ผลของโปรตีนพิษงูแมวเซาซนิด RVV-X และเซรุ่มแก้พิษงูจำเพาะต่อโปรตีนพิษงูแมวเซาซนิด RVV-X ต่อการทำงานของไตและการแข็งตัวของเลือดในหนูขาว

นางสาวมณฑน์มาศ สุนทราวัฒน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EFFECT OF RVV-X AND RVV-X-SPECIFIC ANTIVENOM ON RENAL FUNCTIONS AND COAGULOPATHY IN RATS

Miss Montamas Suntravat

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มณฑน์มาศ สุนทราวัฒน์ : ผลของโปรตีนพิษงูแมวเซาชนิด RVV-X และเซรุ่มแก้พิษงูจำเพาะต่อโปรตีนพิษงูแมวเซาชนิด RVV-X ต่อการทำงานของไตและการแข็งตัวของเลือดในหนูขาว
(EFFECT OF RVV-X AND RVV-X-SPECIFIC ANTIVENOM ON RENAL FUNCTIONS AND COAGULOPATHY IN RATS)
อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.นพ.ดร.อิศรางค์ นุชประยูร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร.นพ. อมรพันธุ์ เสรีมาศพันธุ์, ดร.มาเรียม อยู่สุขสวัสดิ์, 147 หน้า.

ภาวะไตวายเฉียบพลันเป็นภาวะที่พบบ่อย ซึ่งเป็นสาเหตุสำคัญที่ทำให้ผู้ที่ถูกงูแมวเซากัดเสียชีวิต โปรตีนพิษงูแมวเซาชนิด RVV-X เป็นเอนไซม์หลักในพิษงูแมวเซาที่สามารถกระตุ้นกลไกการแข็งตัวของเลือดอย่างรวดเร็ว ซึ่งน่าจะส่งผลให้เกิดการเปลี่ยนแปลงต่อการไหลเวียนของเลือดในไต รวมทั้งการทำงานของไต งานวิจัยนี้ได้ทำการแยกบริสุทธิ์โปรตีนพิษงูแมวเซาชนิด RVV-X จากพิษงูแมวเซารวม โดยใช้ Sephadex G-100 gel filtration และตามด้วย Q-sepharose anion exchange column chromatography ได้โปรตีนพิษงูแมวเซาบริสุทธิ์ชนิด RVV-X ร้อยละ 5 และมีความบริสุทธิ์สูงขึ้น 4 เท่า พบว่าโปรตีนพิษงูแมวเซาชนิด RVV-X ที่แยกบริสุทธิ์ได้ มีคุณสมบัติในการทำให้เลือดแข็งตัว และสามารถตัด human factor X, protein C และย่อยสลาย fibrinogen ได้ ต่อจากนั้น ได้ทำการศึกษาผลของโปรตีนพิษงูแมวเซาบริสุทธิ์ชนิด RVV-X ต่อภาวะการกระจายตัวของลิ่มเลือดในหลอดเลือด การไหลเวียนของเลือดในไต การทำงานของไต และพยาธิสภาพชิ้นเนื้อในหนูขาว (n = 6) หนูถูกฉีดด้วยพิษงูแมวเซารวม (7 μg/kg) และโปรตีนพิษงูแมวเซาบริสุทธิ์ชนิด RVV-X (1.75 μg/kg) ทางหลอดเลือด ต่อจากนั้นได้ทำการศึกษาระดับ D-dimer ในพลาสมาหลังจากฉีดพิษที่เวลาต่างๆ เพื่อบ่งชี้ถึงภาวะการกระจายตัวของลิ่มเลือดในหลอดเลือดของหนูทดลอง พบว่าหนูที่ถูกฉีดด้วย RVV-X มีระดับ D-dimer เพิ่มขึ้นอย่างรวดเร็ว และมีระดับสูงสุดที่เวลา 10 นาที และลดลงชั่วคราว ก่อนจะเพิ่มขึ้นสูงสุดอีกครั้งที่เวลา 30 นาที ขณะที่ระดับ D-dimer ของหนูที่ได้รับพิษงูแมวเซารวมค่อยๆ เพิ่มขึ้น และสูงสุดที่เวลา 45 นาที โดยระดับ D-dimer สูงกว่าที่พบในหนูที่ได้รับ RVV-X ซึ่งคาดว่าเกิดจากการที่พิษงูแมวเซารวมมีโปรตีนตัวอื่นที่สามารถกระตุ้นเลือดแข็งตัว การเปลี่ยนแปลงการไหลเวียนภายในเลือดในไต และการทำงานของไต พิจารณาจากค่า mean arterial pressure (MAP), glomerular filtration rate (GFR), effective renal plasma flow (ERPF), effective renal blood flow (ERBF), renal vascular resistance (RVR), and fractional excretion of all electrolytes (FE_E) หลังจากหนูได้รับพิษงูแมวเซารวมและ RVV-X 10 นาที พบว่าค่า GFR, ERPF, ERBF ลดลงอย่างมีนัยสำคัญ และค่า RVR เพิ่มขึ้นอย่างมีนัยสำคัญ เมื่อเทียบกับกลุ่มควบคุม ซึ่งการเปลี่ยนแปลงดังกล่าวสอดคล้องกับพยาธิสภาพที่พบในชิ้นเนื้อจากไต รวมทั้งไม่พบความแตกต่างของค่าชี้วัดต่างๆ ของการไหลเวียนของเลือดในไต และการทำงานของไตอย่างมีนัยสำคัญในกลุ่มหนูที่ได้รับพิษงูแมวเซารวมและกลุ่มหนูที่ได้รับ RVV-X จากการศึกษาดังกล่าวบ่งชี้ได้ว่า โปรตีนพิษงูแมวเซาบริสุทธิ์ชนิด RVV-X มีบทบาทสำคัญในการทำให้เกิดการเปลี่ยนแปลงของหลอดเลือดในไต รวมทั้งการทำงานของไต จากการกระตุ้นให้เกิดภาวะการกระจายตัวของลิ่มเลือดในหลอดเลือด นอกจากนี้ยังได้ทำการผลิตแอนติบอดีที่จำเพาะต่อโปรตีน rRVV-XH และ rRVV-XL ที่ได้พิสูจน์ว่ามีความจำเพาะต่อ RVV-X แต่ไม่สามารถยับยั้ง factor X activator activity ของ RVV-X ได้ อย่างไรก็ตามแอนติบอดีดังกล่าวสามารถจับกับโปรตีนในพิษงูเขียวหางไหม้ และงูกะปะ แอนติบอดีที่จำเพาะต่อโปรตีน rRVV-XH และ rRVV-XL สามารถนำมาใช้ใน affinity column chromatography เพื่อใช้ในการแยกบริสุทธิ์โปรตีนพิษงูที่มีความสำคัญทางการแพทย์ได้อย่างรวดเร็วขึ้นในอนาคต

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....มณฑน์มาศ สุนทราวัฒน์.....
ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....อิศรางค์ นุชประยูร.....
ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....อมรพันธุ์ เสรีมาศพันธุ์.....
ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

4489663320 : MAJOR MEDICAL MICROBIOLOGY

KEYWORDS: Russell's viper venom-factor X activator / *Daboia russellii siamensis* / renal hemodynamics and functions / disseminated intravascular coagulation / anti-recombinant proteins IgG antibodies

MONTAMAS SUNTRAVAT : EFFECT OF RVV-X AND RVV-X-SPECIFIC ANTIVENOM ON RENAL FUNCTIONS AND COAGULOPATHY IN RATS. THESIS ADVISOR : ASSOC. PROF. ISSARANG NUCHPRAYOON, M.D. Ph.D., THESIS COADVISOR : AMORNPUN SEREEMASPUN, M.D., Ph.D., MARIEM YUSUKSAWAD, Ph.D., 147 pp.

Acute renal failure (ARF) is the most frequent and a serious complication in Russell's viper bite victims. Russell's viper venom-factor X activator (RVV-X) has been identified as a main procoagulant enzyme involving coagulopathy, which might be responsible for changes in renal hemodynamics and functions. In this study, RVV-X was purified four fold from crude Russell's viper venom (cRVV) using a Sephadex G-100 gel filtration and then a Q-sepharose anion exchange column chromatography with approximately 5% yield. Purified RVV-X had coagulant and fibrinogenolytic functions. Then, we studied the effects of purified RVV-X and cRVV on DIC, renal hemodynamics and functions, as well as the accompanying histopathological changes in Sprague-Dawley rats (SD) (n = 6). The plasma D-dimer level was determined after intravenous injection of equipotent sublethal dose of cRVV (7 μg/kg) and purified RVV-X (1.75 μg/kg) in rats at various times. The plasma D-dimer levels increased and reached a peak at 10 min, declined temporarily and then reached another peak at 30 min after purified RVV-X injection, while plasma D-dimer levels of rats given cRVV gradually increased to reach a peak at 45 min. The higher plasma D-dimer level at 45 min of rats given crude venom is supposed to be due to the other coagulants in crude venom promoting coagulopathy. Changes in renal hemodynamic and function were evaluated using mean arterial pressure (MAP), glomerular filtration rate (GFR), effective renal plasma flow (ERPF), effective renal blood flow (ERBF), renal vascular resistance (RVR), and fractional excretion of all electrolytes (FE_E). After 10 min rat receiving both cRVV and purified RVV-X significantly decreased GFR, ERPF, ERBF and significantly increased RVR when compared to the normal saline control group. These changes correlated to the kidney lesions. There were no significant differences in renal hemodynamic and function parameters in rats injected with cRVV and purified RVV-X. These findings indicated that RVV-X plays an important role in renal hemodynamic and function changes through DIC. In addition, we established the rabbit anti-recombinant proteins IgG antibodies recognizing purified RVV-X but did not inhibit factor X activator activity of purified RVV-X. However, these rabbit anti-recombinant proteins IgG antibodies cross reacted with *Cryptelytrops albolabris and Calloselasma rhodostoma* venoms. Further studies are needed to use these antibodies to further develop the affinity column for purification of snake molecules for drug discovery.

Field of study: Medical Microbiology

Academic year: 2009

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Advisor's signature.....Issarang Nuchprayoon.....

Co-advisor's signature.....Mariem Yusuksawad.....

Co-advisor's signature.....

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APTT	activated partial thromboplastin time
BASO	basophil
bp	base pairs
C	degree Celsius
cm	centimeter
cDNA	complementary deoxyribonucleic acid
DAB	3, 3'-diaminobenzidine tetrahydrochloride
DP	diastolic blood pressure
E. coli	Escherichia coli
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EO	eosinophil
ERBF	effective renal blood flow
ERPF	effective renal plasma flow
FE_e	fractional excretion of all electrolytes
FF	filtration fraction
g	gram
GFR	glomerular filtration rate
H&E	hematoxylin and eosin
HBG	hemoglobin
Hct	hematocrit
HR	heart rate
hr	hour (s)
HRP	horseradish peroxidase
IPTG	isopropyl-D-thiogalactopyranoside
kb	kilobase
LB	Luria-Bertani media
LYMPH	lymphocyte

M	molar
mA	milliamps
MAP	mean arterial pressure
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MES	2-(N-Morpholino) ethanesulfonic acid
mg	milligram
min	minute
ml	millilitre
mM	millimolar
MONO	monocyte
N	normal
NEUT	neutrophil
ng	nanogram
nm	nanometer
OD	optical density
PAH	para-aminohippuric acid
PLT	platelet
PP	pulse pressure
RBC	red blood cell
RNase	ribonuclease
RPF	renal plasma flow
rpm	revolution per minute
RVR	renal vascular resistance
SD rat	Sprague-Dawley rat
sec	second
SP	systolic blood pressure
TMB	3,3',5,5'-tetramethyl benzidine
Tris-HCl	tris-(hydroxymethyl)-aminoethane
UV	ultraviolet

U_EV	urinary excretion of electrolytes
µg	microgram
µl	microlitre
v/v	volume/volume
WBC	white blood cell

CHAPTER I

INTRODUCTION

1. Background and rationale

Venomous snakebite is an important medical problem in Southeast Asia. In Thailand, the incidence of snake bite is estimated 13 per 100,000 persons and death rate is 0.01 per 100,000 persons (Department of Disease Control, 2006). Russell's viper (RV) is the major cause of snake bite morbidity and mortality in many Southeast Asian countries including Thailand, Myanmar, India, Sri Lanka, China, Taiwan and Indonesia (Warrell, 1989). The subspecies found in Thailand *Daboia russellii siamensis* is abundant in Eastern and Central regions of Thailand. Symptoms of RV envenoming include edema, pain, thrombocytopenia with increased risk of systemic bleeding from disseminated intravascular coagulation (DIC). Severe RV envenoming can result in death include circulatory collapse, intracranial or massive gastrointestinal hemorrhage, and acute renal failure (ARF) (Mahasandana et al., 1980; Than-Than et al., 1989; Shastry et al., 1977; Thein-Than et al., 1991; Tin-Nu-Swe et al., 1993; Sitprija and Boonpucknavig, 1977; Hutachitta et al., 1986). It was postulated that the pathogenesis of ARF is related to intravascular hemolysis, hypotension, hypovolemia, DIC, and also direct nephrotoxicity of venom.

DIC is associated with consumption of coagulation factors, spontaneous bleeding, seriously prolonged coagulation and intravascular deposition of fibrin. Histopathological studies have shown a sign of fibrin microthrombi deposition in the vital organs, in the renal microvasculature and in the glomerular capillaries in victims of Russell's viper bite with cortical necrosis (Chugh et al., 1984; Than-Than et al., 1989). These findings suggest that DIC plays an important role in snakebite-induced cortical necrosis, and involves in the pathogenesis of ARF. In addition, the presence of fibrin deposition has been believed that could be the cause of procoagulant activity of

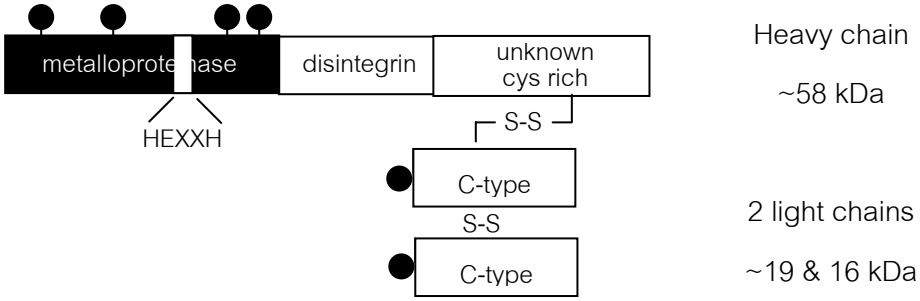
Russell's viper venom (RVV). However, the main toxin procoagulant causing DIC appearance is still unknown.

Direct nephrotoxicity has been assessed by functional and morphologic changes in isolated perfused rat kidney (IPRK) (Willinger et al., 1995). The IPRK changes are indicated by decreases in glomerular filtration rate (GFR) and renal perfusion flow (RPF) but increase in fractional excretion of sodium with a dose-dependent manner. The changes in renal hemodynamic and function are similar to patients with Russell's viper bite. The pathological study demonstrated renal damages including loss of the glomerular filter, lysis of vascular walls, endothelial cells, mesangial cells, vascular smooth muscle cells, and epithelial injury in all tubular segments (Ratcliffe et al., 1989; Thamaree et al., 1994; Willinger et al., 1995). However, the exact pathogenesis of ARF following *D. r. siamensis* envenomation is not well established.

Crude Russell's viper venom (cRVV) contains several toxins including phospholipase, Russell's viper venom-factor X activator (RVV-X), Russell's viper venom-factor V activator (RVV-V), proteinase, and other proteins as yet unidentified. Recently, Suwansrinon and coworkers (2007) studied the effect of Russell's viper fractions on systemic and renal hemodynamics in dogs. They suggested that the changes in hemodynamic were possibly caused by the proteolytic enzyme in the venom. However, the specific component of Russell's viper venom affecting renal hemodynamic alterations remains unclear.

Russell's viper venom-factor X activator (RVV-X)

The main procoagulant enzyme, RVV-X, is a glycoprotein containing 13% carbohydrate with molecular mass of approximately 93 kDa (Gowda et al., 1994). It is composed of three interdisulfide bonded chains, a heavy chain of molecular mass 58 kDa and two light chains of heterogenous molecular mass 19 and 16 kDa as shown in Figure 1. The heavy chain of RVV-X contains metalloproteinase, disintegrin (platelet aggregation inhibitor)-like and cysteine-rich domains. The light chains resemble the C-type lectins (calcium-dependent).



● : N-linked sugar chain

Figure 1 Schematic structure of RVV-X, which composes of heavy chain and two light chains. Heavy chain consists of three domains including metalloproteinase, disintegrin-like, and cysteine (cys)-rich domains. HEXXH is a conserved zinc-binding sequence found in melalloproteinase domain of heavy chain (Gowda et al., 1996).

This protein (RVV-X) activates coagulation factor X by cleaving a specific peptide bond (Arg_{52}-Ile_{53}) of the heavy chain of the clotting factor, and requires calcium ion for the proteolytic activity (Fujikawa et al., 1972; Di Scipio et al., 1977). As a result, the final common coagulation pathway is induced, which leads to rapid formation of blood clots as shown in Figure 2. Thus, RVV-X should be a major lethal factor in Russell's viper venom involving renal hemodynamic and function alterations.

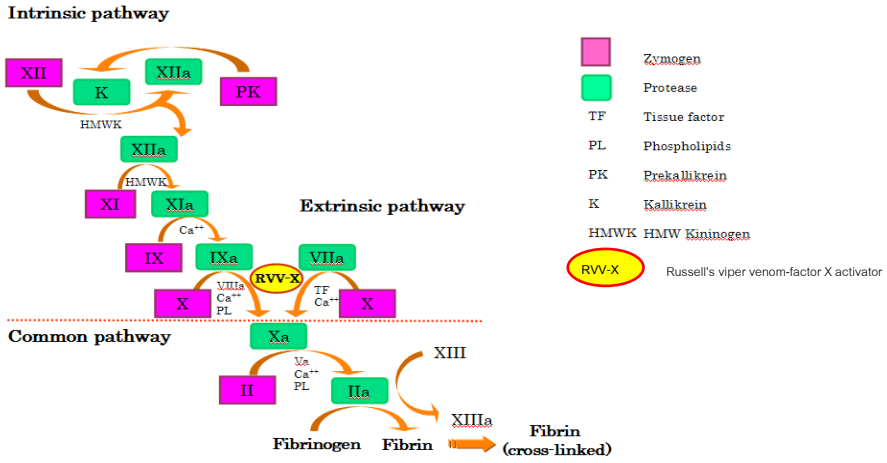


Figure 2 Blood coagulation pathways with RVV-X affecting the final common coagulation pathway.

Our preliminary study was conducted to investigate the effect of purified RVV-X from crude venom on renal histological changes in Sprague Dawley (SD) rats. Using light microscopy, renal tubular necrosis and thrombi in the kidney was observed with a sublethal intravenous injection of purified RVV-X (1.75 μg/kg) (Figure 3). Thus, RVV-X could be a factor in the venom causing DIC and renal dysfunction.

A B

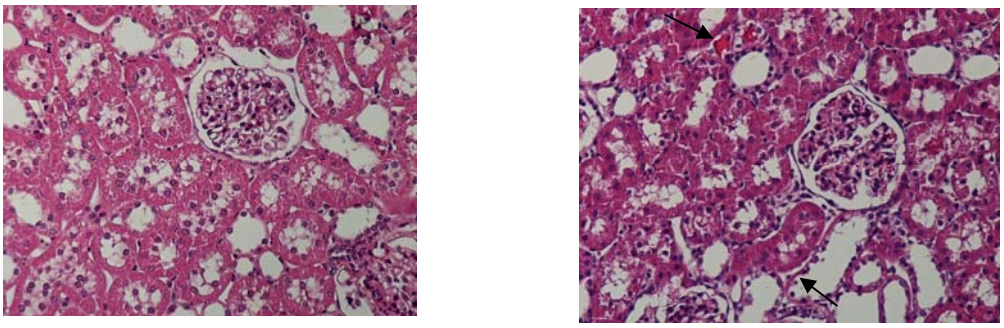


Figure 3 Histological section of kidney stained with H&E (20X magnification) from rats injected with (A) normal saline and (B) purified RVV-X. The arrow indicates the fibrin thrombi and tubular necrotic area.

In the present study, RVV-X was purified from crude *D. r. siamensis* venom, and its procoagulant, fibrinogenolytic activities, and human factor X and protein C cleavage were determined. Then, the effects of cRVV and purified RVV-X on DIC, renal hemodynamics and functions, as well as the accompanying histopathological changes were tested in SD rats. These might lead to the development of new therapeutic strategies and prevention of renal failure in snakebite patients in the future.

Russell's viper venom-specific antibodies

Antivenom therapy remains the mainstay in the treatment of snakebite since antivenom can neutral many of the molecules found in snake venoms. In Thailand, Queen Saovabha Memorial Institute (QSMI) has successfully produced 6 antivenoms against major venomous snakes of Thailand including Russell's viper (Chanhome et al., 2001). Antivenom is a horse serum product that has been purified and the globulin fraction contains antibodies to venom components. Antivenom is a foreign protein and contains molecules, which are not needed in the treatment of snakebites. Antivenom frequently causes early anaphylactoid and serum sickness adverse reactions, which could be severe and life threatening (Theakston and Reid, 1983). To reduce the risk of adverse reactions, toxin-specific antibody production be considered. In addition, toxin-specific antibody could be useful biological reagents used to detect or purify toxins from snake venoms.

Previously, we constructed a cDNA library from RV gland (Nuchprayoon, et al. 2001). cDNAs encoding RVV-X heavy chain (RVV-XH) and one of the light chain (RVV-XL; LC1) were cloned into pTrcHisα-A vector (Sai-gnam, 2007). In this study, we aimed to produce recombinant RVV-XH (rRVV-XH) and recombinant RVV-XL (rRVV-XL) proteins in *E. coli* BL21. These recombinant proteins could be used as antigens for rabbit anti-recombinant proteins antibodies production. Then, the effects of rabbit anti-recombinant proteins antibodies in neutralizing DIC, renal hemodynamics and functions, of cRVV were tested in SD rats. These might produce the high efficacy of RVV-X-specific antibodies for the treatment of snakebites.

2. Objectives

2.1. To study the effects of purified RVV-X and cRVV on DIC, renal hemodynamics and functions in SD rats.

2.2. To study the histopathological changes of SD rats after purified RVV-X and cRVV injection.

2.3. To study the effects of rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies in neutralizing DIC, renal hemodynamics and functions of cRVV.

2.4. To study the Cross-reactivity of rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies against *Cryptelytrops albolabris* and *Calloselasma rhodostoma* venoms using Western blot analysis.

3. Keywords

Russell's viper venom-factor X activator

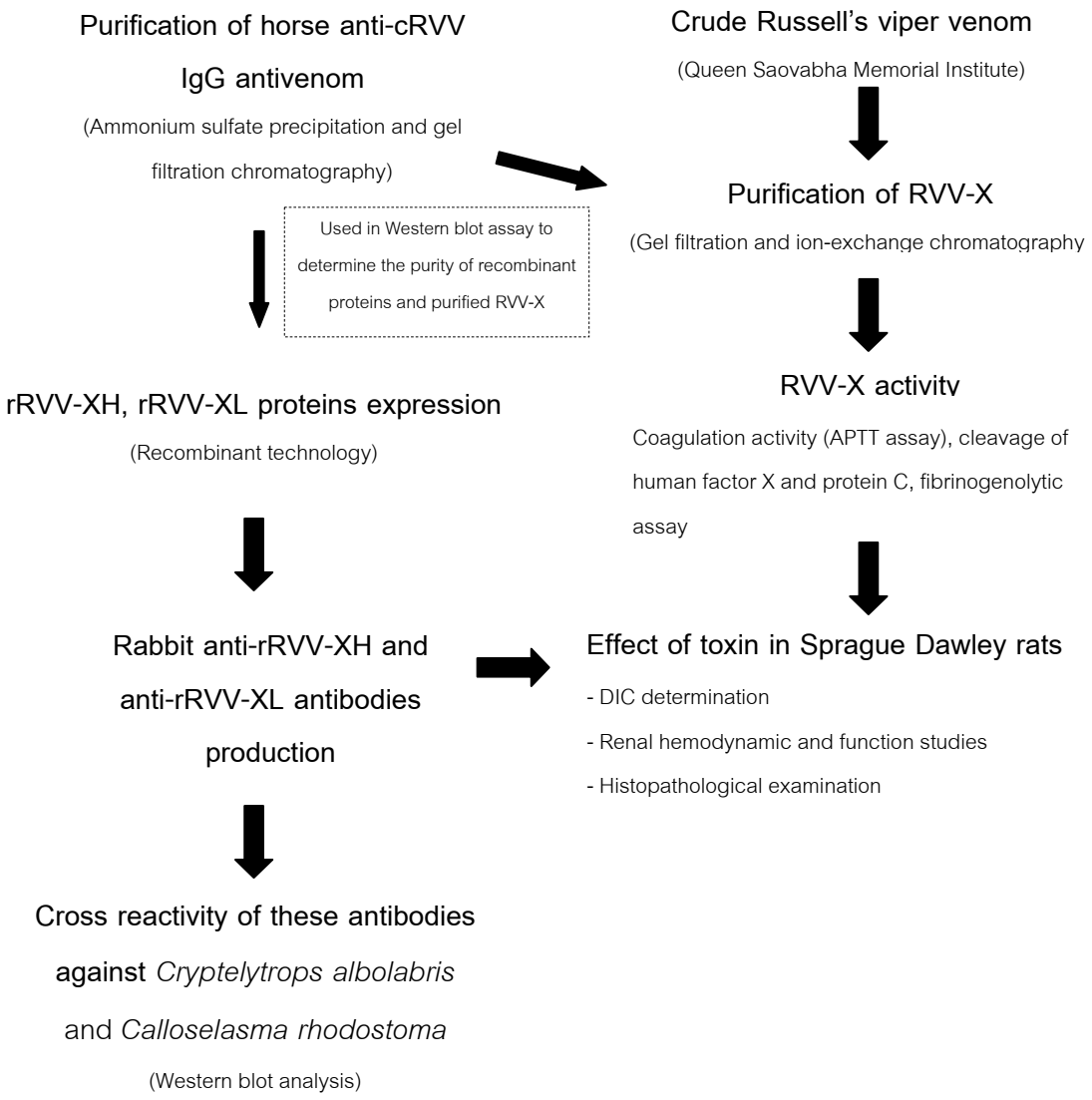
Daboia russellii siamensis

Renal hemodynamics and functions

Disseminated intravascular coagulation

Rabbit anti-recombinant proteins IgG antibodies

4. Conceptual framework



5. Expected benefits and applications

5.1 To develop the new therapeutic strategies of Russell's viper snakebite or other snakebites in the future.

5.2 To establish the rabbit recombinant proteins-specific IgG antibodies to further develop the affinity column for purification of snake molecules for drug discovery.

CHAPTER II

LITERATURE REVIEW

Russell's viper

The Russell's viper (*Daboia* species) is a common venomous snake in the Viperidae family. This snake is usually found in many South Asian countries (Table 1). It is one of the leading causes of snakebite mortality (Swaroop and Grab, 1954; Chugh et al., 1975; Matthai and Date, 1981; World Health Organization, 1981; Jeyarajah, 1984; Myint-Lwin et al., 1985). In India, it is the most common cause of the fatal snakebites and kills five out of 100,000 annually (Phillips et al., 1988). In Myanma, over 1,000 people died each year from RV bite (Aung-Khin, 1980). It also is an important contributor to the fatal snake bite in Pakistan, Thailand, and Sri Lanka (Than-Than et al., 1988). Because of the high prevalence of snakebite from this species, there is an interest in the venom biochemistry and pathological effect of the venom.

Table 1 Distribution of *D. russellii* subspecies in Asia (Warrell, 1989)

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

Clinical manifestations of Russell's viper bites

Venoms of the same snake species from different geographical locations often exhibit different pharmacological or pathological and antigenic properties (Warrell, 1989; Daltry et al., 1996; Aguilar et al., 2007; Salazar et al., 2009). The Russell's viper venom exhibits a striking geographical variation in the venom composition and clinical effects within the same subspecies (Table 2) (Warrell, 1989). Coagulopathy is the most consistent findings from all countries, and was most prominent in Thailand, India, and Myanmar but have been reported from Sri Lanka (Phillips et al., 1988; Than-Than et al., 1988). Pituitary hemorrhage has so far been described only in Southern India and Myanmar. Intravascular hemolysis was most pronounced in Sri Lanka but has been reported from Thailand and India. The neuro-myotoxicity is a dominant clinical feature in Sri Lanka as well as in India. The chemosis and facial edema, which is the result of increased capillary permeability, have so far been described only in Myanmar, where they are common features of severe envenomation. Hypotension and primary shock are most commonly described in Myanmar but have also been mentioned in reports from the other countries (Warrell, 1989). Renal failure has been reported from all countries (Mahasandana et al., 1980; Than-Than et al., 1989; Shastry et al., 1977; Thein-Than et al., 1991; Tin-Nu-Swe et al., 1993; Sitprija et al., 1977; Hutachitta et al., 1986).

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

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

"+" represents detectable symptom.

"–" indicates the absence of detectable symptom.

"?" indicates no clinical data.

Snakebites and acute renal failure (ARF)

ARF is a rapid loss of renal function due to damage to the kidneys. It is a common cause of death in victims envenoming by the viperid snake. In Myanmar and Thailand, 70% of ARF cases was caused by with Russell's viper envenomation (Hla-mon, 1987; Chugh et al., 1984). The incidence rate of ARF is 5% following Russell's viper and sea snakebites in Thailand (Sitprija and Chaiyabutr, 1999). In India, the incidence of ARF is in the range from 13% to 32% following *Echis carinatus* and Russell's viper bites (Shastry et al., 1977; Chugh et al., 1984; Mittal et al., 1986; Mathew and Rajaratnam, 1987; Chugh, 1989). In Nigeria, the incidences of ARF are approximately 1% after *Echis carinatus* bite (Chugh, 1989; Warrell et al., 1977), and 10% following puff adder bite (Warrell and Ormerod, 1975). In Israel, the incidence of ARF is approximately 6.2% following Palestinian viper bite (Efrati and Reif, 1953).

Pathogenesis of ARF

The clinical manifestations of ARF in snakebite patients include oliguria, decrease in GFR, renal perfusion, and increase in fractional excretion of sodium, potassium and early proteinuria (Sitprija et al., 1974; Sitprija and Boonpucknavig, 1977; Than-Than et al., 1989). However, the exact pathogenesis of ARF after snakebite is not well established. Many investigators are convinced that the pathogenesis is related to intravascular hemolysis, hypotension, hypovolemia, and DIC leading to renal ischemia (Sitprija and Boonpucknavig, 1977). However, direct nephrotoxicity of venom could not be excluded.

1. Indirect effect

1.1 Intravascular hemolysis

In India, the incidence of intravascular hemolysis in viper bites with ARF is 49 to 54% (Chugh et al., 1975; 1984; Jeyarajah 1984; Date et al., 1986; Chugh, 1989; Ali et al., 2004). Intravascular hemolysis manifestations are anemia, jaundice, reticulocytosis, raised plasma free hemoglobin, abnormal peripheral blood smear, and hemoglobinuria. Hemolysis after snakebite is probably due to the action of phospholipase A_2 (PLA_2). PLA_2 is commonly found in snake venoms and potentially causes direct hemolysis through esterolysis of red cell membranes. This enzyme is present mainly in elapid venoms (Condrea et al., 1964; Sosa et al., 1979; Wang et al., 1998). In the case of indirect action of PLA_2, the terminal fatty acid radical of plasma lecithin is removed by PLA_2. Then the natural detergent lipid, lysolecithin, is produced causing the lysis of red cell (Collier, 1952; Condrea, 1980). Our previous study was shown that cRVV and purified PLA_2 from crude venom had indirect hemolytic activity using egg yolk as substrate (unpublished data). These results confirmed that PLA_2 might be a main enzyme in venom responsible for intravascular hemolysis in Russell's viper bites.

Although intravascular hemolysis is well-known to be associated with ARF, the exact mechanism of ARF following hemolysis is still unclear. Reineck et al. (1980) studied on the pathophysiology of glycerol-induced myoglobinuric ARF. They found that the decreased GRF and RPF had returned to normal levels within 3 to 6 hr. In

addition, many investigators are convinced that intravascular hemolysis could be the cause of ARF when combined with other severe factors such as dehydration, hemorrhage, or hypotension (Conn et al., 1956; Chugh, et al., 1984; 1989; Athappan et al., 2008).

1.2 Hypotension

Continuous oozing of blood from the fang marks or bleeding into tissues can result in hypotensive shock and circulatory collapse leading to ischemic ARF in snake bite victims (Osman and Gumma, 1974; Warrell 1989). Bleeding is an important manifestation of following viperine snake envenomation (Chugh, 1989; Mahasandana et al., 1980; Mittal, 1994). In India, the incidence of bleeding in viper bites with ARF is reported to range from 8 to 65% (Chugh, 1989; Ali et al., 2004; Athappan et al., 2008). In Thailand, the incidence of systemic bleeding following Russell's viper bite with ARF is approximately 71% (Mahasandana et al., 1980). Bleeding is caused mainly by direct endothelial damage by venom hemorrhagic metalloproteinases, which degrade basement membrane proteins surrounding the blood vessel wall (Bjarnason and Fox, 1988; 1989; 1994; Anderson and Ownby, 1997). In addition, Viperine snake venoms contain components affecting hemostasis (Ouyang et al., 1992; Hutton and Warrell, 1993; Kamiguti and Sano-Martins, 1995). Most of these components are responsible for the activation of coagulation cascade resulting in the consumption of clotting factors leading to incoagulable blood.

Hypotension can also be caused by the releasing bradykinin as reported in Russell's viper bite patients (Minton, 1971; Joseph et al., 2004). Additionally, vasodilatation and increase capillary permeability resulting in hypotension and circulatory collapse have been reported in viper bites (Bicher et al., 1966; Osman and Gumma, 1974). These are known as the cause of ischemic ARF. However, Athappan et al. (2008) reported that some hypotension patients with aggressive treatment still developed ARF. Thus, hypotension did not have a major pathogenetic factor in the development of ARF in viper bites.

1.3 Disseminated intravascular coagulation (DIC)

DIC is a common feature in patients bitten by Viperine snake (Lakier and Fritz 1969; Sitprija et al., 1974; Nicolson et al., 1974; Warrell et al., 1976; 1977; Shastry et al., 1977; Simon and Grace 1981). It is a serious disruption in the body's clotting mechanism by snake procoagulant enzymes. As a result, the coagulation cascade is induced and then numerous small blood clots are produced throughout the body. These small blood clots can block blood supply to organs, which leads to organ dysfunction or organ damage as shown in Figure 4. Clinical features of DIC include thrombocytopenia, hypofibrinogenemia, depletion of clotting factors, and activation of fibrinolysis that may lead to clinical bleeding (Spero et al., 1980; Levi 2004; 2005; 2009).

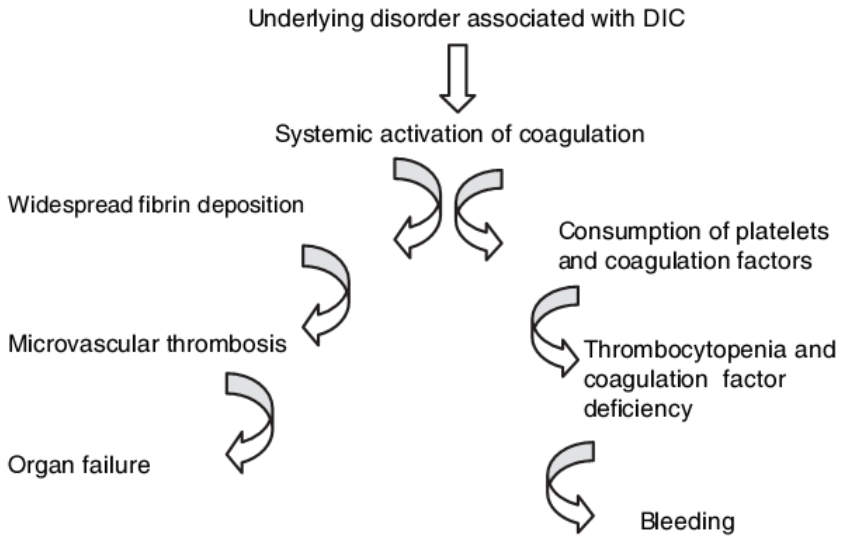


Figure 4 Processes in DIC (Levi et al., 2009)

In India, the incidence of acute DIC in viper bite patients with ARF is 56 to 81% (Chugh 1989; Ali et al., 2004). The incidence of DIC following Russell's viper bite with ARF in Thailand rangs from 37.5% to 71% (Mahasandana et al., 1980; Chugh, 1989; Vijeth et al., 1997). Than-Than et al. (1988) reported that severe Russell's viper bites with DIC are frequently related to spontaneous bleeding and incoagulable blood. Similar results were reported by Athappan et al. (2008). They have found that patients with Russell's viper bites with ARF usually bleed and had low platelet

count. These results have been noticed that bleeding and hypovolemia could also be due to DIC, which may be responsible for death or organ damage through hemorrhage and microvascular fibrin deposition. In addition, the deposition of fibrin thrombi at the site of bite, in the renal microvasculature, in the pituitary, lungs, and kidneys have been reported in fatal cases of Russell's viper bite victims in Myanmar and India (Jayanthi and Gowda, 1988; Than-Than et al., 1989; Soe et al., 1993). The multiple thrombotic occlusions in vessel and the vital organs were reported in patients following Russell's viper bite in Taiwan (Hung et al., 2002). Burdmann et al. (1993) have demonstrated the massive intraglomerular fibrin deposition in Wistar rat kidneys after administration of 0.4 mg/kg of *Bothrops jararaca* venom. The clinical manifestations of ARF in these rats including significant decrease in the GFR, urine output, RPF, and serum fibrinogen levels were also found as similar to those observed in snake bite patients with ARF. DIC could be an important factor causing renal ischemia, and leading to ARF.

In another animal experiment, rhesus monkeys were injected with a sublethal dose of RVV. Acute tubular necrosis was found in more than 50% of monkeys. The fibrin thrombi in glomerular were approximately 50 to 70% (Chugh et al., 1984). It can be concluded from their studies that DIC has an important pathogenetic role in the development of ARF in Russell's viper bites, as a consequence of procoagulant enzymes in snake venoms. However, direct venom nephrotoxicity could not be rejected.

2. Direct nephrotoxicity

Many studies reported that RVV have a direct nephrotoxic action (Sitprija and Boonpucknavig, 1977; Chugh et al., 1975; Ratcliffe et al., 1988). Soe-Soe et al. (1990) demonstrated the tubular damage in rabbit kidney tissues after incubated with 5 mg/ml of RVV for 2 hr. The strong evidence supporting direct nephrotoxic action of RVV was reported by Willinger et al. (1995). They have studied the morphological and functional changes of isolated perfused rat kidney (IPRK). Isolated male Sprague-Dawley (SD) rat kidneys were perfused with 10 μg/ml and 100 μg/ml of RVV at 60 and 80 min, respectively. Significant decreases in RPF, GFR, filtration fraction (FF), and tubular reabsorption of sodium were shown. On morphological analysis, renal cortex

has extensive damage and loss of glomerular epithelial cells. Cultured mesangial cells and renal epithelial cells were also exposed to RVV for 5 min to 48 hr. A 200 μg/ml of RVV induced a complete disintegration of confluent mesangial cell layers. A high doses of RVV (800 μg/ml) caused the microscopically discernible damage of epithelial cell cultures. These results confirmed the direct toxic effect of RVV on the IPRK, directed primarily against glomerular and vascular structures, and on cultured mesangial cells. Thus, direct toxic effect of venom could be a contributing factor to the pathogenesis of ARF.

Renal histopathology

Several investigators reported renal lesions after snake envenoming including glomerulitis, glomerulonephritis, interstitial nephritis, arteritis, tubular necrosis, cortical necrosis, and renal infarct (Oram et al., 1963; Raab and Kaiser, 1966; Sant and Purandare, 1972; Sitprija et al., 1973; 1974; Seedat et al., 1974). Acute tubular necrosis and acute cortical necrosis is the most reported in snakebite patients with ARF.

1. Acute tubular necrosis

Acute tubular necrosis is the most common renal lesion, which is found in 70% to 80% of patients with ARF (Chugh et al., 1984; Chugh, 1989). With on light microscopy, the dilated tubules lined by flattened epithelium are seen in renal biopsies. In severe cases, cell necrosis and desquamation of necrotic cells from the basement membrane were observed. Hyaline, granular, or hemoglobulin pigment casts are found in the tubular lumina. The presence of interstitial edema, hemorrhage, and inflammatory cell infiltration vary in degrees. Intrarenal blood vessels are usually unaffected.

Date and Shastry (1982) reported renal ultrastructure in Russell's viper bites with ARF. Dense intracytoplasmic bodies representing degenerating organelles or protein resorption droplets are observed in the proximal tubules using electron microscopy. There are small areas of denudation of the basement membrane. Distal tubule cells exhibit a dilated endoplasmic reticulum and many degenerating organelles. The interstitium is infiltrated with hyperplastic fibroblasts, plasma cells, eosinophils, and mast cells. Granulated and partially degranulated forms are found in both mast cells

and eosinophils. Blood vessels exhibit normal morphology under light microscopy but ultrastructural abnormalities are notable in large and small caliber vessels. Medullary vessels are swollen, focally necrotic, endothelial cells obliterating the lumen. Smooth-muscle cells present cytoplasmic vacuoles. Severe vascular lesions and the presence of mast cells, eosinophils, and hyperplastic fibroblasts in the interstitium are unique features that have not been seen in acute tubular necrosis form other causes.

2. Acute cortical necrosis

Renal ultrastructures in cortical necrosis following Russell's viper bites have been reported in two patients using electron microscopy (Date and Shastry, 1981). The biopsy from the first victim presented glomeruli with collapsed capillary basement membrane, and denuded foot processes. Swollen rounded cells of endothelium were found in some capillary lumina. Endothelial swelling of small arterioles and necrosis of peritubular capillaries were also seen. The biopsy from the second patient also presented the evidence of extensive tissue destruction. The tubular basement membrane was thickened. The cortical tubules were lined by flattened epithelium with large nuclei and a dilated endoplasmic reticulum. The interstitium presented the fibroblastic proliferation. The urinary space infiltrated with unidentified cells with large cytoplasmic vacuoles.

Snake venom composition

Snake venom is a complex mixture of enzymes, toxins, and all sorts of smaller molecules. Most venomous snake venoms contain hyaluronidase, PLA_2, phosphodierterase, phosphomonoesterase, lecithinase, collagenase, 5'-nucleotidase, NAD nucleosidase, L-amino acid oxidase, ribonuclease,acetyl-cholinesterase, lactate dehydrogenase, deoxyribonuclease, arginine esterase, serine proteinase, and metalloproteinase (Russell, 1980; Warrell, 1989; Meier and Stocker, 1991; Tsai, et al., 1996; Matsui et al., 2000). However, some snake venom components and their functions are not yet characterized. The composition of snake venom differs from species to species. There is also variation within a single species depending on age, diet, habitat, season, temperature, or different isoenzymes (Bonilla et al., 1973; Reid and

Theakston, 1978; Gutierrez et al., 1980; Kornalik and Taborska, 1989; Kornalik, 1991). Some snake venom molecules and their actions are shown in Table 3.

Table 3 Common molecules in snake venoms (modified from Koh et al., 2006)

Compounds	Actions	References
Hyaluronidases	Degradation of hyaluronic acid in the extracellular matrix (ECM) enhancing the diffusion of venom, increasing the potency of other toxins, and causing damages the local tissue	Kemparaju and Girish, 2006
Neurotoxins	Blocking nerve transmission by competitively binding to the nicotinic actylcholine receptor (nAChR) located at the post-synaptic membranes of skeletal muscles and neurons, preventing neuromuscular transmission and thereby asphyxiation	Tsetlin and Hucho, 2004
Phospholipases	Interfering in normal physiological process, trigerring a cascade of inflammatory events	Valentin and Lambeau, 2000
Thrombin-like enzymes	Cleavage only fibrinopeptide A or a few cleave fibrinopeptide B, leading to prevention of clot formation	Laraba-Djebari et al., 1995; Baker and Tu, 1996; Gasmi et al., 1997; Samsa et al., 2002
Disintegrins	Interacting with RGD motif-dependent integrins leading to inhibit platelet aggregation, interacting with leukocyte integrins, and the $\alpha 1\beta 1$ integrins	Huang et al., 1987; 1991; Marcinkiewicz, 2005;
C-type lectin-like proteins	Binding to coagulation factors, to platelet receptors, activating platelets by binding to von Willebrand factor	Clemetson et al., 2005; Lu et al., 2005

Snake venom proteins affecting the hemostatic system

Snake venoms, especially from the family Viperidae, contain numerous proteins that affect the hemostasis, which involves not only clot formation, but also defibrination (Figure 5). The major symptoms from snakebite affecting the hemostatic system are (a) reduced coagulability of blood resulting in an increased risk of systemic bleeding, (b) bleeding due to the damage to blood vessels, (c) secondary effects of increased bleeding, ranging from hypovolemic shock to secondary-organ damage such as intracerebral hemorrhage, anterior pituitary hemorrhage or renal damage, and (d) thrombosis and its sequelae, particularly pulmonary embolism (Numeric *et al*., 2002). These effects cause by snake venom toxins including procoagulant, anticoagulants, platelet-activating and anti-platelet function, fibrinolytic activators, and hemorrhagins, which are summerized in Table 4 (Stocker, 1990; Meier and Stocker, 1991; Ouyang et al., 1992; Hutton and Warrell, 1993; Marsh, 1994). 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 have been classified into various families, such as serine proteinases, metalloproteinases, C-type lectins, disintegrins, and phospholipases.

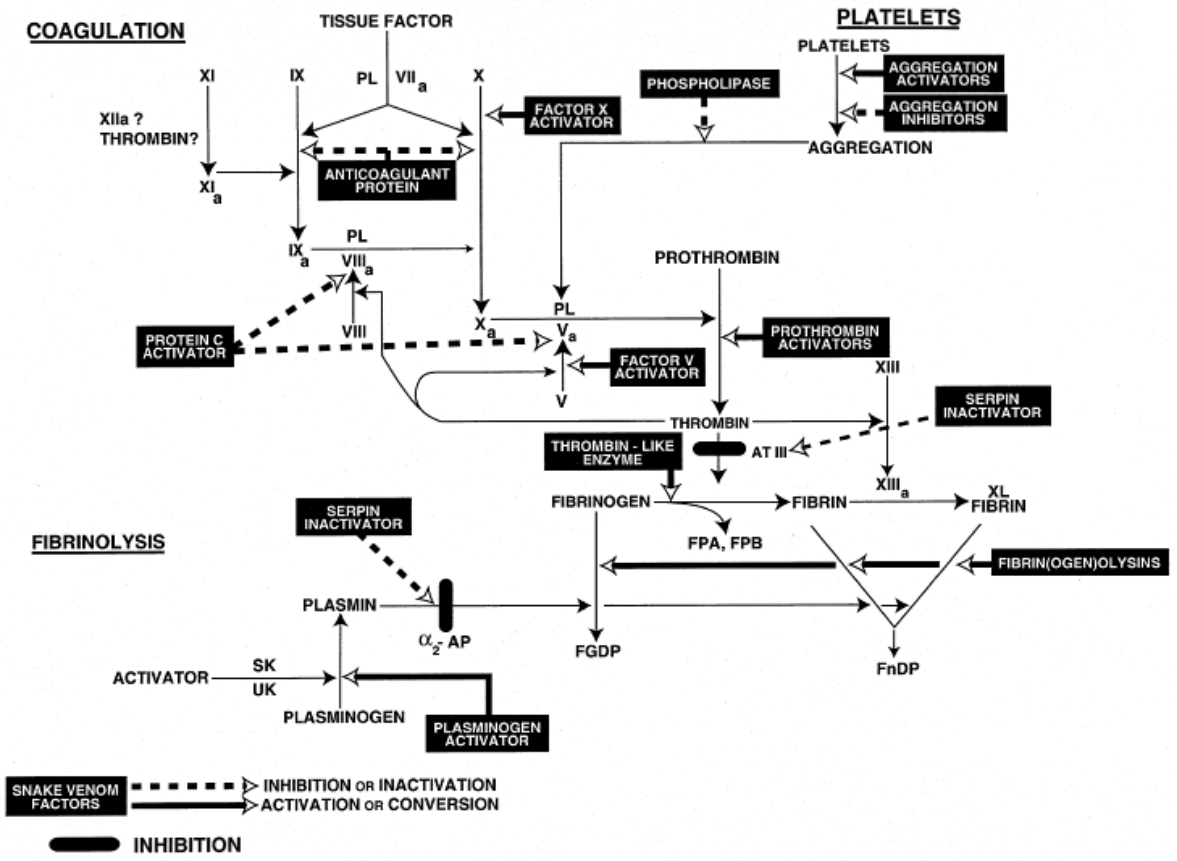


Figure 5 A schematic overview of the coagulation and fibrinolytic pathways. Arrows are not shown from platelets to phospholipids involved in the tissue factor VII_a and the factor IX_a-$VIII_a$ interactions to avoid confusion. Interactions of the venom proteins are indicated in the black boxes. Inhibitory interactions produced by the venom proteins are shown by the wide dashed lines; activation or conversion interactions are shown by the wide solid black lines. The narrow solid lines indicate the normal interactions of the coagulation and fibrinolytic proteins. The solid black ovals in the thrombin and plasmin activity lines indicate inhibition by serine proteinase inhibitors (SERPINS). The diagram is not complete with reference to the multiple sites of interaction of the SERPINS to avoid overcrowding (Markland, 1998).

Table 4 Snake venom proteins active on the hemostatic system (Markland, 1998)

General functional activity	Specific biological activity
Procoagulant	factor V activating
	factor X activating
	factor IX activating
	prothrombin activating
	fibrinogen clotting
Anticoagulant	protein C activating
	factor IX/factor X-binding protein
	thrombin inhibitor
	phospholipase A
Fibrinolytic	fibrin(ogen) degradation
	plasminogen activation
Vessel wall interactive	hemorrhagic
Platelet active	platelet aggregation inducers
	inhibitors of platelet aggregation
Plasma protein inactivators	inhibitors of SERPINS

Russell's viper venom-factor X activator (RVV-X)

The well-known coagulation factor X activator is found in Russell's viper venom. 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). Russell's viper venom-factor X activator (RVV-X) is a 93 kDa glycoprotein with 13% carbohydrate (Gowda et al., 1994). It consists of a heavy chain with molecular mass 58 kDa and two light chains with heterogenous molecular mass 19 and 16 kDa. This protein is classified as a P-IV snake venom metalloproteinase (SVMP, Figure 6).

The mature heavy chain consists of 429 amino acid residues containing four asparagine-linked oligosaccharides. Its entire sequence shares 66-88 % amino acid identical to the closest sequence from *Macrovipera lebetina*, *Echis carinatus*, and *E. ocellatus* (Sai-gnam, 2007). The heavy chain composes of three distinct domains including a metalloproteinase domain, a disintegrin-like domain, and a cysteine-rich domain. The metalloproteinase domain contains the conserved HEXXH metalloprotease catalytic site sequence. 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 123-residue light chain contains one asparagine-linked oligosaccharide. Its sequence shows homology similar to that found in several snake C-type (Ca^{2+}-dependent) lectins. This light chain sequence also shares homology with the anticoagulant proteins factor IX/X-binding protein of *Trimeresurus Flavoviridis* (Takeya et al., 1992; Atoda et al., 1994) and factor X-binding protein of *Deinagkistrodon acutus* (Atoda et al., 1994). 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 Ca^{2+}-dependent manner (Atoda et al., 1994; Sekiya et al., 1995). It is supposed that the two C-type lectin light chains of RVV-X recognizes and binds to the Gla domain of factor X as shown in Figure 7 (Morita, 1998).

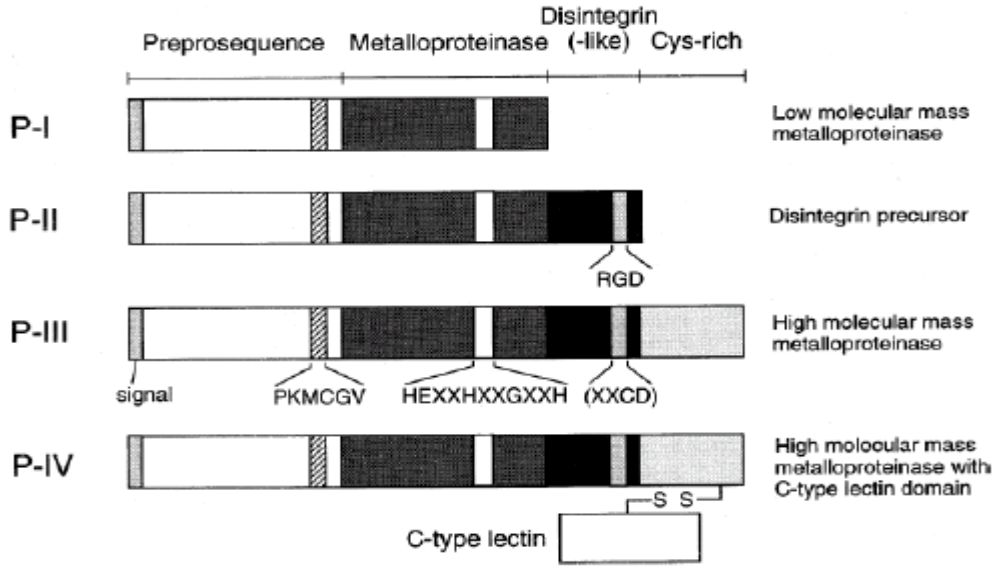


Figure 6 Classification of snake venom metalloproteinases (SVMPs). RVV-X is classified as P-IV SVMP (Kini et al., 2001).

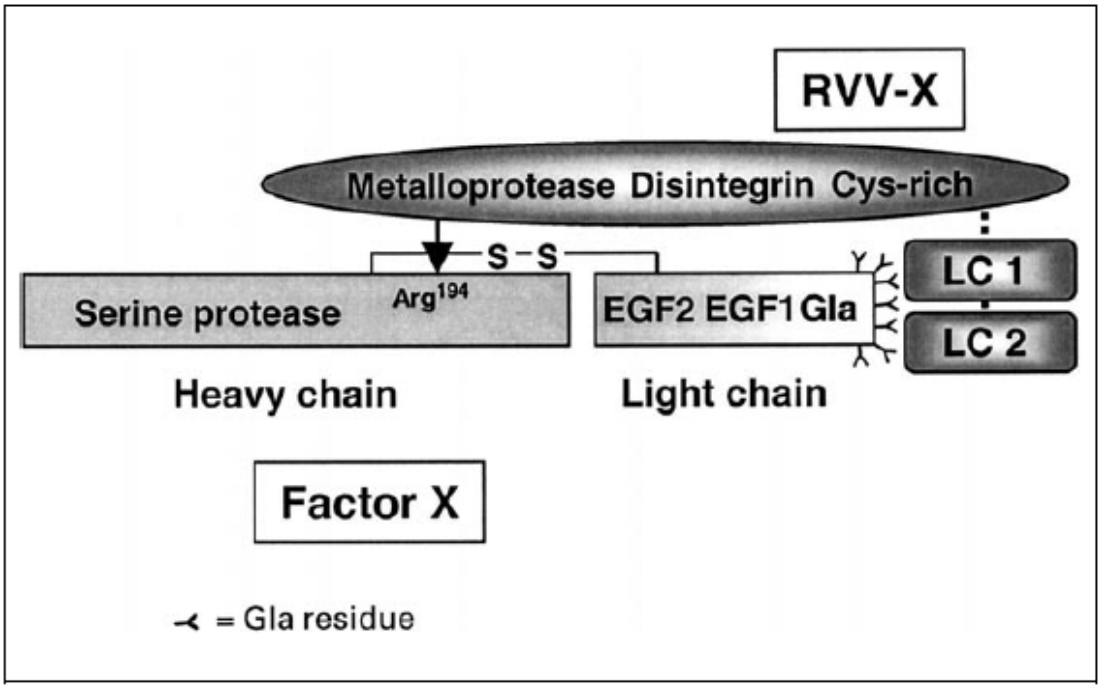


Figure 7 The proposed mechanism of the activation of factor X by RVV-X. The heavy chain of RVV-X contains the metalloprotease domain responsible for the proteolytic cleavage of the target bond in factor X. The two C-type lectin light chains (LC1 and LC2) together form a secondary binding site specific for interaction with the Gla domain of factor X (Tans et al., 2001).

RVV-X activates factor X by cleaving the Arg-Ile bond at position 194 (Figure 7) and is also able to activate factor IX and protein C by specific cleavage of Arg-Ile and Arg-Val bonds (Kisiel et al., 1976a; 1976b). The activation of coagulation factor X and factor IX by RVV-X causes rapidly clot formation. In contrast, its effect on protein C results in a stimulation of fibrinolytic pathway. Many investigators have reported that the activation of clotting and fibrinolytic systems produces the development of DIC (Baglan, 1994; Faulkner, 1995; Carey and Rodgers, 1998). Thus, RVV-X could be an important factor resulting in DIC with subsequent ARF.

Production of toxin-specific polyclonal antibody

Antibody plays a major role in the defense against foreign antigens. The animal-derived polyclonal antibodies are used as therapeutic agents for the treatment of a variety of infectious diseases more than hundred years ago (Lucchesi and Gildersleeve, 1941; Tacket et al., 1984; Kuby, 1994; Casadevall and Scharff, 1994; 1995). Hyperimmune antibodies derived from animals have been used successfully for many years to treat snake or spider bites (Dart et al., 1997). Examples of commercially available polyclonal therapeutics are shown in Table 5. Increased serum titers of toxin-specific antibodies occur after the corresponding antigen immunization in animal.

In most cases antivenoms are produced in animals with crude venoms. Crude venoms may contain some components that are less lethal and may not be needed to neutralize by antivenoms in snakebite victims. Thus, the antibodies against key venom toxins would be less abundant and the antivenom could be less effective. A large volume of antivenom could be needed to effectively neutralize the venom, which may cause serious side effects when large volumes are used. Therefore, reducing the risk of adverse reactions, toxin-specific antibody production may be helpful. Toxin-specific antibodies could be produced to improve the treatment of systemic envenoming by several snakebites. These antibodies could also be used as reagent to be used in ELISA and purify toxins by affinity chromatography.

Table 5 Example of currently available polyclonal therapeutics (modified from Newcombe and Newcombe, 2006)

Marketed polyclonal therapeutic	Product description	Therapeutic application
CroFab[TMa]	Crotalidae (*Crotalus atrox*, *C. adamanteus*, *C. scutulatus*, *Agkistrodon piscivorus*) Polyvalent Immune Fab (Ovine)	Rattlesnake antivenom
DigiFab[TMa]	Digoxin Immune Fab (Ovine)	Digoxin toxicity/oleander poisoning
ViperaTAb[TMa]	Affinity Purified. European Viper Antivenom (Ovine) Fab	Common adder (*Vipera ammodytes*, *V. Aspis*, *V. Berus*) antivenom
Antivenin (*Micrurus fulvius*)[a]	Antivenin (*Micrurus fulvius*) Equine orgin	North American Coral snake antivenom
Antivenin (*Latrodectus mactans*)[a]	Antivenin (*latrodectus mactans*) Equine orgin	Black widow spider antivenom
Thymoglobulin[a]	Anti-Thymocyte globulin (Rabbit)	Immunosuppressive therapy
Antivenin (*Naja kaouthia*)[b]	Antivenin (*N. kaouthia*) Equine orgin	Cobra snake antivenom
Antivenin (*Ophiophagus hannah*)[b]	Antivenin (*N. kaouthia*) Equine orgin	King cobra snake antivenom
Antivenin (*D. r. siamensis*)[b]	Antivenin (*D. r. siamensis*) Equine orgin	Russell's viper snake antivenom
Antivenin (*Bungarus fasciatus*)[b]	Antivenin (*Bungarus fasciatus*) Equine orgin	Branded krait snake antivenom

[a]Data from US Food and Drug Administration, Center for Biologics Evaluation and Research, licensed Establishments and Products (table may not represent a complete list)

[b]Example of antivenin from Queen Saovabha Memorial Institute (Chanhome et al., 2001)

CHAPTER III

MATERIALS AND METHODS

Materials

1. Snake venom proteins

1.1 Lyophilized crude venom samples of Russell's viper (*D. r. siamensis*), *C. albolabris,* and *C. rhodostoma* were obtained from the Queen Saovabha Memorial Institute (QSMI, Thai Red Cross Society, Thailand). These venoms were pooled venom from an underdetermined number of snakes.

1.2 RVV-X was purified from Lyophilized cRVV by gel filtration and ion exchange column chromatography modified from Kisiel et al. (1976).

1.3 rRVV-XH and rRVV-XL proteins

1.3.1 rRVV-XH and rRVV-XL proteins expression in *Escherichia coli*

cDNAs encoding RVV-XH and RVV-XL used in this study were obtained from our previous study in our lab (Sai-gnam, 2007). pTrcHisα-A (Invitrogen^{TM}, CA, USA), a vector containing N-terminal 6X His Tags, was used for expression of recombinant proteins in *E. coli*. Its physical feature and restriction map was shown as below (Figure 8).

1.3.2 Genotypes of *E. coli* strain BL21 (DE3)

BL21 (DE3) *E. coli* B F^{-} *ompT gal [E. coli* B is naturally *dcm and lon] hsdS*$_B$ with DE3, a λ prophage carrying the T7 RNA polymerase gene and lacI^{Q}.

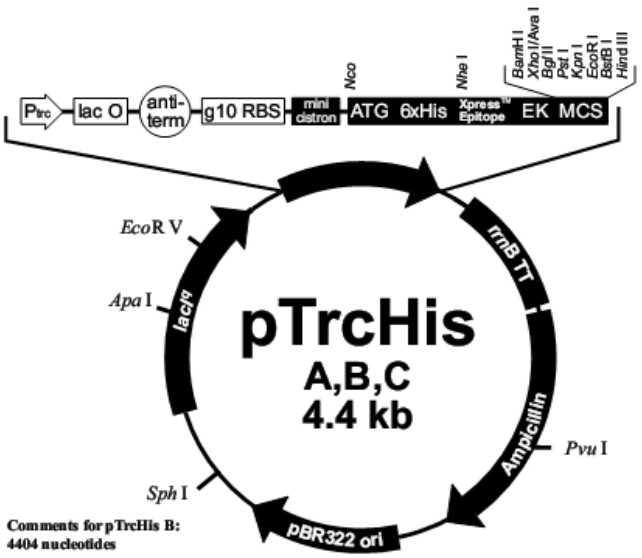
1.3.3. Culture media

Luria Bertani (LB) broth was used for propagating *E. coli* BL21.

1.3.4 Purification of His-Tag proteins

HisTag proteins were purified by BD TALON® immobilized metal affinity resins (BD Biosciences Clontech, CA, USA).

(A)



Comments for pTrcHis B:
4404 nucleotides

trc promoter: bases 191-221
lac operator: bases 228-248
*rrn*B anti-termination sequences: bases 264-333
T7 gene 10 translational enhancer: bases 346-354
Ribosome binding site: bases 370-374
Mini-cistron: bases 383-409
Polyhistidine and enterokinase cleavage site: bases 425-504
Xpress™ epitope: bases 482-505
Multiple cloning site: bases 515-554
*rrn*B transcriptional termination sequence: bases 637-794
Ampicillin resistance ORF: bases 1074-1934
pBR322 origin: bases 2079-2752
lac I^{q} ORF: bases 3281-4365

(B)

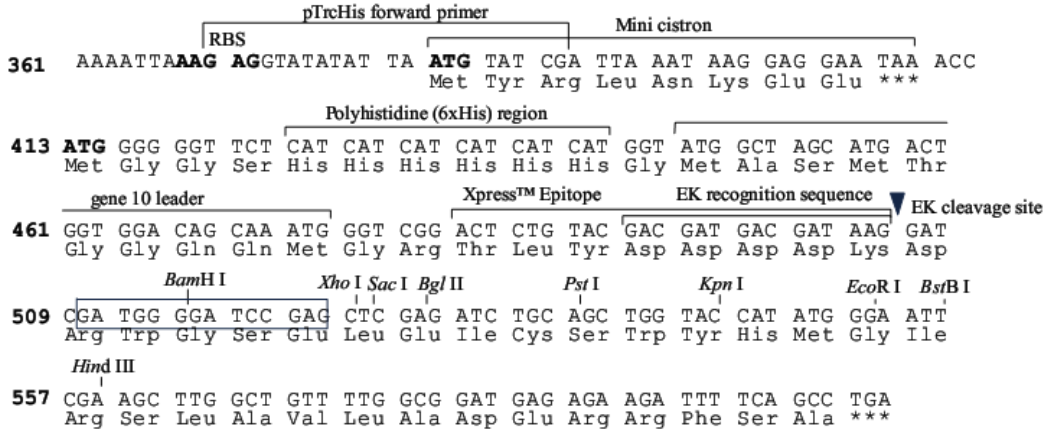


Figure 8 Map and Features of the pTrcHisα-A expression vector. (A) pTrcHisα-A structure showing detailed feature. (B) Multiple cloning sites of pTrcHisα-A.

2 Horse anti-Russell's viper IgG and F(ab')$_2$ antibodies

Horse whole blood containing polyvalent and commercial horse anti-cRVV F(ab')$_2$ antivenom were obtained from QSMI, Thai Red Cross Society, Thailand. Horse anti-cRVV IgG antibody was purified by ammonium sulfate (AS) precipitation (Aalund et al., 1965) and gel filtration chromatography.

3 Rabbit anti-rRVV-XH and anti-rRVV-XL antibodies

Anti-rRVV-XH and anti-rRVV-XL antibodies were produced by immunizing rabbits with rRVV-XH or rRVV-XL proteins.

3.1 Freund's complete and incomplete adjuvants were purchased from Sigma-Aldrich, MO, USA.

3.2 Purification of IgG antibody

IgG antibodies were purified by protein G column affinity resins (KPL, Gaithersburg, MD, USA).

4 Protein concentration

Amicon ultra centrifugal filter units with the molecular weight cut off 10 kDa were purchased from Millipore, MA, USA.

5 Protein detection

5.1 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Mini-PROTEAN 3 Electrophoresis Cell was purchased from Bio-Rad Laboratories, Inc., CA, USA. A XCell SureLock™ system was purchased from Invitrogen, CA, USA.

Acrylamide and Bis-Acrylamide solutions and Precision Plus Protein™ standards (dual color) were purchased from Bio-Rad Laboratories, Inc., CA, USA. SeeBlue Plus2 markers was purchased from Invitrogen™, CA, USA.

5.2 Coomassie Brillient blue staining

Coomassie Brilliant blue G-250 was purchased from USB, USA.

5.3 Silver staining

Silver staining kit protein was purchased from Invitrogen™, CA, USA.

5.4 Western blotting hybridization

Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, Inc., CA, USA) was used for transfer proteins from gel to membrane by electrophoresis. Nitrocellulose membrane (PROTRAN) was purchased from Whatman® GmbH, Germany. 3, 3'- diaminobenzidine (DAB) tetrahydrochloride was purchased from BIO BASIC, Inc., Canada.

5.4.1 Recombinant proteins detection

Mouse anti-His antibody was purchased from Amersham Pharmacia Biotech AB, Sweden.

Horse anti-cRVV IgG antibody was purified from horse whole blood against cRVV.

Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody was purchased from DAKO, CA, USA.

HRP-conjugated sheep anti-horse antibody was kindly provided by R Rutai, Mahidol University, Thailand.

5.4.2 Rabbit rRVV-XH- and rRVV-XL-specific antibodies detection

HRP-conjugated goat anti-rabbit antibody was purchased from Zymed Laboratories, CA, USA.

5.5 Quantitation of rabbit anti-rRVV-XH- and anti-rRVV-XL antibodies with enzyme-linked immunosorbent assay (ELISA)

ELISA plates were purchased from NUNC™, NY, USA.

HRP-conjugated goat anti-rabbit antibody and 3,3',5,5'-tetramethyl benzidine (TMB) were purchased from Zymed Laboratories, CA, USA.

6 Protein determination

PIERCE® BCA protein assay kit was purchased from PIERCE Chemical Company, IL, USA.

7 Factor X activator activity

Factor Xa-specific chromogenic substrate S-2765 was purchased from Chromogenix-Instrumentation Laboratory Company, MA, USA.

8 Coagulation activity using activated partial thromboplastin time (APTT) assay

Normal citrated plasma was purchased from Instrumentation Laboratory Company, MA, USA. Factor X deficiency plasma was purchased from Sigma diagnostics, MO, USA.

9 Fibrinogenolytic assay

Fibrinogen without plasminogen was purchased from Hyphen Biomed, France.

10 Human factor X cleavage assay

Human factor X was purchased from Haematologic Technologies Inc., VT, USA.

11 Human protein C cleavage assay

Human protein C was purchased from Haematologic Technologies, Inc., VT, USA.

12 The fibrin degradation product D-dimer assay

NycoCard® D-dimer was purchased from Axis-Shield PoC, MA, USA.

13 Chemicals

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

14 Animal models

Animals were obtained from the National Laboratory Animal Center (NLAC), Mahidol University.

14.1 Groups of six male Sprague-Dawley (SD) rats (200-250 g).

14.2 Groups of two New Zealand White rabbits (2 kg).

Methods

Part I Effects of rabbit anti-rRVV-XH and anti-rRVV-XL antibodies in neutralizing DIC, renal hemodynamics and functions of cRVV

1. Expression of rRVV-XH and rRVV-XL in *E. coli*

Our previous study, the genes encode for mature RVV-XH and only one chain of light chain (LC1; RVV-XL) were cloned into pTrcHisα-A vector and transformed in *E.coli* DH5α (Sai-gnam, 2007). In this study, the expression of rRVV-XH and rRVV-XL proteins was performed in *E. coli* BL21. rRVV-XH and rRVV-XL proteins were used as antigens for rabbit anti-recombinant proteins antibodies production.

1.1 Preparation of *E. coli* BL21 competent cells by $CaCl_2$ method

A single colony of *E. coli* BL21 was inoculated into 3 ml of LB broth and incubated at 37°C with 200 rpm shaking for 16-20 hr. 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 centrifuged (GS-15R Centrifuge, S4180, Beckman, CA, USA) at 5,000 g for 10 min at 4°C. The pellet was suspended in 5 ml of ice-cold 0.1 M $MgCl_2$. The solution was then centrifuged and resuspended in 5 ml of ice-cold 0.1 M $CaCl_2$. The mixture was left on ice for 30 min to establish competency. After the last 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.

1.2 Transformation of *E. coli* BL21 competent cells

The 200 μl of *E. coli* BL21 competent cells were mixed with 2 μl of recombinant plasmids containing the inserted RVV-XH or RVV-XL DNA fragments. The mixtures were then immediately placed on ice for 30 min. The BL21 competent 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 pTrcHisα-A vectors containing DNA inserts were extracted by alkaline lysis method. Then, PCR sequencing was done to ensure the correct DNA sequences.

1.3 Plasmid DNA extraction by alkaline lysis method

The single colony of bacteria was inoculated in 3 ml LB containing 50 ug/ml amplicilin 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 (Denver Instrument, NY, USA) at 7,200 g 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 III (3 M potassium acetate, 11.5% glacial acetic acid) was added to the mixture and incubated on ice for 5 min. The mixture was centrifuged at 7,200 g for 10 min. The supernatant was decanted to a new tube. The plasmid DNA was recovered from the supernatant by adding 7/10 volume of isopropanol and standing in room temperature for 10 min. The pellet was washed with 500 μl of 70% ethanol, centrifuged at 7,200 g 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 containing 20 μg/ml of RNase).

1.4 RVV-XH and RVV-XL homolog expression by induced with isopropyl beta-thiogalactoside (IPTG)

A single colony of *E. coli* containing rRVV-XH or rRVV-XL plasmids was inoculated into 20 ml of LB broth containing 50 μg/ml of ampicillin. The cultures were incubated at 37°C with 200 rpm shaking for 16-20 hr. The overnight cultures were diluted 1:100 into 1 L of LB broth containing 50 μg/ml ampicillin. The cultures were then incubated at a rotary incubator shaker (200 rpm) in 37°C until an OD_{600} of 0.4-0.6. Then 5 ml of cells was collected to be negative control. A 0.5 mM or 1 mM of IPTG was added into the RVV-XH or RVV-XL cultures, respectively. Then, the cultures were incubated at 37°C with 200 rpm shaking for 3, 5, or 24 hr. The cells were centrifuged at 13,000 rpm for 30 min at 4°C. The cell pellets were lysed by sonication.

1.5 Sonication

Both RVV-XH and RVV-XL pellets were suspended with 30 ml 1X PBS buffer (pH 7.4). The suspensions were removed by centrifugation (GS-15R Centrifuge, S4180, Beckman, CA, USA) at 10,000 g for 30 min. The cell pellets was added to the protease inhibitor cocktail (Sigma-Aldrich, MO, USA). The ratio of protease inhibitor to cell pellet is 1:100. The cell pellets were sonicated 5 times for 10 sec each. The cell lysate was centrifuged at 10,000 g for 30 min at 4°C. Then, the supernatants were decanted. The cell pellets were washed once with 30 ml of triton-X for 4 times. Finally, the washed cell pellets were resuspended into 30 ml of equilibration/wash buffer, pH 7.0 (50 mM sodium phosphate buffer, 6 M guanidine-HCl, 300 mM NaCl).

1.6 Purification of His-tagged recombinant proteins using Immobilized Metal Affinity Chromatography (IMAC)

His-tagged rRVV-XH and rRVV-XL proteins were applied into the TALON®resin column (0.4 x 2.6 cm). The column was previously equilibrated with 30 ml of equilibration/wash buffer, pH 7.0 (50 mM sodium phosphate buffer, 6 M guanidine-HCl, 300 mM NaCl). After samples loading, the column was washed with 20 column volume of the same buffer. Finally, the His-tagged proteins were eluted with 50 ml elution buffer, pH 7.0 (45 mM sodium phosphate buffer, 5.4 M guanidine-HCl, 270 mM NaCl, 150 mM imidazole) into 50 fractions with a flow rate of 0.5 ml/min. Fractionation was carried out at 4°C. The absorbance of all the fractions was monitored at 280 nm using a spectrophotometer (SmartSpec™3000, Bio-rad Laboratories, Inc., CA, USA).

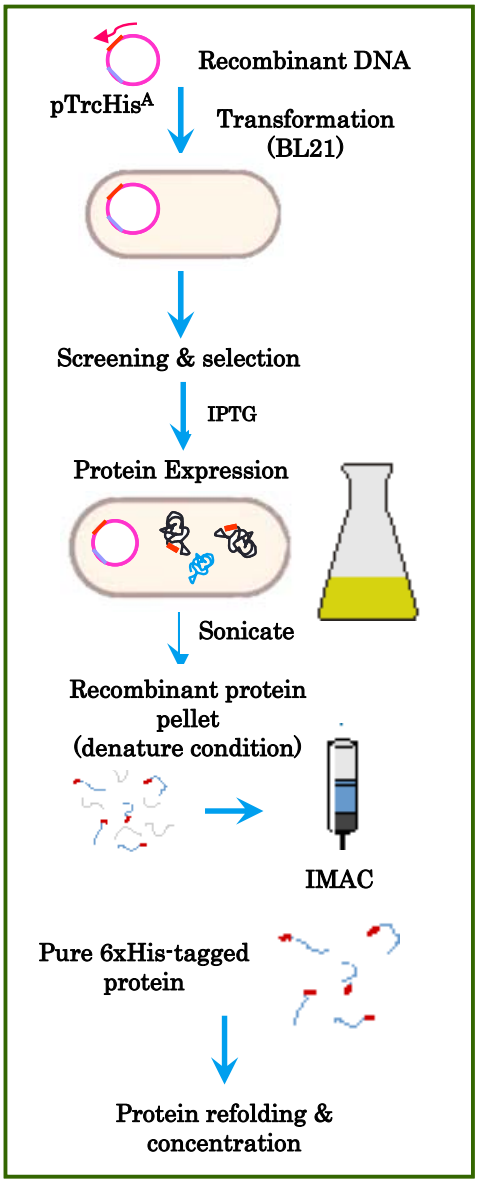


Figure 9 The flow chart protocol for RVV-X expression in *E.coli* BL21.

1.7 Protein concentration

The diluted sample was concentrated by Amicon ultra centrifugal filter units (10-kDa cutoff size, Millipore, MA, USA). The columns were spun at 3,000 g for approximate 1 hr. The remaining culture media less than 500 μl (and the flow-through culture media as well) was kept for further analysis.

1.8 Protein detection

1.8.1 SDS-PAGE analysis

SDS-PAGE was carried out in 12% resolving gel and 4% of stacking gel according to Laemmli (1970) under reducing and non-reducing conditions at 20 mA (constant). The gels were freshly prepared as follow:

12% of resolving gel (per page)

30% acrylamide:Bisacrylamide	2.000 ml
1.875 M Tris-HCl, pH 8.8	1.000 ml
10% SDS	0.050 ml
Distilled water	1.930 ml
10% ammonium persulphate	0.050 ml
TEMED	0.005 ml

4% of stacking gel (per page)

30% Acrylamide:Bisacrylamide	0.250 ml
1.875 M Tris-HCl, pH 6.8	0.165 ml
10% SDS	0.025 ml
Distilled water	2.050 ml
10% ammonium persulphate	0.030 ml
TEMED	0.003 ml

1.8.2 Coomassie blue G250 staining

Gel was stained with 0.25% brilliant blue Coomassie G250 in acetic acid:methanol:water (10:45:45, v/v) for 1 hr. Gel was then destained in acetic acid:methanol:water (10:30:60, v/v).

1.8.3 Western blot analysis

A 250 ng of rRVV-XH and rRVV-XL proteins were separated by 12% reduced or non-reduced SDS-PAGE. Following electrophoretic separation, gels were electroblotted onto 0.45 μm nitrocellulose membrane (Whatman® GmbH, Germany) using Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, USA). The gel and membrane were soaked in transfer buffer, pH 8.3 (20 mM Tris-HCl, 150 mM Glycine, 20% (v/v) methanol, 3 M Tris-HCl, pH8.8, and glycine) for 20 min at room temprature. The proteins are exposed on a thin surface layer for detection as shown in Figure 10. The proteins moved from the gel onto the membrane at 20 volt for 35 min.

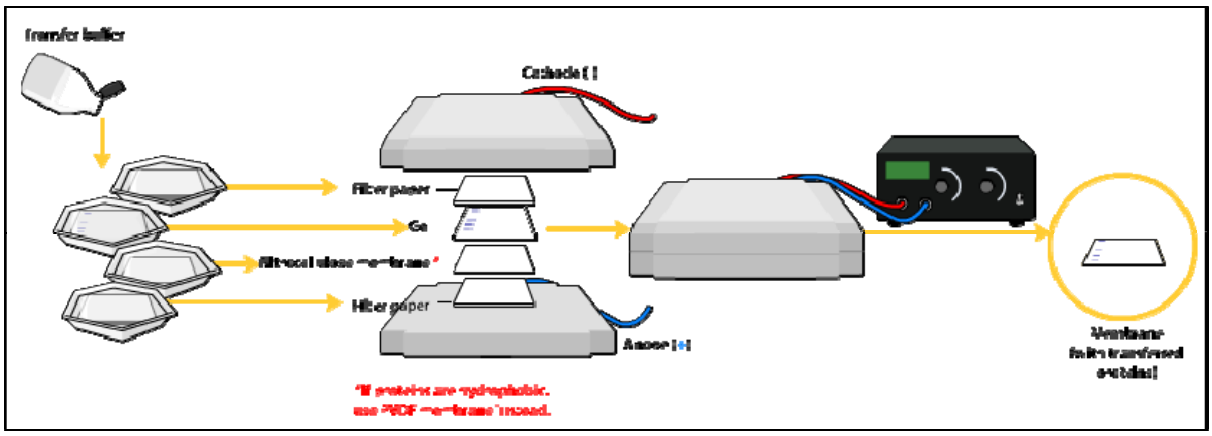


Figure 10 Western blotting process (http://en.wikipedia.org/wiki/Western_blot).

The nitrocellulose membrane was incubated in blocking solution (5% w/v skim milk in PBS buffer, pH 7.4) for 1 hr with gentle agitation and then was washed 3 times with PBS buffer, pH 7.4 for 3 min each time. The membrane was incubated with a 1:5,000 dilution of mouse anti-His antibody (Amersham Pharmacia Biotech AB, Sweden) or a 1:100 dilution of horse anti-cRVV IgG antibody (47.7 mg/ml) in blocking solution for 1 hr at room temperature. The membrane was washed 3 times with PBS buffer, pH 7.4 for 3 min each time. After that, the membrane was then incubated with a 1:1,500 dilution of HRP-conjugated rabbit anti-mouse antibody (DAKO, CA, USA).

The protein bands were visualized by adding the visualizing solution (1.66 mM DAB, 8% $NiCl_2$, and 30% H_2O_2).

1.9 Coagulation activity of rRVV-XH and rRVV-XL proteins using APTT assay

A 100 μl sample of normal citrated plasma (Instrumentation Laboratory Company, MA, USA) or factor X-deficiency plasma (Sigma diagnostics, MO, USA) was added into a 3 ml glass tube. Then, a 100 μl of cRVV (20 μg/ml), rRVV-XH (2.05 mg/ml), or rRVV-XL (0.91 mg/ml) proteins was gently mixed into the tube containing the plasma and incubated at 37°C for 3 min. After that, a pre-warmed 100 μl sample (37°C) of a 0.025 M $CaCl_2$ was added and the clotting time was recorded. Normal plasma negative control was normal citrated human plasma reconstituted with only $CaCl_2$. Factor X deficient plasma negative control was factor X deficient human plasma reconstituted with only $CaCl_2$. The positive control was normal citrated or factor X deficient human plasma activated with cephalin (Instrumentation Laboratory Company, MA, USA) and 0.025 M $CaCl_2$. Each test was performed in triplicate. Their significance was analyzed by the Student's t-test.

2. Production of rabbit anti-rRVV-XH and anti-rRVV-XL antibodies

2.1 Rabbit immunization

For primary immunization, a 250 μg of rRVV-XH or rRVV-XL proteins was diluted to 0.5 ml with sterile normal saline and combined with 0.5 ml of the complete Fruend's adjuvant (CFA). One milliliter of each sample was injected intradermally (i.d.) into 20 sites of 0.05 ml per site on the shaved back of two adult female New Zealand white rabbits (*Oryctolagus cuniculus*). For three-week rest period later, a 100 μg of rRVV-XH or rRVV-XL proteins mixed with Freund's incomplete adjuvant (IFA). All subsequent immunizations were performed every three weeks. Blood was collected form the central ear artery at three-week intervals. The titer of produced antibodies was determined by ELISA. Then, plasma of rabbits containing the rRVV-XH- and rRVV-XL-specific antibodies was purified by protein G column affinity chromatography.

2.2 Quantitation of rabbit anti-recombinant proteins antibodies using ELISA

The antibody titer of rabbit anti-rRVV-XH and anti-rRVV-XL antibodies was determined by an ELISA, which was modified from Rungsiwongse and

Ratanabanangkoon (1991). The polyvinyl microtiter plate (NUNC™, NY, USA) was coated with 50 μl/well of 5 μg/ml of purified RVV-X in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6 at 4°C for 18 hr. The coated plate was washed 4 times for 3 min intervals with washing buffer (0.05% Tween-20 in normal saline), and blocked with blocking buffer (0.15 M PBS, pH 7.4 containing 0.5% BSA). Appropriate starting dilutions of individual sera from immunized animals including positive and negative controls were diluted in 0.15 M PBS, pH 7.4 containing 0.05% Tween-20 and 0.5% BSA. A 50 μl of sample dilution was added to each well and incubated for 1 hr at room temperature. After washing step, 50 μl/well of 1:10,000 diluted HRP-conjugated goat anti-rabbit antibody (Zymed Laboratories, CA, USA) was added and incubated for 1 hr at room temperature. After 4 washes with washing buffer, 100 μl/well of freshly prepared substrate solution (0.01% TMB and 3% H_2O_2 in 0.075 M citrate-phosphate buffer, pH 5.0) was added. The plate was allowed in the dark for 30 min at room temperature. The reaction was stopped by adding 25 μl of 4N sulfuric acid. The absorbance of sample was monitored at 405 nm using an ELISA reader (Multiskan EX, Thermo Labsystems, CA, USA). A horse anti-cRVV IgG antibody was used as the positive control, which was included in every plate to correct for day-to-day or plate-to-plate variations.

2.3 Purification of rabbit anti-recombinant proteins IgG antibodies

Sera of rabbits containing the rRVV-XH and rRVV-XL-specific antibodies were purified by protein G column affinity chromatography. The serum was applied into the protein G (KPL, Gaithersburg, MD, USA) affinity column (0.4 x 2.6 cm). The column was previously equilibrated with 30 ml of wash/binding buffer, pH 7.4 (0.1 M sodium phosphate buffer, 0.15 M NaCl). After samples loading, the column was washed with 30 ml of the same buffer. Finally, the IgG antibodies were eluted with 15 ml of 0.2 M glycine, pH 2.85 into 15 fractions with a flow rate of 0.5 ml/min and immediately added 240 μl of 5X wash/binding buffer. Fractionation was carried out at 4°C. The absorbance of all the fractions was monitored at 280 nm using a spectrophotometer (SmartSpec™3000, Bio-rad Laboratories, Inc., CA, USA).

2.4 Protein detection

2.4.1 SDS-PAGE analysis

SDS-PAGE was carried out in 8% resolving gel and 4% of stacking gel according to Laemmli (1970) under reducing and non-reducing conditions at 20 mA (constant). The gels were freshly prepared as follow:

8% of resolving gel (per page)

30% acrylamide:Bisacrylamide	1.330 ml
1.875 M Tris-HCl, pH 8.8	1.000 ml
10% SDS	0.050 ml
Distilled water	2.600 ml
10% ammonium persulphate	0.050 ml
TEMED	0.005 ml

4% of stacking gel (per page)

30% Acrylamide:Bisacrylamide	0.250 ml
1.875 M Tris-HCl, pH 6.8	0.165 ml
10% SDS	0.025 ml
Distilled water	2.050 ml
10% ammonium persulphate	0.030 ml
TEMED	0.003 ml

2.4.2 Coomassie blue G250 staining

Gel was stained with 0.25% brilliant blue Coomassie G250 in acetic acid:methanol:water (10:45:45, v/v) for 1 hr. Gel was then destained in acetic acid:methanol:water (10:30:60, v/v).

2.5 Binding of rabbit anti-recombinant proteins IgG antibodies to purified RVV-X using Western blot analysis

A cRVV (5 μg), purified RVV-X (250 ng), bovine serum albumin (BSA; 2 μg), and mouse serum (5 μg) were separated by 12% non-reduced SDS-PAGE. Following electrophoretic separation, gels were electroblotted onto 0.45 μm

nitrocellulose membrane (Whatman® GmbH, Germany) using Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, USA). The nitrocellulose membrane was incubated in blocking solution (5% w/v skim milk in PBS buffer, pH 7.4) for 1 hr with gentle agitation and then was washed 3 times with PBS buffer, pH 7.4 for 3 min each time. The membrane was incubated with a 1:1,500 dilution of rabbit anti-rRVV-XH IgG antibody, or a 1:1,000 dilution of rabbit anti-rRVV-XL IgG antibody in blocking solution for 1 hr at room temperature. The membrane was washed 3 times with PBS buffer, pH 7.4 for 3 min each time. After that, the membrane was then incubated with a 1:5,000 dilution of HRP-conjugated goat anti-rabbit antibody (Zymed Laboratories, CA, USA) in blocking solution for 1 hr at room temperature. The protein bands were visualized by adding the visualizing solution (1.66 mM DAB, 8% $NiCl_2$, and 30% H_2O_2).

2.6 Effects of rabbit anti-recombinant proteins IgG antibodies in neutralizing factor X activator activity of purified RVV-X using factor Xa-specific chromogenic substrate S-2765

A 30 μl of rabbit anti-rRVV-XH IgG antibody, anti-rRVV-XL IgG antibody, anti-rRVV-XH and anti-rRVV-XL IgG antibodies, or commercial horse anti-cRVV $F(ab')_2$ antivenom (QSMI, Thailand) at various concentrations were pre-mixed with 30 μl of 2 μg/ml of purified RVV-X at 37°C for 30 min. Twenty five microliters of the normal human citrated plasma (Instrumentation Laboratory Company, MA, USA) dissolved in Tris-EDTA buffer was incubated at 37°C for 3 min. Then, 25 μl of chromogenic substrate S-2765 was added and mixed within 30 sec. A 25 μl of sample mixtures pre-mixed with 0.1 M $CaCl_2$ in equivalent quantities was added and incubated at 37°C for 3 min. The reaction was stopped by 20% acetic acid. The yellow color was monitored at 405 nm. Each test was performed in triplicate. The venom control was prepared by mixing the plasma, chromogenic substrate, 1 μg/ml of purified RVV-X pre-mixing with 0.1 M $CaCl_2$ in equal volume, and 20% of acetic acid. The negative control was prepared by mixing the plasma, chromogenic substrate, 20% of acetic acid and buffer instead of the venom. Commercial horse anti-cRVV $F(ab')_2$ antivenom (QSMI, Thailand) was used as antibody control.

Since Rabbit anti-rRVV-XH antibody, anti-rRVV-XL IgG antibody, or the mixture of rabbit anti-rRVV-XH and anti-rRVV-XL antibodies were unable to neutralize factor X activator activity of purified RVV-X using factor Xa-specific chromogenic substrate S-2765. The effects of rabbit anti-rRVV-XH and anti-rRVV-XL antibodies in neutralizing DIC, renal hemodynamics and functions of cRVV could not be futher investigated.

3. Cross-reactivity of rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies against *C. albolabris* and *C. rhodostoma* venoms using Western blot analysis

A 5 μg of crude venom samples of *D. r. siamensis, C. albolabris*, and *C. rhodostoma* were separated by 12% reduced or non-reduced SDS-PAGE. Following electrophoretic separation, gels were electroblotted onto 0.45 μm nitrocellulose membrane (Whatman® GmbH, Germany) using Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, USA). The nitrocellulose membrane was incubated in blocking solution (5% w/v skim milk in PBS buffer, pH 7.4) for 1 hr with gentle agitation and then was washed 3 times with PBS buffer, pH 7.4 for 3 min each time. The membrane was incubated with a 1:1,500 dilution of rabbit anti-rRVV-XH IgG antibody (4.19 mg/ml) or a 1:1,000 rabbit anti-rRVV-XL IgG antibody (4.14 mg/ml) in blocking solution for 1 hr at room temperature. The membrane was washed 3 times with PBS buffer, pH 7.4 for 3 min each time. After that, the membrane was incubated with a 1:5,000 dilution of HRP-conjugated goat anti-rabbit antibody (Zymed, CA, USA) in blocking solution for 1 hr at room temperature. The protein bands were visualized by adding the visualizing solution (1.66 mM DAB, 8% $NiCl_2$, and 30% H_2O_2).

Part II Effects of purified RVV-X and cRVV on DIC, renal hemodynamic and function, and histopathological changes in SD rats.

1. Purification of horse anti-cRVV IgG antibody

Horse anti-cRVV IgG antibody was purified from horse whole blood against cRVV by ammonium sulfate (AS) precipitation (Aalund et al., 1965) and gel filtration chromatography. One liter of whole blood from QSMI was stored in 4°C for 24 hr. Then, coagulate blood was centrifuged for 10 min at 2,500 g. A 0.1% sodium azide (NaN_3) was added as a preservative to serum sample, which was then stored at -80°C.

1.1 Ammonium sulfate precipitation

Horse anti-cRVV serum was precipitated at 50% AS at 4°C. Briefly, a volume of 20 ml of horse serum (107.16 mg/ml) was premixed with 20 ml of 0.15 M phosphate buffer saline, pH 7.4. Then, a 40 ml of saturated AS was gradually added with stirring at 4°C overnight to complete the precipitation. The precipitate containing horse immunoglobulin was isolated by centrifugation at 10,000 g for 20 min. The precipitate was redissolved in 0.1 M NaCl containing 0.1% NaN_3 and dialyzed at 4°C against the same buffer. A 27.6 ml of redissolved precipitate at the concentration of 41.7 mg/ml was further purified by gel filtration chromatography.

1.2 Gel filtration chromatography

Sephadex G-200 column was used for the second purification step. Two milliliters of clear supernatant at a concentration of 41.7 mg/ml was applied into a Sephadex G-200 gel filtration (26 x 65 cm) column. The column was previously equilibrated with elution buffer (0.1 M NaCl containing 0.1% NaN_3). Fractions of 3 ml were collected by an automatic water fraction collector (ATTO minicollector, Japan) at a flow rate 15 ml/hr. The absorbance of all the fractions was monitored at 280 nm using a UV2100 (UV-VIS recording spectrophotometer, SHIMADZU, Japan). A 14.5 ml of the purified horse anti-cRVV IgG antibody at the concentration of 47.7 mg/ml was aliquoted and stored at -20°C until used. The molecular weight and protein patterns of each fraction were determined by SDS-PAGE. Horse anti-cRVV IgG antibody was used as a primary antibody in Western blot analysis for evaluation of the purity of purified RVV-X.

1.3 Protein detection

1.3.1 SDS-PAGE analysis

SDS-PAGE was carried out in 8% resolving gel and 4% of stacking gel according to Laemmli (1970) under reducing and non-reducing conditions at 20 mA (constant). Gel was stained with 0.25% brilliant blue Coomassie G250 in acetic acid:methanol:water (10:45:45, v/v) for 1 hr. Gel was then destained in acetic acid:methanol:water (10:30:60, v/v).

1.3.2 Quantitation of horse anti-cRVV IgG antibody using ELISA

The amount of horse anti-cRVV IgG antibody was evaluated by an ELISA, which was modified from Rungsiwongse and Ratanabanangkoon (1991). The polyvinyl microtiter plate (NUNC^{TM}, NY, USA) was coated with 50 μl/well of 5 μg/ml of cRVV in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6 at 4°C for 18 hr. The coated plate was washed 4 times for 3 min intervals with washing buffer (0.05% Tween-20 in normal saline), and blocked with blocking solution (0.15 M PBS, pH 7.4 containing 3% casein). Each fraction was diluted 1:100 with 0.15 M PBS, pH 7.4 containing 0.05% Tween-20 and 1% casein. A 50 μl of diluted fractions was then added to each well and incubated for 1 hr at room temperature. After washing step, 50 μl/well of 1:200 diluted HRP-conjugated sheep anti-horse antibody was added and incubated for 1 hr at room temperature. After 4 washes with washing buffer, 100 μl/well of freshly prepared substrate solution (0.01% TMB and 0.003% H_2O_2 in 0.075 M citrate-phosphate buffer, pH 5.0) was added. The plate was allowed in the dark for 30 min at room temperature. The reaction was stopped by adding 25 μl of 4N sulfuric acid. The absorbance of sample was monitored at 405 nm using an ELISA reader (Multiskan EX, Thermo Labsystems, CA, USA). The highest dilution giving an absorbance reading of 0.5 was regarded as the end point titer.

2. Purification of RVV-X from crude venom

Purified RVV-X was purified from lyophilized crude Russell's viper venom (cRVV) by gel filtration and anion exchange chromatography, which was modified from Kisiel et al. (1976).

2.1 Gel filtration chromatography

Samples of 400 mg of lyophilized crude venom were dissolved with 4.0 ml of 0.05 M Tris-phosphate buffer, pH 7.5 containing 1 mM benzamidine-HCl. The suspension was centrifuged at 10,000 g at 4°C for 5 min. Four milliliters of clear supernatant at the concentration of 100 mg/ml was applied into Sephadex G-100 (2.6 x 65 cm) column. The column was previously equilibrated with elution buffer (0.05 M Tris-phosphate buffer, pH 7.5 containing 1 mM benzamidine-HCl). Fractions of 2 ml were collected by an automatic water fraction collector (ATTO minicollector, Japan) at a flow rate 10 ml/hr. The absorbance of all the fractions was monitored at 280 nm using a UV2100 (UV-VIS recording spectrophotometer, SHIMADZU, Japan). Each fraction was screened for factor X activator activity using factor Xa-specific chromogenic substrate S-2765. The molecular weight and protein patterns of each fraction were determined by SDS-PAGE. Fractions containing factor X activator activity were pooled and further purified by Q-Sepharose anion exchange chromatography.

2.2 Anion exchange chromatography

Anion exchange chromatography was used for the second step of purification. The pooled Sephadex G-100 fractions with factor X activator activity was further fractionated by Q-Sepharose anion exchange chromatography. The pooled fractions no. 54-72 from peak I (PI; Figure 13; 82.03 mg) were applied to a Q-Sepharose (0.9 x 10 cm) previously equilibrated with 0.05 M Tris-phosphate buffer (pH 7.5) containing 1 mM benzamidine-HCl. The fractions were eluted with a 0-0.5 M NaCl salt gradient. After the gradient, 1 M NaCl in the same buffer was used to elute tightly bound proteins. One milliliter per tube was collected with a flow rate of 1 ml/min. Each fraction was again screened for factor X activator activity factor Xa-specific chromogenic substrate S-2765. The molecular weight and purity of purified RVV-X were determined by SDS-PAGE and verified by MALDI-TOF mass spectrometry, which was carried out by Bio Service Unit (BSU), National Science Technology Development Agency (NSTDA), Thailand.

2.3 Proteins detection

2.3.1 SDS-PAGE analysis

SDS-PAGE was carried out in 12% resolving gel and 4% of stacking gel according to Laemmli (1970) under reducing and non-reducing conditions at 20 mA (constant). Gel was stained with 0.25% brilliant blue Coomassie G250 in acetic acid:methanol:water (10:45:45, v/v) for 1 hr. Gel was then destained in acetic acid:methanol:water (10:30:60, v/v).

2.3.2 Silver staining

After gel electrophoresis, a protein gel with silver staining was carried out according to the manufacturer's recommendations (Pharmacia Biotech AB, Sweden). 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).

2.3.3 Protein concentration measurement

Protein concentration of purified RVV-X was determined by BCA method following the protocol provided by the manufacture. Bovine serum albumin was used as the standard (The Thermo Scientific, USA).

2.3.4 Purity determination of purified RVV-X was determined by Western blot analysis.

cRVV and purified RVV-X were separated by 12% non-reduced SDS-PAGE. Following electrophoretic separation, gels were electroblotted onto 0.45 μm nitrocellulose membrane (Whatman®, Germany) using Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, USA). The gel and membrane were soaked in transfer buffer, pH 8.3 (20 mM Tris-HCl, 150 mM Glycine, 20% (v/v) methanol, 3 M Tris-HCl, pH8.8, and glycine) for 20 min at room temprature. The proteins are

exposed on a thin surface layer for detection. The proteins moved from the gel onto the membrane at 20 volt for 35 min.

The nitrocellulose membrane was incubated in blocking solution (5% w/v skim milk in PBS buffer, pH 7.4) for 1 hr with gentle agitation and then was washed 3 times with PBS buffer, pH 7.4 for 3 min each time. The membrane was incubated with a 1: 100 dilution of horse anti-cRVV IgG antibody (47.7 mg/ml) in blocking solution for 1 hr at room temperature. The membrane was washed 3 times with PBS buffer, pH 7.4 for 3 min each time. After that, the membrane was incubated with a 1:200 dilution of HRP-conjugated sheep anti-horse antibody in blocking solution for 1 hr at room temperature. The protein bands were visualized by adding the visualizing solution (1.66 mM DAB, 8% $NiCl_2$, and 30% H_2O_2).

3. RVV-X activity

3.1 Coagulation activity of purified RVV-X using activated partial thromboplastin time (APTT) assay

A 100 μl sample of normal human citrated plasma was added into a 3 ml glass tube. Then, a 100 μl of purified RVV-X and cRVV at various concentrations was gently mixed into the tube containing the plasma and incubated at 37°C for 3 min. After that, a pre-warmed 100 μl sample (37°C) of a 0.025 M $CaCl_2$ was added and the clotting time was recorded. Factor X deficient human plasma (Instrumentation Laboratory Company, MA, USA) was used as a negative control substrate for coagulation activity of purified RVV-X. The normal plasma negative control was citrated human plasma reconstituted with only $CaCl_2$. Factor X deficient plasma negative control was factor X deficient human plasma reconstituted with only $CaCl_2$. The positive control was normal citrated or factor X deficient human plasma activated with cephalin (Instrumentation Laboratory Company, MA, USA) and 0.025 M $CaCl_2$. Each test was performed in triplicate.

3.2 Factor X activator activity

Factor X activator activities of cRVV and purified RVV-X were tested using factor Xa-specific chromogenic substrate S-2765. Twenty five microliters of the normal human citrated plasma (Instrumentation Laboratory Company, MA, USA)

dissolved in Tris-EDTA buffer was incubated at 37°C for 3 min. Then, 25 μl of chromogenic substrate S-2765 was added and mixed within 30 sec. The 25 μl of each crude venom or purified RVV-X at various concentrations premixed with 0.1 M $CaCl_2$ in equivalent quantities was added and incubated at 37°C for 3 min. The reaction was stopped by 20% acetic acid. The yellow color was monitored at 405 nm. Each test was performed in duplicate.

3.3 Cleavage of human factor X by purified RVV-X

A 3 μl of purified RVV-X at various concentrations (0.25, 0.5, 1, 2 mg/ml) was incubated with a 3 μl of human factor X at the concentration of 1 mg/ml (Haematologic Technologies Inc., VT, USA) for 30 min at 37°C. A 2 μl of sample mixture was run on a NuPAGE® Novex 4-12% Bis-Tris SDS-PAGE gel (Invitrogen™, CA, USA) under non-reducing conditions. A XCell SureLock™ system with MOPS SDS running buffer (20X) diluted to 1X at a voltage of 200 for 50 min using a Bio-Rad PowerPac Basic was used. SeeBlue Plus2 markers ranging from 3-188 kDa were used as standards. Gel was stained with Simply Blue (Invitrogen™, CA, USA).

3.4 Cleavage of human protein C by purified RVV-X

A 3 μl of purified RVV-X at various concentrations (0.25, 0.5, 1, 2 mg/ml) was incubated with a 3 μl of human protein C at the concentration of 1 mg/ml (Haematologic Technologies Inc.) for 30 min at 37°C. A 2 μl of sample mixture was run on a NuPAGE® Novex 4-12% Bis-Tris SDS-PAGE gel (Invitrogen™, CA, USA) under non-reducing conditions. A XCell SureLock™ system with MOPS SDS running buffer (20X) diluted to 1X at a voltage of 200 for 50 min using a Bio-Rad PowerPac Basic was used. SeeBlue Plus2 markers ranging from 3-188 kDa were used as standards. Gel was stained with Simply Blue (Invitrogen™, CA, USA).

3.5 Fibrinogenolytic activity of purified RVV-X

3.5.1 Effect of various concentrations of purified RVV-X on fibrinogen

A 10 μl of purified RVV-X at various concentrations (0.1, 0.25, 0.5, and 1 mg/ml) was incubated with a 20 μl of purified human fibrinogen (Hyphen Biomed, France) at the concentration of 5 mg/ml for 24 hr at 37°C. The sample mixture was added with a 15 μl of a Novex® Tricine SDS buffer solution (Invitrogen™, CA, USA)

and a 5 μl of reducing agent (Invitrogen™, CA, USA). A 20 μl of sample mixture was run on a Novex® 10-20% Tricine SDS-PAGE gel (Invitrogen™, CA, USA) at 125 V for 90 min. Gel was stained with Simply Blue (Invitrogen™, CA, USA).

3.5.2 Effect of incubation times of purified RVV-X and fibrinogen

A 10 μl of purified RVV-X at the concentration of 1 mg/ml was incubated with a 20 μl of purified human fibrinogen (Hyphen Biomed) at the concentration of 5 mg/ml for 30 min, 1, 2, 4, 8, 24 hr at 37°C. The sample mixture was added with a 15 μl of a Novex® Tricine SDS buffer solution (Invitrogen) and a 5 μl of reducing agent (Invitrogen). A 20 μl of sample mixture was run on a Novex® 10-20% Tricine SDS-PAGE gel at 125 V for 90 min. Gel was stained with Simply Blue (Invitrogen™, CA, USA).

4. Animal experiments

The SD rats were fed on standard rat chow diet with water ad libitum. The rats were divided into six groups: (1) cRVV; (2) purified RVV-X; (3) equine rabies immunoglobulin (ERIG, QSMI, Thailand) with purified RVV-X; (4) commercial horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X; (5) commercial horse anti-cRVV $F(ab')_2$ antivenom; and (6) normal saline solution injection. The animal was fasted for 9-10 hr prior to each experiment. Each rat was weighed and anesthetized with sodium pentobarbital by intraperitoneal injection (60 mg/kg body weight).

4.1 Sublethal dose optimization of cRVV and purified RVV-X in SD rats

The optimum sublethal dose of cRVV was performed by intravenous injections of various concentrations (2.5, 7, 12.5, 25, 50 μg/kg) of cRVV into SD rats (n=2). The dose giving no death with abnormal physiological conditions was used as the sublethal dose for the further experiments. To determine the toxicity of cRVV caused by purified RVV-X, the equipotent sublethal dose of cRVV and purified RVV-X was calculated from the specific factor X activator activity using factor Xa-specific chromogenic substrate S-2765.

4.2 Effect of commercial horse anti-cRVV F(ab')$_2$ antivenom in neutralizing factor X activator activity of purified RVV-X using factor Xa-specific chromogenic substrate S-2765.

A 0.125 μg/ml of purified RVV-X was pre-mixed with an equal volume of commercial horse anti-cRVV F(ab')$_2$ antivenom at various concentrations (0.625, 3.125, 6.25, 15.625, 31.25 μg/ml). The mixtures were then incubated for 30 min at 37°C. Twenty five microliters of the normal human citrated plasma (Instrumentation Laboratory Company, MA, USA) dissolved in Tris-EDTA buffer was incubated at 37°C for 3 min. Then, 25 μl of chromogenic substrate S-2765 was added and mixed within 30 sec. The 25 μl of the sample mixture was pre-mixed with an equal volume of 0.1 M $CaCl_2$, and incubated at 37°C for 3 min. The reaction was stopped by 20% acetic acid. The yellow color was monitored at 405 nm. Negative control was normal citrated human plasma reconstituted with only $CaCl_2$. Venom control was normal citrated activated with purified RVV-X and 0.025 M $CaCl_2$. Antibody control was normal citrated activated with purified RVV-X pre-mixed with equine rabies immunoglobulin (ERIG) and 0.025 M $CaCl_2$. The dose inhibiting factor X activator activity of purified RVV-X was used as the neutralizing dose for the further experiments.

4.3 Disseminated intravascular coagulation (DIC) determination in SD rats

A tracheotomy was performed to insert a tracheal tube to open the airway and to remove some secretion. Right common carotid artery was canulated to collect blood samples. The femoral vein was canulated for the administration of 1 ml of sample. The sample injection dose in each group of SD rats (n=6) was shown in Table 6. Then, a 0.3 ml of blood was collected and diluted with 3.8% of sodium citrate at 5, 10, 15, 20, 30, 45, 60, 120, and 180 min after toxin injections. The plasma D-dimer was determined using the commercial D-Dimer test kit (NycoCard® D-dimer, Axis-Shield PoC, MA, USA).

Table 6 Dosage of samples injection in SD rats (n = 6) for DIC and renal hemodynamic and function experiments

Group	Sample injection	Dosage
1	cRVV	7 μg/kg
2	Purified RVV-X	1.75 μg/kg
3*	(a) ERIG	(a) 437.5 μg/kg
	(b) Purified RVV-X	(b) 1.75 μg/kg
4*	(a) Horse commercial horse anti-cRVV $F(ab')_2$ antivenom	(a) 437.5 μg/kg
	(b) Purified RVV-X	(b) 1.75 μg/kg
5	Commercial horse anti-cRVV $F(ab')_2$ antivenom	437.5 μg/kg
6	Normal saline	-

An asterisk (*) is indicated two steps in SD rats group 3 and 4. SD rats were given with a 1 ml of sample (a) at the rate of 1 ml/min, and suddenly followed by a 1 ml of second sample (b) at the same injection rate.

4.4 Complete blood count (CBC) determination

At the end of the DIC experiment, 1 ml of blood was collected and diluted with 1% of ethylene diamine tetra-acetic acid (EDTA) for determination of CBC using the automated hematology analyzer (Sysmex XS-800i/XS-1000i, Sysmex Corp., Kobe, Japan) at Department of Pediatrics, King Chulalongkorn Memorial hospital, Thailand. Red cell morphology was evaluated on a peripheral blood film. Blood smear was stained with Wright's stain.

4.5 Renal hemodynamics and function studies

The studies of renal hemodynamics and functions were performed as described previously (Yusuksawad et al, 2006; 2007). Briefly, a tracheostomy was performed to insert a tracheal tube to open the air way and to remove some secretion. Right common carotid artery was cannulated to collect blood samples and recorded blood pressure with a physiological recorder (Polygraph RM 6000, Nihon Kohden Corporation, Japan). The femoral vein was cannulated for toxin administration and the

solution of inulin and para-aminohippuric acid (PAH). The urinary bladder was exposed and inserted with polyethylene tube for urine collection.

To replace the body fluid loss during surgery, normal saline solution (NSS) was infused at the rate of 10 ml/kg body weight/hr by a syringe pump (Harvard syringe pump 21, Harvard apparatus Ltd). After the surgery, a mixture of 1 g% inulin and 0.2 g% PAH in NSS was i.v. administrated to the rats at the flow rate of 10 ml/kg body weight/hr instead of NSS throughout the experiments. Following 60 min of stabilization period, each sample dose followed by Table 6 was i.v. given at the rate of 1 ml/min. After toxin injection for 10 min, urine samples were collected two periods of 30 min consecutively. A 0.8 ml of blood sample was collected into a 1.5 ml plastic test tube containing 0.8 μl of heparin (5,000 IU/ml) at the mid-point of each urine collection. The whole blood samples were collected into two capillary tubes and were centrifuged for 10 min using a microhematocrit centrifuge (Z230H, BHG HERMLE, Germany). The hematocrit (Hct) values were measured by a micro-capillary reader (IEC, DAMON/IEC DIVISION). The remaining heparinized whole blood samples in the tube were centrifuged at 2,000 g for 10 min at 25°C to separate the plasma. To access the effective renal plasma flow (ERPF) and glomerular filtration rate (GFR), the renal clearances of PAH and inulin were calculated through the formula:

$$GFR = C_{in} = \frac{U_{in} V}{P_{in}} \qquad (1)$$

GFR = glomerular filtration rate (ml/min/100g body weight)

C_{in} = clearance of inulin (ml/min/100g body weight)

V = urine flow rate (ml/min/100g body weight)

U_{in} = urinary inulin concentration (mg/ml)

P_{in} = plasma inulin concentration (mg/ml)

$$ERPF = C_{PAH} = \frac{U_{PAH}V}{P_{PAH}} \quad (2)$$

ERPF = effective renal plasma flow (ml/min/100g body weight)

C_{PAH} = clearance of PAH (ml/min/100g body weight)

V = urine flow rate (ml/min/100g body weight)

U_{PAH} = urinary PAH concentration (mg/ml)

P_{PAH} = plasma PAH concentration (mg/ml)

$$ERBF = \frac{ERPF}{1- (Hct/100)} \quad (3)$$

ERPF = effective renal blood flow (ml/min)

Hct = hematocrit value (%)

$$FF = \frac{GFR \times 100}{ERPF} \quad (4)$$

FF = filtration fraction (%)

$$V/GFR = \frac{V \times 100}{GFR} \quad (5)$$

V/GFR = fractional excretion of urine

$$RVR = \frac{MAP}{ERBF} \quad (6)$$

RVR = renal vascular resistance (mmHg/ml/min/100g body weight)

MAP = mean arterial pressure (mmHg)

ERPF = effective renal blood flow (ml/min/100g body weight)

To study the renal tubular function, the concentrations of sodium, potassium and chloride in the plasma and urine were carried out by Renal Laboratory research Unit, King Chulalongkorn Memorial hospital, Thailand. Urinary excretion and fractional excretion of the electrolytes were determined through the formula:

$$FE_E = \frac{U_E V / P_E \times 100}{GFR} \quad (7)$$

FE_E = fractional excretion of electrolyte (%)

U_E = concentration of urinary electrolytes (μEq/μl)

P_E = concentration of plasma electrolytes (μEq/μl)

4.6 Histopathological examination

At the end of DIC experiment, the rat was sacrificed immediately under deep anesthesia. Kidney, adrenal, liver, spleen, heart, and lung were fixed in 10% formalin. Tissue sections were cut 2-3 micron thick and stained with Hematoxylin and eosin (H&E) technique. Histopathological examination was carried out by Special and Molecular Pathology Laboratories, Department of pathology, King Chulalongkorn Memorial hospital, Thailand.

4.8 Statistical analysis

All results were given as mean $\pm$ standard deviation (SD). Their significance was analyzed by the Student's t-test. The level of significance was at $P < 0.05$. Their significant differences between sample means more than two groups were analyzed by ANOVA using Least significant difference test (LSD) as post hoc test at $P < 0.05$.

CHAPTER IV

RESULTS

Part I Effects of rabbit anti-rRVV-XH and anti-rRVV-XL antibodies in neutralizing DIC, renal hemodynamics and functions of cRVV

1. Expression of rRVV-XH and rRVV-XL in *E. coli*

The nucleic acid sequences and its deduced amino acid sequences of rRVV-XH (Figure 11) and rRVV-XL (Figure 12) were reported by Sai-gnam (2007). The predicted molecular mass and isoelectric point (pI) of rRVV-XH and rRVV-XL were analysed by ExPASy Proteomics Server, Swiss Institute of Bioinformatics (Table 7).

Table 7 Predicted characteristics of rRVV-XH and rRVV-XL.

Analysis	rRVV-XH	rRVV-XL
Length	429 amino acid	123 amino acid
Molecular weight	47975.43 Da	14104.03 Da
Isoelectric point (pI)	7.43	4.97

```
   1 tcagaagattacagcgagactcattattacccagatggcagagaaattacaacaaaccct
     S  E  D  Y  S  E  T  H  Y  Y  P  D  G  R  E  I  T  T  N  P
  61 ccggttgaggatcactgctattatcatggacgcatccagaatgatgctcactcatctgca
     P  V  E  D  H  C  Y  Y  H  G  R  I  Q  N  D  A  H  S  S  A
 121 agcatcagtgcatgcaatggtttgaaaggacatttcaagcttcgaggggagatgtacttt
     S  I  S  A  C  N  G  L  K  G  H  F  K  L  R  G  E  M  Y  F
 181 attgaacccttgaagctttccaacagtgaagcccatgcagtctacaaatatgaaaacata
     I  E  P  L  K  L  S  N  S  E  A  H  A  V  Y  K  Y  E  N  I
 241 gaaaaagaggatgagatccccaaaatgtgtggggtaacccagactaattgggaatcagat
     E  K  E  D  E  I  P  K  M  C  G  V  T  Q  T  N  W  E  S  D
 301 aagcccatcaaaaaggcctctcagttagtttctacttctgcacaattcaacaaaatattc
     K  P  I  K  K  A  S  Q  L  V  S  T  S  A  Q  F  N  K  I  F
 361 attgagcttgtcataattgtggaccacagcatggccaagaaatgcaattcaactgctaca
     I  E  L  V  I  I  V  D  H  S  M  A  K  K  C  N  S  T  A  T
 421 aatacaaaaatatatgaaattgtcaacagtgcaaatgagatttttaatcctttgaatatt
     N  T  K  I  Y  E  I  V  N  S  A  N  E  I  F  N  P  L  N  I
 481 catgtaacattgattggtgtagaattttggtgcgacagagatttgattaacgtgacatca
     H  V  T  L  I  G  V  E  F  W  C  D  R  D  L  I  N  V  T  S
 541 tcagcagatgaaactttgaactcatttggagaatggagagcgtcagatttgatgactcgg
     S  A  D  E  T  L  N  S  F  G  E  W  R  A  S  D  L  M  T  R
 601 aaaagccatgataatgctctgttattcacggacatgagattcgatttaaacactttggga
     K  S  H  D  N  A  L  L  F  T  D  M  R  F  D  L  N  T  L  G
 661 atcactttcttagctggcatgtgccaggcatatcgttctgtagaaattgttcaggaacaa
     I  T  F  L  A  G  M  C  Q  A  Y  R  S  V  E  I  V  Q  E  Q
 721 gggaacagaaattttaagactgcagttataatggcccatgagctgagtcataatctgggc
     G  N  R  N  F  K  T  A  V  I  M  A  H  E  L  S  H  N  L  G
 781 atgtatcatgacggaaaaaactgtatttgtaatgattcctcatgtgttatgtctcctgtg
     M  Y  H  D  G  K  N  C  I  C  N  D  S  S  C  V  M  S  P  V
 841 ctaagcgatcaaccttccaaattgttcagcaattgtagtattcacgattatcagaggtat
     L  S  D  Q  P  S  K  L  F  S  N  C  S  I  H  D  Y  Q  R  Y
 901 cttactagatataaaccaaaatgcattttcaatccacccttgagaaaagatattgtttca
     L  T  R  Y  K  P  K  C  I  F  N  P  P  L  R  K  D  I  V  S
 961 cctcccgtttgcggaaatgaaatttgggaggagggagaagaatgtgactgtggctctcct
     P  P  V  C  G  N  E  I  W  E  E  G  E  E  C  D  C  G  S  P
1021 gcaaattgccaaaacccgtgctgtgatgctgcaacatgtaaactgaagccaggggcagag
     A  N  C  Q  N  P  C  C  D  A  A  T  C  K  L  K  P  G  A  E
1081 tgtggaaatggactgtgttgttaccaatgcaaaattaagacagcaggaacagtatgccgg
     C  G  N  G  L  C  C  Y  Q  C  K  I  K  T  A  G  T  V  C  R
1141 agagcaagggatgagtgtgacgtccctgaacactgcactggccaatctgctgagtgtccc
     R  A  R  D  E  C  D  V  P  E  H  C  T  G  Q  S  A  E  C  P
1201 agagatcagttgcaacagaatggaaaaccatgccaaaacaacagaggttattgctacaat
     R  D  Q  L  Q  Q  N  G  K  P  C  Q  N  N  R  G  Y  C  Y  N
1261 ggggattgccccatcatgagaaaccaatgtatttctctctttgggtcacgtgcaaatgtg
     G  D  C  P  I  M  R  N  Q  C  I  S  L  F  G  S  R  A  N  V
1321 gctaaagattcatgttttcaggaaaacctgaagggcagttattatggctactgcagaaag
     A  K  D  S  C  F  Q  E  N  L  K  G  S  Y  Y  G  Y  C  R  K
1381 gaaaatggtagaaagattccatgtgcaccacaagatgtaaaatgtggcaggttattctgc
     E  N  G  R  K  I  P  C  A  P  Q  D  V  K  C  G  R  L  F  C
1441 ttaaataattcacctagaaacaagaatccttgcaacatgcactatagctgcatggatcaa
     L  N  N  S  P  R  N  K  N  P  C  N  M  H  Y  S  C  M  D  Q
1501 cataagggaatggttgaccctggaacaaaatgtgaagatggaaaggtctgcaacaacaaa
     H  K  G  M  V  D  P  G  T  K  C  E  D  G  K  V  C  N  N  K
1561 aggcagtgtgttgatgtgaatacagcctaccaatcaaccactggcttctctcagatttga
     R  Q  C  V  D  V  N  T  A  Y  Q  S  T  T  G  F  S  Q  I  *
1621 ttttggagatctttcttccagaaggttcagcttgcctcaagtccaaagagatccatttgc
1681 cttcatcctactaataaatcacccttagcttccagatggcatctaaattctgcaatattt
1741 cttcactatatttaatttgtttacattttgctgtaatcaaacctttttcccgccataaag
1801 ctccaagggtatgtacaacaccagagacttatttgctgtcaagaaaaaaaaaatggccatt
1861 ttatcatttgctaattgcaaagcatatttaatgcaacaagttctgcctttggagctgatg
1921 tattcgaagtaaatgcttcctcttccaaaatatcacactggctttccaagctgtagctgc
1981 ttccatcaataaacaactattctcattctgaaaaaaaaaaaaaaaaaa 2027
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Figure 11 cDNA sequence of RVV-XH. Four hundred and twenty-nine amino acid residues of mature peptide are shown in bold capital letters. Underlined letters represents residues of metalloproteinase domain. Double underlined letters represents residues of disintegrin domain. An asterisk (*) indicates stop codon.

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  1 ccttggaaagcctggagttgcctctgagcagagttgctacctgtggaggccgagggacag

 61 gtgagtgcagctctgccacctgtgagcacctgacagttctctctgcagggaaggaaggaa

121 gaccatggggcgattcatctccgtcagcttcggctgtctggtcgtgtttctctccctgag
        M  G  R  F  I  S  V  S  F  G  C  L  V  V  F  L  S  L  S

181 tggaactgaagctgttttggactgtccctctggttggctctcctatgaacaacattgcta
     G  T  E  A  V  L  D  C  P  S  G  W  L  S  Y  E  Q  H  C  Y

241 caagggcttcaatgacctgaaaaattggactgatgcagagaaattctgcacagaacagaa
     K  G  F  N  D  L  K  N  W  T  D  A  E  K  F  C  T  E  Q  K

301 gaaaggcagccatctggtctccttgcacagcagagaagaagaagagtttgtggtcaacct
     K  G  S  H  L  V  S  L  H  S  R  E  E  E  E  F  V  V  N  L

361 gatctccgaaaatttggaataccctgctacctggattggactgggcaatatgtggaagga
     I  S  E  N  L  E  Y  P  A  T  W  I  G  L  G  N  M  W  K  D

421 ttgcaggatggagtggagcgatcgtggcaatgtcaaatacaaagccttggctgaagaatc
     C  R  M  E  W  S  D  R  G  N  V  K  Y  K  A  L  A  E  E  S

481 ttattgtctcataatgattacacatgaaaaagaatggaagagtatgacctgcaatttcat
     Y  C  L  I  M  I  T  H  E  K  E  W  K  S  M  T  C  N  F  I

541 agcacctgtcgtgtgcaagttctagctgcctgaagatccagctgtgtgaactctggaga
     A  P  V  V  C  K  F  *

601 agcaaagaagccccccacccacccccacctgccgccttctctgctctgccccttcgctc

661 aatggatgctctctgtagccgggatccggttttgccgctcctgatgggtcaggaggtcca

721 ataaattctgcctagcaaaaaaaaaaaaaaaaa
```

Figure 12 cDNA sequence of RVV-XL (LC1). Amino acid residues of signal peptide are shown in underlined capital letters. One hundred and twenty three amino acid residues of mature peptide are shown in bold capital letters. An asterisk (*) indicates stop codon.

The full-length sequences of RVV-XH and RVV-XL cloned into pTrcHisα-A vector were obtained from our previous study. The transformed *E. coli* BL21 were cultured in LB broth. After the protein induction by 0.5 M or 1 M of IPTG, culture media containing *E. coli* cells were collected every 3, 5, and 24 hr to investigate the optimal incubation time for protein expression. The same intensity of rRVV-XH protein band at about 59 kDa was observed in both IPTG concentrations and incubation times (Figure 13). For rRVV-XL protein expression, the greater intensity of protein band at about 18 kDa was found when transformed *E. coli* BL21 induced with 0.5 mM of IPTG for 3 hr (Figure 14). The expression of rRVV-XH and rRVV-XL in *E. coli* BL21 were then activated with 0.5 mM IPTG for 3 hr. After purification of these recombinant proteins using IMAC, rRVV-XH and rRVV-XL were expressed with approximately 0.300 mg/L culture and 0.250 mg/L culture, respectively (Figure 15). For the determination of

binding of horse anti-cRVV IgG antibody on recombinant proteins by Western blot analysis, horse anti-cRVV IgG antibody was used as the primary antibody. Horse anti-cRVV IgG antibody was unable to bind to both recombinant proteins (data not shown).

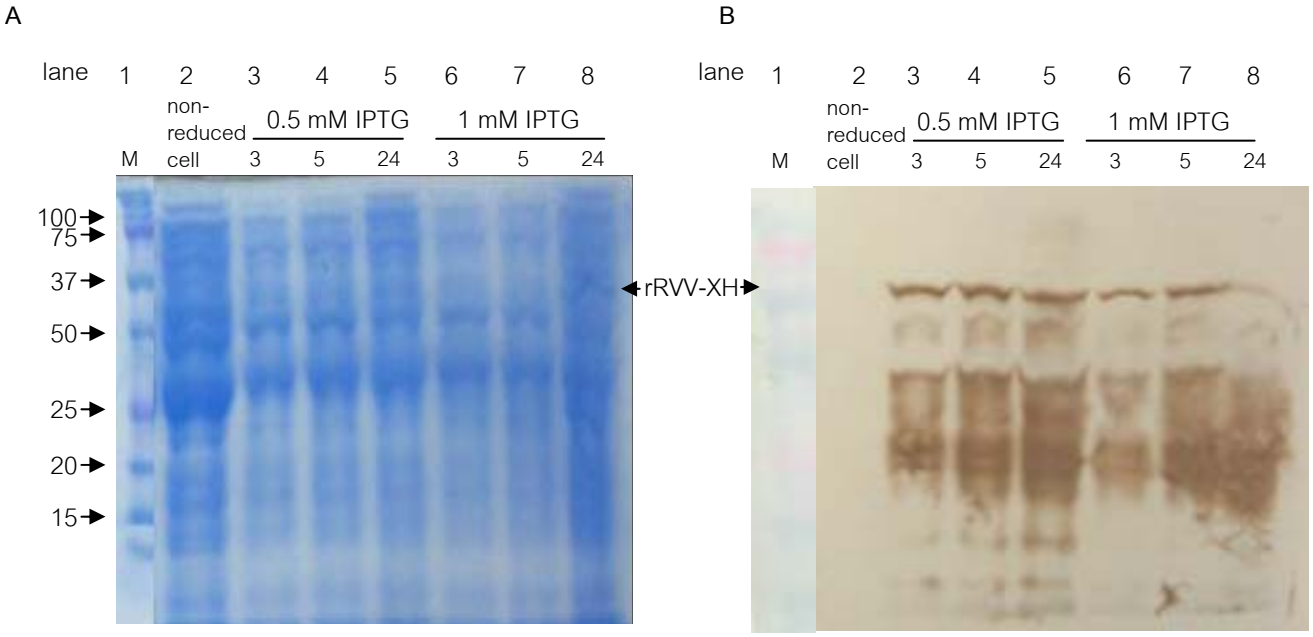

Figure 13 Optimization of IPTG concentration and incubation time for rRVV-XH protein expression. (A) SDS-PAGE analysis of expressed rRVV-XH protein. Samples were run on 12% Bis-Tris gel under reducing at 20 mA (constant). Gel was stained with Coomassie blue G250 and destained. (B) Western blotting developed with anti-His (Amersham Pharmacia Biotech AB, Sweden). Lane 1: Precision Plus Protein™ standards (M, dual color, Bio-Rad Laboratories); lane 2: non-induced cells. Cells were induced with 0.5 mM IPTG for 3, 5, or 24 hr (lane 3-5, respectively). Cells were induced with 1 mM IPTG for 3, 5, or 24 hr (lane 6-8, respectively).

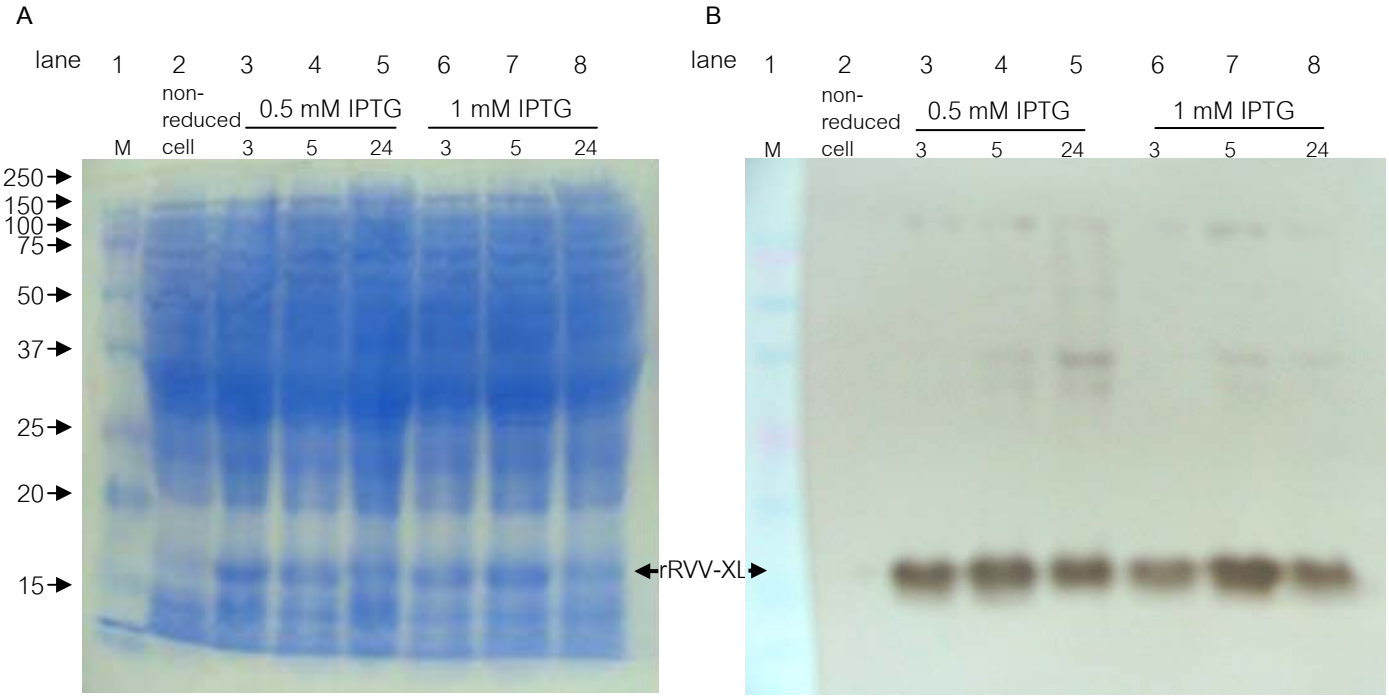

Figure 14 Optimization of IPTG concentration and incubation time for rRVV-XL protein expression. (A) SDS-PAGE analysis of expressed rRVV-XL protein. Samples were run on 12% Bis-Tris gel under reducing at 20 mA (constant). Gel was stained with Coomassie blue G250 and destained. (B) Western blotting developed with anti-His (Amersham Pharmacia Biotech AB, Sweden). Lane 1: Precision Plus Protein™ standards (dual color, Bio-Rad Laboratories); lane 2: non-induced cells. Cells were induced with 0.5 mM IPTG for 3, 5, or 24 hr (lane 3-5, respectively). Cells were induced with 1 mM IPTG for 3, 5, or 24 hr (lane 6-8, respectively).

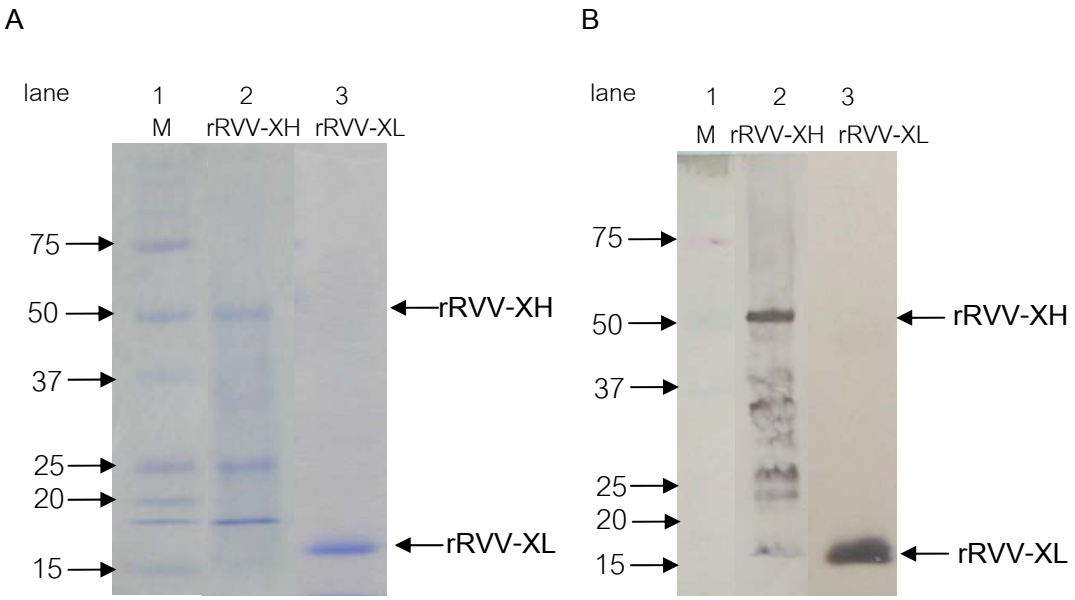

Figure 15 The expression of rRVV-XH and rRVV-XL proteins. (A) SDS-PAGE analysis of expressed recombinant protein. Samples were run on 12% Bis-Tris gel under reducing at 20 mA (constant). Gel was stained with Coomassie blue G250 and destained. (B) Western blotting developed with anti-His (Amersham Pharmacia Biotech AB, Sweden). Lane 1: Precision Plus Protein™ standards (dual color, Bio-Rad Laboratories); lane 2: rRVV-XH; lane 3: rRVV-XL.

2. Coagulation activity of recombinant proteins using APTT assay

rRVV-XH and rRVV-XL proteins were tested for coagulation activity using APTT assay. The average clotting time for normal pooled plasma reconstituted with $CaCl_2$ (normal plasma negative control) was 117.7 ± 2.1 sec (mean ± SD, n = 3). Activation with 2 μg of cRVV significantly shorten the clotting time to 35.6 ± 1.2 sec ($P = 4.96 \times 10^{-7}$). The average clotting time for factor X-deficient plasma reconstituted with $CaCl_2$ (Factor X deficient plasma negative control) was 302.7 ± 6.4 sec (mean ± SD, n = 3). Activation with cRVV significantly shorten the clotting time to 246.7 ± 7.6 sec ($P = 6.28 \times 10^{-4}$). However, there was no significant difference in clotting time between the negative control and rRVV-XH and rRVV-XL (Bold letter, Table 8).

Table 8 Coagulation activity of recombinant proteins using APTT assay

Plasma	Clotting time (sec)				
	cRVV (2 μg)	rRVV-XH (205 μg)	rRVV-XL (91 μg)	Positive control[a]	Negative control[b]
Normal plasma	35.6±1.2*	**112.3±2.5**	**111±6.1**	30.2±1.8	117.7±2.1
Factor X-deficient plasma	246.7±7.6*	-[c]	-[c]	99±2.6	302.7±6.4

Data represent the mean ± SD (n = 3).

Bold letter indicates no significant shortening of clotting time when compared with the negative control ($P > 0.05$). Their significances were analyzed by the Student's t-test.

[a]The positive control was normal citrated or factor X deficient human plasma activated with cephalin and 0.025 M $CaCl_2$.

[b]The negative control was normal citrated or factor X deficient human plasma reconstituted with 0.025 M $CaCl_2$.

[c]Pooled normal citrated plasma did not form clot.

An asterisk (*) indicates statistic significance compared with the purified RVV-X control at $P < 0.05$. Their significance was analyzed by the Student's t-test.

3. Production of rabbit anti-recombinant proteins antibodies

3.1 Quantitation of rabbit anti-recombinant proteins antibodies

The amount of rabbit anti-rRVV-XH and anti-rRVV-XL antibodies was determined by the ELISA (Figure 16). The highest specific antibody against purified RVV-X was observed in rabbits (7H346 and 7H358), which were immunized with rRVV-XH antigen. Animals immunized with rRVV-XL had a much lower ELISA titer.

3.2 Purification of rabbit anti-recombinant proteins IgG antibodies

Rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies were purified from serum of immunized rabbits by a protein G affinity column with approximately 4% and 5% yield, respectively (Table 9). The antibody titer of rabbit anti-rRVV-XH IgG antibody was approximately 5 times higher than that of rabbit anti-rRVV-XL IgG antibody (Table 9). Purified rabbit anti-rRVV-XH- and anti-rRVV-XL antibodies showed a broad band at about 150 kDa using SDS-PAGE under non-reducing conditions (Figure 17).

3.3 Binding of rabbit anti-recombinant proteins IgG antibodies to purified RVV-X using Western blot analysis

Purified RVV-X with molecular mass of 90 kDa from crude venom was recognized by both of rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies using Western blot analysis (Figure 18). Rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies were unable to bind to bovine serum albumin (BSA) and normal mouse serum.

3.4 Effects of rabbit anti-recombinant proteins IgG antibodies in neutralizing factor X activator activity of purified RVV-X using factor Xa-specific chromogenic substrate S-2765.

Rabbit anti-rRVV-XH antibody, anti-rRVV-XL IgG antibody, or the mixture of rabbit anti-rRVV-XH and anti-rRVV-XL antibodies were unable to neutralize factor X activator activity of purified RVV-X using factor Xa-specific chromogenic substrate S-2765 (Figure 19). Only commercial horse anti-cRVV $F(ab')_2$ antivenom at the concentration of 31.25 μg/ml completely neutralized factor X activator activity of a 0.125 μg/ml of purified RVV-X.

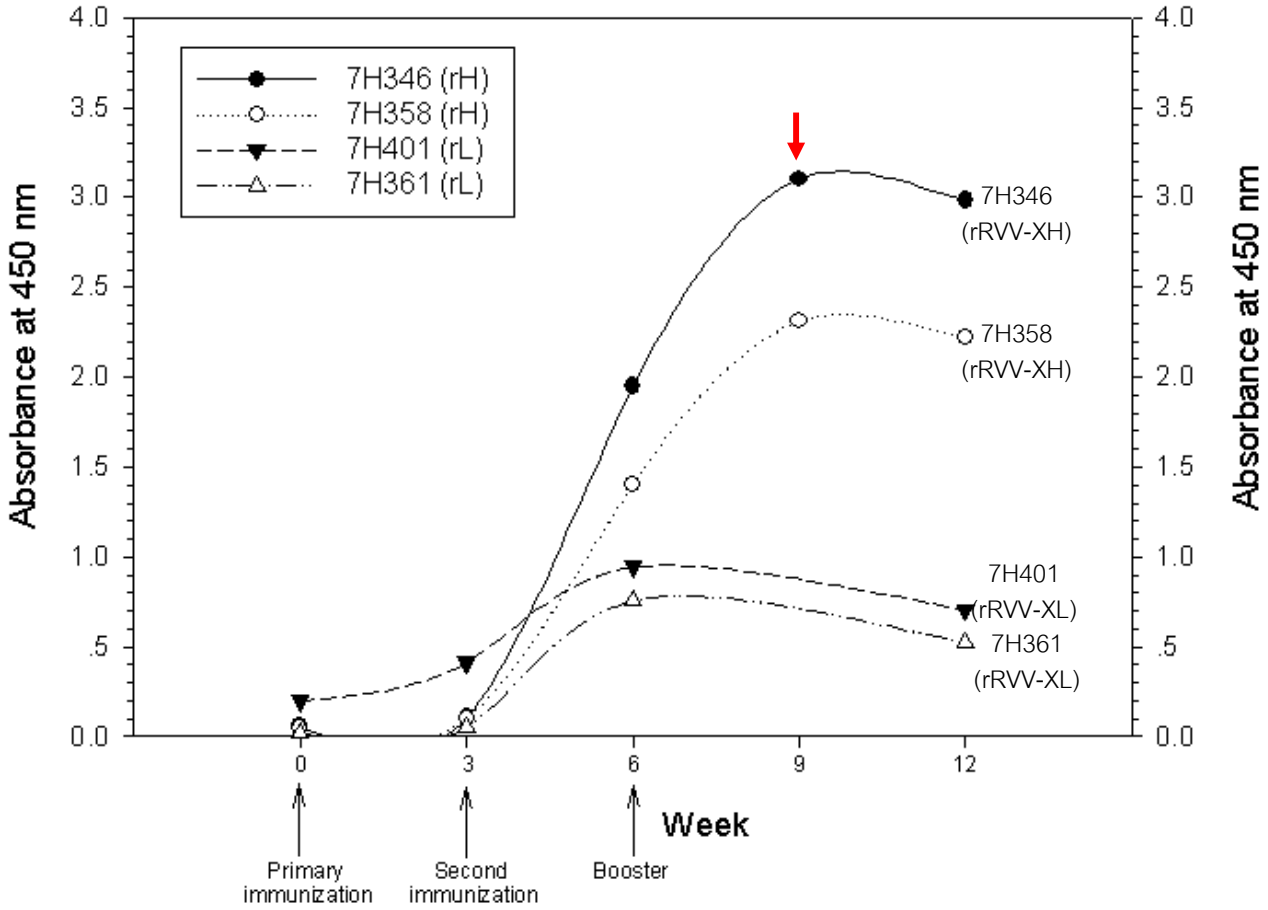

Figure 16 Determination of the specific antibody activity using the ELISA after immunization of two rabbits each with rRVV-XH and rRVV-XL. Arrow indicates the highest specific antibody activity.

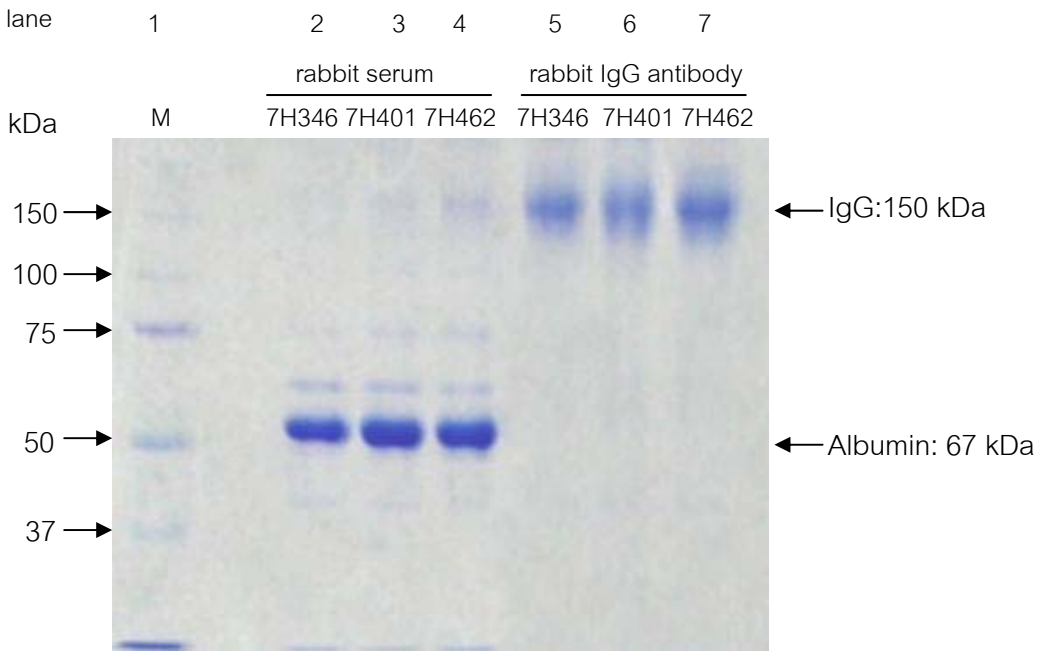

Figure 17 SDS-PAGE analysis of purified recombinant proteins-specific IgG antibody from rabbit anti-recombinant proteins serum. Samples (5 μg) were run on 8% Bis-Tris gel under non-reducing conditions at 20 mA (constant). Gel was stained with Coomassie blue G250 and destained. Lane 1: Precision Plus Protein™ standards (dual color, Bio-Rad Laboratories); lane 2: rabbit anti-rRVV-XH serum (7H346); lane 3: rabbit anti-rRVV-XL serum (7H401); lane 4: rabbit normal serum (7H462); lane 5: rabbit anti-rRVV-XH IgG antibody (7H346); lane 6: rabbit anti-rRVV-XL IgG antibody (7H401); lane 7: rabbit normal IgG antibody.

Table 9 Purification of rabbit anti-recombinant proteins IgG antibodies from rabbit anti-recombinant proteins serum

Sample	Volume (ml)	Protein concentration (mg/ml)[a]	Total protein (mg)[b]	Total activity (U)[c]	Specific antibody activity (U/mg)[d]	Recovery of protein (%)[e]	Purification (fold)[f]
Rabbit anti-rRVV-XH serum	10	59.34	593.40	6200	10.45	100	1.00
Rabbit anti-rRVV-XH IgG antibody	5.64	4.19	23.63	10000	423.16	3.98	40.50
Rabbit anti-rRVV-XL serum	10	51.06	510.60	1500	2.94	100	1.00
Rabbit anti-rRVV-XL IgG antibody	6.36	4.14	26.33	2300	87.35	5.16	29.73

[a]Protein concentration was determined by BCA method.

[b]The total protein (mg) was calculated by multiplying (total volume; ml) x (venom protein concentration; mg/ml).

[c]The highest dilution giving an absorbance reading of 2 was regarded as the end point titer. Total activity (U) was calculated by dividing protein concentration (mg/ml) by the end point titer concentration (mg/ml).

[d]Specific activity (U/mg) was calculated by total activity (U) by total protein (mg) in each sample.

[e]Recovery of protein was defined as the total protein recovered of at each step of purification.

[f]Purification factor was the number of times that specific antibody activity increased over crude rabbit serum immunized with recombinant proteins.

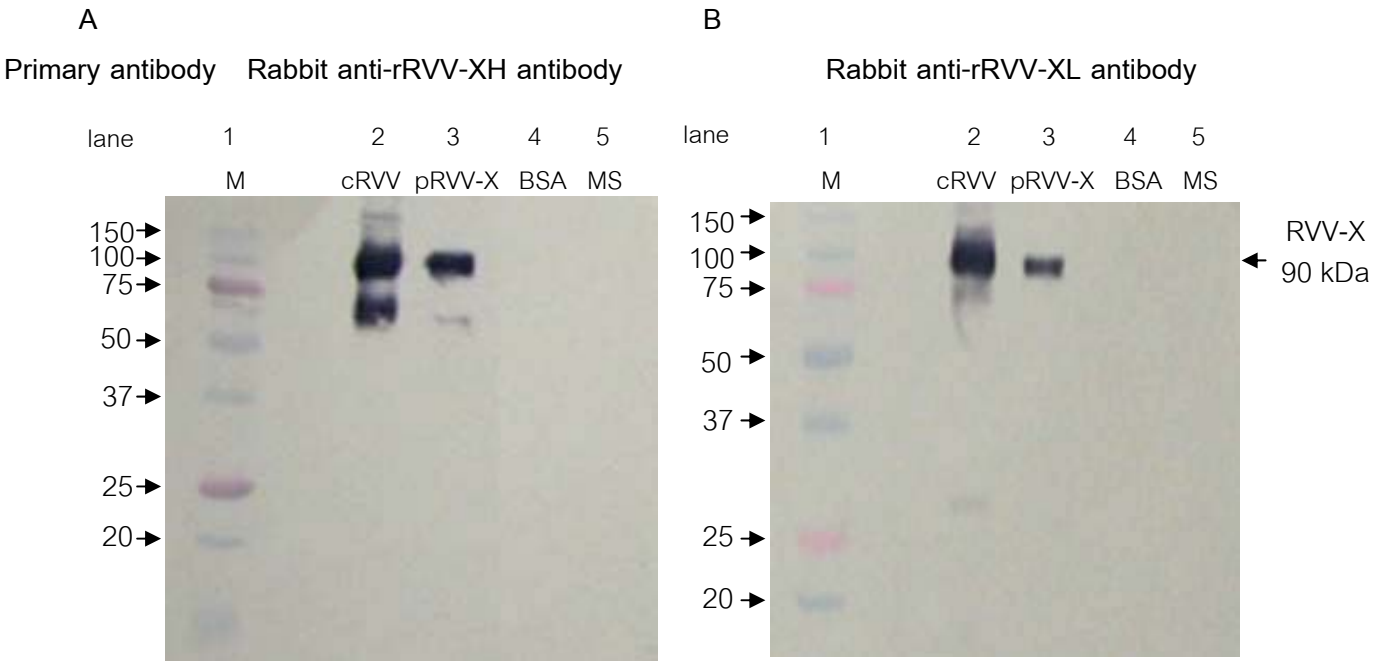

Figure 18 Binding of rabbit anti-recombinant proteins IgG antibodies to purified RVV-X using Western blot analysis. (A) Western blotting developed with a 1:1,500 dilution of rabbit anti-rRVV-XH IgG antibody. (B) Western blotting developed with a 1:1,000 dilution of rabbit anti-rRVV-XL IgG antibody. Lane 1: Precision Plus Protein™ standards (dual color, Bio-Rad Laboratories); lane 2: cRVV (5 μg); lane 3: purified RVV-X (pRVV-X; 250 ng); lane 4: bovine serum albumin (BSA; 2 μg); lane 5: normal mouse serum (MS, 5 μg).

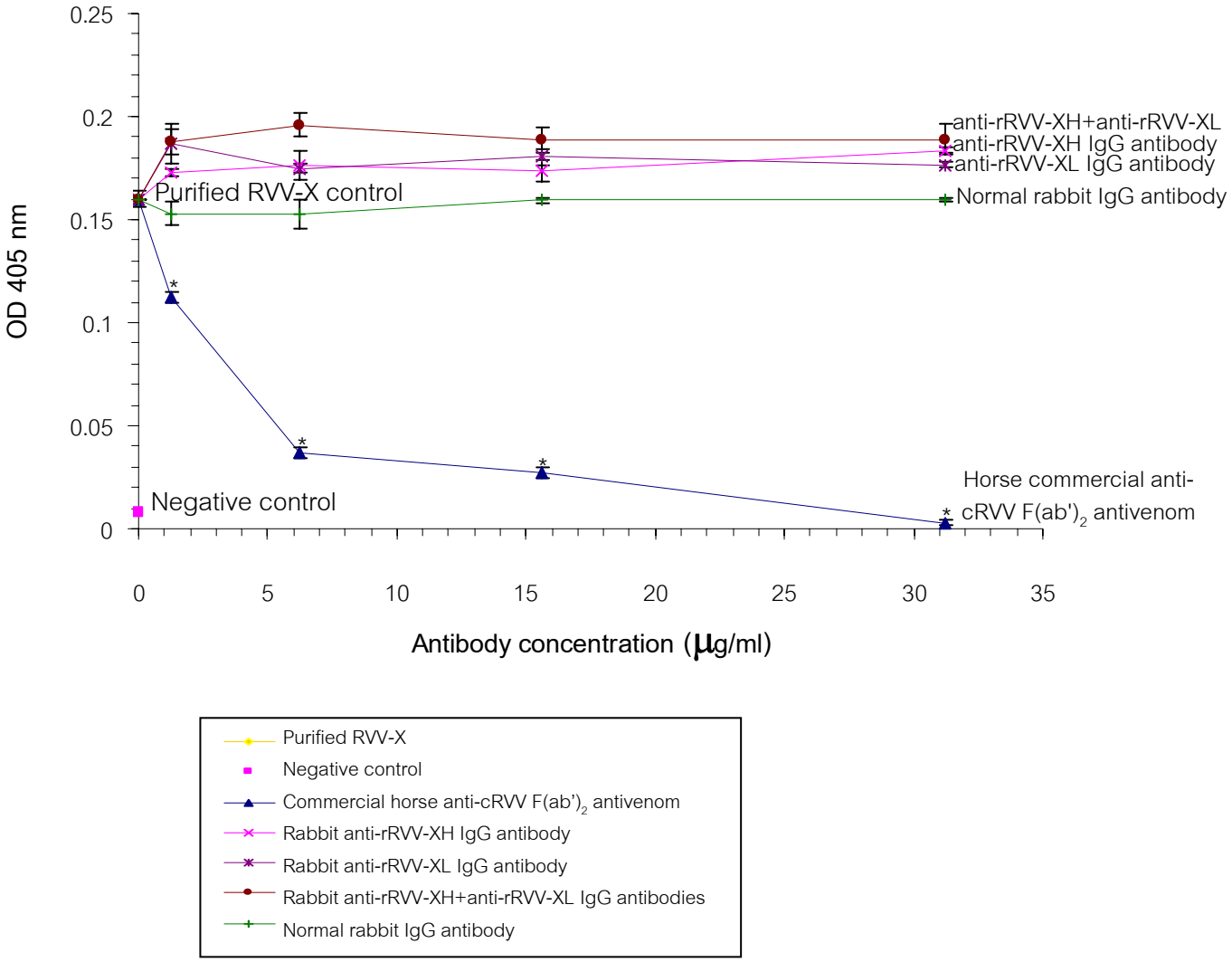

Figure 19 Effects of rabbit anti-recombinant proteins IgG antibodies in neutralizing factor X activator activity of purified RVV-X using factor Xa-specific chromogenic substrate S-2765. A factor Xa-specific chromogenic substrate S-2765 was used to measure the decrease in factor X activator activity of purified RVV-X. The negative control was normal citrated human plasma reconstituted with only $CaCl_2$. Venom control was normal citrated plasma activated with purified RVV-X and 0.025 M $CaCl_2$. Antibody control was normal citrated plasma activated with purified RVV-X pre-mixed with commercial horse anti-cRVV $F(ab')_2$ antivenom (QSMI, Thailand) and 0.025 M $CaCl_2$. The mean and SD was calculated (n = 3). An asterisk (*) indicates statistic significance compared with the purified RVV-X control at $P < 0.05$. Their significance was analyzed by the Student's t-test.

4. Cross-reactivity of rabbit anti-rRVV-XH and anti-rRVV-XL antibodies against *C. albolabris* and *C. rhodostoma* venoms using Western blot analysis

Purified RVV-X with molecular mass of 90 kDa from *D. r. siamensis* venom was recognized by both of rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies using Western blot analysis (Figure 20). In addition, rabbit anti-rRVV-XH IgG antibody was able to bind to a 18 kDa protein band of *C. rhodostoma* venom, and different protein bands with a molecular mass range between 28-70 kDa of *C. albolabris* venom. Rabbit anti-rRVV-XL IgG antibody recognized protein bands of crude venoms of *C. rhodostoma* and *C. albolabris* at about 25 kDa and 23 kDa, respectively.

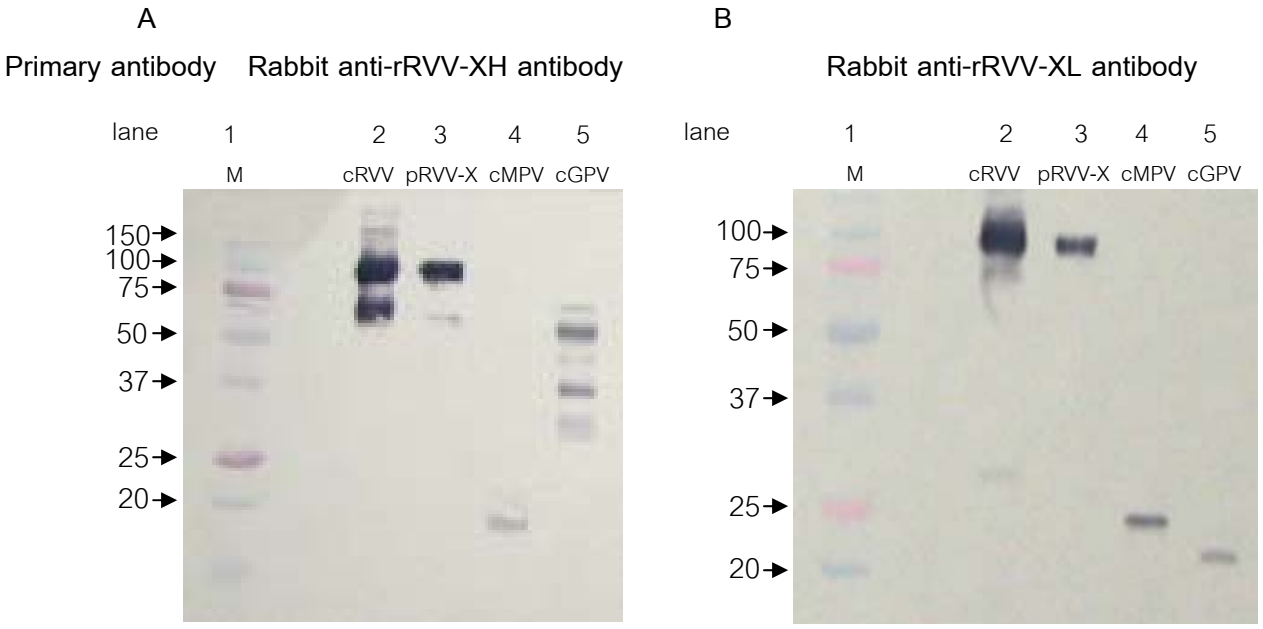

Figure 20 Cross-reactivity of rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies against *C. rhodostoma* and *C. albolabris* venoms using Western blot analysis. (A) Western blotting developed with a 1:1,500 dilution of rabbit anti-rRVV-XH IgG antibody. (B) Western blotting developed with a 1:1,000 dilution of rabbit anti-rRVV-XL IgG antibody. Lane 1: Precision Plus Protein™ standards (dual color, Bio-Rad Laboratories); lane 2: cRVV (5 μg); lane 3: purified RVV-X (pRVV-X; 250 ng); lane 4: *C. rhodostoma* venom (cMPV; 5 μg); lane 5: *C. albolabris* venom (cGPV; 5 μg).

Part II Effects of purified RVV-X and cRVV on DIC, renal hemodynamic and function, and histopathological changes in SD rats.

1. Purification of horse anti-cRVV IgG antibody

Horse anti-cRVV IgG antibody was purified from horse anti-cRVV serum by a two-step procedure with approximately 32% yield (Table 10).

This antibody was used in a Western blot assay to determine the purity of purified RVV-X. Gamma globulin rich precipitated fraction from horse anti-cRVV serum was obtained from AS precipitation followed by gel filtration with a Sephadex G-200 column. The chromatographic profile is shown in Figure 21. The second peak (PII) had antibody activity (dashed line, Figure 21) as determined in an ELISA. The second peak (fractions no. 62-74) was pooled and 0.5 ml was aliquoted into 1.5 ml plastic tubes, and frozen at -20°C until used.

Purified horse anti-cRVV IgG antibody had a broad band at about 150 kDa using SDS-PAGE under non-reducing conditions (Figure 22). It had the antibody titer of 8,000 (Figure 23) and had a threefold increase in specific antibody activity (Table 9).

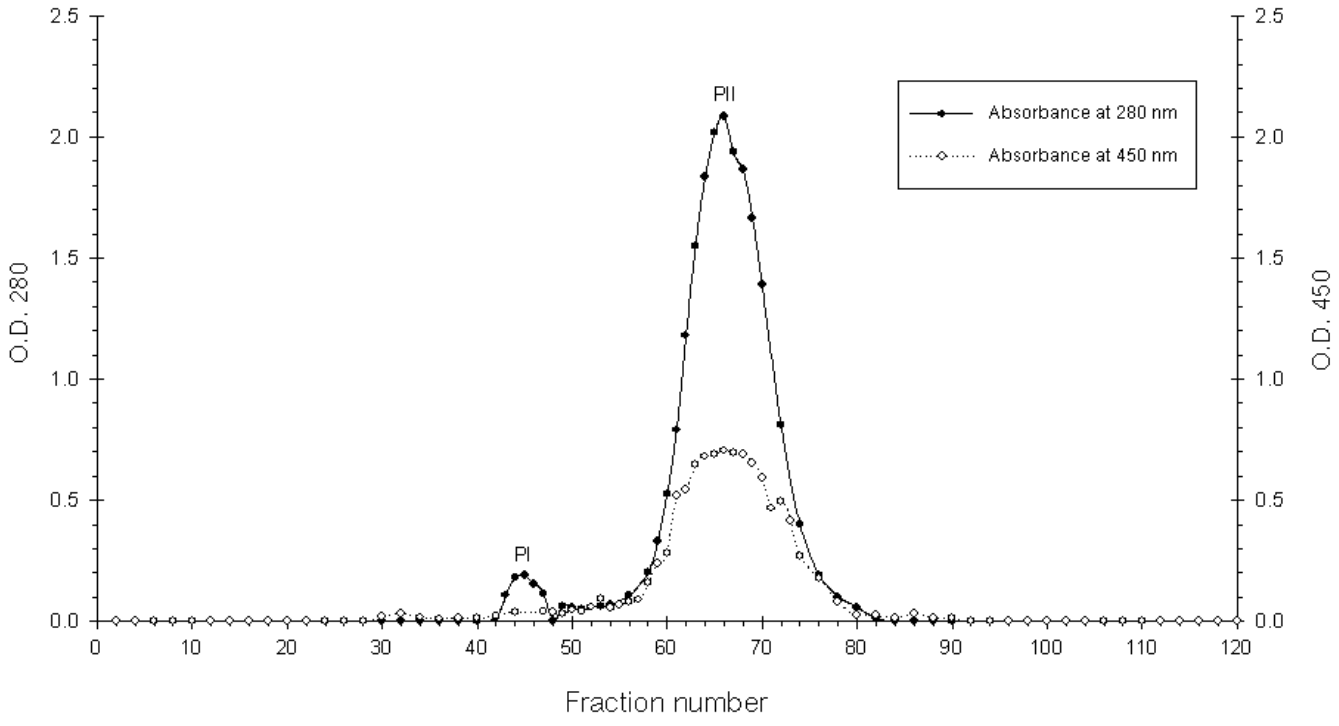

Figure 21 Gel filtration chromatographic profile of AS precipitated horse anti-cRVV antibody on Sephadex G-200 column (2.6 x 65 cm). The flow rate was 15 ml/h using 0.1 M NaCl containing 0.1% NaN_3. Fractions of 3 ml were collected. Solid line indicates absorbance at 280 nm. Antibody activity was expressed as the absorbance at 450 nm in ELISA (dashed line).

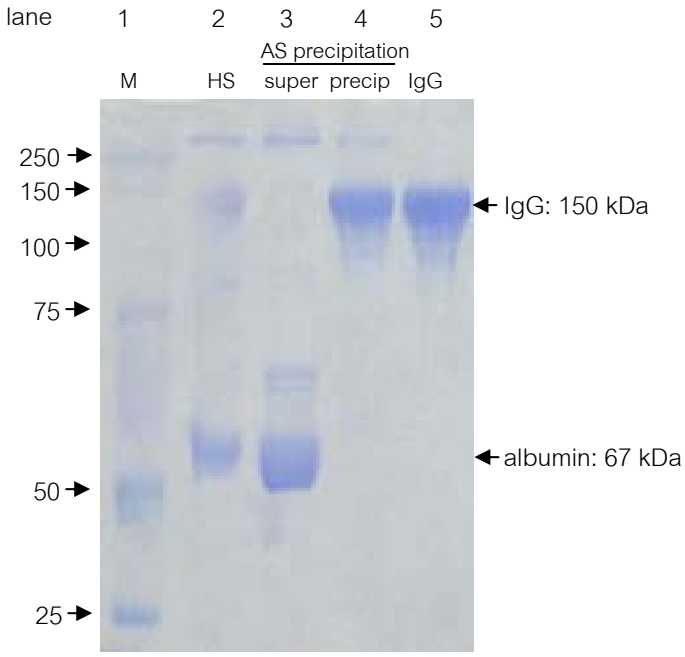

Figure 22 SDS-PAGE analysis of horse anti-cRVV IgG antibody from two purification steps. Molecular weight markers and samples (10 μg) from each purification step were run on 8% Bis-Tris gel under non-reducing conditions at 20 mA (constant). Gel was stained with Coomassie blue R250 and destained. Lane 1: Precision Plus Protein™ standards (dual color, Bio-Rad Laboratories); lane 2: crude Horse anti-cRVV serum (HS); lane 3: supernatant (super) after ammonium sulfate (AS) precipitation; lane 4: precipitate (precip) after AS precipitation; lane 5: horse anti-cRVV IgG antibody (IgG) from Sephadex G-200 column.

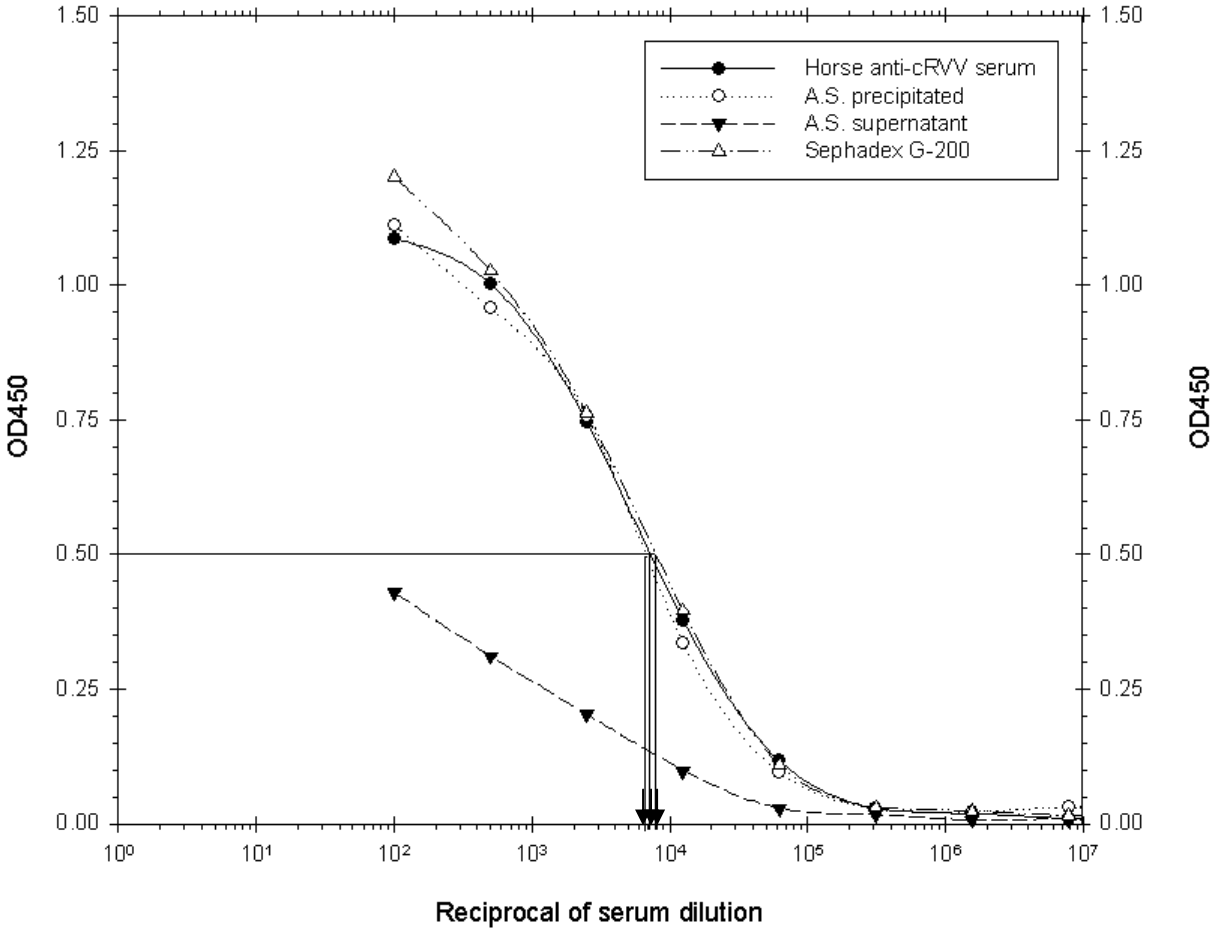

Figure 23 ELISA activity of each sample from two purification steps. The highest dilution giving an absorbance reading of 0.5 was regarded as the end point titer (arrow).

Table 10 Purification of horse anti-cRVV IgG antibody from horse anti-cRVV serum

Sample	Volume (ml)	Protein concentration (mg/ml)[a]	Total protein (mg)[b]	Total activity (U)[c]	Specific activity (U/mg)[d]	Recovery of protein (%)[e]	Purification (fold)[f]
Horse anti-cRVV serum	20	107.16	2,143.20	7,500	3.50	-	1.00
AS precipitate	27.6	41.7	1,150.92	7,000	6.08	53.70	1.74
AS supernatant	79.5	8.44	670.98	0.00	0.00	31.31	0.001
Sephadex G-200	14.5	47.7	691.65	8,000	11.57	32.27	3.31

[a]Protein concentration was determined by BCA method.

[b]The total protein (mg) was calculated by multiplying (total volume; ml) x (venom protein concentration; mg/ml).

[c]The highest dilution giving an absorbance reading of 0.5 was regarded as the end point titer. Total activity (U) was calculated by dividing protein concentration (mg/ml) by the end point titer concentration (mg/ml).

[d]Specific activity (U/mg) was calculated by total activity (U) by total protein (mg) in each sample.

[e]Recovery of protein was defined as the total protein recovered of at each step of purification.

[f]Purification factor was the number of times that specific antibody activity increased over crude horse serum immunized with cRVV.

2. Purification of RVV-X

RVV-X was purified from cRVV by a two-step procedure with approximately 5% yield, and had a fourfold increase in specific activity (Table 11). Crude venom was initially fractionated by Sephadex G-100 column. Four different peaks (PI-PIV) were obtained from the gel filtration separation. Factor X activator activity was in peak I (PI) using a chromogenic substrate S-2765 assay (—o—, Figure 24). The pooled fractions from PI were then purified by Q-Sepharose anion exchange chromatography. Two different peaks were observed. The second peak, the fractions from 47-58 (PII), had factor X activator activity using a chromogenic substrate S-2765 assay (—o—, Figure 25).

PII showed a diffuse protein band at approximately 90 kDa using SDS-PAGE staining with Coomassie blue G250. The same peak (PII) using reduced SDS-PAGE had a heavy chain with molecular weight 55 kDa and two light chains with molecular weight about 18 and 16 kDa (Figure 26). The same protein banding patterns were revealed by SDS-PAGE following silver nitrate staining (Figure 27). The molecular weight of purified RVV-X was 90,713 Da, which was verified by Mass spectrometry (Figure 28).

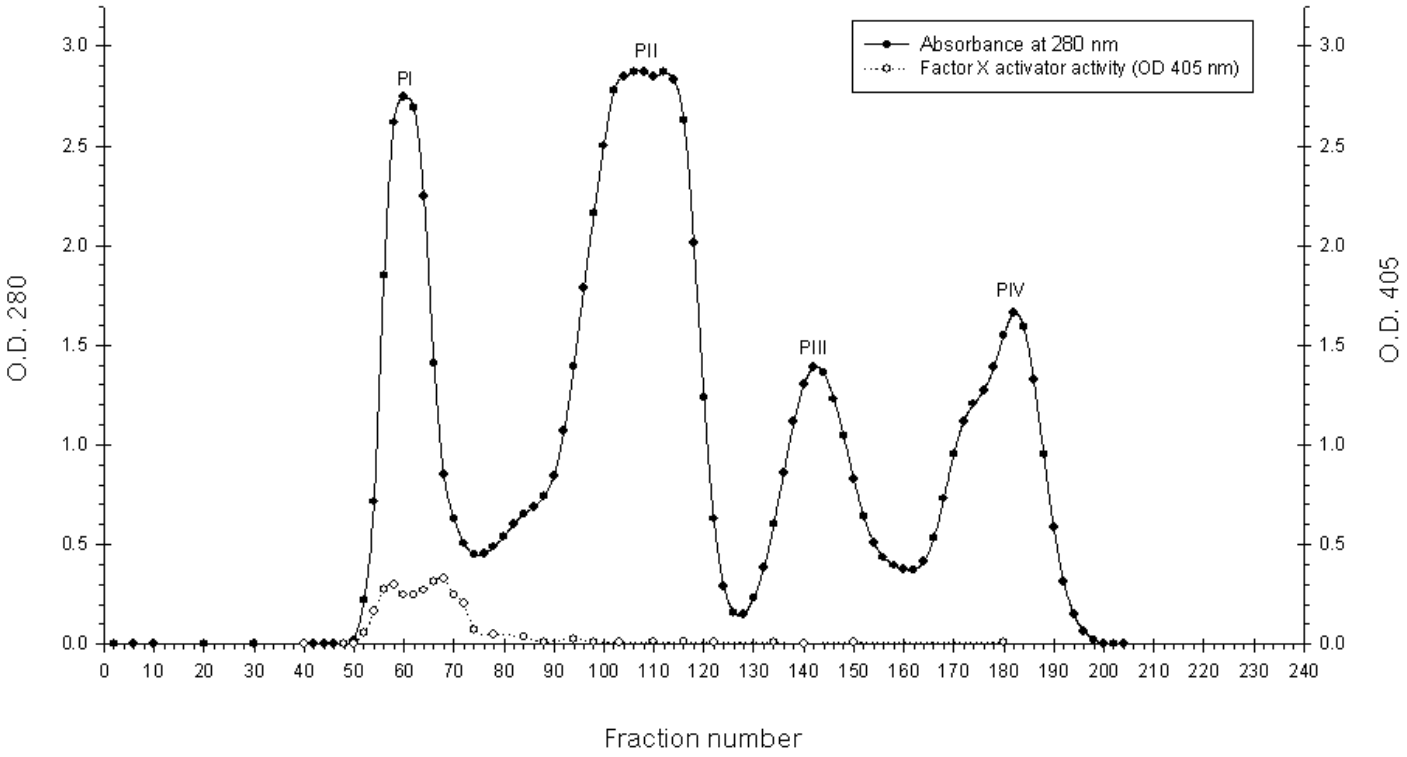

Figure 24 Gel filtration (GF) chromatographic profile of cRVV on Sephadex G-100 column (2.6 x 65 cm). The flow rate was 10 ml/h using 0.05 M Tris-phosphate buffer, pH 7.5 containing 1 mM benzamidine-HCl. Fractions of 2 ml were collected. The absorbance of the fractions was monitored at 280 nm using a UV2100 (UV-VIS recording spectrophotometer, SHIMADZU) as shown in the figure (—●—). Factor X activator activity using chromogenic substrate S-2765 is expressed as the absorbance at 405 nm (—o—).

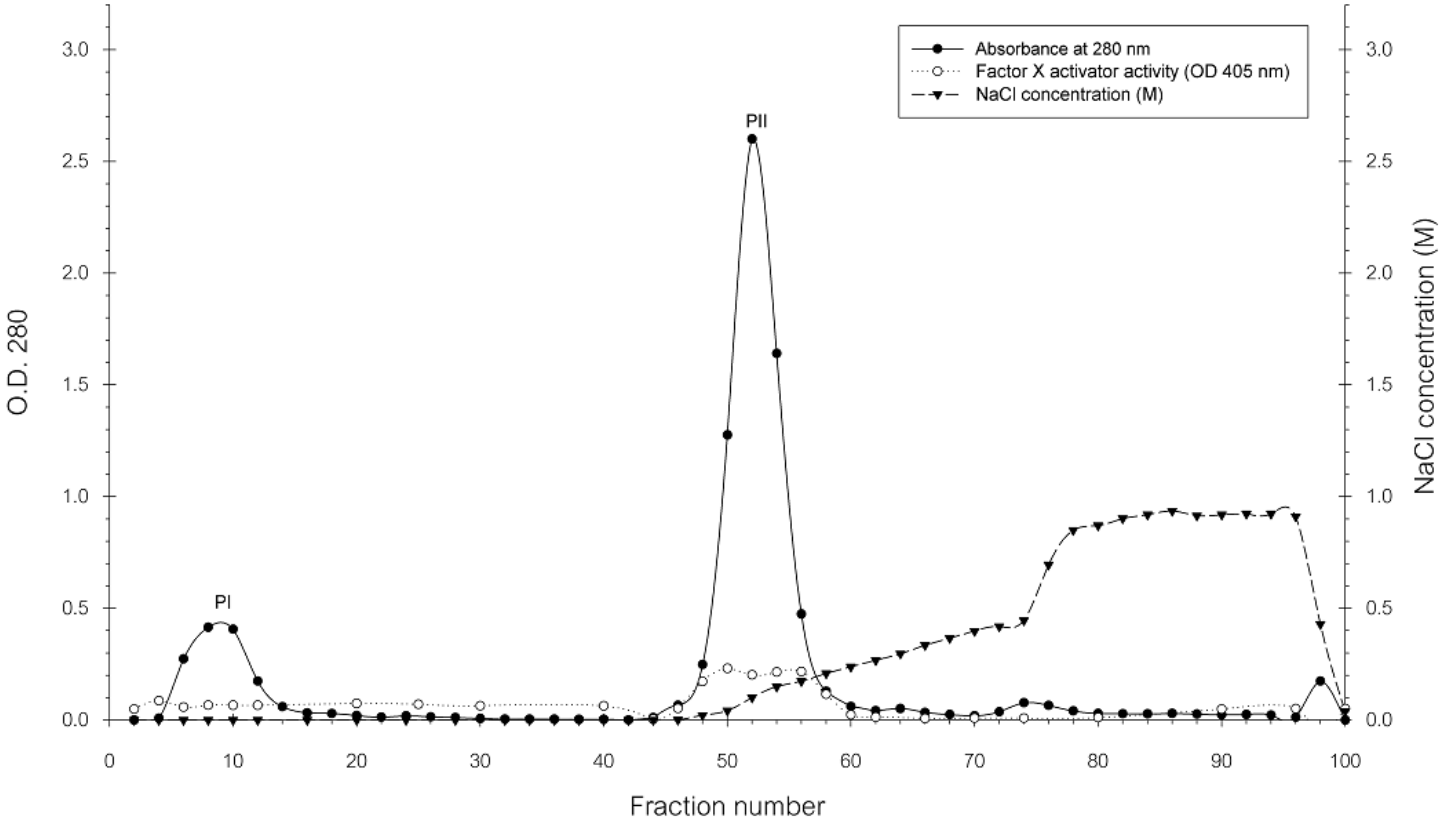

Figure 25 Q-Sepharose anion exchange chromatographic profile from peak I from Sephadex G-100 chromatography column. The fractions were eluted with a 0-0.5 M NaCl salt gradient. One milliliter per tube was collected at the flow rate of 1 ml/min. The absorbance of the fractions was monitored at 280 nm using a UV2100 (UV-VIS recording spectrophotometer, SHIMADZU) as shown in the figure (—●—). Factor X activator activity using chromogenic substrate S-2765 is expressed as the absorbance at 405 nm (—o—). NaCl concentration indicates as (—▼—)

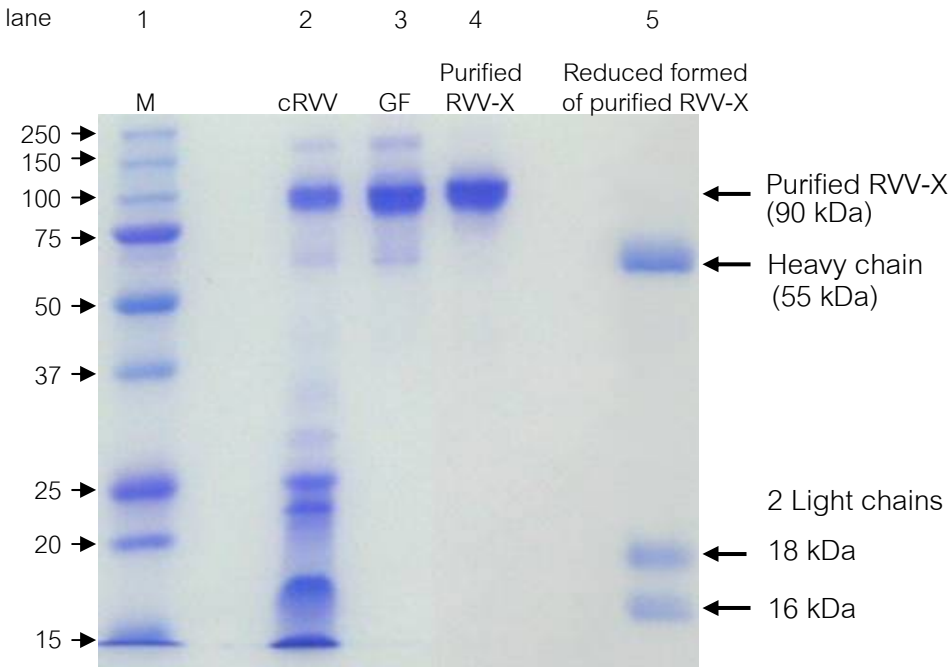

Figure 26 SDS-PAGE analysis of purified RVV-X from purification step. Sample from each purification step were run on 12% Bis-Tris gel under non-reducing and reducing conditions at 20 mA (constant). Gel was stained with Coomassie blue G250 and destained. Lane 1: Precision Plus Protein™ standards (dual color, Bio-Rad Laboratories); lane 2: cRVV (15 μg); lane 3: peak 1 (PI) from GF (7 μg); lane 4: Peak II (PII) from Q-Sepharose anion exchange (purified RVV-X; 7 μg); lane 5: reduced form of purified RVV-X (7 μg).

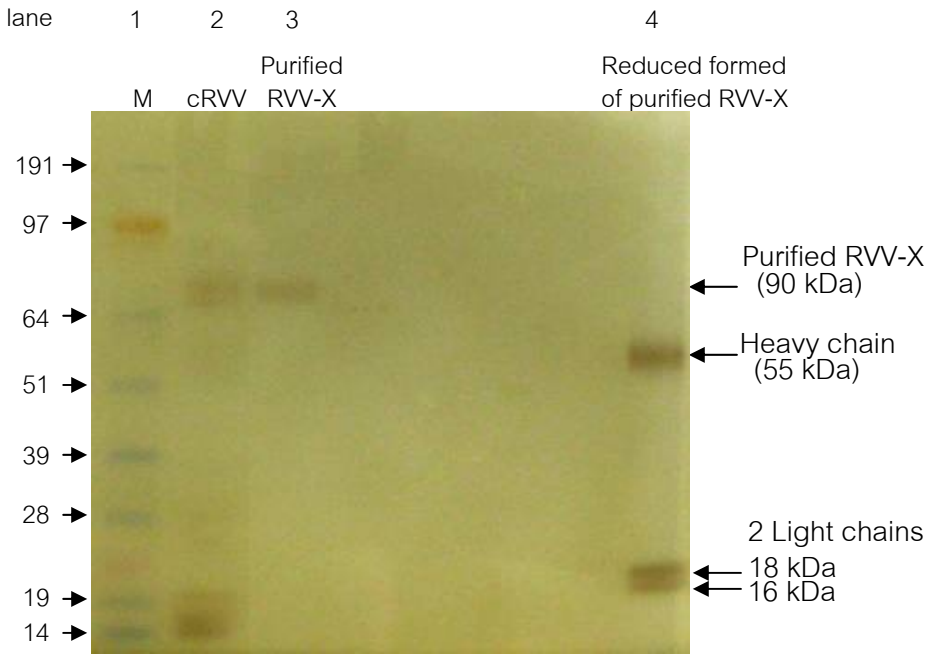

Figure 27 SDS-PAGE of purified RVV-X following silver nitrate stain. cRVV and purified RVV-X were run on 4-12% Bis-Tris NuPAGE gel at 200 V for 50 min. Gel was stained with silver stain. Lane 1: SeeBlue Plus2 Markers (Invitrogen™); lane 2: cRVV (5 μg); lane 3: non-reduced form of purified RVV-X (1 μg); lane 4: reduced form of purified RVV-X (1.5 μg).

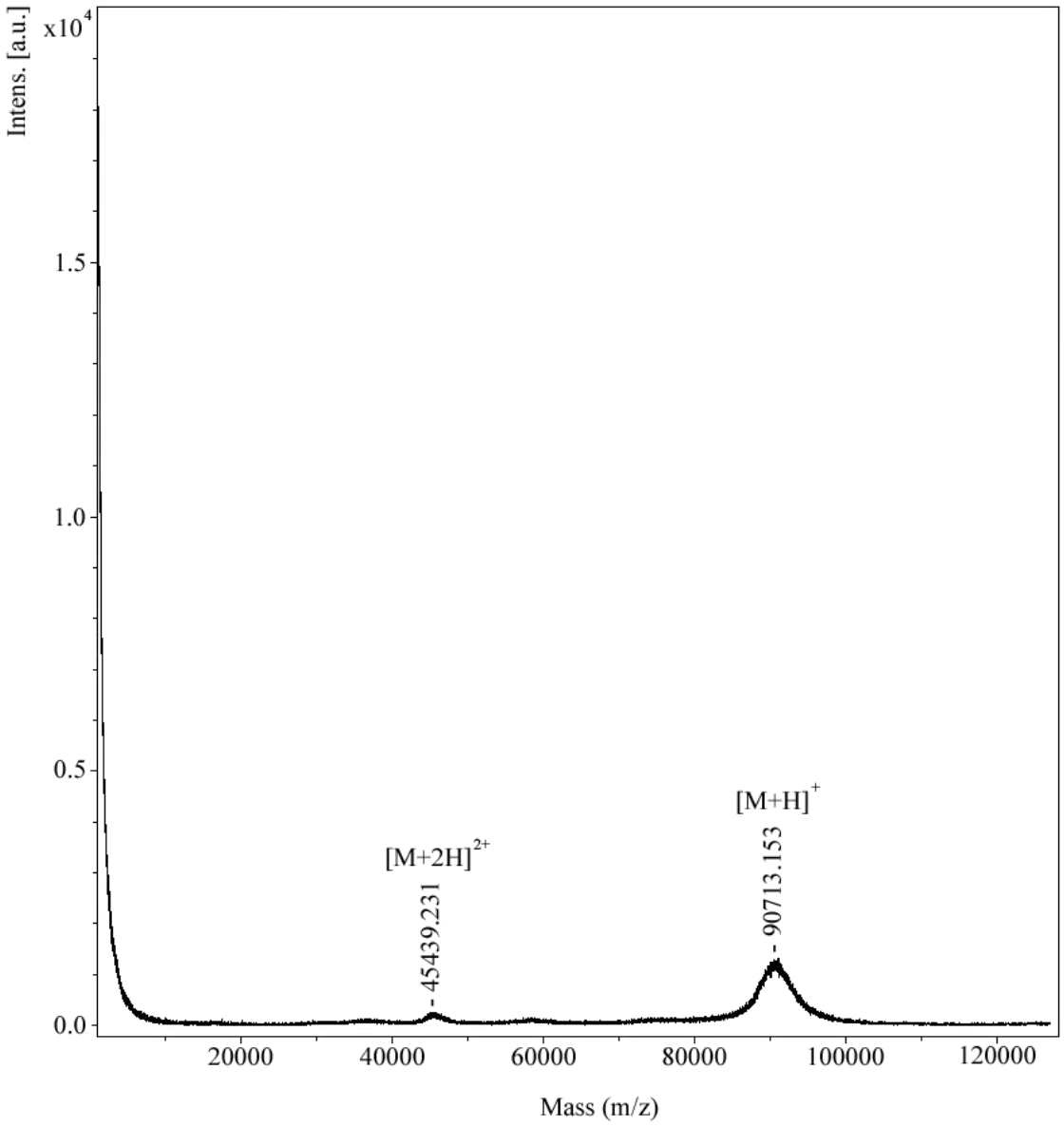

Figure 28 Matrix-assisted laser desorption mass spectrum of purified RVV-X. The measured molecular mass of purified RVV-X was 90,713 Da. The single-charged monomer ($[M+H]^{+}$) and the double-charged monomer ($[M+2H]^{2+}$) are indicated.

Table 11 Purification of RVV-X from crude venom

Purification Step	Volume (ml)	Protein concentration (mg/ml)[a]	Total protein (mg)[b]	Specific activity (nkat/ng)[c]	Recovery of protein (%)[d]	Purification factor (fold)[e]
Crude *D. r. siamensis* venom	4.15	100.33	416.25	0.80	100.00	1.00
Sephadex G-100	38.00	2.16	82.03	1.50	19.71	1.88
Q-sepharose	12.00	1.60	19.20	3.20	4.61	4.00

[a]Protein concentration was determined by BCA method.

[b]The total protein (mg) was calculated by multiplying (total volume; ml) x (venom protein concentration; mg/ml).

[c]One katal (kat) was the amount of enzyme that converts one mole of chromogenic substrate S-2765 per second. One nanokatal (nkat) was 1 x 10^{-9} mole of product released per second. Specific activity was calculated by 1 nkat by total protein (ng) in each sample. One nkat was calculated by the following equation: $[\text{OD 405 nm} / (\varepsilon \times T \times 10^{-9})]$, where the enzyme activity is measured at 405 nm, ε is the extinction coefficient of chromogenic substrate S-2765 (1.27 x 104 mol^{-1}. L. cm^{-1} at Emax = 405 nm), T is reaction time in second.

[d]Recovery of protein was defined as the total protein recovered of at each step of purification.

[e]Purification factor was the number of times that specific factor X activator activity increased over *D. r. siamensis* venom.

3. Purity determination of purified RVV-X by Western blot analysis

For the determination of purified RVV-X purity by Western blot analysis, horse anti-cRVV IgG antibody was used as the primary antibody. Purified RVV-X was showed a single protein band at about 90 kDa on the Coomassie-stained SDS-PAGE gel (Figure 29A) and Western blotting (Figure 29B).

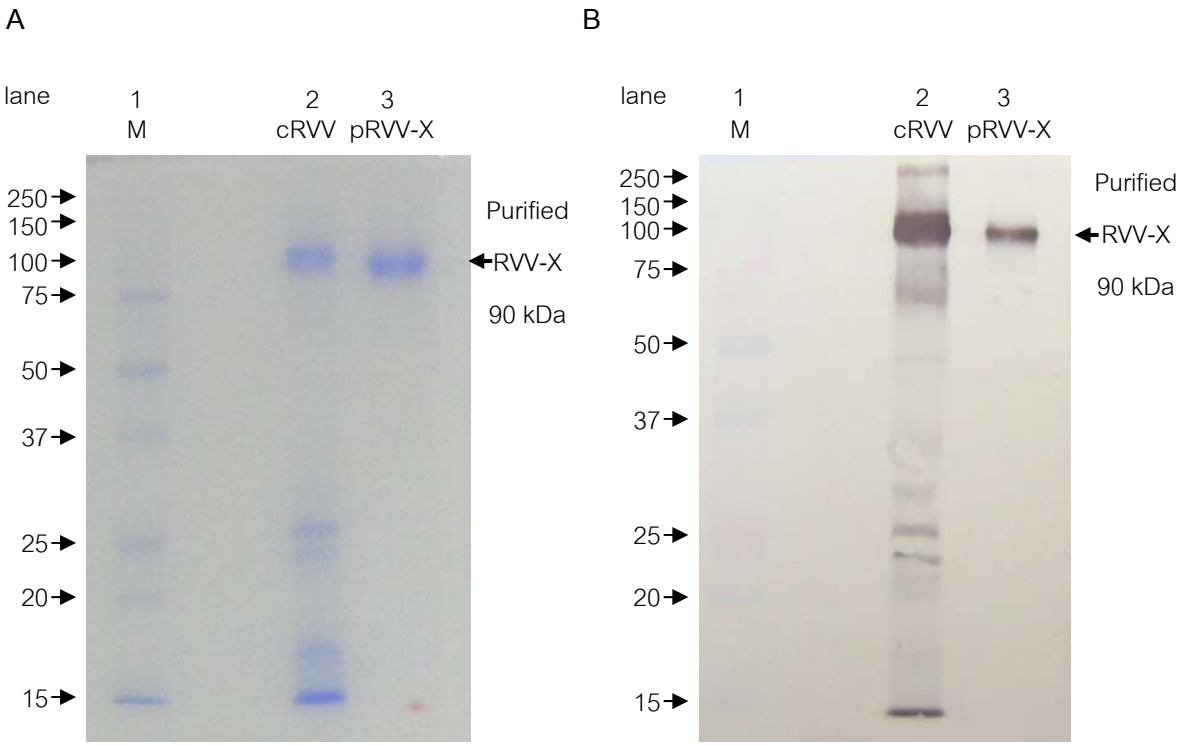

Figure 29 Purity determination of purified RVV-X (pRVV-X) by SDS-PAGE (A) and Western blotting (B). (A) SDS-PAGE analysis of purified RVV-X. Samples were run on 12% Bis-Tris gel under non-reducing conditions at 20 mA (constant). Gel was stained with Coomassie blue G250 and destained. Lane 1: Precision Plus Protein™ standards (dual color, Bio-Rad Laboratories); lane 2: cRVV (15 μg); lane 3: pRVV-X (4 μg). (B) Western blotting developed with horse anti-cRVV IgG antibody. Lane 1: Precision Plus Protein™ standards (dual color, Bio-Rad Laboratories); lane 2: cRVV (5 μg); lane 3: pRVV-X (232 ng).

4. RVV-X activity

4.1 Coagulation activity of purified RVV-X using APTT assay

cRVV and purified RVV-X activities were measured with normal human citrated plasma using APTT assay. Purified RVV-X significantly shorten the clotting time of normal human citrated plasma and factor X deficient plasma in a dose-dependent manner (Figure 30). The coagulation activity of purified RVV-X was compared to crude venom. The clotting time with 3 ng of purified RVV-X was approximately 1.7 times shorter than the clotting time with cRVV.

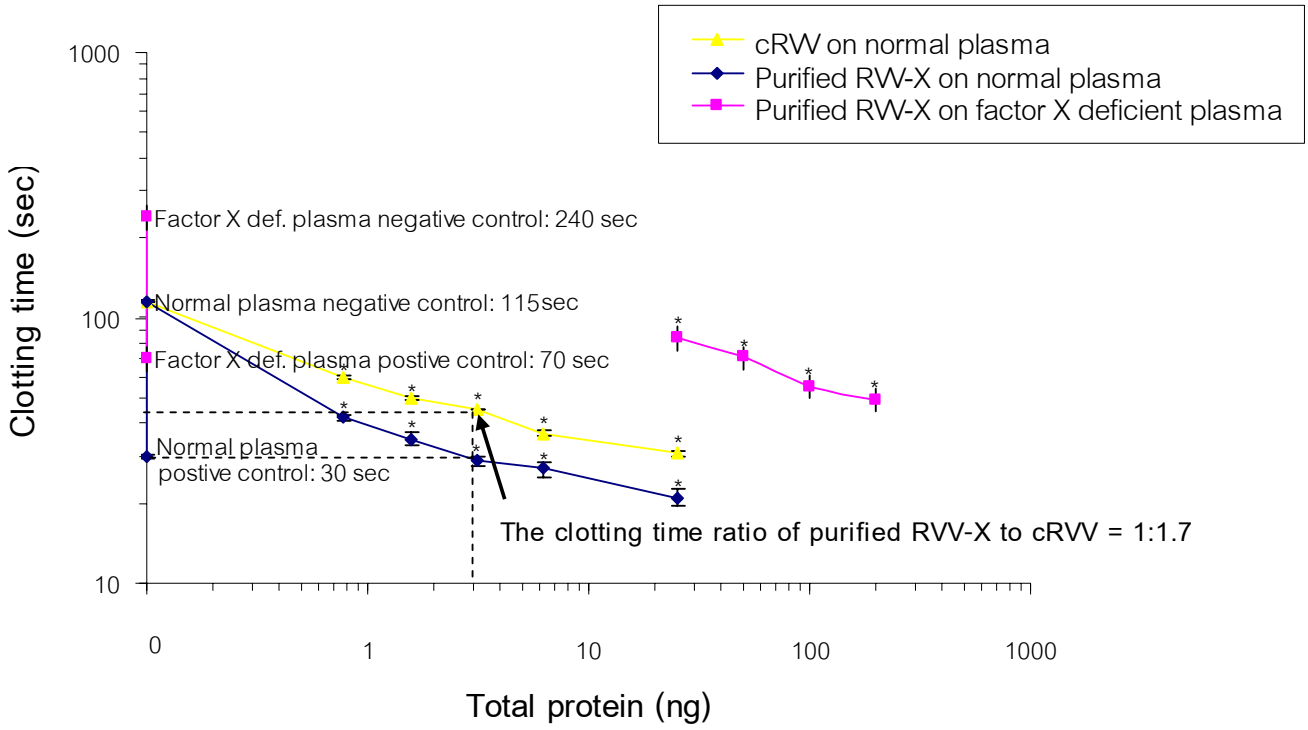

Figure 30 Effect of purified RVV-X and cRVV at various concentrations on clotting time of human normal citrated plasma using APTT assay. Data represent the mean ± SD (n = 3). An asterisk (*) indicates statistic significance compared with the negative control plasma at $P < 0.05$. Their significance was analyzed by the Student's t-test.

4.2 Factor X activator activity

The factor X activator activity of cRVV and purified RVV-X was examined by assessing the hydrolysis of chromogenic substrate S-2765. Significant factor X activator activity was observed after activating the substrate with cRVV and purified RVV-X as shown in Table 10. The specific factor X activator activity of purified RVV-X was 4 times higher than crude venom.

4.3 Cleavage of human factor X by purified RVV-X

Human factor X had molecular weight at about 59 kDa under non-reducing conditions (lane 2, Figure 31). Purified RVV-X had molecular weight at about 90 kDa (lane 3, Figure 21). Cleavage of human factor X with purified RVV-X was observed and had cleavage product at about 48 kDa (lane 4 through 7). The lower molecular weight cleavage products were not observed. The intensity of human factor X protein band (59 kDa) was inversely proportional to the purified RVV-X concentration.

4.4 Cleavage of human protein C by purified RVV-X

Human protein C had a major protein band at about 62 kDa and a less intensity protein band at about 58 kDa (lane 3, Figure 32). After the addition of 2 μg and 1 μg of purified RVV-X (lane 4 and 5, respectively), the cleavage products with protein bands at about 48 kDa and 52 kDa were observed. Very little changes in these protein bands with a faint band of cleavage product at about 52 kDa were showed after the addition of 0.5 μg and 0.25 μg of purified RVV-X (lane 7 and lane 8, respectively).

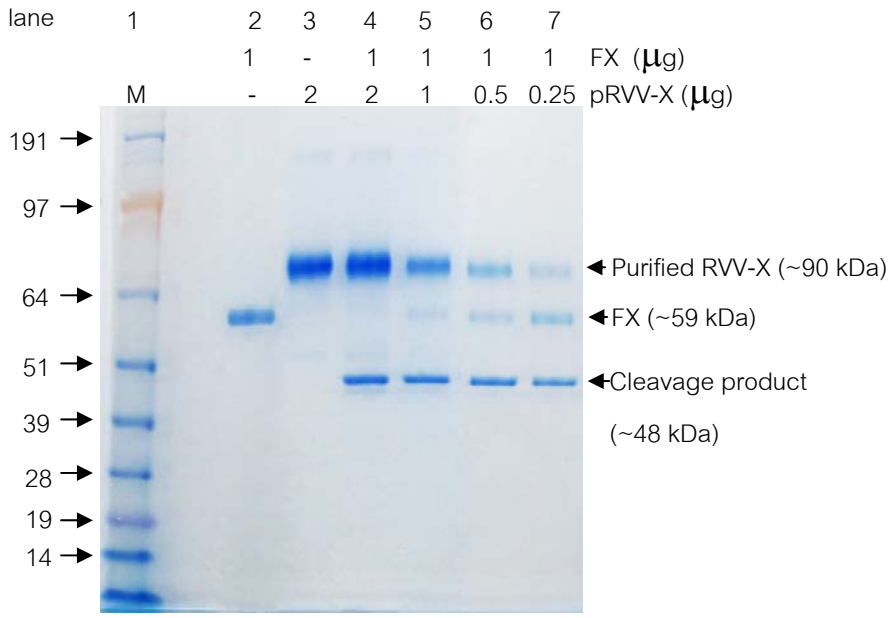

Figure 31 SDS-PAGE of cleaved human factor X (FX) by pRVV-X at various concentrations. FX pre-mixed with an equal volume of pRVV-X at various concentrations (0.25, 0.5, 1, 2 mg/ml) for 30 min at 37°C were run on 4-12% Bis-Tris NuPAGE gel under non-reducing conditions at 200 V (constant) for 50 min. Gel was stained with RapidStain. Lane 1: SeeBlue Plus2 Markers (Invitrogen™); lane 2: FX alone (1 μg); lane 3: pRVV-X alone (2 μg). FX were mixed with 2, 1, 0.5, or 0.25 μg of pRVV-X (lane 4-7, respectively).

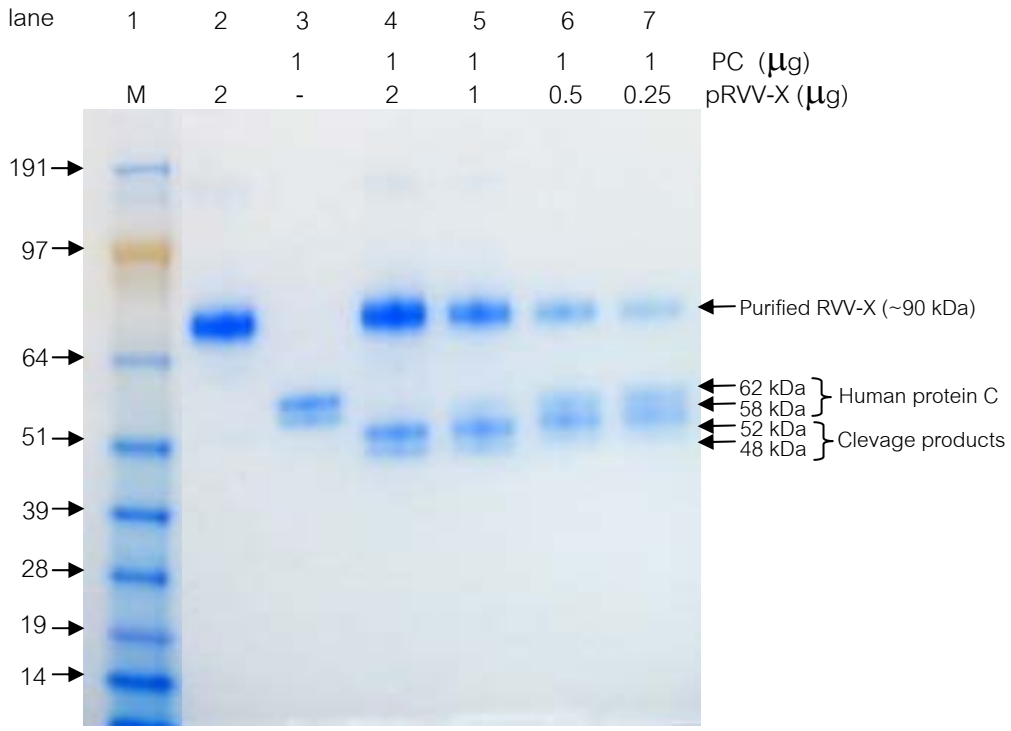

Figure 32 SDS-PAGE of cleaved human protein C (PC) by pRVV-X at various concentrations. PC pre-mixed with an equal volume of pRVV-X at various concentrations (2, 1, 0.5, 0.25 mg/ml) for 30 min at 37°C were run on 4-12% Bis-Tris NuPAGE gel under non-reducing conditions at 200 V (constant) for 50 min. Gel was stained with RapidStain. Lane 1: SeeBlue Plus2 Markers (Invitrogen™); lane 2: pRVV-X alone (2 μg); lane 3: PC alone (1 μg). PC were mixed with 2, 1, 0.5, or 0.25 μg of pRVV-X (lane 4-7, respectively).

4.5 Fibrinogenolytic activity of purified RVV-X

4.5.1 Effect of various concentrations of purified RVV-X on fibrinogen

Human fibrinogen contained three polypeptide subunits (Aα, Bβ, and γ chains) with molecular weight at about 65, 55 and 47 kDa, respectively, on a Novex® 10-20% Tricine SDS-PAGE gel under reducing condition (lane 4, Figure 33). The Aα chain with a molecular weight of 65 kDa was not observed when human fibrinogen incubated for 24 hr at 37°C with purified RVV-X at the concentration of 1, 0.5, and 0.25 mg/ml (Figure 23, lane 4 through 6). A faint band at about 65 kDa was showed when human fibrinogen incubated with purified RVV-X at the concentration of 0.1 mg/ml (Figure 33, lane 7). The Bβ chain with a molecular weight of 55 kDa was not observed when human fibrinogen was incubated with purified RVV-X at the concentration of 1 mg/ml (Figure 33, lane 4) but the presence of this band were inversely proportional to purified RVV-X concentration. However, the γ chain with a molecular weight of 47 kDa were seen when human fibrinogen was incubated with all purified RVV-X concentrations. Thus, 1 mg/ml of purified RVV-X was further determined its effect on fibrinogen at various incubation times. There were many smaller cleavage products when purified RVV-X was used at all concentrations.

4.5.2 Effect of incubation times of purified RVV-X and fibrinogen

The Aα chain with a molecular weight of 65 kDa was completely cleaved when human fibrinogen was incubated with purified RVV-X at 37°C for 2 hr (Figure 34, lane 6). While, the Bβ chain of human fibrinogen with a molecular weight of 55 kDa was completely cleaved after the additional of purified RVV-X at 37°C for 24 hr (Figure 34, lane 9). However, the γ chain with a molecular weight of 47 kDa was left intact even after 24 hr incubation.

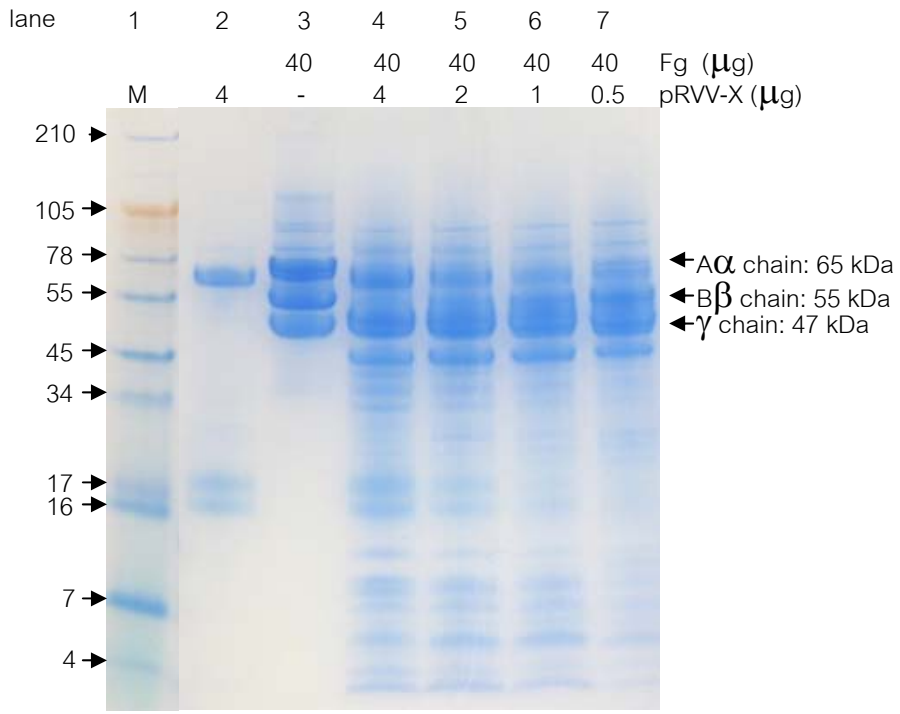

Figure 33 SDS-PAGE of human fibrinogen (Fg) when mixed with purified RVV-X at various concentrations. Fg pre-mixed with an equal volume of purified RVV-X at various concentrations (0.1, 0.25, 0.5 and 1 mg/ml) for 24 hr at 37°C were run on a Novex® 10-20% Tricine SDS-PAGE gel under reducing conditions at 125 V (constant) for 90 min. Gel was stained with RapidStain. Lane 1: SeeBlue Plus2 Markers (M) (Invitrogen™); lane 2: purified RVV-X alone (4 μg); lane 3: Fg alone (40 μg). Fg were mixed with 4, 2, 1, 0.5 μg of pRVV-X (lane 4-7, respectively)

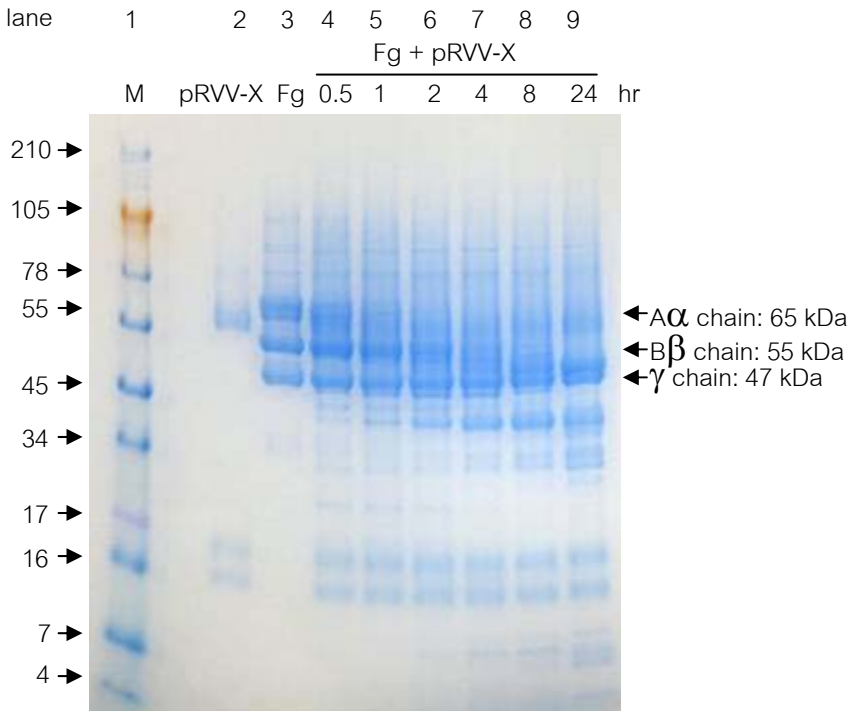

Figure 34 Time-dependent effect of purified RVV-X on fibrinogen. Human fibrinogen premixed with an equal volume of purified RVV-X at the concentration of 1 mg/ml for 30 min, 1, 2, 4, 8, 24 hr at 37°C were run on a Novex® 10-20% Tricine SDS-PAGE gel under non-reducing conditions at 125 V (constant) for 90 min. Gel was stained with RapidStain. Lane 1: SeeBlue Plus2 Markers (M) (Invitrogen™); lane 2: purified RVV-X (4 μg); lane 3: Fg (40 μg); lane 4-9: Fg was incubated with pRVV-X for 30 min, 1, 2, 4, 8, 24 hr, respectively.

5. Animal experiments

5.1 Sublethal dose optimization of cRVV and purified RVV-X in SD rats

To evaluate the optimum sublethal dose of cRVV, two of SD rats were injected with cRVV intravenously at various concentrations (2.5, 7, 12.5, 25, 50 μg/kg). All of 2 rats died within 8-10 min when i.v. injected with a 50 μg/kg of cRVV. One rat died within 2 hr when i.v. injected with a 25 μg/kg of cRVV. All rats injected with a 2.5, 7, 12.5 μg/kg of cRVV survived and showed irregular breathing. Thus, a 7 μg/kg of cRVV was used as an injected dose for the determination of DIC, renal hemodynamics and functions, and histopathological studies.

Based on the specific factor X activator activity, the equipotent sublethal dose of purified RVV-X for animal experiments was approximately four times less than that of cRVV. Thus, a 1.75 μg/kg of purified RVV-X was used for an equipotent sublethal dose in animal experiments.

5.2 Effect of commercial horse anti-cRVV $F(ab')_2$ antivenom in neutralizing factor X activator activity of purified RVV-X using factor Xa-specific chromogenic substrate S-2765.

A concentration of 31.25 μg/ml of commercial horse anti-cRVV $F(ab')_2$ antivenom completely neutralized factor X activator activity of a 0.125 μg/ml of purified RVV-X as the ratio of 250:1 (Figure 35).

A 437.5 μg/kg of commercial horse anti-cRVV $F(ab')_2$ antivenom was further used as a neutralizing dose in animal experiments. The specificity of horse commercial anti-cRVV $F(ab')_2$ antivenom was evaluated by using ERIG as the antibody control. All concentrations of irrelevant horse serum (ERIG) were incapable of neutralizing factor X activator activity of a 0.125 μg/ml of purified RVV-X.

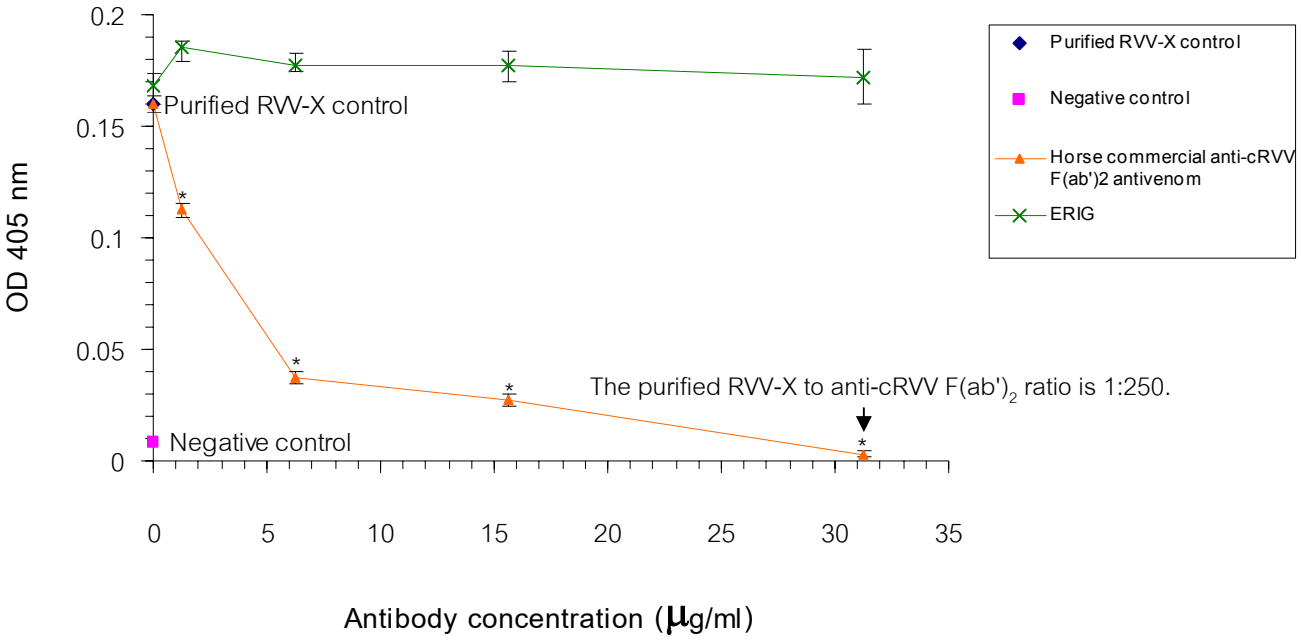

Figure 35 Effect of commercial horse anti-cRVV $F(ab')_2$ antivenom in neutralizing factor X activator activity of purified RVV-X using factor Xa-specific chromogenic substrate S-2765. Negative control was normal citrated human plasma reconstituted with only $CaCl_2$. Venom control was normal citrated activated with purified RVV-X and 0.025 M $CaCl_2$. Antibody control was normal citrated activated with purified RVV-X pre-mixed with ERIG and 0.025 M $CaCl_2$. Data represent the mean $\pm$ SD (n = 3). An asterisk (*) indicates statistic significance compared with the purified RVV-X control at $P < 0.05$. Their significance was analyzed by the Student's t-test.

5.3 Disseminated intravascular coagulation (DIC) determination

D-dimer is the hallmark of DIC (Adam et al., 2009). The plasma D-dimer level in SD rats serially measured after injection of cRVV and purified RVV-X. The average plasma D-dimer level of rats given crude venom was gradually increased to reach a peak at 45 min (Figure 36, 37). The average plasma D-dimer increased and reached a peak at 15 min, declined temporarily and then reached another peak at 30 min after purified RVV-X injection (Figure 36, 38).

Horse anti-cRVV $F(ab')_2$ antivenom effectively prevent DIC caused by purified RVV-X. Rats given horse anti-cRVV $F(ab')_2$ antivenom immediately following purified RVV-X injection has minimal increased in the plasma D-dimer level to about 1 mg/L at 5 min and was decreased to reach baseline at 1 hr. In constrast, rats given an irrelevant horse serum (ERIG) following purified RVV-X developed very high level of D-dimer (Figure 36) at 20 min.

As negative controls, rats given NSS and horse anti-cRVV $F(ab')_2$ antivenom did not develop DIC (Figure 36).

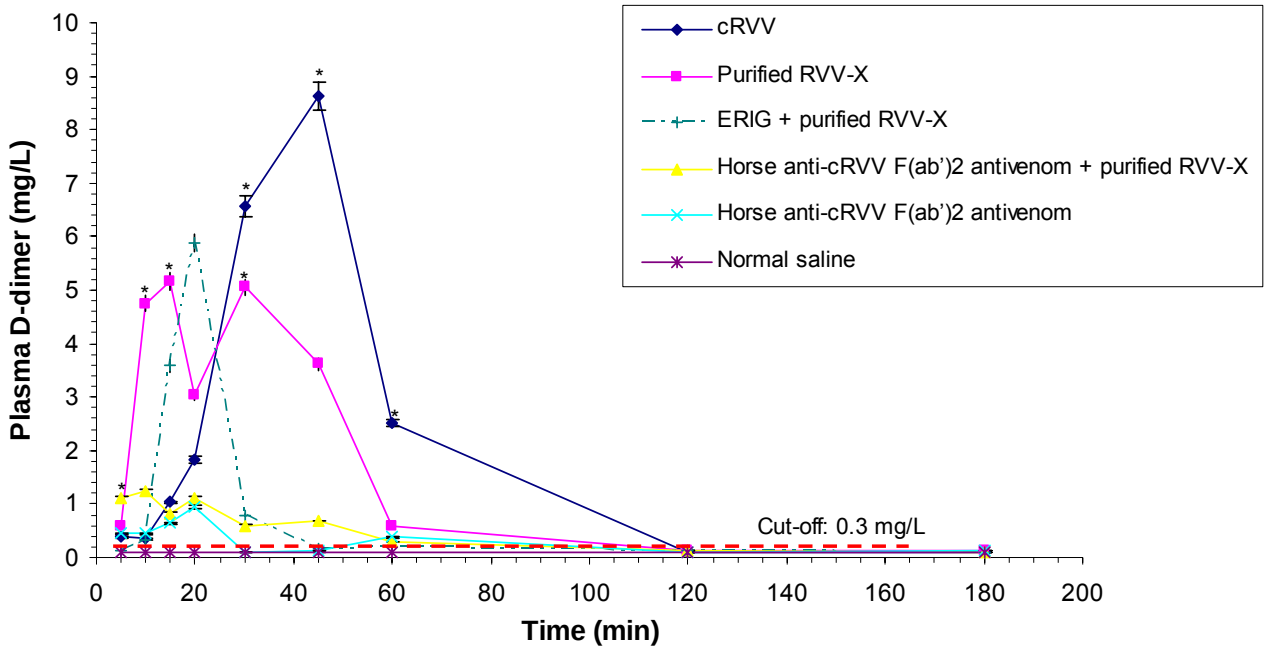

Figure 36 The average plasma D-dimer level in each group of rats at various times. Negative control group was rats given only normal saline. Antibody control group was rats given only horse anti-cRVV $F(ab')_2$ antivenom. Data represent the mean $\pm$ SD (n = 6). An asterisk (*) indicates statistic significance compared with the negative control at $P < 0.05$. Their significance was analyzed by ANOVA using LSD as post hoc test at $P < 0.05$. A dashed line indicates the plasma D-dimer cut-off level at 0.3 mg/L.

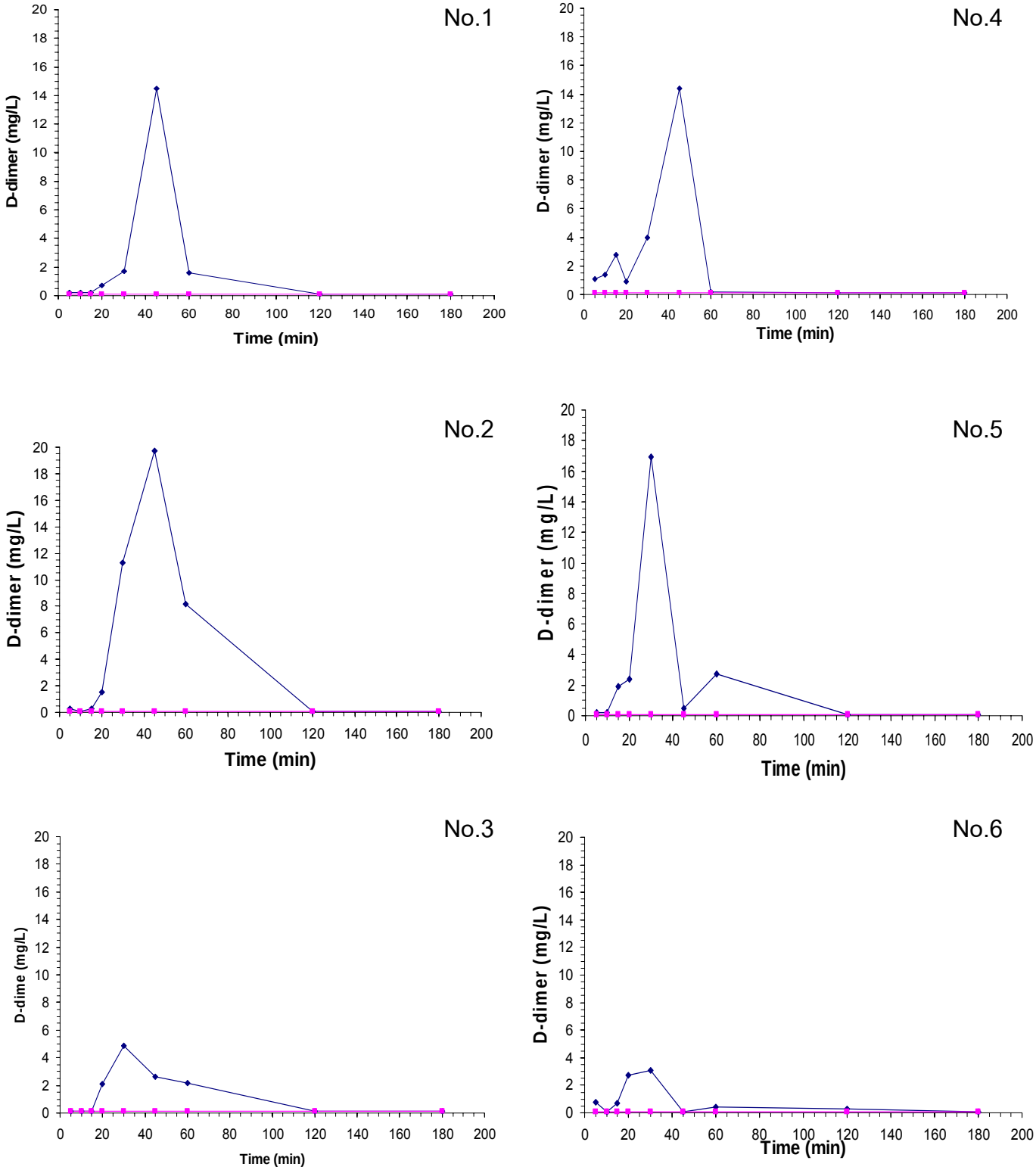

Figure 37 The plasma D-dimer level of each individual rat injected with crude venom (7 μg/kg; n = 6). The plasma D-dimer level of normal saline group indicated as (-■-).

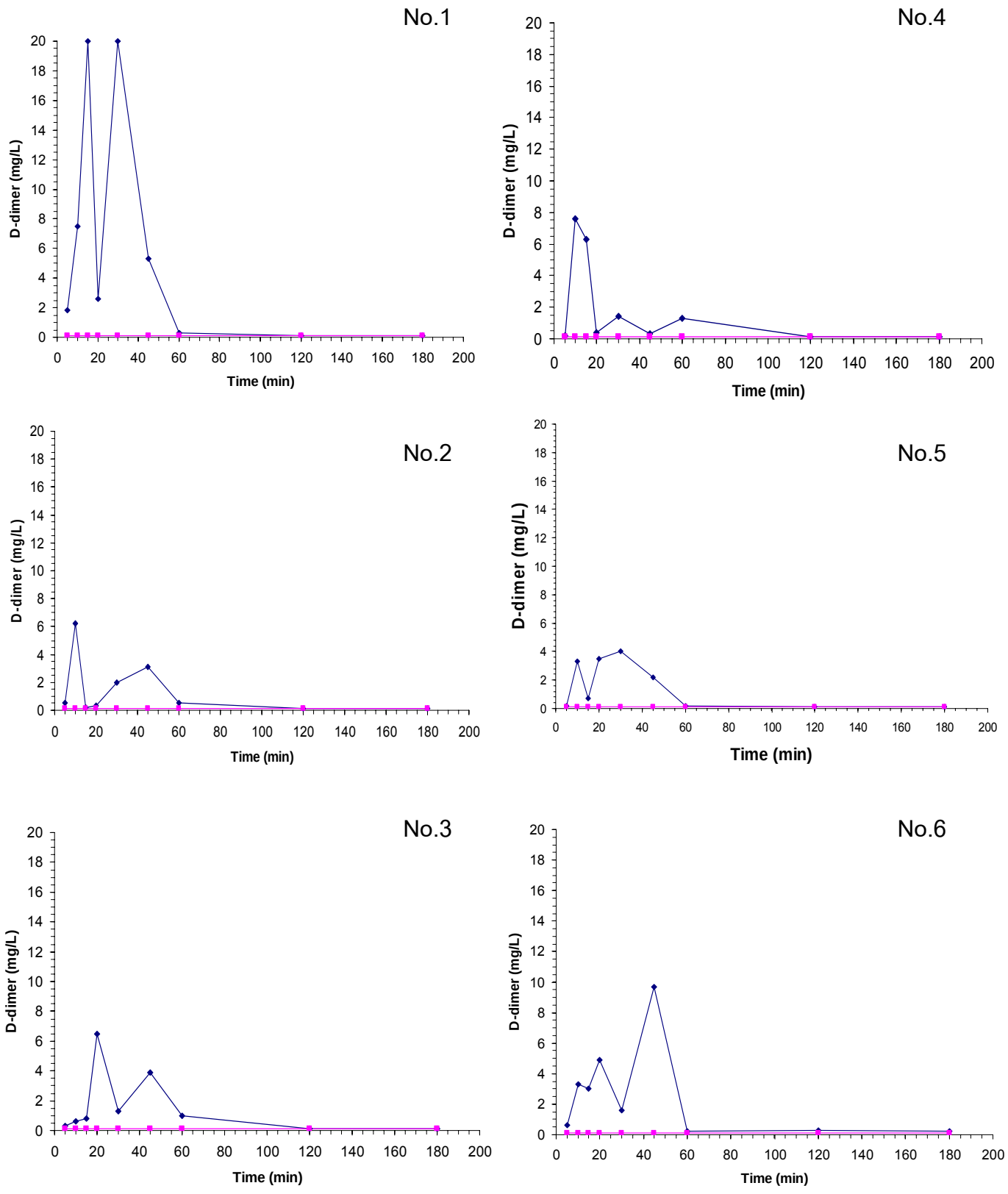

Figure 38 The plasma D-dimer level of each individual rat injected with purified RVV-X (1.75 μg/kg; n = 6). The plasma D-dimer level of normal saline group indicated as (–■–).

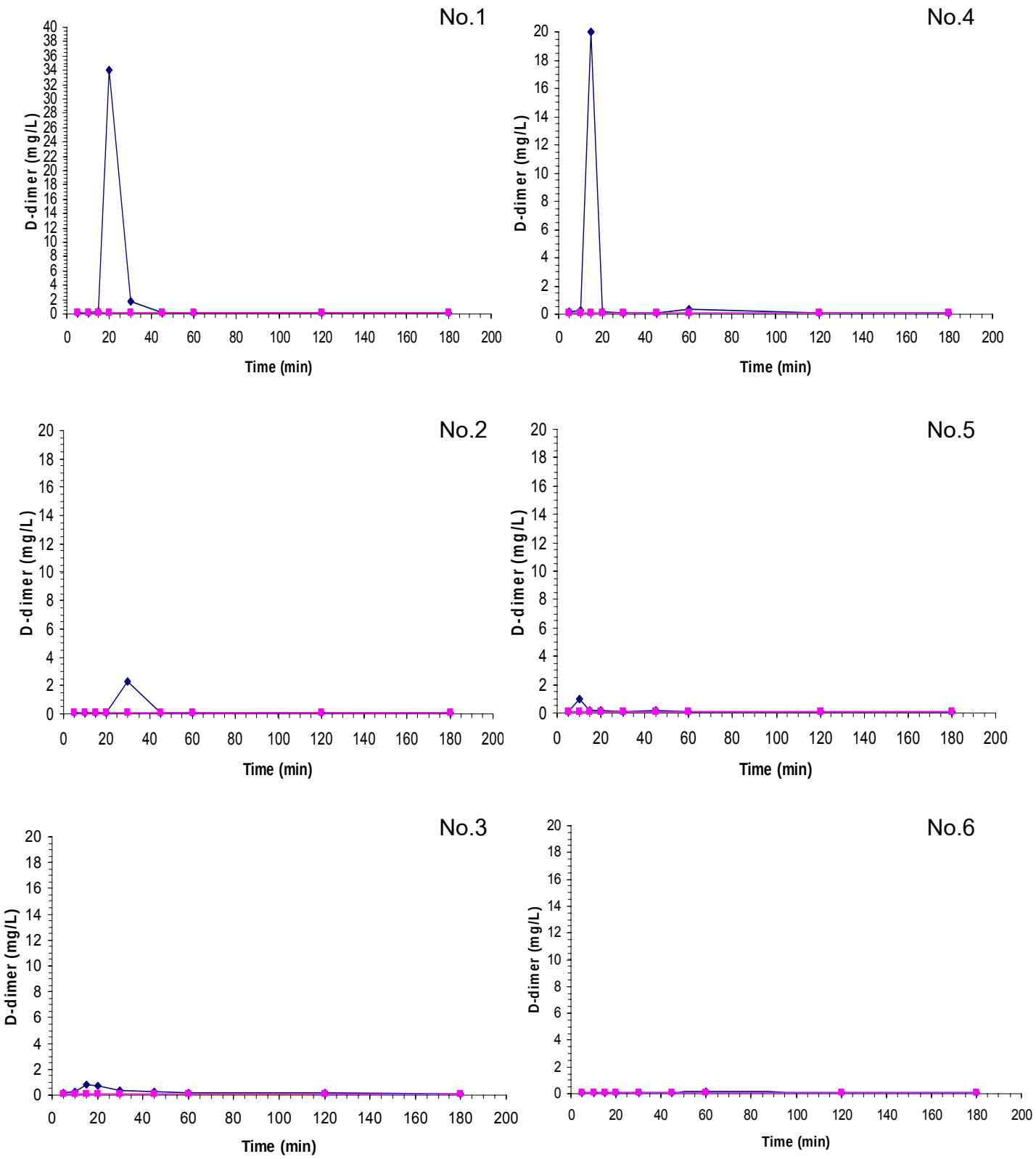

Figure 39 The plasma D-dimer level of each individual rat (n = 6) injected with ERIG (437.5 μg/kg) and purified RVV-X (1.75 μg/kg). The plasma D-dimer level of normal saline group indicated as (–■–).

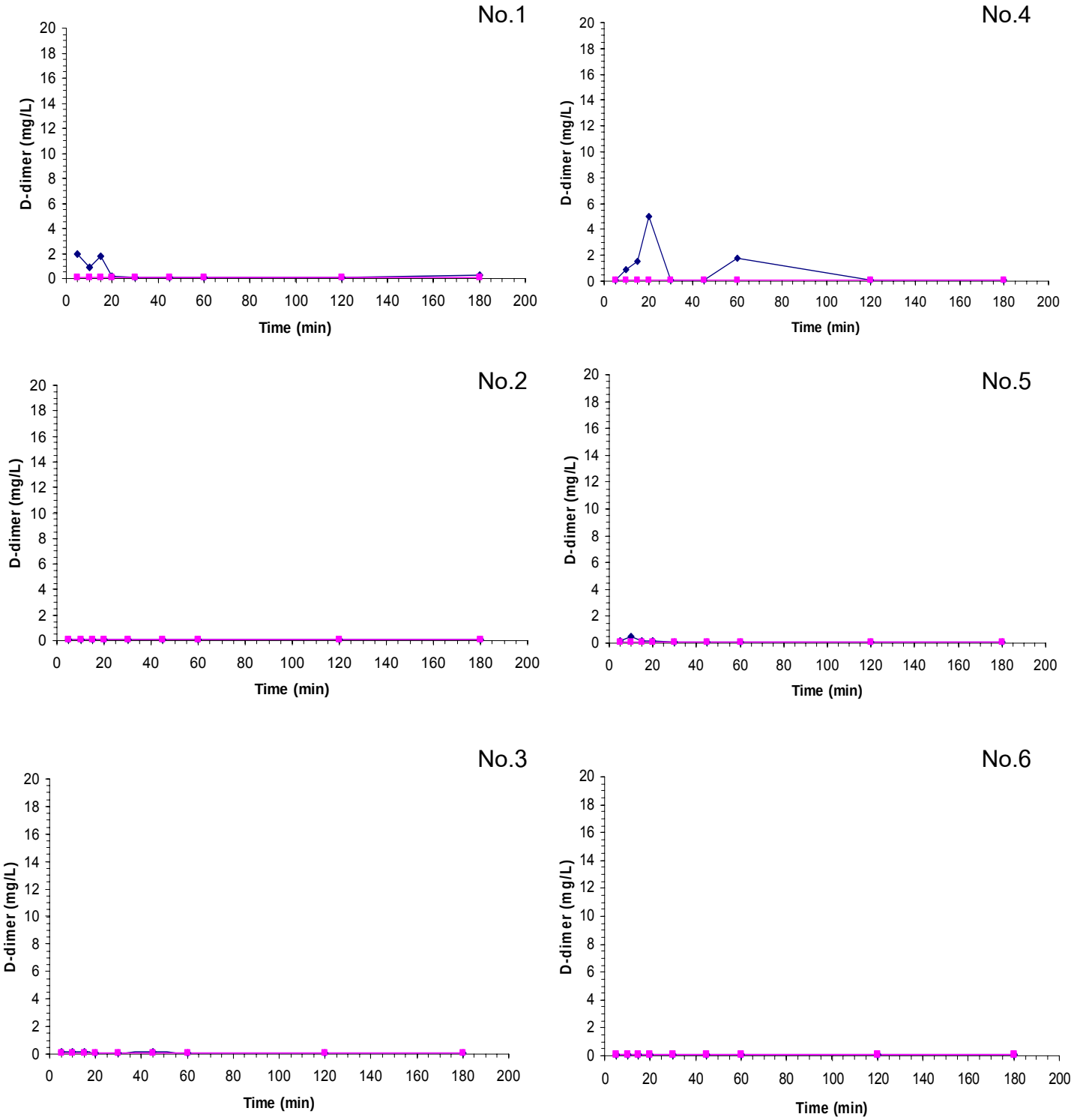

Figure 40 The plasma D-dimer level of each individual rat injected with horse anti-cRVV $F(ab')_2$ antivenom (437.5 μg/kg; n = 6). The plasma D-dimer level of normal saline group indicated as (-■-).

5.4 RVV and purified RVV-X induced thrombocytopenia in SD rats

The effects of cRVV, purified RVV-X, ERIG and purified RVV-X, and horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X on blood counts were summarized in Table 12. Significantly decreased platelet count was observed in three groups of rats given cRVV, purified RVV-X, and ERIG and purified RVV-X. There were significant decrease in RBC and significant increases in MCV and MCH in all groups of rats.

Red cell fragment (schistocyte) was found in peripheral blood film of rats 3 hr after injection of cRVV, purified RVV-X, and ERIG and purified RVV-X (Figure 41).

Table 12 Hematological data in rats 3 hr after injection of cRVV, purified RVV-X, ERIG and purified RVV-X, horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X, and horse anti-cRVV $F(ab')_2$ antivenom

Parameters	cRVV	Purified RVV-X	ERIG + purified RVV-X	Horse anti-cRVV $F(ab')_2$ antivenom + purified RVV-X	Horse anti-cRVV $F(ab')_2$ antivenom	Normal saline
WBC (10^3/ul)	5.30±1.85^{a}	3.93±1.33^{ab}	4.90±1.76^{ab}	3.47±1.39^{b}	3.31±0.29^{b}	5.59±1.98^{a}
RBC (10^6/ul)	5.95±0.60^{b}	5.44±0.36^{b}	5.57±0.64^{b}	5.51±0.46^{b}	5.80±0.44^{b}	6.66±0.56^{a}
HGB (g/dL)	11.75±1.27^{ab}	10.88±0.66^{b}	10.88±1.26^{b}	10.80±0.85^{b}	11.37±0.80^{ab}	12.35±1.17^{a}
Hct (%)	34.30±3.9^{ab}	32.20±2.10^{b}	32.13±3.49^{b}	32.25±2.40^{b}	34.20±1.99^{ab}	36.42±3.30^{a}
MCV (fL)	57.55±1.08^{b}	59.13±1.11^{b}	57.72±1.22^{b}	58.60±1.73^{b}	59.00±1.88^{b}	54.72±2.82^{a}
MCH (pg)	19.75±0.31^{b}	19.98±0.19^{b}	19.53±0.32^{b}	19.62±0.34^{b}	19.62±0.43^{b}	18.55±0.74^{a}
MCHC (g/dL)	34.28±0.35^{a}	33.78±0.62^{ab}	33.85±1.20^{ab}	33.47±0.50^{ab}	33.2±0.57^{b}	33.92±0.88^{ab}
PLT (10^3/ul)	133±44.08^{b}	238±136.25^{b}	247±170.79^{b}	466±163.97^{a}	629±45.31^{c}	568±59.84^{ac}
NEUT (%)	38.5±7.5^{b}	40.2±5.0^{b}	43.5±6.8^{b}	36.6±13.1^{bc}	28.6±6.2^{c}	57.2±3.3^{a}
LYMPH (%)	57.9±11.2^{b}	57.6±5.1^{b}	51.7±7.5^{b}	60.7±13.8^{bc}	68.9±6.5^{c}	38.5±5.6^{a}
MONO (%)	3.0±4.2^{ab}	1.7±1.1^{a}	4.2±1.8^{b}	2.2±1.0^{ab}	2.0±0.9^{ab}	3.3±1.3^{ab}
EO (%)	0.7±0.2^{a}	0.5±0.2^{a}	0.7±0.6^{a}	0.7±0.6^{a}	0.6±0.2^{a}	0.4±1.6^{a}
BASO (%)	-	-	-	-	-	-

All results were presented as mean ± SD (n = 6).

WBC: white blood cell; RBC: red blood cell; HBG: hemoglobin; Hct: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet; NEUT: neutrophil; LYMPH: lymphocyte; MONO: monocyte; EO: eosinophil; BASO: basophil

Statistically significant differences were indicated by comparing renal hemodynamic parameters among groups at $P < 0.05$ (ANOVA).

Values within the same row with different superscripts (a, b, c) were significantly different at $P < 0.05$ (ANOVA).

Values followed by the identical superscripts within the same column were not significantly different ($P > 0.05$).

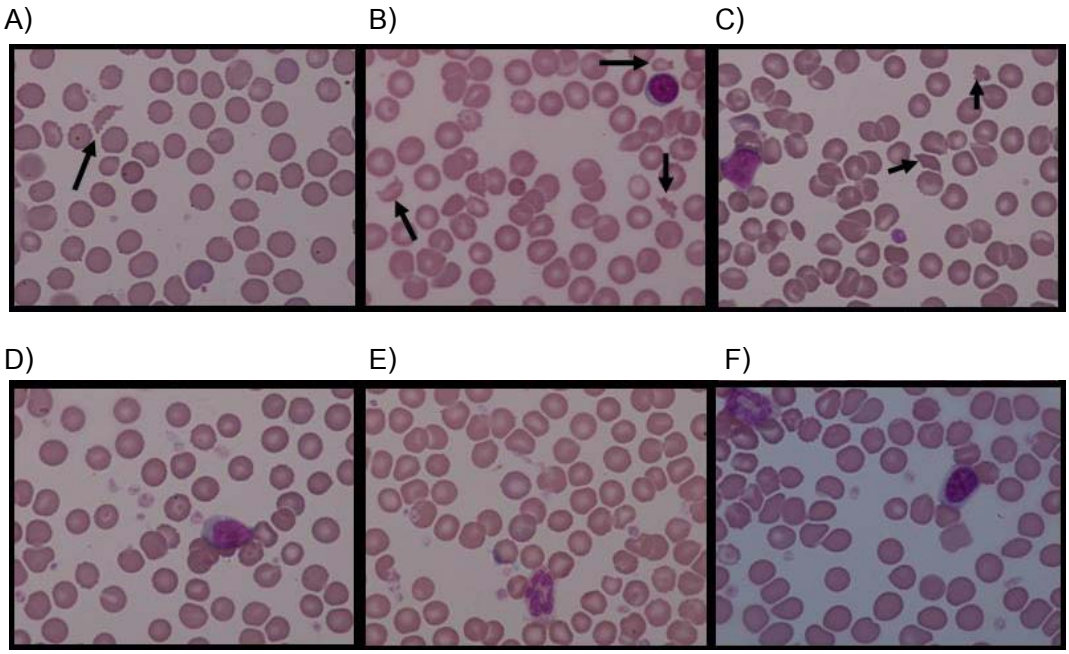

Figure 41 Peripheral blood film (100X magnification) of rats 3 hr after injection of (A) cRVV, (B) purified RVV-X, (C) ERIG and purified RVV-X, (D) horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X, (E) horse anti-cRVV $F(ab')_2$ antivenom, and (F) normal saline. Blood film was stained with Wright's stain. The arrow indicates schistocytes (red cell fragments).

5.5 Renal hemodynamic and function studies

5.5.1 Changes of general hemodynamic parameters

The hemodynamic effects of cRVV, purified RVV-X, ERIG and purified RVV-X, and horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X were summarized in Table 13. Significantly decreased DP was observed in rats injected with crude venom. SP, MAP, and HR were decreased in two groups of rats given cRVV and purified RVV-X without statistical significance ($P > 0.05$). There were no significant differences in SP, DP, MAP, PP, and HR between normal saline control group and three groups of rats given ERIG and purified RVV-X, horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X, and horse anti-cRVV $F(ab')_2$ antivenom groups.

Table 13 General hemodynamics in rats 10 min after injection of cRVV, purified RVV-X, ERIG and purified RVV-X, horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X, and horse anti-cRVV $F(ab')_2$ antivenom

Group	SP (mmHg)	DP (mmHg)	MAP (mmHg)	PP (mmHg)	HR (beat/min)	Hct
cRVV	119.80 ± 16.20^{a}	77.25 ± 19.63^{b}	95.62 ± 15.05^{b}	36.27 ± 4.74^{a}	293 ± 42.63^{b}	40.67 ± 2.42^{a}
Purified RVV-X	120.59 ± 12.83^{ac}	85.12 ± 10.25^{ab}	96.94 ± 10.92^{ab}	35.47 ± 4.98^{a}	306 ± 20.08^{ab}	42.00 ± 1.10^{a}
ERIG + purified RVV-X	126.37 ± 5.50^{ab}	95.88 ± 9.32^{a}	106.05 ± 7.06^{ab}	30.49 ± 9.00^{a}	324 ± 48.45^{ab}	44.96 ± 2.19^{b}
Horse anti-cRVV $F(ab')_2$ antivenom + purified RVV-X	134.75 ± 6.70^{b}	94.12 ± 3.50^{a}	107.66 ± 4.43^{a}	40.64 ± 3.94^{a}	331 ± 15.06^{ab}	42.42 ± 2.01^{a}
Horse anti-cRVV $F(ab')_2$ antivenom	132.20 ± 6.53^{bc}	93.20 ± 5.37^{a}	106.20 ± 5.65^{ab}	39.00 ± 2.64^{a}	333 ± 26.50^{a}	43.00 ± 1.90^{ab}
Normal saline solution	126.47 ± 8.92^{ab}	90.20 ± 9.36^{a}	102.29 ± 8.48^{ab}	36.27 ± 7.66^{a}	319 ± 34.03^{ab}	41.17 ± 2.04^{a}

All results were presented as mean $\pm$ SD (n = 6).

SP: systolic blood pressure; DP: diastolic blood pressure; MAP: mean arterial pressure; PP: pulse pressure; HR: heart rate; Hct: hematocrit

Statistically significant differences were indicated by comparing renal hemodynamic parameters among groups at $P < 0.05$ (ANOVA).

Values within the same column with different superscripts (a, b, c) were significantly different at $P < 0.05$ (ANOVA).

Values followed by the identical superscripts within the same column were not significantly different.

5.5.2 Changes of renal globular functions and hemodynamics

Renal hemodynamic and function changes were observed within 10 min (Table 14) after an equipotent sublethal dose of cRVV, purified RVV-X, and ERIG and purified RVV-X injection. There were significant decreases in GFR, ERPF, and ERBF, and significant increases in RVR in three groups of rats injected with cRVV, purified RVV-X, and ERIG and purified RVV-X as compared with the normal saline control group. Significantly increased FF was observed in rats injected with crude venom. However, there were no significant differences in all hemodynamic parameters between cRVV group and two groups of rats given purified RVV-X, and ERIG and purified RVV-X. There were no significant differences in all hemodynamic parameters between normal saline control group and two groups of rats receiving horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X, and horse anti-cRVV $F(ab')_2$ antivenom groups.

Table 14 Renal hemodynamics and functions in rats 10 min after injection of cRVV, purified RVV-X, ERIG and purified RVV-X, horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X, and horse anti-cRVV $F(ab')_2$ antivenom

Group	GFR (ml/min/100 g BW)	ERPF (ml/min/100 g BW)	ERBF	FF (%)	RVR (mmHg/ml/min/100g BW)
cRVV	0.43 ± 0.23^{b}	1.14 ± 0.91^{b}	1.88 ± 1.46^{b}	61.45 ± 58.39^{b}	77.93 ± 26.81^{b}
Purified RVV-X	0.50 ± 0.24^{b}	1.69 ± 0.93^{b}	2.93 ± 1.63^{b}	33.46 ± 8.50^{ab}	81.16 ± 67.60^{b}
ERIG + purified RVV-X	0.42 ± 0.36^{b}	0.99 ± 0.35^{b}	1.82 ± 0.72^{b}	47.56 ± 18.95^{ab}	71.52 ± 24.39^{b}
Horse anti-cRVV $F(ab')_2$ antivenom + purified RVV-X	0.77 ± 0.14^{a}	2.62 ± 0.42^{a}	4.54 ± 0.67^{a}	31.25 ± 6.15^{a}	25.62 ± 3.68^{a}
Horse anti-cRVV $F(ab')_2$ antivenom	0.79 ± 0.18^{a}	3.05 ± 0.64^{a}	5.36 ± 1.13^{a}	26.65 ± 6.89^{a}	21.06 ± 6.01^{a}
Normal saline	0.82 ± 0.10^{a}	2.79 ± 0.36^{a}	4.75 ± 0.62^{a}	30.00 ± 1.95^{a}	23.67 ± 3.18^{a}

All results were presented as mean $\pm$ SD (n = 6).

MAP: mean arterial pressure; GFR: glomerular filtration rate; ERPF: renal plasma flow; ERBF: effective renal blood flow; FF: filtration fraction; RVR: renal vascular resistance.

Statistically significant differences were indicated by comparing renal hemodynamic parameters among groups at $P < 0.05$ (ANOVA).

Values within the same column with different superscripts (a, b) were significantly different at $P < 0.05$ (ANOVA).

Values followed by the identical superscripts within the same column were not significantly different ($P > 0.05$).

5.5.3 Changes of renal tubular functions

The effects of cRVV, purified RVV-X, ERIG and purified RVV-X, and horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X on renal tubular function were summarized in Table 15. Significantly decreases in V and $U_{Na}V$ were observed in three groups of rats given cRVV, purified RVV-X, and ERIG and purified RVV-X as compared with the normal saline control group. U_KV were significantly decreased in two groups of rats injected with cRVV and ERIG and purified RVV-X but not significant decreased in rat given purified RVV-X. Reduction of $U_{Cl}V$ and $U_{Mg}V$ were showed in three groups of rats receiving cRVV, purified RVV-X, and ERIG and purified RVV-X without statistical significance ($P > 0.05$). There were no significant differences in V/GFR, FE_{Na}, FE_K, FE_{Cl}, and FE_{Mg} between normal saline control group and all groups of rats.

Table 15 Renal tubular functions in rats 10 min after injection of cRVV, purified RVV-X, ERIG and purified RVV-X, horse anti-cRVV F(ab')$_2$ antivenom and purified RVV-X, and horse anti-cRVV F(ab')$_2$ antivenom

Group	V	V/GFR (%)	$U_{Na}V$ (μEq/min/100g BW)	U_KV (μEq/min/100g BW)	$U_{Cl}V$ (μEq/min/100 g BW)	$U_{Mg}V$ (μEq/min/100 g BW)	FE_{Na} (%)	FE_K (%)	FE_{Cl} (%)	FE_{Mg} (%)
cRVV	1.67 ± 0.70^{b}	0.56 ± 0.25^{a}	0.18 ± 0.08^{b}	0.34 ± 0.19^{b}	0.48 ± 0.57^{ab}	1.28 ± 0.74^{b}	0.41 ± 0.14^{ab}	32.46 ± 14.36^{a}	0.92 ± 0.61^{a}	18.02 ± 6.99^{a}
Purified RVV-X	2.58 ± 1.35^{bc}	0.48 ± 0.11^{a}	0.30 ± 0.21^{b}	0.38 ± 0.22^{ab}	0.40 ± 0.21^{b}	1.53 ± 0.63^{ab}	0.39 ± 0.15^{ab}	30.16 ± 10.82^{a}	0.75 ± 0.15^{a}	16.82 ± 5.20^{a}
ERIG + purified RVV-X	2.25 ± 1.97^{bc}	0.53 ± 0.10^{a}	0.23 ± 0.28^{b}	0.27 ± 0.16^{b}	0.41 ± 0.39^{b}	1.26 ± 0.82^{b}	0.32 ± 0.21^{a}	26.81 ± 9.46^{a}	0.81 ± 0.33^{a}	20.26 ± 8.68^{a}
Horse anti-cRVV F(ab')$_2$ antivenom + purified RVV-X	4.12 ± 0.90^{a}	0.52 ± 0.31^{a}	0.60 ± 0.27^{ac}	0.61 ± 0.16^{a}	0.82 ± 0.31^{ab}	2.10 ± 0.50^{ab}	0.61 ± 0.39^{b}	28.12 ± 5.98^{a}	1.03 ± 0.55^{a}	13.79 ± 4.14^{a}
Horse anti-cRVV F(ab')$_2$ antivenom	3.80 ± 1.23^{ac}	0.92 ± 0.11^{a}	0.49 ± 0.35^{ab}	0.87 ± 0.33^{c}	0.90 ± 0.46^{a}	2.20 ± 1.08^{a}	0.47 ± 0.32^{ab}	28.43 ± 6.47^{a}	1.09 ± 0.54^{a}	14.13 ± 5.63^{a}
Normal saline	4.24 ± 0.82^{a}	0.51 ± 0.06^{a}	0.70 ± 0.15^{a}	0.62 ± 0.11^{a}	0.73 ± 0.11^{ab}	1.92 ± 0.79^{ab}	0.58 ± 0.09^{ab}	29.68 ± 3.50^{a}	0.80 ± 0.09^{a}	13.91 ± 5.61^{a}

All results were presented as mean ± SD (n = 6).

V: urine flow rate; U_EV: urinary excretion of electrolytes; FE_E: fractional excretion of electrolytes

Statistically significant differences were indicated by comparing renal hemodynamic parameters among groups at $P < 0.05$ (ANOVA).

Values within the same column with different superscripts (a, b, c) were significantly different at $P < 0.05$ (ANOVA).

Values followed by the identical superscripts within the same column were not significantly different ($P > 0.05$).

5.6 Histopathological examination

Tissue samples from kidney, lung, spleen, adrenal, liver, and heart were taken from all groups of rats at the end of DIC experiment. The tissue sections were stained with H&E technique. The tissue sections from all rats were determined, but not shown. The kidney showed minimal histopathological necrotic changes in the tubular area. Some fibrin thrombi were found in rat kidney when cRVV, purified RVV-X, and ERIG and purified RVV-X was given (Figure 42A-C).

In the lungs, the minimal intrapulmonary hemorrhage, inflammatory cell infiltration, and vasculitis were found in the same groups of rats (Figure 43A-C). Large cells were seen in the spleen of all groups of rats but not in the normal saline control group (Figure 44A-C). Other organs including adrenal, liver, and heart appeared normal (Figure 45-47).

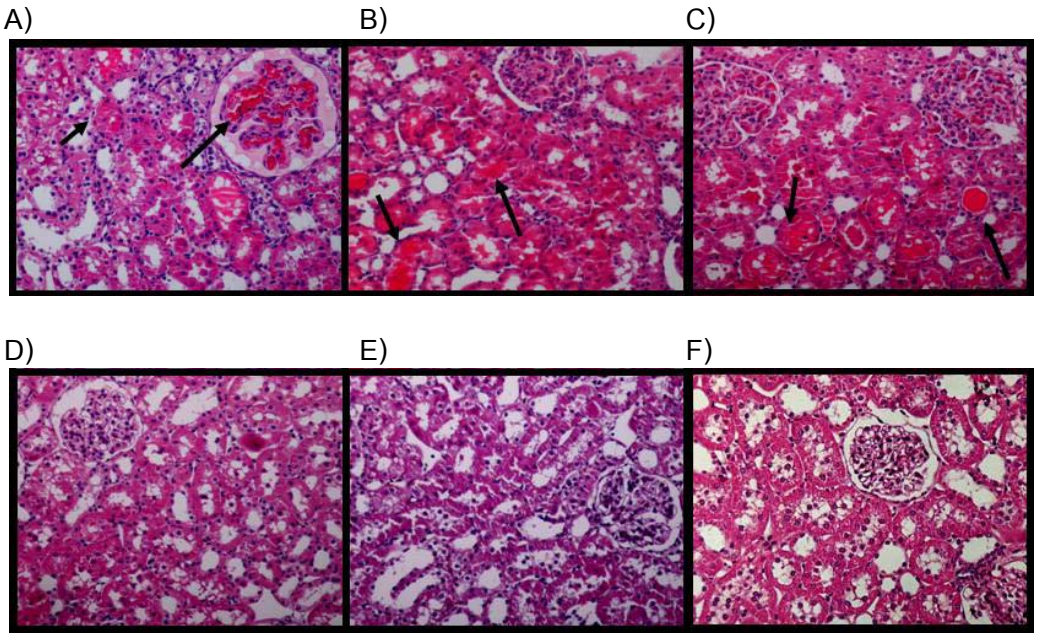

Figure 42 Histological section of kidney stained with H&E (20X magnification) of rats 3 hr after injection of (A) cRVV, (B) purified RVV-X, (C) ERIG and purified RVV-X, (D) horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X, (E) horse anti-cRVV $F(ab')_2$ antivenom, and (F) normal saline. The arrow indicates the fibrin thrombi.

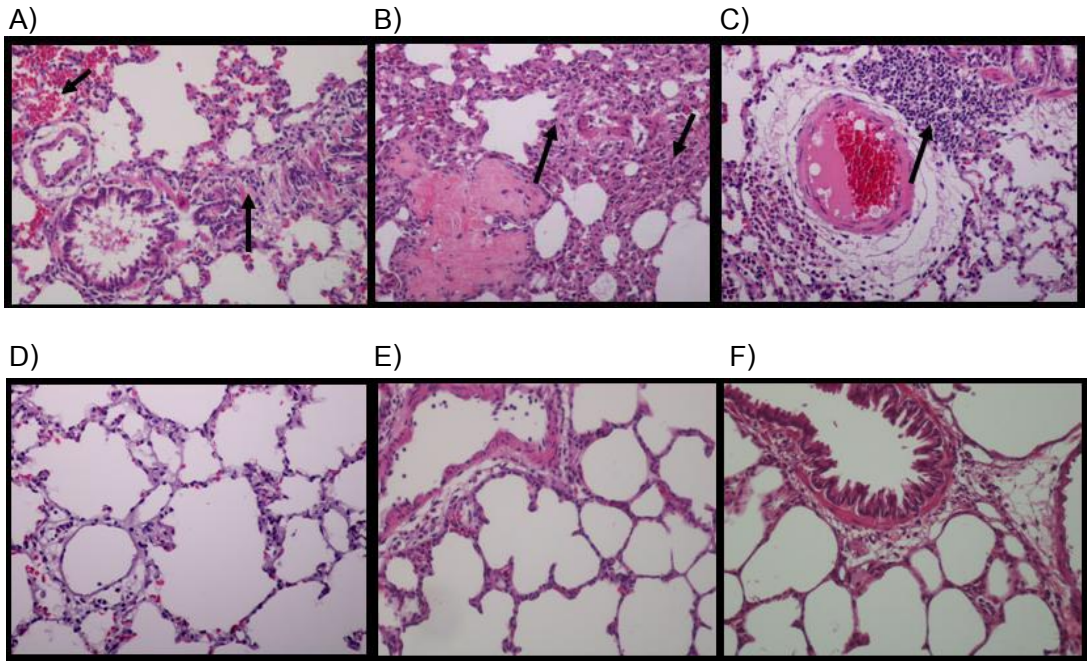

Figure 43 Histological section of lung stained with H&E (20X magnification) of rats 3 hr after injection of (A) cRVV, (B) purified RVV-X, (C) ERIG and purified RVV-X, (D) horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X, (E) horse anti-cRVV $F(ab')_2$ antivenom, and (F) normal saline. The arrow indicates the hemorrhage, inflammatory cell infiltration, and vasculitis.

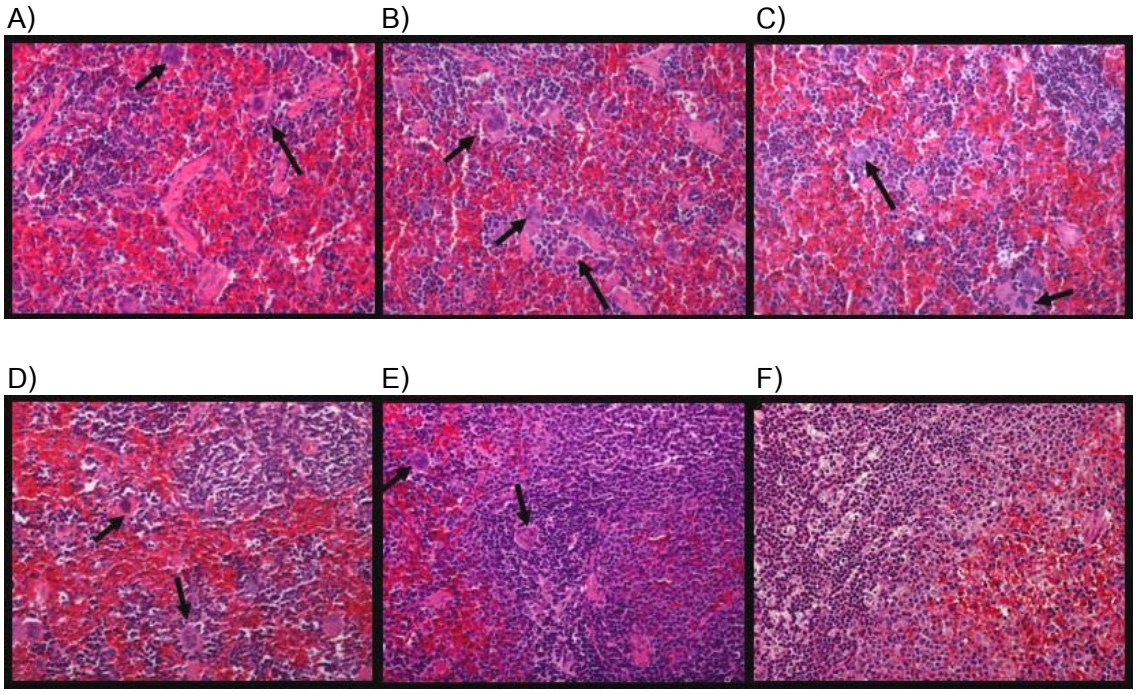

Figure 44 Histological section of spleen stained with H&E (20X magnification) of rats 3 hr after injection of (A) cRVV, (B) purified RVV-X, (C) ERIG and purified RVV-X, (D) horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X, (E) horse anti-cRVV $F(ab')_2$ antivenom, and (F) normal saline. The arrow indicates the large cells in both red and white pulb of spleen.

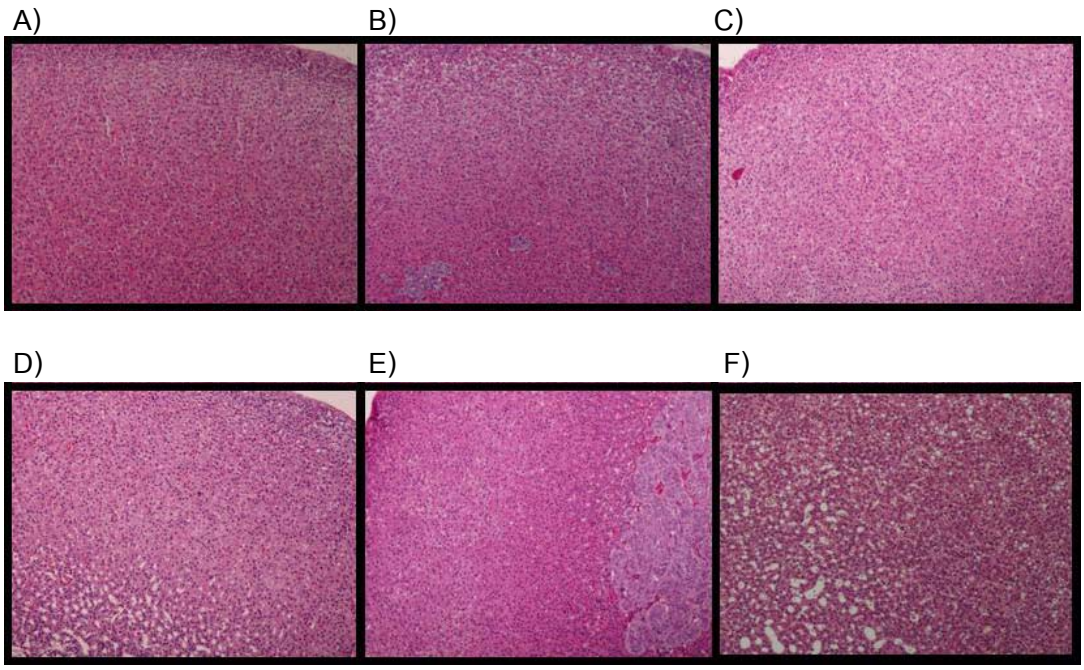

Figure 45 Histological section of adrenal stained with H&E (10X magnification) of rats 3 hr after injection of (A) cRVV, (B) purified RVV-X, (C) ERIG and purified RVV-X, (D) horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X, (E) horse anti-cRVV $F(ab')_2$ antivenom, and (F) normal saline.

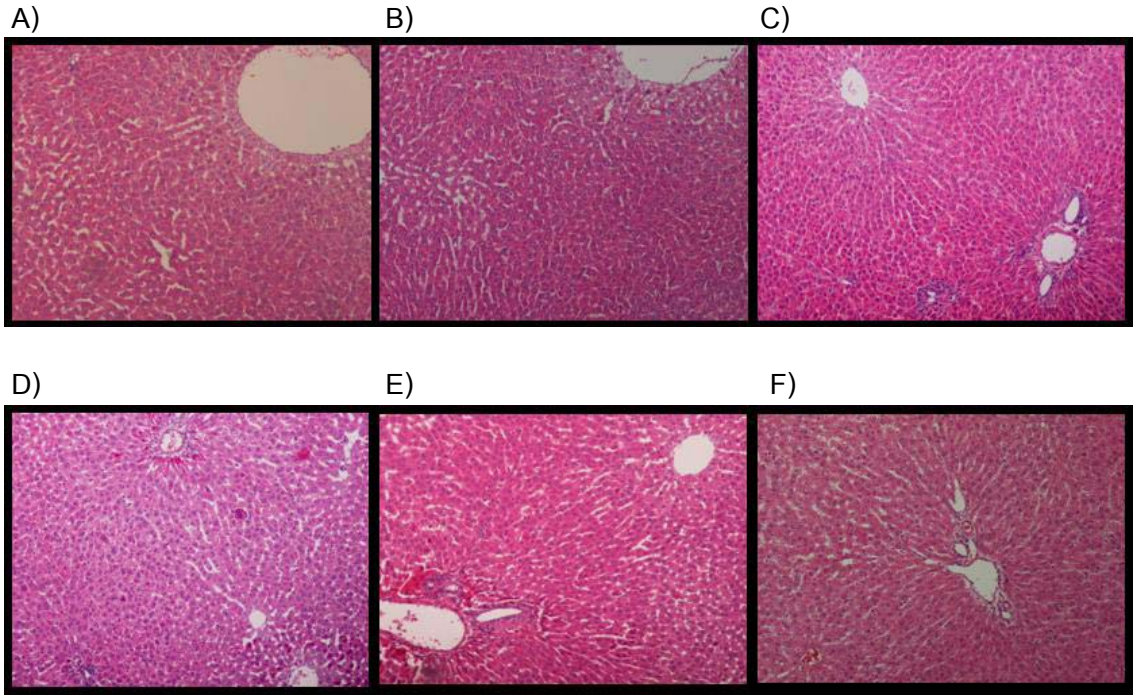

Figure 46 Histological section of liver stained with H&E (20X magnification) of rats 3 hr after injection of (A) cRVV, (B) purified RVV-X, (C) ERIG and purified RVV-X, (D) horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X, (E) horse anti-cRVV $F(ab')_2$ antivenom, and (F) normal saline.

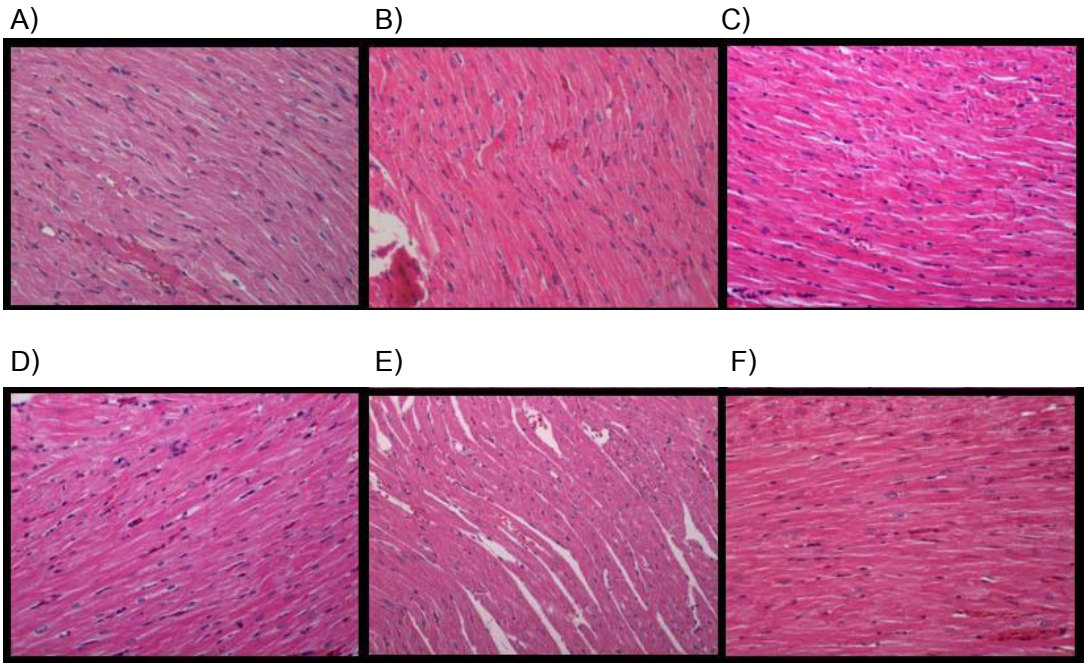

Figure 47 Histological section of heart stained with H&E (20X magnification) of rats 3 hr after injection of (A) cRVV, (B) purified RVV-X, (C) ERIG and purified RVV-X, (D) horse anti-cRVV $F(ab')_2$ antivenom and purified RVV-X, (E) horse anti-cRVV $F(ab')_2$ antivenom, and (F) normal saline.

CHAPTER V

DISCUSSION

Acute renal failure (ARF) is the most frequent and serious complication in Russell's viper bite victims. Although, the pathogenesis of ARF have been conducted in several studies (Chugh et al., 1984; Than-Than et al., 1989; Ratcliffe et al., 1989; Thamaree et al., 1994; Willinger et al., 1995; Tin-Nu-Swe et al., 1993; Suwansrinon et al., 2007), the specific component of Russell's viper venom causing ARF remains obscure.

In this study, we separately expressed rRVV-XH and rRVV-XL from (LC1) proteins in *E. coli* BL21 for the production of the rabbit recombinant proteins-specific antibodies. The expression yield of rRVV-XH and rRVV-XL in *E. coli* BL21 was low and had no coagulation function (Table 8). Gowda et al. (1996) have demonstrated that the conformational changes in the RVV-X molecule by removal of the core sugars with N-glycanase causes a virtually complete loss of factor X activating capacity. In addition, horse anti-cRVV IgG antibody was unable to recognize both recombinant proteins (data not shown). These results strongly suggest that the expression of RVV-X including one heavy chain and two light chains with the correct conformation could be necessary for its coagulant activity.

However, these recombinant proteins were useful for rabbit immunization. We found that these recombinant proteins were immunogenic in rabbits. The antibody specific activities for purified rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies increased by a factor of 40 and 30 respectively when purified (Table 9), which demonstrates the ability of rRVV-XH and rRVV-XL to produce antibody response in rabbits. It is not surprising that rRVV-XH had a much higher specific activity antibody response than rRVV-XL by a factor 4.8, since the molecular weight of rRVV-XH is approximately 3 times higher than rRVV-XL. Higher molecular weight antigens have more antigenic determinant sites and would produce higher titers of antibodies.

Rabbit anti-rRVV-XH IgG antibody recognized the same protein bands with molecular weight of about 55 and 90 kDa of cRVV and purified RVV-X using Western

blot analysis (Figure 18). The protein band at 90 kDa is the intact protein of RVV-X. The protein band at 55 kDa is possible to be the heavy chain of RVV-X. Two light chains of RVV-X might be cleaved due to the long-term storage. Kroon et al. (1992) have demonstrated that the monoclonal antibody OKT3 stored at 2-8°C for 9 months was degraded by the oxidation of cysteine and several methionines.

Although, these rabbit anti-recombinant proteins IgG antibodies can bind to purified RVV-X, neither of rabbit anti-recombinant proteins IgG antibodies or mixing both antibodies were capable of neutralizing the factor X activator activity of purified RVV-X. This suggests that at least antibody was produced to antigenic site for both recombinant proteins but neutralization of RVV-X might require correct conformation of the whole RVV-X molecule. Morita (1998) suggested that the two light chains of RVV-X serves as an exosite by which RVV-X recognizes and binds to the Gla domain of factor X (Figure 7). Our study, only one light chain of RVV-X (LC1) was expressed and immunized in rabbit. The construction of three chains of RVV-X in one expression cassette might be needed to produce a neutralizing antibody for RVV-X.

The fact that rabbit anti-recombinant proteins IgG antibodies cross reacted with *C. albolabris* and *C. rhodostoma* venoms is not surprising as snake venom metalloproteinase (SVMP) (Jia et al., 1996; Ramos and Selistre-de-Arujo, 2006; Fox and Serrano, 2008) and C-type lectin (Lu et al., 2005; Morita, 2005) are common venom components of most vipers. Rabbit anti-rRVV-XH IgG antibody was able to bind to a 18 kDa protein in *C. rhodostoma* venom and a number of proteins in *C. albolabris* venom. A snake venom metalloproteinase, kistomin (25 kDa), has been identified in *C. rhodostoma*, can cleave platelet glycoprotein VI and impairs platelet functions (Hsu et al., 2008). A 60 kDa metalloproteinase alborhagin, platelet membrane glycoprotein VI (GPVI) agonist, was also found in *T. albolabris* venom (Andrews et al., 2001; Wijeyewickrema et al., 2007). In addition, previous studies have reported that *C. rhodostoma* venom had factor X activator activity (Dambisya et al., 1994; Yamada et al., 1997). Factor X activators are present in the snake venoms of genera Bothrops (Nahas et al., 1979; Hofmann and Bon, 1987), Vipera (Komori et al., 1990; Samel and Siigur, 1995), and Cerastes (Franssen et al., 1983; Farid et al., 1993). These molecules vary widely in molecular weights from 12 to 102 kDa. These findings suggest that these

venom components with immunological cross-reactivity to anti-rRVV-XH IgG antibody could be metalloproteinases and/or factor X activator.

Rabbit anti-rRVV-XL IgG antibody was able to bind to low molecular weight proteins in *C. rhodostoma* and *C. albolabris* venoms. Previous studies have reported that *C. rhodostoma* venom contains a C-type lectin aggretin (28 kDa), which is a potent platelet aggregation inducer (Huang et al., 1995; Navdaev et al., 2001). The venom C-type lectin with similar function, a 25 kDa alboaggregin-B, was also found in *C. albolabris* venom (Peng et al., 1991; 1992; Yoshida et al., 1993; Usami et al., 1996). These venom components with immunological cross-reactivity to anti-rRVV-XL IgG antibody could be the C-type lectin molecule. Once purified, characterized, and cloned of venom components with immunological cross-reactivity to anti-recombinant proteins IgG antibodies could have potential applications in medicine. By this approach, anti-rRVV-XH and rRVV-XL IgG antibodies could be used to purify snake venom molecules by affinity chromatography as a first step in purification of factor X activator and other cross reacting molecules.

In this study, purified RVV-X was investigated to determine its effects on renal hemodynamics and functions, renal histopathology, and DIC. RVV-X was purified from cRVV, and then the purity and molecular weight of purified RVV-X were determined. RVV-X was purified fourfold from crude RVV with approximately 5% yield of total venom protein. The purification process was summarized in Table 10. The molecular weight of purified RVV-X is similar to RVV-X that was reported by Gowda (1994), who showed that RVV-X (Mr = 92,880 Da) contains a heavy chain of 57,600 Da, and two light chains of 19,400 and 16,400 Da, respectively. Purified RVV-X had a clotting time 1.7 times shorter than that of crude venom. The coagulant activity of crude venom was the result of RVV-X in crude venom. Purified RVV-X also activated factor X deficient plasma, which may be due to the presence of factor X at very low levels. In addition, purified RVV-X was able to activate human factor X (Figure 31) and protein C (Figure 32). Interestingly, purified RVV-X was capable of hydrolyzing both Aα and Bβ chains of fibrinogen, but Bβ chain was much slower than the Aα chain (Figure 33 and 34). This is the first report of the fibrinogenolytic activity of purified RVV-X from crude *D. r. siamensis* venom. These results confirmed that purified RVV-X possesses both coagulant and

anticoagulant properties, which were consistent with the findings of other studies (Kisiel et al. 1976a; 1976b; Hutton and Warrell, 1993). Factor X activator from *Vipera berus berus* was also found to have the multiple actions on azocasein, gelatin, and Aα chain of fibrinogen (Samel et al., 2003).

Interestingly, the short term study of purified RVV-X on DIC has been shown two peaks of the plasma D-dimer levels. The finding could be caused by the coagulant and anticoagulant properties of purified RVV-X. Purified RVV-X directly converts FX and FIX to activated form and then rapidly activates the final common pathway of coagulation (Furie and Furie, 1976; Kisiel et al., 1976; Jackson and Nemerson, 1980). The plasma D-dimer level immediately increased within 10 min after purified RVV-X injection (pink line, Figure 36). Decreased peak plasma D-dimer levels at 20 min could be resulted of the anticoagulant activity of purified RVV-X, which might be less potent than its coagulant activity. An increased plasma D-dimer levels to reach a peak at 30 min could be due to the recovery of coagulation activation of purified RVV-X. However, two different peaks of plasma D-dimer level in rats given purified RVV-X are probably due to the fibrinolytic mechanisms in host. Interestingly, plasma D-dimer level in rats receiving irrelevant antibody (ERIG) following purified RVV-X was shown a single peak at 20 min (green line, Figure 36), which differed from that in rats given only purified RVV-X. Used of normal horse IgG antibody instead of antibody against rabies (ERIG) might be further determined. Plasma D-dimer level of rats given crude venom was gradually increased to reach a peak at 45 min (blue line, Figure 36). The results suggest that crude venom contains anticoagulants or platelet aggregation inhibitors resulting in the prevention of coagulation process, but the higher plasma D-dimer levels were probably due to other coagulants promoting coagulopathy. Previous studies have indicated that *D. r. siamensis* contains RVV-X and Russell's viper venom-factor V (RVV-V), which accelerate the clotting of plasma (Hanahan et al., 1972; Kisiel et al., 1976; Takeya et al., 1992; Gowda et al., 1994). This venom also contains PLA_2, disintegrin, which inhibit platelet aggregation involving the coagulation disturbance (Prasad et al., 1996; Ondee, 2008). However, the greatly varies between plasma D-dimer level of each individual rat in the same group is presumed to be due to factors such as the genetic characteristics of the sample population and environmental factors (Figure 37-40). The thrombocytopenia

and fragmented red blood cells (schistocytes) were seen on blood smear (Figure 41). These results confirm the DIC in rats after injection of purified RVV-X, cRVV, and ERIG and purified RVV-X.

Suwansrinon et al. (2007) have reported that hemodynamic changes were possibly caused by the metalloproteinases in the venom. Metalloproteinases can activate the releasing of vasoactive mediators such as TNF-α (Moura-da-silva et al., 1996). Vasoactive substances have been suggested to correlate with the alterations in renal functions and hemodynamics (Cumming et al., 1988; Lieberthal et al., 1989; Chan et al., 1994; Lin et al., 2003). In the present study, purified RVV-X, which is a member of the venom metalloproteinase, caused the changes in renal hemodynamics and functions. The change in arterial blood pressure of the rats injected with cRVV or purified RVV-X was in the normal range (Table 13) and similar to those observed in the dogs injected with cRVV as previously described (Tungthanathanich et al., 1986; Suwansrinon et al., 2007). The blood pressure of the rats in the experiments is expected to maintain the autoregulation of GFR and ERBF. However, the decreases in ERPF and GFR were apparent in the present study. It indicates that purified RVV-X may affect the renal hemodynamics via other factors. The changes in renal hemodynamics in rats induced by purified RVV-X were similar to those changes reported in several studies of cRVV injection (Tungthanathanich et al., 1986; Suwansrinon et al. 2007). The decreases in GFR and ERPF and the increase in RVR of rats given cRVV, purified RVV-X plus ERIG and purified RVV-X alone (Table 14) are possibly due to the obstruction of renal microvasculature by fibrin thrombi and the glomerular capillary damage. The results are supported by the determination of DIC *in vitro* (Figure 36) and the pathological study (Figure 42). The highest increase in the average FF of cRVV group is supposed to be attributable to more increased vasoconstriction of the efferent arterioles than the afferent arterioles in the kidneys that may be caused by other molecules in crude venom (Lonigro et al., 1973; Terragno et al., 1977; Huang, 1984).

Decreases in the fractional excretion of all electrolytes (FE_E) in rats injected with cRVV have been reported in several studies (Tungthanathanich et al., 1986; Thamaree et al., 1994; Suwansrinon et al. 2007). In the present study, the decreases in urinary excretion of all electrolytes (U_EV) in rats receiving cRVV, purified RVV-X plus ERIG, and

purified RVV-X alone ars presumed to be due to damage in some renal tubular areas, which were correlated to renal tubular lesions (Figure 42). In addition, FE_{Mg} of the rats injected with the venoms tended to be increased indicating the appearance of tubular epithelial injury in the rats injected with cRVV, purified RVV-X plus ERIG and purified RVV-X alone (Table 15) (Futrakul et al., 2006). This finding is supported by the study of renal tubular lesions (Figure 42).

Histopathologically, minimal changes of tubular necrotic area and some fibrin deposition were found in the kidney of three groups of rats receiving cRVV, purified RVV-X, and ERIG and purified RVV-X (Figure 42A-C). These findings were in accordance with previous reports of victim autopsy (Chugh et al., 1984; Than-Than et al., 1989; Soe-Soe et al., 1993).

In addition, the inflammation reaction including inflammatory cell infiltration and vasculitis was observed in lung of the same groups of rats (Figure 43A-C). Similar results of cell infiltration have been observed in Bothrops, Tityus, and Androctonus envenomations (Lomonte et al., 1993; Farsky et al., 1997; Bertazzi et al., 2005; Adi-Bessalem et al., 2008). The activation of pro-inflammatory cytokines, especially TNF-α, IL1-β, and IL-6, plays an important role in the cell recruitment and the activation of mediators resulting in the later inflammatory response (Faccioli et al., 1990; Meki and Mohey El-Dean, 1998; D'Suze et al., 2004). Furthermore, the later inflammatory responses also play an important role in the pathophysiological disturbance (Daisley et al., 1999; Mazzei de Dàvila et al., 2002; Adi-Bessalem et al., 2008). Interestingly, our hematological data (Table 12) indicated that after 3 hr administration of cRVV, purified RVV-X, and ERIG and purified RVV-X was showed the decreases in the inflammatory neutrophils in the circulation. These findings were correlated to the infiltration of these inflammatory neutrophils in lung. Neutrophils are usually the first-responder of inflammatory cell to reach the site of inflammation.

Recently, Swirski et al. (2009) have been indicated that spleen is an organ for storage and rapid deployment of monocytes. The splenic monocytes are a resource that the body exploits to regulate inflammation. In this study, we found many large cells in spleen of all groups of rats with the exception of normal saline group (Figure 44A-E).

These results are supposed to be due to the immune response of rats against the foreign antigens.

CHAPTER VI

CONCLUSION

Recombinant proteins were separately expressed in *E. coli* BL21 and had no coagulant activities. We suggest that the expression of RVV-X including one heavy chain and two light chains with the correct conformation could be necessary for its coagulant activity. Post translational modification may also be necessary and eukaryotic vectors may be needed.

The recombinant proteins (RVV-XH and RVV-XL) were used to raise antibodies in rabbit. These rabbit anti-recombinant proteins antibodies did not inhibit purified RVV-X as measured in a chromogenic substrate assay. Rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies specifically bind to purified RVV-X and no other molecules in crude Russell's viper venom; however, these same antibodies cross-reacted with molecules in other venoms (*C. albolabris* and *C. rhodostoma*). It can be concluded that other venoms have molecules with similar antigenic determinants.

Despite non-specificity of anti-rRVV-XH and anti-rRVV-XL IgG antibodies, these antibodies could potentially be used in affinity chromatography to purify RVV-X molecules from crude venom. These antibodies are potential useful to identify factor X activator-like, metalloproteinase, or C-type lectin molecules from other snake venoms.

Based on literatures and this study, RVV-X appears to be the main procoagulant enzyme in cRVV involving coagulopathy, which might be responsible for changes in renal hemodynamics and functions. In present study, we studied the effects of purified RVV-X and cRVV on DIC, renal hemodynamics and functions, as well as the accompanying histopathological changes in SD rats. We found that SD rats receiving purified RVV-X showed DIC and simultaneously caused of renal dysfunction. These changes were correlated with the renal pathological changes. Priming rat with commercial horse anti-cRVV $F(ab')_2$ antivenom containing anti-RVV-X antibody could completely neutralize DIC, ameliorate renal dysfunction, and kidney lesion in the rats injected with purified RVV-X. In addition, other tissue lesions induced by purified RVV-X

were attenuated by anti RVV-X antibody injection. These findings suggest that RVV-X could be a major factor causing renal dysfunction through DIC, contributing the pathogenesis of ARF following Russell's viper snakebite. However, the exactly mechanism of the pathogenesis of ARF caused by RVV-X-induced renal dysfunction is considered and should be further investigated.

This research provides a better understanding of the actions of RVV-X on DIC, renal hemodynamics and functions. These might lead to the development of new therapeutic strategies of Russell's viper snakebite or other snakebites in the future. In addition, we established the rabbit recombinant proteins-specific IgG antibodies to further develop the affinity column for purification of snake molecules for drug discovery.

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APPENDICES

APPENDIX A

Research Instruments

Automatic adjustable micropipette (Eppendorf, Germany)

Automated hematology analyzer (Sysmex XS-800i/XS-1000i, Japan)

Balance (Precisa, Switzerland)

Beaker (Pyrex)

Combs (Bio-Rad Laboratories, USA)

Cover slip

DNA Thermal cycler 2400 (Perkin Elmer, Cetus USA)

Electrophoresis Chamber set (Bio-Rad Laboratories, USA)

ELISA reader (Multiskan EX, ThermoLabsystems, CA, USA)

Flask (Pyrex)

Fraction collector (ATTO minicollector, Japan)

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)

Micro-capillary reader (IEC, DAMON/IEC DIVISION)

Microcentrifuge (Eppendorf, USA)

Micro-hematocrit centrifuge (Z230H, BHG HERMLE, Germany)

Microscope (Olympus)

Microscope slides

Needle (Nipro)

Reagent bottles (Duran)

Semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, USA)

Spectrophotometer (Bio-Rad Laboratories, USA; SHIMADZU, Japan)

Syringe (Nipro)

Syringe pump (Harvard syringe pump 21)

Thermometer (Precision, Germany)

Vortex (scientific Industry, USA)

Water bath

APPENDIX B

Reagent for ammonium sulfate precipitation

Saturated $(NH_4)_2 SO_4$ solution

$(NH_4)_2 SO_4$	50	g
Distilled water	100	ml

Solution was heat 50°C and left overnight before use.

APPENDIX C

Reagent for ELISA

1. 0.15 M Phosphate buffered saline (PBS), pH 7.4

NaCl	8.0	g
KCl	0.20	g
Na_2HPO_4	1.15	g
KH_2PO_4	0.20	g

Dissolve in distilled water, adjust to pH 7.4 with HCl and adjust to 1000 ml final volume with distilled water.

2. 0.05 M Sodium carbonate/bicarbonate buffer, pH 9.6 (coating buffer)

Na_2CO_3	1.59	g
$NaHCO_3$	2.93	g
Distilled water to	1000	ml

3. Phosphate buffered saline-Tween (PBST)

Tween 20	0.5	ml
PBS to	1000	ml

4. 0.5% BSA in PBST (diluting buffer)

Bovine serum albumin	0.5	g
PBST	100	ml

5. 0.05% Tween 20 in normal saline (washing solution)

NaCl	9.0	g
Tween 20	0.5	ml
Distilled water to	1000	ml

6. 0.075 M Phosphate-citrate buffer, pH 5.0 (substrate buffer)

Na_2HPO_4	7.19	g
Citric acid	5.19	g
Distilled water to	1000	ml

7. Substrate solution

1% TMB	120	µl
Substrate buffer	12	ml
3% H_2O_2	12	µl

8. 4 N H_2SO_4

H_2SO_4	10.99	ml
Distilled water to	100	ml

APPENDIX D

Reagent for SDS-PAGE

1. 10% Sodium dodecyl sulfate (SDS) (w/v)

Sodium dodecyl sulfate	1	g
Distilled water to	10	ml

2. 10% Ammonium persulfate (APS) (w/v)

Sodium dodecyl sulfate	1	g
Distilled water to	10	ml

3. 1.875 M Tris-HCl, pH 8.8 (Resolving gel buffer)

Tris base	11.35	g

Dissolve in distilled water, adjust to pH 8.8 with concentrated HCl and adjust to 50 ml final volume with distilled water.

4. 1.875 M Tris-HCl, pH 6.8 (Stacking gel buffer)

Tris base	11.35	g

Dissolve in distilled water, adjust to pH 6.8 with concentrated HCl and adjust to 50 ml final volume with distilled water.

5. Electrode buffer

Tris base	3	g
SDS	1	g
Glycine	14.4	g
Distilled water to	1000	ml

6. Sample buffer (2X concentration)

10% SDS	2	ml
Glycerol	1	ml
1.875 M Tris-HCl, pH 6.8	0.334	ml
2-mercaptoethanol	0.5	ml
1% Bromophenol blue	0.5	ml

Dissolve and adjust the volume to 5 ml with distilled water.

7. 8% of resolving gel (1 page)

30% acrylamide:bisarylamide	1.330	ml
1.875 M Tris-HCl, pH 8.8	1.000	ml
10% SDS	0.050	ml
Distilled water	2.600	ml
10% ammonium persulphate	0.050	ml
TEMED	0.005	ml

After TEMED was added, the gel solution was immediately loaded on the vertical gel electrophoresis and overlay with water. Then the gel solution was allowed to polymerize for 60 min.

8. 12% of resolving gel (1 page)

30% acrylamide:bisarylamide	2.000	ml
1.875 M Tris-HCl, pH 8.8	1.000	ml
10% SDS	0.050	ml
Distilled water	1.930	ml
10% ammonium persulphate	0.050	ml
TEMED	0.005	ml

After TEMED was added, the gel solution was immediately loaded on the vertical gel electrophoresis and overlay with water. Then the gel solution was allowed to polymerize for 60 min.

9. 4% of stacking gel (1 page)

30% Acrylamide:Bisarylamide	0.250	ml
1.875 M Tris-HCl, pH 6.8	0.165	ml
10% SDS	0.025	ml
Distilled water	2.050	ml
10% ammonium persulphate	0.030	ml
TEMED	0.003	ml

After TEMED was added, the gel solution was immediately loaded on the vertical gel electrophoresis. The comb was gently inserted on the top of the gel to make wells for sample application. Then the gel solution was allowed to polymerize for 60 min.

10. Staining solution

Coomassie brilliant blue G250	0.25	g
Methanol	45	ml
Glacial acetic acid	10	ml
Distilled water	45	ml

11. Destaining solution

Methanol	30	ml
Glacial acetic acid	10	ml
Distilled water	60	ml

BIOGRAPHY

Miss Montmas Suntravat was born on August 11, 1980 in Bangkok, Thailand. She was graduated with the Bacherlor degree of Science in Medical Technology from Chulalongkorn University in 2000. She received scholarship from the Royal Golden Jubilee Ph.D. program for her study in Doctor of Philosophy program in Medical Microbiology at Chulalongkorn University in 2000. This scholarship covered her research working at the National Natural Toxins Research Center (NNTRC), Texas A&M University-Kingsville, USA, for 1 year in 2009.

Award:

1. Outstanding poster presentation in RGJ-Ph.D. Congress XI, Pattaya, Thailand, April 1-3, 2010.

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4. **Suntravat M**, Nuchprayoon I. A study of recombinant Russell's viper venom-factor X activator (RVV-X)-specific antibody: Neutralization and cross-reactivity with *Cryptelytrops albolabris* and *Calloselasma rhodostoma* venoms. (manuscript preparation)