การศึกษาการแสดงออกของยีนในต่อมพิษงูแมวเซาและการโคลนยีน การแสดงออกและการศึกษาการทำงานของ FACTOR X ACTIVATOR ของงูแมวเซา

นายอาคม ใสงาม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2550

GENE EXPRESSION ANALYSIS OF RUSSELL'S VIPER VENOM AND MOLECULAR CLONING, EXPRESSION AND FUNCTIONAL STUDY OF RV FACTOR X ACTIVATOR

Mr. Arkhom Saingam

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Biomedical Sciences

(Interdisciplinaray Program)

Graduate School

Chulalongkorn University

Academic Year 2007

Thesis Title	GENE EXPRESSION ANALYSIS OF RUSSELL'S VIPER VENOM AND MOLECULAR CLONING, EXPRESSION AND FUNCTIONAL STUDY OF RV FACTOR X ACTIVATOR
By	Mr. Arkhom Saingam
Field of study	Biomedical Sciences
Thesis Advisor	Associate Professor Issarang Nuchprayoon, M.D., Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

..... Dean of the Graduate School
(Assistant Professor M.R. Kalaya Tingsabadh, Ph.D.)

THESIS COMMITTEE

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

..... Thesis Advisor
(Associate Professor Issarang Nuchprayoon, M.D., Ph.D.)

..... Member
(Associate Professor Ponlapat Rojnuckarin, M.D., Ph.D.)

..... Member
(Thaweesak Tirawatnapong, Ph.D.)

..... Member
(Assistant Professor Apinunt Udomkit, Ph.D.)

อาคม ใสงาม : การศึกษาการแสดงออกของยีนในต่อมพิษงูแมวเซาและการโคลนยีน การแสดงออกและการศึกษาการทำงานของ FACTOR X ACTIVATOR ของงูแมวเซา (GENE EXPRESSION ANALYSIS OF RUSSELL'S VIPER VENOM AND MOLECULAR CLONING, EXPRESSION AND FUNCTIONAL STUDY OF RV FACTOR X ACTIVATOR) อ. ที่ปรึกษา : รศ. นพ. ดร. อิศรางค์ นุชประยูร, 98 หน้า.

งูแมวเซา (Russell's viper) เป็นงูพิษที่พบได้ทั่วไปในหลายภูมิภาคของเอเชีย พิษของงูแมวเซามีความรุนแรง โดยอาจทำให้ผู้ถูกกัดเสียชีวิตได้ และงูแมวเซาในแต่ละภูมิภาคจะมีพิษที่แตกต่างกัน งานวิจัยนี้มีวัตถุประสงค์คือ 1) วิเคราะห์องค์ประกอบของพิษงูแมวเซาโดยเทคนิค Expressed Sequence Tags (ESTs) 2) นำพิษงูที่เป็นประโยชน์มาประยุกต์ใช้ทางการแพทย์ 3) ผลิตแอนติบอดี ที่มีความจำเพาะต่อพิษที่สำคัญๆ ดังได้กล่าวแล้ว ข้อมูลการแสดงออกของยีนจากต่อมพิษงู สามารถใช้เป็นเป็นแนวทางในการศึกษาความเป็นพิษของงูได้ ดังนั้น ห้องสมุดยีนจึงได้สร้างขึ้นจากต่อมพิษงู เพื่อที่จะศึกษาองค์ประกอบพิษของมัน จากการสุ่มจำนวน 135 โคลน มาหาลำดับเบส พบว่า 77 โคลน (57%) มีความเหมือนกับฐานข้อมูลยีนใน genbank โดยยีนที่พบมากคือ phospholipase A2, factor X activator and factor V activator, BPP-CNP homolog ซึ่งล้วนมีบทบาทต่อระบบ hematostasis ทั้งสิ้น ในขณะเดียวกันได้ทำการโคลนและแสดงออกของแฟคเตอร์เทนแอกติเวเตอร์ (RVV-X) ใน *Pichia pastoris* ข้อมูลจากการทำ Northern analysis พบว่า mRNA ของ RVV-X heavy chain และ light chain มีขนาดประมาณ 2.5 kb และ 1.0 kb ตามลำดับ การแสดงออกของ RVV-X ใน pichia yeast ให้ผลค่อนข้างต่ำ มีเพียง RVV-X light chain ที่สามารถพบได้เมื่อทำ SDS-PAGE และสามารถจับได้กับ anti-His (C-term) antibody และยังจับได้กับ antibody ของม้าที่ได้จากการกระตุ้นด้วยพิษงูแมวเซา นอกจากนี้ ยังได้นำ RVV-X มาทำการทดลองเพื่อสร้าง antibody ในหนูทดลองโดยเทคนิค DNA immunization พบว่า RVV-X ส่วนที่เป็น disintegrin domain สามารถสร้าง antibody ได้ดีในหนูทดลอง โดยสรุปคืองานวิจัยนี้ได้รายงานชนิด องค์ประกอบของพิษงูแมวเซา และมีความพยายามที่จะนำโปรตีนพิษที่สำคัญทางการแพทย์ไปใช้ รวมทั้งยังเสนอวิธีการสร้างแอนติบอดีที่มีความจำเพาะต่อพิษโดยวิธี DNA immunization อีกด้วย

สาขาวิชาชีวเวชศาสตร์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษา.....

458 96910 20 : MAJOR BIOMEDICAL SCIENCES

KEY WORD : GENE EXPRESSION / RUSSEL'S VIPER / EXPRESSED SEQUENCE TAGS / FACTOR X ACTIVATOR / DNA IMMUNIZATION

ARKHOM SAINGAM : GENE EXPRESSION ANALYSIS OF RUSSELL'S VIPER VENOM AND MOLECULAR CLONING, EXPRESSION AND FUNCTIONAL STUDY OF RV FACTOR X ACTIVATOR. THESIS ADVISOR : ASSOC. PROF. ISSARANG NUCHPRAYOON, M.D., Ph.D., 98 pp.

Russell's viper, *Daboia russelli*, is a venomous snake widely distributed in several parts of Asia. RV bites from different geographical areas cause variety of signs and symptoms with severe injury or even death in human. This study was aim at (1) characterization the toxin composition of Thai RV venom by Expressed Sequence Tags (ESTs) analysis, (2) utilization of the medical useful toxin by DNA recombinant technology, and (3) production of toxin-specific antibody by DNA immunization. ESTs are an important and rapid tool to characterize the pattern of gene expression of organism. cDNA library was constructed from the snake venom glands to study the active genes in the venom. A total 135 ESTs have been generated from size-selected clones at random. Fifty-seven percents (77/135) of ESTs were significant matching to the entries in the database. Of these, several ESTs involved in the toxicity of the venom were obtained. e.g. phospholipase A2, factor X activator and factor V activator, BPP-CNP homolog, etc. Some of cDNA sequences of these genes showed a variation from the other reports from RV from other region. These ESTs data could provide a resource of the expressed pattern relating to the clinical pathology of RV bite. In the mean time, factor X activator (RVV-X), one of RV key toxin, was cloned, characterized, and expressed in *Pichia pastoris*. Northern analysis indicated that the mRNA of RVV-X heavy chain and light chain were about 2.5 kb and 1.0 kb, respectively. However, the expression of RVV-X was unappreciated in *Pichia* yeast. Only heterologous RVV-X light chain could be seen in SDS-PAGE while RVV-X heavy chain could not be detected. RVV-X light chain could also be detected by anti-His (C-term) antibody and RVV-immunized horse antibodies. Moreover, to generate toxin-specific antibody, RVV-X was cloned into pVaxSec mammalian expression vector for DNA immunization in mice. RVV-X disintegrin domain showed to be a good DNA immunogen in antibody production in mice while RVV-X with other domains gave unsatisfied results. Antibody from RVV-X disintegrin domain-immunized mouse serum could bind to the RVV-X in both reduced and non-reduced forms. In summary, this work has identified the Thai RV venom composition by characterization the RV transcripts and could produce heterologous RVV-X light chain in *Pichia pastoris*. In addition, RVV-X-specific antibody was successfully produced by DNA immunization in mice which may be developed for the large-scale antibody production in other mammals.

Field of study Biomedical Sciences Student's signature.....

Academic year 2007 Advisor's signature.....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my advisor, Associate Professor Dr. Issarang Nuchprayoon for his competent supervision, guidance, encouragement, patience, and criticism which have inspired me to accomplish my study.

I am very grateful to my thesis co-advisor, Dr. Robert Harrison, for his kindly supervising and taking care when I visited Liverpool School of Tropical Medicine, Liverpool, United Kingdom. My grateful appreciation is extended to Associate Professor Dr. Surang Nuchprayoon, Professor Dr. Apiwat Mutirangura, Associate Professor Dr. Ponlapat Rojnuckarin, Dr. Thaweesak Tirawatnapong and Assistance Professor Dr. Apinunt Udomkit, for their valuable suggestions and criticisms.

I am particularly indebted to the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program, Snake Bite and Venom Research Unit, Chulalongkorn Medical Research Center, Faculty of Medicine, Chulalongkorn University for supporting equipments and other utilities.

I am also indebted to all members in Lymphatic Filariasis Research Unit, and Snake Bite and Venom Research Unit for their encouragement, sincerity, and friendship.

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

TABLE OF CONTENTS

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

Figure **Page**

LIST OF ABBREVIATIONS

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

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

CHAPTER I

INTRODUCTION

1. Background and Rationale

Russell's viper (RV), *Daboia russelli*, is a venomous snake widely distributed in East and Southeast Asia. At least 5 subspecies of RV have been recognized based on minor differences 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). In Thailand, RV is often found in central and eastern part. The RV stout body is tan with a series of multi-coloured blotches of variable shape and size, adult RV is up to 150 cm in length, **Figure 1**. The prevalence of snake bite in Thailand from Ministry of Health is approximate 7,000 each year (สุชัย, 2542). However, there is no formally recorded for RV bite.

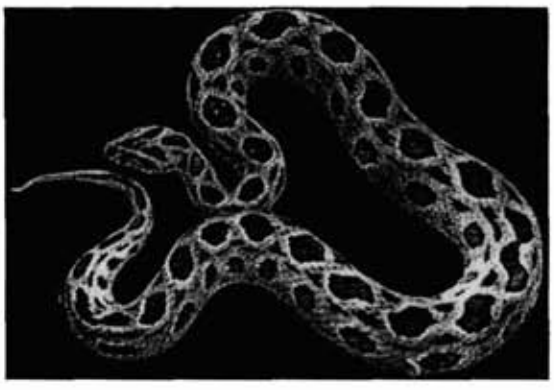


Figure 1. Morphology of Russell's viper snake.

The venom of RV is potentially fetal and known to affect haematostasis system of its preys. Signs and symptoms of RV bite include disseminated intravascular coagulation (DIC), haemolysis, rhabdomyolysis, neurotoxicity, platelet damage, oedema formation, vasodilatation causing hypotention, release of autacoids such as histamine, etc. (Slater *et al.*, 1988; Kini *et al.*, 1986). RV venom is a mixture of several poisonous substances and could contain as much as 70% of the protein phospholipase A2 of which at least seven isoenzymes (Denis *et al.*, 1994). Other enzymes include L-amino oxidase, endonuclease, phosphodiesterase, 5'-nucleotidase, phosphomonoesterase, paraoxonase, hyaluronidase, and a variety of proteinases (Pukrittayakamee *et al.*, 1990). Although some proteins in RV venom have been isolated and identified, most components have not been characterized yet.

1.1. Study of Gene Expression in RV Venom Gland

There are some genetic variations among RVs, lead to the differences in venom composition and cause broad symptoms or effects to preys. Their antivenom raised against each sometime could not cross neutralize the toxin (Woodhams *et al.*, 1990). Prediction of the clinical signs from snake bite required well-identified toxin components of the venom. However, isolation and characterization of hundreds of toxins in snake venom is difficult. Study of expressed sequence tags (ESTs) is a rapid and valuable as the first glimpse for studying gene expression. ESTs approach is an alternative of protein characterization which is more complicate and time consuming. Recently, there are only a few records of nucleotide or protein sequences of RV in online database. Therefore, the ESTs data of RV venom from this research not only give insight in venom biology but hopefully in clinical management of snake bite also.

1.2. Russell's Viper Factor X Activator, RVV-X

RVV-X, a potent activator of factor X purified from RV venom, is a well characterized proteinase. Their protein sequences including one heavy chain and one light chain have been reported in 1992 by direct amino acid sequencing (Takeya *et al.*, 1992). It specifically activates factor X as a result of a single cleavage at the same internal Arg-Ile bond in factor X as do factors IXa and VIIa (Fujikawa *et al.*, 1972). RVV-X catalyzed factor X activation, however, is not inhibited by diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride but is inhibited by EDTA, thereby suggesting that RVV-X is not a serine proteinase but a metalloproteinase (Kisiel *et al.*, 1976). Unlike vitamin K-dependent clotting factors, RVV-X does not require a negatively charged surface such as phospholipids for factor X activation, but does require exogenous Ca^{2+} and the amino-terminal Gla domain of factor X for enhanced activation.

RVV-X is a glycoprotein containing 13% carbohydrate with an apparent M.W. of 79,000. It is composed of two disulfide-bonded chains, a heavy chain of M.W. 59,000 and heterogeneous light chains with M.W. 18,000 and 21,000. The amino acid sequence of heavy chain and one of the light chain (LC1) has been characterized. The mature heavy chain consists of 427 amino acid residues containing four asparagine-linked oligosaccharides. Its entire sequence is similar to that of the high molecular mass hemorrhagic protein, HRlB, isolated from the venom of *Trimeresurus flavoviridis*. The heavy chain compose of three distinct domains, metalloproteinase, disintegrin (platelet aggregation inhibitor)-like, and unknown cysteine-rich domains. On the other hand, light chain LC1 consists of 123 amino acid residues containing one asparagine-linked oligosaccharide and shows sequence homology similar to that found in the so-called C-type (Ca^{2+}-dependent) lectins.

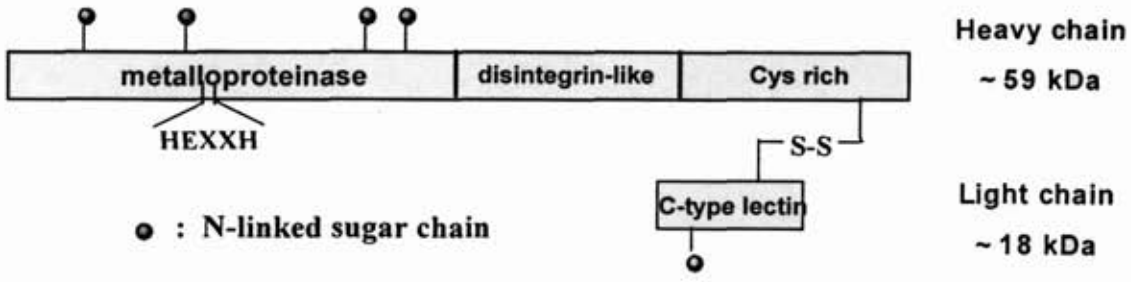


Figure 2. Schematic structure of RVV-X which composes of heavy chain and light chain. Heavy chain can be divided in three domains; metalloproteinase, disintegrin-like, and Cys rich domains. **HEXXH** is a conserved zinc-binding sequence found in melalloproteinase domain of heavy chain.

1.3. Treatment of RV bite

In Thailand, Queen Saovabha Memorial Institute has been successfully produced 6 antivenoms against Thai major venomous snakes including Russell's viper (Chanhome *et al.*, 2001). The antivenoms were purified from horses' sera after repeated immunized by whole venom from snake. Although the using of conventional antivenoms is life-saving, they contain an immunoglobulin pool of unknown antigen specificity and known redundancy, which necessitates the delivery of large volumes of heterologous immunoglobulin to the envenomed victim. Therefore, it also increases the risk of anaphylactoid and serum sickness adverse effects. To overcome this problem, toxin-specific antibody production is to be considered. However, since snake venom is the mixtures of hundreds of toxins, which are not easy to be isolated or purified, and has limited amount as well. There was the recent exploit molecular sequence analysis and DNA immunization tools to design more rational toxin-targeted

antivenom. Thus, this work are try to demonstrate the using of DNA immunization against a key toxin in RV venom, RVV-X.

2. Objectives

1. To study gene expression profile of Russell's viper venom by Expressed Sequence Tags (ESTs) analysis.
2. To produce active recombinant RVV-X in *Pichia pastoris*.
3. To produce the specific antibody against RVV-X by DNA immunization.

3. Keywords

Gene expression

Russell's viper

Expressed Sequence Tags

Factor X activator

DNA immunization

4. Expected Benefits & Application

Information in gene expression of RV venom would provide more understanding in molecular biology of snake venom which may finally lead to the better treatment of RV bite. The venom expressed profile could also be used to compare Thai RV to other RV from various parts of Asia. Active recombinant RVV-X may be developed in medical used, e.g. in blood clotting test. It also might be developed as bio-drug for treating people such as in hemophilia patients. In addition, the using of toxin-specific antibody could be the alternative way to reduce the

possibility of adverse reaction or side effect from conventional antivenom treatment in some patients. It also improves the efficacy of antivenom use since almost of antibodies are raised from key toxins.

CHARTER II

REVIEW OF RELATED LITERATURES

1. Russell's viper

Russell's viper (*Daboia russelli*) is distributed in wide parts of Asia **(Table 1).** There is no doubt that this species of snake kills large numbers of people in Asia each year. In India, it is the most common cause of the fatal snake bites and kills five out of 100,000 annually (Phillips *et al.*, 1988). In Myanmar, it kills over 1,000 people each year and was during some time the fifth most common cause of death. It also is an important contributor to the fatal snake bite in Pakistan, Thailand, and Sri Lanka (Than-Than *et al.*, 1988). Where it is abundant it is one of the worst snake bite hazards to farmers working in the fields (Phillips *et al.*, 1988). Because of the high frequency of snake bite from this species, there is also an interest in venom research and medical research related to snake bites. This snake is also of medical interest because of the properties of its venom. Its coagulant/anticoagulant properties the venom of this species has been used in investigations of the human blood clotting cascade.

Table 1. The distribution of Russell's viper in several parts of Asia

(Koh *et al.*, 2006).

Subspecies	Distributions
Daboia russelli russelli	Pakistan, India & Bangladesh
Daboia russelli formosensis	Taiwan
Daboia russelli limitis	Indonesia (Java, Komodo, Flores, Lomblen, Endeh)
Daboia russelli pulchella	Sri Lanka
Daboia russelli siamensis	Burma, Endeh, Vietnam, Laos, China (Kwangtung), Thailand & Cambodia

This species of snake is a typical ambush predator. It sits still and waits for prey passing by. The fangs are as with all vipers, long and hollow like a syringe, and when delivering a bite the venom is injected deep into the tissues of the prey. The prey often dies within minutes of being bitten with their hearts and vessels distended with coagulated blood. Larger animals however display the opposite effect i.e. the blood coagulation is seriously prolonged when bitten (Than-Than *et al.*, 1988). Because of the snake somewhat lethargic disposition it often remains immobile when humans are working in the field and relying on its camouflage to remain undetected. The snake is attracted to cultured fields because of the rodents searching for the crops. Therefore, it is very easy to step on the snake by accident. When thread upon it defends itself with a bite. The amount of venom injected varies with the size of snake. Not all bite victims are envenomed, in a study conducted in Sri Lanka 73% of the victims were envenomed (Phillips *et al.*, 1988). Venoms of the same snake species from different geographical regions often exhibit different pharmacological or pathological and antigenic properties. *Daboia russelli* is not an exception in this regard and symptoms after envenomation differ between subspecies (**Table 2**). and also within the different subspecies. Studies carried out in India, Thailand, Burma, and Sri Lanka have revealed a fascinating geographical variation in the clinical manifestations of *Daboia russelli* bite (Nijaguna *et al.*, 1999). Some of the most important symptoms of *Daboia russelli* bites are ascribed to an enzyme, phospolipase A2. This enzyme induces neurotoxicity, myotoxicity, edema, and hemorrhage (Uma, 2000).

Table 2. Broad spectrum of signs & symptoms of Russell's viper bites (Koh *et al.*, 2006).

Species	Signs & Symptoms
Daboia russelli russelli	Acute kidney disorders, anuria, vomiting, nausea, abdominal pain, local swelling from injured part with further spreading, nose bleed, blood vomiting, hematurie, hypotonia, thrombus. Neurological and muscular symptoms possible in South India.
Daboia russelli formosensis	Light local swelling, light local pains, acute Kidney disorders.
Daboia russelli limitis	No clinical data, but deaths reported
Daboia russelli siamensis	*Symptoms Burma* : Vomiting, hypotonia (resulting in syncope) hypovolaeia with circulatory shock, local swelling, blood vomiting, nose bleeds, hematuria, intracerebral bleedings, subarachoidal bleedings, pains in epigastrum, thrombus. As a consequence a chronic insufficiency in the kidneys and pituary gland can occur. *Symptoms China* : Local swelling, necrosis, systemic bleedings, oligurie
Daboia russelli pulchella	Local pain, blurred or double vision, bleedings, difficulty swallowing, generalized muscle aching, black urine, pain in lymphnodes draining the bite site, faithless and loss of consciousness.

1.1. Composition in snake venom

The composition of snake venom differs from species to species. There is also variation within a single species depending on age, season and temperature. It is a complex mixture of enzymes, toxins, and all sorts of smaller molecules. The most important components are the substances with a cytotoxic effect, the neurotoxins and the coagulants. Some toxins have multiple effects. The function of some components is still a mystery. For example, "nerve growth factor" was isolated from cobra venom. This protein, discovered by Rita Levi-Montalcini and Stanley Cohen (Nobel Prize 1986), plays a major role in the growth of nerve tissue, yet why this molecule is present at high concentration in venom in the first place remains an open question. Possibly it promotes the absorption of venom by releasing various mediators from

mastocytes. The common compounds and their actions found in several snake venoms were shown in **Table 3**.

Table 3. Common compounds in snake venom.

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
Proteases	Catalyze reactions that disrupt protein peptide bonds in tissues, causing blood vessel wall damage and hemorrhaging and muscle-fiber deterioration.	Vipers and Pit vipers
Thrombin-like enzymes	Inhibit blood clotting	Vipers, pit vipers, and a few rare elapids
Peptide bradykinin potentiators	Enhancement of one of the body's natural responses to injury by dilating and increasing permeability of blood vessels, stimulates pain receptors and contracts some smooth muscles. This allows the venom to diffuse quickly into the bloodstream, increases bleeding and inhibits the ability to flee	*Bothrops* and *Crotalus* genera
Polypeptide toxins	Disrupts nerve-impulse transmission, causing heart or respiratory failure	Mambas, *Vipera, Crotalus, Bungarus, Naja, Laticaua, Hydrophis* all with different types of toxin.
Nerve growth factor	Stimulates the growth of nerve cells	*Agkistrodon, Crotalus*

Source: *Snakes in Question* by Carl H. Ernst and George R. Zug, 1996

1.2. Toxins affecting the haemostatic system

The major symptoms from snake bite affecting the haemostatic system are (a) reduced coagulability of blood, resulting in an increased tendency to bleed, (b) bleeding due to the damage to blood vessels, (c) secondary effects of increased bleeding, ranging from hypovolaemic shock to secondary-organ damage, such as intracerebral haemorrhage, anterior pituitary haemorrhage or renal damage, and (d) direct pathologic thrombosis and its sequelae, particularly pulmonary embolism (Numeric *et al.*, 2002). More recently, snake venoms have been used in the development of platelet aggregation and blood-clotting inhibitors. Venoms from vipers and some Australian snakes are rich sources of proteases that strongly affect the haemostatic mechanism (White *et al.*, 2005). Coagulant enzymes include activators of the blood coagulation factors II (prothrombin), V and X, while anti-coagulants include protein C activators, inhibitors of prothrombin complex formation and fibrinogenases (based on their specificity for the alpha, beta and gamma chains of fibrinogen). Intermediates between the true coagulants and true anticoagulants are the thrombin-like enzymes which bring about clotting *in vitro* but defribination (anticoagulation) *in vivo*. Snake venoms (including the disintegrins, a group of RGD containing proteins) also affect platelets by inducing or inhibiting platelet aggregation, fibrinolytic activators and haemorrhagins (alpha-fibrinogenases) to cause haemorrhage by acting via platelets or proteolysis of the blood vessel wall (March *et al.*, 1944). Snake venom proteins that affect the blood coagulation cascade are summarized in **Figure 3**. It appears that for every factor involved in the blood coagulation cascade, there is a counterpart among the snake venom compounds that could either activate or inactivate the factors. These activators or inhibitors usually belong to various families such as serine proteases, metalloproteinases, C-type lectins, disintegrins, and phospholipases.

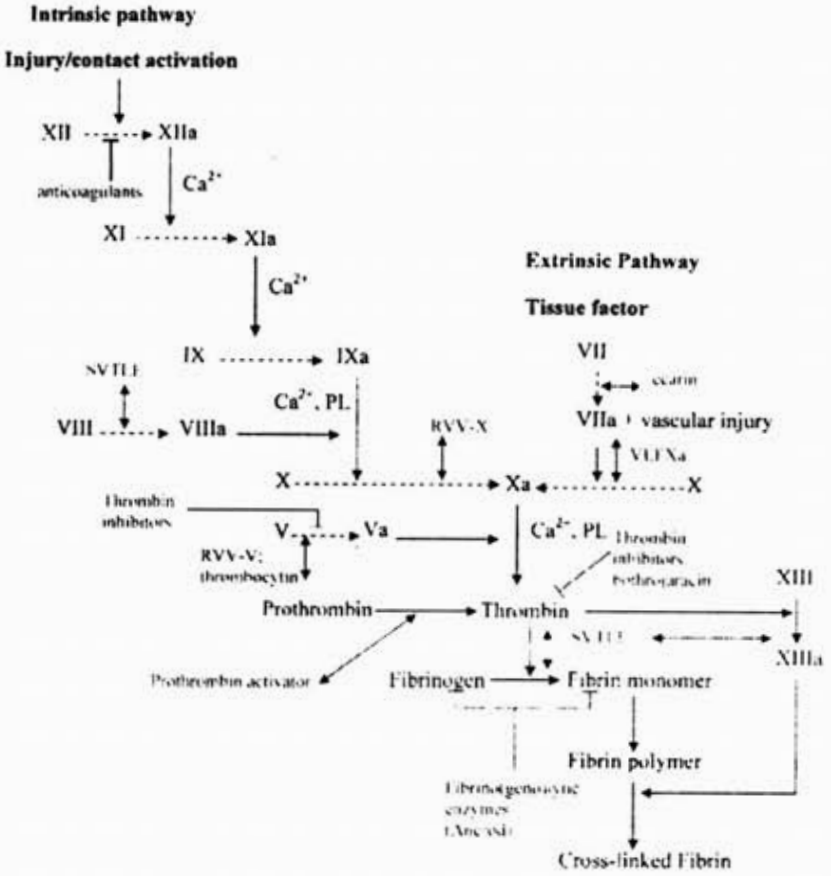


Figure 3. Blood coagulation pathways and the steps in which snake venom proteins interfere. SVTLE, snake venom thrombin-like enzymes; RVV-V, Russell's viper venom factor V activator; RVV-X, Russell's viper venom factor X activator; VLFXa, *Vipera lebetina* factor X activator. Activation by venom protein is denoted by ⟶ , inhibition by ---l. (Koh *et al.*, 2006)

1.3. Russell's Viper factor X activator (RVV-X)

By far the strongest and most important factor X activator is the one found in the venom of Russell's viper. Venom preparations of the major two subspecies, *Daboia russelli russelli* and *Daboia russelli siamensis,* show potencies that are approximately the same and that are at least ten- to a hundred-fold higher than in any other venom (Yamada *et al.*, 1997). The purified factor X activator (RVV-X) contains

some 13% carbohydrate and runs as a single band with 79,000 kDa on SDS-PAGE (Kisiel *et al.*, 1976). It comprises from a heavy chain and 2 light chains, **Figure 4**. The 427- residue heavy chain (classified as SVMP class 4) was found to contain three distinct domains: a metalloproteinase domain containing the conserved HEXXH metalloprotease catalytic site sequence, a disintegrin (platelet aggregation inhibitor) domain, and a cysteine-rich region, the function of which is as yet not completely clear. The disintegrin domain is presumably responsible for the strong antiplatelet-aggregating effect of RVV-X, although the specific RGD sequence is missing (Takeya *et al.*, 1992). The light chain (123 residues) shares homology with several snake C-type (Ca^{2+}-dependent) lectins.

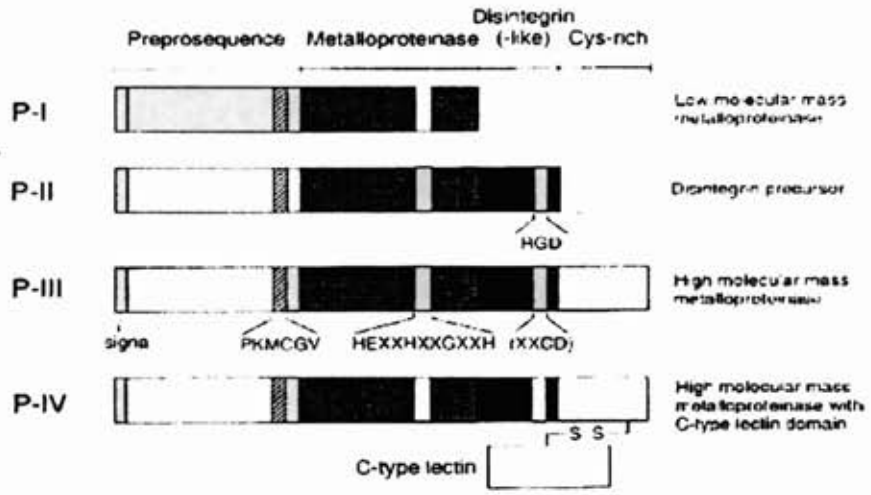


Figure 4. Classification of Snake venom metalloproteinases (SVMPs). RVV-X is classified as P-IV SVMP (Kini *et al.*, 2001).

RVV-X activates factor X by cleaving the Arg-Ile bond at position 194 (**Figure 5**) and is also able to activate factor IX and protein C by specific cleavage of Arg-Ile and Arg-Val bonds (Kisiel *et al.*, 1976). The molecule contains six N-linked oligosaccharides, four of which are located in the heavy chain and one in each light chain. Removal of easily accessible sugars does not appear to change the functional

capacity of RVV-X, but removal of the core sugars with N-glycanase causes a virtually complete loss of factor-Xactivating capacity, apparently as a result of major conformational changes in the molecule (Gowda *et al.*, 1996). The complete amino acid sequence of the heavy chain and the smallest of the two light chains have been determined by direct protein sequencing. Hence, cDNA sequences of RVV-X have not yet been found in the databases. These latter findings are of great interest and can explain some of the mechanistic properties of RVV-X described earlier. The factor IX/X- and X- binding proteins consist of two C-type lectin subunits held together by a single disulfide bond, probably as the result of domain swapping between the two subunits (Mizuno *et al.*, 1997; Mizuno *et al.*, 1999). These C-type lectin heterodimers specifically bind to the Gla domain of factor IX and factor X in a Ca2+-dependent manner (Atoda *et al.*, 1994; Sekiya *et al.*, 1995) as a result of which the binding of these clotting factors to negatively charged membranes is likely blocked (Mizuno *et al.*, 2001).

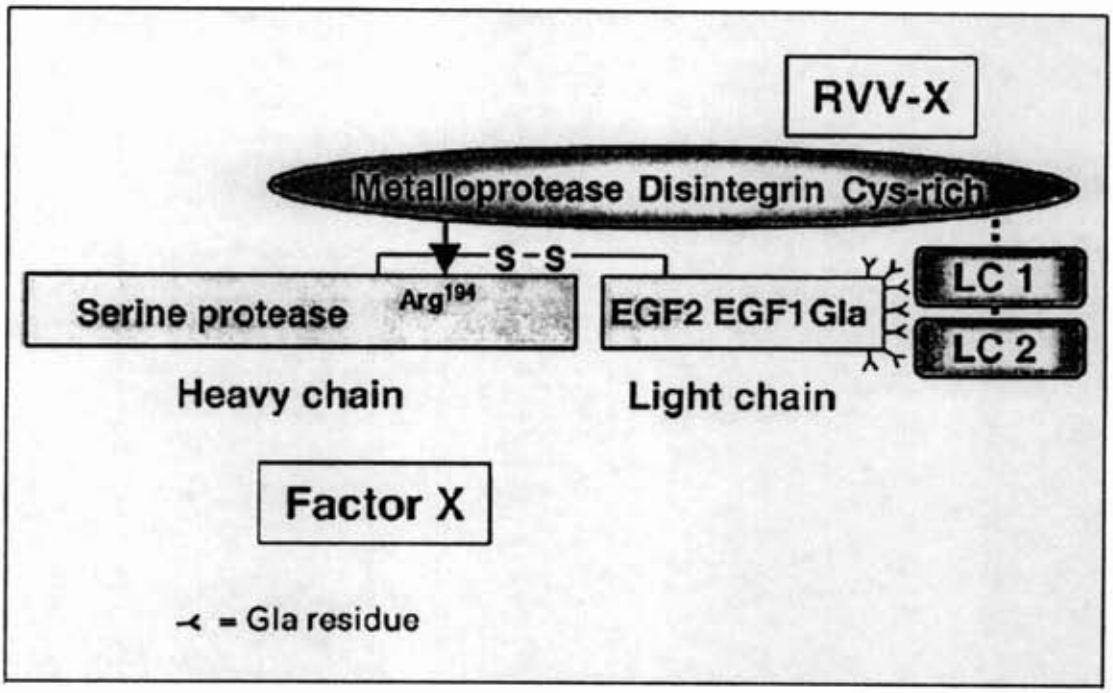


Figure 5. The proposed mechanism of the activation of factor X by RVV-X. The heavy chain of RVV-X contains the metalloprotease domain responsible for the proteolytic cleavage of the target bond in factor X. The two C-type lectin light chains (LC1 and LC2) together form a secondary binding site specific for interaction with the Gla domain of factor X (Tans *et al.*, 2001).

2. Protein expression in *Pichia pastoris*

Pichia pastoris is a species of methylotrophic yeast. *Pichia* is widely used for protein expression using recombinant DNA techniques. Hence it is used in biochemical and genetic research in academia and the biotechnical industry. A number of properties make *Pichia* suited for this task: *Pichia* has a high growth rate and is able to grow on a simple, inexpensive medium. *Pichia* can grow in either shake flasks or a fermentor, which makes it suitable for both small and large scale production.

Pichia pastoris has two alcohol oxidase genes, 4*AOX*1 and *AOX*2, which have a strongly inducible promoter. These genes allow *Pichia* to use methanol as a carbon and energy source. The *AOX* promoters are induced by methanol and are repressed by e.g. glucose. Usually the gene for the desired protein is introduced under the control of the *AOX*1 promoter, which means that protein production can be induced by the addition of methanol. In a popular expression vector, the desired protein is produced as a fusion product to the secretion signal of the α-mating factor from *Saccharomyces cerevisiae* (baker's yeast). This causes the protein to be secreted into the growth medium, which greatly facilitates subsequent protein purification. There are commercially available plasmids, that have these features incorporated. pPicZα-A is one of *Pichia* expression plasmid from Invitrogen that has several advantages. It contains α-factor which facilitates the expressed protein to be secreted into the culture media that make protein purification easier. Zeocin resistance marker in pPicZα-A also be the benefit for the recombinant selection of both *E. coli* and *Pichia* yeast. AOX promoter also allows the protein expression by the induction of methanol. which is cheap and effective inducer.

2.1. Comparison to other expression systems

In standard molecular biology research, the bacterium *Escherichia coli* is the most frequently used organism for production of recombinant DNA and proteins. This is due to *E. coli* fast growth rate, good protein production rate, and undemanding growth conditions. Protein expression in *E. coli* is usually faster than in *Pichia pastoris* for several reasons: Competent *E. coli* cells can be stored frozen, and thawed immediately before use, whereas *Pichia* cells have to be produced immediately before use. Expression yields in *Pichia* vary between different clones, and usually a large number of clones needs to be screened for protein expression before a good producer is found. Optimal induction times of *Pichia* are usually on the order of days, whereas *E.*

coli usually reaches optimal yields within hours of induction. The major advantage of *Pichia* over *E. coli* is that Pichia is capable of producing disulfide bonds and glycosylations in proteins. This means that in cases where disulfides are necessary, *E. coli* might produce a misfolded protein, that is usually inactive or insoluble.

2.2. Snake toxin expression in *Pichia* yeast

Numerous strains of *Pichia pastoris* with a wide range of expression vector have been used to express many heterologous snake toxins, **Table 4**. However, the degree of success was also dependent upon several parameters such as the culture vessel, e.g. pH, temperature, and O_2 availability, and they are dependent on the residual concentrations of methanol. Therefore, appropriate controls are necessary for monitoring the effectiveness of the expression system.

Table 4. Snake venom toxins which successfully expressed in *Pichia pastoris*.

(Raveendra *et al.*, 2007)

Proteins	Number of Residues	No. of cysteines (disulfide bonds)	Vector / Strain	Yield[a] (mg/l)
Disintegrins				
Rhodostomin	68	12(6)	pPIC*ZαA* X33	10–15
Halystatin	76	14(7)	pPIC9 GS115	ND[b]
Echistatin	49	8(4)	pPIC*ZαA* X33	2–5
Three-fingered toxins				
Dendroaspin	59	8(4)	pPIC*ZαA* X33	5–10
Erabutoxin b	61	8(4)	pPIC*ZαA* X33	12–18
α-bungarotoxin	74	10(5)	pPIC9K GS115	0.1
γ-bungarotoxin	68	10(5)	pPIC*ZαA* X33	2–5
κ-bungarotoxin	68	10(5)	pPIC9 GS115	0.1
m1-Toxin1	66	8(4)	pPIC3.5 GS115	0.1
Proteases				
Batroxobin (thrombin-like)	231	12(6)	pPIC9 GS115	7
Cerastobin (thrombin-like)	299	12(6)	pPIC9K GS115	3.5
Protein C activator	231	12(6)	pPIC*ZαA* X33	150
Vasoconstrictor endothelin receptor agonists				
Sarafotoxin	21	4(2)	pPIC9 GS115	ND[b]
Phospholipase A2				
IA-PLA2	126	14(7)	pPIC9 SMD1168	0.4–30
C-Type lectin-like protein				
AFC I ($\alpha\beta$-heterodimer)	α-129	7(7)	pPIC*ZαA*,	1–4
	β-123	7	pPIC9K GS115	

3. Toxin-specific antibody production by DNA immunization

DNA immunization is a proposed experimental technique for producing the specific antibody against desired protein by introducing the naked DNA to trigger an immunological response. Conventional antivenom preparations are relatively crude preparations which produce many antibodies against non-toxic as well as toxic molecules in snake venom. Nowadays, antivenom is prepared from big animal, e.g. horse. Crude venom is repetitively injected into the animal to immunize and boost the immune response. The antibodies were then purified from the horse serum and might be modified to improve their effectiveness such as digested by papain to generate $F(ab')_2$. However, there are some draw backs of the treatment using conventional

antivenom including its side-effect and ineffectiveness. Since the raised antibodies were immunized from the whole venom so some of them recognize non-toxic compounds which are present in high number the venom as well. Thus the antibodies against key venom toxins are dilute out in the pool of antibodies used. A large volume of antivenom have to be given to the patient which may cause serious side-effects.

DNA immunization could introduce the new approach to produce the toxin-specific antibody with cheaper and more effectiveness. In this approach, DNA fragments encoding the key venom toxins can be engineered into the expression vector to immunize the animal. The successful in venom toxin specific antibody production has been reported (Harrison *et al.*, 2000). In this work, a part of DNA encoding jararhagin, toxin from Brazilian pit viper which causes bleeding and prevents blood from clotting, were immunized in the experimental mice. The DNA was delivered into the mouse abdomen by using a gene gun delivery system. The gold beads coated with DNA are shot into the mouse skin with needle-free and pain-free. The immunized mice shown to produced the potent anti-jararhagin antibodies in hemorhage neutralizing assay with quite effective as sera made from whole venom in viper venom. Other research has been reported the using of DNA immunization against SVMPs of *Echis ocellatus* (Wagstaff *et al.*, 2006). This work also demonstrated the possibility of DNA to be engineered into multiepitope string to improve the antibody production. Thus, DNA immunization could potentially be developed venom toxin-specific antibodies to improve the treatment of systemic envenoming by several snake bites.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1. Expressed Sequence Tags Analysis

1.1.1. Venom glands of Russell's viper snake

Adult Russell's viper was captured from AngThong province. The venom glands were dissected under anesthetized condition by expertised veterinarian.

1.1.2. ZAP Express cDNA library of Russell's viper venom glands

ZAP express cDNA Synthesis Kit and ZAP express cDNA Gigapack III Gold Cloning Kit (Stratagene). ZAP Express vector map is shown in **Figure 6**.

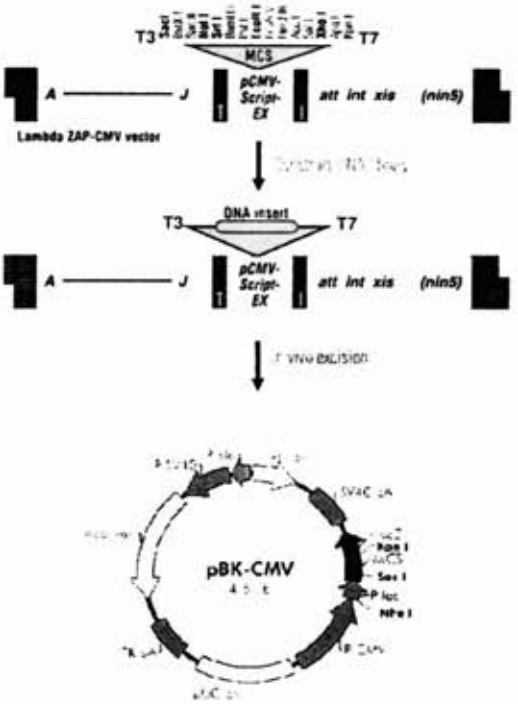


Figure 6. Feature of Lamda ZAP express vector and the excised pBK-CMV phagemid.

1.1.3. Genotypes of *Escherecia coli* Strain

XL1-Blue MRF' strain; Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gryA96 relA1 lac [F' proAB lacI^{q}ZΔM15 Tn10(Tet^{r})].

XLOLR strain; Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 thi-1 recA1 gryA96 relA1 lac [F' proAB lacI^{q}ZΔM15 Tn10(Tet^{r})] Su^{-} (nonsuppressing) λ^{r} (lambda resistant).

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

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

1.1.4. DNA sequencing reaction

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

1.1.5. Synthetic oligonucleotides (or primers)

Oligonucleotides were purchased from Bio Service Unit, NSTDA, Thailand. The DNA sequences were shown in **Table 5**.

Table 5. Oligonucleotide DNA sequences.

	Primer name	Sequence (5'-3')	Bases
pPicZA, Pichia expression			
	pPic-HF	GAA TTC TTA GTT TCT ACT TCT G	22
	pPic-HR	TCT AGA TGA ATC TGA GAG AG	20
	pPic-HF	GAA TTC TTA GTT TCT ACT TCT G	22
	pPic-MPR2	TCT AGA TGG GGT GGA TTG AAA ATG	24
	pPic-DisF2	GAA TTC TTG AGA AAA GAT ATT G	22
	pPic-HR	TCT AGA TGA ATC TGA GAG AG	20
	pPic-LF	GAA TTC GTT TTG GAC TGT CCC T	22
	pPic-LR	TCT AGA TGG AAC TTG CAC ACG	21
pVaxSec, DNA immunization			
	pVS-RVHF	GGA TCC TTA GTT TCT ACT TCT GCA	24
	pVS-RVHR	CTC GAG TCA AAT CTG AGA GAA GC	23
	pVS-RVHF	GGA TCC TTA GTT TCT ACT TCT GCA	24
	pVS-MPR2	CTC GAG TCA GGG TGG ATT GAA AAT G	25
	pVS-DisF2	GGA TCC TTG AGA AAA GAT ATT G	22
	pVS-RVHR	CTC GAG TCA AAT CTG AGA GAA GC	23
	pVS-LightF2	GGA TCC GTT TTG GAC TGT CCC TC	23
	pVS-RVLR	CTC GAG TCA GAA CTT GCA CAC	21
yeast transformant screening			
	Alpha factor	TAC TAT TGC CAG CAT TGC TGC	21
	3' AOX1	GCA AAT GGC ATT CTG ACA TCC	21
sequencing primers			
	T3	AAT TAA CCC TCA ACT AAA GGG	21
	T7	GTA ATA CGA CTC ACT ATA GGG C	22

1.1.6. Restriction endonuclease and modified enzymes

All Restriction endonucleases (EcoRI, XbaI, BamHI, XhoI, etc.) were purchased from Biolabs.

Taq DNA polymerase (Promega), T4 DNA ligase (USB)

1.1.7. DNA Purification from gel slice

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

1.1.8. Proteins detection

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

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

1.1.9. Coomassie Brillient Blue staining

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

1.1.10. Silver staining

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

1.1.11. Western blotting hybridization

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

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

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.

1.1.12. Concentration of protein

Vivaspin concentrators were purchased from Vivascience Ltd., Germany.

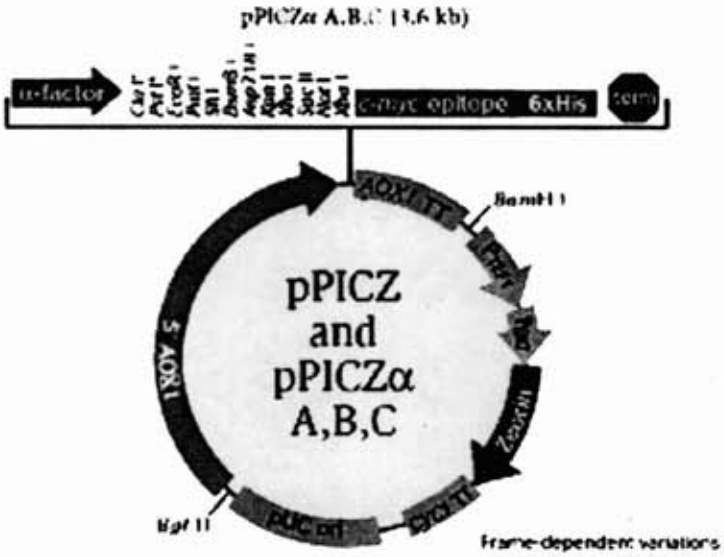
1.1.13. Purification of His-Tag protein

HisTag proteins were purified by MagneHis Protein Purification System (Promega, USA).

1.2. Protein expression in *Pichia pastoris*

pPicZα-A was purchase from Invitrogen. Its physical feature and restriction map was shown in **Figure 7**.

(A)



(B)

Figure 7. Map and Features of the pPicZα-A expression vector. (A) pPicZα-A structure showing detailed feature. (B) Multiple cloning sites of pPicZα-A, cloning site of *Eco*R I and *Xba* I were shown in boxes.

1.2.1. *Pichia* yeast strain

X-33, GS115 and KM71H were come along with EasySelect *Pichia* Expression Kit.

1.2.2. Culture media

All culture media were purchased from Fermentus, USA.

1.3. DNA immunization

1.3.1. pVaxSec Expression vector

pVaxSec was genetically modified by Dr. Harrison. Its feature was shown in **Figure 8**.

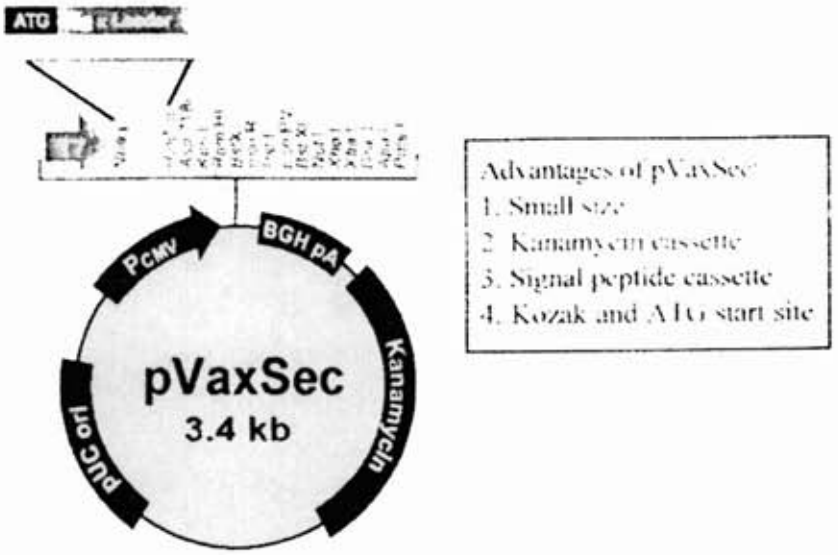


Figure 8. Feature of a modified expression plasmid pVaxSec. pVaxSec was modified by Dr. Harrison and his colleagues. It was generated by the insertion of the consensus KOZAK and IgK leader sequences from pSecTagB (Invitrogen) into the multiple cloning site of pVax (Invitrogen).

1.3.2. BalB/c mice

Thirty of female BalB/c mice were available at Faculty of Medicine, University of Liverpool. The mice were approximately 3 months old with average weighing of 20 gm.

1.3.3. Helios Gene gun

Helios gene gun and its accessories were purchased from Bio-Rad, **Figure 9**.

(A) (B)

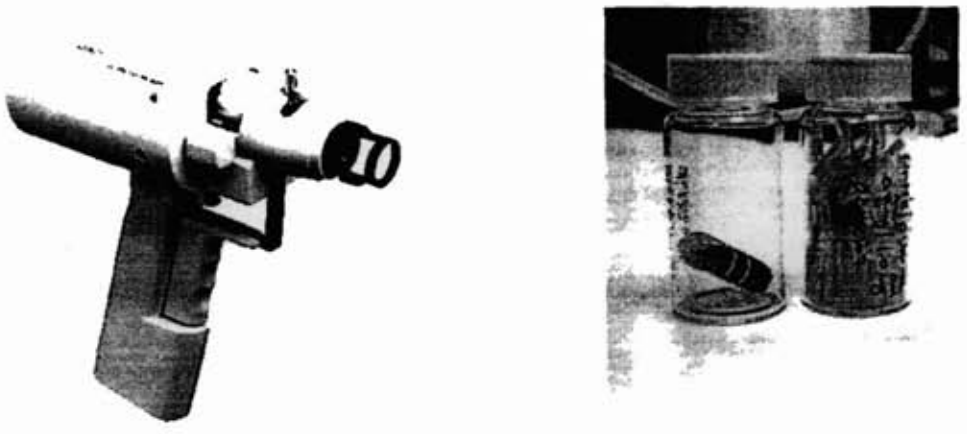


Figure 9. Devices used in DNA immunization experiments. (A) Helios gene gun, (B) Gene gun's bullets (half-inch plastic tubes) containing inner coated DNA-gold microcarrier.

1.4. Chemicals

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

2. Methods

2.1. cDNA library construction & ESTs analysis

2.1.1. Animal specimens

An adult Russell's viper, *D. russelli siamensis*, was obtained from Queen Saovabha Memorial Institute, Thailand. Venom glands were dissected from the snake under ether anesthesia by the veterinarian and kept immediately in liquid nitrogen until use.

2.1.2. Poly $(A)^+$ RNA isolation

Venom glands were ground by mortar and pestle under liquid nitrogen. Total RNA was isolated by TRIzol LS reagent (Gibco BRL) according to manufacturer's recommendation. Total RNA was then purified for poly $(A)^+$ RNA by PolyAT Tract system (Promega) according to manufacturer's recommendation.

2.1.3. cDNA library construction

A cDNA library was constructed using ZAP express cDNA Synthesis Kit and ZAP express cDNA Gigapack III Gold Cloning Kit (Stratagene) according to manufacturer's recommendation. Briefly, 5 μg of poly $(A)^+$ RNA was used as substrate to construct the double-stranded cDNA. The cDNA was then modified for ligating to the vector arms. The ligated DNA was packed with packaging extract to generate the infectious phage particle. The quality of constructed cDNA library was assessed by determining the library titer (plaque-forming unit) and the average size of cDNA inserts.

2.1.4. *In vivo* single-clone excision of the pBK-CMV phagemid vector from the ZAP Express vector

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

The interested plaques were cored from the agar plate and transferred to a sterile microcentrifuge tube containing 500 µl SM buffer (50 mM Tris-HCl pH 7.5, 0.1 M NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, 0.01% gelatin) and 20 µl of chloroform. The content was vortexed to release the phage particles into the SM buffer, and incubated overnight at $4^{O}C$. The XL1-Blue MRF' cells and XLOLR cells were grown separately overnight in LB broth (1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract, pH 7.0) with 10 mM $MgSO_4$ and 0.2% (w/v) maltose, and in NZY broth (0.5% w/v NaCl, 0.2% w/v $MgSO_4 \cdot 7H_2O$, 0.5% w/v yeast extract, 1% NZ amine (casein hydrolysate), pH 7.5), respectively. Following inoculation, the cells were gently spun down at 6,000 rpm for 2 min and supernatant was discarded. The pellet was suspended in 10 mM $MgSO_4$ to an OD_{600} of 1.0. One µl of the ExAssist helper phage was added into the mixture containing 60 µl of XL1-Blue MRF' cells at an OD_{600} of 1.0 and 75 µl phage stock of interested plaque in SM buffer. The reaction was incubated for $37^{O}C$ for 15 min, and then 750 µl of NZY broth was add and incubated at $37^{O}C$ for 2-3 hrs with shaking. After incubation, the mixture of bacteria and phage was heated at $65\text{-}70^{O}C$ for 20 min and spun down at 600 rpm for 2 min. The supernatant was decanted into a

new clean tube. This stock contains the excised pBK-CMV phagemid vector packaged as filamentous phage particle. The mixture of 100 µl phage supernatant and 200 µl freshly grown XLOLR cells was incubated at 37 °C for 15 min. Three hundred µl of NZY broth was added and incubated at 45 °C for 45 min. After incubation, the XLOLR cells were spread on LB-kanamycin agar plate (50 µg/ml) and incubated overnight at 37°C.

2.1.5. Double strand phagemid DNA extraction by alkaline lysis method

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

2.1.6. Digestion of restriction endonucleases and analysis

About 500 ng of phagemid DNA was double-digested with 5 units of *Eco*R I and *Xho* I according to manufacturer's protocols (New England Biolabs), 1X reaction buffer (provided), 1X BSA and sterile distilled water to a final volume of 20 µl. The digestion was incubated at 37°C for 3 hrs. After digestion, digested phagemids

were fractionated on 1.2% agarose gel electrophoresis. Clones which containing of over 0.6 kb inserted cDNA were selected for sequencing.

2.1.7. Screening of *RVV-X* clones from ZAP Express cDNA library

2.1.7.1. Plaque-lift hybridization

2.1.7.1.1. Plating

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

2.1.7.1.2. Lifting

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

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

2.1.7.1.3. Probe labeling

The *RVV-X* DNA fragment which isolated from previous work was PCR amplified and labeled by North2South® Direct HRP Labeling and Detection Kit as follows. One hundred ng of *RVV-X* DNA fragments in 10 µl water were denatured at 95 °C for 5 min and snapped cool for 5 min. Ten µl of North2South® Direct Stabilized HRP Label and 10 µl of North2South® Direct Reaction Buffer were added. After incubation at 37°C for 30 min, 30 µl of North2South® Direct Enzyme Stabilization Solution was added and mixed.

2.1.7.1.4. Hybridization

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

To pre-hybridization, equal volume of North2South® Direct Hybridization Buffer Component 1 and 2 were combined in a clean plastic plate at least 0.1 ml per cm^2 membrane. The hybridization solution was incubated at 55°C in a hybridization oven (HYBAID). After warming the hybridization solution at least 5 min, the blot-membrane was placed and pre-hybridized with gentle rotation for at least 15 min. Following the pre-hybridization step, about 5-10 ng of HRP-labeled DNA probe per ml of hybridization solution was added and incubated 1-4 hrs with gentle

rotation at 55°C. After hybridization, the membrane was washed 3 times with 0.5 ml per cm^2 membrane washing buffer 1 (2X SSC, 0.1% SDS) at 55°C for 5 min per each wash, performed same process with washing buffer 2 (2X SSC) at room temperature, and proceed to detection step.

2.1.7.1.5. Detection

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

2.1.7.2. DNA Sequencing

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

2.1.7.3. Alignment and computational searching sequences analysis

The cDNA sequences and predicted amino acid sequences will be compared to the sequences against the National Center for Biotechnology Information (NCBI) non-redundant nucleotide and protein database using BLAST program (Altschul *et al.*, 1997). Matches with E value of 10^{-5} or less were considered to be significant. Alignments of interesting sequences were made by using CLUSTAL X multiple alignment program.

2.2. Expression of recombinant RVV-X in *Pichia pastoris*

In previous attempt, we try to express RVV-X in *E. coli* using pTrcHis expression vector (Invitrogen). The expression level of recombinant RVV-X heavy chain and light chain were obtained in fair amount (mg protein/L culture media). Nevertheless, the recombinant RVV-X could not activate human factor X in blood clotting test. The explanation might due to the lacking of glycosylation on the RVV-X proteins since they are synthesized in bacteria. Therefore, RVV-X was expressed in eukaryotic cells instead. *Pichia* yeast is selected as the host for expression because of its several advantages.

The mature RVV-X heavy chain consisting of 431 amino acid residues was divided into 2 subdomains, Metalloproteinase domain (MP) and Disintegrin domain (DC). MP domain is started from residue 1-205 (LVSTS...IFNPP) while DC domain started from residue 206-233 (LRKDI...GFSQI). 2.2.1. Isolation of mature RVV-X heavy chain and light chain cDNAs

2.2.1. Isolation of mature RVV-X heavy chain and light chain cDNA

The gene encode for mature RVV-X was isolated from RVV cDNA library by plaque-lift hybridization or by using degenerate PCR. For RVV-X heavy chain, EST clone number **RVV012** which contains a part of RVV-X heavy chain was used as probe in plaque-lift hybridization. RVV-X light chain cDNA was isolated by degenerate PCR. The size of full-length mRNA encoding for RVV-X heavy chain and light chain were determined by Northern blot analysis.

2.2.2 Subcloning to Expression Vector

Each DNA encoding desired expressed proteins were amplified by PCR with their primer paired as mentioned in Materials and Methods, **Table 5.** 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 MgCl2, 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 PCR products were then cloned into pCR2.1-TOPO vector prior to subcloned into pPicZα-A vector.

2.2.2.1. Ligation of PCR Products into Plasmid Vector

After *EcoR* I and *Xba* I-digestion of pPicZα-A vector and the digested DNA from recombinant pCR2.1-TOPO 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 pPicZα-A 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 pPicZα-A vector is approximate 3.3 kb and supplied at 50 ng/µl. The amount of the DNA insert was calculated from the following equation:

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

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

2.2.2.2. 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 OD_{600} of 0.4-0.5. The cell culture was chilled on ice for 10 min prior to pipetting the 10 ml aliquots of the culture into 15 ml sterile polypropylene tubes. The cells were pelleted by centrifugation at 4,000 rpm for 10 min at 4°C. After that, the pellet was suspended in 5 ml of ice-cold 0.1 M $MgCl_2$, centrifuged, resuspended in 5 ml of ice-cold 0.1 M $CaCl_2$ and left on ice for 30 min to establish competency. Finally, after centrifugation, the pellet was resuspended in 750 µl of 15% v/v glycerol and 0.1 M $CaCl_2$. The cells were kept in 200 µl aliquots at -80°C until required.

2.2.2.3. Transformation of *E. coli* Competent Cells

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

After transformation, the pPicZα-A vectors containing DNA inserts were extracted by alkaline lysis method. Then, PCR sequencing was proceeded to ensure the correct DNA sequences.

2.2.3. Transformation of recombinant pPicZα-A into *Pichia pastoris*

Recombinant pPicZα-A were chemically transformed into *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 °C for 3-7 days. The growing yeast colonies were picked to test for the recombinant pPicZα-A integration by PCR.

2.2.4. Methanol induction of Pichia pastoris

Six types of the transformed *Pichia pastoris*; 1) positive control which secrete 67 kDa of human serum albumin, 2) RVV-X whole heavy chain, 3) RVV-X MP domain, 4) RVV-X DC domain, 5) RVV-X light chain, and 6) empty pVaxSec control, were cultured in BMGY and BMMY media. After the protein induction by methanol, culture media containing yeast cells were collected to investigate the protein

expression at every 24 hr for 5 days. The summary flow chart for RVV-X express in *Pichia pastoris* was shown in **Figure 10**.

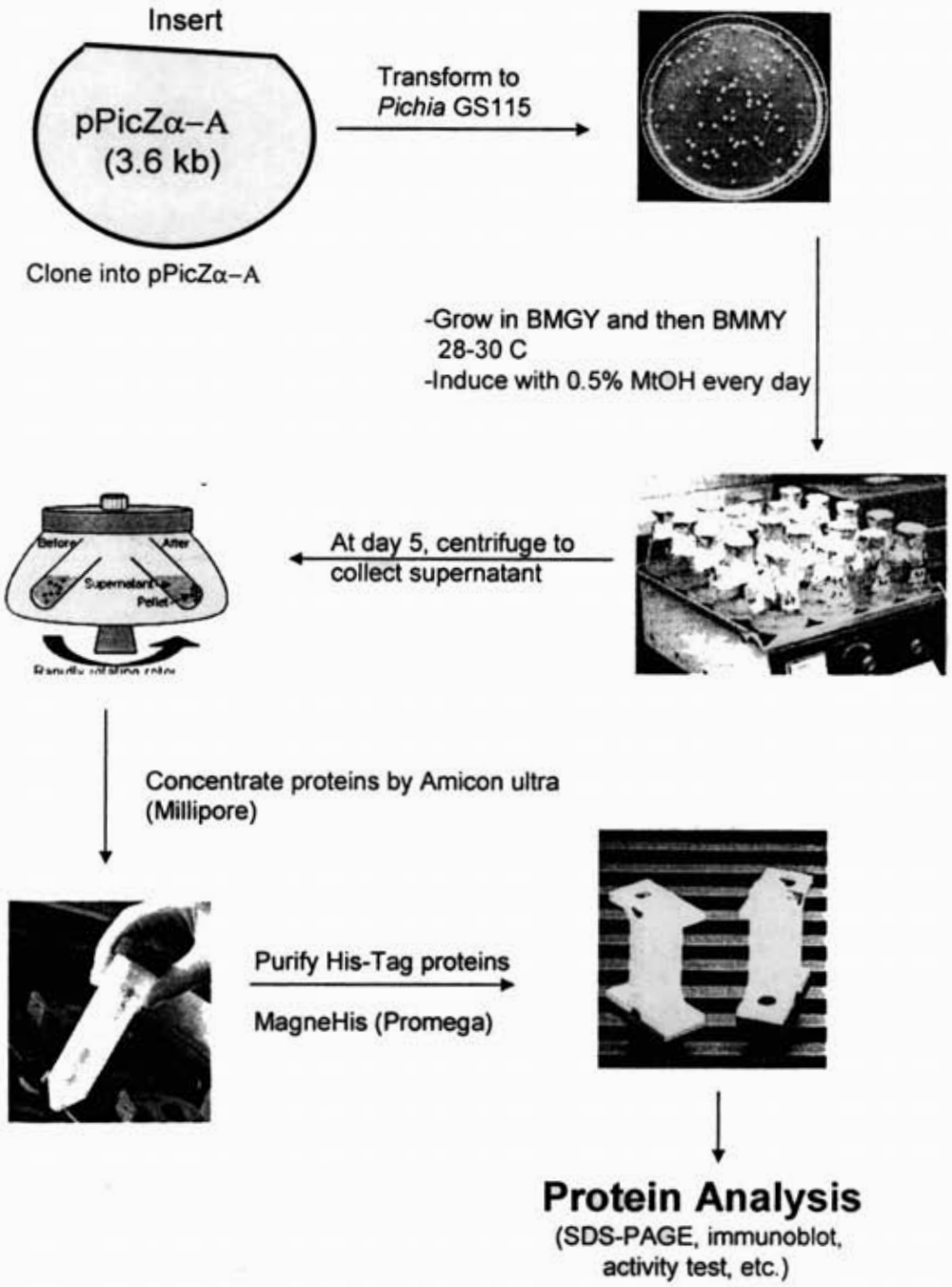


Figure 10. The flow chart protocol for RVV-X expression in *Pichia pastoris*.

2.2.5. Purification of recombinant RVV-X protein

2.2.5.1. Vivaspin column

Approximate 20 ml of each collected culture media from day 5 methanol induction were poured into Vivaspin columns (Amicon ultra, Millipore). The columns were spin at 3,000 g for approximate 1 hr. The remaining culture media less than 500 ul (and the flow-through culture media as well) was kept for further analysis.

2.2.5.2. purification of His-Tag protein

Concentrated proteins were further purified by MagneHis Protein Purification System (Promega, USA), **Figure 11**. MagneHis. Ni-Particles were vortexed to a uniform suspension. Thirty microliters of MagneHis. Ni-Particles was added to 1.0ml of culture medium after removing cells. The suspension was mixed well (approximately 10 times), and incubated for 2 minutes at room temperature. The tube was placed in the appropriate magnetic stand for approximately 30 seconds to capture the MagneHis. Ni-Particles with the magnet. The supernatant was carefully removed using a pipette. After removing the tube from the magnet. 500 µl of MagneHis. Binding/Wash Buffer containing 500 mM NaCl was added to the MagneHis. Ni-Particles and pipet to mix. The tube was placed in magnetic stand for approximately 30 seconds to capture the MagneHis. Ni-Particles with the magnet. The supernatant was carefully removed using a pipette. The washing step was repeated for 2 times. A hundred microliters of MagneHis. Elution was added, incubate for 1.2 minutes at room temperature. Placed the tube on the magnetic strand again. The supernatant contains purified protein was carefully removed by using a pipette. The eluted protein was analyzed by SDS-PAGE or by functional assay.

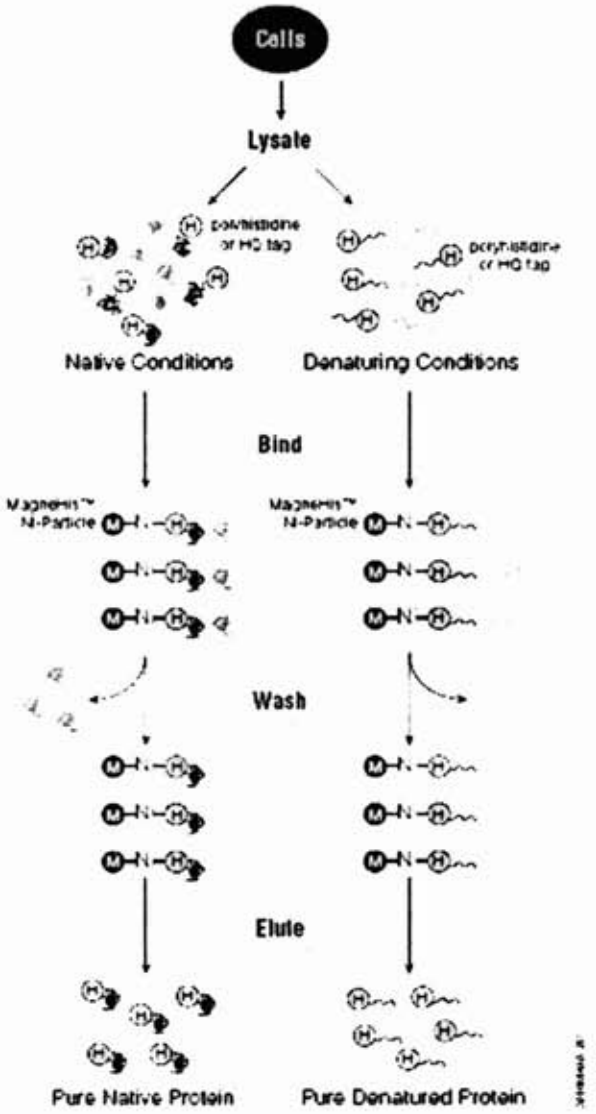


Figure 11. Diagram for HisTag protein purification by MagneHis protein purification system.

2.2.6. Western blot by anti-His (C-terminal) antibodies

Each blotted membrane was blocked with 5% non-fat dry milk (Carnation) in 1X-PBS/0.1% Tween-20 at 4 ^{o}C overnight. The blot was then incubated with rabbit anti-His (C-Terminal) HRP-conjugated antibodies diluted in the blocking buffer (1:3,000) at room temperature for 2.5 h. After incubation of the primary antibodies, the blot was washed in four changes of 1X-PBS/0.1% Tween-20 for 5 min. The blot was then incubated with DAB substrate (Pierce) for 5 min.

2.2.7. Coomassie blue G-250 staining

The Coomassie blue staining was achieved as described (Neuhoff *et al.*, 1988). A protein gel was fixed with 50% methanol and 10% acetic acid for 10 min, and then washed with distilled water for 5 min. For staining, the gel was covered overnight with the staining solution, 8% ammonium sulfate, 0.8% phosphoric acid, 0.08% Coomassie blue G-250 (Bio-Rad), and 20% methanol, and de-stained with Milli-Q water.

2.2.8. Silver staining

After gel electrophoresis, a protein gel with silver staining was carried out according to the manufacturer's recommendations (Bio-Rad). A protein gel was incubated in the fixative enhancer solution (10% fixative enhancer concentrate, 50% methanol, and 10% acetic acid) for 20 min with gentle agitation. After decanting of the fixative enhancer solution, a protein gel was rinsed in deionized distilled water for 10 min, and the rinse step was repeated. For staining step, a protein gel was incubated in the staining solution (5% silver complex solution containing NH_4NO_3 and $AgNO_3$, 5% reduction moderator solution containing tungstosilicic acid, 5% image development solution containing formaldehyde, and 50% development accelerator solution containing Na_2CO_3).

2.2.9. Western blot analysis by RVV-immunized horse antibodies

A PVDF membrane blotted with proteins was blocked with 5% non-fat dry milk (Carnation) in 1X-tris-buffered saline (1X-TBS; 10 mM Tris, pH 7.4, 137 mM NaCl)/0.1% Tween-20 at 4°C overnight. The blot was then incubated with C-terminal anti-His or RVV-immunized horse antibodies diluted in the blocking buffer (1:150) at room temperature for 2.5 h. After washing in four changes of 1X-TBS/0.1% Tween-20 for 5 min, the secondary antibody, HRP-conjugated goat anti-horse IgG conjugate (Pierce) diluted in the blocking buffer (1:20,000), was applied, and followed by incubation for 1 h at room temperature. The blot was then washed in four changes of 1X-TBS/0.1% Tween-20 for 10 min, and the blot was then incubated with DAB substrate (Pierce) for 5 min.

2.2.10. Enzymatic activity testing of recombinant RVV-X

To determining activity of RVV-X is to measure its ability to activate human factor X resulting to plasma clot. RVV-X activity can be assessed by measuring partial thromboplastin time (PTT). A hundred microliters of cryophilized plasma and 100 ul of 25 mM $CaCl_2$ were mixed with RVV-X or blank control. The mixture solution was immediately incubated in 37 C water bath and vigorously mixed until observing the clot. The time that cryophilized plasma started clotting was recorded as PTT.

2.3. DNA immunization

2.3.1. Preparation of RVV-X DNA constructs for gene gun DNA immunization

To provide comparative immunological data to the sera raised by immunization with the RVV-X DNA construct, mice were immunized with DNA encoding the RVV-X heavy chain (HC), metalloproteinase (MP) domain, disintegrin (DC) domain, and RVV-X light chain (LC). DNA encoding mature protein sequences were amplified by PCR with complementary 5' and 3' primers, and the amplicons cloned into pCR2.1 TOPO (Invitrogen) followed by subcloning into pVaxSec at *Bam* HI and *Xho* I sites. Following sequence verification, transformed colonies were grown in 500 ml of LB medium, and plasmid DNA purified using MegaPrep plasmid DNA purification kit (Qiagen, Crawley, United Kingdom). Purified pVaxSec plasmid (vehicle control) and pVaxSec/HC, pVaxSec/MP, pVaxSec/DC, and pVaxSec/LC were precipitated onto 1.6-μm gold beads and coated on the inner surface of half-inch lengths of plastic (Tefzel) tubing according to the manufacturer's protocol (Bio-Rad, Hercules, California, United States).

2.3.2. Gene gun transfer of DNA immunogen to experimental mice

The quantity of DNA gold beads was adjusted to provide individual tubing (shots) of 1 μg DNA/0.5 mg Au. The abdomens of anaesthetized female BALB/c mice (18-20 g) were shaved and each subjected to four "shots" expelled under a burst of helium gas at 350 psi into the epidermal layer using the Helios Gene Gun (Bio-Rad). Groups of six mice were immunized with the four pVaxSec constructs or vector alone as follows; 1st immunization day 0 and day 2; 2nd immunization day 14 and day 16; 3rd immunization day 21nd day 23 – each immunization consisted of a total

of 1ug DNA administered using 2 shots of 0.5 ug gold/shot. Mice sera were examined after the final immunization. Horse Antivenom antibodies control was also prepared by hyperimmunized horses with increasing doses of Russell's viper venom. The collected horse sera was further purified for IgG by ammonium sulfate precipitation. The schematic protocol of RVV-X DNA immunization in mice was shown in **Figure 12.**

2.3.3. Western blot analysis of mice sera antibodies

Russells's viper venom was separated by 12 % reduced or non-reduced SDS-PAGE. Following electrophoretic separation, gels were electroblotted onto 0.45 μm nitrocellulose (Bio-Rad) by semi-dry blotting device (Biorad). Membranes were blocked in PBS with 5% nonfat skimmed milk for 1 hr, followed by washes in PBS then incubation in test antisera at 1/200 dilution in 5% nonfat skimmed milk in PBS for 3 hrs at room temperature. Membranes were washed in PBS then incubated with a 1/1,000 dilution of HRP-conjugated goat anti-mouse immunoglobulins (Dako Cytomation, Cambridgeshire, United Kingdom) in PBS for 1 h at room temperature, then washed in PBS and developed using DAB peroxidase.

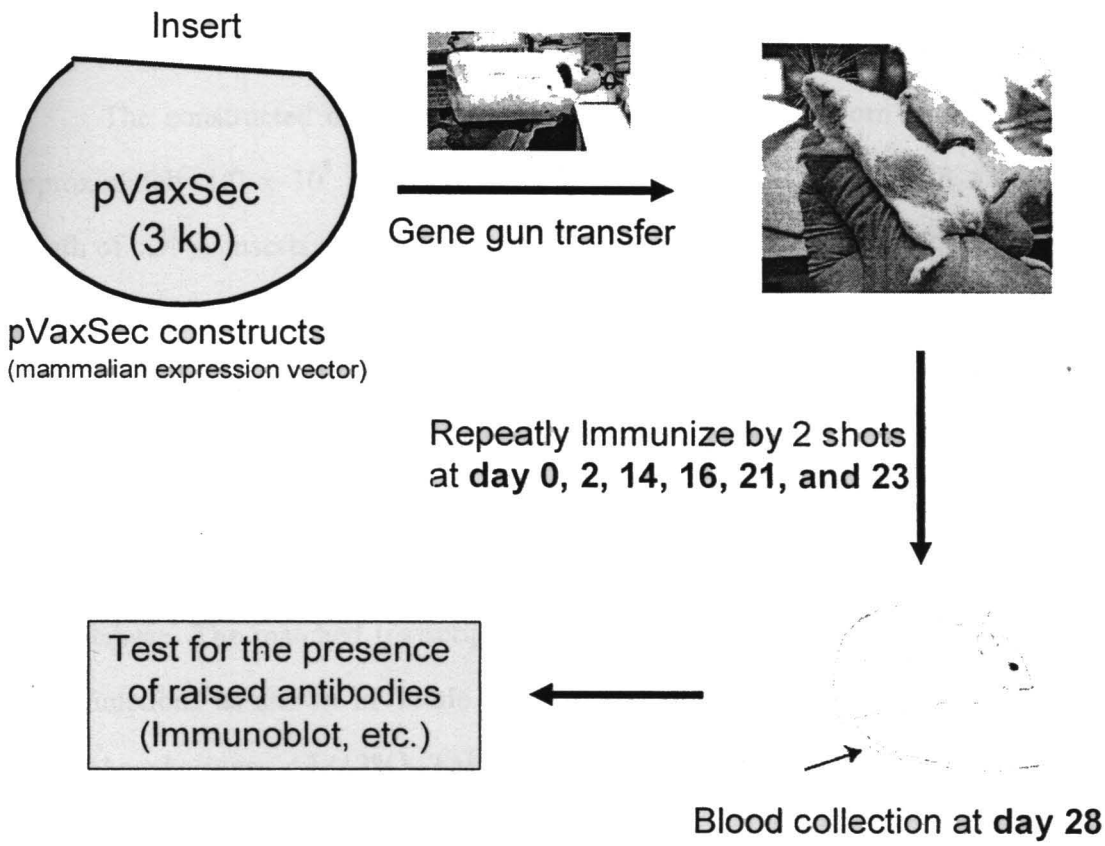


Figure 12. Schematic protocol of RVV-X DNA immunization in mice.

CHAPTER IV

RESULTS

1. cDNA Library construction and analysis

1.1. Characteristic of RVV's cDNA library

The constructed cDNA library from Russell's viper venom glands contained approximately 1.0 x 10^6 plaque-forming units per microgram of DNA vector. The length of cDNA inserts were varying from 0.6 kb to over 2.0 kb (average length = 0.9 kb), ensure that every clones contains at least part of coding sequence. Double stranded pBK-CMV phagemids from bacteriophage phage excisions were digested by restriction endonuclease showing their cDNA inserts, **Figure 13**. A total 135 expressed sequence tags (ESTs) have been generated from size-selected clones at random. Fifty-seven percent (77/135) of ESTs were significant matched to the reported sequences in the database. The matched transcripts could be categorized into groups according to their functions as shown in **Table 6**. The most abundant transcripts found in RVV were *phospholipase A2* (13%), **Table 7**. The other putative proteins found were also related to the haemostatic alteration including BPP-CNP, factor V activator, factor X activator, etc.

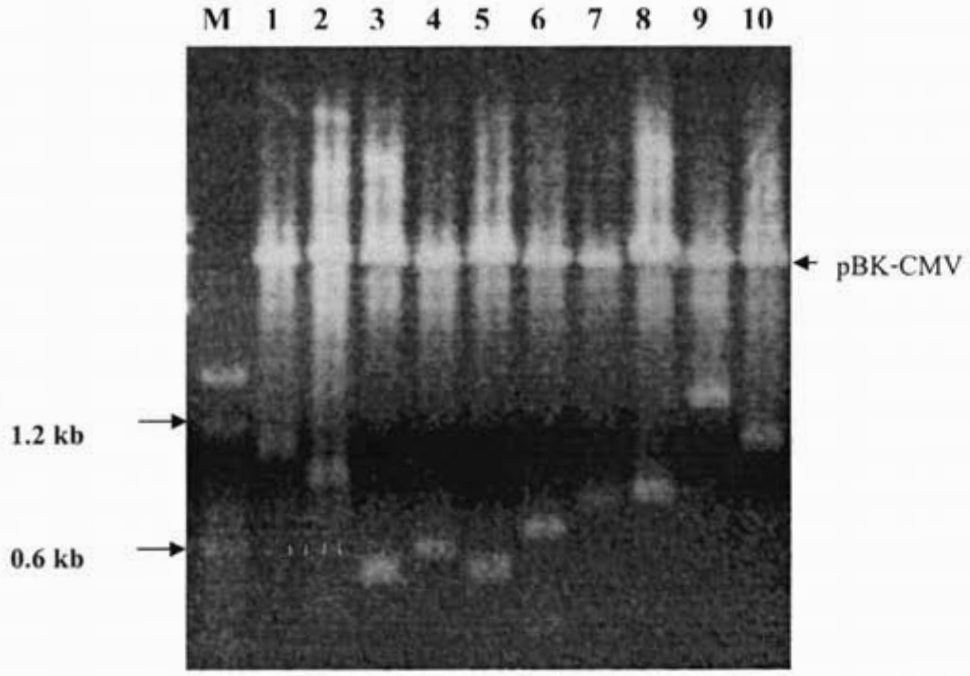


Figure 13. Examples of *Eco*R I and *Xho* I digested pBK-CMV phagemids showing various size of cDNA inserts.

Table 6. Classification of RVV transcripts which shown significant matching to the entries in the database.

Clone ID	Length/kb	Putative protein (or gene*)	Access. No.	Species
Cell signaling and comunication (10)				
57	1.7	tyrosine phosphatase lamda	Z21960	Gallus gallus
99	0.8	dual specificity phosphatase 12	BC006286	Homo sapiens
135	0.9	similar to dendritic cell protein	14771338	Homo sapiens
202	0.8	Similar to dendritic cell protein	BC005598	Mus musculus
445	0.6	synaptogyrin 2	AJ002308	Homo sapiens
460	0.6	phosphatase 2, catalytic subunit, alpha isoform (PPP2CA)	XM_037928	Homo sapiens
482	1.4	N-ras upstream protein	AB020692	Homo sapiens
514	0.9	Nori-2 protein	AB028045	Mus musculus
293	1.6	Integrin BETA-1 precursor (CD29)	U27351	Felis catus
313	1.7	RAN binding protein 7	XM_049130	Homo sapiens
Cell structure and motility (10)				
82	1.6	lysosomal membrane glycoprotein A	M59365	Gallus gallus
105	0.6	myosin regulatory light chain	L03785	Homo sapiens
109	1.0	Putative enigma homolog	AK009464	Mus musculus
431	1.0	Steroid membrane binding protein	AF173937	Homo sapiens
441	1.0	troponin:ISOTYPE=T	1810398A	Oryctolagus cuniculus
455	0.8	steroid membrane binding protein	AF173937	Homo sapiens
268	1.0	Myosin X	NM_012334	Homo sapiens
301	1	Fast skeletal muscle troponin T isoform	AF226598	Mitu tomentosa
310	1.1	Cell adhesion protein retina cognin - chicken (fragment)	A47300	Gallus gallus
605	0.6	Telethonin	AJ223854	Mus musculus
Cell/organism defense (39)				
12	0.6	Phospholipase A2 RV-7	S29299	Daboia russelli
15	1.1	Factor X activating enzyme (heavy chain)	A42972	Daboia russelli
26	0.9	BPP-CNP precursor homolog*	GI4127226	Agkistrodon blomhoffi
47	0.6	Phospholipase A2 RV-7	S29299	Daboia russelli
49	0.8	Factor X activating enzyme (heavy chain)	A42972	Daboia russelli
50	0.6	Phospholipase A2 RV-7	S29299	Daboia russelli
54	0.6	atrolysin A	S41607	Crotalus atrox
56	0.6	Phospholipase A2 RV-7	S29299	Daboia russelli
84	1.6	ecto-5'-nucleotidase	L12059	Mus musculus
92	0.6	Phospholipase A2 RV-7	S29299	Daboia russelli
104	0.9	snake venom factor V activator gamma	B32121	Daboia russelli
106	0.4	Phospholipase A2 RV-4	S29298	Daboia russelli
107	0.6	adipocyte-derived leucine aminopeptidase	AF106037	Homo sapiens
108	0.6	Factor X activating enzyme (heavy chain)	A42972	Daboia russelli
117	0.8	BPP-CNP precursor homolog*	GI4127226	Agkistrodon blomhoffi
141	1.6	salmobin	AF015727	Gloydius halys
157	0.5	Phospholipase A2 RV-7	S29299	Daboia russelli
159	0.7	Phospholipase A2 RV-7	S29299	Daboia russelli
160	0.7	Phospholipase A2 RV-7	S29299	Daboia russelli
161	0.7	Phospholipase A2 RV-7	S29299	Daboia russelli
162	0.7	Phospholipase A2 RV-7	S29299	Daboia russelli
164	0.6	Phospholipase A2 RV-4	S29298	Daboia russelli
165	1.5	kinin-releasing and fibrinogen-clotting serine proteinase	AB004067	Bothrops jararaca
166	0.8	factor V activating enzyme precursor	AF163973	Macrovipera lebetina
168	1.0	lebetase Le3	X97894	Macrovipera lebetina

Table 6. (continued)

Clone ID	Length/kb	Putative protein (or gene*)	Access. No.	Species
186	0.7	gTgPLA2-V gene for phospholipase A2, complete*	GI994789	Trimeresurus gramineus
204	1.0	kinin-releasing and fibrinogen-clotting serine proteinase	AB004067	Bothrops jararaca
208	0.6	Snake BP-II gene for phospholipase A2	D13384	Trimeresurus flavoviridis
211	0.6	Phospholipase A2 RV-7	S29298	Daboia russelli
500	0.9	lebetin 2 isoform alpha	S71381	Vipera lebetina
541	1.1	5'-nucleotidase	X55740	Homo sapiens
247	1.3	mRNA for BPP-CNP*	AB020810	Agkistrodon blomhoffi
266	0.8	mRNA for BPP-CNP*	AB020810	Agkistrodon blomhoffi
275	0.8	Batroxobin gene	X12747	Bothrops atrox
276	1.1	Phospholipase A2 RV-4	S29298	Daboia russelli
299	0.6	Phospholipase A2 RV-7	S29299	Daboia russelli
600	0.5	Phospholipase A2 RV-4	S29298	Daboia russelli
602	0.7	mRNA for BPP-CNP*	AB020810	Agkistrodon blomhoffi
607	0.6	Phospholipase A2 RV-7	S29299	Daboia russelli
Gene and protein expression (7)				
1	0.8	far upstream element-binding protein	A53184	Homo sapiens
77	0.8	far upstream element-binding protein	A53184	Homo sapiens
96	0.7	ganglioside expression factor 2	BC005985	Homo sapiens
111	0.6	PC4, p15	X79805	Homo sapiens
137	1.5	cellular nucleic acid binding protein	AF242550	Rattus norvegicus
298	1.0	procollagen C-endopeptidase enhancer 2	XM_031556	Homo sapiens
552	1.1	protein disulfide-isomerase (EC 5.3.4.1) precursor	J05185	Mus musculus
Metabolism (8)				
42	0.8	breed Landrace mitochondrion	AF304202	Sus scrofa
43	0.6	NADH dehydrogenase subunit 1	AB008539	Dinodon semicarinatus
53	0.6	ribosomal protein S10	2113200G	Homo sapiens
94	0.6	ribosomal protein L9 (AA 1-192)	X51706	Rattus rattus
167	0.9	Human golgi-associated MP1 adapter protein	AF070659	Homo sapiens
212	0.7	cytochrome b	AF217837	Paranaja multifasciata
260	0.8	NADH dehydrogenase subunit 1	AF407497	Varanus flavescens
601	1.0	Cytochrome c oxidase subunit II	NP_008422	Dinodon semicarinatus
Unclassified (3)				
20	2.0	Unnamed protein	AK023676	Homo sapiens
145	1.0	HRIHFB2072	AB015335	Homo sapiens
180	0.8	Unknown (protein for MGC:10813)	BC003545	Homo sapiens
Microsatellite sequence (2)				
51	0.7	GA repeat		
210	0.7	A repeat		

Table 7. The most abundant transcripts found in RV venom glands.

	putative protein (or gene*)	No. clones
1	Phospholipase A2 (RV-7 form)	13 (9.6%)
2	BPP-CNP precursor homolog *	5 (3.7%)
3	Phospholipase A2 (RV-4 form)	4 (3.0%)
4	Factor X activating enzyme (heavy chain)	3 (2.2%)
5	Factor V activating enzyme	2 (1.5%)
	Kinin-releasing and fibrinogen-clotting serine proteinase	2 (1.5%)
	Nucleotidase	2 (1.5%)
	Dendritic cell protein	2 (1.5%)
	Tyrosine phosphatase	2 (1.5%)
	NADH dehydrogenase subunit 1	2 (1.5%)

1.2. Molecular cloning and expression of RVV-X

1.2.1. Characterization of RVV-X heavy chain and light chain mRNA transcripts

The characteristic of the mRNA transcripts of RVV-X heavy chain and light chain were investigated by Northern blot analysis. RVV-X heavy chain and light chain fragments were amplified by degenerate PCR and then labeled by North2South kit. The strong band around 2.5 kb of RVV-X heavy chain was observed when probed with RVV-X heavy chain fragment, **Figure 14**. While the strong band of around 1.0 kb was observed when probed with RVV-X light chain fragment **Figure 14.** Therefore, Northern analysis indicated that the full-length mRNA of RVV-X heavy chain and light chain were 2.5 kb and 1.0 kb, respectively.

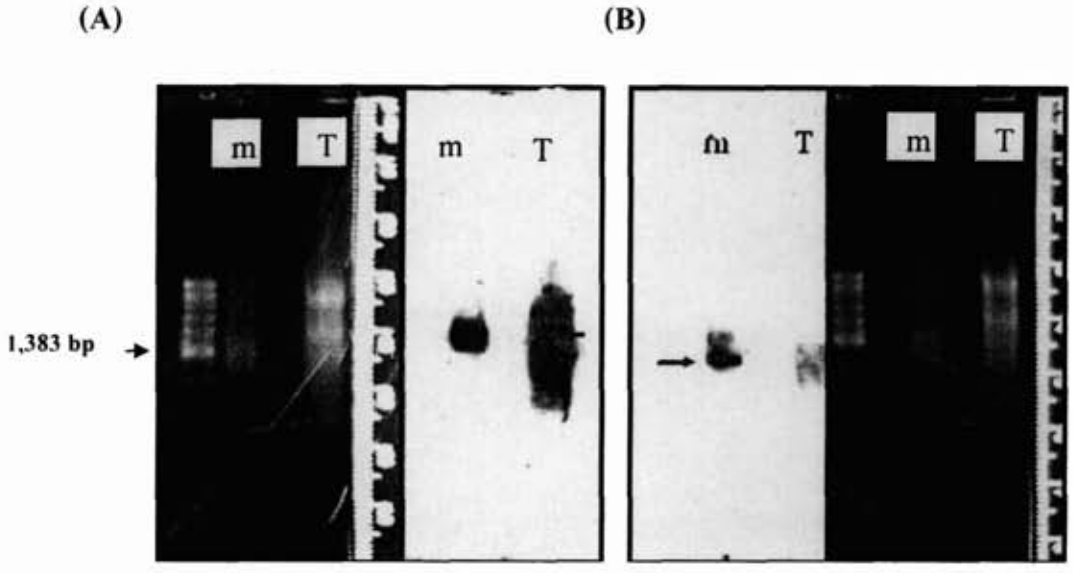


Figure 14. Northern blot analysis of RVV-X heavy chain (A) and light chain (B). (A) The blot was probed by labeled RVV-X heavy chain DNA fragment, (B) The blot was probed by labeled RVV-X light chain DNA fragment. RNA ladder Marker from Promega. T; 1.0 ug of total RNA was loaded in each lane, m; 10 ng of mRNA was loaded in each lane.

1.2.1.1. Sequence analysis of RVV-X heavy chain

The sequence of RVV-X heavy chain was obtained from cDNA library screening by plaque lift hybridization technique. The nucleic acid sequences from several clones were compared. However, almost of the clones seem to be truncated at their 5' ends. In the mean time, to get the longest cDNA sequence at 5' end, RACE was performed. **Figure 15** showed the nucleic acid sequences and its deduced amino acid sequence of RVV-X heavy chain. The pre-pro sequence (but not yet completed) prior to the mature of heavy chain were also shown.

1.2.1.2. Sequence analysis of RVV-X light chain

The full-length sequence of RVV-X light chain was characterized by RACE technique to get the full-length clone. The cDNA sequence and predicted amino acids of light chain was shown in **Figure 16**.

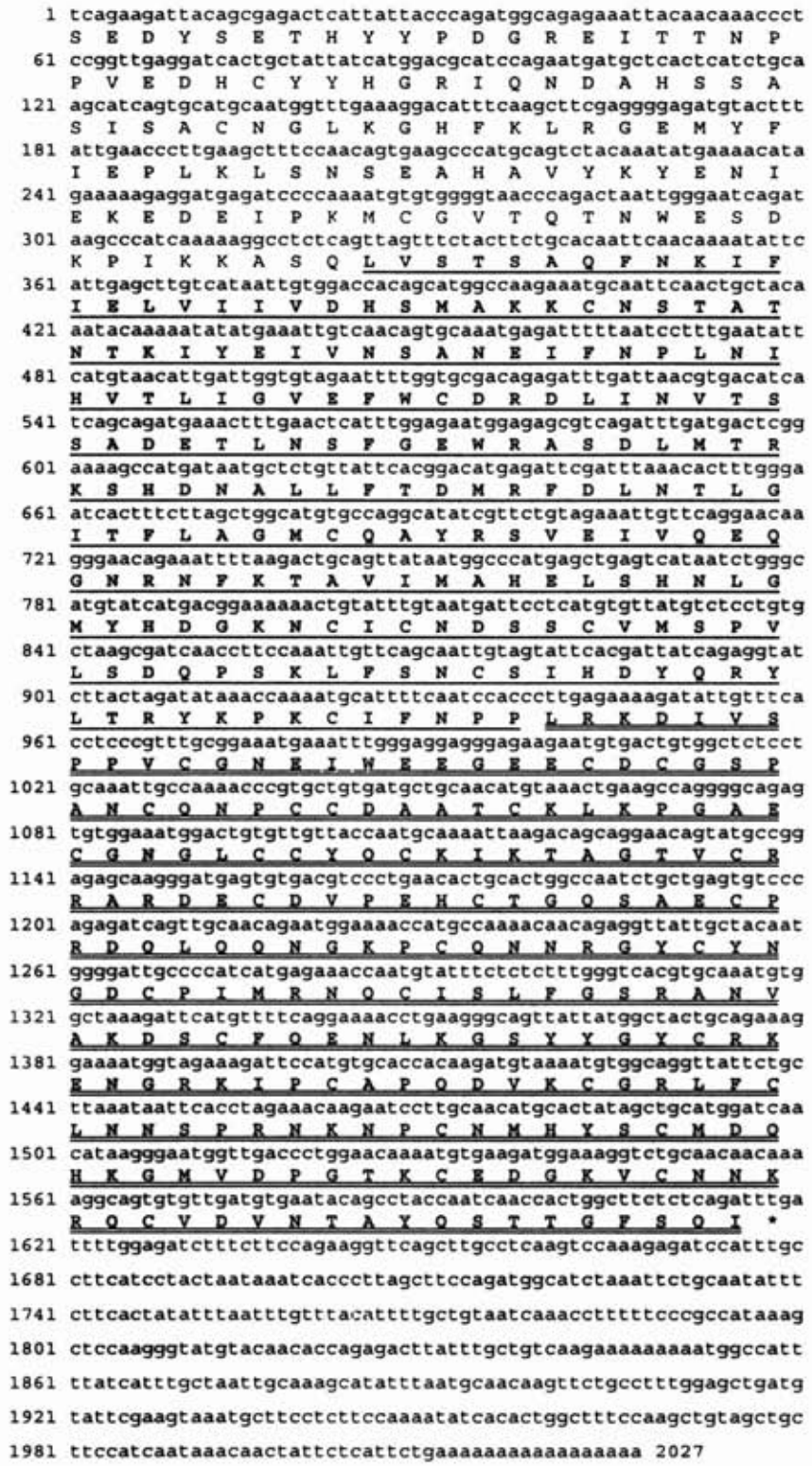


Figure 15. cDNA sequence of RVV-X heavy chain. Four hundred and thirty one amino acid residues of mature peptide were shown in bold capital letters. Underlined letters represents residues of metalloproteinase domain. Double underlined letters represents residues of disintegrin domain. * represents stop codon.

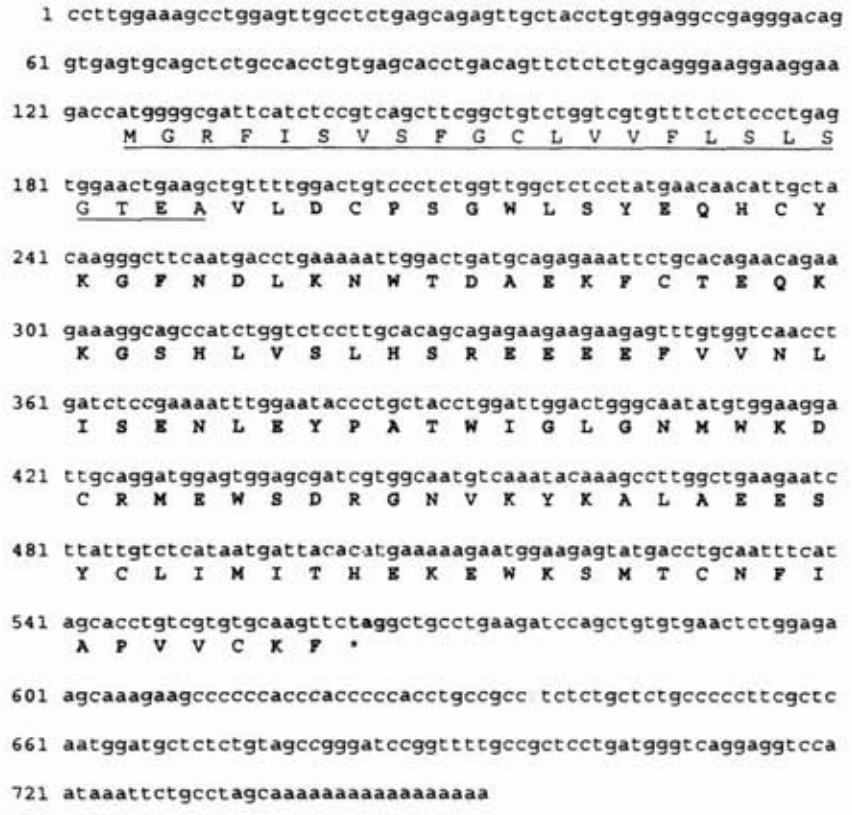


Figure 16. cDNA sequence of RVV-X light chain. Amino acid residues of signal peptide were shown in underlined capital letters. One hundred and twenty three amino acid residues of mature peptide were shown in bold capital letters. * represents stop codon.

1.2.2. Protein expression of RVV-X in *Pichia pastoris*

As mentioned in Materials and Methods, RVV-X was cloned separately in 4 types. To perform RVV-X protein expression in *Pichia pastoris*, RVV-X was cloned into pPicZα-A expression cassettes into 4 types; 1) whole RVV-X heavy chain, 2) Metalloproteinase domain, 3) Disintegrin domain, and 4) whole RVV-X light chain. PCR primers were designed to amplify the cDNA sequences of desired DNA fragments. The PCR product were then cloned into TA cloning vector, pCR2.1-TOPO (**Figure 17**), prior to subcloned into pPicZα-A vector (**Figure 18**). The correct sequences were confirmed by DNA sequencing to ensure the in-frame reading protein translation.

1.2.2.1. Transformation of recombinant pPicZα-A into *Pichia pastosis*

Four of recombinant pPicZα-A and empty pPicZα-A vector control were transformed into *Pichia pastoris*. Yeast transformants which be able to grow in Zeocin-YPDS plates were screened for the DNA integration. Alpha factor primer and 3' AOX primers were used to PCR amplify the integration into the yeast genome. The presence of recombinant pPicZα-A integration in the *Pichia* genomes were shown in **Figure 19**. The size of PCR products of RVV-X/HC, RVV-X/MP, RVV-X/DC, and RVV-X/LC are 1,592, 914, 968, and 668 bp, respectively.

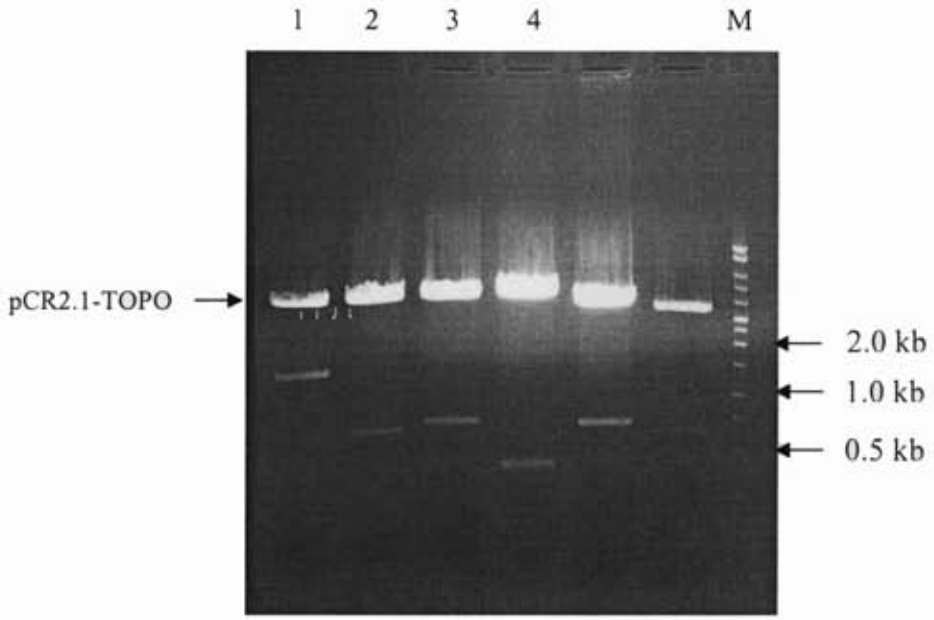


Figure 17. *Eco*R I and *Xba* I digestion of recombinant pCR2.1-TOPO. Lane 1: RVV-X heavy chain, lane 2: metalloproteinase domain, lane 3: disintegrin domain, and lane 4: RVV-X light chain.

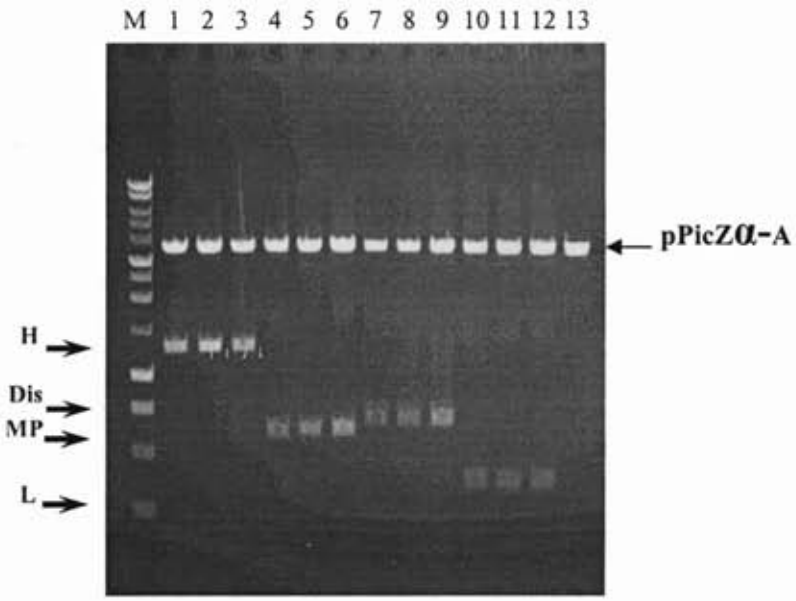


Figure 18. ***Eco*R I and *Xba* I digestion of recombinant pPicZα-A.** Lane 1-3: RVV-X heavy chain, lane 4-6: metalloproteinase domain, lane 7-9: disintegrin domain, lane 10-12: RVV-X light chain, and lane 13: empty pPicZα-A.

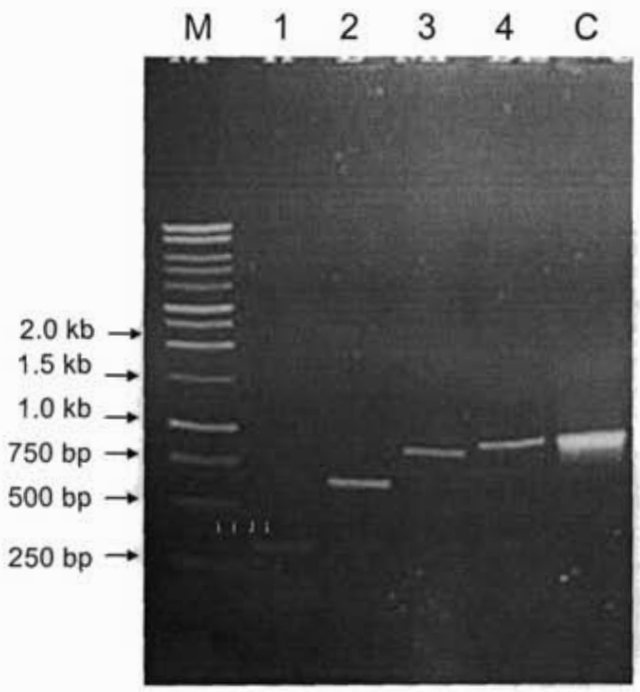


Figure 19. PCR screening of yeast transformants. Lane 1: RVV-X heavy chain, lane 2: RVV-X light chain integration, lane 3: metalloproteinase domain integration, lane 4: disintegrin domain integration, Lane C: PCR control.

1.2.2.2. Protein expression of recombinant RVV-X in *Pichia pastoris*

The transformed *Pichia pastoris* were cultured in BMGY and BMMY media. After the protein induction by methanol, culture media containing yeast cells were collected to investigate the protein expression at every 24 hour for 6 days. **Figure 20** showed the protein expression of each transformed yeast cells at day 6. No protein bands were observed in all lanes except in the control yeast cell. This demonstrated that the expressed proteins may be quite low to be directly detected by SDS-PAGE coomassie blue staining. Therefore, the culture media were approximately 25 times concentrated by Vivaspin columns, the concentrated culture media were loaded on SDS-PAGE as shown in **Figure 21**. As shown in the figure, some of unexpected bands were observed in the culture media, therefore, the proteins were further purified by MagneHis system to get only the expressed proteins which contain Histidine tagging in their C-terminals. The MagneHis purified proteins were run on SDS-PAGE and stained by silver chloride for more protein detection sensitivity, **Figure 22**.

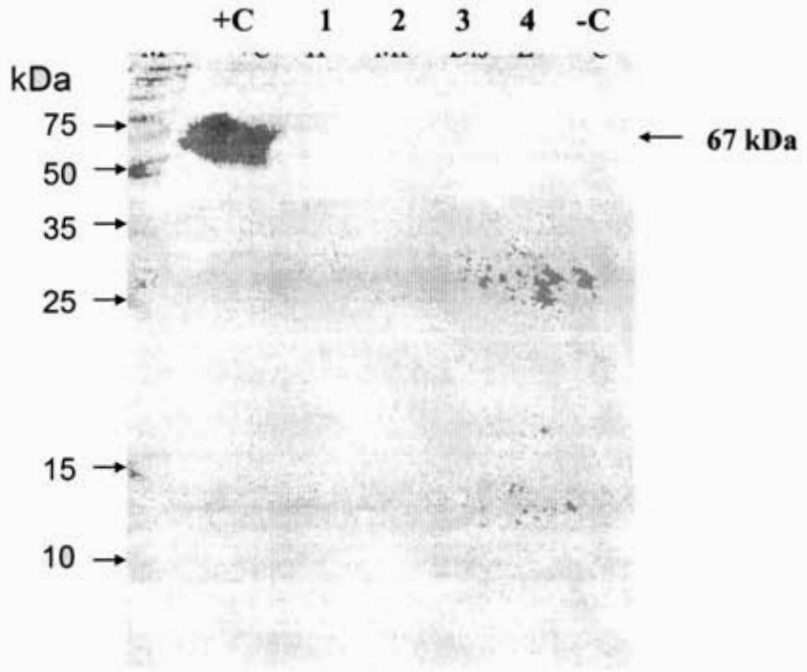


Figure 20. Coomassie blue-stained SDS-PAGE electrophoresis of yeast culture media after induction by methanol at Day 5. Lane +C: albumin control (67 kDa), lane 1: RVV-X heavy chain, lane 2: metalloproteinase domain, lane 3: disintegrin domain, lane 4: RVV-X light chain, lane –C: transformed yeast cell with empty pPicZα-A.

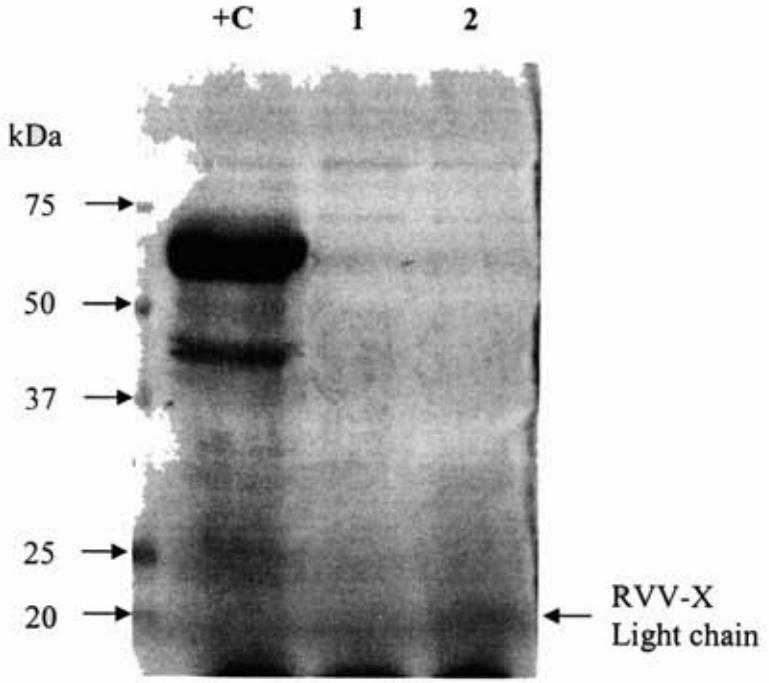


Figure 21. SDS-polyacrylamide gel electrophoresis of (25 times) <u>concentrated</u> yeast culture media after induced by methanol at Day 5. Lane +C : albumin control (67 kDa), lane1: RVV-X heavy chain, lane 2: RVV-X light chain.

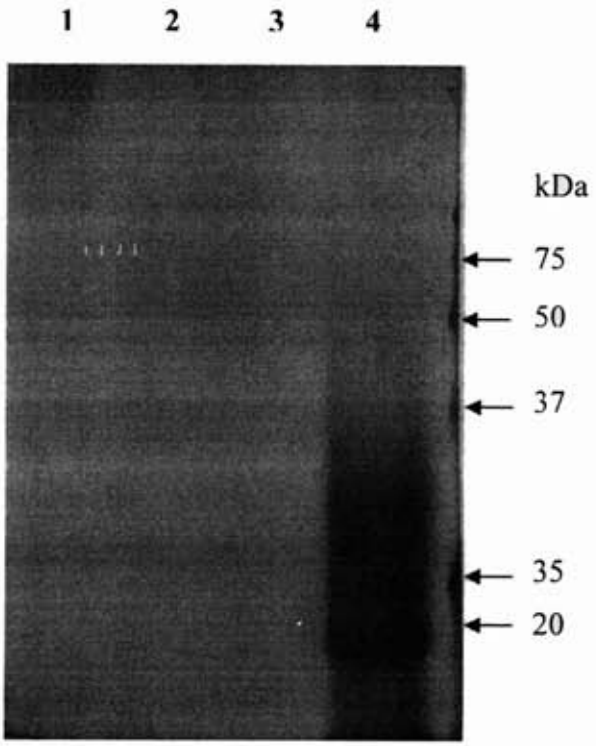


Figure 22. Silver-stained of SDS-polyacrylamide gel electrophoresis of (25 times) concentrated yeast culture media after induced by methanol at Day 5 and purified by MagneHis column. Lane 1: RVV-X heavy chain, lane 2: metalloproteinase domain, lane 3: disintegrin domain, and lane 4: RVV-X light chain.

1.2.2.3. Detection of recombinant proteins by Western blotting analysis

There were several attempts to detect recombinant proteins by using antibodies against blotted proteins from SDS-PAGE. Nevertheless, none of them were detected by anti-His (C-term) antibody and RVV immunized-horse serum antibodies. Therefore, the recombinant proteins were analyzed by dot blot analysis instead. Concentrated culture media by Vivaspin column from *Pichia* cultures were blot onto nitrocellulose membrane. Then C-terminal anti-His antibody was added. Signal of substrate development after DAB adding was shown in **Figure 23**.

To check the affinity binding between expressed protein and RVV-immunized horse antibody, the expressed proteins were blot onto nitrocellulose and performed as mentioned above but using purified horse IgG instead of C-terminal anti-His antibody, **Figure 24**.

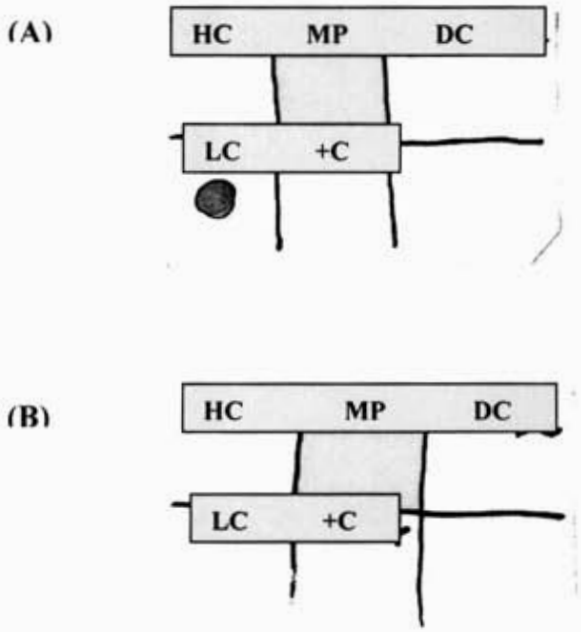


Figure 23. Dot blot analysis of concentrated culture media using C-terminal anti-His antibody. After (A) and before (B) MagneHis-purification. HC : RVV-X heavy chain, MP: metalloproteinase domain, DC: disintegrin domain, LC: RVV-X light chain, and +C: human serum albumin from yeast control.

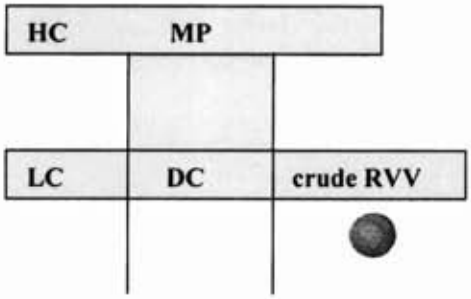


Figure 24. Dot blot analysis of MagneHis-purified culture media using RVV-immunized horse IgG antibodies. HC: RVV-X heavy chain, MP: metalloproteinase domain, DC: disintegrin domain, LC: RVV-X light chain, and crude RVV as positive control.

1.2.2.4. Enzymatic activity testing of recombinant RVV-X

Almost of recombinant RVV-X expression in *Pichia pastoris* yielded undetectable of expressed proteins or very low in RVV-X light chain. However, there was the attempt to test its enzyme activity since RVV-X activity test by PTT is very sensitive which can detect as low as 1 ng of purified RVV-X. Nevertheless, none of recombinant RVV-X (using eluted solution from MagneHis columns, the final step in protein purification) gave the detectable enzyme activity as shown in **Table 8**.

Table 8. Enzymatic activity testing of recombinant RVV-X by PTT.

	Purified RVV-X	**Recombinant RVV-X**	**clotting time** (min:sec)
1	-	-	3:10 (±0:05)
2	+	-	0:31 (±0:03)
3	-	RVV-X/HC	3:00 (±0:15)
4	-	RVV-X/MP	2:45 (±0:30)
5	-	RVV-X/DC	3.20 (±0:20)
6	-	RVV-X/LC	3.22 (±0:20)

1.3. DNA immunization

1.3.1. Construction of pVaxSec expression cassettes

RVV-X was also cloned separately in 4 types as in pPicZα-A cloning; 1) pVaxSec/HC, 2) pVaxSec/MP, 3) pVaxSec/DC, and 4) pVaxSec/LC. PCR primers were designed to amplify the cDNA sequences of each desired DNA fragments, **Figure 25**. The PCR products were then cloned into TA cloning vector, pCR2.1-TOPO (**Figure 26**), prior to subcloned into pVaxSec vector (**Figure 27**). The correct sequences were confirmed by DNA sequencing to ensure the in-frame reading protein translation.

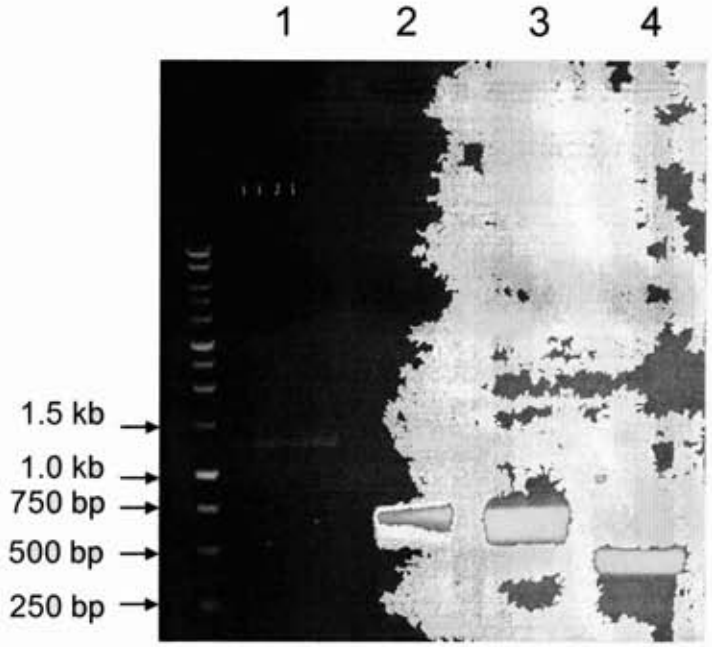


Figure 25. PCR products of RVV-X for cloning into pVaxSec. Lane 1: RVV-X heavy chain, lane 2: metalloproteinase domain, lane 3: disintegrin domain, lane 4: RVV-X light chain.

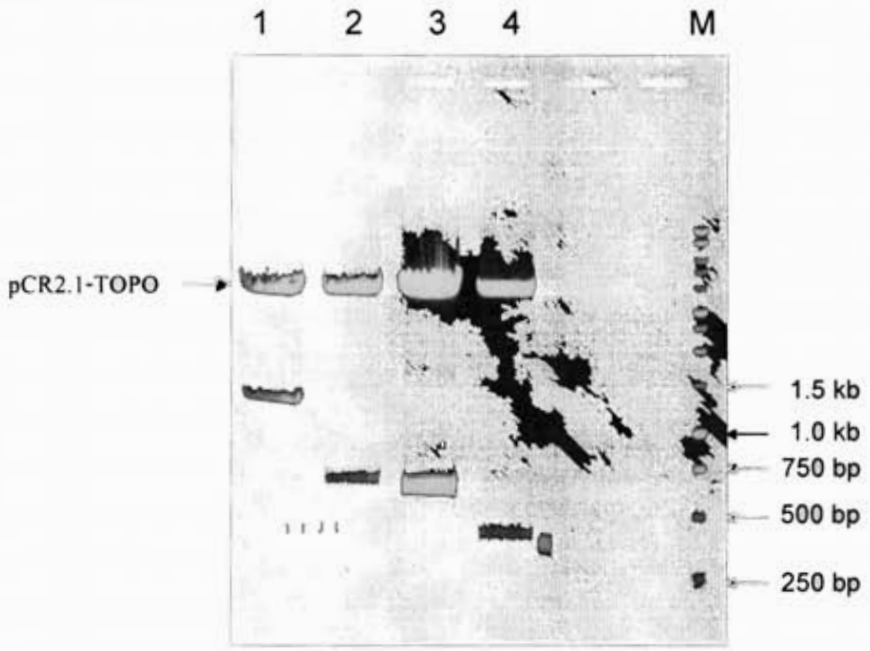


Figure 26. *Bam* HI and *Xho* I digestion of recombinant pCR2.1-TOPO. Lane 1: RVV-X heavy chain, lane 2: disintegrin domain lane, 3: metalloproteinase domain, lane 4: RVV-X light chain, and M: DNA marker.

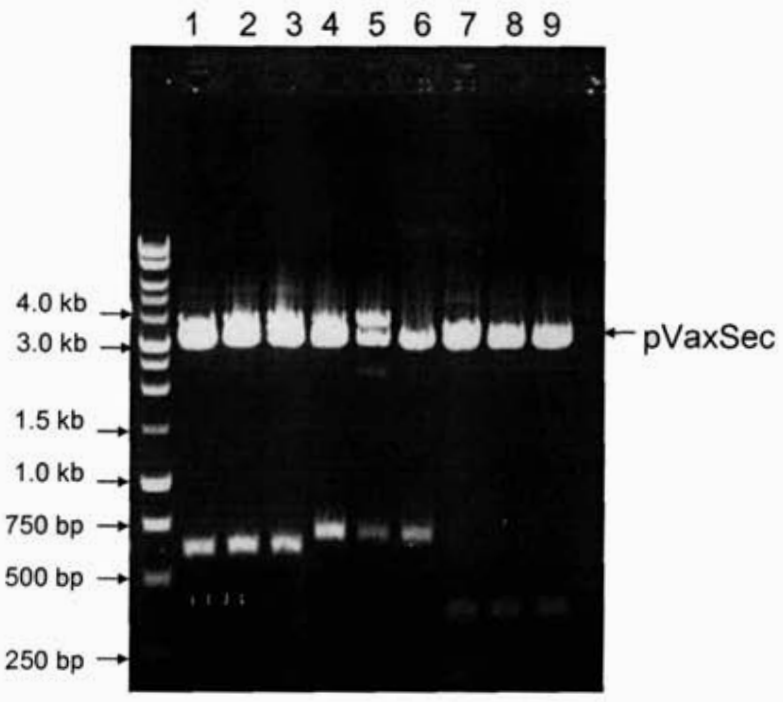


Figure 27. *Bam* HI and *Xho* I digestion of recombinant pVaxSec. Lane 1-3: metalloproteinase domain, lane 4-6: disintegrin domain, and lane 7-9: RVV-X light chain.

1.3.2. DNA immunization in mice by gene gun transfer

DNA immunized cassettes were prepared into 5 types, pVaxSec/HC, pVaxSec/MP, pVaxSec/DC, pVaxSec/LC, and pVaxSec empty vector. Experimental mice for each DNA immunized were 6, thus 30 mice required for this work. However, before the blood collection, 2 mice died (1 from pVaxSec/DC and 1 from pVaxSec/HC). Approximate 1.0 ml of blood was collected from each mouse at day 28 after the first immunization which can then prepared for approximate 0.5 ml of mouse serum.

1.3.3. Immunoblot

The mice sera from DNA immunization experiments were tested by immunoblot to determine the presence of the raised antibodies. Crude RVV (**Figure 28**) and purified RVV-X (**Figure 29**) were run on SDS-PAGE under reducing condition and hybridized to the raised antibodies from each mice group. The affinity binding of raised antibody against non-reduced form of purified RVV-X was also determined, **Figure 30.**

The immunoblot result indicated that antibodies from pVaxSec/DC immunized mice were able to bind to RVV-X in crude venom and purified RVV-X. It also bound to both form of reduced or non-reduced purified RVV-X.

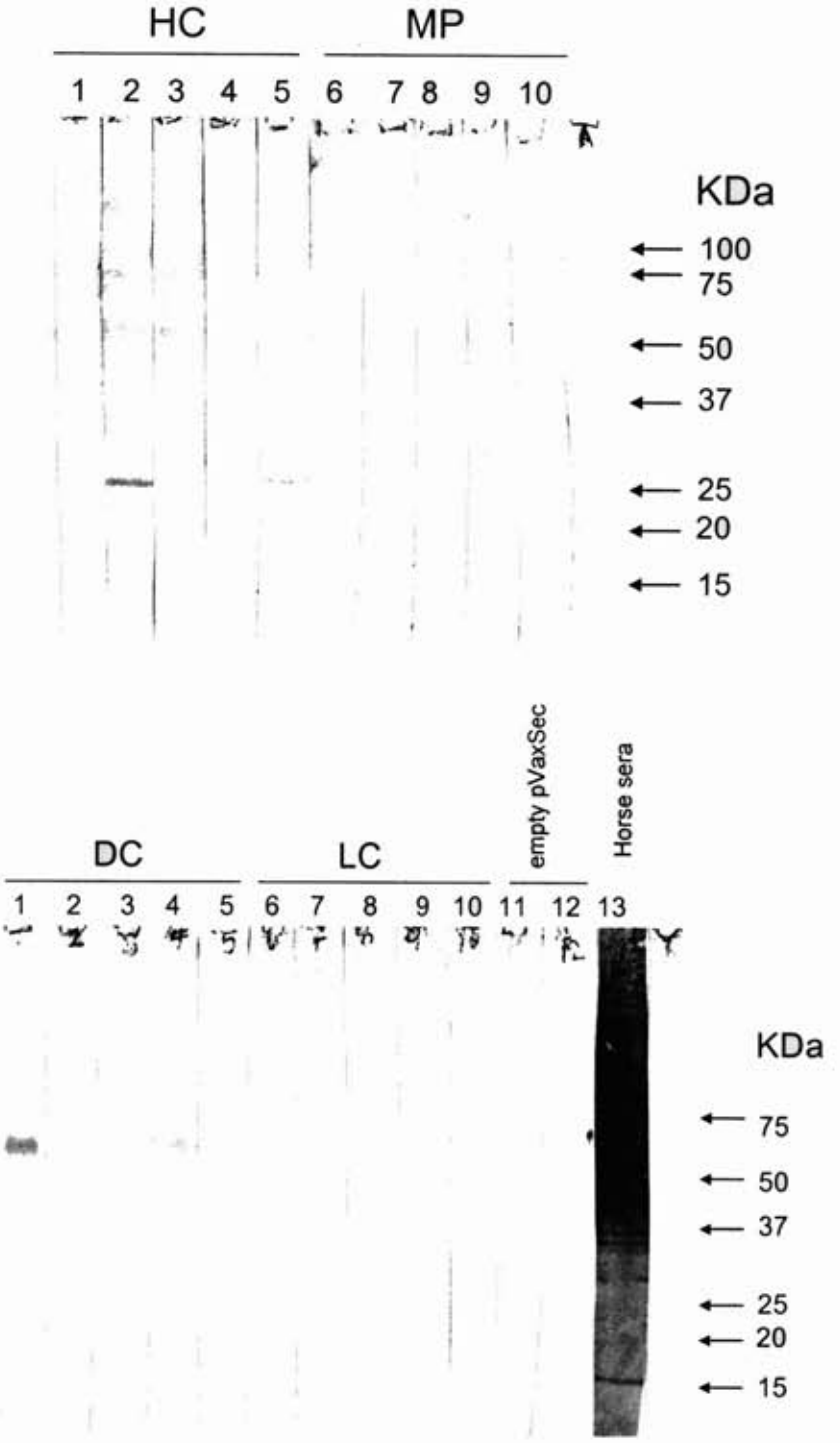


Figure 28. Immunoblot analysis of <u>reduced-crude RVV</u> against DNA immunized mice sera. Each DNA immunized group consists of 5 mice sera, except empty pVaxSec control (2 mice sera).

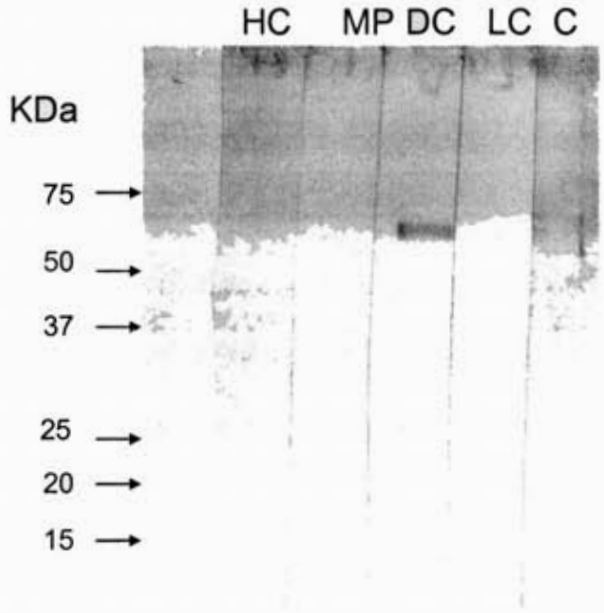


Figure 29. Immunoblot analysis of purified <u>reduced RVV-X</u> against DNA immunized mice sera. Lane C: immunized by empty pVaxSec, lane LC: by RVV-X light chain, lane DC: by RVV-X disintegrin domain, lane MP: by RVV-X metalloproteinase domain, lane HC: by RVV-X heavy chain.

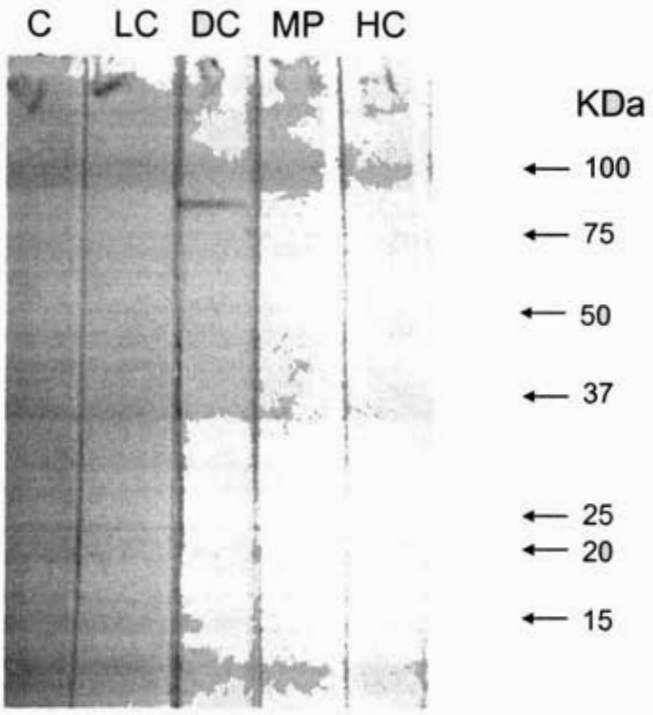


Figure 30. Immunoblot analysis of purified <u>non-reduced RVV-X</u> against DNA immunized mice sera. Lane C: immunized by empty pVaxSec, lane LC: by RVV-X light chain, lane DC: by RVV-X disintegrin domain, lane MP: by RVV-X metalloproteinase domain, lane HC: by RVV-X heavy chain.

1.3.4. Neutralization of raised antibodies against RVV-X induced plasma clotting

The mice sera from pVaxSec/DC immunized group were tested for PTT.

Table 9. Neutralization of RVV-X-induced plasma clot by antibodies (PTT test).

	Purified RVV-X	horse IgG	DC serum	Mouse serum	clotting time (min:sec)
1	-	-	-	-	3:10 (±0:05)
2	+	-	-	-	0:31 (±0:03)
3	+	+	-	-	3:10 (±0:15)
4	+	-	+	-	< 0:20
5	+	-	-	+	<0:20
6	+	+	+	-	2:20 (±0:20)

From **Table 9**, the clotting time of normal plasma was 3:10 min (tube 1) while RVV-X induced plasma clotting was shorten to 0:31 (tube 2). When horse IgG which purified from crude RVV-immunized horse serum was added, the clotting time was prolonged to 3:10 min (tube 3) indicating that neutralization of RVV-X activity. When mice serum from pVaxSec/DC immunized group were added (tube 4 and 5) the clotting time were shorten compared to tube 2 and 3. This result suggesting the interferences from mouse serum can shorten (instead of prolong) the clotting time.

Therefore, the mouse IgG must be purified from mouse serum before performing PTT assay to remove any impurities or even mouse clotting factors. However, the remaining pVaxSec/DC mice sera were less than 200 ul which is too small amount to be purified for the repeated PTT assay.

CHAPTER V

CONCLUSION AND DISCUSSION

1. Proteins in Russell viper 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. By using Expressed Sequence Tags analysis, hundreds of venom protein compounds have been identified and also elucidate their nucleotide or deduced protein sequences. 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. A few years ago, there were a few records of nucleotide or protein sequences from RV venom in Genbank database. Recently, tens to hundreds of nucleotide or protein sequences of RV have been reported but many from the study of phospholipase A2 or mitochondria genes. This work has also reported several novel genes waiting for further characterize and study.

1.1 Characteristic of some key toxins found in RV venom

Phospholipase A2

Phospholipase A2, a multifunctional enzyme, one of the key toxins in several snake venoms. Dried weight of PLA2 may up to 12% of the whole venom. PLA2 sequences obtained in this study were also distinguished into 2 forms as previously reported (Tsai *et al.*, 1996), RV-4 and RV-7. By ESTs analysis and plaque-lift hybridization, we have identified 8 clones of RV-4 and 18 clones of RV-7. RV-4 itself was nerotoxic, whereas RV-7 had much lower enzymatic activity and was not toxic.

Since RV bite in Thailand did not show neurotoxic property (Warrell *et al.*, 1986) this may due to the high proportion of RV-7 form compare to RV-4 form (in Taiwan RV, *Daboia russelli formosensis*, RV-4 and RV-7 proteins seem to be equally presence). The signal peptides of PLA2 of both forms were identical with 16 residues.

BPP-CNP

BPP-CNP stands for Bradykinin Potentiating Peptide-C-type Natriuretic Peptides. This protein has been first described from *Bothrops jararaca* venom in the middle of 1960s. It was the first natural inhibitor of the angiotensin-converting enzyme displaying strong anti-hypertensive effects in human subjects. However, there was no previous report of BPP-CNP precursors in RV venom. The presence of BPP-CNP in RV venom indicates the using of these small peptides in affecting blood pressure of the prey. Moreover, there was the effort to develop BPPs as the early drugs aimed to control unbalanced cardiovascular functions (Ondetti *et al.*, 1982).

Factor V activator and Factor X activator (RVV-V and RVV-X)

ESTs encode the two major procoagulant factors in RVV which activate factor V and X (Than-Than *et al.*, 1988) were obtained in this library. The presence of these two proteases supports the observation of disseminated intravascular coagulation in the bitten patients (Warrell *et al.*, 1989). RVV-V and RVV-X induce the coagulation of mammalian plasma. RVV-X activates factors X and IX by limited proteolysis in a calcium-dependent manner, whereas RVV-V activates factor V in a calcium independent manner.

Other proteins found in snake venom

The common gene expressed found in the RV venom include genes for ribosomal proteins, membrane proteins, and several transcription regulators. Other interesting RV genes showed similarity to those genes encoding for;

Axotrophin : Axotrophin (axot) is a newly characterised stem cell gene and mice that lack axotrophin are viable and fertile, but show premature neural degeneration and defective development of the corpus callosum. It also involved in immune regulation. Both T cell proliferation and T cell-derived leukaemia inhibitory factor (LIF) were suppressed by axotrophin in a gene-dose-dependent manner (Metcalfe *et al.*, 2005).

Nucleotidase : as found in many snakes, nucleotidase was also present in RV venom. Apart from proteases and phospholipase A2s (PLA2s), 5'nucleotidase is known to affect hemostasis by inhibiting platelet aggregation (Dhananjaya *et al.*, 2006).

Alpha lebetin : A divergent natriuretic toxin from the viper *Macrovipera lebetina* (Barbouche *et al.*, 1996). Synthetic L1alpha and L1beta inhibited collagen-induced platelet aggregation in the nanomolar range. The activity of L1 peptides was also tested in vivo: their intravenous administration strongly inhibited collagen-induced thrombocytopenia in rats (Barbouche *et al.*, 1998).

Atrolysin : A haemorrhagic metalloproteinase atrolysin A derived from the venom of Crotalus atrox venom. The cysteine-rich domain of the haemorrhagic metalloproteinase atrolysin A was shown to inhibit collagen-stimulated platelet aggregation and to interact with MG-63 osteosarcoma cells via integrin alpha2beta1 to inhibit adhesion to collagen I. By solid-phase binding assays, atrolysin A also binds to collagen I and to vWF (von Willebrand factor) via exosites in the cysteine-rich domain (Serrano *et al.*, 2005).

Batroxobin (defibrase) : a thrombin-like serine protease derived from the venom of Bothrops atrox (Wu *et al.*, 2005).

Salmobin : the thrombin-like enzyme obtained from Korean snake (Agkistrodon halys) venom (Jeong *et al.*, 2001).

By the way, ESTs data reported here were derived from a small number of cloned genes. In order to obtain more reliable proportion of gene expressed in the venom more ESTs are needed. These ESTs data could provide resource for study gene expressed pattern in RV, develop assay to identify RV snake like PCR, and may serve for gene mapping in this organism in the future.

1.2. Sequence analysis of RVV-X

The amino acid alignment between RVV-X heavy chain found from this study (*Daboia russelli siamensis*) showed 4 amino acid residues longer (**PSQI**- at C terminal end)compared to previous report of RVV-X heavy chain obtained from direct protein sequencing from *Daboia russelli siamensis* venom as well, **Figure 31**. While amino acid alignment between RVV-X light chain reported from this study was identical to the previous report of RVV-X LC1 obtained from direct protein sequencing from *Daboia russelli siamensis* venom, **Figure 32**.

The RVV-X heavy chain reported here share 66-88 % amino acid identical to the closest sequence from *Macrovipera lebetina*, *Echis carinatus*, and *E. ocellatus*. This indicate that RVV-X heavy chain from *Daboia russelli* is quite unique and classified in P(IV) of snake venom metalloproteinase. Recently, several isoforms of C-type lectins (CTLs) from RV have been reported in the Genback. RVV-X light chain is also classified to a CTL, they are closely related to other CTLs reports with 77-100 % amino acid identity. As seen in **Figure 32**, most diverse regions are located in N-terminal ends of these CTLs. Since several CTLs have been reported from the same

species of *Daboia russelli siamensi,* it raises the question which CTLs are the 2 light chains of RVV-X. It is possible that 2 light chains of RVV-X may form with several combinations of these CTLs resulting in the broad M.W. of RVV-X besides from the degree of carbohydrate moiety.

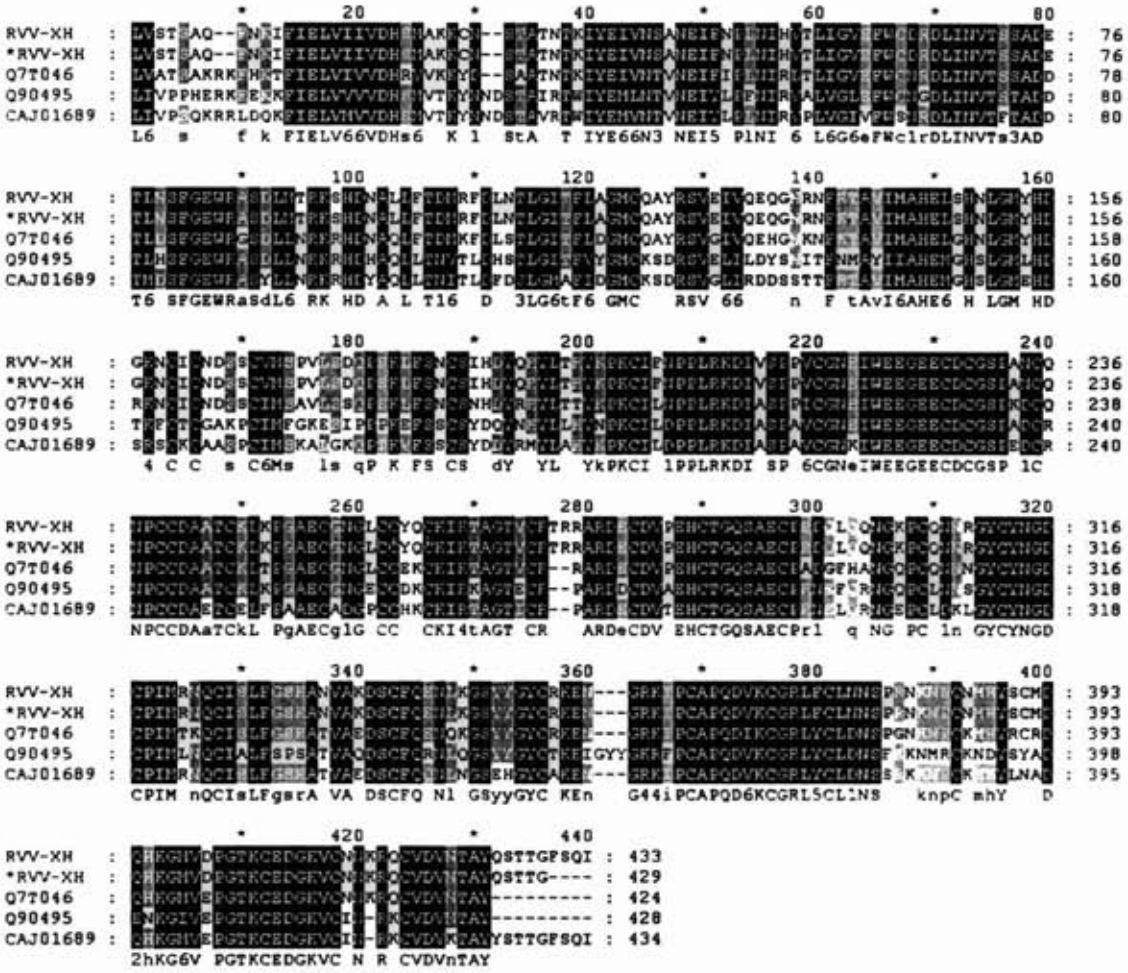


Figure 31. Amino acid alignment of mature RVV-X heavy chain from this study and previous reports. **RVV-XH**, from this study; ***RVV-XH**, from *Daboia russelli siamensis* by direct protein sequencing; **Q7T046,** from *Macrovipera lebetina* (VLFXA heavy chain); **Q90495,** from *Echis carinatus* (saw-scaled viper) Ecarin precursor; and **CAJ01689,** from *Echis ocellatus* (Group III snake venom metalloproteinase).

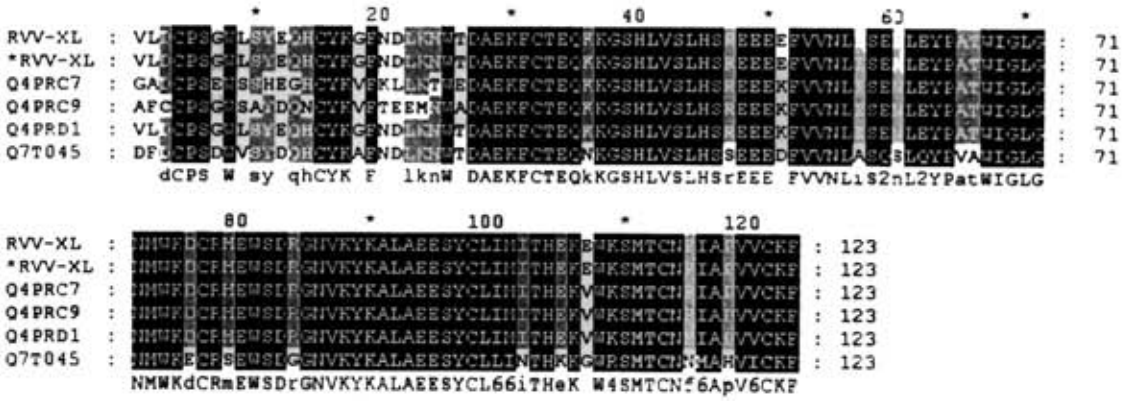


Figure 32. Amino acid alignment of mature RVV-X light chain from this study and previous reports. **RVV-XL**, from this study; ***RVV-XL**, from *Daboia russelli siamensis* by direct protein sequencing; **Q4PRC7,** one of C-type lectin from *Daboia russelli siamensis*; **Q4PRC9,** one of C-type lectin from *Daboia russelli siamensis*; **Q4PRD1,** LC1 from *Daboia russelli siamensis*; and **Q7T045**, LC1 from *Macrovipera lebetina*.

2. Protein expression in *Pichia* yeast

The expression yield of heterologous RVV-X in *Pichia pastoris* was undetectable or very low. The RVV-X heavy chain or its two domains; metalloproteinase and disintegrin, could not be detected by SDS-PAGE or antibody detection. Only RVV-X light chain could be detected after concentrated by Vivaspin column. In many attempts, several parameters which may affect the expression yield were changed including *Pichia* strains (KM71H, X33, and GS115), culture media, pPicZα-A, etc. However, the protein expression of RVV-X still unsatisfied. Since the yeast control which constructed to secrete human serum albumin in the culture media gave high level of protein expression, indicating that expression condition are eligible for *Pichia* expression (methanol induction, culture media, incubation temperature, protein detection system, etc.). Therefore, the explanation for unsuccessfully *Pichia* expression might due to the nature of RVV-X itself or even the transformed yeasts. The expression of metalloproteinase protein might harmful to the yeast cell itself leads to low or undetectable protein expression. Interestingly, with several reports of successfully snake toxins expressed in *Pichia pastoris*, there was no report of the expression of snake metalloproteinase as the summary of in **Table 4** in Chapter II. Most of the shown proteinases are belong to serine-proteinase.

3. RVV-X specific antibody production

Using DNA immunization approach, RVV-X specific antibody could be obtained from mice sera immunized by pVaxSec/DC. While other RVV-X domain constructs could not generate RVV-X specific antibody in immunized mice. This observation is corresponding to the previous report from the study of SVMPs of *Echis ocellatus* (Wagstaff *et al.*, 2005) in which DC domain give better antibody production compare to the whole heavy chain (MPDC) or MP domain **Figure 33A**. The explanation might due to the presence of antigenic epitopes which are frequently found in DC domain than MP domain **Figure 33B**. The antigenic profile prediction of RVV-X heavy chain and light chain were shown in **Figure 34** and gave similar pattern to the reported SVMP's, **Figure 33B**.

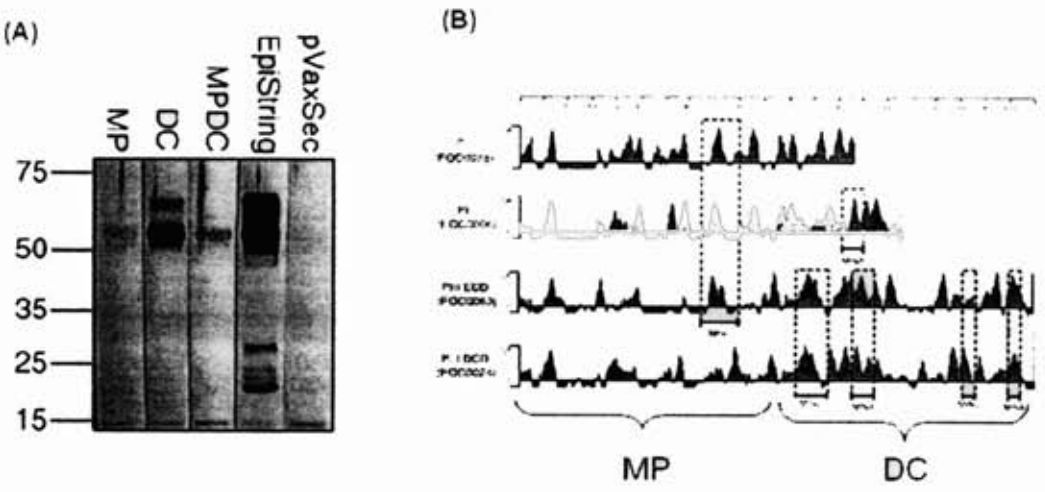
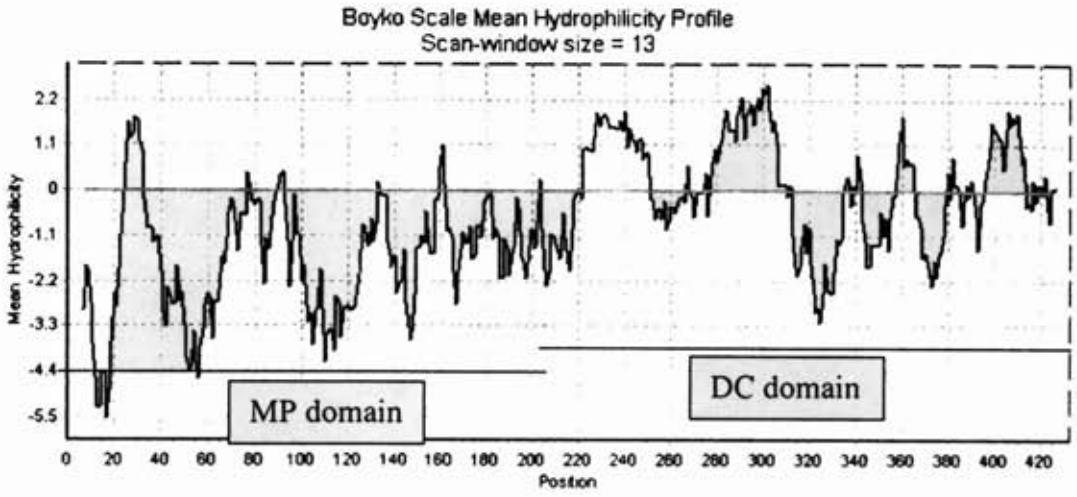


Figure 33. DNA immunization by SVMP multi-epitopes string. (A) Immunoblot of mice sera antibody against crude *Echis ocellatus* venom. (B) Antigenic profile prediction of *E. ocellatus* SVMPs (Wagstaff *et al.*, 2005).

RVV-X heavy chain



RVV-X light chain

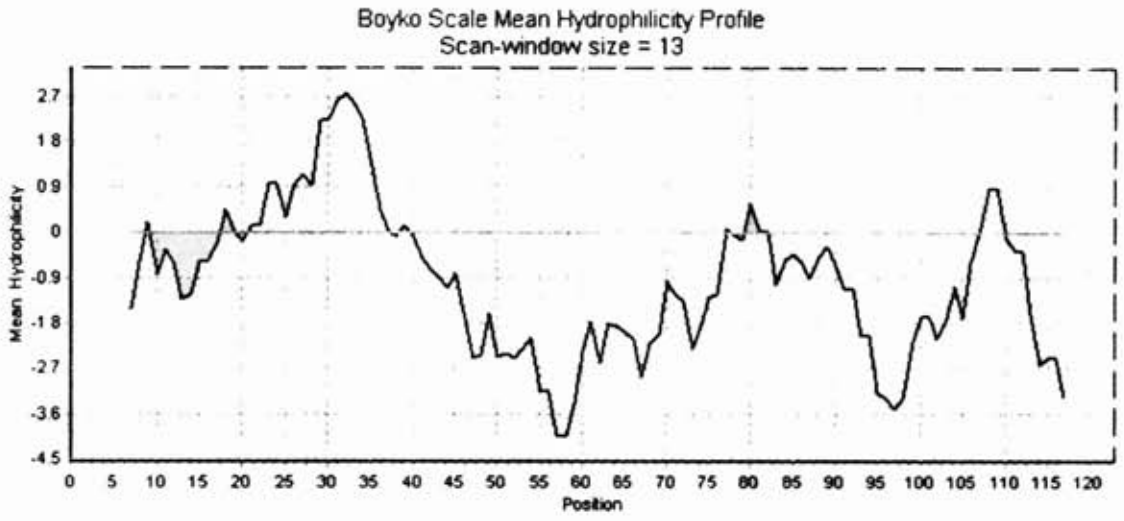


Figure 34. The antigenic profile prediction of RVV-X heavy chain (upper) and RVV-X light chain (lower). These plots were determined by Boyko Scale Mean Hydrophilicity Profile.

The avidity between antibody from pVaxSec/DC immunized mice sera and RVV-X (both reduced and non-reduced forms) implies the RVV-X neutralization property of this antibody. However, in order to perform RVV-X neutralization assay, IgG antibody must be purified from pVaxSec/DC mice sera to remove all unwanted compounds such as mouse clotting factors or any impurities. Nevertheless, the mice sera were remain in limited amount to be purified for IgG by protein G column. The bigger animals like rabbit or horse are the next target for DNA immunized by snake venom toxin to get more antibody production.

To improve the antibody production by DNA immunization, some parameter may be changed. In protein translation, snake codon usage are a bit different from mammalian codon usage (**Table 10-11**), the significant codon bias are shown in bold letters. However, the codon usage data of *Daboia russelli* are limited reports with only 10 CDS. Alteration of snake codon adjusted to mammalian host codon bias (e.g. mouse or horse) in DNA immunized expression vector might yield better antibody production. As shown in antigenic profile prediction, only some parts of protein sequence may be able to trigger the immune response. Therefore, construction of DNA immunized cassette might need only the particular DNA encoding the strong antigenic peptide instead of constructing by the whole DNA molecule. By this approach, it allows several cDNA encoding several key snake toxins to be constructed in one expression cassette as a multi-epitopes string. This method showed to be the effective way to produce multi-toxin specific antibodies in one-shot DNA immunization. With carefully designed, particular DNA encoding the strong antigenic peptide of key toxins that are highly conserved among major venomous snakes could be generated for producing the pool of antibodies which can cross neutralize many snake toxins in the future.

Table 10. Codon usage of Russell's viper *Daboia russellii siamensis*. Derived from 10 CDS's (1,472 codons)

fields: [triplet] [frequency: per thousand] ([number])

UUU 18.3(27)	UCU 10.9(16)	UAU 15.6(23)	UGU 18.3(27)
UUC 46.9(69)	UCC 29.2(43)	UAC 25.1(37)	**UGC 42.8**(63)
UUA 4.1(6)	UCA 6.8(10)	UAA 3.4(5)	UGA 0.0(0)
UUG 17.0(25)	UCG 2.0(3)	UAG 3.4(5)	UGG 39.4(58)
CUU 6.8(10)	**CCU 5.4**(8)	CAU 17.0(25)	CGU 4.1(6)
CUC 16.3(24)	**CCC 5.4**(8)	CAC 8.8(13)	CGC 4.1(6)
CUA 0.0(0)	CCA 4.8(7)	CAA 6.1(9)	CGA 5.4(8)
CUG 31.2(46)	CCG 2.0(3)	**CAG 13.6**(20)	CGG 4.8(7)
AUU 6.8(10)	ACU 10.9(16)	AAU 25.1(37)	AGU 12.2(18)
AUC 20.4(30)	ACC 17.0(25)	AAC 23.1(34)	AGC 20.4(30)
AUA 10.2(15)	ACA 10.2(15)	AAA 36.7(54)	AGA 6.8(10)
AUG 27.9(41)	ACG 6.1(9)	AAG 36.7(54)	AGG 7.5(11)
GUU 8.2(12)	GCU 12.9(19)	GAU 29.2(43)	GGU 8.2(12)
GUC 29.9(44)	GCC 13.6(20)	GAC 17.7(26)	GGC 21.7(32)
GUA 6.1(9)	GCA 16.3(24)	GAA 45.5(67)	GGA 20.4(30)
GUG 20.4(30)	GCG 6.1(9)	GAG 27.9(41)	GGG 19.0(28)

Coding GC 48.03% 1st letter GC 43.89% 2nd letter GC 39.47% 3rd letter GC 60.73%

Table 11. Codon usage of mouse *Mus musculus*. Derived from 44265 CDS's (20,326,463 codons)

fields: [triplet] [frequency: per thousand] ([number])

UUU 17.1(346577)	UCU 16.1(326745)	UAU 12.1(245823)	UGU 11.1(226235)
UUC 22.0(447802)	UCC 18.1(367625)	UAC 16.3(330873)	**UGC 12.1**(246546)
UUA 6.6(133165)	UCA 11.7(236824)	UAA 0.7(14002)	UGA 1.2(24809)
UUG 13.3(270889)	UCG 4.3(86648)	UAG 0.6(11863)	UGG 12.4(251860)
CUU 13.3(269749)	**CCU 18.4**(373009)	CAU 10.4(210521)	CGU 4.7(95291)
CUC 20.3(412247)	**CCC 18.3**(371735)	CAC 15.3(310356)	CGC 9.5(192518)
CUA 8.0(162477)	CCA 17.1(348549)	CAA 11.7(236875)	CGA 6.6(134316)
CUG 40.0(812149)	CCG 6.2(126015)	**CAG 34.2**(694579)	CGG 10.3(210309)
AUU 15.4(313042)	ACU 13.6(276739)	AAU 15.5(315322)	AGU 12.5(254165)
AUC 22.9(465043)	ACC 19.1(388937)	AAC 20.5(417613)	AGC 19.7(400017)
AUA 7.2(145637)	ACA 15.9(322804)	AAA 21.7(440296)	AGA 11.7(238615)
AUG 22.9(465092)	ACG 5.7(116777)	AAG 33.9(688311)	AGG 12.0(243598)
GUU 10.6(216432)	GCU 20.1(408274)	GAU 21.1(428155)	GGU 11.4(232559)
GUC 15.6(316130)	GCC 26.3(534775)	GAC 26.4(536436)	GGC 21.5(437378)
GUA 7.4(149792)	GCA 15.8(321979)	GAA 26.9(546690)	GGA 16.8(341114)
GUG 28.8(585727)	GCG 6.5(132545)	GAG 39.7(806693)	GGG 15.3(310795)

Coding GC 52.21% 1st letter GC 55.43% 2nd letter GC 42.21% 3rd letter GC 58.99%

4. Conclusion

This work tried to study the Russell's viper venom in many aspects. We started from the identification of RV venom by ESTs approach to get more data of molecular biology of RV venom. From ESTs data, we found many toxins affecting the hematostasis after snake bites and some of them still unknown functions. One of those toxins, RVV-X, found to be the key toxin in RV bite and also has a potential to be adopted in medical use. Therefore, RVV-X was selected to be further characterize, clone and express in eukaryotic system, *Pichia pastoris*. Nevertheless, the heterologous RVV-X expression in *Pichia* gave unsatisfied result. So, it needs further effort to solve the expression problem. In the mean time, for better treatment of RV bite patients, specific antibody against RVV-X was successfully produced by DNA immunization in mice model. This preliminary data may be used in toxin-specific antibody production for better antivenom used or for special purpose such as toxin purification.

REFERENCES

ภาษาไทย

สุชัย สุเทพารักษ์. งูพิษกัดและการดูแลผู้ป่วย. วารสารเวชศาสตร์สิ่งแวดล้อม 2542; 171-181.

ภาษาอังกฤษ

Altschul, Stephen F., Thomas L, *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997; 25:3389-3402.

Atoda H., Yoshida N., Ishikawa M, Morita T. Binding properties of the coagulation factor IX/factor X-binding protein isolated from the venom of *Trimeresurus flavoviridis*. Eur J Biochem 1994; 224:703–708.

Barbouche R., Marrakchi N., Mansuelle P., Krifi M., Fenouillet E., Rochat H., el Ayeb M. Novel anti-platelet aggregation polypeptides from Vipera lebetina venom: isolation and characterization. FEBS Lett. 1996; 392(1):6-10.

Barbouche R., Marrakchi N., Mabrouk K., Krifi M.N., Van Rietschoten J., Fenouillet E., El Ayeb M., Rochat H. Anti-platelet activity of the peptides composing the lebetin 1 family, a new class of inhibitors of platelet aggregation. Toxicon. 1998; 36(12):1939-47

Chanhome L., Jintakune P., Wilde H., Cox M.J. Venomous snake husbandry in Thailand. Wilderness Environ Med 2001; 12(1):17-23.

Dennis, E.A. Diversity of group types, regulation, and function of phospholipase A2. J Biol Chem. 1994; 269:13057-13060.

Dhananjaya B.L., Nataraju A., Rajesh R., Raghavendra Gowda C.D., Sharath B.K., Vishwanath B.S., D'Souza C.J. Anticoagulant effect of Naja naja venom 5'nucleotidase: demonstration through the use of novel specific inhibitor, vanillic acid. Toxicon. 2006; 15;48(4):411-21.

Fujikawa K., Legaz M.E., Davie E.W. Mechanism of activation by protein from Russell's viper venom. Biochemistry. 1972; 11,4892-4899.

Gowda D.C., Jackson C.M., Kurzban G.P., McPhie P., Davidson E.A. Core sugar residues of the N-linked oligosaccharides of Russell's viper venom factor X-activator maintain functionally active polypeptide structure. Biochemistry. 1996; 35: 5833–5837.

Harrison R.A., Moura-da-Silva A.M., Laing G.D., Wu Y., Richards A., Broadhead A., et al. Antibody from mice immunised with DNA encoding the carboxyl-disintegrin and cysteine rich domain (JD9) of the haemorrhagic metalloprotease, Jararhagin, inhibits the main lethal component of viper venom. J Clin Exp Immunol. 2000; 121:358–63.

Jeong H., Yoo S., Kim E. Cell surface display of salmobin, a thrombin-like enzyme from Agkistrodon halys venom on Escherichia coli using ice nucleation protein. Enzyme Microb Technol. 2001; 28(2-3):155-160.

Kini R.M. and Iwanaga S. Structure-function relationships of phospholipase1: prediction of presynaptic neurotoxicity. Toxicon. 1986; 24,527-541.

Kini R.M., Morita T., Rosing J. Classification and nomenclature of prothrombin activators isolated from snake venoms. Thromb Haemost. 2001; 86(2):710-1.

Koh D.C.I., Armugam A., Jeyaseelan K. Snake venom components and their applications in biomedicine. Cell. Mol. Life Sci. 2006; DOI 10.1007/s00018-006-6315-0.

Kisiel W., Hermodoson M.A, Davie E.W. Factor X activating enzyme from Russell's viper venom: isolation and characterization. Biochemistry. 1976; 15,4901-4906.

Marsh N.A. Inventory of haemorrhagic factors from the snake venoms. Thromb. Haemost. 1994. 71, 793–797.

Maung-Maung-Thwin, Gopalakrishnakone P., Yuen P. and Tan C.H. A major lethal factor of the venom of Burmese Russell's viper *(Daboia Russelli Siamensis)*: Isolation, N-terminal sequencing and biological activities of daboiatoxin. Toxicon. 1995; 63-76.

Metcalfe S.M., Muthukumarana P.A., Thompson H.L., Haendel M.A., Lyons G.E. Leukaemia inhibitory factor (LIF) is functionally linked to axotrophin and both LIF and axotrophin are linked to regulatory immune tolerance. FEBS Lett. 2005; 31:609-14.

Mizuno H., Fujimoto Z., Koizumi M., Kano H., Atoda H., Morita T. Structure of coagulation factors IX/Xbinding protein, a heterodimer of Ctype lectin domains. Nat Struct Biol. 1997; 4:438–441.

Mizuno H., Fujimoto Z., Koizumi M., Kano H., Atoda H., Morita T. Crystal structure of coagulation factor IXbinding protein from habu snake venom: Implication of central loop swapping based on deletion in the linker region. J Mol Biol. 1999; 289:103–112.

Mizuno H., Fujimoto Z., Atoda H., Morita T. Crystal structure of an anticoagulant protein in complex with the Gla domain of factor X. Proc Natl Acad Sci. 2001; 98:7230–7234.

Numeric P., Moravie V., Didier M., Chatot-Henrey D., Cirille S., Bucher B., Thomas L. Multiple cerebral infarctions following snakebite by *Bothrops carribbaeus*. Am. J. Trop. Med. Hyg. 2002; 67:287–288.

Ondetti M.A., Cushman D.W. Enzymes of the renin-angiotensin system and their inhibitors. Annu Rev Biochem. 1982; 51:283–308.

Phillips R.E., Theakston R.D., Warrell D.A., Galigedara Y., Abeysekera D.T., Dissanayaka P., Hutton R.A., Aloysius D.J. Paralysis, Rhabdomyolyis and Haemolysis caused by bites of *Russell's viper* (*Vipera russelli pulchella*) in Sri Lanka: Failure of indian (Haffkine) antivenom. Quartely Journal of Medicine. 1988; 257:691-716.

Prasad N.B., Uma B., Bhatt S.K.G., Gowda V.T. Comparative characterization of Russell's viper *(Daboia/Vipera russelli)* venoms from different regions of the Indian peninsula. Biochimica et Biophysica Acta. 1999; 1428:121-136,

Pukrittayakamee, S., Warrel, D.A., Desakorn, V., McMichael, A.J., White, N.J., Bunnag, D. The hyaluronidase activities of venoms of some South East Asian snakes. Toxicon. 1998; 26:629-637.

Raveendra A., Chiu-Yueh C., Chun-Ho C., Yi-Chun C., Chu-Chun C., Yuan-Ping C., *et al.* Expression of snake venom toxin in *Pichia pastoris*. Toxin Reviews. 2007; 26:169 – 187.

Sekiya F, Yamashita T, Morita T. Role of calcium(II) ions in the recognition of coagulation factors IX and X by IX/X-bp, an anticoagulant from snake venom. Biochemistry. 1995; 34:10043–10047.

Serrano S.M., Jia L.G., Wang D., Shannon J.D., Fox J.W. Function of the cysteine-rich domain of the haemorrhagic metalloproteinase atrolysin A: targeting adhesion proteins collagen I and von Willebrand factor. Biochem J. 2005; 391:69-76.

Shiu J.H. and Chuang W.J. Expression of snake venom toxins in *Pichia pastoris*. Toxin Reviews. 2007; 26:169 – 187.

Slater N.T., Freedman J.E., Larson-Prior L.J. Russell's viper venom proteins: molecular probes for neurotransmitter receptors: a review. Comparative and Biochemical Physiology. 1988; 91:51-60.

Takeya H., Nishida, S., Miyata T., *et al.* Coagulation factor X activating enzyme from Russell's viper venom (RVV-X)-a novel metalloproteinase with disintegrin (platelet aggregation inhibitor)-like and C-type lectin-like domains. J Biol Chem. 1992; 267:14109-14117.

Tans G. and Rosing J. Snake Venom Activators of Factor X: An Overview. Haemostasis. 2001; 31:225–233

Than-Than, *et al.* Heamostatic disturbances in patients bitten by Russell's viper (*vipera russelli siamensis*) in Burma. Br J Haematol. 1988; 69:513-520.

Tsai I.H., Lu P.J. and Su J.C. Two types of Russell's viper revealed by variation in phospholipase A_2 from venom of the subspecies. Toxicon. 1996; 34:99-109.

Uma B., Veerabasappa G. Molecular mechanism of lung hemorrhage induction by VRV-PL-VIIIa from Russel's viper (*Vipera russelli*) *venom.* Toxicon. 2000; 38:1129-1147.

Wagstaff S.C., Laing G.D., Theakston R.D.G., Papaspyridis C., Harrison R.A. Bioinformatics and multiepitope DNA immunization to design rational snake antivenom. PLoS Med. 2006; 3(6): e184. DOI: 10.1371/journal.pmed.0030184

Warrell D.A. Snake venoms in science and clinical medicine. Russell's viper: biology, venom and treatment of bites. Trans R Soc Trop Med Hyg. 1989; 83:732-740.

White, J. Snake venom and coagulopathy. Toxicon. 1997. 45:951–967.

Woodhams B.J., Wilson S.E., Xin B.C., & Hutton R.A. Differences between the venoms of two sub-species of Russell's viper: *Vipera russelli pulchella* and *Vipera russelli siamensis*. Toxicon. 1990; 28:427-33.

Wu G., Huang F.P. Effects of venom defibrase on brain edema after intracerebral hemorrhage in rats. Acta Neurochir Suppl. 2005; 95:381-7.

APPENDICES

CHEMICAL AGENTS AND INSTRUMENTS

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 rack (Autopack,USA)

Pipette tip (Axygen, USA)

Plastic wrap

Polypropylene conical tube (Elkay, USA)

Power supply model

pH meter (Eutech Cybernataics)

Microcentrifuge (Eppendorf, USA)

Microscope (Olympus)

Reagent bottles (Duran)

Spectrophotometer (BIO-RAD, USA)

Thermometer (Precision, Germany)

Vortex (scientific Industry, USA)

Water bath

Cover slip

Microscope slides

Needle (Nipro)

Syringe (Nipro)

B. 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

Personal data

Full name:	Mr. Arkhom Saingam
Institutional Affiliation:	Inter-department of Biomedical Sciences Graduate School, Chulalongkorn University, Bangkok, Thaialnd
Birth date and Place:	9/22/75; Sisaket, Thailand
Citizenship:	Thai

Education and Professional Training

1993-1996	Khon Kaen University Bangkok, Thailand	B.Sc. (Chemistry)
1997-1999	Mahidol University Bangkok, Thailand	M.Sc. (Mol. Gen & Gen En)
2002-2007	Chulalongkorn University Bangkok, Thailand	Ph.D. (Biomedical Sciences)
2006-2007	Snake Venom Research Unit, Liverpool School of Tropical Medicine, United Kingdom	Visiting Graduate