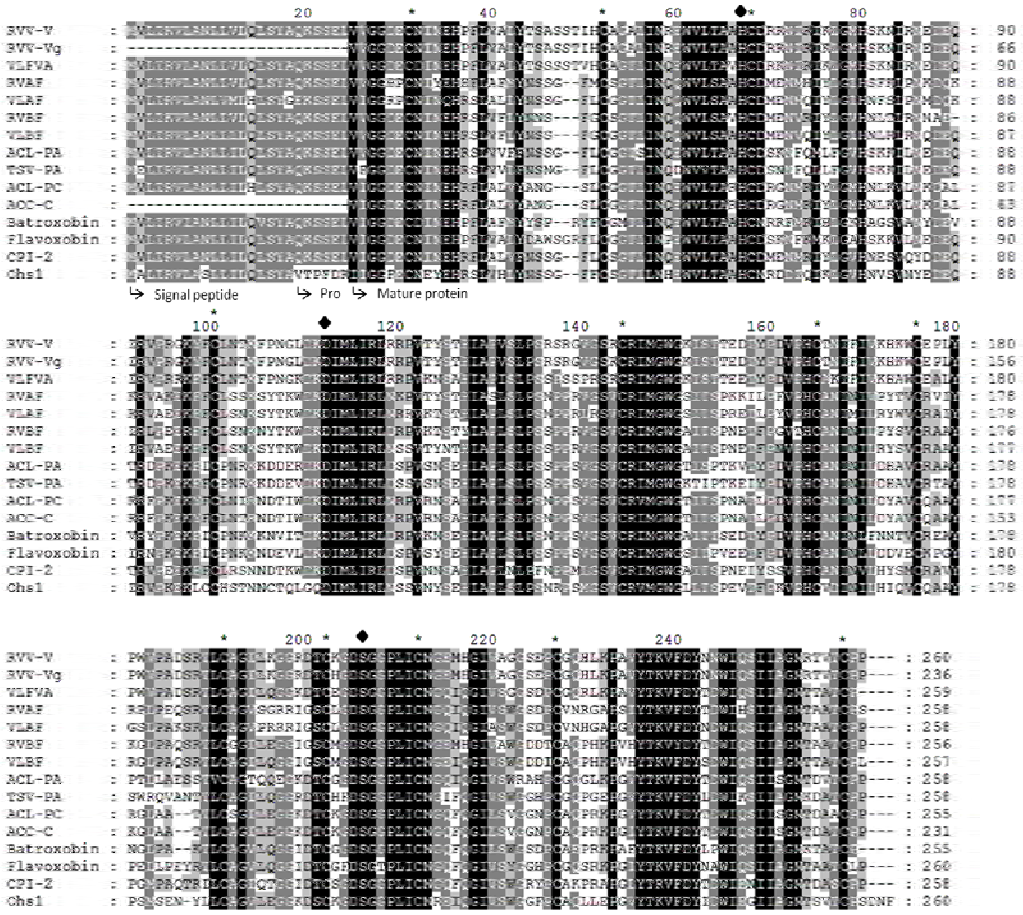


Fig. 11. Multiple sequence alignment of amino acid sequences of snake venom serine proteinases. The alignment was performed by Clustal W program. Arrows indicate the boundaries of signal peptide, activation (pro-) peptide and mature protein. Catalytic triad residues were shown with solid diamond. Asterisks indicated the 12 conserved cysteins. Abbreviations: RVV-V: factor V activator from Russell's viper (from this study); RVV-Vg: RVV-V gamma; VLFVA: factor V activator from *Macrovipera lebetina*; RVAF: Russell's viper serine alpha-fibrinoginase (from this study); VLAF: serine alpha-fibrinogenase from *Macrovipera lebetina*; RVBF: Russell's viper serine beta-fibrinoginase (from this study); VLBF: serine beta-fibrinogenase from *Macrovipera lebetina*; APL-PA: plasminogen activator from *A. p. leucostoma*; TSV-PA: plasminogen activator from *Viridovipera stejnegeri*; APL-C: protein C activator from *A. p. leucostoma*; ACC-C: protein C activator from *Agkistrodon contortrix contortrix*; CPI-2: Capillary permeability-increasing enzyme 2 from *Gloydius ussuriensis*; Batroxobin from *Bothrops atrox*; Flavoxobin from *Trimeresurus flavoviridis*; Ohs1: serine proteinase from *Ophiophagus hannah*

1.2. RVV-Vγ cDNA analysis

Because only RVV-Vγ amino acid sequence has been reported, the cDNA of the RVV-V from this study was most identical with the cDNA of the factor V activator from *Macrovipera lebetina* (VLFVA) (94% identity) (Siigur et al., 1999; Siigur et al., 1998). The identity of protein coding sequence, 5'- and 3'-UTR between the RVV-Vγ and the VLFVA cDNAs were 92, 95 and 97%, respectively. In the protein-coding region, the identity was highest in the activation peptide-coding region (100%). The signal peptide and the mature protein coding regions were 98% and 91% identical, respectively, compared with 94 and 83% identity in the translated amino acids.

It was found that the snake venom cDNA encoding mature protein has evolved in accelerated manner than that encoding untranslated region (Deshimaru et al., 1996; Nakashima et al., 1995). To prove this hypothesis in RVV-V cDNA, number of nucleotide substitutions per synonymous site (K_S) for the mature protein coding region and numbers of nucleotide substitutions per site (K_N) for the 3' UTR of the RVV-Vγ cDNA were calculated in comparison with other snake venom serine proteinases (Table 5). The K_N values for the UTR were less than the K_S values in all pairs, including the pair of the closely related RVV-Vγ and VLFVA. The $K_N/K_S < 1$ indicated that the mature protein coding region of the RVV-V cDNA evolved more rapidly than the conserved UTR.

Table 5. K_N and K_S values of RVV-V cDNA compared with snake venom serine proteinases from GenBank data base

cDNA pairs	K_S (Mature)	K_N (3'UTR)
RVV-V vs. VLFVA[a]	0.085	0.012
RVV-V vs. TSV-PA[b]	0.267	0.078
RVV-V vs. CPI-2[c]	0.299	0.078
RVV-V vs. VLAF[d]	0.269	0.111
RVV-V vs. batroxobin[e]	0.305	0.102
RVV-V vs. Flavoxobin[f]	0.308	0.089

[a]VLFVA: factor V activator from *Macrovipera lebetina*

[b]TSV-PA: plasminogen activator from *Viridovipera stejnegeri*

[c]CPI-2: capillary permeability-increasing enzyme-2 from *Gloydius ussuriensis*

[d]VLAF: serine alpha-fibrinogenase from *Macrovipera lebetina*

[e]Batroxobin from *Bothrops atrox*

[f]Flavoxobin from *Trimeresurus flavoviridis*

1.3. Consensus PCR

According to the high conservation of the 5'- and 3' UTR, there was a probability to obtain the undiscovered serine proteinases by consensus PCR. RT-PCR was performed using the primers designed from the consensus regions from the 5'- and 3' UTR of the RVV-Vγ and snake venom serine proteinase cDNAs. A RT-PCR product of approximately 800 bp was cloned and sequenced. Similarly to the study of Siigur, *et al* (1999), only the RVV-Vγ cDNA was amplified while the alpha- and beta-like isoforms of RVV-V were not observed (Siigur et al., 1999; Tokunaga et al., 1988). Besides RVV-Vγ, two serine proteinase cDNAs were obtained, designed as Russell's viper alpha-fibrinogenase homologue (RVAF) (Fig. 12A) and Russell's

viper beta-fibrinogenase homologue (RVBF) (Fig 12B). Deduced amino acid sequence of RVAF and RVBF had highest identity with the serine alpha-fibrinogenase precursor (VLAF) (80%) and the serine beta-fibrinogenase precursor (VLBF) (85%), respectively, from *Macrovipera lebetina* (Siigur et al., 2003). Both RVAF and RVBF have not purified from RVV. Only a spot of RVV β-fibrinogenase like protein was found from a proteomic analysis study (Risch et al., 2009). Therefore, this is the first report of the present of the alpha-fibrinogenase in Russell's viper venom. Moreover, this study has discovered two novel serine proteinase cDNAs from Russell's viper transcripts. These two cDNAs have also been submitted in GenBank (Accession no. HQ270464 and HQ270465 for RVAF and RVBF, respectively).

To explore the biochemical properties of the novel genes, the pI values and molecular weight before glycosylation as well as putative glycosylation sites were predicted. RVAF had pI value of 9.2 and molecular weight of 25.8 kDa. ORF of the RVAF, encoded 258 amino acids, shared 80% amino acid identity and 90% nucleotide identity with VLAF. One putative N-linked glycosylation site (NXS/T) at the Asn44 and two O-linked glycosylation sites at the position Thr255 and Ser258 contributed to posttranslational modification and altered the molecular weight and pI (Siigur et al., 2003). Deduced 256 amino acid sequence of the RVBF was 85% identical with the VLBF and nucleotide sequence of both genes shared 92% identity. The RVBF had pI of 6.7, molecular weight of 25.40 kDa, 2 N-linked glycosylation sites (Asn78 and Asn101) and 1 O-linked glycosylation site (Thr 253). VLAF and VLBF hydrolyzed α- and β-chain of fibrinogen, respectively, inhibiting clotting

function of fibrinogen by thrombin (Samel et al., 2002). The RVBF was previously found in the proteomic analysis of Russell's viper venom from Myanmar (Risch et al., 2009). The observed molecular weight of RVBF was approximately 60 kDa, which indicated the role of heavy glycosylation (Risch et al., 2009). Experimental investigations are required to confirm this predicted data.

A

```
1    ATG GTG CTG ATC AGA GTG CTA GCA AAC CTT CTG GTA CTA CAG CTT TCT TAC GCA CAA AAG TCT TCT GAA CTG GTC GTT GGA GGT CAT CCA   90
1     M  V  L  I  R  V  L  A  N  L  L  V  L  Q  L  S  Y  A  Q  K  S  S  E  L  V  V  G  G  H  P   30

91   TGT AAC ATA TAT GAA CAT CAT TTC CTT GCA TTC ATG TAT AAC TCT AGC GGA TTT ATG TGC AGT GGG ACT TTG ATC AAC CAG CAA TGG GTG   180
31    C  N  I  Y  E  H  H  F  L  A  F  M  Y  N  S  S  G  F  M  C  S  G  T  L  I  N  Q  Q  W  V   60

181  CTC AGT GCT GCA CAC TGC GAC ATG GAA AAT ATG CAT ATA TAC CTT GGT TTG CAT AGC TTC AAG CTA CCA AAT AAG GAT CAG AAG AAA AGA   270
61    L  S  A  A  H  C  D  M  E  N  M  H  I  Y  L  G  L  H  S  F  K  L  P  N  K  D  Q  K  K  R   90

271  GTC GCA AAG GAG AAG TTC TTT TGT CTC AGT AGC AAA AGC TAC ACC AAA TGG GAC AAG GAC ATC ATG TTG ATC AAG CTG AAC AAA CCT GTT   360
91    V  A  K  E  K  F  F  C  L  S  S  K  S  Y  T  K  W  D  K  D  I  M  L  I  K  L  N  K  P  V   120

361  ACC TAC AGT ACA CAC ATC GCG TCT CTC AGC TTG CCT TCC AAC CCT CCC CGT GTG GGC TCA GTT TGC CGT ATT ATG GGA TGG GGC TCA ATT   450
121   T  Y  S  T  H  I  A  S  L  S  L  P  S  N  P  P  R  V  G  S  V  C  R  I  M  G  W  G  S  I   150

451  ACA TCT CCT AAA AAG ATT TTG CCC TTT GTG CCT CAT TGT GCT AAC ATT AAC ATA GTC CCT TAT ACG GTG TGT CGA GTA ATT TAC AGA CCG   540
151   T  S  P  K  K  I  L  P  F  V  P  H  C  A  N  I  N  I  V  P  Y  T  V  C  R  V  I  Y  R  P   180

541  TTA CCG GAA CAA AGC AGA ACA TTG TGT GCA GGT GTC TCA GGA AGA CGC ATA GGT TCA TGT CTG GGT GAC TCT GGG GGA CCA CTC ATC TGT   630
181   L  P  E  Q  S  R  T  L  C  A  G  V  S  G  R  R  I  G  S  C  L  G  D  S  G  G  P  L  I  C   210

631  AAT GGA CAA ATC CAG GGC ATT GTA TCT TGG GGG AGC GAC CCT TGT GTC AAT CGT GGT GCA CCT AGC ATA TAC ACC AAG GTC TTT GAT TAT   720
211   N  G  Q  I  Q  G  I  V  S  W  G  S  D  P  C  V  N  R  G  A  P  S  I  Y  T  K  V  F  D  Y   240

721  ACT GAC TGG ATC CAC AGC ATT ATT GCA GGA AAT ACA GCT GCA ACT TGC CCG TCG TGA   777
241   T  D  W  I  H  S  I  I  A  G  N  T  A  A  T  C  P  S  *
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B

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1    ATG GTG CTG ATC AAA GTG CTA GCA AAC CTT CTG GTA CTA CAG CTT TCT TAC GCA CAA AAG TCT TCT GAA CTG GTC GTT GGA GGT GAT GAA   90
1     M  V  L  I  K  V  L  A  N  L  L  V  L  Q  L  S  Y  A  Q  K  S  S  E  L  V  V  G  G  D  E   30

91   TGT AAC ATA AAT GAA CAT CGT TCC CTT GTA TTC TTG TAT AAC AAT AGC TTT GGC TGC AGT GGG ACT TTG ATC AAC CAG CAA TGG GTG CTC   180
31    C  N  I  N  E  H  R  S  L  V  F  L  Y  N  N  S  F  G  C  S  G  T  L  I  N  Q  Q  W  V  L   60

181  AGC GCT GTA CAC TGC GAC ATG GAA AAT GTG CGG ATA TAC CTT GGT GTG CAT AAC CTC ACT CTA CGA AAT AAT GCG GAG ATA AGA CTC CCA   270
61    S  A  V  H  C  D  M  E  N  V  R  I  Y  L  G  V  H  N  L  T  L  R  N  N  A  E  I  R  L  P   90

271  GAG GAG AGG TTC TTT TGT CTC AGT AAC AAA AAC TAT ACC AAA TGG GAC AAG GAC ATC ATG TTG ATC AAG CTG GAC AGA CCT GTT AAA ACC   360
91    E  E  R  F  F  C  L  S  N  K  N  Y  T  K  W  D  K  D  I  M  L  I  K  L  D  R  P  V  K  T   120

361  AGT ACA TAC ATC GCG CCT CTC AGC TTG CCT TCC AGT CCT CCC CGT GTG GGC TCA GTT TGC CGT ATT ATG GGA TGG GGT GCA ATC ACA TCT   450
121   S  T  Y  I  A  P  L  S  L  P  S  S  P  P  R  V  G  S  V  C  R  I  M  G  W  G  A  I  T  S   150

451  CCT AAT GAG ACT TTT CCC GGT GTA ACT CAT TGT GCT AAC ATC AAC ATA CTC CCT TAT TCA GTG TGT CGA GCA GCT TAC AAA GGG TTA CCG   540
151   P  N  E  T  F  P  G  V  T  H  C  A  N  I  N  I  L  P  Y  S  V  C  R  A  A  Y  K  G  L  P   180

541  GCA CAA AGC AGA ACA CTG TGT GGA GGT ATC CTG GAA GGA GGC ATA GGT TCA TGT ATG GGT GAC TCT GGG GGA CCG CTC ATC TGT AAT GGA   630
181   A  Q  S  R  T  L  C  G  G  I  L  E  G  G  I  G  S  C  M  G  D  S  G  G  P  L  I  C  N  G   210

631  GAA ATG CAC GGC ATT GTA GCT TGG GGG GAC GAT ACT TGT GCC CAA CCT CAT AAG CCT GTC CAC TAC ACC AAG GTC TAC GAT TAT ACT GAC   720
211   E  M  H  G  I  V  A  W  G  D  D  T  C  A  Q  P  H  K  P  V  H  Y  T  K  V  Y  D  Y  T  D   240

721  TGG ATC CAG AGC ATT ATT GCA GGA AAT ACA GCT GCG ACT TGC CCA CCG TGA   771
241   W  I  Q  S  I  I  A  G  N  T  A  A  T  C  P  P  *
```

Figure 12 (previous page). cDNA sequences of RVAF and RVBF from Russell's viper cDNA. His-Asp-Ser residues of catalytic triad were boxed. The single underlined sequences indicated the signal and activation peptides. A, RVAF; B, RVBF.

1.4. Multiple sequence alignment

To investigate the characteristics of the deduced amino acid sequences of RVV-Vγ (from this study), RVAF and RVBF, multiple sequence alignment of such tree proteins and other snake venom serine proteinases was performed as shown in Figure 11. The hydrophobic residue-rich signal peptides, residue 1-18, were highly conserved among snake venom serine proteinases. Interestingly, the signal sequences from Viperidae snakes also shared high identity with that of Ohs1, a serine proteinase from *Ophiophagus hannah*, an Elapidae snake. This indicated the conservation of venom-secreting system since early development of snake venom gland (Fry et al., 2006). The putative activation peptides (residue 19-24) were highly conserved among Viperidae snake venom serine proteinases. However, no identical amino acid was observed between the activation peptides of Osh1 and Viperidae proteinases, indicating the different proteolytic pathways for proteinase activation (Fig. 11). The catalytic triad of snake venom serine proteinases, His43-Asp88-Ser182 (numbering based on that of the mature RVV-Vγ), and the surrounding regions were strongly conserved. Such His-Asp-Ser residues are strongly conserved in serine proteinases involved in a wide variety of physiological processes, including blood coagulation, fibrinolysis and immune response (Hedstrom, 2002a, b). All Viperidae proteinases in the alignment contained 12 highly conserved cysteines, which formed 6 disulfide

bridges: Cys7-Cys141, Cys28-Cys44, Cys120-Cys188, Cys152-Cys167, Cys178-Cys203 and Cys76-Cys234 (Serrano and Maroun, 2005; Siigur et al., 1999; Vitorino-Cardoso et al., 2006). Additionally, two additional cysteine residues, Cys75 and Cys82, was found in Ohs1, as well as in NaSP and BmSP, two Elapidae serine proteinases from *Naja atra* and *Bungarus multicinctus*, respectively (Jin et al., 2007). Therefore, multiple sequence alignment indicated that RVV-Vγ, RVAF and RVBF are expressed as zymogen, and they contain the unique features of snake venom serine proteinase: catalytic triad and twelve conserved cysteines.

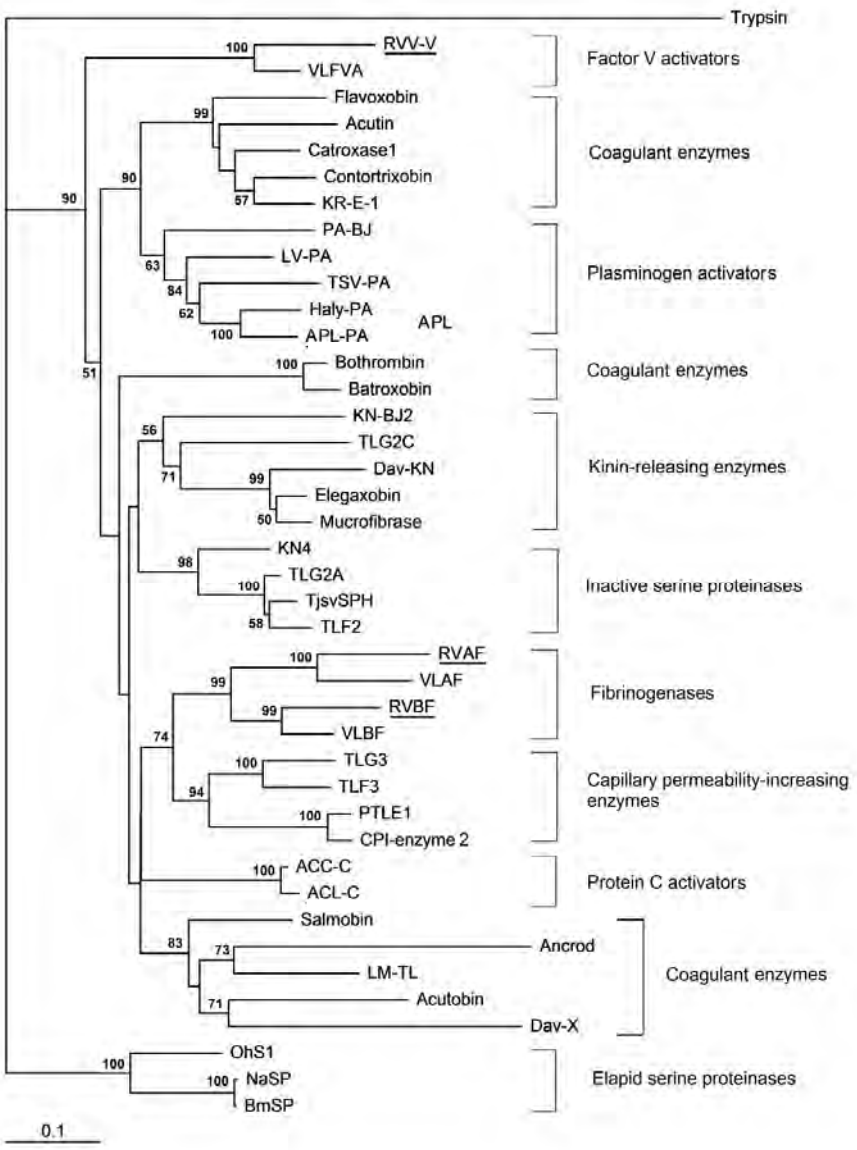
1.5. Phylogenetic analysis

To improve our understanding of the phylogenetic relationship of snake venom serine proteinases, the amino acid sequences of mature proteins of RVV-Vγ, RVAF, RVBF and other serine proteinases from Viperidae and Elapid snakes from GenBank database (Table 4) were used for construction of phylogenetic tree, a useful tool for classification of structure and function of proteins and DNAs, using the neighbour-joining algorithm (Fig. 13A). The sequence of human trypsin was used as outgroup. Only the bootstrap values of higher than 0.50 were shown on the tree. There are some algorithms that can be used for phylogenetic tree construction including the popular nighbor-joining, maximum parsimony and maximum likelihood. Both maximum parsimony and maximum likelihood algorithms are based on all characters (patterns of sequence) to build all possible trees, giving the best score tree. However, these algorithms are not suitable for construction of a tree from a number of sequences. The nighbor-joining (NJ) algorithm was chosen because it is based on

analysis of genetic distance to construct only one tree. Building a NJ tree is suitable for construction of a tree from many of amino acid or nucleotide sequences.

The analyzed serine proteinases were clustered in the tree based on their functions: factor V activator, coagulant enzymes, plasminogen activators, kinin-releasing enzymes, inactive serine proteinases, fibrinogenase enzymes, capillary permeability-increasing enzymes, protein C activators and Elapidae serine proteinases. The serine proteinases from Elapidae snakes were clustered separately from that from Viperidae snakes since early evolutionary speciation of colubroid snakes (Fry et al., 2006). The cluster of RVV-Vγ and VLFVA isolated from other Viperidae proteinases with bootstrap value of 100%, suggesting that the factor V activator genes have evolved in independent way. The fibrinogenase cluster contained RVAF, RVBF, VLAF and VLBF with bootstrap value of 99%. Alpha and beta serine fibrinogenases shared high similarity. Parallel evolution of factor V activators and fibrinogenases enzymes indicated close relationship between Russell's viper and *Macrovipera lebetina* despite of geographic difference. The amino acid NJ tree for the snake venom serine proteinases was supported by the DNA NJ tree (Fig. 13B). Since the RVAF and RVBF were grouped with the other fibrinogenases (VLAF and VLBF), implying that both RVAF and RVBF may have specific activity against alpha and beta chains of fibrinogen, respectively. It is unpractical to differentiate the activity of snake venom serine proteinases by amino acid sequences. So phylogenetic tree is a useful tool for characterization of the snake venom serine proteinases according to their functions.

13A


Trypsin
RVV-V
VLFVA
Factor V activators
Flavoxobin
Acutin
Catroxase1
Contortrixobin
KR-E-1
Coagulant enzymes
PA-BJ
LV-PA
TSV-PA
Haly-PA
APL-PA
APL
Plasminogen activators
Bothrombin
Batroxobin
Coagulant enzymes
KN-BJ2
TLG2C
Dav-KN
Elegaxobin
Mucrofibrase
Kinin-releasing enzymes
KN4
TLG2A
TjsvSPH
TLF2
Inactive serine proteinases
RVAF
VLAF
RVBF
VLBF
Fibrinogenases
TLG3
TLF3
PTLE1
CPI-enzyme 2
Capillary permeability-increasing enzymes
ACC-C
ACL-C
Protein C activators
Salmobin
Ancrod
LM-TL
Acutobin
Dav-X
Coagulant enzymes
OhS1
NaSP
BmSP
Elapid serine proteinases
0.1

13B

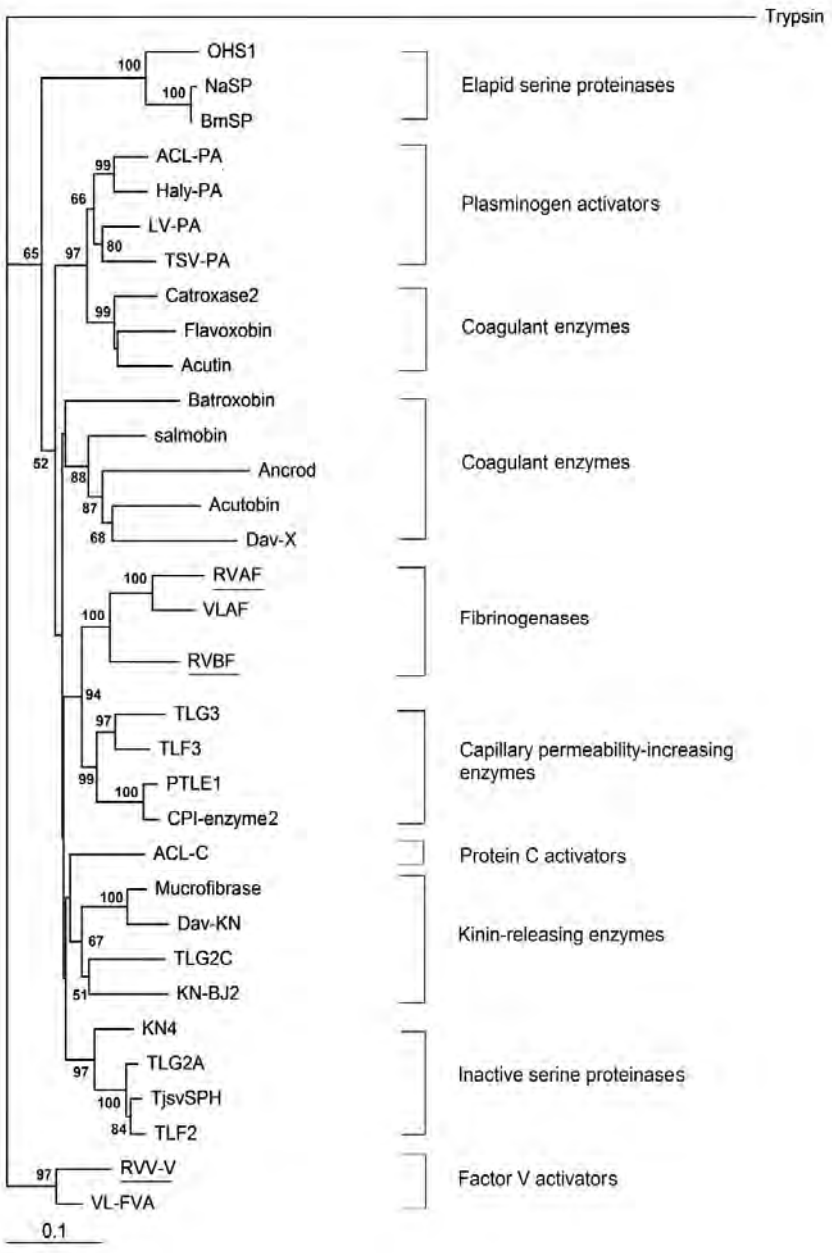


Figure 13. Phylogenetic tree of snake venom serine proteinases, obtained from neighbor-joining analysis. The proteins analyzed in this study were underlined. Bootstrap values (1000 replicates) above 50% were shown at the node. The scale bar indicated substitutions per site. A) NJ tree based on amino acid sequence (previous page); B) NJ tree based on DNA sequence

1.6. K_A/K_S values

Some snake venom proteins showed accelerated evolution (positive selection) to gain more variety of the toxins (Deshimaru et al., 1996; Nakashima et al., 1995). To investigate the positive selection hypothesis in RVV-V, RVAF and RVBF, ratio of number of nucleotide substitutions per nonsynonymous site (K_A) and number of nucleotide substitutions per synonymous site (K_S) of RVV-Vγ, RVAF, RVBF, APL-PA and APL-C were computed according to Nei and Gojobori method (Nei and Gojobori, 1986), as shown in Table 6. K_A values indicate nucleotide substitutions that alter the corresponding amino acid compositions. K_S values indicate nucleotide substitutions that do not alter the corresponding amino acid compositions. K_A/K_S values of the investigated genes compared with other Viperidae proteiases were close to or greater than one. This indicated accelerated evolution in the protein-coding regions of serine proteinases from Russell's viper and *A. p. leucostoma* venoms. Rapid evolution was also demonstrated in snake venom serine proteinases from *Trimeresurus flavoviridis*, *T. gramineus* and *Deinagkistrodon actus* (Deshimaru et al., 1996; Nikandrov et al., 2005), as well as in snake venom disintegrins (Soto et al., 2007), phospholipase A_2 (Soto et al., 2007) and Kunitz-BPTI protein (Zupunski et al., 2003). In contrast, K_A/K_S values of the Elapidae proteinases were close to one, indicating that Elapidae serine proteinases have evolved under neutral selection manner (Table 6). In conclusion, the K_A/K_S values indicated that the Viperidae serine proteinases have evolved in positive selection manner: nucleotide substitutions preferred to change the amino acids to obtained new genes with new functions.

Table 6. K_A[a] and K_S[b] values of snake venom serine proteinases.

cDNA pairs	K_A	K_S	K_A/K_S	*p* value
RVV-V vs. RVAF	0.265	0.172	1.541	0.005
RVV-V vs. RVBF	0.234	0.157	1.490	0.013
RVV-V vs. VLAF	0.260	0.159	1.635	0.001
RVV-V vs. TSV-PA	0.228	0.165	1.382	0.034
RVV-V vs. KN-BJ2	0.249	0.179	1.391	0.024
RVV-V vs. CPI-2	0.253	0.180	1.406	0.020
RVAF vs. ACL-PA	0.222	0.149	1.490	0.016
RVAF vs. VL-FVA	0.244	0.166	1.470	0.012
RVAF vs. Flavoxobin	0.242	0.162	1.494	0.011
RVAF vs. Haly-PA	0.226	0.147	1.537	0.007
RVAF vs. RVS-2 vs. TLG3	0.195	0.140	1.393	0.026
RVAF vs. Acutobin	0.263	0.201	1.308	0.038
RVAF vs. Dav-KN	0.227	0.159	1.428	0.026
RVBF vs. ACL-PA	0.201	0.139	1.446	0.026
RVBF vs. VL-FVA	0.218	0.154	1.416	0.034
RVBF vs. Flavoxobin	0.225	0.165	1.364	0.039
RVBF vs. Haly-PA	0.199	0.127	1.567	0.010
RVBF vs. TLF3	0.153	0.102	1.500	0.038
RVBF vs. Acutobin	0.227	0.162	1.401	0.031
RVBF vs. TLG2C	0.216	0.153	1.412	0.023
Ohs1 vs. NaSP	0.094	0.115	0.817	1.000
Ohs1 vs. BmSP	0.092	0.109	0.844	1.000
NaSP vs. BmSP	0.002	0.020	0.100	1.000
Ohs1 vs RVV-V	0.257	0.277	0.928	1.000
NaSP vs. RVV-V	0.256	0.298	0.859	1.000
BmSP vs. RVV-V	0.255	0.288	0.885	1.000

[a] K_A: Numbers of non-synonymous substitutions per non-synonymous site
[b] K_S: Number of nucleotide substitutions per synonymous site

2. Production of recombinant RVV-V

2.1. Expression of rRVV-V in *E. coli* with pET32a

(i) Molecular cloning of RVV-V cDNA into pET32a

To produce rRVV-V by pET32a, the mature protein-encoding sequence of RVV-V cDNA was amplified with a pair of primer containing *Nco*I and *Xho*I restriction sites (Fig 5 and Table 3). An approximately 700 bp PCR product was cloned into pGEM-T easy vector (Fig 14). The *Nco*I and *Xho*I digested DNA fragments were subsequently subcloned into pPicZα-A vector (Fig 15). The correct sequences were confirmed by DNA sequencing to ensure the in-frame reading protein translation.

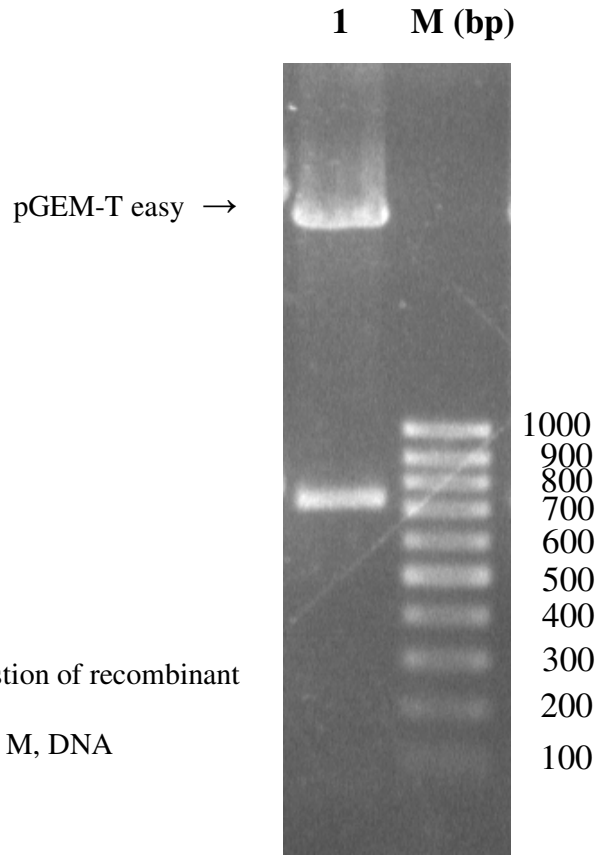


Figure 14. *Nco*I and *Xho*I digestion of recombinant pGEM-T easy. Lane 1, RVV-V; M, DNA ladder.

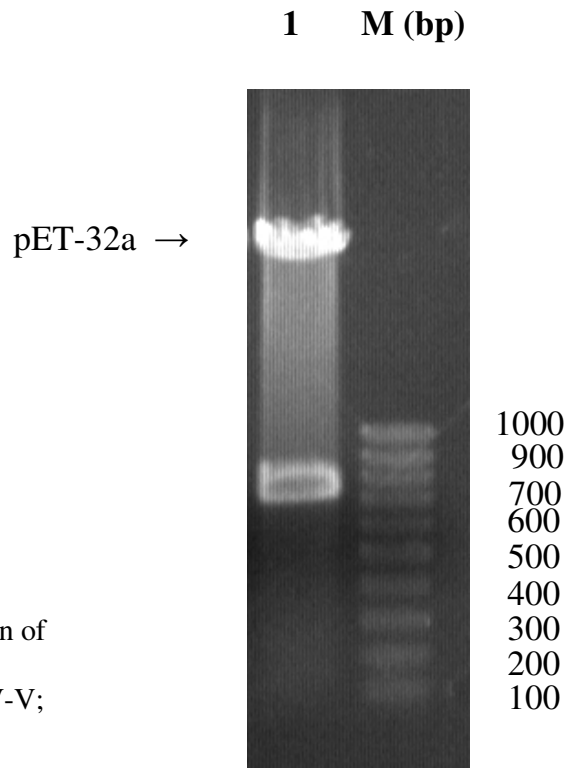


Figure 15. *Nco*I and *Xho*I digestion of recombinant pET32a. Lane 1, RVV-V; M, DNA ladder.

(ii) Effect of temperature on level of expressed rRVV-V

Since the expression level of a recombinant protein could be influenced by temperature of inducing conditions, the optimized temperature yield highest level for production of rRVV-V was elucidated. As shown in Figure 16, the IPTG-induced recombinant protein was expressed in 25, 30 and 37^{o}C. A 39 kDa protein was appeared in the lysate of the induced cells but not in that of the uninduced. According to 6x histidine introduced into the rRVV-V and rRVV-VΔ, the proteins could also be interacted by anti-His tag antibody (Fig 17), implying that the apparent protein was rRVV-V. Although 37^{o}C is appropriate temperature for growing many kinds of prokaryotic organism including *E. coli*, it seems that protein expression in 30^{o}C yields

highest level of both proteins. Therefore, the future expression will be performed in 30°C.

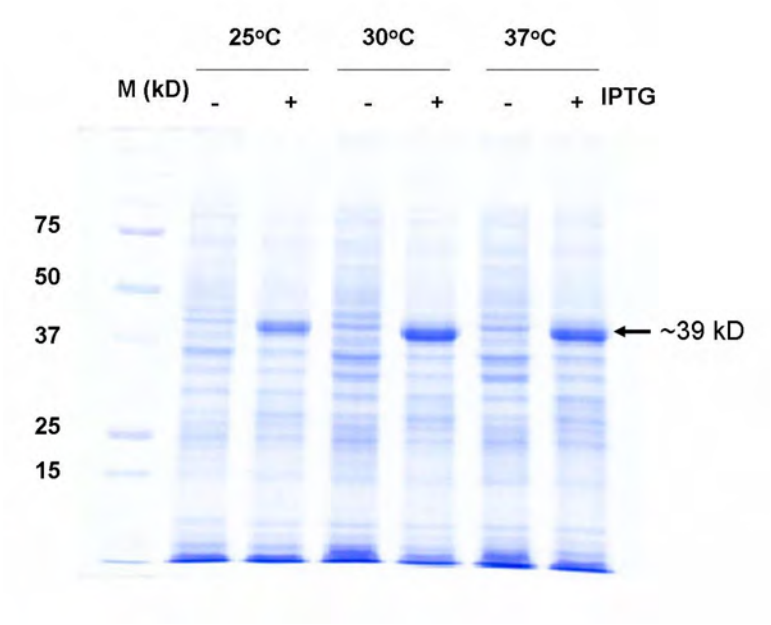


Figure 16. Expression of rRVV-V on three temperatures; 25, 30 and 37°C. The arrow indicates the recombinant protein expressed after induction by 0.5 mM IPTG.

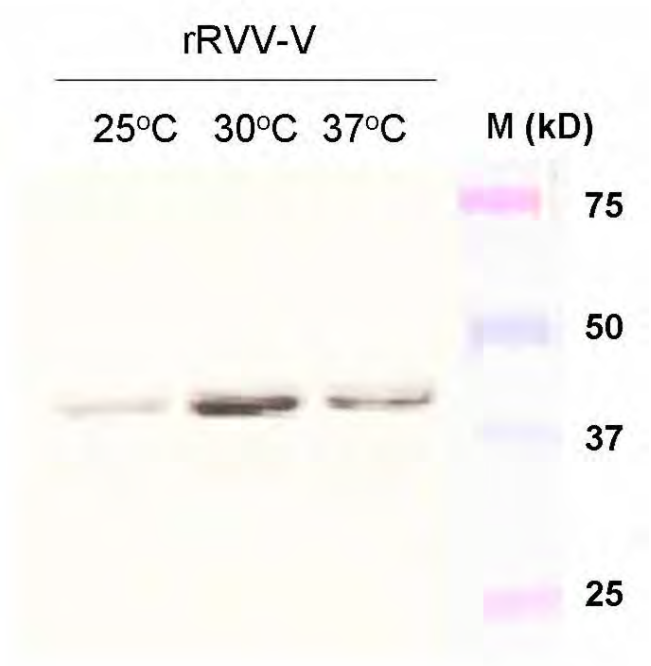


Figure 17. Immunoblotting of the lysates of the transformant *E. coli*.

(iii) Production of soluble rRVV-V with co-expresson of molecular chaperone

Since molecular chaperones are proteins adapted to assist *de novo* protein folding and facilitate expressed polypeptide's proper conformation, molecular chaperones were co-expressed from pG-KJE8 by induction with L-arabinose and tetracyclin. Soluble rRVV-V was observed in the supernatant of *E. coli* cell lysate (Fig 18, lane 2). Affinity chromatography using TALON® Metal Affinity Resins was performed and the purified protein was then concentrated (Fig 18, lane 3). Western blotting was also performed (Fig 19).

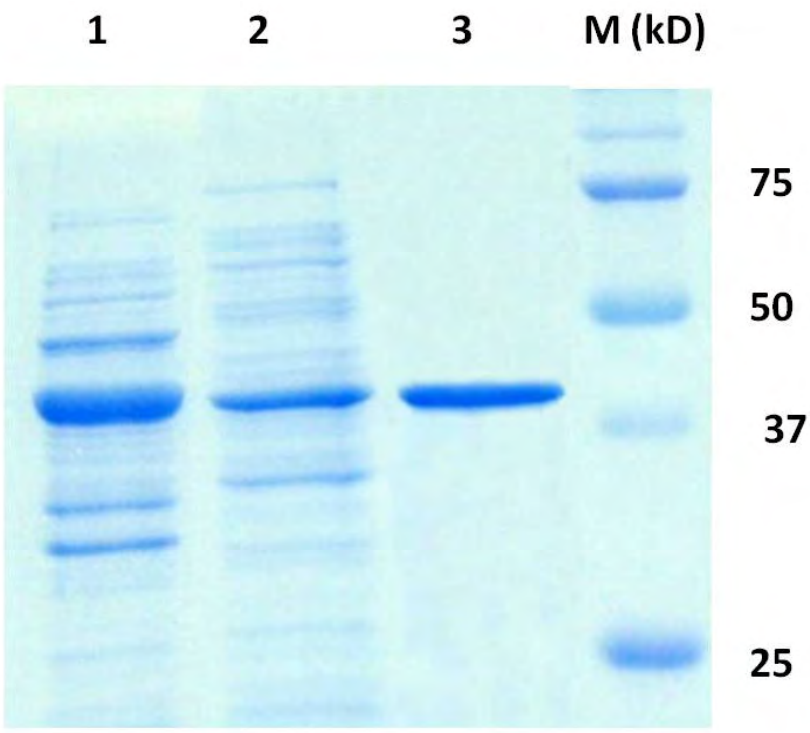


Figure 18. Expression of soluble rRVV-V and affinity purification. Lane 1, Cell lysate of rRVV-V-expressing *E. coli* cells; Lane 2, Supernatant of the cell lysate after centrifugation; Lane 3, purified rRVV-V from affinity chromatography with TALON® Metal Affinity Resins; M, Protein ladder

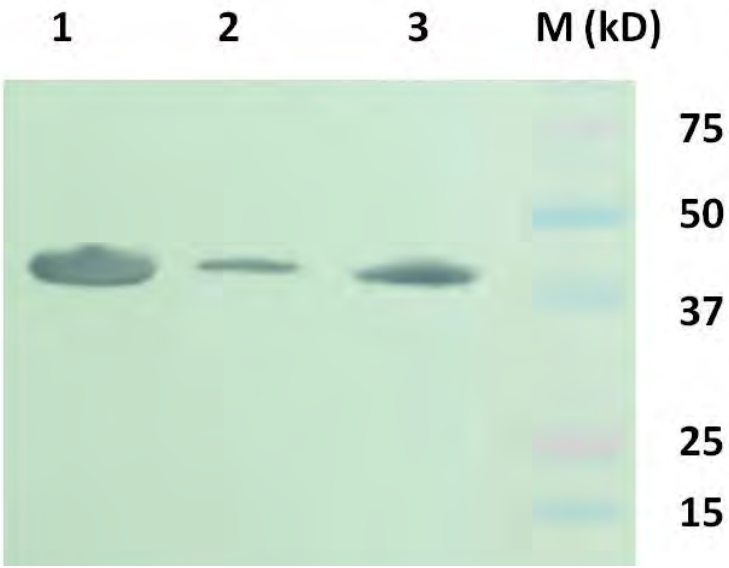


Figure 19. Immunoblotting of the cell lysate and purified rRVV-V. Lane 1, Cell lysate of rRVV-V-expressing *E. coli* cells; Lane 2, Supernatant of the cell lysate after centrifugation; Lane 3, purified rRVV-V from affinity chromatography with TALON® Metal Affinity Resins; M, Protein ladder

(iv) Arginine esterase activity

The purified rRVV-V (Fig. 19, lane 3) was tested for its arginine esterase activity with Benzoylarginine ethyl ester (BAEE), a low molecular wieght arginine ester substrate. However, it was shown that the rRVV-V lacked of activity (Fig 20).

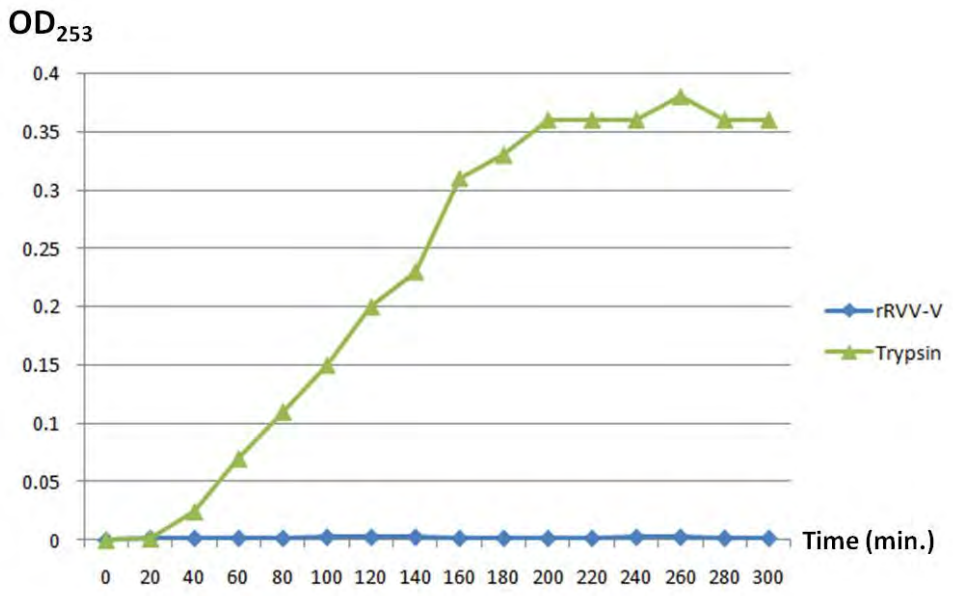


Figure 20. Arginine esterase assay. Diamond line represents the purified rRVV-V; Triangle line represents trypsin.

2.2. Expression of rRVV-V in *P. pastoris* with pPicZα-A

(i) Molecular cloning of RVV-V cDNA into pPicZα-A

To clone rRVV-V into pPicZα-A, the mature protein-encoding sequence of RVV-V cDNA was amplified with a pair of primer containing *EcoR*I and *Xba*I restriction sites (Fig 6 and Table 3) and cloned into pGEM-T easy vector. The *EcoR*I and *Xho*I digested DNA fragments were subsequently subcloned into pPicZα-A

vector (Fig 21). The correct sequences were confirmed by DNA sequencing to ensure the in-frame reading protein translation.

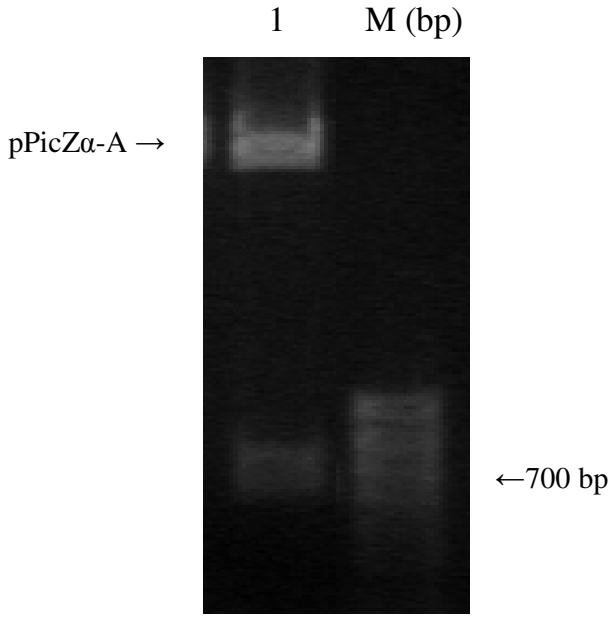


Figure 21. *EcoR*I and *Xba*I digestion of recombinant pPicZα-A. Lane 1, RVV-V; M, DNA ladder.

(ii) Transformation of recombinant pPicZα-A into Pichia pastosis

The recombinant pPicZα-A-RVV-V was transformed into *Pichia pastoris* using Pichia EasyComp(tm) Transformation Kit. Yeast transformants which were able to grow in Zeocin-YPDS plates were screened for the DNA integration. The pPic-VF and pPic-VR primers were used for PCR amplify the integration into the yeast

genome. The presence of recombinant pPicZα-A integration in the Pichia genomes were shown in Figure 22.

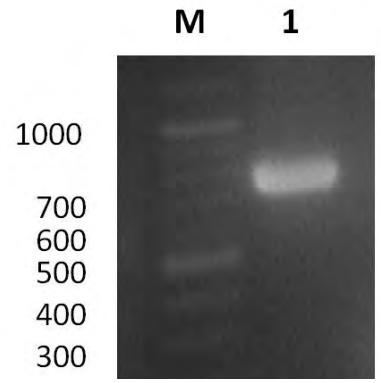


Figure 22. PCR screening of yeast transformants. Lane 1, RVV-V; M, DNA ladder.

(iii) Induction of protein expression in *P. Pastoris* by methanol

The transformed *Pichia pastoris* were cultured in BMGY and BMMY media. After the protein induction by methanol, culture media were collected to investigate the protein expression at every 24 hour for 4 days. Unfortunately, no protein bands were observed in all lanes (Fig 23). Although the culture media were concentrated by Vivaspin columns, the rRVV was still undetectable, as shown in Figure 24.

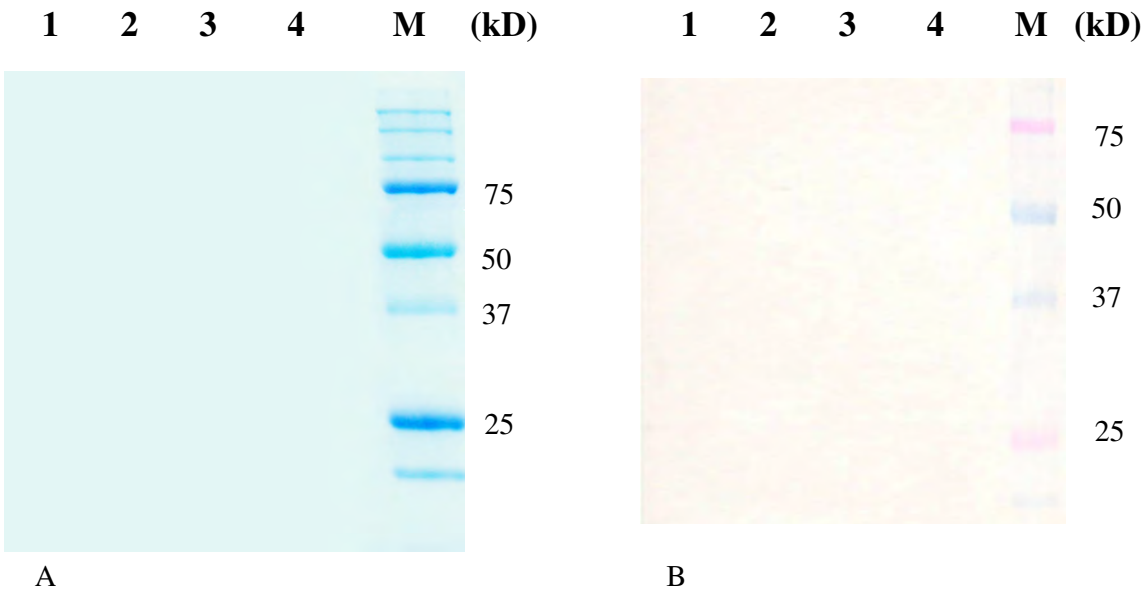


Figure 23. SDS-PAGE analysis of culture media from methanol-induced *P. pastoris*. Lane 1, 24 hrs; Lane 2, 48 hrs; Lane 3, 72 hrs; Lane 4, 96 hrs; M, Protein ladder. A, Coomassie blue staining; B, Western blot analysis

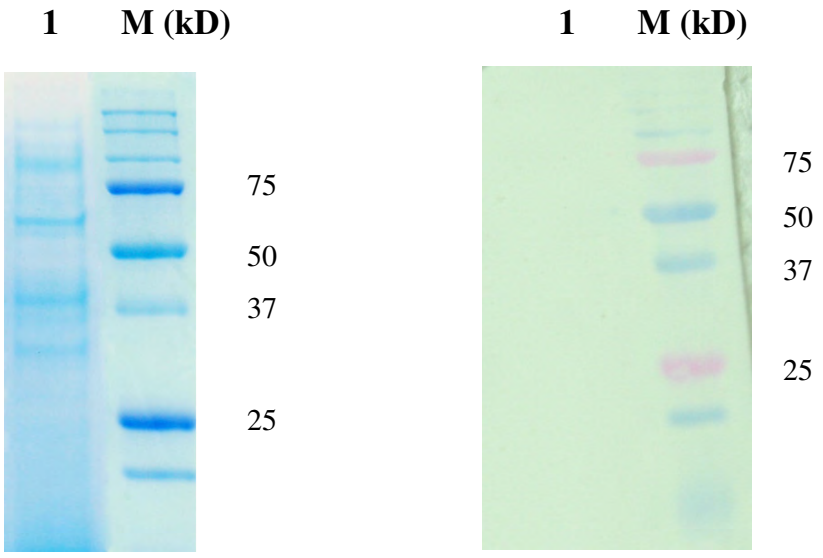


Figure 24. SDS-PAGE analysis of concentrated culture media from methanol-induced *P. pastoris*. Lane 1, Concentrated culture media; M, Protein ladder A, Coomassie blue staining; B, Western blot analysis.

(iv) Arginine esterase activity

The concentrated rRVV-V was tested for its arginine esterase activity with Benzoylarginine ethyl ester (BAEE). Unfortunately, no activity was observed. There was no change in OD253.

2.3. Expression of rRVV-V in *P. pastoris* with pPinkα-HC

(i) Molecular cloning of RVV-V cDNA into pPinkα-HC

Instead of using antibiotic for selection, pPinkα-HC system use *ADE* gene for selection, in which the transformants can get more integrant DNA than using drugs. To clone rRVV-V into pPinkα-HC, the mature protein-encoding sequence of RVV-V cDNA was amplified with a pair of primer containing *SmaI* and *FseI* (Fig 7 and Table 3). The PCR product was cloned into pGEM-T easy and the recombinant pGEM-T plasmids were restriction digested by *SmaI* and *FseI*. The digested rRVV-V cDNA was then cloned into the pPinkα-HC that was linearized by digestion with *Stu*I and *Mly*I. The recombinant pPinkα-HC plasmids were digested with *BanHI* at 37 ^{o}C for 4 hrs and electrophoresed in 1% agarose gel (Fig 25).

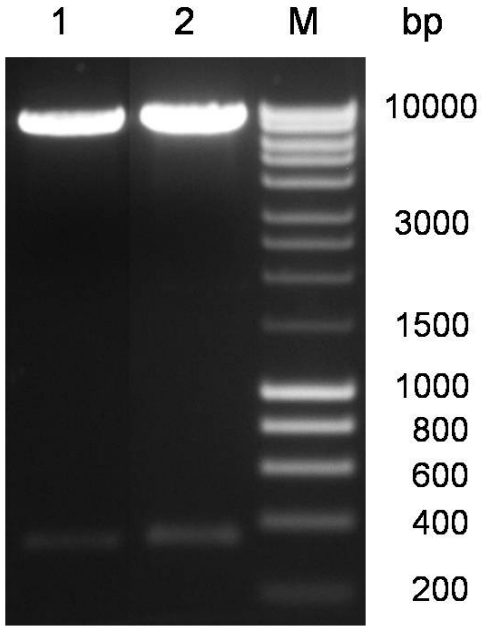


Figure 25. Restriction enzyme digestion of the recombinant pPinkα-HC. The recombinant pPinkα-HC plasmids were digested with *BanHI* at 37 ^{o}C for 4 hrs and

electrophoresed in 1% agarose gel. The 380 bp DNA fragments were observed. Lanes 1: RVV-V; Lanes 2: PA

(ii) Transformation of recombinant pPinkα-HC into *Pichia pastosis*

The recombinant pPinkα-HC-RVV-V was transformed into *Pichia pastoris* using Biorad electroporation system. The transformants, which were able to grow in adenine dropout medium with white color, were selected for rRVV-V expression. The presence of recombinant pPinkα integration in the Pichia genomes were determined by PCR (Fig 26).

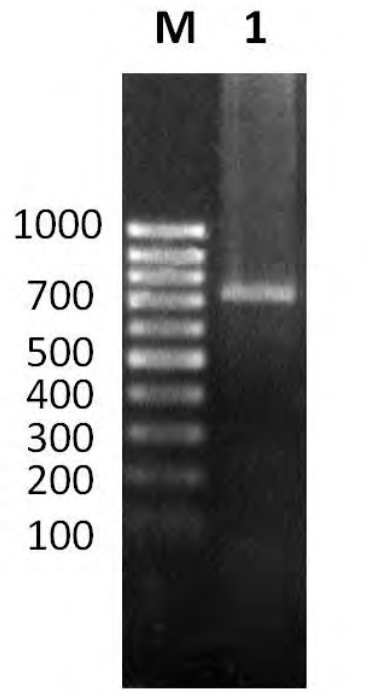


Figure 26. PCR screening of pPinkα-HC transformant. Lane 1, RVV-X heavy chain; M, DNA ladder.

(iii) Induction of protein expression in *P. Pastoris* by methanol

The white colonies of transformed *Pichia pastoris* were cultured in BMGY and BMMY media. After the protein induction by methanol, culture media were collected to investigate the protein expression at every 24 hour for 2 days. Many protein bands were observed (Fig 27).

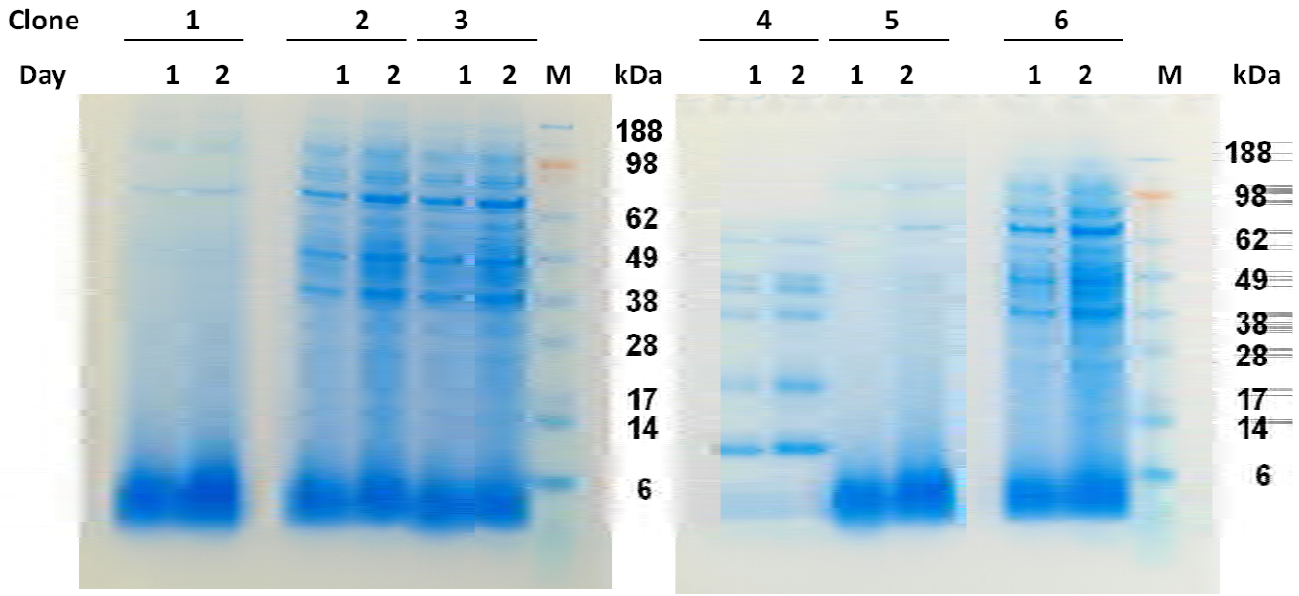


Figure 27. SDS-PAGE analysis of culture media from methanol-induced *P. pastoris* transformed with pPinkα-HC.

(iv) Arginine esterase activity

Although a number of protein bands were observed, all culture media tested for arginine esterase activity with Benzoylarginine ethyl ester (BAEE) showed negative result.

CHAPTER V

DISCUSSION AND CONCLUSION

1. Sequence analysis of RVV-V and other serine proteinases from snake venom

The venoms from *Daboia russelli* are diverse and complex and compose of hundreds of toxic proteins. Identification of these compounds is technically difficult and takes time. Many proteins found in RV venom play role in hematostasis alteration while some are responsible for normal cell functions. However, many proteins could not yet be identified since there are limited toxin sequences in the databases to date. Although RVV-V has been purified and its amino acid sequence has veen reported, its cDNA is still unknown. This is the first study that cloned and characterized the cDNA sequence of RVV-V that has not reported yet. In addition, to our knowledge, this is the first report of the cDNAs encoding alpha- and beta- fibrinogenases in Russell's viper venom which have not been found in Russell's viper venom.

1.1 Molecular cloning of RVV-V cDNA

RVV-V cDNA contains all characteristics of eukaryotic cDNA: a start codon for methionine (ATG), stop codon (TGA), polyadenylation signal (AATAAA) and poly (A) tail. The pre-pro peptide indicated that the RVV-V is expressed and secreted to the venom glands as a zymogen. The translated signal peptide sequence of the RVV-V was rich in hydrophobic amino acid residues which were highly conserved

among snake venom serine proteinases (Fig. 11). However, genetic polymorphism between the sequence in this study and that in GenBank since one amino acid substitution (H203K) was observed (Fig. 11). RVV-V cDNA showed high identity with the cDNA of VLFVA, the factor V activator from *Macrovipera lebetina*. The highest identity was found in the activation peptide-coding region (100%). Analysis of number of nucleotide substitutions per synonymous site (K_S) for the mature protein coding region and Numbers of nucleotide substitutions per site (K_N) for the 3' UTR of the RVV-Vγ cDNA indicated that the mature protein coding region of the RVV-V cDNA evolved more rapidly than the conserved UTR. This observation was also found in serine proteinases and phospholipase A_2s from *Trimeresurus flavoviridis* and *T. gramineus*, and serine proteinases from *Deinagkistrodon actus* (Deshimaru et al., 1996; Nakashima et al., 1995; Nikandrov et al., 2005; Tani et al., 2002). Thus, the RVV-V cDNA revealed new information that RVV-V is expressed as a zymogen which requires proteolytic cleavage for activation. RVV-V cDNA was submitted in GenBank (Accession No HQ270463).

1.2 Molecular cloning of two novel genes from RVV transcripts

Consensus RT-PCR successfully amplified two novel cDNAs from Russell's viper gland cDNA. However, only the RVV-Vγ cDNA was amplified. The alpha- and beta isoforms of RVV-V were purified along with the gamma isoform by Tokunaka (1988). In the study of VLFVA, only one isoform was also observed. Two novel cDNAs, RVAF and RVBF, were highly similar to alpha-fibrinogenase and beta-fibrinogenase of *Macrovipera lebetina*, respectively. Expressions of the homologous

factor V activators, fibrinogenase enzymes, as well as the factor X activators, the P-IV metalloproteinases, in the Russell's viper and *Macrovipera lebetina* venom gland indicated evolutionary relation between both species despite of geographic difference (Chen et al., 2008; Siigur et al., 1999; Siigur et al., 2004). A spot of RVBF was found from a proteomic study of Russell's viper venom, but both RVAF and RVBF have not been purified yet. The cDNAs of RVAF and RVBF, therefore, have been firstly reported in this work and submitted in Genbank (Accession No. HQ270464 and HQ270465 for RVAF and RVBF, respectively). Discovery of novel genes in Russell's viper venom can improve understanding about RVV envenomation, as well as RVV bite therapy.

1.3 Comparison of RVV-V, RVAF and RVBF by Multiple Alignment

Multiple sequence alignment also showed some characteristics of snake venom serine proteinases (Fig 11). All proteinases contained conserved catalytic triad (His-Asp-Ser) which works together in catalytic reaction. The twelve conserved cysteines, which form six disulfide bridges for stabilizing the toxin, were also found in all proteinases. Besides, the amino acid sequence at the residue 81-84, which is located in solvent-exposed loop (Zhang et al., 1997), represented activity-related characteristic (Fig. 2). The sequence FPNG found only in the RVV-V and the VLFVA may contribute to the factor V activation (Siigur et al., 1999). TSV-PA ontained the charged peptide KDDE (95-98, numbering based on that of a-chymotrypsin). Substitution of D97V in TSV-PA resulted in 125-fold decrease in plasminogen activation, which might be the effect of the proximity of the DDE loop to the catalytic site (Zhang et al., 1997). When TSV-PA bind substrates, Asp97 of

TSV-PA electrostatically interacted with the residues vicinal to the cleavage peptide bond of plasminogen (Lys556-Lys557) (Zhang et al., 1997). Although α- and β- hydrolyze different chains of fibrinogen, the unique sequence at the residue 81-84 shared in VLAF, VLBF, RVAF and RVBF may influence binding of the fibrinogenases and fibrinogen. Only the polar residue Ser81 was replaced by another polar amino acid, Asn81, in RVBF.

D176G (mature RVV-Vγ numbering; 189 in Chymotrypsinogen numbering), a residue located in the bottom of the primary specificity pocket near the active site, was found only in RVAF, RVBF, VLAF and VLBF (Siigur et al., 2003). This substitution was associated with lack of arginine esterase activity of VLAF, although other unknown factors may involve to the present of this activity in VLBF (Siigur et al., 2003; Siigur et al., 1991). In addition to the RRR (residue 172-174) that distinguished VLAF from VLBF (Siigur et al., 2003), the PHK (residue 206-208) presented in VLBF and RVBF may also distinguish the beta serine fibrinogenases from the alpha isoform.

1.4 Phylogenetic analysis of RVV-V, RVAF and RVBF

Phylogenetic tree approach can be successfully performed to classify these proteinases according to their functions. RVV-Vγ and VLFVA were isolated from other Viperidae proteinases , indicating that the factor V activator genes have evolved in unique way. The fibrinogenase cluster contained RVAF, RVBF, VLAF and VLBF with strong bootstrap value of 99%. Since the snake venom serine proteinases share high homology in both nucleotide and amino acid sequences, they lack the unique

sequences which can be used for classify their fuctions. So phylogenetic tree become a powerful tool to classify these proteins and help in predicting the function of newly discovered serine proteinase cDNAs, giving the direction for further functional assays. More biochemical experiments should be performed to confirm the presence of RVAF and RVBF in Russell's viper venom.

1.5 Investigation of positive selection in RVV-V, RVAF and RVBF

Ratio of number of nucleotide substitutions per nonsynonymous site (K_A) and number of nucleotide substitutions per synonymous site (K_S) can be used for compare evolution rate between genes. K_A/K_S values of the investigated genes, which were close to or greater than one, indicated that the protein-coding regions of serine proteinases from Russell's viper have evolved in accelerated manner. On the other hand, slower evolutionary rates may consequently result in less diversity of Elapidae serine proteinases than that of Viperidae, since a number of Viperidae serine proteinases have been reported (Serrano and Maroun, 2005). It has been proposed that evolution of snake venom toxin families is a result of gene recruitment, in which alternate genes emerged from ancestor gene by altered gene expression, followed by mutations, gene duplication, and functional constraint (Fry and Wuster, 2004; Soto et al., 2007). According to this hypothesis, serine proteinases were recruited before Colubroid radiation, and accelerated evolution has subsequently taken place especially in Viperidae serine proteinases to gain variety of functions while some characteristics, such as the catalytic triad and disulfide bridges, have been retained. The reasons underlying positive selection of the snake venom serine proteinases might be according to (a) the importance of snake venom toxins to kill pray and

protect the snakes themselves, in which the high virulence venoms have been evolved, and (ii) the snake venom proteins are secreted proteins which are not important for the snake cells; so they have capability to gain mutations without affecting the snake.

2. Production of recombinant RVV-V

Snake venoms are complex mixture of enzymes and peptides which cause medical emergencies. However, some molecules in the venom have potential applications in medicines. Purification techniques have been used to isolate and characterize useful molecules. Many of these molecules are in a low concentration and in many cases it is easier to clone the molecules in bacteria or yeast. In this study, the effort to produce recombinant RVV-V (rRVV-V) has done on two microorganisms, *Escherichia coli* and *Pichia pastoris*.

2.1 Production of rRVV-V in *E. coli*

Although expression of heterologous proteins in *E. coli* offers many advantages including inexpensive cost, rapid biomass accumulation, capability for high-cell density fermentation, and simple process scale up, lack of post-translational machinery and production of inactive protein due to the formation of inclusion bodies are the major challenge. IPTG-induced protein expression was performed in 25, 30 and 37^{o}C and the rRVV-V was obtained. Although 37^{o}C is appropriate temperature for growing many kinds of prokaryotic organism including *E. coli*, it seems that protein expression in 30^{o}C yields highest. The increased expression at lower growth temperatures may be associated with increased expression of a number of chaperones

in *E. coli* (Sahdev et al., 2008). To improve the strategy of rRVV-V expression in *E. coli*, molecular chaperones were included. Chaperones can help in recombinant protein refolding and involve in preventing protein aggregation. This gets benefits since the renaturing processes for recover the inactive inclusion bodies can be escaped. With co-expression of molecular chaperones, small amount of the soluble rRVV-V was obtained. However, the purified rRVV-V did not exhibited activity to cleave the arginen esterase substrate. So, rRVV-V mat require more post-translational modifications that does not exist in *E. coli*.

2.2 Production of rRVV-V in *P. pastoris*

The yeast, *Pichia pastoris,* was used for produce the rRVV-V. Since *P. pastoris* is an Eukaryotic organism, it provide post-translational modifications for the expressed recombinant protein and it is easy to handle like other microorganisms. High yields in production of recombinant proteins were also reported. However, in this study, the expression yield of heterologous RVV-V in *Pichia pastoris* was undetectable or very low. In many attempts, several parameters which may affect the expression yield were changed including *Pichia* strains (X33, and pPink strains), induction time, expression vector, etc. However, the protein expression of RVV-V still unsatisfied. The explanation for unsuccessfully *Pichia* expression might due to the nature of RVV-V itself or even the transformed yeasts. The expression of the rVV-V may be harmful to the yeast cell itself leads to low or undetectable protein expression. Further improved strategies are required for produce the active rRVV-V, including production of specific antibodies against the venom toxins.

3. Conclusion

In conclusion, this study has successfully cloned and characterized the undiscovered RVV-V cDNAs from Russell's viper gland transcripts. In addition, two novel cDNAs, RVAF and RVBF, were also firstly observed in Russell's viper venom gland by consensus PCR, since neither RVAF nor RVBF have been purified. RVV-V, RVAF, RVBF, as well as other snake venom serine proteinases, contained the characteristics of snake venom serine proteinases: highly conserved catalytic triad and 12 cysteins, which form six disulfide bonds. Accelerated evolution was also observed. Phylogenetic tree approach can be successfully performed to classify RVV-V, RVAF, RVBF and other proteinases according to their functions. Characterization of these proteinases can provide information for further studies, including specific activity assays and site-direct mutagenesis. The recombinant expression of RVV-V was performed in *E. coli* and yeast. Although the functional rRVV-V was obtained in neither *E. coli* nor *Pichia pastoris,* more improved strategies for produce the active protein in high yield are required in further studies.

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APPENDICES

APPENDIX A

RESEARCH INSTRUMENTS

Automatic adjustable micropipette (Eppendorf, Germany)

Balance (Precisa, Switzerland)

Beaker (Pyrex)

Combs (BIO-RAD, USA)

DNA Thermal cycler 2400 (Perkin Elmer, Cetus USA)

Electrophoresis Chamber set (BIO-RAD, USA)

Flask (Pyrex)

Heat block (Bockel)

Parafilm (American National Can, USA)

Pipette boy (Tecnomara, Switzerland)

Pipette tip (Axygen, USA)

Polypropylene conical tube (Elkay, USA)

pH meter (Eutech Cybernataics)

Microcentrifuge (Eppendorf, USA)

Microscope (Olympus)

Reagent bottles (Duran)

Spectrophotometer (BIO-RAD, USA)

Thermometer (Precision, Germany)

Vortex (scientific Industry, USA)

Needle (Nipro)

APPENDIX B

RESEARCH REAGENTS

Absolute ethanol (Merck)

Acetic acid (Merck)

Agar (Scharlau)

Agarose (USB, Spain)

Ampicillin (M&H manufacturing)

Bromphenol blue (Sigma, USA)

Chloroform (Merck)

Disodium ethylenediamine tetraacetic acid: EDTA (Merck)

Ethidium bromide (Sigma)

Glucose (Merck)

Glycerol (Phamacia Amersham)

Hydrochloric acid (Merck)

IPTG (USB)

Isoamyl alcohol (Merck)

Phenol (Sigma, USA)

Sodium acetate (Merck)

Proteinase K (Phamacia Amersham)

Sodium hydroxide (Merck)

Sucrose (Sigma, USA)

Tris base (USB)

Triton X-100 (Sigma, USA)

Tween 20 (Sigma, USA)

100 bp DNA ladder (NEB, USA)

Deoxynucleotide triphosphates (dNTPs) (Invitrogen, USA)

Oligonucleotide primer (IDT, USA)

Taq DNA polymerase (Invitrogen, USA)

BIOGRAPHY

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Abstracts and presentations:

- **Pattadon Sukkapan**, Issarang Nuchprayoon. Production of antivenom by immunization with DNA encoding snake venom serine protease. 14th International Congress of Immunology, Kobe, Japan, August 22-27, 2010. (Poster presentation)
- **Pattadon Sukkapan**, Issarang Nuchprayoon. Molecular cloning and expression of factor V activator (RVV-V) from Russell's viper venom. 8th IST Asia – Pacific Meeting on Animal, Plant and Microbial Toxins, Hanoi, Vienam, December 2-6, 2008. (Poster presentation)
- **Pattadon Sukkapan**, Issarang Nuchprayoon. Molecular cloning of full-length cDNA encoding factor V activator from Russell's viper venom. The Fifth Princess Chulabhorn International Science Congress Evolving Genetics and Its Global Impact. Bangkok, Thailand, August 16-20 2004. (Poster presentation)