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Studies on Physiological Effects, Toxinokinetics and Biological Characteristics of the Venom of *Bungarus candidus* From Different Parts of Thailand

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Animal Physiology
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Thesis Title	STUDIES ON PHYSIOLOGICAL EFFECTS, TOXINOKINETICS AND BIOLOGICAL CHARACTERICTICS OF THE VENOM OF *BUNGARUS CANDIDUS* FROM DIFFERENT PARTS OF THAILAND.
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การศึกษางูทับสมิงคลา 4 กลุ่ม แบ่งเป็นงูจากภาคตะวันออกเฉียงเหนือ ภาคใต้ ภาคตะวันออกของประเทศไทย และงูเพาะเลี้ยงเกิดจากพ่อแม่พันธุ์ภาคใต้ จากการศึกษาเปรียบเทียบลักษณะสัณฐานวิทยาของตัวงู พบว่าลำตัวมีสีเป็นปล้องดำสลับขาว แตกต่างกันในเชิงความกว้างและจำนวน ปริมาณน้ำพิษที่รีดได้ต่อตัวงูมีความสัมพันธ์กับน้ำหนักตัวมากกว่าความยาวลำตัว การตรวจการออกฤทธิ์ของเอนไซม์ต่างๆ ในพิษงู พบว่ามีฟอสฟอไลเปสเอทู อะเซทิลโคลินเอสเทอเรส แอล-อะมิโนแอซิดออกซิเดส และไฮยาลูโรนิเดส สูง อัลคาลายน์ฟอสฟอโมโนเอสเทอเรส ปานกลาง และมีฟอสฟอไดเอสเทอเรส และโปรตีนเอส ต่ำ การเปรียบเทียบองค์ประกอบโปรตีนในพิษงูบน Tricine SDS-PAGE พบว่าพิษงูจากธรรมชาติทั้ง 3 กลุ่มมีแถบโปรตีนแตกต่างจากกลุ่มงูเพาะเลี้ยงที่น้ำหนักโมเลกุล 18.1 – 41.3 kDa การเปรียบเทียบองค์ประกอบโปรตีนของพิษงูโดยวิธี RP-HPLC พบว่าพิษงูมีองค์ประกอบโปรตีนเป็นเอกลักษณ์ของงูเฉพาะตัว โดยพิษงูจากภาคตะวันออกเฉียงเหนือคล้ายคลึงกับพิษงูจากภาคตะวันออก และพิษงูจากกลุ่มงูเพาะเลี้ยงคล้ายคลึงกับพิษงูจากภาคใต้ แสดงถึงความเชื่อมโยงทางวิวัฒนาการของพิษงูมีความเกี่ยวข้องกับแหล่งภูมิศาสตร์ที่งูอาศัยอยู่ในธรรมชาติ

การทดสอบฤทธิ์ของพิษงูทับสมิงคลาต่อความเปราะของเม็ดเลือดแดงกระต่ายซึ่งเก็บในสารกันเลือดแข็งต่างกัน แสดงผลเป็นค่าความเข้มข้นของสารละลายโซเดียมคลอไรด์ (% NaCl) ที่ทำให้ปริมาณเม็ดเลือดแดงแตก 50% (mean corpuscular fragility; MCF) พบว่าพิษงูทำให้เม็ดเลือดแดงที่เก็บในสารกันเลือดแข็ง heparin มีค่า MCF (0.73 – 0.74 % NaCl) แตกต่างอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับเม็ดเลือดแดงที่เก็บในสารกันเลือดแข็ง EDTA (MCF 0.48 % NaCl) การทดสอบฤทธิ์ของพิษงูต่อกล้ามเนื้อร่วมประสาทของหนูขาวพบว่าพิษงูจากธรรมชาติทั้ง 3 กลุ่มที่ความเข้มข้น 3.5 และ 7 µg/ml ออกฤทธิ์ยับยั้งการหดตัวของกล้ามเนื้อร่วมประสาทในเวลาที่แตกต่างอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับพิษของงูเพาะเลี้ยง การทดสอบการยับยั้งฤทธิ์ของพิษงูต่อกล้ามเนื้อร่วมประสาทด้วยเซรุ่มแก้พิษงูทับสมิงคลา พบว่าการให้เซรุ่มทันทีหรือการบ่มพิษงูกับเซรุ่ม (1: 4.8 w / v) ในหลอดทดลองก่อนทดสอบกับกล้ามเนื้อร่วมประสาทที่ได้รับพิษงู มีผลยับยั้งการออกฤทธิ์ของพิษงูได้ดีกว่าการให้เซรุ่มหลังจากพิษงูออกฤทธิ์ยับยั้งการหดตัวของกล้ามเนื้อร่วมประสาทไปแล้ว 50%

การทดสอบฤทธิ์ของพิษงูทับสมิงคลาในกระต่าย เพื่อสังเกตการณ์เปลี่ยนแปลงทางสรีรวิทยาของระบบไหลเวียนเลือดทั่วไป ระบบไหลเวียนเลือดในไต และพิษจลนศาสตร์ พบว่าค่าเฉลี่ยความดันเลือด และอัตราการเต้นของหัวใจลดลงอย่างชัดเจนภายใน 2-5 นาทีหลังการฉีดพิษ ซึ่งค่าเฉลี่ยความดันเลือด ลดลงเพียงระยะสั้นจากนั้นค่อยๆ ปรับสูงขึ้นภายใน 30 นาทีแต่ยังอยู่ในระดับต่ำกว่าปกติ การลดลงของความดันเลือดและอัตราของปริมาณเลือดจากหัวใจตลอดระยะการให้พิษทำให้การไหลเวียนเลือดในไต อัตราการกรองผ่านไต และสัดส่วนปริมาณเลือดไหลสู่ไตต่อปริมาณออกจากหัวใจ มีแนวโน้มลดลง ในขณะที่แรงต้านทานในหลอดเลือดไตเพิ่มขึ้นในช่วง 150 นาทีหลังการฉีดพิษ การเปรียบเทียบพิษจลนศาสตร์ของพิษงูที่ความเข้มข้น 50 และ 150 µg/kg โดยการตรวจวัดปริมาณของพิษงูในพลาสมากระต่ายที่เวลาต่างๆ โดยวิธี Sandwich ELISA พบว่าพิษงูทับสมิงคลามีการกระจายของพิษจลนศาสตร์เป็นแบบ bi-compartmental model และค่าพารามิเตอร์ต่างๆ ของพิษจลนศาสตร์ของพิษงูทั้งสองระดับความเข้มข้นไม่มีความแตกต่างกัน นอกจากนี้ยังตรวจพบพิษงูระดับต่ำในปัสสาวะกระต่ายในช่วง 5 – 120 นาทีหลังการฉีดพิษ แสดงว่ามีการขับถ่ายพิษงูบางส่วนผ่านทางระบบไต จากผลการศึกษาชี้ให้เห็นว่าพิษงูทับสมิงคลามีความเป็นพิษต่อเซลล์โดยตรง พิษต่อระบบประสาทและระบบควบคุมการทำงานของร่างกาย โดยเฉพาะอย่างยิ่งต่อการทำงานของระบบหัวใจหลอดเลือดและการทำงานของไต

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KEYWORDS: PHYSIOLOGICAL EFFECTS/ TOXINOKINETICS/ BIOLOGICAL CHARACTERISTICS/ SNAKE VENOM/ *BUNGARUS CANDIDUS*

LAWAN CHANHOME: STUDIES ON PHYSIOLOGICAL EFFECTS, TOXINOKINETICS AND BIOLOGICAL CHARACTERISTICS OF THE VENOM OF *BUNGARUS CANDIDUS* FROM DIFFERENT PARTS OF THAILAND. THESIS ADVISOR: PROF. NARONGSAK CHAIYABUTR, Ph.D., THESIS CO-ADVISOR: PROF. VISITH SITPRIJA, Ph.D., 205 pp.

The studies in four groups of *Bungarus candidus* from northeastern, southern and eastern Thailand, and captive born group were carried out. The determinations of morphology of *Bungarus candidus* from different localities of Thailand and a captive-born group revealed color variation of the typical black and white bands from individual to individual. The same total length of snakes was shown the difference in the body weight without the correlation to the sexes. The liquid venom yield per individual snake was mainly correlated to its body weight. The biological characteristics of all venom groups demonstrated the different enzymatic activities of the high activities of phospholipase A_2, acetylcholinesterase, L-amino acid oxidase and hyaluronidase; the moderate activity of alkaline phosphomonoesterase and the low activities of phosphodiesterase and protease. The SDS-PAGE profiles for protein compositions showed the quantitative differences of the protein bands at the molecular weight from 7.1 to 41.3 kDa in all venom groups and from 18.1 – 41.3 kDa between the wild-caught and a captive-born venom groups. All major venom proteins on RP-HPLC were subjected to individual variation within each geographic population.

In vitro studies for the effect of venom on hemolysis and the osmotic fragility of rabbit red blood cells revealed the significant difference of MCF values between venom-treated heparinized blood (0.73 – 0.74% NaCl) and venom-treated EDTA blood (0.48% NaCl). The study for neurotoxic effect in mouse phrenic nerve diaphragm preparation demonstrated that at the venom concentration of 3.5 and 7 µg/ml, all wild-caught venom groups were significantly more potent in inhibited indirectly evoked twitches blockade than that of a captive-born venom group. For the studies of neutralization, the neurotoxicity of *B. candidus* venom was effectively attenuated by administration of antivenom promptly (t_0) with venom or by pre-incubation of venom and antivenom (1:4.8 w/v). The administration of antivenom at t_{50} blockade produced the different percentage of reversal of the twitch blockade.

In vivo studies in rabbits for the effect of venom on cardiovascular functions, renal hemodynamics and toxinokinetics demonstrated an immediate drop in systemic arterial blood pressure (MAP) and heart rate. The reduction of MAP persisted for a short duration and then gradually improved to approach the control level within 30 min. The falls in renal blood flow and glomerular filtration rate with an increase in renal vascular resistance (RVR) accompanied with hypotension and the reduction of cardiac output were apparent until 150 min after envenomation. The renal fraction tended to decrease, but not significantly. The venom kinetic study in rabbits after intravenous injection of *B. candidus* venom at the doses of 50 and 150 µg/kg showed that venom kinetics were fitted in bi-compartmental open model without significant differences between two venom groups for the rate constants (α and β), the half-life ($T_{1/2}$), the mean residence time (MRT), the volume of distribution (Vd) of α phase and β phase, the volume of distribution at steady state (Vdss) and total body clearance (CL_T). The significant differences revealed in the values of A and B intercepts and the area under the curve (AUC). The venom in urine was detected by ELISA technique at the given times between 5 – 120 min after envenomation. These findings demonstrate that body responses to envenomation by *B. candidus* bite not only affect to cell injury directly but also neurotoxic effect and systemic effects, particularly on cardiovascular and renal functions.

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LIST OF ABBREVIATIONS

AChE = acetylcholinesterase
BC = *Bungarus candidus* snake
BC-AV = monospecific *Bungarus candidus* antivenom
BC-CB = the captive-born *Bungarus candidus* snake
BC-E = *Bungarus candidus* snake from eastern Thailand
BC-NE = *Bungarus candidus* snake from northeastern Thailand
BC-S = *Bungarus candidus* snake from southern Thailand
BCV = *Bungarus candidus* venom
BSA = bovine serum albumin
BTC = butyrylthiocholine
BuTx = bungarotoxin
$CaCl_2$ = calcium chloride
Cin = clearance of inulin
Cl^- = chloride ion
C_{H2O} = free water clearance
cm = centrimeter
C_{OSM} = Osmolal clearance
CO = cardiac output
CO_2 = carbon dioxide
C_{PAH} = clearance of *p*-aminohippuric acid
CPK = creatine phosphokinase
DS = direct stimulation to the diaphramatic muscle
EDTA = ethylenediaminetetraacetic acid
ELISA = enzyme linked immunosorbent assay
ERBF = effective renal blood flow
ERPF = effective renal plasma flow
FE_E = fractional excretion of electrolytes
FF = filtration fraction
GFR = glomerular filtration rate

g %	=	gram per deciliter
Hb	=	hemoglobin
Hct	=	hematocrit
HR	=	heart rate
H_2O_2	=	hydrogen peroxide
HYA	=	hyaluronidase
Hz	=	hertz, the number of complete cycles per second
IgG	=	immunoglobulin G
IS	=	indirect stimulation to the phrenic nerve
K^+	=	potassium ion
kg	=	kilogram
KCl	=	potassium chloride
KH_2PO_4	=	potassium dihydrogen phosphate
LAAO	=	L-amino acid oxidase
LDH	=	lactate dehydrogenase
LD_{50}	=	lethal toxicity
LVW	=	left ventricular work
Na^+,	=	sodium ion
NaCl	=	sodium chloride
$NaHCO_3$	=	sodium carbonate
ng	=	nanogram
nm	=	nanometer
NSS	=	normal saline solution
MAP	=	mean arterial blood pressure
MCF	=	the mean corpuscular fragility
MCV	=	the mean corpuscular volume
$MgSO_4$	=	magnesium sulfate
mg	=	milligram
min	=	minute
ml	=	milliliter

mmHg = millimeters of mercury, a unit of pressure equal to 0.001316 atmosphere
mM = millimolar
mm = millimeter
mOsm = milliosmol
ms = millisecond
O_2 = oxygen
OPD = o-phenylenediamine dihydrochloride
PBS = phosphate buffer saline
Pd = diastolic blood pressure
PDE = phosphodiesterases
per. comm.. = personal communication
PME = phosphomonoesterases
P_E = concentration of plasma electrolytes
Pin = plasma inulin concentration (mg/ml)
PLA_2 = phospholipase A_2
PNDp = the phrenic nerve-diaphragm preparation
P_{OSM} = plasma osmolality
P_{PAH} = plasma *p*-aminohippuric acid concentration
PRO = Proteases
Ps = systolic blood pressure
RF = renal fraction
rpm = round per minute
RVR = renal vascular resistance
SGOT = serum glutamic oxaloacetic transaminase
SGPT = serum glutamic pyruvic transaminase
Sr^{2+} = strontium ion
$SrCl_2$ = Strontium chloride
SV = stroke volume
TPR = total peripheral resistance
T-PBS = phosphate buffer saline pH 7.2 containing 0.05 % of Tween

Tween20	=	polyoxyethylene-sorbitan monolaurate
U_E	=	concentration of urinary electrolytes
Uin	=	urinary inulin concentration (mg/ml)
U_{PAH}	=	urinary *p*-aminohippuric acid concentration
U_{OSM}	=	urine osmolality
V	=	urine flow
v/v	=	volume by volume
w/v	=	weight by volume
µEq	=	micro equivalent
µg	=	micro gram
µl	=	micro liter
^oC	=	degree Celsius
%	=	percentage
α	=	alpha
β	=	beta
γ	=	gamma
κ	=	kappa

CHAPTER I

GENERAL INTRODUCTION

Venomous snakes produce their venoms from specialized venom glands, which are evolutionarily related to maxillary salivary glands. Snake venoms are heterogeneous compounds of proteins and peptides that have marked variations in biological functions, induction of toxicities, including immobilization and digestion of the prey (Kochva, 1987; Koh, Armugam, and Jeyaseelan, 2006). Differences in biological properties of snake venoms have been widely studied. Interspecies and intraspecies variations of snake venoms have been reported, which based on the differences in clinical observations and then through rudimentary experiments with conflicting results (Chippaux, William, and White, 1991). Several reports have described the variation in venom biological properties which depend on geographical differences (Jayanthi and Gowda, 1988; Daltry, Wüster, and Thrope, 1997; Shashidharamurthy et al., 2002; Tsai et al., 2003; Salazar et al., 2007; Shashidharamurthy and Kemparaju, 2007), the season of venom collection (Chaudhuri, Maitra, and Ghosh, 1971; Monteiro et al., 1998; Magro et al., 2001), sexes and the age of snakes (Tun-Pe *et al.,* 1995; Daltry et al*.,* 1996 & 1997; Saldarriaga et al., 2003; Furtado, Travaglia-Cardoso, and Rocha, 2006; Menezes et al., 2006). Because of the interspecies and intraspecies variations in the snake venom components, the treatment of envenoming is best achieved by the administration of monospecific or polyspecific antivenoms. Monospecific antivenoms are used preferentially if the envenomed patient properly identifies the snake responsible for the symptoms characterized within the region (Jones, Lee, and Landon, 1999).

Bungarus candidus (Malayan krait) is a medically important venomous snakes distributed widely throughout Southeast Asia. However, few data are available on the comparative determination of the morphology of *B. candidus* snakes from different parts of Thailand. The knowledge of geographical and individual intraspecific variability of snake venom may be implied to the selection of pooled venom for antivenom manufacturer and the approval of snakebite treatment (Gutiérrez et al., 2009). Thus, the initial work of this thesis was concerned with the study of the

morphology of *B. candidus* snake from different parts of Thailand and its venom collection (Chapter IV).

Envenomation by snakebites represents a serious public health problem in tropical countries due to their high incidence, severity and sequelae. Several studies have reported that the envenoming by snakebites is a complex process containing many components which may affect local injury, neurological, hematological, cardiovascular and renal functions disturbances after snakebites in the victim. It has been known that more than 39 different enzyme activities have been detected in snake venoms. A number of studies are available for several enzyme activities in snake venoms which act differentially of the body responses (Kumar and Elliot, 1973; Tu and Hendon, 1983; Tan and Tan, 1987 & 1988; Pukrittayakamee et al., 1988; Dixon and Harris, 1999; Du and Clemetson, 2002; Harris, 2003; Koh et al., 2006). Some of these enzymes are characteristic constituents of venoms from certain snake families or genera. Protease including kallikreins, hemorragins, enzymes affecting the blood clotting cascade and hydrolases of arginine esters are predominantly found in the venoms of Viperidae. Whereas phospholipase B (or lysophospholipase) and acetylcholinesterase are mainly found in the venoms of Elapidae, with the latter enzyme specifically in cobra (*Naja, Hemachatus*) and krait (*Bungarus*) venoms. The other enzymes such as phospholipase A_2 (PLA_2), L-amino acid oxidase, 5'-nucleotidase, several phosphoesterases, and hyaluronidase are found in most venom (Mebs, 1998). However, the biological components of *B. candidus* venom from snakes distributed in different geography are not clear. The studies of the biological characteristics of venoms, the lethal toxicity**,** the enzymatic activities, and the molecular weight of protein components of the venoms of *B. candidus* snakes from three different localities in Thailand and the captive-born snakes were clarified in Chapter V.

The Elapid snake venoms are mainly consisting of neurotoxins which cause the well known feature of systemic neurotoxicity in envenoming by *B. candidus* bite (Warrell et al., 1983; Pochanugool et al., 1997; 1998). The onset of neurotoxic symptoms can appear within minutes or delay for hours. Few data are available for the details of the fatalities of *B. candidus* envenoming especially the knowledge of the biological components in venom. The first clinical data envenoming by *B. candidus*

from eastern Thailand and northwestern Malaysia was described (Warrell, et al., 1983), with one patient responded to the treatment by Haffkine's polyvalent antivenom. The survey throughout Thailand in 1980, *B. candidus* was responsible for 28% of fatal snake bites which respiratory failure is the major cause of death (Looareesuwan, Viravan, and Warrell, 1988). Some of the patients gradually recovered after respiratory support and other supportive treatment (Pochanugool, et al., 1997; Kanchanapongkul, 2002). The decreased parasympathetic activity in 3 victims envenoming by the Malayan krait from northeastern Thailand manifested by mydriasis, prolong hypertension and tachycardia (Laothong and Sitprija, 2001). The autonomic disturbances of transient hypertension, tachycardia, lacrimation, sweating, and salivation also manifested in 66% of patients with moderate to severe envenoming by common krait (*Bungarus caeruleus*) in Sri Lanka (Kularatne, 2002).

The secreted phospholipases A_2 are the most common enzyme found in various snake venoms isolated from the snakes in Families Elapidae, Viperidae, and Crotalidae. The venom PLA_2s possess a digestive function and the wide array of pharmacological actions such as antiplatelet, anticoagulant, hemolytic, neurotoxic, myotoxic, edema-inducing, hemorrhagic, cytolytic, cardiotoxic, and muscarinic inhibitor activities (Harris, 1991; Kini, 2003). However snake venom PLA_2s mostly express their pharmacological effects on their own and some exhibit the full potency of their pharmacological effects only when they form a complex, for example β-bungarotoxin (Kini, 2003). Therefore more data are required to elucidate the effects of *B. candidus* venom on the cytotoxicity and the neurotoxicity (Chapter VI).

Recently, the observation throws some doubts on the action of *B. candidus* venom in additional clinical sign of cardiovascular and kidney function disorders (Wirat Leeprasert, 2008 personal communication). Acute renal failure is a frequent complication observed in victims of snakebites. The pathogenesis of acute renal failure after snake bites usually appears to be multifactorial. However, little is known about the sequential pathophysiological changes of renal function after envenomation. More information is required to study the alterations in general circulation and renal hemodynamics induced by the *B. candidus* venom in the experimental animal (Chapter VII). However, few data are available in aspects of physiological effects and toxinokinetics of the *B. candidus* venom which mainly studied on its compositions,

sequence of amino acid, toxic and pharmacological properties (Abe, Alema, and Miledi, 1977; Tan, Poh, and Tan, 1989; Chu, Li, and Chen, 1995; Dixon and Harris, 1999; Kuhn et al., 2000; Torres et al., 2001; Tsai, Hsu, and Wang, 2002; Khow et al., 2003; Kuch et al., 2003). The envenomed victims by *B. candidus* bite responded irregularly to the treatment by monovalent specific *B. candidus* antivenom depending on the severity of neurotoxicity and the given time of antivenom treatment. Thereafter the study of the toxinokinetics of *B. candidus* venom was elucidated in experimental animals in Chapter VIII.

Review of the literature for the previous work concerned with *B. candidus* snake and the action of its venom is in Chapter II. The methodology used in this work is described in Chapter III. The overall effects of *B. candidus* venom on physiological changes both *in vitro* and *in vivo* studies are discussed and concluded in Chapter IX.

CHAPTER II

REVIEW OF THE LITERATURE

Since the main emphasis of the work described in this thesis has been directed towards the snake. This review deals largely publications relevant to *Bungarus candidus* and its venom.

2.1 Characteristics of *Bungarus candidus* Linnaeus, 1758 (Malayan krait)

There are 196 species and subspecies of snakes presently indigenous to Thailand, and 59 species and subspecies would qualify as venomous snakes in Families Elapidae and Viperidae, of which the approximately 17 species and subspecies are aware as medically important (Chanhome and Pauwels, 2007). *Bungarus candidus* Linnaeus, 1758 (Malayan krait) is the smallest species of genus *Bungarus* (the Kraits) found in Thailand. The other two species are *Bungarus fasciatus* (Banded krait) and *Bungarus flaviceps* (Red-headed krait). The maximum length of *B. candidus* is 155 cm, with adults averaging 100 cm (David and Vogel, 1996). The body and tail are cross-banded with black bands, the ventral surface is white. The head is black or grayish black above, the color extending posterior to the first black mark on the nape so that an indistinct V-shaped mark is present. *B. candidus* is distinguished from all of the similar-colored non-venomous snakes by its enlarged vertebrals (Soderberg, 1973). This nocturnal and terrestrial snake feeds on cold-blooded vertebrates, primarily other snakes but also lizards, toads and frogs. It is oviparous, laying 4–10 eggs per clutch. This snake is widely distributed without geographical or biological zone in northeastern, eastern, southern and western Thailand. Its foreign range includes southern Vietnam, Laos, Cambodia, and peninsular Malaysia and extends through Singapore and the Indonesian Islands of Sumatra, Java, Bali, and Sulawesi. (Chanhome et al., 1998; 2001; Cox, 1991; Cox et al., 1998; David and Ineich, 1999; David and Vogel, 1996; Jintakune and Chanhome, 1996; Manthey and Grossmann, 1997).

2.2 Envenoming by snakebites

Venomous snakes are widely distributed throughout the world and are found on every continent except frozen Antarctica and the colder elevations of the remaining continents. Envenoming by snakebite is an important public health problem in the rural tropics where the human populations experience high morbidity and mortality. Many of the snakebite victims in these areas mainly seek for traditional treatments; therefore the mortality rate is probably underestimated. Clinical features of envenomed victims are involved in different organs and tissues. Severe cases mostly result from bites by snakes of the families Viperidae (pitvipers and true vipers) and Elapidae (cobras, kraits, mambas, coral snakes, Australian species and sea snakes) (WHO, 2007). The greatest impact of venomous snakes is felt in South Asia, Southeast Asia, and sub-Saharan Africa, where estimates of at least 421,000-1,841,000 envenomations and 20,000-94,000 deaths occur annually (Kasturiratne et al., 2008).

Toxic constituents in snake venom can cause a wide array of physiological effect to envenomed victims from local reaction with pain and swelling to severe systemic response and vital organ involvement (Sitprija and Gopalakrishnakone, 1998). Early systemic symptoms and signs of envenoming by Elapid snakes manifest tremor, marked salivation, including drowsiness. Thereafter the neurologic manifestations are usually evidenced by ptosis, dysarthria, dysphagia, dyspnea and respiratory paralysis (Gold, Dart, and Barish, 2002). Myotoxic venom is also observed in Australian Elapid snakes such as *Notechis scutatus, Oxyuranus scutellatus, Psuedechis colletti, P. affinis, P. porphyriacus, Pseudonaja textiles*, including some of the kraits such as *Bungarus multicinctus* and *Bungarus caelureus.* Myotoxin in these snake venoms causes rhabdomyolysis (Sitprija and Gopalakrishnakone, 1998). Therefore, not only neurotoxicity but also myotoxicity should be bewared in some case of snakebite by Elapid snakes.

Envenoming by the Malayan krait (*Bungarus candidus*)

Bungarus candidus venom possesses a powerful neurotoxin that causes severe neurotoxicity (Warrell et al., 1983; Pochanugool et al., 1997; 1998). The onset of neurotoxic symptoms can appear within minutes or be delayed for hours. Few data are available for the details of the fatalities of *B. candidus* envenoming because bites

mainly occur in rural areas at night. Krait bites happen with a quick snapping motion by the snake and initial lack of pain with little or no local edema or discoloration in the victim. Most envenomed victims awaken with suspicious morning weakness, and loin or abdominal pain. There is a slow onset of serious weakness and it is difficult to identify the site of the bite. Therefore, medical attention is usually delayed (Goonetilleke and Harris, 2002).

Paralysis of respiratory muscles is a potentially fatal manifestation of this snake's bite. It is especially notorious for rapid development of respiratory failure and peripheral sensory neuropathy in some case. Patients with respiratory failure were successfully managed with the help of mechanical ventilation. This emphasizes the importance of anticipation of this complication and timely intervention. The treatment of patients with neurotoxic envenomation with anticholinesterases is debatable as some studies show favorable results while others do not (Sereviratne and Dissanayake, 2002).

The first clinical data of five cases of envenomation by *B. candidus* from eastern Thailand and northwestern Malaysia was described (Warrell, *et al*., 1983). One patient responded to the treatment with Haffkine's polyvalent antivenom produced from the venoms of *Bungarus caeruleus, Naja naja, Echis carinatus* and *Vipera russeli* (presently known as *Daboia russelii*). In a survey of 15 provincial hospitals throughout Thailand between October and December 1980, *B. candidus* was responsible for 13 of 46 cases (28%) of fatal snake bites with the major cause of death as follow; respiratory failure (13/13; 100%), pneumonia (6/13; 46%) and septicemia (2/13; 15%) (Looareesuwan *et al.,* 1988). Some of the patients gradually recovered after receiving respiratory support and other supportive treatment (Pochanugool, et al., 1997; Kanchanapongkul, 2002). Laothong and Sitprija (2001) suggested the clinically dominated symptoms by presynaptic neurotoxin in three victims envenoming by *B. candidus* from northeastern Thailand.

Early systemic manifestations of envenoming by *B. candidus* included headache, nausea, vomiting and myalgia. Within six hours after bites, patients developed ptosis, opthalmoplegia, diplopia, had difficulty swallowing, increased myalgia, blurred vision, occulomotor palsies, dysarthria, dysphagia, diffuse muscle weakness to generalized flaccid paralysis, and ended up with respiratory failure which

was the major cause of death. Physical examination revealed normal pulse, respiratory rate, blood pressure and temperature. The laboratory findings yielded normal results of hematocrit, complete blood count, prothrombin time, urinalysis, blood urea nitrogen (BUN), creatinine, SGOT, SGPT, alkaline phosphatase, serum bilirubin and serum electrolytes (Na^+, K^+, and Cl^-). The bite sites were virtually painless with adjacent minimal erythema and mild or no swelling (Pochanugool et al., 1997; Kanchanapongkul, 2002). The interval time of bite to hospital admission was 1–7 hours with the progressive paralysis upon arrival to the hospitals. The interval time of bite to death was 3–288 hours (Looareesuwan et al., 1988).

In comparison with the other species of *Bungarus* snakes, autopsy study was performed in the human deaths envenomed by *Bungarus caeruleus* (Common krait) bite in Sri Lanka. In the acute deaths that died within 24 hours, one showed the histological changes of acute renal tubular necrosis and pan lobular microvesicular fatty changes in the liver, and the other showed petichial haemorrhages in the myocardium, renal cortex and adrenal gland macroscopically. One of the late deaths revealed congestion of kidneys with cortical haemorrhage microscopically (Kularatne and Ratnatunge, 2001). Inoculation of the *B. caeruleus* crude venom in rat model experiments revealed biochemical alterations such as the increase of serum aspartate aminotransferase, creatine kinase, lactate dehydrogenase, alkaline phosphatase, alanine aminotransferase, urea concentrations and induced hyperglycemia. Histopathological changes were also found, such as hemorrhage, multifocal areas of myocardial fiber necrosis and constriction of blood vessels in the heart; congestion of the vessels, hemorrhage and necrosis of proximal tubules in the kidney; congestion and hemorrhage in the liver (Kiran, More, and Gadag, 2004; Mirajkar, More, and Gadag, 2005). A study of histopathological changes induced by the venom of *Bungarus ceylonicus* in mice revealed significant inflammatory and necrotic changes in the liver and brain, and congestion in kidneys (Nanayakkara et al., 2007).

2.3 The principle of neurotoxins in the *Bungarus candidus* venom

Snake venoms consist of numerous components such as enzymes, non-enzymatic polypeptide toxins, and non-toxic proteins (Warrell, 1996) that have different biochemical mechanisms evolved to immobilize prey and serve as a

digestive strategy that commences degradation of prey tissues internally. Snake venoms also are used in defense against predators. The principal effects of snake venoms are varied from presenting neurotoxicity, cardiotoxicity, nephrotoxicity, myotoxicity, coagulopathy, vascular endothelial damage, local reactions and muscular weakness to the envenomed patients (Warrell et al., 1983).

Studies of *B. candidus* venom, revealed that the most lethal components are the phospholipase A_2 of which presynaptically acting toxins like the beta-bungarotoxin (Bon and Saliou, 1983; Tan, et al.,1989; Tsai, et al., 2002; Khow, et al., 2002; 2003) and postsynaptically acting toxins like the alpha-bungarotoxin (Nirthanan, et al., 2002; Kuch, et al., 2003) from *B. multicinctus*, that is close phylogenetic relationship to *B. candidus* (Slowinski, 1994).

Two highly lethal toxin fractions with phospholipase A_2 (PLA_2) activity and two polypeptide toxins were identified from a commercial *B. candidus* venom of unknown origin (Tan et al., 1989). These toxins exhibited high hyaluronidase, acetylcholinesterase and phospholipase A_2; moderately high L-amino acid oxidase; low proteinase, 5'nucleotidase, alkaline phosphomonoesterase and phosphodiesterase. The X-ray crystal and Nuclear Magnetic Resonance (NMR) structure of a three-finger toxin, named "bucandin" from Balinese *B. candidus* venom were demonstrated 63 amino acid residues, which had an additional disulfide in the first loop and the unique property of enhancing presynaptic acetylcholine release (Kuhn et al., 2000; Torres et al., 2001).

The PLA_2 cDNAs from the venom gland of *B. candidus* (Bali, Indonesia origin) were amplified and cloned (Tsai et al., 2002). About 20 PLA_2 clones were found to encode the β-bungarotoxin A-chains. Two of the cDNA clones encoded a novel PLA_2 which was designated as "Bc-PL", having 25 amino acid residues and belongs to group IB of the PLA_2 subfamily. It is structurally 61% identical to the myotoxic/cardiotoxic PLA_2 from king cobra (*Ophiophagus hannah*) venom from Kunming, China and the hemorrhagic/myotoxic PLA_2 from Australian tiger snake (*Notechis scutatus scutatus*) venom. Therefore this is the first case in which a group IB PLA_2 was found in krait venom (Tsai et al., 2002).

Three β-bungarotoxin like basic neurotoxins, T1-1, T1-2, and T2, with PLA_2 activity were isolated from pooled *B. candidus* venom from southern Thailand (Khow

et al., 2003). The lethal toxicity of these neurotoxins amounted to 30.5% of that of crude venom, of which T1-1 and T1-2 are comparable to those of β1-BuTx from *B. multicinctus* venom (Kondo et al., 1982a) and the major neurotoxin from *B. flaviceps* venom (Khow et al., 2002). T2 exhibits comparable lethal toxicity of β2-BuTx (Kondo et al., 1982b) and ceruleotoxins from *B. fasciatus* venom (Bon and Saliou, 1983). These toxins consist of two polypeptide chains with molecular weights of 15.5–16.5 and 8–8.5 kDa, respectively. The amino terminal sequences of the two chains exhibited similarity with those of the A-chains and B-chains of β-BuTxs in the venom of *B. multicinctus*.

The major postsynaptic neurotoxin was purified and identified from the venom of *B. candidus* from Java (Kuch et al., 2003). This toxin was indistinguishable from α-bungarotoxin which was isolated from *B. multicinctus* venom. A phylogenetic study revealed a close relationship between *B. candidus* and *B. multicinctus* (Slowinski, 1994; Kuch et al., 2003). These outstanding studies of the toxins from *B. candidus* venom are subsequent comparable to the toxins previously identified from *B. multicinctus* as follow:

2.3.1. Presynaptic beta-bungarotoxin (β-BuTx)

Presynaptic neurotoxins have been identified in the venoms of the four major families of venomous snakes, namely Elapidae, Hydrophiidae, Viperidae, and Crotalidae. These toxins display varying PLA_2 activities primarily involved in the digestion of prey. Interestingly, the neurotoxic activity is not related directly to phospholipase activity (Harris, 1997). Presynaptic neurotoxin generally produces neuromuscular blockade by inhibiting the release of acetylcholine from the nerve terminal. Their activity is characterized by a triphasic change on evoked acetylcholine release that effect to the amplitude of end plate potential, initially a decrease phase (partial inhibition of acetylcholine release), followed by a transient increase phase (facilitation of acetylcholine release), and then complete blockade phase (progressive decline of neurotransmission) (Rowan, 2001).

β-Bungarotoxin (β-BuTx), the main presynaptic PLA_2 neurotoxin from the venom of *B. multicinctus* (Taiwan banded krait), consists of two covalently bonded subunits, the A chain (14 kDa) and B chain (7 kDa), cross-linked by an interchain disulfide bond (Abe et al., 1977; Kondo et al., 1982a; 1982b). A chain, a single

polypeptide of 120 amino acid residues, is the active subunit responsible for PLA_2 activity and neurotoxic effect (Chang and Yang, 1988; Chu et al., 1993). Whereas B chain, a smaller polypeptide of 60 amino acid residues, might be responsible for the blockade of certain voltage-gated K^+ channels that do not involve A chain (Benishin, 1990). The study in *Xenopus* nerve-muscle cultures demonstrated that both A and B chains are indispensable parts of β-BuTx and functionally involved in neurotoxic effects, the facilitation of neurotransmitter release (Liou et al., 2004).

The β-BuTx family from *B. multicinctus* venom contains more than 16 isotoxins, of which each isotoxin shows two important structural features (Chu et al., 1995). The two subunits of each isotoxin interact to stabilize the antigenic determinants of intact toxin. The correlation of the isotoxins with a common PLA_2 subunit but with different non-PLA_2 subunits is important in the role of each subunit in the PLA_2 activity-dependent and the PLA_2 activity-independent neurotoxic effects, both of which block the neuromuscular transmission.

Tseng and Lin-Shiau (2003a; 2003b) demonstrated that the β-BuTx-mediated increase in the influx of Ca^{2+} in the cultured primary cerebellar granular neurons (CGNs) induces an imbalance in mitochondrial homeostasis, leading to mitochondrial dysfunction. Moreover, the $[Ca^{2+}]_I$ elevation is a secondary messenger for triggering reactive oxygen species (ROS) production, which induces neuronal death. These findings establish a direct link between β-BuTx-induced-neurotoxicity and Ca^{2+} and mitochondrial dysfunction.

The presynaptically neurotoxic mechanism of β-BuTx was investigated and it was discovered that β-BuTx inhibits phosphorylation of synapsin I, the growth-associated protein 43 (GAP-43), and the myristolated alanine-rich C-kinase substrate (MARCKS). Inhibition of phosphorylation may be associated with its blockade of Ach release, suggesting that the PLA_2 activity of β-BuTx may not be essential for its action (Ueno and Rosenberg, 1995; 1996). Inoculation of the sublethal doses of β-BuTx from the venom of the Taiwan banded krait (*B. multicinctus*) into the hind limb of rats caused the depletion of transmitter from the motor nerve terminals and the degeneration of the motor nerve terminal and intramuscular axons (Dixon and Harris, 1999; Prasarnpun, Walsh, and Harris, 2004). This observation can explain the

severity of the neuromuscular paralysis and the difficulty in management of victims envenomed by kraits and other related snakes of the family Elapidae.

Patients bitten by snakes whose venoms contain large quantities of presynaptically active neurotoxins suffered profound neuromuscular paralysis and were extremely difficult to manage. They required long periods in intensive care (Laothong and Sitprija, 2001; Goonetilleke and Harris, 2002; Kanchanapongkul, 2002) because they did not respond to antivenom (Warrell et al., 1983; Looareesuwan et al.,1988; Viravan et al.,1992) or to procedures expected to relieve neuromuscular blockade such as anticholinesterase and diaminopyridine (Trevett et al., 1995).

2.3.2. Postsynaptic alpha-bungarotoxin (α-BuTx)

Postsynaptic α-neurotoxin has been investigated more than any other snake toxins. They are the major components of the snake venoms of the families Elapidae (e.g. cobras, kraits) and Hydrophiidae (sea snakes). The common target of α-neurotoxin is the muscle nicotinic acetylcholine receptor (nAChR) (Endo and Tamiya, 1991), a ligand-gated ion channel on the postsynaptic fold of the neuromuscular junction with the subunit stoichiometry of α2βγδ (Karlin, 1993). α-Neurotoxins prevent the binding of acetylcholine (Ach) and the subsequent Ach-induced ion flow, resulting of neuromuscular inhibition and causing paralysis in the envenomed victims. They display different binding kinetics and different affinity for subtypes of nAChR (Hodgson and Wickramaratna, 2002).

α-Neurotoxins are polypeptides with molecular weights ranging from 7 to 8 kDa and have a characteristic three-dimensional (3D) structure called the three-finger structure. To date, approximately 100 α-neurotoxins have been isolated and sequenced (Endo and Tamiya, 1991). These toxins are classified as long or short α-neurotoxins depending on the number of amino acids present. Short α-neurotoxins contain 60-62 amino acids and four disulfide bridges in common positions. Long α-neurotoxins are formed by 66-74 residues and contain another fifth disulfide bond. Long toxins associate and dissociate much more slowly than short α-neurotoxins. In the muscle-type AChR (in addition to α 7, α 8, and α 9 neuronal subtypes), the most profusely used long α-neurotoxin is the α-bungarotoxin (Arias, 2000).

α-Bungarotoxin (α-BuTx) isolated from the venom of Taiwan banded krait (*B. multicinctus*) (Mebs et al., 1972) binds to acetylcholine receptors and has been used in

experiments to demonstrate reduction in acetylcholine receptor sites in postsynaptic membrane (Drachman *et al.,* 1976). Alpha-cobra toxin which has a similar action produces features of myasthenia gravis in the experimental animals (Satyamurti, Drachman, and Slone, 1975). α-BuTx represents an interesting structural intermediate between long postsynaptic neurotoxin, particularly *κ*-neurotoxin, from the venom of *Naja haji haji* (Aird et al, 1999). It has been valuable ligands in characterizing and purifying muscle nicotinic receptors. α-BuTx also have C-termini 7–9 residues longer than that of gamma-bungarotoxin (γ-BuTx). Other differences include the N-terminal three residues and residues 53–62. In the latter segment, γ-BuTx residues Pro-53, Ser-54, Thr-60, and Phe-62 are unique.

Bucain, isolated from the venom of *Bungarus candidus*, is structurally classified as a three-fingered α-neurotoxin possessing a positively charged AChR-binding site (Murakami, Kini, and Arni, 2009). This toxin has molecular masses of approximately 6,000-8,000 Da and encompasses the potent curaremimetic neurotoxins which confer lethality to *Elapidae* and *Hydrophidae* venoms (Watanabe et al., 2002).

2.3.3. Postsynaptic gamma-bungarotoxin (γ-BuTx)

The structure of γ-BuTx, a toxin from *B. multicinctus* venom, was determined using mass spectrometry and Edman degradation. The toxin has a mass of 7,524.7 Da and consists of 68 amino acid residues. γ-BuTx is represented structurally intermediate between long postsynaptic neurotoxins, particularly kappa-bungarotoxin and the elapid toxin of unknown pharmacology, such as CM-11, from the venom of *Naja haji haji*. Its C-terminal nine residues are identical to those of the *κ*-neurotoxins. Its disulfide bond locations appear identical to those of several elapid toxins of unknown pharmacology and its hydrophobicity profile is also strikingly similar. However, with an LD50 of 0.15 mg/g intravenously in mice, γ-BuTx is 30-150 fold more toxic than other members of this latter class. Its toxicity is comparable to those of nicotinic acetylcholine receptor antagonists (Aird et al., 1999).

2.3.4. Postsynaptic kappa bungarotoxin (*κ*-BuTx)

Kappa-bungarotoxin (*κ*-BuTx), a postsynaptic neurotoxin purified from the venom of *B. multicinctus*, exhibits a potent effect to block a transmission at the neuronal nicotinic receptor in several neuronal systems. The three known κ-

neurotoxins are reported, namely kappa 2-bungarotoxin and kappa 3-bungarotoxin from *B. multicinctus* and kappa-flavitoxin from *B. flaviceps* (Chiappinelli et al., 1987; Chiappinelli and Wolf, 1989; Chiappinelli et al., 1990). *κ*-BuTx is actually more potent at neuronal nicotinic receptors than at muscle nicotinic receptors, the toxin is a valuable ligand for comparing the active sites of neuronal and muscle nicotinic receptors. The amino acid sequence of *κ*-BuTx shows greatest homology to the curaremimetic postsynaptic long neurotoxins of which α-BuTx is also a member. However, there are some striking differences between *κ*-BuTx and other members of this group which may explain its unusual ability to block neuronal acetylcholine receptors.

2.3.5. The criteria to differentiate the post-synaptic bungarotoxins

The criteria to differentiate the post-synaptic bungarotoxins involve; a) The structure of toxins, b) The amino acid sequences, c) The molecular weight and d) The site of action (binding site) at neuromuscular junction.

Alpha-bungarotoxin (α-BuTx) is polypeptide with molecular weights ranging from 7 to 8 kDa. It is a long α-neurotoxins formed by 66-74 residues and contains another fifth disulfide bond. Long toxins associate and dissociate much more slowly than short α-neurotoxins. The common target of α-BuTx is the muscle nicotinic acetylcholine receptor (nAChR), preventing the binding of acetylcholine (Ach) and the subsequent Ach-induced ion flow, resulting in neuromuscular inhibition and causing paralysis. It displays different binding kinetics and different affinity for subtypes of nAChR. α-BuTx also has C-termini 7–9 residues longer than that of *γ*-BuTx. Other differences include the N-terminal three residues and residues 53–62. In the latter segment, *γ*-BuTx residues Pro-53, Ser-54, Thr-60, and Phe-62 are unique.

Gamma-bungarotoxin (γ-BuTx) has a molecular weight of 7524.7 Da and consists of 68 amino acid residues. Eight of the cysteine residues in *γ*-BuTx occupy the same positions as those of the *κ*-neurotoxins, however the remaining two are dissimilar, implying that the three-dimensional topography of *γ*-BuTx differs from that of the *κ*-neurotoxins. Specifically, the disulfide linking Cys-30 and Cys-34 is missing. *γ*-BuTx also possesses three N-terminal residues that *κ*-toxins lack. In addition, a nine-residue segment (residues 7–15) differs from the *κ*-toxin sequences, although Phe-8 of *γ*-BuTx is quite hydrophobic, as is Ile-8 of the *κ*-toxins. This

segment contains the putative variant disulfide bond between Cys-6 and Cys-11. *γ*-BuTx lacks Arg-37 that thought to be essential for the activity of *κ*-toxins. This arginine residue is common to all *α*-neurotoxins as well, and has been implicated as essential to an effective blockade of peripheral nicotinic receptors. *γ*-BuTx possess an arginine at position 39, where all *κ*-toxins possess a proline, and where most *α*-toxins possess a lysine (position 41). *γ*-BuTx also has an arginine at position 33. Most *α*- and all *κ*-toxins have a glycine in position 40, and also possess a disulfide bond near the tip of loop II (Cys-30–Cys-34) that is lacking in *γ*-BuTx. In *α*- and all *κ*-neurotoxins this disulfide bond secures an interior three-residue loop to the descending side of loop II. It is impossible to predict the conformation of loop II of *γ*-BuTx, but it is possible that Arg-33 occupies a position in three-dimensional space relatively close to that of Arg-32 or Lys-32 in κ_1-bungarotoxin.

Kappa-Bungarotoxin (*κ*-BuTx) consists of a single polypeptide chain of 66 amino acids with a molecular weight of 7,313 Da (Grant and Chiappinelli, 1985). It contains 10 cysteinyl residues, presumably arranged in five disulfide bonds, and is completely devoid of methionine and tryptophan. *κ*-BuTx is more potent at neuronal nicotinic receptors than at muscle nicotinic receptors due to the differences of individual amino acid side-chains in the binding of *κ*-BuTx to the receptor. The complete amino acid sequence of *κ*-BuTx is clearly distinguished from the related long-type postsynaptic α-neurotoxins in having a much-reduced C-terminal tail, the lack of the α-neurotoxin-invariant Trp-26, having an invariant prolinyl residue adjacent to the invariant Arg-34/Gly-35, and a leucine residue at position 57 (Grant and Chiappinelli, 1985; Chiappinelli et al., 1996). The shortened COOH-terminal tails of *κ*-BuTx may well account for their relatively weak binding to the nicotinic receptor found at the neuromuscular junction. The α-neurotoxin-invariant Trp-26 is an important determinant of binding the toxin to muscle AChR, therefore the lack of the invariant Trp-26 in *κ*-BuTx may lead to weak binding to muscular nicotinic AchR.

The dimerization of *κ*-BuTx is important in accounting for the potency of *κ* -BuTx in autonomic ganglia. *κ*-BuTx binds to both α3-containing receptors in autonomic ganglia and α1-containing muscle receptors. There are two distinct regions of the α3-subunit where *κ*-BuTx interacts. The first region is located near N-terminus

of the subunit (51-70), while the second region is located between residues 183 and 201. The Arg-34 and Pro-36 residues in κ -BuTx are critical for binding α3-containing neuronal AChR.

2.4 Enzymes in snake venom

Enzymes are the first clearly recognized components of snake venoms. Several enzymes are discovered and involved in many levels of venom action. They may serve as spreading factors, or they may produce such as bradykinin and lysolecithins in tissues of preys or predators. Some enzymes potentiate the toxic action of others. The analysis of a single enzyme may, therefore, not fully reveal its biofunction of snake venom (Zeller, 1977). Enzymes mainly found in snake venoms are listed as follow;

2.4.1. Phospholipase A_2 enzymes (PLA_2s)

Phospholipase A_2 enzymes (PLA_2s) catalyse the hydrolysis of glycerophospholipids at the sn-2 acyl bond releasing lysophospholipids and free fatty acids (Lambeau and Lazdunski, 1999). PLA_2s enzymes were isolated from a number of snake venoms (Harris, 2003; Koh et al., 2006). Snake venom PLA_2 enzymes share similarity in structure and catalytic activity with mammalian PLA_2 enzymes (Gelb et al., 1995). In contrast to mammalian PLA_2 enzymes which are generally nontoxic, snake venom PLA_2 enzymes exhibit a diverse devastation by interfering with the normal physiological processes of the victim and inducing a wide variety of pharmacological effects such as neurotoxicity (presynaptic and postsynaptic neurotoxicities), myotoxicity (local myonecrosis and systemic myotoxicity), cardiotoxicity, anticoagulant effects, platelet aggregation initiation and inhibition, hemolytic activity, hemoglobinurea inducing activity, internal hemorrhage, convulsant activity, hypotension activity, edema inducing activity, organ or tissue damage (liver, kidney, lungs, testis, pituitary damage) (Yang, 1994; Kini, 2003). PLA_2s from different venoms or even the same venom can display in different pharmacological activities, indicating to the complication between structure and pharmacological activities of this enzyme. On the other hand, several PLA_2 enzymes can exhibit the same pharmacological effects through different mechanisms (Kini, 2003). For example, different neurotoxic PLA_2 enzymes could bind to different target proteins and exhibit similar presynaptic neurotoxic symptoms. Moreover the

interactions of PLA_2 enzymes and cardiotoxins contribute significantly to the enzymatic and pharmacological activities of PLA_2 enzymes (Condrea, Barzilay, and Mager, 1970).

2.4.2. Acetylcholinesterase (AChE)

Acetylcholinesterase (AChE) is a neurotransmitter esterase that breaks the acetate ester bond found in acetylcholine. Its main site of action is in the synapse and some vesicle-contained acetylcholine may be degraded. The end result is an inability to enervate smooth muscles and an inability to relax striated muscles resulting in spasmodic paralysis and sometimes a concurrent drop in blood pressure and difficult breathing.

Elapid snake venoms usually contain a strong AChE activity (Tan and Tan, 1988; Kumar and Elliot, 1973) which can vary significantly among the different venoms from the same species (Tan and Tan, 1987). Purified AChE from snake venoms has not contributed any lethal toxicity in its own right. It acts in co-operation with or as a facilitator for other venom components by hydrolyzing acetylcholine in terms that retard the normal physiology or defensive response of the victims. It provides a more subtle contribution to the poisoning or digestion mechanism.

2.4.3. L-amino acid oxidase (LAAO)

L-amino acid oxidase (LAAO) is widely found in many organisms including venomous snakes of the families Viperidae, Crotalidae and Elapidae. LAAO is a flavoprotein consisting of two identical subunits, with a molecular mass of approximately 110-150 kDa, which catalyses the stereospecific oxidative deamination of L-amino acid substrate to an α-keto acid along with the production of ammonia and hydrogen peroxide. The enzyme is highly specific for L-amino acids, and generally hydrophobic amino acids (e.g. L-leucine, L-phenylalanine, L-tryptophan, L-methionine and L-isoleucine) are the best substrates (Du and Clemetson, 2002). The purified enzymes are glycoproteins with 3-4% carbohydrate content. Deglycosylation of the enzyme did not alter the enzymatic activity but appeared to alter its pharmacological activities. The amino acid sequences of snake venom LAAOs showed a high degree of homology.

X-ray structural analysis of LAAO revealed a dynamic active site which consisted of three domains in each subunit: a FAD-binding domain, a substrate-

binding domain and a helical domain (Pawelek et al., 2000). LAAOs exhibit moderate lethal toxicity. Recent studies showed that LAAOs are multifunctional enzymes exhibiting edema-inducing and hemolysis (Ali et al., 2000), platelet aggregation activating (Li, Yu, and Lian, 1994; Ali et al., 2000) or platelet aggregation inhibiting (Sakurai et al, 2001; Takatsuka et al., 2001), apoptotic inducing (Torii, Naito, and Tsuruo, 1997) as well as anti-bacterial effect (Stiles, Sexton, and Weinstein, 1991; Tõnismägi et al., 2006), anti-coagulant effect (Sakurai et al. 2003) and anti-HIV effects (Zhang et al., 2003). All of these effects are thought to be at least partly related to H_2O_2 production because catalase, an H_2O_2 scavenger, inhibits the actions of venom LAAOs (Du and Clemetson, 2002). A better understanding of the pharmacological actions of LAAOs will facilitate the application of snake venom LAAOs in the design of anti-cancer and anti-HIV drugs as well as drugs for the treatment of infectious diseases caused by parasites such as leishmaniasis (Tan and Fung, 2008). The isolated L-amino acid oxidase from the venom of *Gloydius blomhoffi*, M-LAO, prolongs the APTT, PT, and fibrinogen clotting time and cleaves the Aα-chain of fibrinogen. LAO inhibitors or catalase did not inhibit these effects. The M-LAO fraction also contained a small amount of 39 kDa metalloproteinase. The prolongation of clotting time and degradation of fibrinogen were inhibited by a metalloproteinase inhibitor. Therefore, the anticoagulant activity of the M-LAO fraction was due to the 39 kDa metalloproteinase (Fujisawa, Yamazaki, and Morita, 2009).

2.4.4. Proteases (PRO)

Proteases or proteolytic enzymes are mainly found in the snake venoms from vipers and some Australian snakes. Two types of proteases, serine endopeptidases (or serine protease) and metalloendopeptidases (or metalloprotease), found in snake venom causing hemorrhagic edema and coagulation disorders (Murzaeva, Malenev, and Bakiev, 2000; White, 2005). These two classes differ in their mechanism of action and they target different amino acid sequences in fibrin(ogen), but each perform the same role in nature. These enzymes facilitate the spread of the toxic components throughout the circulation by breaking down fibrin rich clots and help to prevent further clot formation by their action on fibrinogen (Swenson and Markland, 2005).

Snake venom metalloproteases (SVMPs) act mainly as hemorrhagic factors, also called hemorrhagin, because they degrade the proteins from the endothelial basal membrane (Ramos and Selistre-de-Araujo, 2006). SVMPs can also inhibit the platelet aggregation and trigger the release of cytokines which are associated with the proteolytic digestion of basal membrane related to the induction of hemorrhage (Laing and Moura-da-Silva, 2005; Schattner et al., 2005). The proteolytic activity of SVMP induces the hemorrhage, inflammation and necrosis that characterize the local lesion usually observed in envenoming by Viperid snakes (Laing et al., 2003).

2.4.5. Phosphodiesterases (PDEs)

Phosphodiesterases (PDEs) catalyze the hydrolysis of phosphodiesters to phosphomonoesters. They are found in a wide variety of tissues and organisms as intracellular and extracellular PDEs (Conti et al., 1995). Intracellular PDEs play a role in signal transduction by regulating the cellular concentrations of cyclic nucleotides (Bentley and Beavo, 1992). Extracellular PDEs known as exonucleases, exist in venoms and their role in envenomation is mostly by attacking nucleic acids. The prime substrate for PLA_2 is phosphatidyl choline (PC) which upon cleavage is converted to lysophosphatidyl choline (lysoPC). LysoPC of various acyl chains were demonstrated to enhance considerably the activity of snake venom PDE. Lysophosphatidic acid (LPA) and its cyclic form (cLPA) were found to inhibit PDE in a non-competitive (LPA) or competitive (cLPA) manner. This finding contributes to the progression and subsidence of the poisoning profile that upon venom inception PLA_2 and PDE act in tandem, i.e. PLA_2 causes cell lysis and liberates lysoPC, which acts further to activate the PDE in its deleterious DNA and RNA degradation (Mamillapalli et al., 1998).

Snake venom PDE purified from *Crotalus adamanteus* represented the chelator inhibition and the atomic absorption analysis indicated that snake venom PDE is a zinc metalloprotein enzyme. Snake venom PDE shares a number of mechanisms in common with the nucleotidyl transferases (Pollack, Uchida, and Auld, 1983). PDE breaks the phosphate bonds that provide the backbone for nucleic acid rendering DNA and RNA useless in the effected cell causing apoptosis. PDE accounts for the negative cardiac reactions in victims, most notably a rapid drop in blood pressure.

2.4.6. Hyaluronidase (HYA)

Hyaluronidase (HYA) is an invariant factor in the venom of snake, bee, spider, scorpion, lizard, stonefish, wasp and hornets (Girish et al., 2004). This enzyme is generally referred to as a spreading factor which is involved in the diffusion of target-specific toxins from the site of the bite into the general circulation. HYA potentiates the toxicity of venom by increasing the influx of systemic toxin.

2.5 Differences in toxicity of snake venom

Snake venom contains more than one toxin, and in combination the toxins have more potent effect than the sum of their individual effect. Differences in venom composition and toxicity can vary among individuals of the same species (Braud, Bon, and Wisner, 2000; Salazar et al., 2007; Shashidharamurthy et al., 2002; Shashidharamurthy and Kemparaju, 2007; Tsai et al., 2003), and even in the same litter. Such variations are found in all species of snakes and are often greater among geographically different populations. The varying clinical symptoms of Russell's viper bites in Southeast Asia (Warrell, 1985 & 1989) were implied to analyze the differences of the venom composition and toxicity of Russell's viper venom from three localities in India (Jayanthi and Gowda, 1988). Venom toxicity may also vary over time or season in the same individual (Monteiro et al., 1997 & 1998; Magro et al., 2001). Venoms collected immediately after the molting period and after prolonged fasts are ten times more toxic than after a plentiful meal or before the molt (Chaudhuri et al., 1971). The venom of newborn and small juvenile snakes appears to be more potent than those adults of the same species (Tun-Pe et al., 1995), but some studies presented no significance of age-dependency in the biological properties and protein composition (Tan, Armugam, and Mirtschin, 1992; Tan, Ponnudurai, and Mirtschin, 1993a; 1993b).

This review of the literature reveals that most of the studies of the neurotoxins of genus *Bungarus* have been based upon studies of the venom of *B. multicinctus.* Therefore, advanced research into the venom of *B. candidus* might yield results that add significant new information and result in improved clinical therapeutics for those victims envenomed by *B. candidus.* It is hoped that this will be a beneficial result for the citizens of the Kingdom of Thailand and for people living within the range of *B. candidus.*

CHAPTER III

MATERIALS AND METHODS

The present studies were based on four series of separate experiments in both *in vitro* and *in vivo* studies. The first series of study was conducted on the study of morphology of *Bungarus candidus* snake (Chapter IV). The second and third series were *in vitro* studies which were carried out to investigate the biological characteristics of the venom of *B. candidus* snake and the trial experiments for effects of venom on neurotoxicity in mouse phrenic nerve-diaphragm preparation and hemolytic effect in rabbit red blood cell (Chapter V and VI). The forth series of *in vivo* studies were conducted in experimental animals (Chapter VII and VIII). In this chapter, materials and methods used in the experiments are described in details.

Experimental protocols

Four series of experiments performed in both *in vitro* and *in vivo* studies are presented in Figure 3.1.

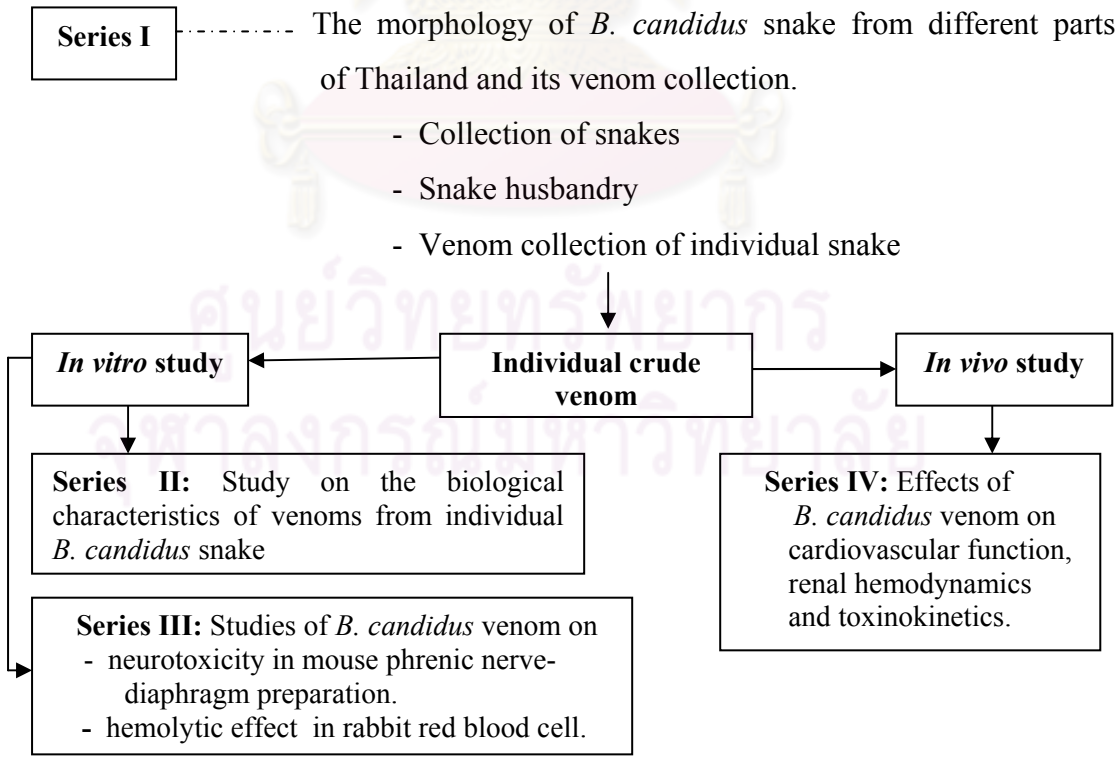


Figure 3-1 Schematic diagrams illustrating the series of studies in both *in vitro* and *in vivo*.

SERIES I: The morphology of *Bungarus candidus* snake from different parts of Thailand and its venom collection.

1.1 Collection of *Bungarus candidus* snakes

The fifteen *Bungarus candidus* snakes were used in this study, which were collected from eastern, northeastern and southern Thailand including captive born snakes. They were categorized into 4 groups;

Group 1: three snakes were collected from Chantaburi and Chonburi Provinces representing the eastern population (BC-E).

Group 2: three snakes were collected from Nakhon Ratchasima Province representing the northeastern population (BC-NE).

Group 3: four snakes were collected from Nakhon Si Thammarat Province representing the southern population (BC-S).

Group 4: five captive born snakes (BC-CB) from Snake Husbandry Unit of Snake Farm at Queen Saovabha Memorial Institute (QSMI).

Total length, snout-vent length, and body weight for each snake were recorded.

1.2 Snake husbandry

All snakes were kept individually in plastic cages (60 x 60 x 40 cm) equipped with the secure locking in the snake husbandry unit of QSMI snake farm, which were controlled the temperature at 26±1^{o}C and at 60-70% of relative humidity in the daytime. All cages had a hiding box and a water bowl (Chanhome et al., 2001). Snakes were fed once weekly with the non-venomous snakes (*Enhydris* sp.) for the wild-caught snakes (Group 1–3), and mice for the captive-born snakes (Group 4). The data of the snout-vent length, the total length, the body weight, and sexes of all snakes were recorded. The age of the captive-born group was also recorded at the period of venom collection.

1.3 Venom collection

Snake venom was individually extracted once a month from each snake by directly attaching a microhaematocrit tube on each fang and transferred to a 1.5 ml microcentrifuge tube. The liquid (fresh) venom was weighed immediately before frozen at –20˚C and lyophilized by Freeze Dryer (Modulyo, EDWARDS). The dry

(lyophilized) venom was weighed, pooled and stored in -20 ˚C for further study. The weights of liquid and dry venoms of individual snakes were recorded. Animal care and procedures used for venom extraction were in accordance with guidelines of the Animal Ethics Committee of QSMI.

SERIES II: Comparative studies of the biological characteristics of venoms from individual *Bungarus candidus* snakes

2.1 Animals

Swiss albino mice weighed 18-20 g were supplied from the laboratory animal unit of the Queen Saovabha Memorial Institute. All animals were housed under 12 h light/dark cycles at room temperature of 25±2ºC, standard foods and water were supplied *ad libitum* until the end of experiments.

2.2 Snake venom

The venoms of *B. candidus* from three different parts of Thailand including a group of captive-born snakes were extracted according to the details described in the experiment Series I.

2.3 Determination of lethal toxicity (LD_{50})

The lethal toxicity was determined by intravenous injection of 0.2 ml of serially 1.4 fold-diluted venom solutions into the tail vein of mice. Five groups of eight mice for each venom sample were tested and observed throughout the period of the experiments. The control group was performed using normal saline solution. The endpoint of lethality of the mice was determined after 24 hours. The LD_{50} was calculated by the method of Reed-Muench (1938) with 95% confidence limits by the method of Pizzi (1950). The experimental protocol was approved by the institutional committee for ethics in animal experimentation of QSMI in accordance with the guideline of the National Research Council of Thailand.

2.4 Determinations of enzymatic activities

All reagents for enzymatic determinations were analytical grade. The *B. candidus* venom (1 mg/ml) was dissolved in 10 mM Tris-buffer (pH 7.4) for all enzymatic activity determinations. All assays were performed in duplicated test.

2.4.1 Phospholipase A_2 activity

Phospholipase A_2 activity was determined by the indirect hemolytic method using human erythrocyte-egg yolk-agarose plate (Marinetti, 1965; Gutierrez et al., 1988) with some modifications. Agarose gel (30 ml. of 1%) warmed at 50°C, was mixed with 1 ml of egg yolk suspension, 0.5 ml. of erythrocyte suspension and 0.25 ml. of 10mM $CaCl_2$ at 50°C. The mixture was poured into a plastic Petri dish. The gel was punched to make several small wells 2 cm apart using a 3 mm puncher. A series of 2-fold diluted venom solutions (5µl) was added to the wells. The plate was placed at room temperature for 24 h and then the cross diameters of hemolytic zones of each well were measured. The straight calibration curve and the parallel line analysis (WHO, 1995) for the log dose-diameter relationship was established to determine the hemolytic dose (HLD, in µg) causing a hemolytic zone (clear zone) of 10 mm in diameter.

2.4.2 Acetylcholinesterase activity

Acetylcholinesterase activity was determined using the diagnostic kit "BTC SIGMA" based on the use of the butyrylthiocholine (BTC) as substrate. The content of 1 bottle of buthylthiocholine was dissolved in 10 ml. of distilled water. A mixture of 500 µl of substrate and 5 µl of venom sample was introduced into t cuvette, and cover the cuvette was covered with Parafilm. The content of the cuvette was immediately mixed thoroughly, then the cuvette was put in the spectrophotometer where absorption at 405 nm was read immediately (time 0) and thereafter at appropriate time intervals. The initial rate of enzyme reaction was used to calculatie the enzymatic activity. One unit of enzyme activity was arbitrarily defined as the difference in absorption at 405 nm/min/mg.

2.4.3 Protease activity

Protease or proteolytic activity was determined by means of the hide powder azure hydrolyzing activity (Omori-Satoh et al., 1995). Hide powder azure 10 mg suspended in 1 ml of 30 mM borax buffer (pH 9.0) was incubated with 100 µl of venom sample at 37°C for 1 h. The reaction was stopped with 100 µl of 100 mM EDTA, and then centrifuged at 3000 rpm for 10 minutes. The supernatant was measured at 595 nm against a control without venom solution. One unit of the enzyme

activity was defined as the amount of venom hydrolyzing the substrate at a rate of 1.0 absorbance unit per minute.

2.4.4 Alkaline phosphomonoesterase activity

Alkaline phosphomonoesterase activity was determined by the modified methods (Lo, Chen, and Lee, 1966; Tan and Tan, 1988). Venom sample (100 µl) was added to the substrate mixture containing 500 µl of 500 mM glycine buffer (pH 8.5), 500 µl of 10 mM *p*-nitrophenylphosphate and 300 µl of 10 mM $MgSO_4$. The mixture was incubated at 37^{o}C for 30 minutes, and then 2 ml of 200 mM sodium hydroxide was added and placed for 20 minutes. The absorbance of the mixture was read at 440 nm. One unit of enzyme activity was defined as the amount of enzyme caused an increase of 0.001 absorbance unit per minute.

2.4.5 Phosphodiesterase activity

Phosphodiesterase activity was determined by the modified methods (Lo et al., 1966; Tan and Tan, 1988). Venom sample (100 µl) was added to a substrate mixture containing 500 µl of 170 mM veronal buffer (pH 9.0), 500 µl of 2.5 mM Ca-bis-*p*-nitrophenylphosphate and 300 µl of 10 mM $MgSO_4$. The hydrolysis of the substrate was measured by the increasing rate of absorbance at 440 nm. One unit of enzyme activity was defined as the amount of venom caused the increment of 0.001 absorbance unit per minute.

2.4.6 L-amino acid oxidase activity

L-amino acid oxidase activity was determined with the modified technique (Worthington Enzyme Manual, 1977; Tan and Tan, 1988). 50 µl of 0.007% peroxidase (510 NIH units/mg) was added to the substrates containing 1 ml of 200 mM triethanolamine buffer (pH 7.6), 0.1% L-leucine and 0.0065% *o*-dianisidine and incubated for 3 minutes at room temperature, and then 100 µl of venom sample was added. The absorbance at 426 nm of the mixture was read. One unit of enzyme activity was defined as the amount of venom causing an increment of 0.001 absorbance unit per minute.

2.4.7 Hyaluronidase activity

Hyaluronidase activity was determined with the modified method (Xu et al., 1982; Tan and Tan, 1988). The substrate solution containing 200 mM acetate buffer (pH 5.0), 150 mM sodium chloride, and 200 µg hyaluronic acid was mixed

with 100 µl of the venom sample and incubated at 37^{o}C for 1 hour. The reaction was terminated by the addition of 2 ml of 2.5% cetyltrimethylammonium bromide in 2 % sodium hydroxide solution and placed for 30 minutes at room temperature. The enzyme activity was expressed as National Formulary Unit per milligram (NFU/mg) at the absorbance of 400 nm.

2.5 Determinations of protein components and the molecular weight

Measurements of protein components and the molecular weight of all venoms were performed using tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE). Venoms (12 µg) were run under non-reducing conditions on a 16% Tricine SDS-PAGE gel with the modified method (Schägger and von Jagow, 1987; Schägger, 2006). Electrophoresis was carried out at the voltage of 30 V for 25 min, 100 V for 90 min and 150 V for 90 min using a Hoefer power supply PS500 XT. The Kaleidoscope Prestained Standard (BioRad) with molecular weights ranging from 7.1–210 kDa was used as standard markers.

Gels were stained with 0.2% Coomassie blue (CBB R-250) in 7: 46.5: 46.5 ml acetic acid: methanol: distilled water, for 30 min with agitation. Destaining was done with 25% methanol-12.5% acetic acid mixture with agitation. Several changes of the destaining solution were made until the background was clear. The result of protein bands on the electrophorogram was observed and recorded by taking a photograph.

2.6 Fractionation of *Bungarus candidus* venom by Reverse-phase High Performance Liquid Chromatography (RP-HPLC).

Fresh venom from individual *B. candidus* snake was obtained. The protein concentration of the venom was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard. Each venom was dissolved in deionized water to give a protein concentration of 800 µg/ml. Eighty micrograms of total venom proteins was fractionated by reverse-phase HPLC using a preparative HPLC system (Water 600E) and a Symmetry 300 C 18 column (250 x 4.6 mm, 5 µm particle size) with photodiode array detector (Waters 2996) at OD 206 and 280 nm. The column was equilibrated with 85:15 (v/v) of Mobile A (5% acetonitrile + 0.1% trifluoroacetic acid, pH 2.6) and Mobile B (95% acetronitrile + 0.1% trifluoroacetic acid) and was eluted with a linear gradient of 15-50% acetonitrile at a flow rate of 1 ml/min and a

total elution volume of 60 ml. Acetone and acenaphthene were used as external reference standards for the calibration of the column.

2.7 Isolation and purification of the major neurotoxins

2.7.1 Cation-exchange chromatography

Fifty milligrams each of *B. candidus* lyophilized venom from the northeast (BC-NE) and the south (BC-S) of Thailand was dissolved in 2 ml of 50 mM phosphate buffer (pH 6.25), applied to the Resource^{TM} S Column equilibrated with the buffer, and then eluted with a linear gradient of 0–0.3 M NaCl at a flow rate of 10 ml/h. Absorbance was measured at 280 nm, and fractions of 2 ml were collected (Khow et al., 2003).

2.7.2 Adsorption chromatography on hydroxyapatite

The major toxic fractions obtained on cation-exchange chromatography were pooled, concentrated in collodion bag *in vacuo*, and dialyzed against 0.05 mM phosphate buffer then applied to a hydroxyapatite column. The column was eluted at the flow rate of 15 ml/h with a linear gradient of 0.05–0.35 M phosphate buffer (pH 6.25). Fractions of 1.5 ml were collected (Khow et al., 2003).

2.7.3 Two dimensional gel (2D) electrophoresis

The proteins of each fraction obtained from adsorption chromatography were separated by 2-D electrophoresis using an IPGphor (Amersham Bioscience, Uppsala, Sweden) instrument. For the first dimension, isoelectric focusing (IEF), protein sample was prepared in 8M urea. 2% CHAPS and 2% IPG buffer to obtain a sample concentration of 0.46 mg/ml for BC-S and 0.61 mg/ml for BC-NE. Then 130 µl of the prepared protein sample was loaded on a 7 cm IPG strip (pH range 3-10) using the following focusing conditions: 200 V for 1 min (1V/h), 3500 V for 1 h 30 min (2800 V/h) and 3500 V for 1 h 15 min (4500 V/h). For the second dimension, electrophoretic separation (SDS-PAGE) was done in a 10 cm, 12.5% polyacrylamide gel. 4 µl of molecular weight marker was applied. Coomassie blue was employed for protein staining. The spots of interest were analyzed by MALDI-TOF mass spectrometer model reflex V (Brucker Daltonik GmbH). Protein identifications were obtained from MASCOT (MatrixScience) and were matched with peptide mass fingerprints in a protein database. Protein scores are significant when p value is less than 0.05.

SERIES III: ***In vitro* study: Neurotoxic and hemolytic effects of the *Bungarus candidus* venom.**

The experiments of this series were performed:

- to investigate the neurotoxicity of the crude venom of *B. candidus*, using the mouse phrenic nerve-diaphragm preparation (Harvey et al., 1994).

- to study the hemolytic effect of *B. candidus* venom on osmotic fragility in rabbit red blood cells.

3.1 The neurotoxicity studies on mouse phrenic nerve-diaphragm preparation (PNDp).

3.1.1 Antivenom

The monospecific *B. candidus* antivenom (Lot no. BC4701) obtained from Queen Saovabha Memorial Institute (Bangkok, Thailand), was raised in horses hyperimmunised with the *B. candidus* venom.

3.1.2 The phrenic nerve-diaphragm preparation was obtained from mice sacrificed by cervical dislocation (Nirthanan et al., 2003). The hemi-diaphragm associated with phrenic nerve was isolated and mounted under a tension of 1 g in a vertical chamber of organ bath containing 35 ml of Krebs solution (pH 7.4) of the following composition (mM): NaCl, 118.4; KH_2PO_4, 1.2; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.4; $NaHCO_3$, 25 and glucose, 11.1, aerated with 95% O_2 and 5% CO_2, at 37^0C For indirect stimulation, the phrenic nerve was stimulated using stimulating electrode (Suction electrode, A-M Systems,Inc.) connected to a stimulator (Grass S48) at a frequency 0.2 Hz, in pulses of 0.2 ms duration and the supramaximal voltage (4× thereshold; 1-10 V). Direct stimulation to the muscle was achieved by electrical stimulation (0.2 Hz, 0.2 ms, 10-30 V). Isometric twitch tension of muscle was recorded by a force displacement transducer coupled to Polygraph recorder (Grass Model 79). The PNDps were allowed to stabilize for at least 20 min before the experiments. The experiments were carried out as follow:

3.1.2.1 The control experiments were tested on Krebs solution alone or Krebs solution plus antivenom. The indirect stimulation to the phrenic nerve was applied for 60 minutes (Figure 3-2).

3.1.2.2 To determine the twitch responses of PNDps to the various concentrations of the *B. candidus* crude venom (BCV; 1, 3.5 and 7.0 µg/ml Krebs solution) (Figure 3-2).

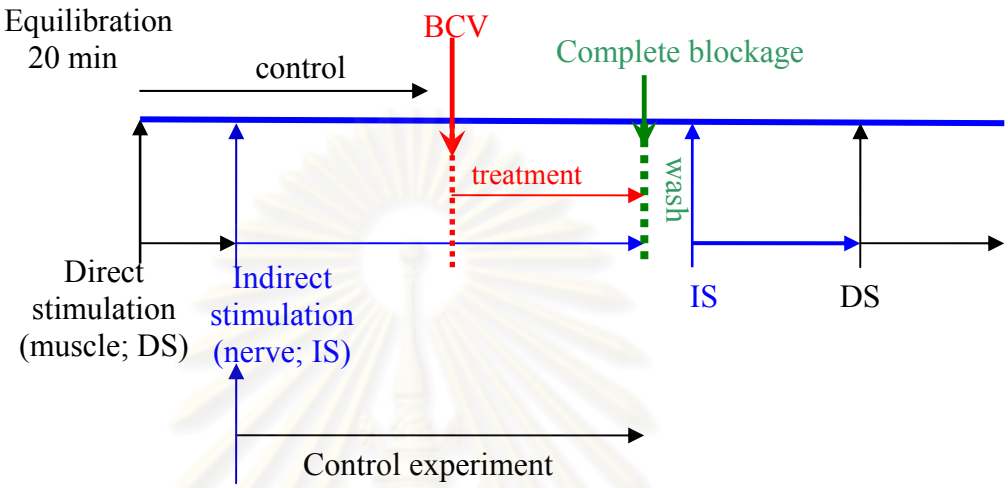


Figure 3-2 Schematic diagrams illustrating the experiments of 3.1.2.1 (Control experiment) and 3.1.2.2 (BCV; 1, 3.5 and 7.0 µg/ml Krebs solution).

3.1.2.3 To determine the twitch responses of PNDps when treatment with monospecific *B. candidus* antivenom (BC-AV; 17 µl/ml Krebs solution) promptly with the addition of the *B. candidus* crude venom (BCV; 3.5 µg/ml Krebs solution) (Figure 3-3).

3.1.2.4 To determine the twitch responses of PNDps when treatment with the mixture of monospecific *B. candidus* antivenom (BC-AV 600 µl) and the *B. candidus* crude venom (125 µg) incubated at 37^0C for 30 min prior to addition into the organ bath (Figure 3-3).

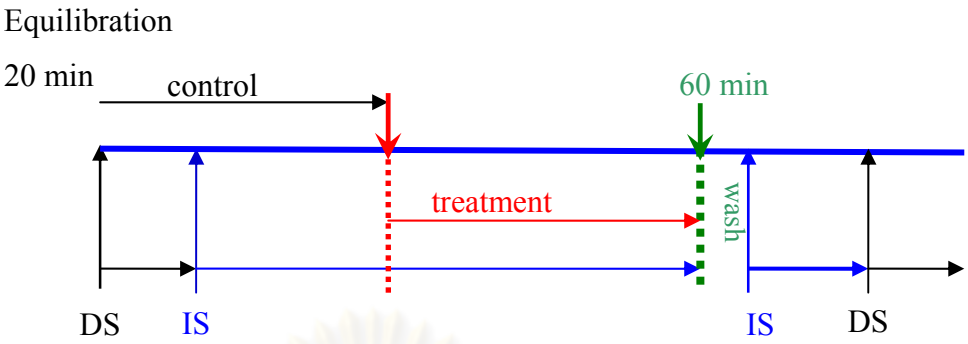


Figure 3-3 Schematic diagrams illustrating the experiments 3.1.2.3 (BCV / BC-AV) and 3.1.2.4 (BCV+BC-AV_{30min})

3.1.2.5 To determine the twitch responses of PNDps if treatment with monospecific *B. candidus* antivenom (BC-AV; 17µl/ml Krebs solution) when 50% twitch tension blocked by the *B. candidus* crude venom (BCV; 3.5 µg/ml Krebs solution) (Figure 3-4).

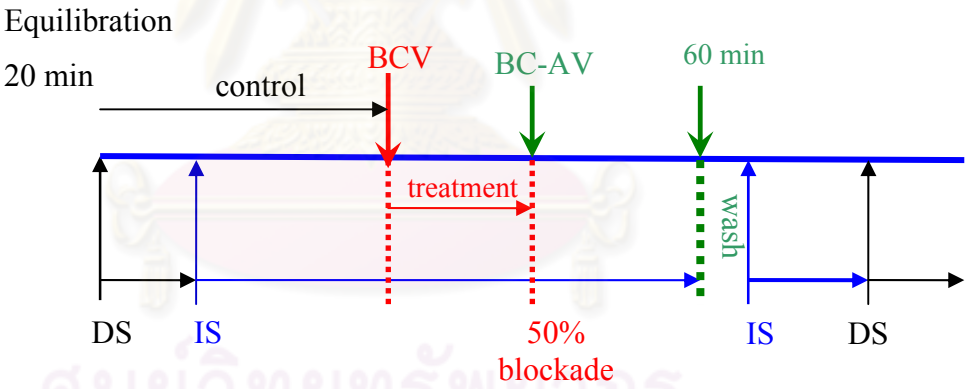


Figure 3-4 Schematic diagrams illustrating the experiments 3.1.2.5

3.1.2.6 Substituting strontium (Sr^{2+}) in Krebs solution

Strontium chloride ($SrCl_2$) was substituted for calcium chloride ($CaCl_2$) on an equimolar basis in Krebs solution. Sr^{2+}-containing Krebs solution was perfused in organ bath for the same experiment as 3.1.2.2.

3.2 The hemolytic studies on mean corpuscular volume and osmotic fragility test of the rabbit red blood cells

3.2.1 Blood collection

Blood samples were drawn from the marginal ear vein of New Zealand white rabbit (weight 3.0–3.5 kg) and collected into either heparinized or EDTA tubes as anticoagulant, respectively.

3.2.2 Experimental protocol

Various concentrations of *B. candidus* crude venom (0.7 and 2.0 µg/50 µl of 0.9% saline solution) were added into 1 ml of heparinized or EDTA rabbit whole blood, respectively. The control blood was added by 50 µl of normal saline solution (NSS-treated whole blood). The *B. candidus* venom-treated whole blood was gently mixed and hold at 37^{o}C for 30 minutes. Thereafter, one part of the venom-treated whole blood was determined for mean corpuscular volume and hematocrit by Automated, an in vitro Diagnostic Medical Equipment (Model Mythic18 (IVD) C2 Diagnostics; Montrellier, France).

3.2.2.1 Determination of osmotic fragility of *B. candidus* venom-treated red blood cells was carried out according to the modified method of Dacie and Lewis (1984). Briefly, 0.02 ml of the venom-treated whole blood in either low dose (0.7 µg/ml blood) or high dose (2.0 µg/ml blood) of *B. candidus* venom and the NSS-treated whole blood was added into the glass tubes in varying of concentrations of 5 ml buffer saline (0.1–0.9 %) and in the distilled water. The tubes were gently mixed and incubated at room temperature for 30 minutes. Then the samples were centrifuged at 2,000 rpm for 5 minutes. Supernatants were collected for measurement of plasma hemoglobin.

3.2.2.2 Determination of plasma hemoglobin

Plasma hemoglobin was determined by the method of Simmons (1968). 200 µl of supernatant was added to the mixture containing 1 ml of 1 % benzidine in glacial acetic acid and 1 ml of 1% hydrogen peroxide. The mixture was well mixed and placed for 30 minutes at room temperature. 10 ml of 10 % acetic acid was added and mixed by gently inversion the tube, then read at the absorbance of 515 nm. The standard whole blood hemoglobin was determined using the supernatant from the rabbit whole blood added into the distilled water. Hemolysis or plasma hemoglobin

was expressed as percentage of the absorbance in distilled water. The conventional osmotic fragility curve was drawn by plotting the percentage of hemolysis or plasma hemoglobin against decreasing saline concentrations. The saline concentration at which 50% of the red blood cells lysed was used to express the results as the mean corpuscular fragility (MCF; g%). The stability of red blood cells was evaluated as percentage of the quotient of the difference between the MCF value of the test and control samples (Chikezei, Uwakwe, and Monago, 2009). Percentage of Stability of red cell was determined as following:

$$\text{Plasma hemoglobin (mg\%)} = \frac{\text{OD of the test supernatant}}{\text{OD of the standard}} \times \text{standards value}$$

$$\text{\% stability of red blood cell} = \frac{\text{MCFtest} - \text{MCFcontrol}}{\text{MCFcontrol}} \times 100$$

MCF = mean corpuscular fragility; the saline concentration at which 50% of the red blood cells were lysed.

SERIES IV: Effects of *Bungarus candidus* venom on cardiovascular function, renal hemodynamics and toxinokinetics.

The effects of *B. candidus* venom on cardiovascular function and renal excretion will be performed on the experimental animals as follow:

4.1 Animals

Adult male New Zealand white rabbits weighing 2.5–3.5 kg, used for the experiments were supplied from the laboratory animal unit of Queen Saovabha Memorial Institute and housed in the conventional animal facilities. They were cared for in accordance with the guideline for experimental animals suggested by the National Research Council of Thailand.

4.2 The experimental protocol

4.2.1 Determination of the sublethal dose of *Bungarus candidus* venom in rabbits.

Before the experiment, the minimal lethal doses of *B. candidus* venom were determined in twelve anesthetized rabbits. The *B. candidus* venom in lyophilized

form from the Queen Saovabha Memorial Institute, Thai Red Cross Society, was used for the study (1 mg of lyophilized crude venom dissolved in 0.9% saline solution). A single dose of the venom was injected intravenously at the dosage of 150 μg/kg in 6 rabbits; 75 μg/kg in 6 rabbits. Blood samples were collected before and 5, 10, 20, 30, 60, 90, 120, and 150 min after venom injection for determination of plasma venom concentration by enzyme linked immunosorbent assay (ELISA). The general condition was measured for heart rate and arterial blood pressure.

4.2.2 The effects of *Bungarus candidus* venom on cardiovascular function and renal hemodymanics.

Twelve rabbits were divided into 3 groups of four animals each. Animals in group 1 were received normal saline as the control group. Animals in group 2 were received the *B. candidus* snakes venom from the northeast (BC-NE) and group 3 were received the *B. candidus* snakes venom from the south (BC-S). The animals were anesthetized with pentobarbital sodium (25 mg/kg) by intravenous injection. Supplemental doses were given as required to maintain surgical anesthesia throughout the experiment. The animals were tracheotomized, and an endotracheal tube was inserted to free the airway. The carotid artery was catheterized with polyethylene tubes for recording cardiac output, arterial blood pressure and heart rate (Grass Polygraph Model 79 E, Grass Instrument CO, USA) and for blood sample collections. The jugular vein was catheterized with polyethylene tubes for fluid infusion. A left flank incision was made via a retroperitoneal approach, and the left ureter was cannulated with a polyvinyl catheter for urine collection and for renal clearance studies. The experimental study in each group was divided into pretreatment and treatment periods. Both pretreatment and treatment period, measurements of renal and cardiovascular functions were performed.

4.2.2.1 Renal plasma flow and glomerular filtration rate

Measurements of renal plasma flow and glomerular filtration rate were performed using the standard techniques (Smith, 1962) with the modification. For pretreatment period, the normal saline solution containing 1.2% *p*-aminohippuric acid (PAH) and 5% inulin was infused via the jugular vein as the priming dose. Plasma levels of PAH and inulin were maintained by sustained infusion of isotonic saline solution containing the PAH (0.12%) and inulin (0.5%) at the rate of

0.5 ml/min. After the 45 min for equilibration, urine was collected for 10 min and an arterial blood sample was obtained at the midpoint of urine collection. For treatment period, the animals were administered intravenously with *B. candidus* venom (1 mg of lyophilized crude venom dissolved in 0.9 % saline solution) at the dosage of 50 μg/kg. Cardiovascular functions and renal hemodynamics were carried out by collecting arterial blood samples (1 ml) at the baseline (before) and 30, 60, 90, 120, and 150 min after venom injection. Urine samples were collected at 10 minute duration.

4.2.2.2 Cardiac output

Cardiac output was performed by dye dilution technique using Evan blue (T-1824) in stead of Indocyanin green dye (Chaiyabutr, Faulkner, and Peaker, 1980). 1 ml of dye solution (0.03% T-1824 in normal saline) was injected into the jugular vein and serial blood samples were collected from the carotid artery within 2 seconds after dye injection. Serial samples of arterial blood were collected 0.8 ml/second for a period of 8 seconds by means of a peristaltic pump. The concentration of dye in the plasma was determined in every second from the beginning of the curve by spectrophotometry at the absorbance of 620 nm. The sum of all concentration was calculated for cardiac output (CO) using the standard formula (Burton, 1965).

4.2.3 Determinations of *Bungarus candidus* venom in plasma and urine samples.

Enzyme linked immunosorbent assay (ELISA) was used to detect the concentration of the venom in blood and urine samples of rabbits. Blood samples were collected from carotid artery into heparinized tubes at regular intervals from 2 to 150 min (2, 5, 10, 20, 30, 60, 90, 120, and 150 minutes) after intravenous injections of minimal lethal dose of venom. Urine samples were collected before and 5, 10, 20, 30, 60, 120 and 150 minutes after venom injection. Then the values of venom appearance (ng/ml) with time-course (minute) for each individual animal were plotted on a semi logarithmic graph paper. The extrapolation of log concentration-time curves obtained were used for analysed the venom kinetic parameters.

The procedure of sandwich-ELISA for venom determination

The assay were performed in 96-well polystyrene microtiter plates (MaxiSorp^{TM}, NUNC) coated with 50 μl/well of 1:100 dilution of rabbit anti-*Bungarus candidus* venom in carbonate buffer 0.05 M pH 9.6 and incubated at 37^{o}C

for 2 hours. The plates were washed three times with 200 µl each of phosphate buffer saline pH 7.2 containing 0.05% of Tween 20 (T-PBS) (Sanofi Pasteur PW40). The blocking solution (3% bovine serum albumin in carbonate coating buffer) was added and the plates were incubated overnight at 4^{o}C and washed three times with T-PBS. A volume of 50 µl of plasma, urine samples and 1:2 serial dilution of *B. candidus* venom standard (stock venom 4 mg/ml in normal rabbit plasma) were added in duplicate to the wells and incubated at 37^{o}C for 1 hour. After washing the plates, a volume of 50 µl of 1:4 dilution of horse IgG anti-*Bungarus candidus* venom in 0.5% BSA-PBS (pH 7.2) was added in each well and incubated at 37^{o}C for 2 hours. The plates were subsequently washed and 50 µl of rabbit anti-horse IgG peroxidase (Sigma Chemical Co., USA) in 1:10000 dilution 0.5% BSA-PBS was added and incubated at 37^{o}C for 1 hour. The plates were washed and 50 µl of OPD (1mg/tablet, DAKO) dissolved in 3 ml of distilled water and 1.25 µl H_2O_2 was added. After incubation of the plates in the dark at room temperature for 20 min, the reaction was stopped by the addition of 50 µl of 0.5 M sulfuric acid. The plates were measured at 492 nm using ELISA reader (Molecular Device Emax precision microplate reader).

4.3 Chemical analysis

All blood and urine samples were determined inulin, PAH, packed cell volume, osmolality, sodium, potassium and chloride. Determination of PAH and inulin were carried out by standard method (Smith, 1962) with the modification. Osmolality was determined by the freezing-point depression method (The Advanced^{TM} Osmometer Model 3D3). Sodium and potassium were measured by flame photometry (Clinical Flame Photometer 410C), chloride with a Chloride Analyzer 925. Plasma lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) were determined by immunoturbidimetric assay using COBAS INTEGRA 800 (Roche Diagnostics GmbH, Manheim) and measured the increase in absorbance at 340 nm.

4.4 Calculations

Inulin clearance and *p*-aminohippurate clearance were performed for the glomerular filtration rate (GFR) and effective renal plasma flow (ERPF), respectively. Renal blood flow (RBF) was calculated from ERPF and packed cell volume. Total peripheral resistance (TPR) and renal vascular resistance (RVR) were calculated from

mean arterial blood pressure (MAP), cardiac output (CO) and RBF using the standard formula (Burton, 1965). Filtration fraction was obtained by dividing GFR by ERPF.

Derived calculations

The measurements of parameters for renal hemodynamics and cardiovascular function were calculated using the following equations:

Glomerular filtration rate (**GFR**; ml/min) = **Cin = Uin V/Pin**

Cin = Inulin clearance (ml/min)

V = urine flow (ml/min)

Uin = urinary inulin concentration (mg/ml)

Pin = plasma inulin concentration (mg/ml)

Effective renal plasma flow (**ERPF**; ml/min) = $\mathbf{C_{PAH} = U_{PAH}\ V/P_{PAH}}$

C_{PAH} = clearance of PAH (ml/min)

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

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

Effective renal blood flow (**ERBF**; ml/min) = **RPF/ 1-(Hct/100)**

Hct = hematocrit value (%)

Filtration fraction (**FF**; %) = **GFR x 100/ ERPF**

Fractional excretion of electrolytes (**FE**; %) = $\mathbf{(U_E\ V/P_E)\ x\ 100/\ GFR}$

U_E = concentration of urinary electrolytes (μEq/ml)

P_E = concentration of plasma electrolytes (μEq/ml)

Renal vascular resistance (**RVR**; mmHg/ml/min/kg body weight) = **MAP/ ERBF**

Mean arterial blood pressure (**MAP**; mmHg) = **Pd** + 1/**3 (Ps – Pd)**

Pd = diastolic blood pressure (mmHg)

Ps = systolic blood pressure (mmHg)

Osmolal clearance ($\mathbf{C_{OSM}}$; ml/min) = $\mathbf{(U_{OSM} \times V)/ P_{OSM}}$

U_{OSM} = urine osmolality (mOsm)

P_{OSM} = plasma osmolality (mOsm)

Free water clearance ($\mathbf{C_{H2O}}$; ml/min) = $\mathbf{V - C_{OSM}}$

Renal fraction (**RF**; %) = **ERBF x 100 / CO**

CO = cardiac output (ml/min/kg)

Stroke volume (**SV**; ml/beat/kg) = **CO / HR**

HR = heart rate (beats/min)

Total peripheral resistance (**TPR**; mmHg/ml/min/kg) = **MAP / CO**

Left ventricular work (**LVW**; kgm/min) = **CO** x **MAP** x 13.6 x 1.055 x 10^{-3}

Statistical analysis

All data were expressed as mean ± standard deviation (SD), except the data of the studies of neurotoxic effect of *B. candidus* venom were expressed as mean ± standard error of mean (SEM) (Chapter VI). Mean values in Chapter V were determined by analysis of variance (ANOVA) and significance of variables among groups of studies was determined by the Duncan's multiple range tests. Mean values in Chapter IV and VI were determined by ANOVA and significant difference test was performed by Newman-Keuls test. The data in Chapter VII were analyzed by ANOVA, post hoc tests for comparisons of each value of post-treatment periods against the value of pre-treatment period using Bonferroni t-test. Mean values in Chapter VIII were analyzed for statistical significance using unpaired *t*-test. The significant level was determined at $p < 0.05$.

Ethical considerations

All the experimental procedures used in the present study were carried out in accordance with the principles and guidelines of the Faculty of Veterinary Science,

Chulalongkorn University. The protocol in experimental animals was submitted to the Animal Ethics Committee of the Queen Saovabha Memorial Institute (QSMI), The Thai Red Cross Society for approval before conducting experiments. These guidelines were in compliance with the National Research Council of Thailand guidelines for care and use of animals in research.

CHAPTER IV

THE MORPHOLOGY OF *Bungarus candidus* SNAKE FROM DIFFERENT PARTS OF THAILAND AND ITS VENOM COLLECTION

INTRODUCTION

Bungarus candidus Linnaeus, 1758 (Malayan krait) is a prominent neurotoxic Elapid snake, which is commonly found in Southeast Asia including Thailand (Cox, 1991; Chanhome et al., 1998). This snake is the smallest species of the genus *Bungarus* (the Kraits) found in Thailand, the other two species being *B. fasciatus* (Banded krait) and *B. flaviceps* (Red-headed krait). The maximum length of *B. candidus* is 155 cm, with adults averaging 100 cm (David and Vogel, 1996). The body and tail are cross-banded with black blotches, the ventral surface is white. The head is black or grayish black above, the color extending posteriorly to the first black mark on the nape so that an indistinct V-shaped mark is present (Soderberg, 1973).

B. candidus venom possesses a powerful neurotoxin that causes severe neurotoxicity (Warrell et. al., 1983; Pochanugool et. al., 1997; 1998). The onset of neurotoxic symptoms can appear within minutes or be delayed for hours. Few data are available for the details of the fatalities of *B. candidus* envenoming because bites mainly occur in rural areas at night. Krait bites happen with a quick snapping motion by the snake and initially envenomation does not result in pain, and there is little or no local edema or discoloration in the victim. Most envenomed victims awaken with suspicious morning weakness, loin or abdominal pain, but also there is a slow onset of serious weakness but it is difficult to identify the site of the bite. Therefore, medical attention is usually delayed (Goonetilleke and Harris, 2002)

B. candidus is found mainly in northeastern, eastern, southern and western Thailand. It is interesting to compare snake from these three different parts of Thailand in terms of its morphology and venom yield. These data will be useful as part of a database for treatment of envenomed victims from this snake.

MATERIALS AND METHODS

4.1 Collection of Bungarus candidus snakes

B. candidus snakes were collected from eastern, northeastern and southern Thailand (Figure 4-1) where have been reported the incidence of *B. candidus* bite. A group of captive born snakes was also included in the study group. The study group was divided into four groups. Total length, snout-vent length, sexes and body weight for each snake were recorded.

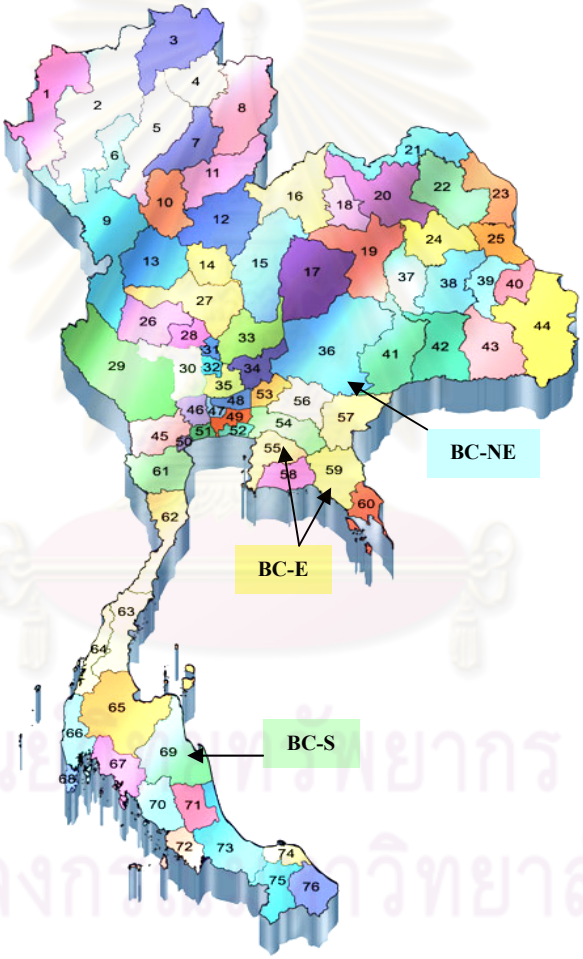


Figure 4-1 Map of Thailand presents the localities where *B. candidus* snakes were collected: BC-NE group from Nakhon Ratchasima (36), BC-E group from Chonburi (55) and Chanthaburi (59), BC-S group from Nakhon Si Thammarat (69).

4.2 Snake husbandry

All snakes were kept individually in securely locked plastic cages furnished with a hiding box and a water bowl (Chanhome et al., 2001). All cages were placed in

a room with a controlled daytime temperature of 26±1°C and daytime relative humidity at 60-70 %. All snake groups were fed once weekly. The snout-vent length, total length and body weight were measured, and the sexes of all snakes were recorded. The ages of the captive born group were also recorded at the period of venom collection.

4.3 Venom collection

Snake venom was individually extracted once a month for one year from each snake. The liquid (fresh) venom was weighed immediately before frozen at -20˚C and lyophilized. The dry (lyophilized) venom was weighed, pooled and stored in -20˚C for further study. The weights of liquid and dry venoms of individual snakes were recorded.

Statistical analysis

The results were expressed as the mean ± SD of the number of snakes or the number of venom extraction in each snake group. Differences were compared using ANOVA followed by Newman-Keuls test with $p<0.05$ indicating significance.

RESULTS

Snake and venom collection

The sexes, snout-vent lengths, total lengths, body weights of each snake, and the average weights of liquid (fresh) and dry (lyophilized) venoms extracted from each snake are presented in Table 4-1. Three males of *B. candidus* were collected from Chanthaburi and Chon Buri Provinces (BC-E), two males and one female from Nakhon Ratchasima Province (BC-NE), whereas the BC-S group consisting of three males and one female from Nakhon Si Thammarat Province. The five snakes of the BC-CB group consisted of four males and one female that were hatched from three different clutches of eggs from the southern-origin parents. They were two, four, and five years old during venom extraction for the present study. The average size and the average weight of liquid and dry venoms of snakes in all groups are presented in Table 4-2. The BC-NE group possessed the highest body weight in the average of 398.33 ± 215.02 gm (range 185.0-615.0 gm) and was correlated with the average

weight of liquid venom 116.90 ± 69.07 mg/snake (range 27.1-255.8 mg/snake) and the average weight of dry venom 30.45 ± 17.18 mg/snake (range 7.6-67.7 mg/snake) when compared with other groups. The percentage of dry matter of venom was in the average of 27.90 ± 3.94% (BC-E), 26.59 ± 1.79% (BC-NE), 27.53 ± 4.55% (BC-S), and 27.15 ± 6.87% (BC-CB). The percentage of liquid venom yield per snake body weight in gram was 0.032 ± 0.013 %, 0.026 ± 0.009 %, 0.023 ± 0.012 %, and 0.023 ± 0.010 % for BC-E, BC-NE, BC-S and BC-CB venom groups, respectively (Table 4-2).

Morphology of Bungarus candidus snake

According to the observation of live *B. candidus* snake in this study, the color and pattern of all snake groups are shown in Figures 4-2 to 4-5. The comparative characteristics of all snake groups are presented in Table 4-3. In general, the dorsal surfaces of the body and tail have a series of black and white bands. The black bands do not cross the ventral surface. The dorsal surface of the head is black and extends for several head lengths on to the nape. The ventral surface of the body, chin, and the labials are white. The white bands are speckled with black flecks which are darker in BC-NE group than the others.

DISCUSSION

All snakes in each group were selected as adults with total lengths of the three females ranging from 93.0 – 119.0 cm. and of the 12 males ranging from 93.0–139.5 cm. (Table 4-1). In comparison of the same total length between male and female (BC-NE 3 = BC-CB 5 = 93.0 cm; BC-S 3 = BC-S 4 = 119.0 cm.) were shown the difference in the body weight without the correlation to the sexes. In this regard, males possess longer tail length than females. The liquid venom yield per individual snake was mainly correlated to its body weight. The percentage of venom yield per gram of snake body weight (0.023–0.032%) and the percentage of dry matter of venom (26.59–27.90%) revealed no differences among groups (Table 4-2).

The procedure of venom collection using a hematocrit tube attached directly to the snake fangs gains high venom yield specifically in Elapid snakes which mainly have short and small fangs. Moreover, this procedure is sanitary controlled the debris

or saliva mixed into the venom. This procedure needs careful and proper snake handling and is also dependent on the responsiveness of the snake during venom collection. Therefore some of the heavier snakes may give a lower amount of venom yield than that of lighter ones. The experiments demonstrated that the physical displacement of fang sheath towards the base of fang either by a container during venom collection or by the victim's skin surface during fang penetration is a prerequisite for venom release. The flow of venom during bites by most venomous snakes is significantly influenced by the amount of pressure placed by the contraction of skeletal muscles in contact with the venom glands (Young et al., 2001; 2003).

All snake groups were kept in the same environment in captivity during venom extraction. Though the liquid and dry venoms per snake were individually different, however the mean values of percentages of dry matter of venom or the percentages of venom yield per gram of snake body weight showed no differences among groups with narrow ranges and no association with body weight. These values are useful for estimating the dosage range of antivenom treatment in case of *B. candidus* envenomation if the victim can take the snake to the physicians.

A

B

C

D

Figure 4-2 *Bungarus candidus* from northeastern Thailand. The head is solid black extending back about 1.5–2 times the head length to the first white band. Some have a narrow faint white line at the base of the head. The white bands are grayish because of the prominent black specks.

A **B**

C

D

Figure 4-3 Captive born *Bungarus candidus* from southern parents. The head is grayish-black or jet black, the color extending back about 1.5–2 times the head length to the first white band. A faint, narrow white band at the base of the head results in a black, arrowhead shape on the nape. Black specks are not prominent; the white bands are distinctly white.

A

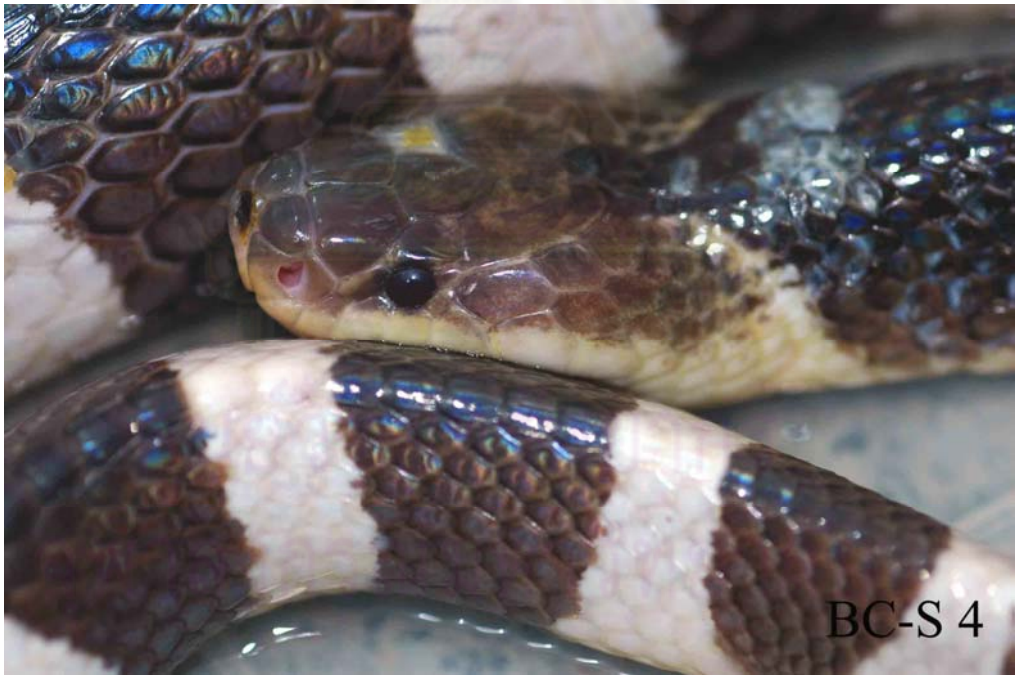
B

C

D

Figure 4-4 *Bungarus candidus* from eastern Thailand. The head is grayish-black becoming more distinctly black extending back about 1.5–3 times the head length to the first white band. Some have a faint white line at the base of head resulting in a black, arrowhead shape on the nape (A and B). White bands are distinctly white, black specks barely noticeable (C).

A



B

Figure 4-5 *Bungarus candidus* from southern Thailand. The head is distinctly grayish-black with a narrow, white band at the base of the head that creates a jet black arrow-shaped mark on the nape. Black specks are more prominent on anterior white body bands but become less prominent posteriorly.

Table 4-1 Fifteen *Bungarus candidus* snakes from three localities in Thailand plus a captive-born group; the age, sex, snout-vent length, total length, body weight, number of venom extractions and average weight of fresh and dry venoms are shown for each snake.

Snake code	Locality	Age (year)	Sex	SVL (cm.)	ToL (cm.)	Body weight (g)	No. of extraction	Weight of fresh venom (mg)	Weight of dry venom (mg)
BC-E 1	Chanthaburi	-	Male	101.0	115.5	200	8	70.58 ± 15.54 (48.70-88.10)	17.57 ± 4.06 (11.38-23.06)
BC-E 2	Chonburi	-	Male	91.0	101.5	195	8	65.79 ± 34.88 (29.60-144.10)	18.43 ± 10.08 (7.70-40.70)
BC-E 3	Chonburi	-	Male	106.0	120.5	305	4	67.10 ± 22.73 (33.30-82.60)	22.68 ± 7.39 (12.00-28.10)
BC-NE 1	Nakhon Ratchasima	-	Male	110.0	123.0	395	7	97.23 ± 34.75 (49.10-152.60)	26.41 ± 9.25 (13.91-40.37)
BC-NE 2	Nakhon Ratchasima	-	Male	123.0	139.5	615	9	135.93 ± 65.72 (58.80-255.80)	33.53 ± 16.82 (14.97-67.70)
BC-NE 3	Nakhon Ratchasima	-	Female	85.0	93.0	185	5	46.98 ± 15.19 (27.10-61.60)	13.00 ±3.81 (7.60-15.80)

Table 4-1 (cont.) Fifteen *Bungarus candidus* snakes from three localities in Thailand plus a captive-born group; the age, sex, snout-vent length, total length, body weight, number of venom extractions and average weight of fresh and dry venoms are shown for each snake.

Snake code	Locality	Age (year)	Sex	SVL (cm.)	ToL (cm.)	Body weight (g)	No. of extraction	Weight of fresh venom (mg)	Weight of dry venom (mg)
BC-S 1	Nakhon Si Thammarat	-	Male	104.0	117.0	245	8	37.74 ± 13.16 (19.50-62.50)	10.00 ± 3.49 (5.07-16.50)
BC-S 2	Nakhon Si Thammarat	-	Male	91.0	113.0	165	4	9.23 ± 12.60 (5.80-34.10)	3.45 ± 4.89 (1.70-13.10)
BC-S 3	Nakhon Si Thammarat	-	Male	104.0	119.0	285	8	101.49 ± 30.83 (43.60-133.50)	25.46 ± 8.21 (10.18-34.90)
BC-S 4	Nakhon Si Thammarat	-	Female	105.0	119.0	255	8	65.41 ± 13.77 (51.60-93.60)	17.46 ±3.68 (13.17-24.63)
BC-CB 1	Captive-born	5	Male	85.0	96.4	185	6	17.85 ± 7.14 (7.90-29.30)	5.05 ± 2.03 (2.30-8.30)
BC-CB 2	Captive-born	4	Male	99.0	112.0	260	8	69.39 ± 14.97 (35.30-86.20)	13.93 ± 3.28 (7.81-19.07)
BC-CB 3	Captive-born	4	Male	94.0	105.6	245	7	64.00 ± 15.56 (39.50-83.90)	20.00 ± 5.05 (12.41-27.12)
BC-CB 4	Captive-born	4	Female	92.0	102.9	180	8	56.81 ± 13.30 (42.80-80.00)	13.76 ± 3.63 (9.79-20.29)
BC-CB 5	Captive-born	2	Male	83.0	93.0	145	4	37.38 ± 23.94 (11.00-61.60)	14.25 ± 11.15 (5.29-29.60)

Table 4-2 The size of *Bungarus candidus* snakes and their venom from three localities of Thailand and a captive-born group.

Snake group	Locality	Snake size			No. of Snake	Weight of liquid venom (mg/snake)	Weight of dry venom (mg/snake)	% Dry matter of venom	% Venom yield per gram of snake BW
		SVL (cm)	ToL (cm)	BW (gm)					
BC-E	East	99.33 ± 7.64	112.50 ± 9.85	233.33 ± 62.12	3	67.97 ± 24.98	18.93 ± 7.48	27.90 ± 3.94	0.032 ± 0.013
		(91.0-106.0)	(101.5-120.5)	(195.0-305.0)	(3M)	(29.6-144.1) (n = 20)	(7.7-40.7)	(22.71-37.02)	(0.011-0.074)
BC-NE	Northeast	106.00 ± 19.31	118.50 ± 23.57	398.33 ± 215.02	3	116.90 ± 69.07	30.45 ± 17.18	26.59 ± 1.79	0.026 ± 0.009
		(85.0-123.0)	(93.0-139.5)	(185.0-615.0)	(2M 1F)	(27.1-255.8) (n = 21)	(7.6-67.7)	(23.24-30.21)	(0.010-0.042)
BC-S	South	101.00 ± 6.68	117.0 ± 2.83	237.50 ± 51.23	4	60.41 ± 36.68	15.85 ± 9.09	27.53 ± 4.55	0.023 ± 0.012
		(91.0-105.0)	(113.0-119.0)	(165.0-285.0)	(3M 1F)	(5.8-133.5) (n = 20)	(1.7-34.9)	(23.35-40.48)	(0.004-0.047)
BC-CB	Captive-	90.60 ± 6.58	101.98 ± 7.52	203.00 ± 48.04	5	51.95 ± 23.52	13.60 ± 6.71	27.15 ± 6.87	0.023 ± 0.010
	born	(83.0-99.0)	(93.0-112.0)	(145.0-260.0)	(4M 1F)	(7.9-86.2) (n = 33)	(2.3-29.6)	(18.29-48.09)	(0.004-0.042)

All data of snake size and venom are presented in mean ± SD. SVL = the snout to vent length; ToL = total length in centimeters; BW = body weight in grams; M = male; F = female. % Dry matter of venom is defined as the percentage of the weight of dry venom to liquid venom. % Venom yield is defined as the percentage of the weight of liquid venom (in gram) to the body weight of snake (in gram). n = total number of venom collections in each group.

Table 4-3 Characteristics of color and patterns of *Bungarus candidus* from different parts of Thailand and a captive born group.

Characteristics	BC-NE	BC-E	BC-S	BC-CB
Head	Solid black extending back about 1.5-2 times the head length to the first white band. Some have a narrow faint white line at the base of the head.	Grayish-black becoming more distinctly black extending back about 1.5-3 times the head length to the first white band. Some have a faint white line at the base of head resulting in a black, arrowhead shape on the nape.	Very grayish-black with a fair, narrow, white "near band" at the base of the head, resulting in a jet black arrow shaped mark on the nape.	grayish-black or jet black, the color extending back about 1.5-2 times the head length to the first white band. A faint, narrow white band at the base of the head results in a black, arrowhead shape on the nape.
Body color / pattern	25-29 total black bands with first 3-6 anteriormost the widest. White bands have many black specks giving a grayish appearance. Ventral surface (belly) is white.	29-33 total black bands with first 3 anteriormost the widest. Black and white bands are wider anteriorly. White bands are distinctly white, black specks barely noticeable. The edges of black and white bands are zig zag. Ventral surface is white.	25-34 total black bands with first 2-3 anteriormost the widest. The black specks are more prominent in the anterior white bands but become less prominent posteriorly. Ventral surface is white.	28-36 total black bands with first 3-5 anteriormost the widest. White bands are mainly equal wide or narrower than black bands. Black and white bands mostly equal in width. Black specks are not prominent; the white bands are distinctly white. Ventral surface is white.

CHAPTER V

THE COMPARATIVE STUDY ON THE BIOLOGICAL CHARACTERISTICS OF VENOMS FROM INDIVIDUAL *Bungarus candidus* SNAKES

INTRODUCTION

Venomous snakes produce venoms as the weapon for their survivals in term of defense, forage and digestion. Snake venoms with their cocktail of bioactive molecules such as proteins, peptides and enzymes exhibit marked variations in potency and induction of toxicities (Koh et al., 2006). Various situations have been reported to affect the biological properties of snake venoms, e.g. geographical differences (Daltry et al., 1997; Shashidharamurthy et al., 2002; Tsai et al., 2003; Salazar et al., 2007; Shashidharamurthy & Kemparaju, 2007), season of venom collection (Monteiro et al., 1998; Magro et al., 2001), diet (Tan et al., 1992; 1993a; 1993b), sexes (Furtado et al., 2006; Menezes et al., 2006) and age of snakes (Tun-Pe et al., 1995; Saldarriaga et al., 2003).

A number of studies are available for several enzyme activities in snake venoms which act differentially of the body responses. For examples, the syndrome of neuromuscular paralysis in victims has been demonstrated from the action of presynaptically active phospholipase A_2 in the snake venom (Dixon and Harris, 1999). Phospholipase A from a number of snake venoms exhibit wide varieties of myotoxic, cardiotoxic or neurotoxic effects (Harris, 2003; Koh et al., 2006). The potentiating hemorrhagic effect and necrosis has been shown by the effect of hyaluronidase activity (Tu and Hendon, 1983; Pukrittayakamee et al., 1988). L-amino acid oxidase in snake venoms plays a role in inducing apoptosis, hemorrhagic effects and cytotoxicity (Ahn, Lee, and Kim, 1997; Du and Clemetson, 2002). In addition, the activity of acetylcholinesterase in Elapid snake venoms has been shown vary significantly among the different venoms from the same species (Tan and Tan, 1987; Tan and Tan, 1988; Kumar and Elliot, 1973). However, those results need to be verified in the severity of lethal toxicity among venoms relating to the heterogeneous

components in the venom whether depending on type or the concentration of components.

Based upon the studies of *Bungarus candidus* venom, the most lethal components are the phospholipase A_2 of which presynaptically acting toxins like beta-bungarotoxin (Bon and Saliou, 1983; Tan et al., 1989; Tsai et al., 2002; and Khow et al., 2003) and postsynaptically acting toxins like alpha-bungarotoxin (Nirthanan et al., 2002; Kuch et al., 2003) of *B. multicinctus*. Preliminary investigation of biological properties of the commercial *B. candidus* venom and venom fractions has revealed high hyaluronidase, acetylcholinesterase and phospholipase A activities, moderate high L-amino acid oxidase. The major lethal fractions have possessed phospholipase toxin (Tan et al., 1989). The similarity of neurological symptoms has been reported in victims envenoming by *B. candidus* from different geographical areas. The recovery of victims has required the proper time of artificial ventilation and prolonged periods in intensive care (Pochanugool et al., 1997; Dixon and Harris, 1999; Kanchanapongkul, 2002). However, the observations for recovery in different victims are still inconsistency. The treatment using anticholinesterase has been reported to negatively respond in the victim (Looareesuwan et al., 1988; Kanchanapongkul, 2002). Variations in the results of these observations would associate with heterogeneous components in the venom. There is no information whether the different contents of any enzymes in the *B. candidus* venom concern with the variations of clinical features in term of the onset of neurotoxicity, abnormal physical signs, reversible or irreversible of neurological signs and the interval time of bite to death. A few data are available for biological characteristics of the venom of *B. candidus* snake inhabiting in different parts of Thailand, although the complication of clinical signs have been observed in victims with *B. candidus* envenomation (Looareesuwan et al., 1988; Pochanugool et al., 1997; 1998; Laothong and Sitprija, 2001; Kanchanapongkul, 2002; Leeprasert and Kaojarern, 2007). Therefore, the purpose of the present study is to evaluate the venoms of *B. candidus* snakes from three different localities in Thailand and the captive-born snakes in aspects of the lethal toxicity**,** the enzymatic activities, and the molecular weight of protein components. This information may contribute to better insight into the patho-physiological and biochemical basis for solving potential problem in snake bite.

MATERIALS AND METHODS

Animals

Swiss albino mice weighing 18-20 g were obtained from the laboratory animal unit of the Queen Saovabha Memorial Institute. All animals were housed under 12 h light/dark cycles at room temperature of 25±2 ºC, standard foods and water were supplied *ad libitum* until the end of experiments.

Snake venom

The venoms of *Bungarus candidus* from three different parts of Thailand including a group of captive-born snakes were used (details in Chapter IV).

The experimental protocols

5.1 Determination of lethal toxicity (LD_{50})

The lethal toxicity was determined in mice. Five groups of eight mice for each venom sample were tested and observed throughout the quarantine period and experiments. The control group was performed using normal saline solution. All groups were housed in cage and observed throughout the quarantine period of experiments. The LD_{50} was calculated by the method of Reed-Muench (1938) and 95 % confidence limits by the method of Pizzi (1950).

5.2 Determinations of enzymatic activities

All reagents for enzymatic determinations were analytical grade. The *Bungarus candidus* venom was dissolved in 10 mM Tris-buffer (pH 7.4) at the concentration of 1 mg/ml for all enzymatic activity determinations. All assays were performed in duplicated test.

5.2.1 Phospholipase A_2 activity

Phospholipase A_2 activity was determined by the indirect hemolytic method using the preparation of human erythrocyte-egg yolk-agarose plate (Marinetti, 1965; Gutierrez et al., 1988) with the modified method. The cross diameter of hemolytic zones were measured after placing the plate at room temperature for 24 h. The straight calibration curve and the parallel line analysis (WHO, 1995) for the log

dose-diameter relationship was established to determine the hemolytic dose (HLD, in micrograms) causing a hemolytic zone (clear zone) of 10 mm in diameter.

5.2.2 Acetylcholinesterase activity

Acetylcholinesterase activity was determined using the diagnostic kit "BTC SIGMA" based on the use of the butyrylthiocholine (BTC) as substrate. The initial rate of enzyme reaction was used for calculation of the activity. One unit of enzyme activity was arbitrarily defined as the difference at 405 nm / min / mg.

5.2.3 Protease activity

Protease or proteolytic activity was determined by mean of the hide powder azure hydrolyzing activity (Omori-Satoh et al., 1995). One unit of the enzyme activity was defined as the amount of venom hydrolyzing the substrate at a rate of 1.0 absorbance at 595 nm against a control without venom solution.

5.2.4 Alkaline phosphomonoesterase activity

Alkaline phosphomonoesterase activity was determined by a modified method (Lo et al., 1966; Tan and Tan, 1988). One unit of enzyme activity was defined as the amount of enzyme caused the increase of 0.001 absorbance unit per min at the absorbance of 440 nm.

5.2.5 Phosphodiesterase activity

Phosphodiesterase activity was determined by a modified method (Lo et al., 1966; Tan and Tan, 1988). One unit of enzyme activity was defined as the amount of venom caused the increment of 0.001 absorbance unit per min at the absorbance of 440 nm.

5.2.6 L-amino acid oxidase activity

L-amino acid oxidase activity was determined with the modified technique (Worthington Enzyme Manual, 1977; Tan and Tan, 1988). One unit of enzyme activity was defined as the amount of venom caused the increment of 0.001 absorbance unit per min at the absorbance of 426 nm.

5.2.7 Hyaluronidase activity

Hyaluronidase activity was determined with the modified method (Xu et al., 1982; Tan and Tan, 1988). The enzyme activity was expressed as National Formulary Unit per milligram (NFU/mg) at the absorbance of 400 nm.

5.3 Determinations of protein components and the molecular weight

Protein components and the molecular weight of all *B. candidus* venoms and the other Elapid snake venoms were performed on tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) according to the detail in Chapter III.

5.4 Fractionation of Bungarus candidus venom by Reverse-phase High Performance Liquid Chromatography (RP-HPLC)

Fresh venom from individual *Bungarus candidus* snake was obtained. The protein concentration of the venom was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard. Each venom was dissolved in deionized water to give a protein concentration of 800 µg/ml. Eighty micrograms of total venom proteins was fractionated by reverse-phase HPLC.

5.5 Isolation and purification of the toxins

5.5.1 Cation-exchange chromatography

Fifty milligrams each of crude *Bungarus candidus* venom from the northeast (BC-NE) and the south (BC-S) of Thailand was dissolved in 2 ml of 50 mM phosphate buffer (pH 6.25), applied to the Resource ^{TM} S Column equilibrated with the buffer, and then eluted with a linear gradient of 0–0.3 M NaCl at the flow rate of 10 ml/h. Absorbance was measured at 280 nm, and fractions of 2 ml were collected.

5.5.2 Adsorption chromatography on hydroxyapatite

The major toxic fractions obtained on cation-exchange chromatography were pooled, concentrated in collodion bag *in vacuo*, and then dialyzed against the phosphate buffer. The column was eluted with a linear gradient of 0.05–0.35 M phosphate buffer (pH 6.25). Fractions of 1.5 ml were collected at the flow rate of 15 ml/h.

5.5.3 Two dimensional gel (2D) electrophoresis

The proteins of fraction obtained from adsorption chromatography were separated by 2-D electrophoresis using an IPGphor (Amersham Bioscience, Uppsala, Sweden) instrument. The spots of interest were analyzed by MALDI-TOF mass spectrometer model reflex V (Brucker Daltonik GmbH). Protein identifications

were obtained from MASCOT (MatrixScience) and were matched with peptide mass fingerprints in a protein database. Protein scores are significant when *p* value is less than 0.05.

Statistical analysis

All mean values are presented as mean ± SD. The results were evaluated by analysis of variances (ANOVA); the significant differences among groups was compared by Duncan's multiple range test, with $p<0.05$ indicating significance.

RESULTS

Determinations of lethal toxicity and enzymatic activities

The mean values of the lethal toxicity and enzymatic activities among Malayan krait venom groups are presented in Table 5-1. The lethal toxicity among groups showed no significant difference. Determinations of enzymatic activities of *B. candidus* venom among the wild-caught groups (BC-E, BC-NE, and BC-S) and the captive-born group (BC-CB) revealed no significant differences in phospholipase A_2, protease, phosphodiesterase and hyaluronidase activities. L-amino acid oxidase activity of BC-CB (1771 ± 837 unit/min/mg) were significantly lower than those of BC-S (2986 ± 321 unit/min/mg), BC-E (2900 ± 552 unit/min/mg) and BC-NE (2587 ± 418 unit/min/mg)($p<0.05$). The acetylcholinesterase activity of BC-CB (1.459 ± 0.216 unit/min/mg) was significantly higher than that of BC-E (0.633 ± 0.080 unit/min/mg) ($p<0.05$). The alkaline phosphomonoesterase activity of BC-CB (303 ± 164 unit/min/mg) was significantly lower than that of BC-NE (1385 ± 1207 unit/min/mg) ($p<0.05$).

Among groups of the wild-caught *B. candidus* venom, the enzymatic activities exhibited significant differences in acetylcholinesterase, alkaline phosphomonoesterase and hyaluronidase activities. The acetylcholinesterase activity of BC-E venom (0.633 ± 0.080 unit/min/mg) were significantly lower ($p<0.05$) than those of BC-S (1.426 ± 0.325 unit/min/mg) and BC-NE (1.206 ± 0.259 unit/min/mg). The alkaline phosphomonoesterase of BC-NE venom (1385 ± 1207 unit/min/mg) was significantly higher ($p<0.05$) than those of BC-E (372 ± 257 unit/min/mg) and BC-S

(385 ± 173 unit/min/mg), whereas the hyaluronidase activity of BC-E (481 ± 32 NFU) was significantly higher ($p<0.05$) than that of BC-NE (157 ± 112 NFU).

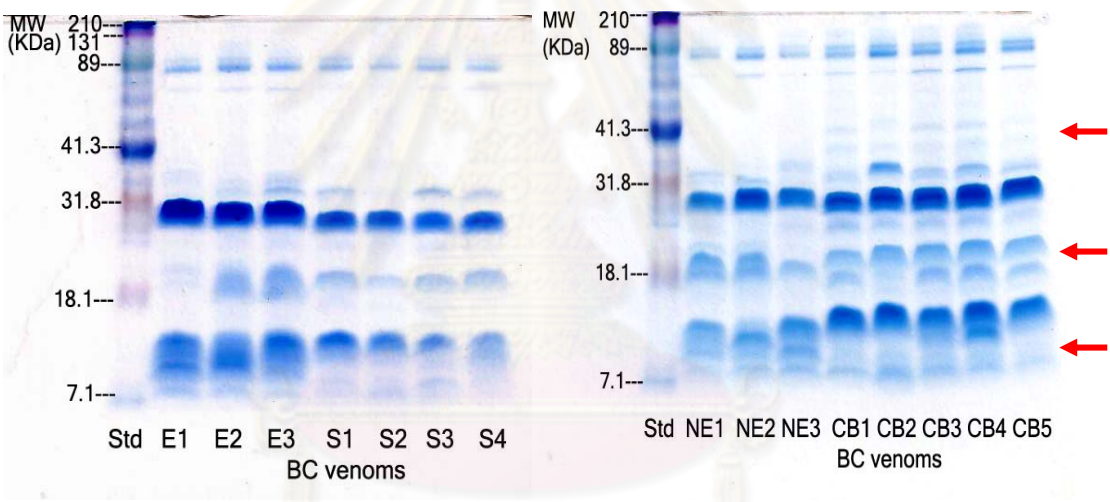
Table 5-1 Mean values of lethal toxicity and enzymatic activities of each *Bungarus candidus* venom group.

Snake group	**BC-E** (n = 3)	**BC-NE** (n = 3)	**BC-S** (n = 4)	**BC-CB** (n = 5)
LD_{50} (µg / g)	0.053 ± 0.013	0.064 ± 0.015	0.061 ± 0.013	0.070 ± 0.024
HLD (µg)	0.12 ± 0.11	0.04 ± 0.03	0.05 ± 0.02	0.07 ± 0.02
AChE (Unit / min / mg)	0.633 ± 0.080 [a]	1.206 ± 0.259 [b]	1.426 ± 0.325 [b]	1.459 ± 0.216 [b]
PRO (Unit / mg)	0.033 ± 0.009	0.029 ± 0.019	0.054 ± 0.025	0.057 ± 0.010
PME (Unit / min / mg)	372 ± 257 [a]	1385 ± 1207 [b]	385 ± 173 [a]	303 ± 164 [a]
PDE (Unit / min / mg)	19 ± 5	21 ± 14	23 ± 6	17 ± 3
LAAO (Unit / min / mg)	2900 ± 552 [a]	2587 ± 418 [ab]	2986 ± 321 [a]	1771 ± 837 [b]
HYA (NFU)	481 ± 32 [a]	157 ± 112 [b]	243 ± 190 [ab]	292 ± 206 [ab]

[a, b] Means within a row with different superscripts between groups of each measurement differ significantly ($p < 0.05$). LD_{50} = Lethal toxicity (µg of venom / g of mouse body weight); HLD = Indirect hemolytic dose representing phospholipase A_2 activity which is the amount of venom (µg) produced a hemolytic (clear) zone of 10 mm in diameter; AChE = Acetylcholinesterase activity; PRO = Proteolytic activity; PME = Alkaline phosphomonoesterase activity; PDE = Phosphodiesterase activity; LAAO = L-amino acid oxidase activity; HYA = Hyaluronidase activity; NFU = National Formulary Unit.

Molecular weight determination of protein components

Pattern of tricine SDS-PAGE under non-reducing condition of each *B. candidus* venom is presented in Figure 5-1. The overall marked dense protein bands were quantitative different in the region of molecular weight from 7.1 to 41.3 kDa. The number of protein bands at the molecular weight from 18.1–41.3 kDa was distinct in captive-born group (BC-CB) as compared with the southern group (BC-S) and the other two groups (BC-E and BC-NE groups). The protein pattern of *B. candidus* venom was different from the other Elapid venoms, e.g. *Naja kaouthia, Ophiophagus hannah* and *Bungarus fasciatus* (Figure 5-2).



(A) (B)

Figure 5-1 Tricine SDS-PAGE pattern of *Bungarus candidus* venom under non-reducing condition. Twelve microgram of venom solution (1mg/ml) from eastern (E), northeastern (NE), southern (S) regions and captive-born (CB) groups were applied to each lane for electrophoresis. Kaleidoscope prestained standard (lane Std) was used as molecular weight markers in kDa, from top to bottom: myosin (210), β-galactosidase (131), bovine serum albumin (89), carbonic anhydrase (41.3), soybean trypsin inhibitor (31.8), lysozyme (18.1), and aprotinin (7.1). Arrows (red) indicate the marked difference in the variations of protein pattern. The panel A, comparison of eastern and southern venom groups; the panel B, comparison of northern and captive-born venom groups.

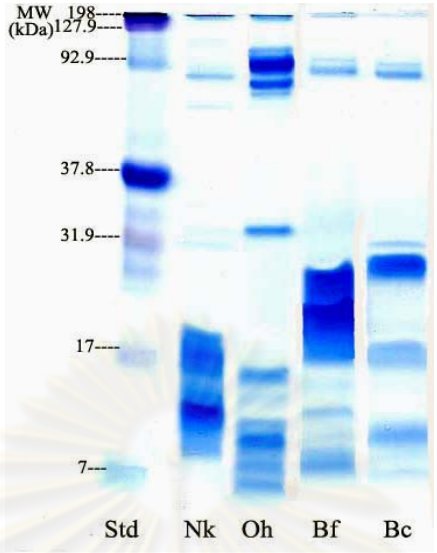


Figure 5-2 Tricine SDS-PAGE pattern of Elapid snake venoms under non-reducing condition. Kaleidoscope prestained standards (BIO-RAD Catalog 161-0324, Control 310006180; lane Std) was used as molecular weight markers in kDa, from top to bottom: myosin (198), β-galactosidase (127.9), bovine serum albumin (92.9), carbonic anhydrase (37.8), soybean trypsin inhibitor (31.9), lysozyme (17), and aprotinin (7). Nk = *Naja kaouthia*; Oh = *Ophiophagus hannah*; Bf = *Bungarus fasciatus*; Bc = *Bungarus candidus*.

Fractionation of Bungarus candidus venom by Reverse-phase High Performance Liquid Chromatography (RP-HPLC)

The RP-HPLC chromatographic profiles of the venom of individual snake of each group of *B. candidus* are shown in Figures 5-3A, 5-4A, 5-5A and 5-6A for eastern (BC-E), northeastern (BC-NE), southern (BC-S) and captive born (BC-CB) snakes, respectively. The venom protein peaks were aligned according to the retention times while each peak was designated by a capital letter (A to Z) as shown in Figures 5-3B, 5-4B, 5-5B 5-6B and also in Table 5-2. From these figures and table, it appeared that, overall, the profiles of BC-E and BC-NE contained less protein peaks than those of BC-S and BC-CB. These results were supported by that of SDS-PAGE shown in Figure 5-1.

Another point of interest is the venom protein profiles of BC-E and BC-NE were quite similar but were different from those of BC-S and BC-CB (Figure 5-7). It should be mentioned that the BC-CB were the offspring of snakes from the south of the country and it is therefore not surprising the BC-CB and BC-S profiles were almost identical. From these observations, RP-HPLC seems to be a better method than SDS-PAGE (Figure 5-1) to differentiate the venoms of BC-S from those of BC-NE and BC-E.

Table 5-2 also reveals the individual differences regarding the numbers and the percentage abundance of protein peaks in RP-HPLC of venoms of the same group. For example, among the 4 BC-S snakes, BC-S1 venom contained 16 while BC-S2 showed 19 peaks under RP-HPLC (Figure 5-5A and Table 5-2). Moreover, peaks J and K of these two venoms, while eluted at almost the same retention times, showed several fold differences in protein abundance.

Table 5-2. Retention time and percent abundance of protein peaks under RP-HPLC of various *B. candidus* venoms.

Snake Gr.	Northeastern BC							Eastern BC							Southern BC								Captive born BC									
	NE1		NE2		NE3			E1		E2		E3			S1		S2		S3		S4			CB1		CB2		CB3		CB4		CB5	
Peak	RT	Area (%)	RT	Area (%)	RT	Area (%)	Peak	RT	Area (%)	RT	Area (%)	RT	Area (%)	Peak	RT	Area (%)	RT	Area (%)	RT	Area (%)	RT	Area (%)	Peak	RT	Area (%)	RT	Area (%)	RT	Area (%)	RT	Area (%)	RT	Area (%)
A	5.4	1.74	5.2	0.90	5.0	1.87	A	5.0	3.56	5.2	3.66	5.2	2.70	A			5.3	1.19			5.2	1.06	A	5.5	0.96					4.9	2.85		
B	6.2	6.43	5.8	4.33	5.6	10.75	B	5.6	13.30	5.8	8.78	5.9	6.35	B									B										
C							C	7.8	0.67					C					8.2	0.77	8.3	1.15	C					6.2	0.96	6.9	0.61		
D					13.7	2.02	D							D	14.3	8.84	14.3	13.55	14.2	6.69	13.6	5.44	D	14.5	13.65	14.3	15.65	13.7	0.87	11.9	8.88	14.8	16.03
E	15.6	2.11	16.2	7.38	15.2	1.69	E	15.4	1.34	15.8	0.81	16.0	0.83	E	16.1	1.32	16.1	1.91	16.0	1.39	15.9	1.25	E	16.7	2.00	16.2	0.96	16.7	7.32	15.1	1.35	16.8	1.17
F	16.5	1.50	17.2	1.28	17.2	1.30	F	16.7	2.61	17.7	1.26	17.2	1.35	F	17.0	1.69					17.3	0.85	F	17.8	1.46	17.7	0.56	18.0	0.87			17.7	1.36
G							G	18.5	1.73	18.8	1.01			G			18.9	0.94					G			19.0	1.05	19.4	2.27			19.3	0.76
H							H							H									H			20.4	0.89						
I							I	22.3	0.24			22.5	0.83	I									I										
J	23.8	3.83			23.6	1.15	J	23.5	3.18	23.9	6.77	23.8	5.48	J	23.1	1.31	23.0	6.16	23.2	10.47	23.8	3.51	J					24.3	16.44	23.6	3.18		
K			24.4	5.97	24.5	3.57	K	24.2	2.53					K	24.4	14.89	24.2	6.79	24.0	14.01	24.4	16.83	K	24.5	18.15	24.8	15.16	25.2	2.43	24.4	15.23	24.6	11.22
L					26.0	7.29	L	25.9	6.03					L	26.3	5.49	26.0	5.70	26.0	7.45	26.0	5.10	L	26.2	2.90	25.9	5.44	27.1	4.78	25.8	4.96	26.6	5.22
M	26.5	20.23	26.5	14.62	26.9	23.78	M	26.5	18.57	26.2	16.24	26.4	11.46	M	26.7	6.77	27.2	6.29			26.9	4.24	M	26.9	4.35	26.9	2.53			26.9	5.80	27.4	3.64
N			27.8	0.97			N			27.1	12.69	26.7	14.33	N	28.6	1.69	28.5	1.55	28.4	1.34	28.3	1.22	N	28.5	1.65	28.3	1.27	29.4	1.05	28.2	1.30	28.8	1.41
O	29.4	5.49	28.9	5.42	29.8	8.83	O					29.1	5.62	O	29.7	11.25	29.5	9.89	29.5	8.45	29.5	7.45	O	29.5	8.23	29.5	7.12	30.4	9.88	29.4	7.07	30.0	8.94
P	29.8	3.04	30.0	2.52			P	29.8	4.19	30.0	14.63	29.8	3.49	P	31.0	8.43	30.8	7.48	30.8	8.01	29.9	3.64	P	30.8	7.64	30.0	3.75	31.6	10.23	29.9	4.70	30.9	2.63
Q	31.0	5.78	30.7	2.43			Q	30.7	1.17			30.5	2.96	Q	31.4	8.88	31.3	6.44	31.3	6.08	30.8	9.63	Q	31.3	8.84	31.0	8.31	30.9	7.19	30.8	7.76	31.4	4.85
R	32.1	34.95	32.3	27.48	32.0	19.05	R	32.0	3.64	32.0	15.79	32.0	21.65	R			32.1	6.60					R	32.1	4.49	31.4	7.54	32.0	4.71			31.8	7.44
S							S			32.5	3.67	32.6	6.23	S	32.8	13.12	32.8	6.29	32.7	15.34	32.6	7.38	S	32.8	10.17	32.1	4.68	32.9	13.86	32.7	5.95	32.6	3.80
T			33.2	7.08	32.9	3.19	T	32.8	5.21	33.0	3.08			T	33.8	6.49	33.7	6.70	33.7	6.77	33.7	13.71	T	33.7	5.57	32.4	5.97	33.6	6.42	33.7	15.02	33.5	13.06
U	33.8	7.03	33.9	12.61	34.1	6.25	U	34.1	4.87	34.0	6.35	33.6	5.62	U	34.9	3.68	34.8	5.02	34.8	4.25	34.9	6.81	U	34.8	3.07	33.7	7.46	34.6	4.39	34.9	5.76	34.5	6.69
V	35.4	5.90	35.3	4.69	35.9	9.27	V	35.8	27.16	35.8	5.26	35.7	11.08	V	35.6	4.59	35.6	5.10	35.2	4.89	35.7	4.51	V	35.6	4.34	35.0	2.74	35.7	4.86	35.6	4.07	35.5	3.49
W			36.9	2.32			W							W	36.6	1.54	36.5	1.51	36.5	2.72	36.5	4.85	W	36.5	1.62	35.8	5.43	36.5	1.48	36.5	4.54	36.1	4.83
X							X							X			37.9	0.90	37.3	1.37	37.5	1.37	X	37.3	0.39	37.0	1.71			36.9	0.97	37.1	1.98
Y							Y							Y									Y	38.0	0.52	37.9	1.42					38.5	1.48
Z	39.8	1.97					Z							Z									Z			39.0	0.36						
#	14		15		14			17		14		15			16		19		16		19			19		21		18		18		19	

Note: RT, retention time (min)

#, total number of protein peaks

BC-E VENOMS

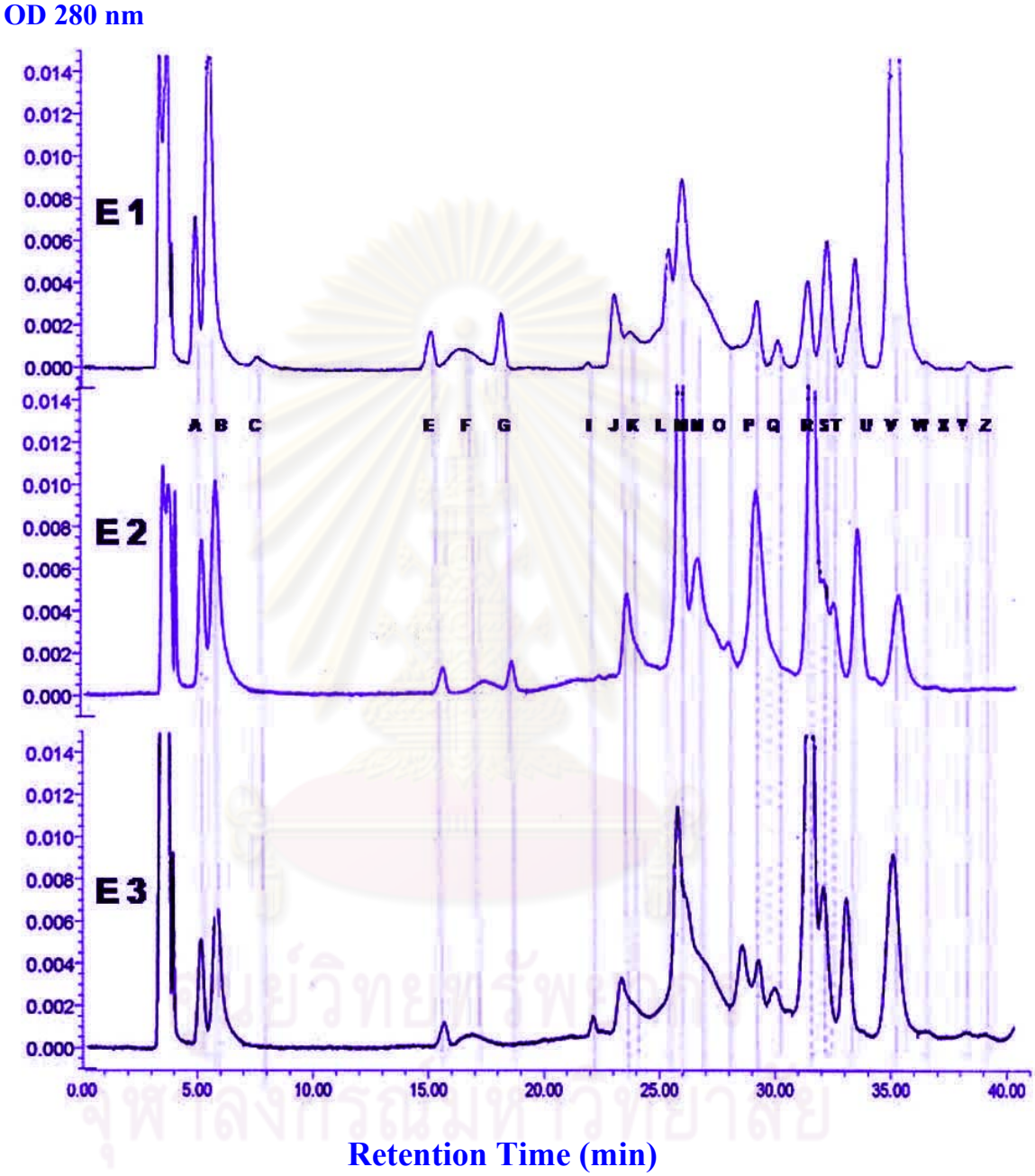


Figure 5-3A. RP-HPLC profiles of individual snake venoms from the eastern *B. candidus* (BC-E) group with the protein peaks (A to Z) aligned according to their retention times (vertical dot line).

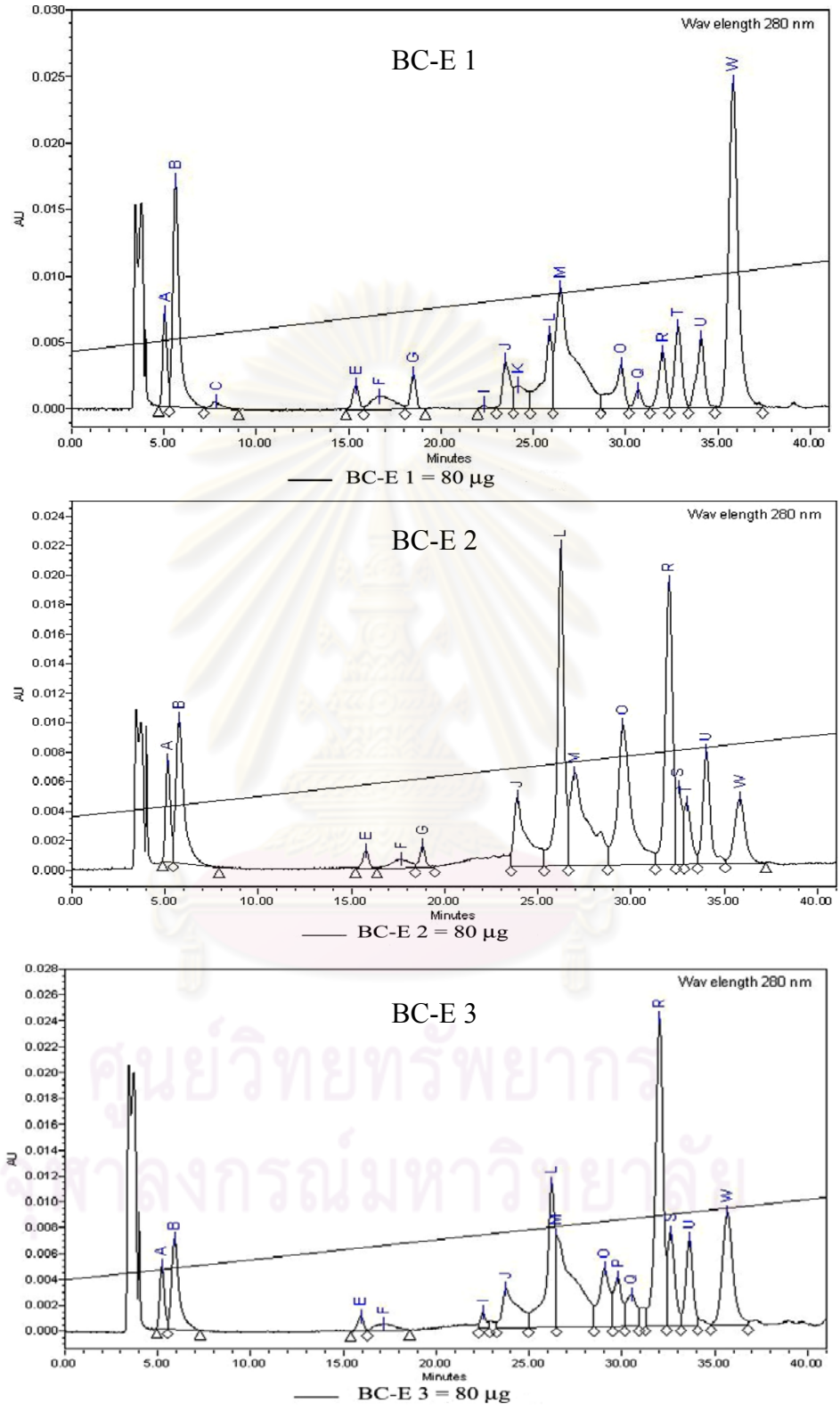


Figure 5-3B. RP-HPLC profiles of individual snake venom from the eastern *B. candidus* (BC-E) group showing the areas of protein peaks (A to Z).

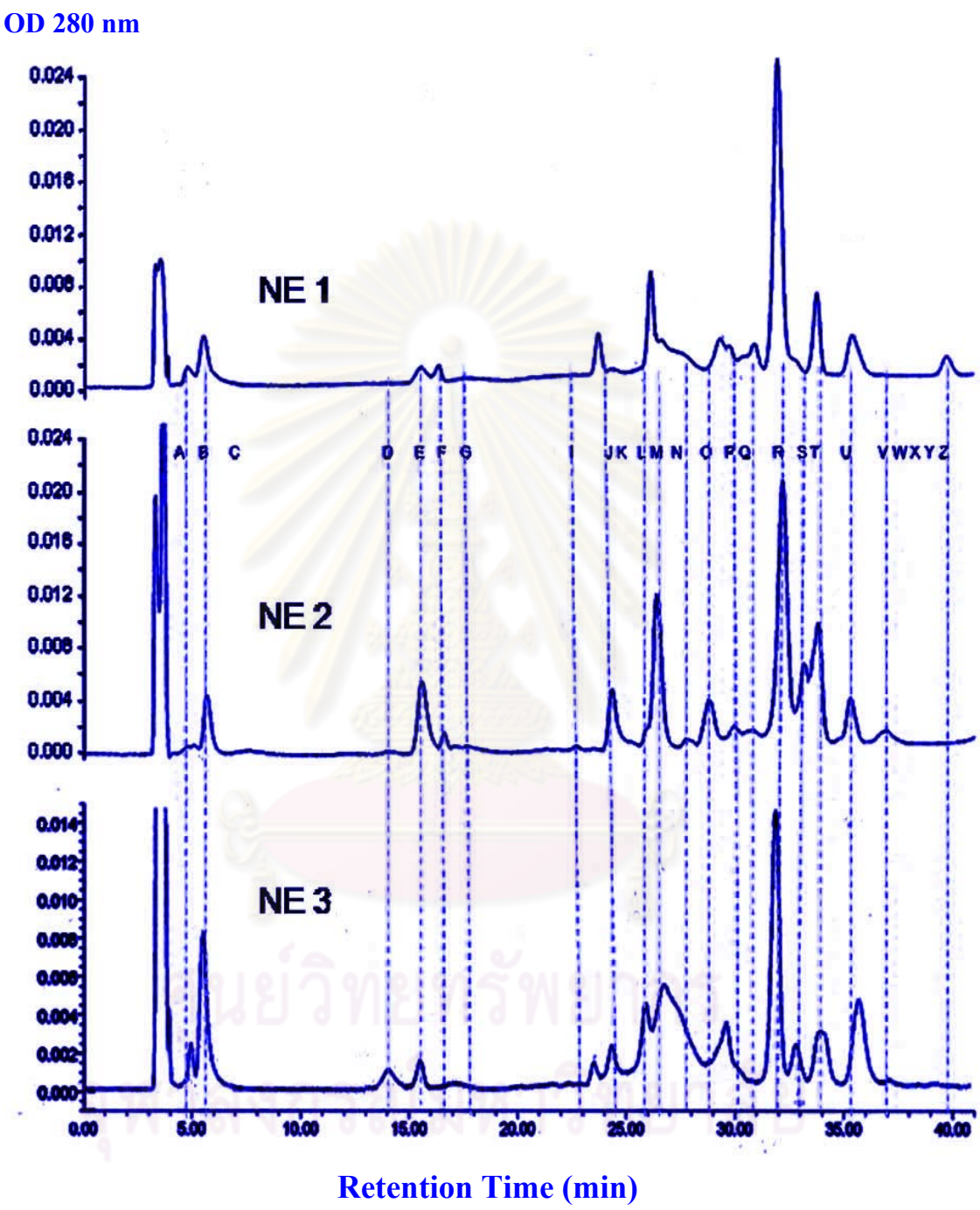


Figure 5-4A. RP-HPLC profiles of individual snake venoms from the northeastern *B. candidus* (BC-NE) group with the protein peaks (A to Z) aligned according to their retention times (vertical dot line).

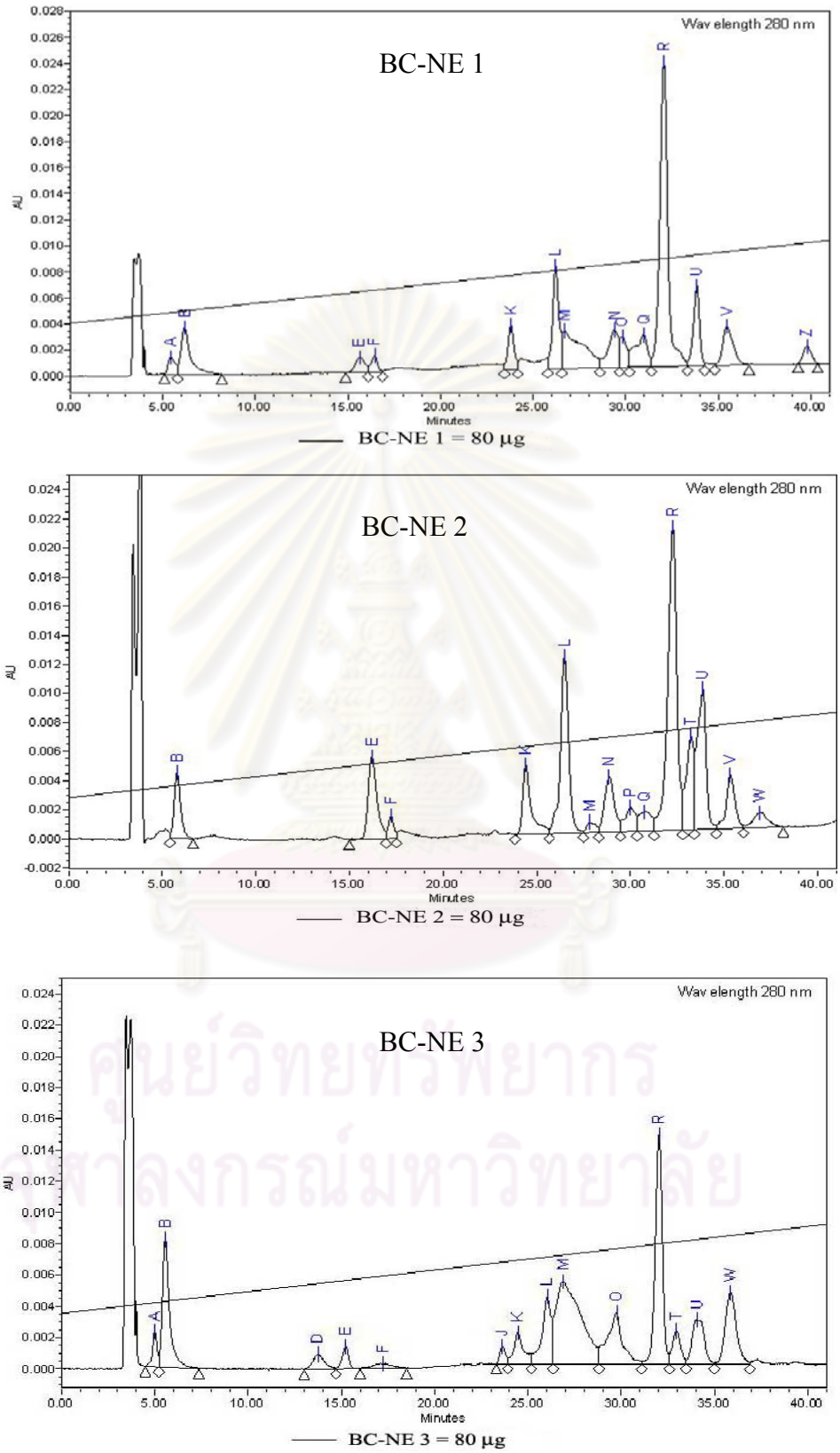


Figure 5-4B. RP-HPLC profiles of individual snake venom from the northeastern *B. candidus* (BC-NE) group showing the areas of protein peaks (A to Z).

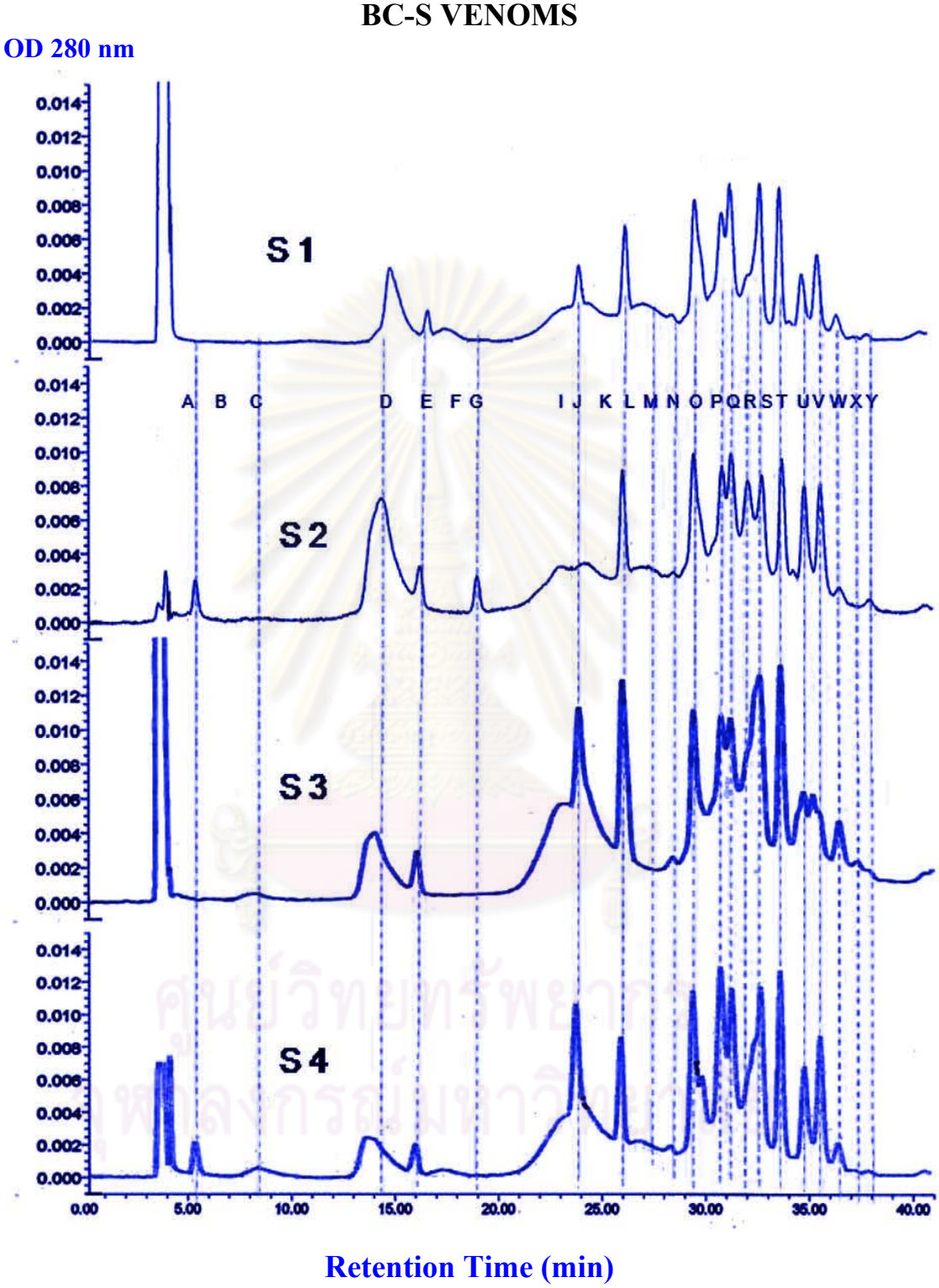


Figure 5-5A. RP-HPLC profiles of individual snake venoms from the southern *B. candidus* (BC-S) group with the protein peaks (A to Z) aligned according to their retention times (vertical dot line).

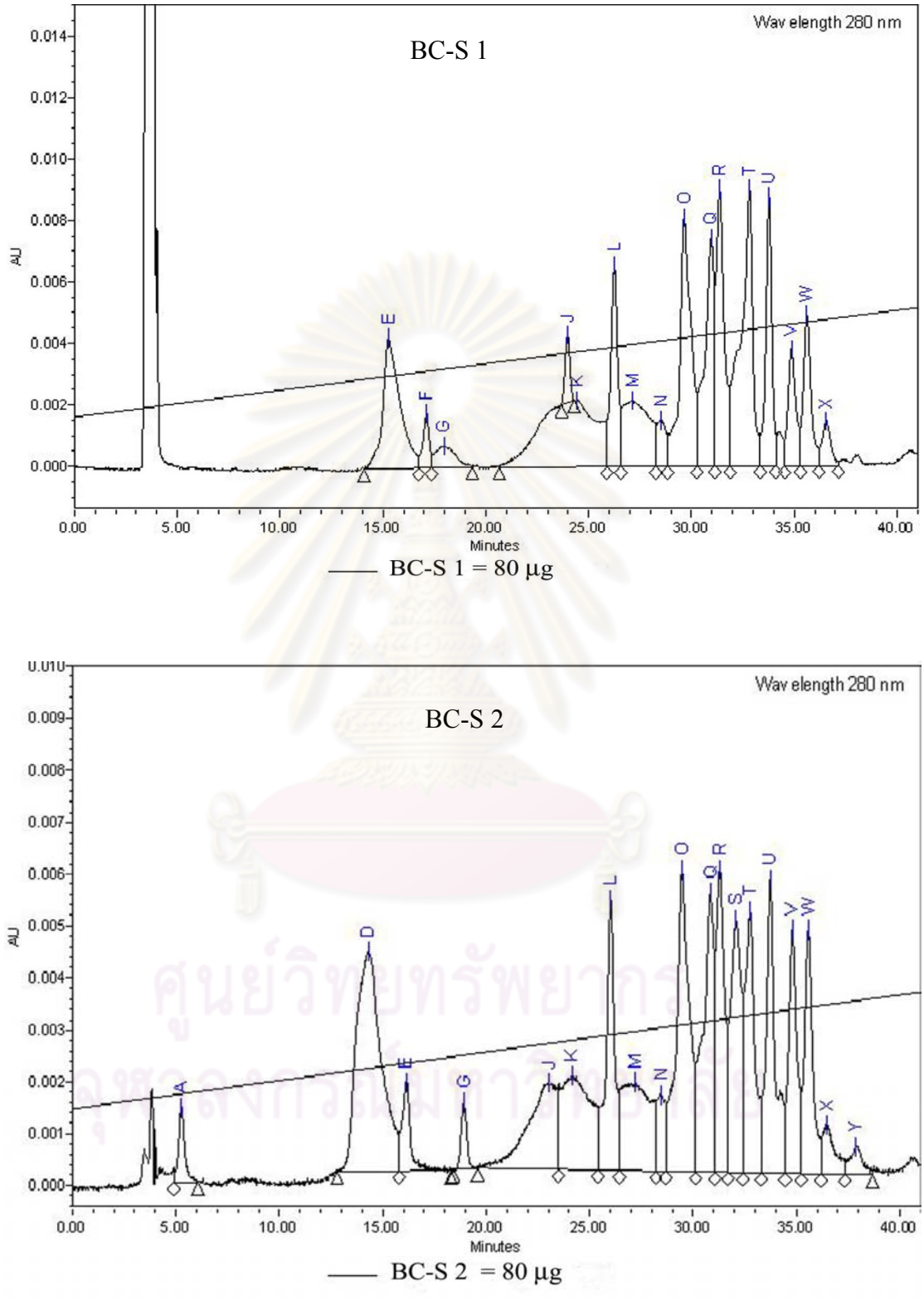


Figure 5-5B. RP-HPLC profiles of individual snake venom from the southern *B. candidus* (BC-S) group showing the areas of protein peaks (A to Z).

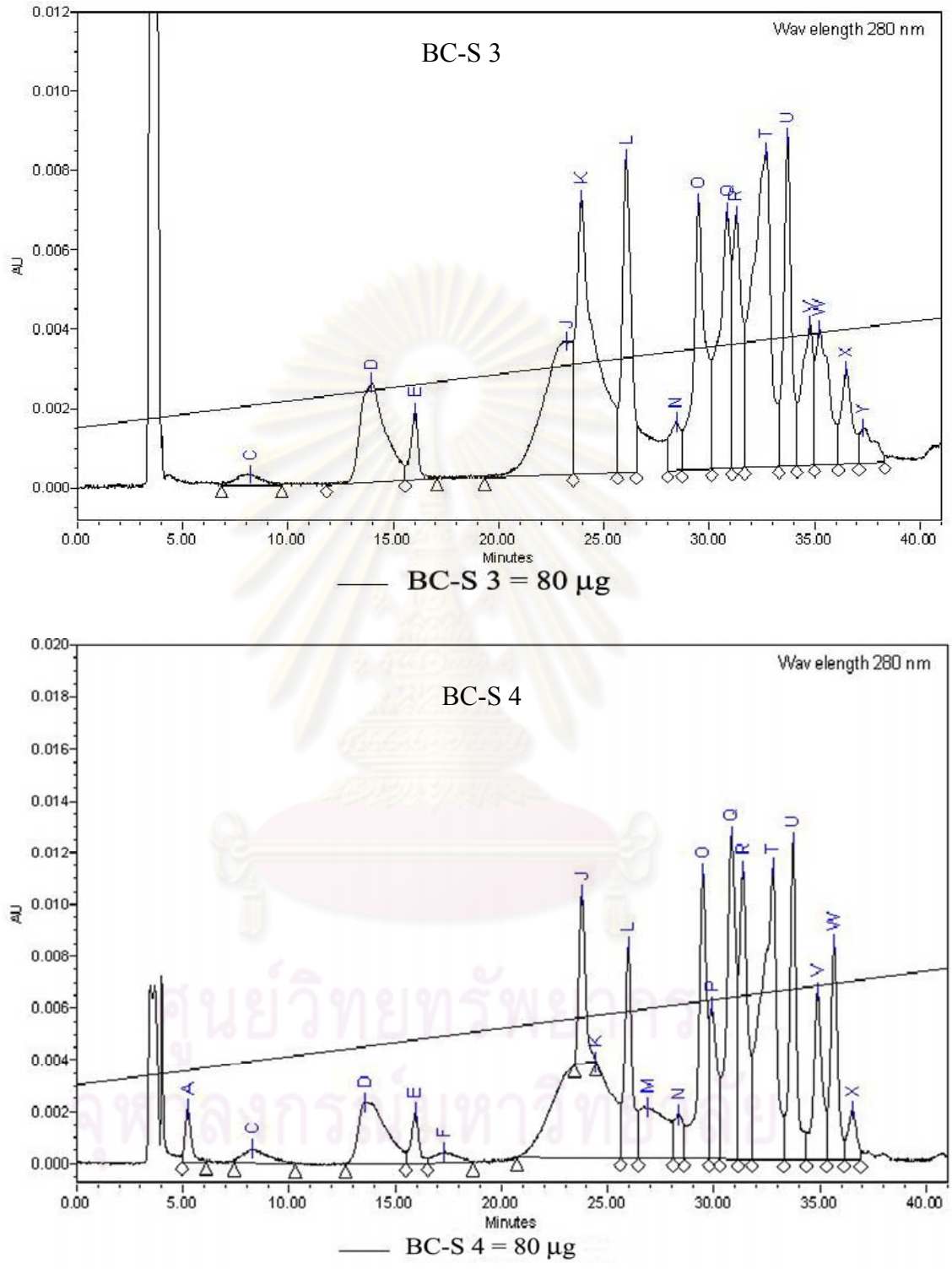


Figure 5-5B. (Continue) RP-HPLC profiles of individual snake venom from the southern *B. candidus* (BC-S) group showing the areas of protein peaks (A to Z).

BC-CB VENOMS

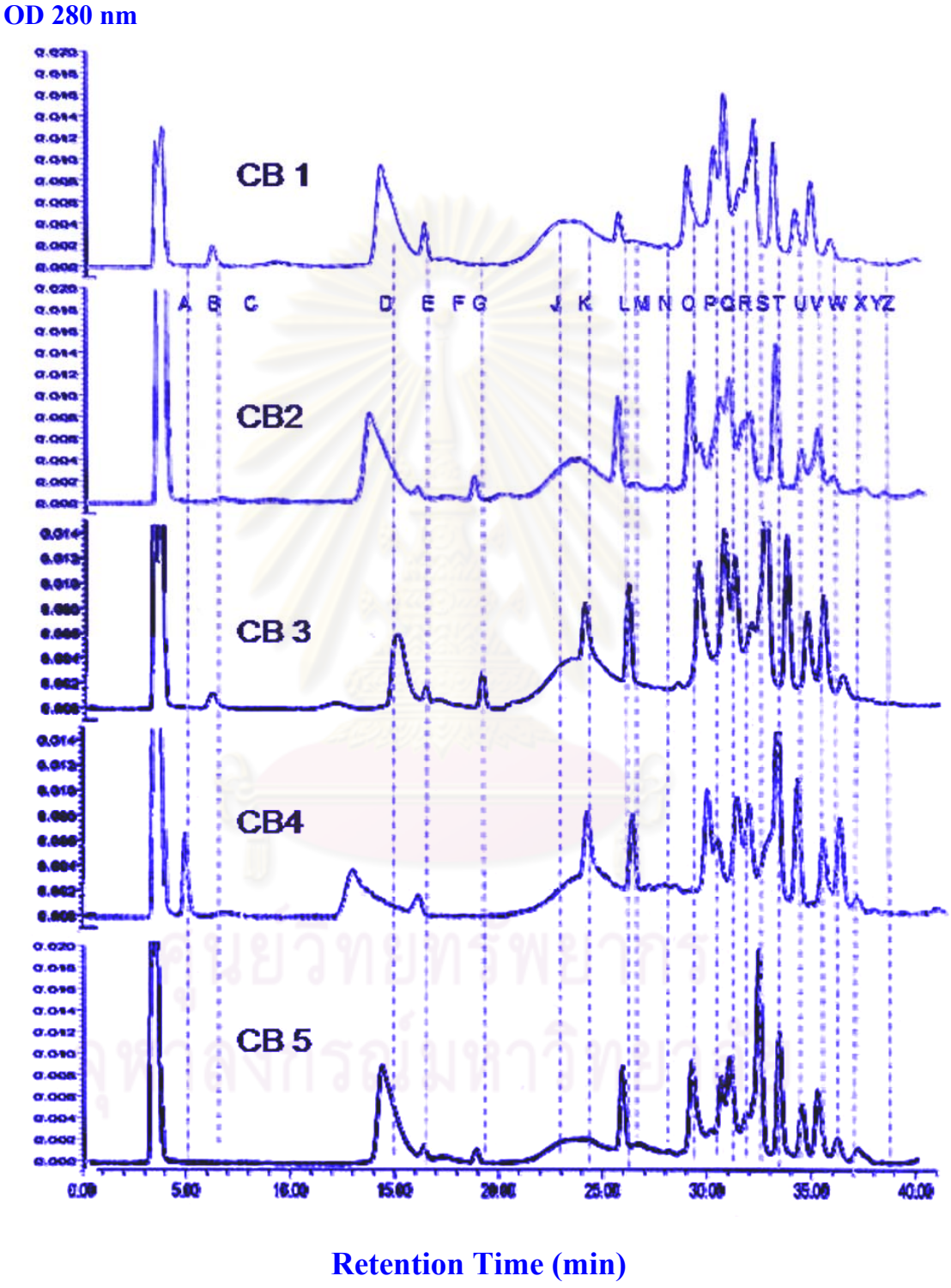


Figure 5-6A. RP-HPLC profiles of individual snake venoms from the captive-born *B. candidus* (BC-CB) group with the protein peaks (A to Z) aligned according to their retention times (vertical dot line).

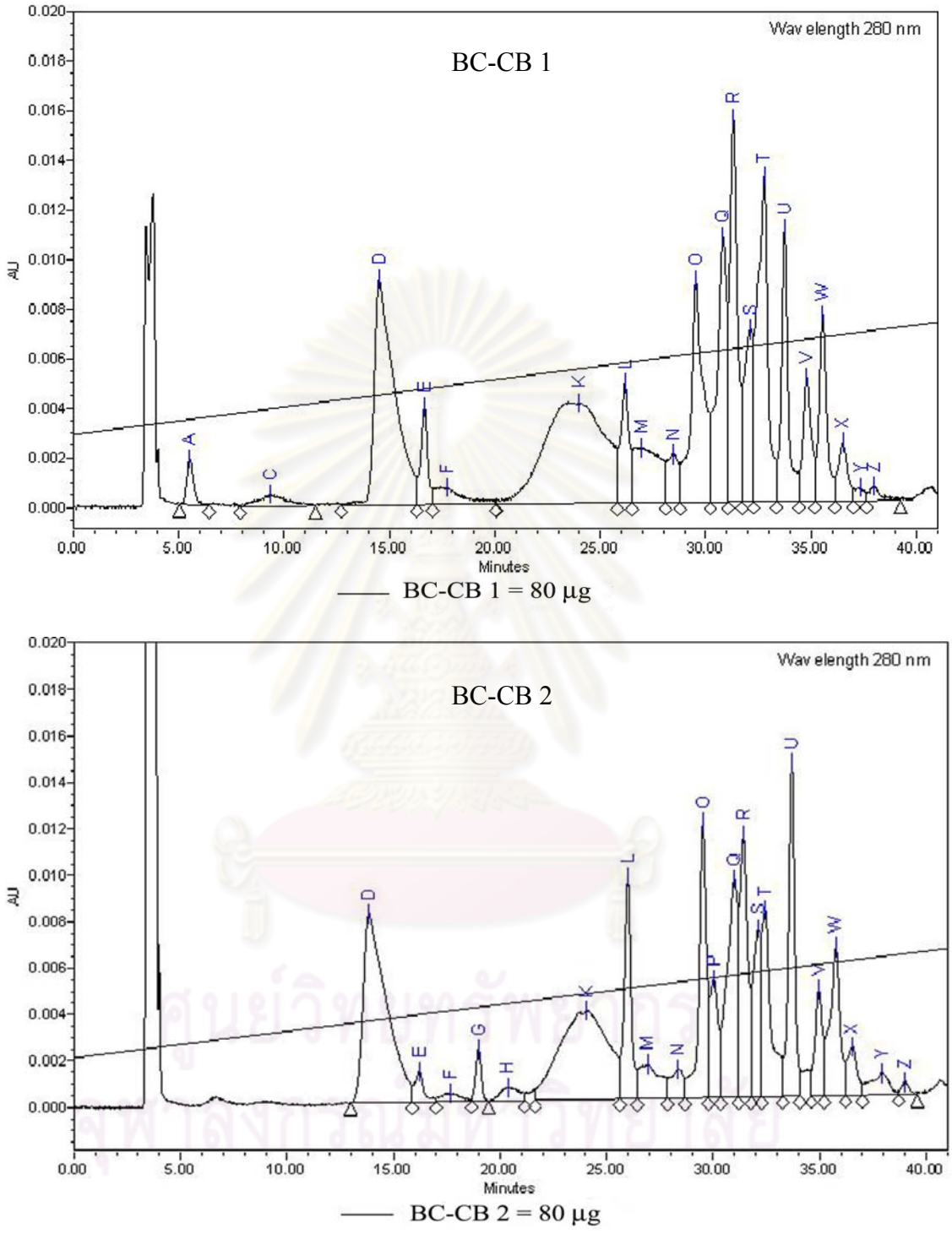


Figure 5-6B. RP-HPLC profiles of individual snake venom from the captive born *B. candidus* (BC-S) group showing the areas of protein peaks (A to Z).

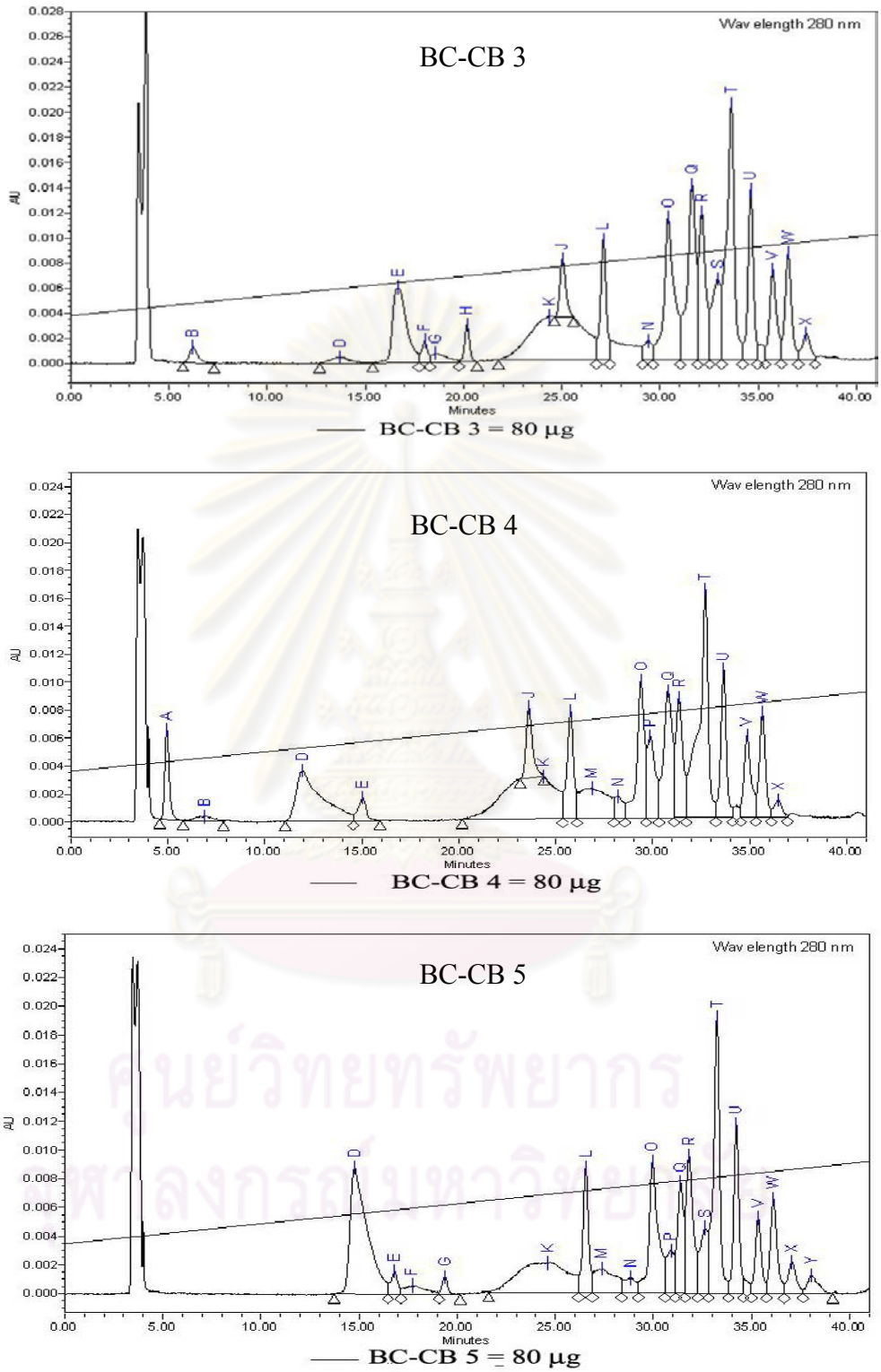


Figure 5-6B. (Continue) RP-HPLC profiles of individual snake venom from the captive born *B. candidus* (BC-S) group showing the areas of protein peaks (A to Z).

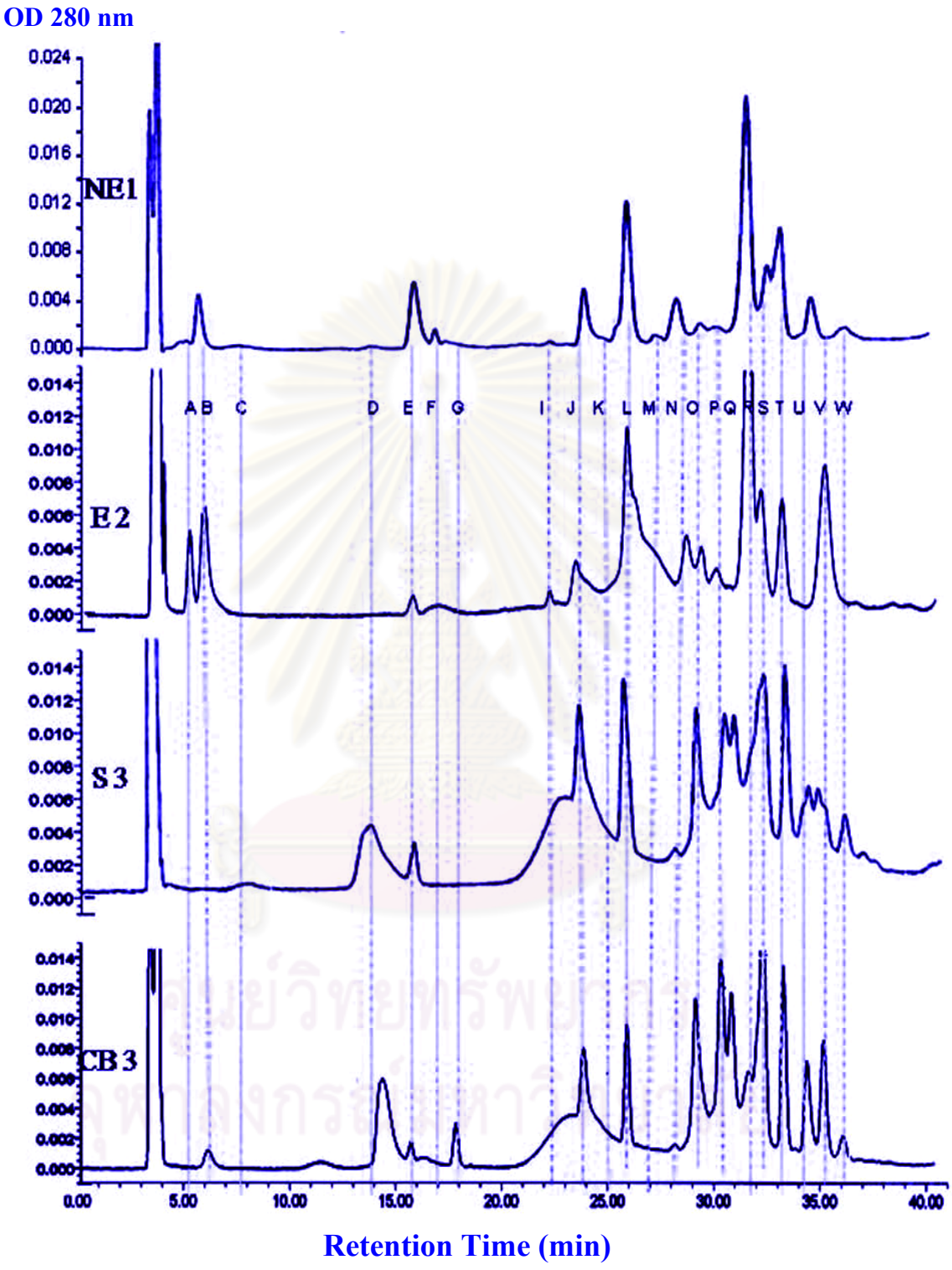


Figure 5-7 RP-HPLC profiles of *B. candidus* venoms from snakes of different origins with the protein peaks (A to Z) aligned according to their retention times (vertical dot line). NE, northeastern; E, eastern; S, southern; CB, captive-born.

Isolation and purification of the toxins

On cation-exchange chromatography of the BC-NE2 and BC-S3 crude venoms, 102 and 103 fractions were obtained and pooled into 15 and 18 fractions, respectively (Figures 5-8 and 5-10). Distribution of protein, lethal toxicity and PLA_2 activity of BC-NE2 and BC-S3 are summarized in Table 5-3 and 5-5. The pooled fractions of 5, 6, 12, 13, 14, and 15 exhibited the lethal toxicity which more potent than the BC-NE2 crude venom (2 LD_{50} = 2.18 μg/ 20 gm mouse body weight). Whereas the lethal toxicity of the pooled fractions which more potent than the BC-S3 crude venom (2 LD_{50} = 1.62 μg/20 gm mouse body weight) was observed for the last six pooled fractions (13–18). Fractions 12–14, the major toxic fractions with high protein contents obtained on cation-exchange chromatography of BC-NE2, were contained 67% in total of the recovered lethal toxicity and thus were chosen for adsorption chromatography. On the other hand, the pooled fractions 15 -17 from cation-exchange chromatography of BC-S3 contained 51% in total of the recovered lethal toxicity were chosen.

Adsorption chromatography of fractions 12–14 (BC-NE2) and fractions 15–17 (BC-S3) on hydroxylapatite column resulted in an elution pattern comprising four peaks each. Lethal toxicity detected in all peaks was potent than the BC-NE2 and BC-S3 crude venoms. Four peaks were designated as N1 (tube no. 84–92), N2 (tube no. 93–107), N3 (tube no. 108–115), and N4 (tube no. 116–123) for the BC-NE crude venom (Fig 5-9) and as SO1 (tube no. 100–116), SO2 (tube no. 117–128), SO3 (tube no. 129–143) and SO4 (tube no. 144–156) for the BC-S3 crude venom (Figure 5-11). Table 5-4 and 5-6 summarize the purification of toxins from the BC-NE2 and BC-S3 crude venoms on cation-exchange chromatography and adsorption chromatography on hydroxylapatite, respectively. Only fractions N2, N3, SO1 and SO2 were determined PLA2 activity. Fractions N2 and N3 were achieved 4- and 5- fold purification of the BC-NE2 crude venom, whereas SO1 and SO2 were achieved 4- and 10-fold purification of the BC-S3 crude venom. The total lethal toxicity of these toxins amounted to 19.3% of the BC-NE2 and to 9.9% of BC-S3 crude venoms.

Two dimensional gel (2D) electrophoresis

The protein peaks of N2 and SO2 from the adsorption chromatography on hydroxylapatite were chosen and applied separately to 2D gel electrophoresis. The

protein spots of interest were performed (Figure 5-12) and analyzed by MALDI-TOF mass spectrometer. The protein identifications obtained from the MASCOT search (MatrixScience at http://www.matrixscience.com/) were matched with peptide mass fingerprints in a protein database as follow;

N2-1 is matched to chain A of β2-bungarotoxin from *Bungarus multicinctus* which acted as a potassium channel binding by Kunitz modules and targeted phospholipase action. The nominal mass (M_r) is 14,235 Da and pI value 7.57 (Figure 5-13). The amino acid sequence coverage 89% matched peptides is shown in bold red,

1 **NLINFMEMIR** YTIPCEK**TWG EYADYGCYCG AGGSGRPIDA LDRCCYVHDN**
51 **CYGDAEK**KHK **CNPKTQSYSY KLTKRTIICY GAAGTCARIV CDCDRTAALC**
101 **FGNSEYIEGH KNIDTAR**FCQ

N2-2 is matched to phospholipase A_2, β bungarotoxin A1 chain precursor (phosphatidylcholine 2-acylhydrolase) from *Bungarus candidus*. The nominal mass (M_r) is 16,045 Da and pI value 7.59 (Figure 5-14). The amino acid sequence coverage 41% matched peptides is shown in bold red,

1 AVCVSLLGAA NIPPHPLNLI NFMEMIRYTI PCEKTWGEYA DYGCYCGAGG
51 SGRPIDALDR **CCYVHDNCYG DAEKK**HKCNP KTQSYSYKLT KR**TIICYGAA**
101 **GTCARIVCDC DRTAALCFGD SEYIERHKNI DTAR**FCQ

SO2-1, 2-2, 2-3 and 2-4 are matched to phospholipase A_2, β bungarotoxin A3 chain precursor (phosphatidylcholine 2-acylhydrolase) from *Bungarus candidus*. The nominal mass (M_r) is 15,880 Da and pI value 6.30 (Figures 5-15, 5-16, 5-17 and 5-18). The amino acid sequence coverage 64% matched peptides is shown in bold red,

1 AVCVSLLGAA NIPPHPLNLI NFMEMIRYTI PCEK**TWGEYT NYGCYCGAGG**
51 **SGRPIDALDR CCYVHDNCYG DAANIRDCNP K**TQSYSYKLT KR**TIICYGAA**
101 **GTCARVVCDC DRTAALCFGD SEYIEGHKNI DTAR**FCQ

Table 5-3 Distribution of protein, lethal toxicity and PLA_2 activity in the pooled fractions of BC-NE 2 obtained on Resourse^{TM} column chromatography.

Fraction (Tube no.)	Protein (mg)	Lethal toxicity		PLA_2 activity	
		LD_{50} (µg)	Total LD_{s50}	HLD(µg)	Total HLD_s
1 (3 - 7)	14.26	>2.18	-	0.092	155,000
2 (8 - 14)	9.22	>2.18	-	0.002	4,610,000
3 (15 - 18)	1.21	>2.18	-	0.004	302,500
4 (28 - 31)	0.75	>2.18	-	0.020	37,500
5 (32 - 35)	2.49	1.37	1,818	0.160	15,563
6 (36 - 40)	1.54	0.78	1.974	0.043	35,814
7 (41 - 44)	2.14	>2.18	-	0.230	9,304
8 (45 - 50)	3.12	>2.18	-	0.230	13,565
9 (51 - 54)	1.48	>2.18	-	0.160	9,250
10 (55 - 59)	2.68	>2.18	-	0.450	5,956
11 (60 - 64)	2.04	>2.18	-	0.750	2,720
12 (65 - 68)	1.87	0.43	4,349 (21)[a]	0.840	2,226 (0.04)[b]
13 (69 - 74)	1.65	0.38	4,342 (21)[a]	0.400	4,125 (0.08)[b]
14 (75 - 79)	1.68	0.32	5,250 (25)[a]	0.145	11,586 (0.22)[b]
15 (100 - 102)	1.02	0.32	3,188	0.050	20,400
Total	47.15		20,921		5,235,509
Crude BC-NE 2	50	1.09	45,872	0.008	6,250,000

[a, b] Figures in parentheses are the percentage of recoveries calculated to the total toxicity and PLA_2 activity obtained on Resource^{TM} S column chromatography. The pooled fraction 12, 13, and 14 were pooled and subjected to the next step of purification (adsorption chromatography on hydroxylapatite).

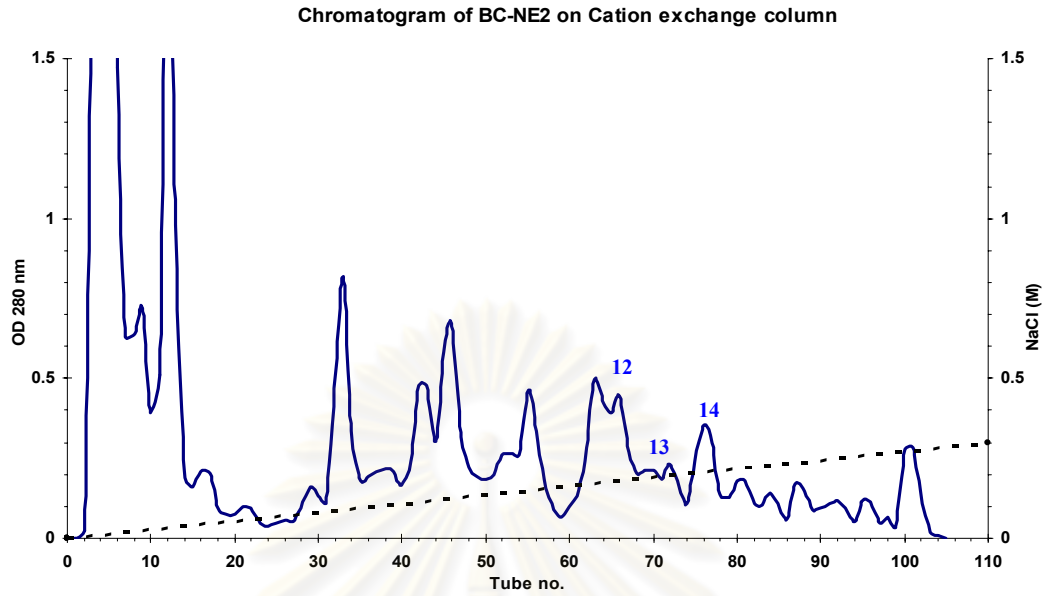


Figure 5-8 Chromatogram of the BC-NE 2 crude venom on Resource^{TM} S column chromatography, 102 fractions were obtained and pooled into 15 fractions (details in Table 5.3). The pooled fractions 12, 13, and 14 were chosen and applied to adsorption chromatography on hydroxylapatite.

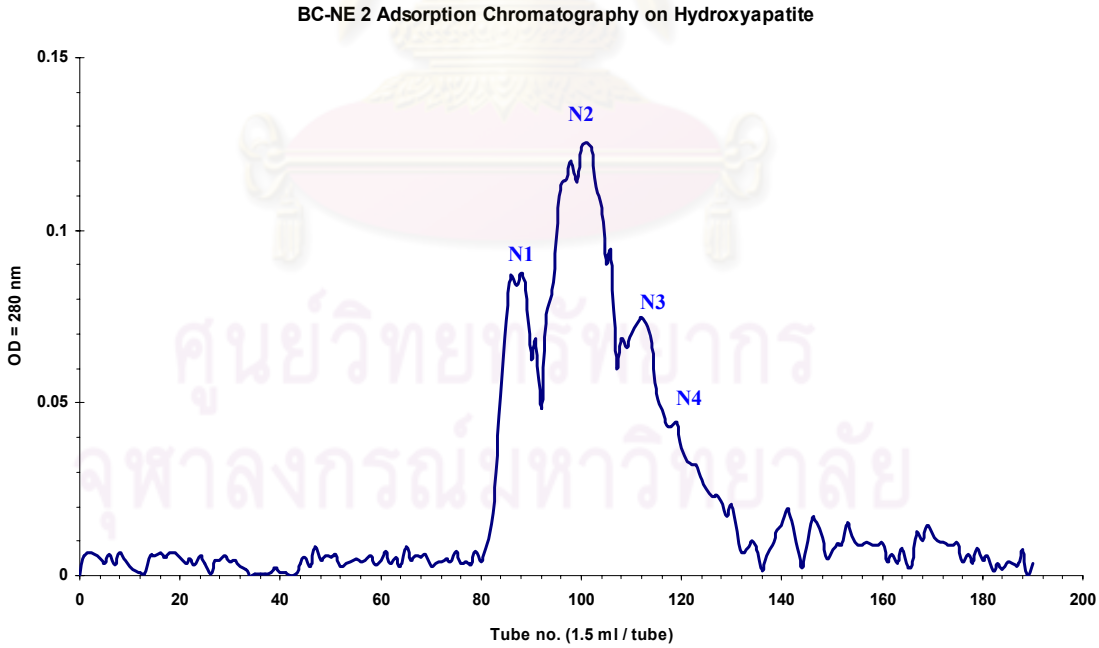


Figure 5-9 Adsorption chromatography of the pooled fractions 12, 13, and 14 on hydroxylapatite presenting four peaks designated as N1, N2, N3, and N4 which were detected the lethal toxicity and the PLA_2 activity summarized in Table 5-4.

Table 5-4 The purification of toxins from BC-NE 2 crude venom on cation-exchange (Resource™ S column) chromatography and adsorption chromatography on hydroxylapatite.

Step	Protein (mg)	Lethal toxicity		PLA_2
		LD_{50} (µg)	Total LD_{50}s	HLD (µg)
Crude venom	50.00	0.94 (0.72 - 1.68)	53,192	0.008
Resource™ S				
Fraction 12	1.87	0.43 (0.27 - 0.68)	4,349	0.840
Fraction 13	1.65	0.38 (0.14 - 1.02)	4,342	0.400
Fraction 14	1.68	0.32 (0.16 - 0.62)	5,250	0.145
Hydroxylapatite				
Fraction N1	0.73	0.17 (0.09 - 0.31) (5.5)[a]	4,294 (8.0)[b]	Not tested
Fraction N2	1.73	0.24 (0.12 - 0.47) (3.9)[a]	7,208 (13.6)[b]	0.390
Fraction N3	0.61	0.20 (0.12 - 0.34) (4.7)[a]	3,050 (5.7)[b]	0.400
Fraction N4	0.36	0.30 (0.16 - 0.58) (3.1)[a]	1,200 (2.3)[b]	Not tested

[a, b] Figures in parentheses are the purification factors and the percent recoveries calculated to crude venom. The purification factor is defined as LD_{50} of crude venom divided by LD_{50} of each fraction.

$$\text{The percent recovery} = \frac{\text{Total } LD_{50} \text{ of each fraction}}{\text{Total } LD_{50} \text{ of crude venom}} \times 100$$

Total LD_{50} is defined as protein of crude venom or of each fraction (in µg) divided by LD_{50} of crude venom or of each fraction (in µg).

Table 5-5 Distribution of protein, lethal toxicity and PLA_2 activity in the pooled fractions of BC-S 3 obtained on Resourse™ column chromatography.

Fraction (Tube no.)	Protein (mg)	Lethal toxicity		PLA_2 activity	
		LD_{50} (µg)	Total LD_{s50}	HLD(µg)	Total HLD_s
1 (3 – 7)	21.11	>1.62	-	0.380	55,553
2 (8 - 10)	2.74	>1.62	-	0.007	391,429
3 (11 – 13)	3.09	>1.62	-	0.043	71,860
4 (14 – 18)	2.30	>1.62	-	0.066	34,848
5 (19 – 21)	0.38	>1.62	-	0.040	9,500
6 (28 – 31)	0.72	>1.62	-	0.098	7,347
7 (40 – 43)	1.10	>1.62	-	0.150	7,333
8 (44 – 48)	0.87	>1.62	-	0.340	2,559
9 (49 – 54)	1.61	>1.62	-	0.530	3,038
10 (57 - 59)	0.58	>1.62	-	0.380	1,526
11 (60 - 62)	0.53	>1.62	-	0.440	1,205
12 (63 - 67)	1.00	>1.62	-	0.860	1,163
13 (68 - 72)	1.10	0.51	2,157	0.740	1,486
14 (73 - 76)	1.04	0.58	1,793	0.500	2,080
15 (77 - 78)	0.60	0.28	2,143 (11)[a]	0.440	1,364 (0.22)[b]
16 (79 - 85)	1.71	0.40	4,275 (21)[a]	0.21	8,143 (1.30)[b]
17 (86 - 89)	0.58	0.15	3,867 (19)[a]	0.078	7,436 (1.20)[b]
18 (100 - 103)	1.81	0.30	6,033	0.110	16,455
Total	42.87		20,268		624,325
Crude BC-S 3	50	0.81	61,728	0.101	495,050

[a, b] Figures in parentheses are the percentage of recoveries calculated to the total toxicity and PLA_2 activity obtained on Resource™ S column chromatography. The pooled fraction 15, 16, and 17 were pooled and subjected to the next step of purification (adsorption chromatography on hydroxylapatite).

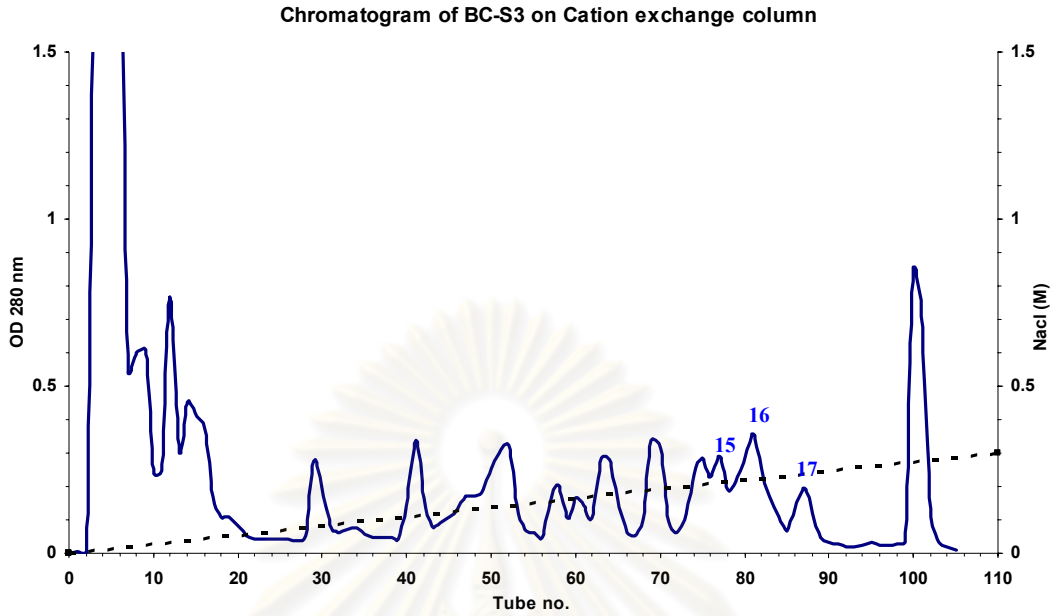


Figure 5-10 Chromatogram of the BC-S 3 crude venom on Resource^{TM} S column chromatography, 103 fractions were obtained and pooled into 18 fractions (details in Table 5-5). The pooled fractions 15, 16, and 17 were chosen and applied to adsorption chromatography on hydroxylapatite.

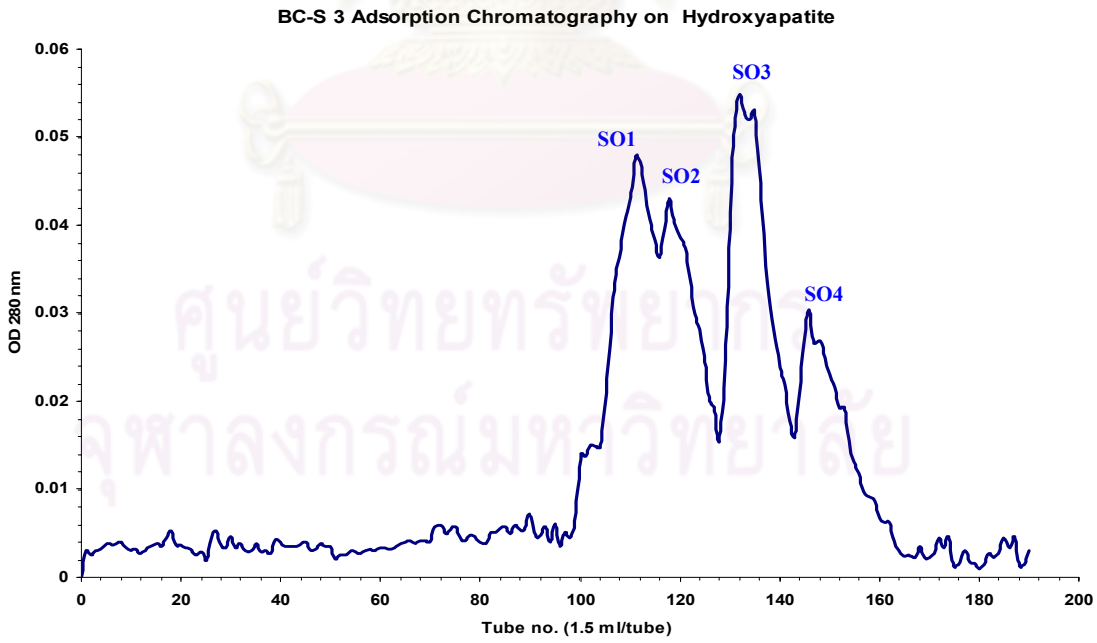


Figure 5-11 Adsorption chromatography of the pooled fractions 15, 16, and 17 on hydroxylapatite presenting four peaks designated as SO1, SO2, SO3, and SO4 which were detected the lethal toxicity and the PLA_2 activity summarized in Table 5-6.

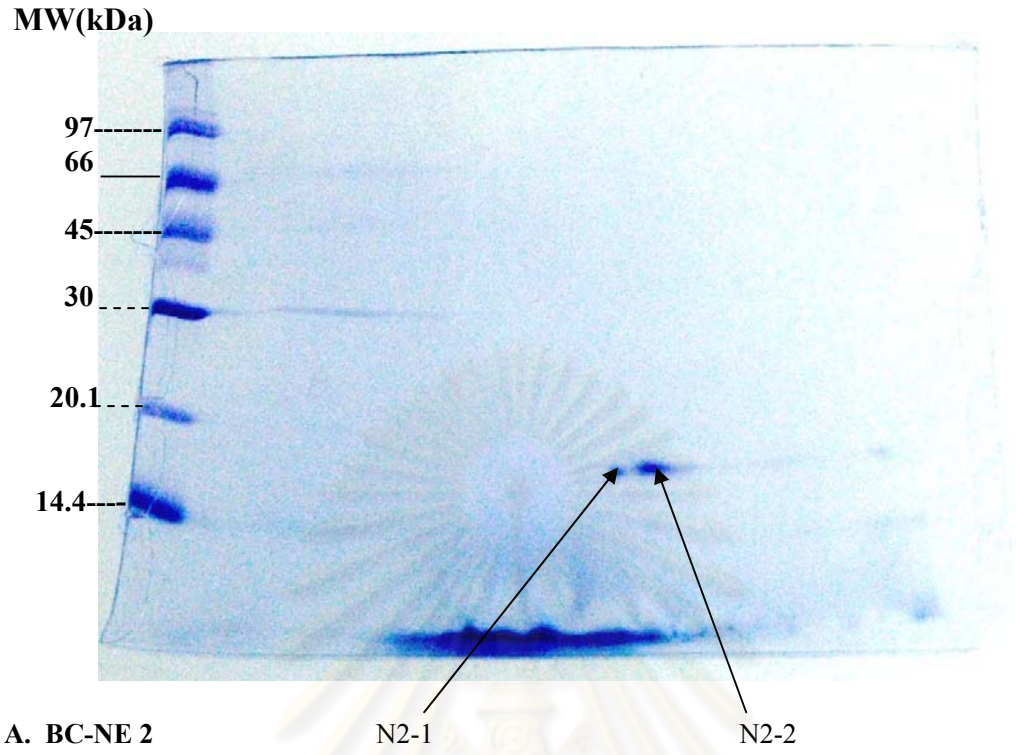
Table 5-6 The purification of toxins from BC-S 3 crude venom on cation-exchange (Resource™ S column) chromatography and adsorption chromatography on hydroxylapatite.

Step	Protein (mg)	Lethal toxicity		PLA_2 activity
		LD_{50} (µg)	Total LD_{50}s	HLD (µg)
Crude venom	50.00	0.97 (0.77 - 1.22)	51,546	0.101
Resource™ S				
Fraction 15	0.60	0.28 (0.26 - 0.31)	2,143	0.440
Fraction 16	1.71	0.40 (0.26 - 0.60)	4,275	0.210
Fraction 17	0.58	0.15 (0.11 - 0.20)	3,867	0.078
Hydroxyapatite				
Fraction SO1	0.48	0.23 (0.12 - 0.45) (4.2)[a]	2,087 (4.1)[b]	0.043
Fraction SO2	0.30	0.10 (0.05 - 0.19) (9.7)[a]	3,000 (5.8)[b]	0.088
Fraction SO3	0.48	0.56 (0.33 - 0.97) (1.7)[a]	857 (1.7)[b]	Not tested
Fraction SO4	0.27	0.56 (0.33 - 0.97) (1.7)[a]	482 (0.9)[b]	Not tested

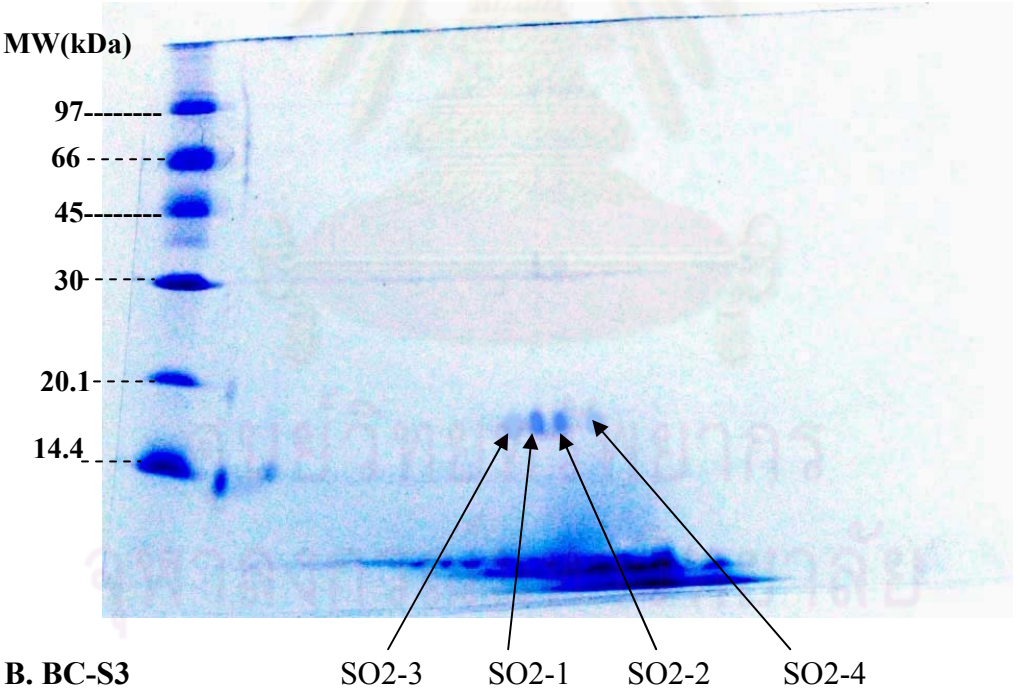
[a, b] Figures in parentheses are the purification factors and the percent recoveries calculated to crude venom. The purification factor is defined as LD_{50} of crude venom divided by LD_{50} of each fraction.

$$\text{The percent recovery} = \frac{\text{Total } LD_{50} \text{ of each fraction}}{\text{Total } LD_{50} \text{ of crude venom}} \times 100$$

Total LD_{50} is defined as protein of crude venom or of each fraction (in µg) divided by LD_{50} of crude venom or of each fraction (in µg).

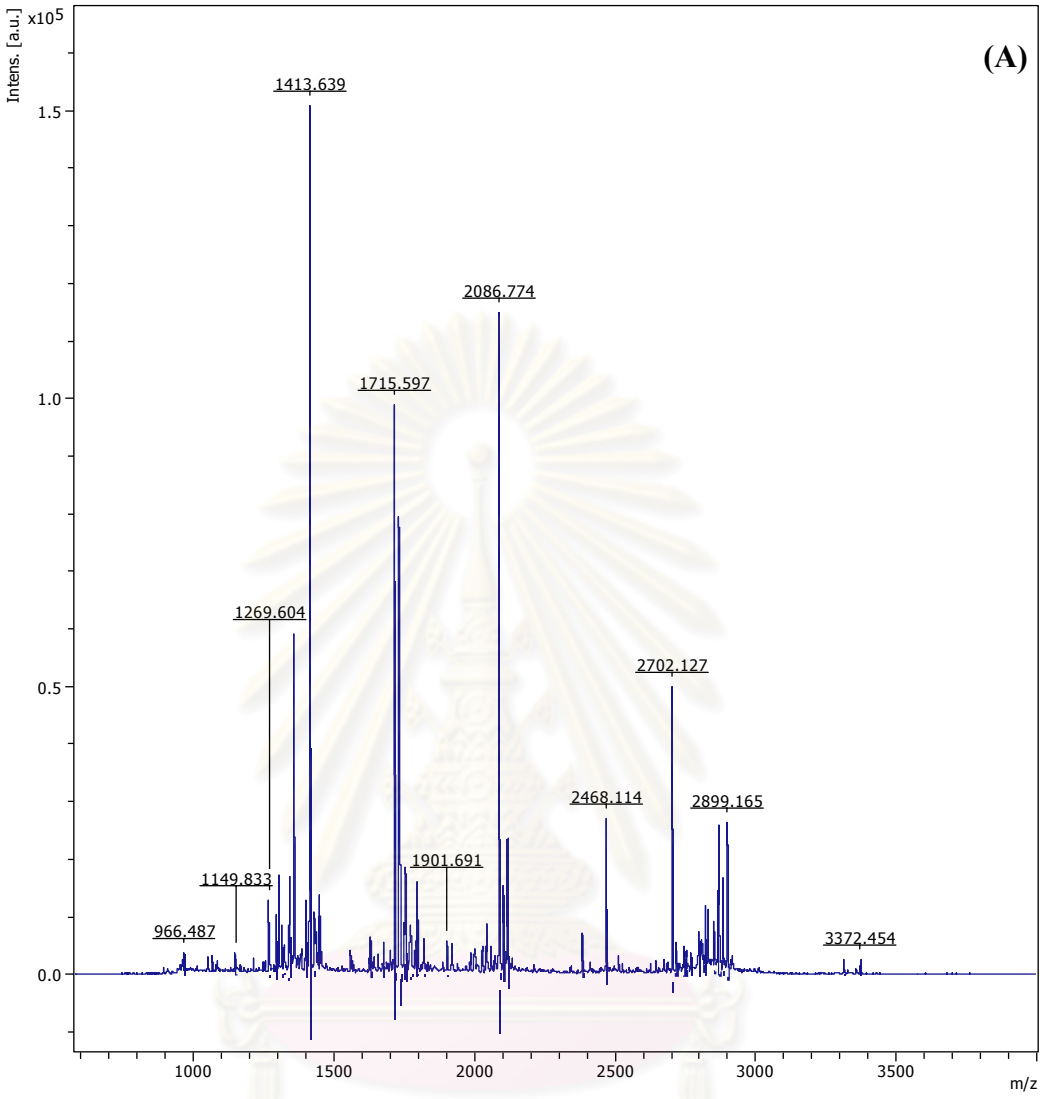


A. BC-NE 2



B. BC-S3

Figure 5-12 2D gel electrophoresis of the fractionated proteins obtained from adsorption chromatography on hydroxylapatite, *Panel A*) BC-NE 2, and *Panel B*) BC-S 3. Molecular weight markers in kDa; Phosphorylase b (97), albumin (66), ovalbumin (45), carbonic anhydrase (30), trypsin inhibitor (20.1), and α-lactalbumin (14.4).



(B). **N2-1** Match to: gi|1431755 Score: 48 Expect: 14

Chain A, Structure of Beta2-Bungarotoxin: Potassium Channel Binding By Kunitz Modules And Targeted Phospholipase Action. Nominal mass (M_r): 14235; Calculated pI value: 7.57

Taxonomy: *Bungarus multicinctus*. Sequence Coverage: 89**%**

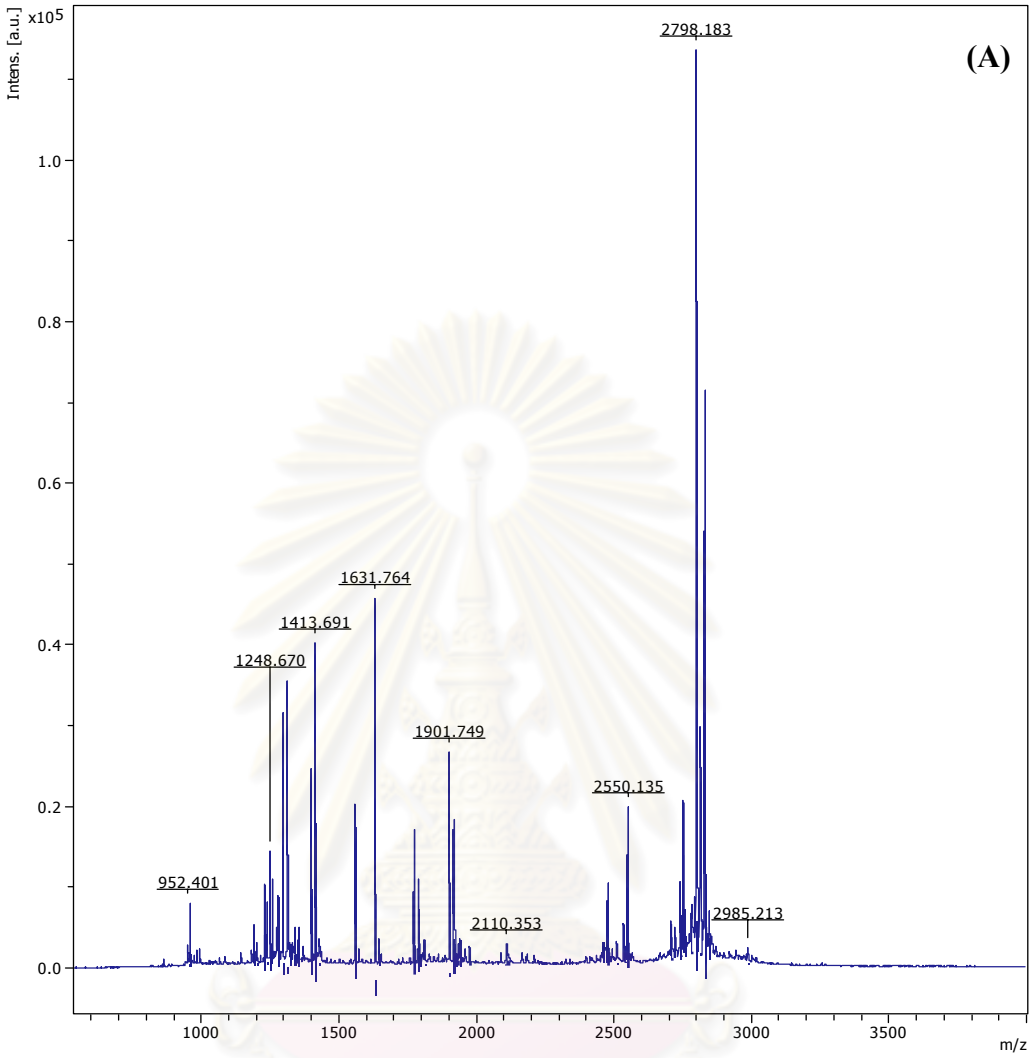
Matched peptides shown in bold red,

1 **NLINFMEMIR** YTIPCEK**TWG EYADYGCYCG AGGSGRPIDALDRCCYVHDN**

51 **CYGDAEK**KHK **CNPKTQSYSY KLTKRTIICY GAAGTCARIV CDCDRTAALC**

101 **FGNSEYIEGH KNIDTAR**FCQ

Figure 5-13 (A) Determination of the molecular masses of protein N2-1 isolated in 2D gel electrophoresis panel A (Figure 5-12); (B) MS/MS-derived amino acid sequence through MATRIX SCIENCE (MASCOT Search)



(B). **N2-2** Match to: gi|82212017 Score: 48 Expect: 15
Phospholipase A2, beta bungarotoxin A1 chain precursor (Phosphatidylcholine 2-acylhydrolase)

Nominal mass (M_r): 16045; Calculated pI value: 7.59

Taxonomy: *Bungarus candidus*. Sequence Coverage: 41%

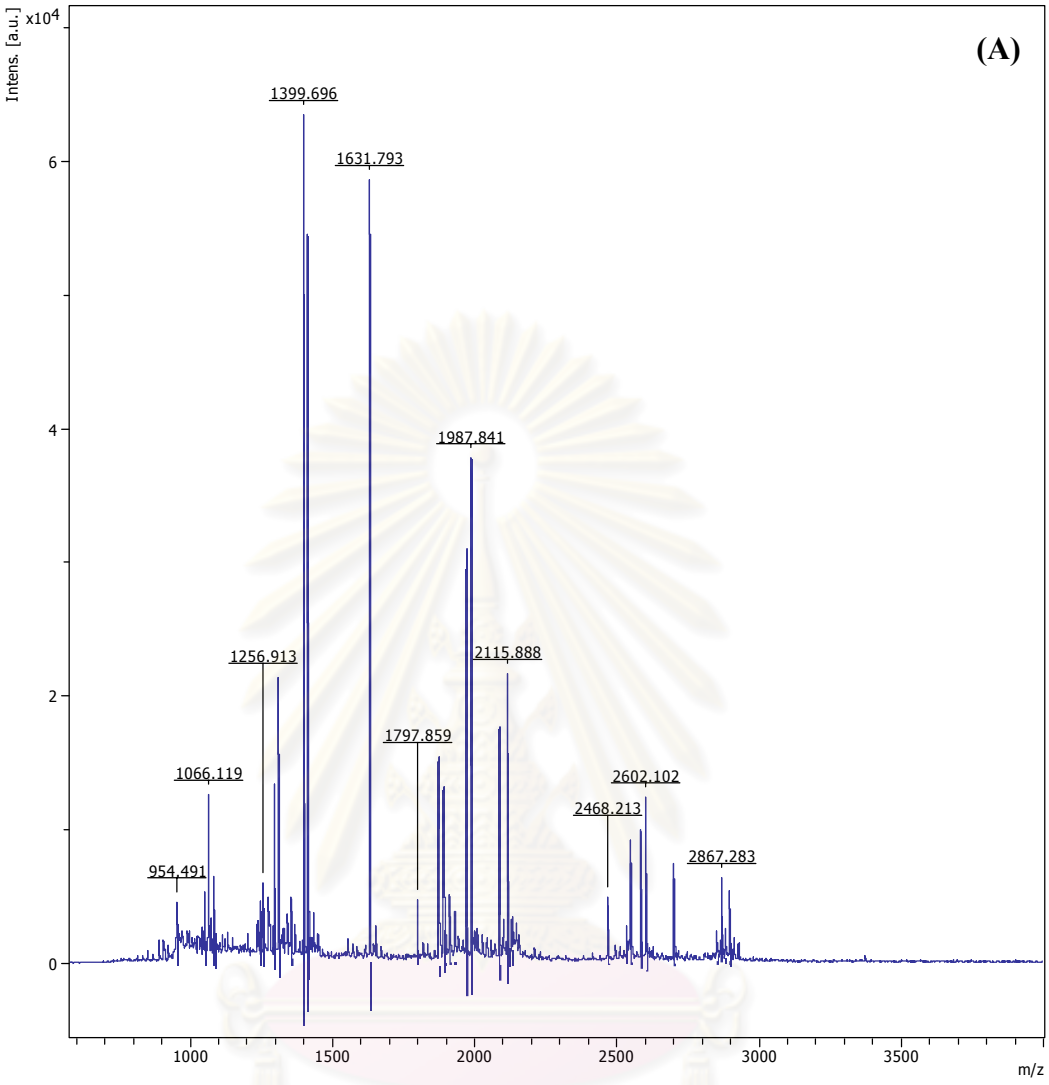
Matched peptides shown in bold red,

1 AVCVSLLGAA NIPPHPLNLI NFMEMIRYTI PCEKTWGEYA DYGCYCGAGG

51 SGRPIDALDR **CCYVHDNCYG DAEKK**HKCNP KTQSYSYKLT KR**TIICYGAA**

101 **GTCARIVCDC DRTAALCFGD SEYIERHKNI DTAR**FCQ

Figure 5-14 (A) Determination of the molecular masses of protein N2-2 isolated in 2D gel electrophoresis panel A (Figure 5-12); (B) MS/MS-derived amino acid sequence through MATRIX SCIENCE (MASCOT Search).



(B). SO2-1 Match to: gi|82206358 Score: 77 Expect: 0.02

Phospholipase A2, beta bungarotoxin A3 chain precursor (Phosphatidylcholine 2-acylhydrolase)

Nominal mass (M_r): 15880; Calculated pI value: 6.30

Taxonomy: *Bungarus candidus*. Sequence Coverage: 64**%**

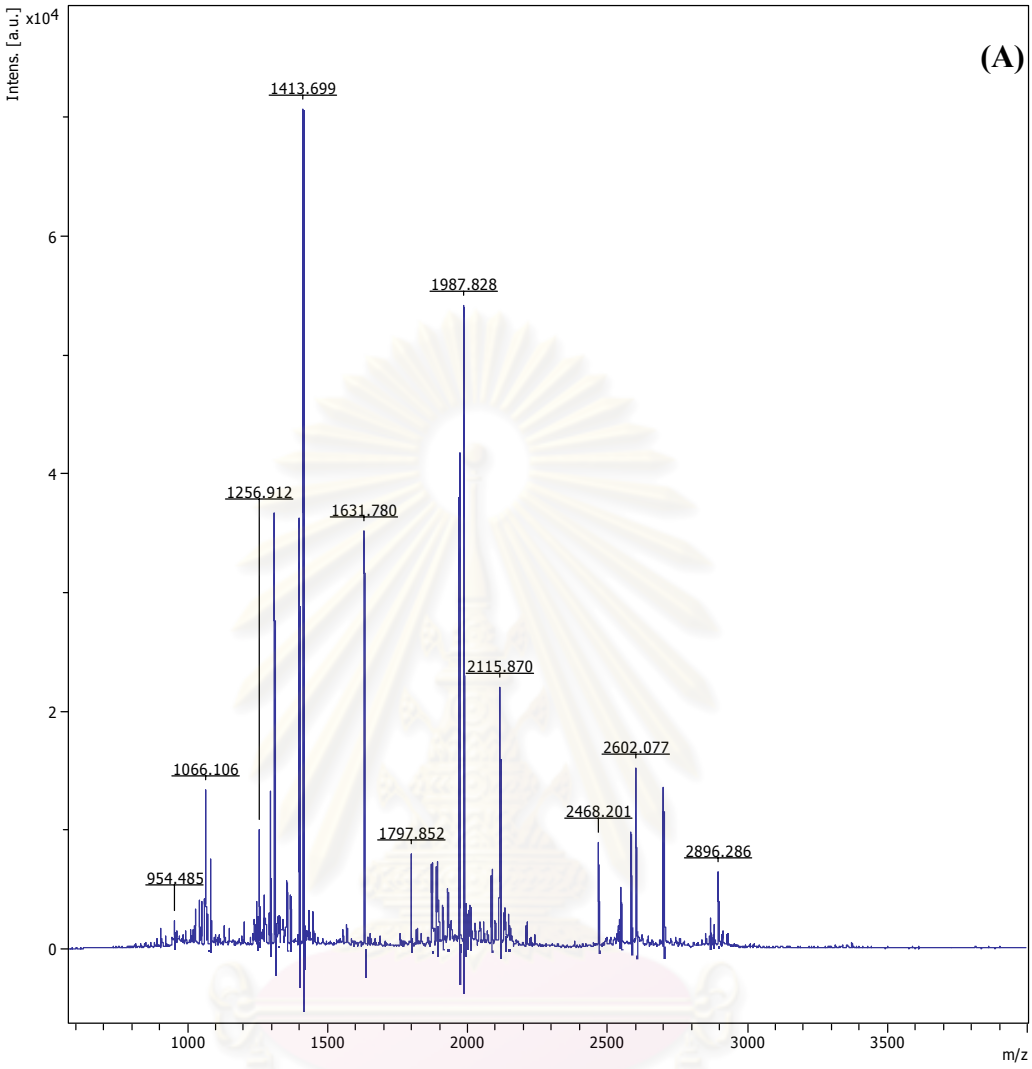
Matched peptides shown in bold red,

1 AVCVSLLGAA NIPPHPLNLI NFMEMIRYTI PCEK**TWGEYT NYGCYCGAGG**

51 **SGRPIDALDR CCYVHDNCYG DAANIRDCNP K**TQSYSYKLT KR**TIICYGAA**

101 **GTCARVVCDC DRTAALCFGD SEYIEGHKNI DTAR**FCQ

Figure 5-15 (A) Determination of the molecular masses of protein SO2-1 isolated in 2D gel electrophoresis panel B (Figure 5-12); (B) MS/MS-derived amino acid sequence through MATRIX SCIENCE (MASCOT Search).



(B). SO2-2 Match to: gi|82206358 Score: 74 Expect: 0.034

Phospholipase A2, beta bungarotoxin A3 chain precursor (Phosphatidylcholine 2-acylhydrolase)

Nominal mass (M_r): 15880; Calculated pI value: 6.30

Taxonomy: *Bungarus candidus*.Sequence Coverage: 64**%**

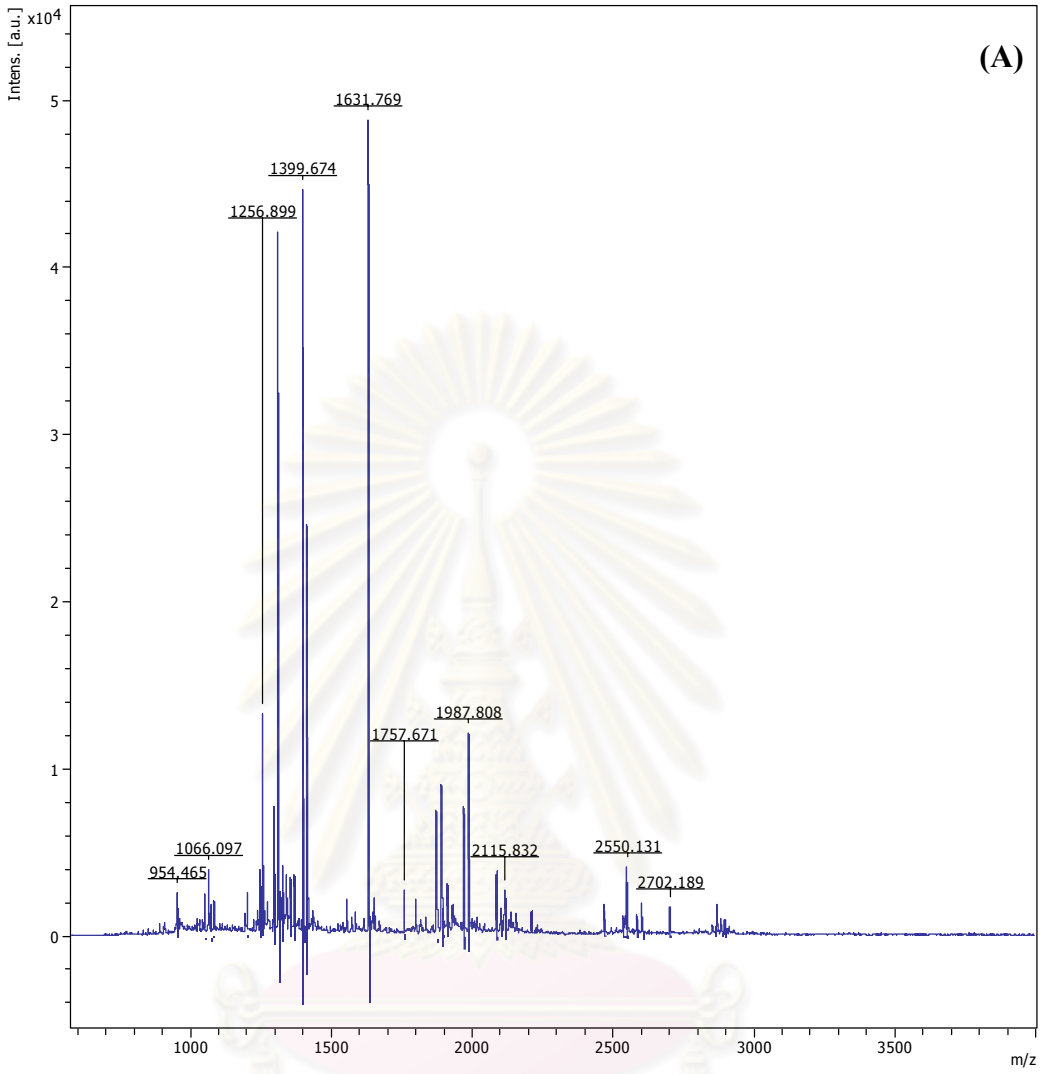
Matched peptides shown in bold red,

1 AVCVSLLGAA NIPPHPLNLI NFMEMIRYTI PCEK**TWGEYT NYGCYCGAGG**

51 **SGRPIDALDR CCYVHDNCYG DAANIRDCNP K**TQSYSYKLT KR**TIICYGAA**

101 **GTCARVVCDC DRTAALCFGD SEYIEGHKNI DTAR**FCQ

Figure 5-16 (A) Determination of the molecular masses of protein SO2-2 isolated in 2D gel electrophoresis panel B (Figure 5-12); (B) MS/MS-derived amino acid sequence through MATRIX SCIENCE (MASCOT Search).



(B). SO2-3 Match to: gi|82206358 Score: 63 Expect: 0.48

Phospholipase A2, beta bungarotoxin A3 chain precursor (Phosphatidylcholine 2-acylhydrolase)

Nominal mass (M_r): 15880; Calculated pI value: 6.30

Taxonomy: *Bungarus candidus*. Sequence Coverage: 64**%**

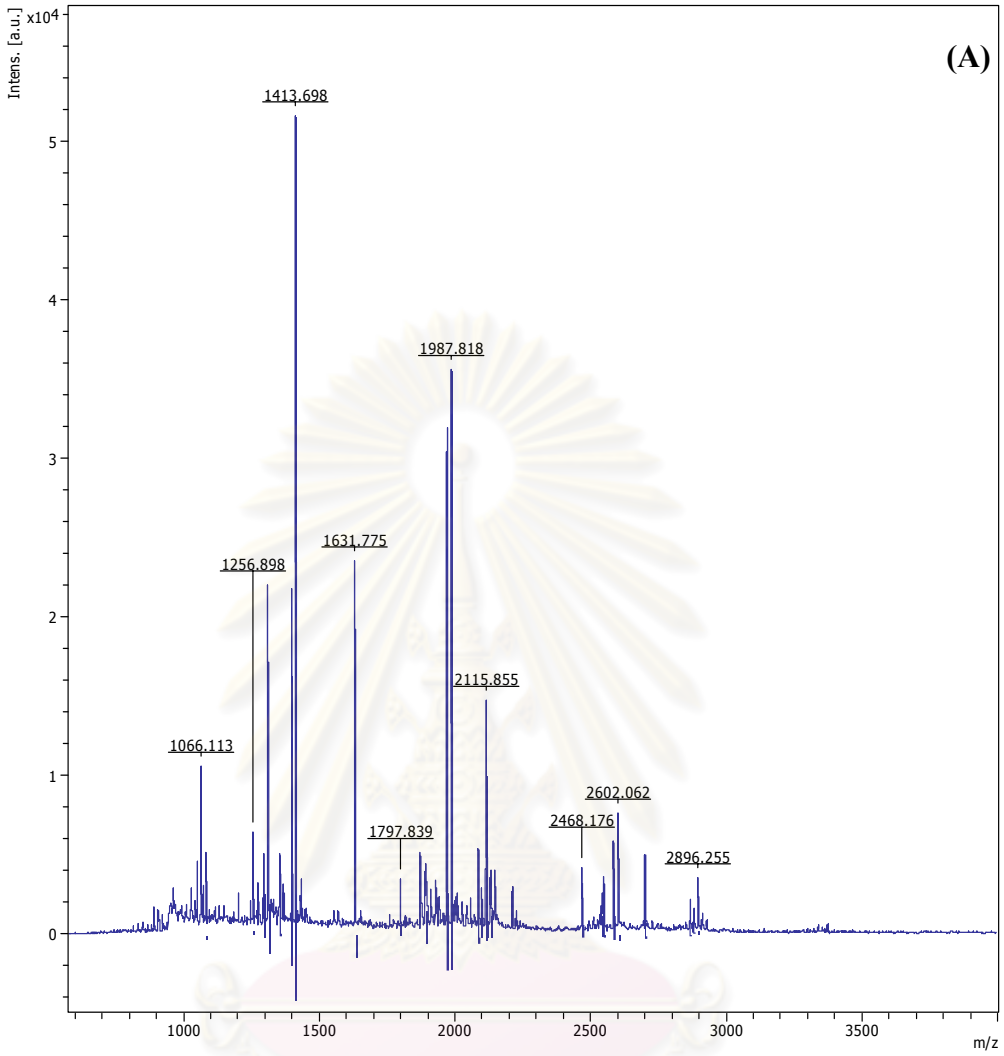
Matched peptides shown in bold red,

1 AVCVSLLGAA NIPPHPLNLI NFMEMIRYTI PCEK**TWGEYT NYGCYCGAGG**

51 **SGRPIDALDR CCYVHDNCYG DAANIRDCNP K**TQSYSYKLT KR**TIICYGAA**

101 **GTCARVVCDC DRTAALCFGD SEYIEGHKNI DTAR**FCQ

Figure 5-17 (A) Determination of the molecular masses of protein SO2-3 isolated in 2D gel electrophoresis panel B (Figure 5-12); (B) MS/MS-derived amino acid sequence through MATRIX SCIENCE (MASCOT Search).



(B). SO2-4 Match to: gi|82206358 Score: 84 Expect: 0.0037

Phospholipase A2, beta bungarotoxin A3 chain precursor (Phosphatidylcholine 2-acylhydrolase)

Nominal mass (M_r): 15880; Calculated pI value: 6.30

Taxonomy: *Bungarus candidus*. Sequence Coverage: 64**%**

Matched peptides shown in bold red,

1 AVCVSLLGAA NIPPHPLNLI NFMEMIRYTI PCEK**TWGEYT NYGCYCGAGG**
51 **SGRPIDALDR CCYVHDNCYG DAANIRDCNP K**TQSYSYKLT KR**TIICYGAA**
101 **GTCARVVCDC DRTAALCFGD SEYIEGHKNI DTAR**FCQ

Figure 5-18 (A) Determination of the molecular masses of protein SO2-4 isolated in 2D gel electrophoresis panel B (Figure 5-12); (B) MS/MS-derived amino acid sequence through MATRIX SCIENCE (MASCOT Search).

DISCUSSION

In the present study, values of the enzymatic activity of *Bungarus candidus* venom in all groups were in the range of previous values that studied in *B. candidus* and other species of *Bungarus* snakes, such as *B. caeruleus, B. multicinctus and B. fasciatus* (Tan and Ponnudurai, 1990). The enzymatic activities in the *B. candidus* venom exhibit the high activities of phospholipase A_2, acetylcholinesterase, L-amino acid oxidase and hyaluronidase, the moderate activity of alkaline phosphomonoesterase and the low activities of phosphodiesterase and protease. However, the present results demonstrate that variations in different types of enzymatic activities and protein compositions occurred in the venoms of *B. candidus* snakes from three different localities of Thailand and the captive-born snakes. The geographical variations of the compositions and toxicity in snake venoms from the same species have been well documented (Daltry et al., 1997; Tsai et al., 2003; Shashidharamurthy et al., 2002; Shashidharamurthy and Kemparaju, 2007; Salazar et al., 2007). In the present study, no differences in lethal toxicity of *B. candidus* venoms were apparent among groups of snakes from different locations (Table 5-1). However, the present findings reveal that the lethal toxicity of BC-S venom (0.061 μg/g mouse) was lower than those of pooled venom of *B. candidus* from the same southern region (0.160-0.175 μg/g mouse) (Khow et al., 2003; Chanhome et al.,1999), whereas there were no differences in phospholipase A_2 activity. These differences in terms of the response to lethal toxicity indicate that the toxicity is not solely due to the activity of phospholipase A_2. In contrary to the present findings in different type of snake, the high lethal toxicity of the *Vipera russelii* venom was attributed to the high phospholipase activity. The reverse relationship between lethal toxicity and proteolytic activity have also been reported in *Vipera russelii* venom (Jayanthi and Gowda, 1988) and *Crotalus atrox* venom (Minton and Weinstein, 1986) collected from different localities of India and the United States. Therefore, the lethal toxicity of *B. candidus* venom would be induced by variety of enzyme components. A synergistic action of different toxic and nontoxic components within venom may take place for its overall toxicity (Tu, 1991).

The present study in BC-CB venoms showed the lowest activities of L-amino acid oxidase without the difference in lethal toxicity when compared with other groups, although L-amino acid oxidase has been postulated to be toxic in inducing apoptosis, hemorrhagic and cytotoxic effects (Du and Clemetson, 2002). It is possible that during venom collection, an appearance of white to pale yellow color of BC-CB venom might be accounted for the low activity of L-amino acid oxidase. This result might be supported by other findings in Russell's viper venom that the low activity of L-amino acid oxidase would associate with the colorless venom of juvenile snake. The variations of clinical symptoms following the bites of adult and young Russell's viper were also noted (Tun-Pe et al. 1995). Moreover, differences of L-amino acid oxidases activity between the wild-caught groups and captive-born group may attribute to the different types of feeding diets in the present study. However, the phospholipase A_2 and protease activities of BC-CB venom were not different from other groups. The presence of high activities of acetylcholinesterase and hyaluronidase in BC-CB venom might suffice for inducing lethal toxicity (Girish and Kemparaju, 2006; Yingprasertchai, Bunyasrisawat, and Ratanabanangkoon, 2003).

The value of hyaluronidase activities of BC-NE venoms was lower than those of other groups. However, the lower hyaluronidase activities found in the present study would not affect to its lethal toxicity, although the hyaluronidase contents of *B. candidus* venom have been reported to be exceptionally high in comparison with the other elapid venoms (Tan and Tan, 1988; Pukrittayakamee et al., 1988). The hyaluronidase activity has been known as a spreading factor by disrupting the connective tissues and potentiating hemorrhagic effect and necrosis (Tu and Hendon, 1983), and accelerating venom absorption and diffusion in contributing to systemic envenomation (Pukrittayakamee *et al*., 1988; Girish et al., 2004). It has been reported that snake venoms contain both nonspecific and specific phosphomonoesterase. Nonspecific phosphomonoesterase is frequently referred to as a phosphatase which has two activities depending on its optimum pH designated as an acid phosphatase (pH 5) and an alkaline phosphatase (pH 9.5). Both phosphatases generally hydrolyze phosphomonoesterase bonds (Rael, 1998). Thus, the marked high values of phosphomonoesterase in BC-NE venom might be relevant to induce the lethal toxicity. In BC-E venom, the high hyaluronidase values, the low value of

acetylcholinesterase with remaining in high lethal toxicity were apparent. These results suggest that the lethal toxicity of the BC-E venom would be shared by enzymatic activities among components in the venom.

Determining the profile for the diversity of protein bands in the range of molecular weight from 7.1 to 41.3 kDa (Figure 5-1) showed the quantitative differences in all venom groups. The distinction of the protein bands at the molecular weight from 18.1–41.3 kDa in captive-born group compared with the other three snake venom groups were possibly the differences in their food preference, of which the captive-born snakes were fed on mice. The measured values could be an indicator of type of toxin, taking into consideration the number of signs responses to the venom toxin. The dense protein bands at the molecular weight approximately 22–25 kDa may be comparable to the presynaptic neurotoxin isolated from *B. candidus* venom (Khow et al., 2003). In addition, the difference in quantitative of the marked dense bands in the region of molecular weight of 7.1 to 18.1 are probably compounded of α-bungarotoxin (Kuch et al., 2003), candoxin (Nirthanan et al., 2003; Paaventhan et al., 2003) and bucain (Watanabe et al., 2002) isolated from the venom of Malayan kraits originally from the other Southeast Asia countries. All these toxins mainly produced postsynaptic neuromuscular blockade. However, studies of the toxin purification or venom fractions of BC-NE and BC-S venom in experimental animals would be further investigated.

All major venom proteins from *B. candidus* appeared on reverse-phase HPLC separations (RP-HPLC) were subjected to individual variation within each geographic population. While qualitative and quantitative differences were observed among members of the same group, it appeared that sex of the snakes (BC-NE3, BC-S4 and BC-CB4 were female) was not involved in these differences. Furthermore, since all the snakes were kept under the same conditions and fed with live snakes (except BC-CB which were fed with mice), these subtle differences in RP-HPLC profiles were likely to be due to other factors, e.g., genetic background.

It is well established that venoms of snakes of the same species or even same sub-species but captured from different countries or regions, often are different pharmacologically and immunologically. Thus, an antivenom prepared against one

species of snake in one country may not be effective when used to treat envenomation by that same species in a different country. It is therefore recommended that in the production of snake antivenom for therapeutic use in a certain geographic area, the snake venom(s) used as immunogen should originate from the same locality. As a quality control measure, the antivenom producer should therefore provide a certificate mentioning the Latin name of the species and its geographic origin, some relevant biochemical activities of the venom together with pictures of SDS-PAGE or HPLC profiles of the venom proteins. In this regard, since SDS-PAGE of the venoms of all 4 groups did not show obvious differences in protein bands (Figure 5-1), it may not be possible to use SDS-PAGE to identify the geographic origins of BC venoms studied here. In this respect, RP-HPLC may be more useful, for example, to identify BC-S from BC-NE and BC-E.

The isolation and purification of toxins from BC-NE2 and BC-S3 venoms revealed the different isoforms of β-bungarotoxin chain A unit which acts as phospholipase A_2. Fraction N2 on adsorption chromatography (Figure 5-9) exhibited the lethal toxicity at 0.24 (0.12 – 0.47) μg / 20 gm mouse body weight which achieved about 4-fold purification and 14 % recovery to BC-NE2 crude venom (Table 5-4). On 2D gel electrophoresis and MS/MS-derived amino acid sequence through the MASCOT search (MATRIX SCIENCE), it was consisted of two different isoforms of β-bungarotoxin. The first was designated as toxin N2-1 which the amino acid sequence coverage 89% matched peptide to β2-bungarotoxin chain A purified from *Bungarus multicinctus* venom (Kwong et al., 1995). The second was designated as toxin N2-2 which the amino acid sequence coverage 41% matched peptide to β-bungarotoxin chain A1 purified from *B. candidus* venom. Fraction SO2 on adsorption chromatography (Figure 5-11) exhibited the lethal toxicity at 0.10 (0.05–0.19) μg/20 gm mouse body weight which achieved about 10-fold purification and 6% recovery to BC-S3 crude venom (Table 5-6). On 2D gel electrophoresis this fraction was separated into 4 protein spots which designated as toxin SO2-1, SO2-2, SO2-3, and SO2-3. From MS/MS-derived amino acid sequence through the MASCOT search (MATRIX SCIENCE), the amino acid sequence of all toxins were coverage 64% matched peptide to β-bungarotoxin chain A3 purified from *B. candidus* venom. β-

bungarotoxin is a presynaptic neurotoxin composed of two subunits linked by disulfide bond. The chain A subunit has the phospholipase A_2 activity which catalyzes the calcium-dependent hydrolysis of the 2-acyl groups in 3-sn-phosphoglycerides. The chain B is a K^+ channel binding subunit which is a member of the Kunitz protease inhibitor superfamily. The β-bungarotoxin family consists of a group of isotoxins which is the main constituent in the venoms of *Bungarus* snakes (Chu et al., 1995).

CONCLUSION

The results of the present findings suggest that the lethal toxicity of the venom is synergistic action of various components of the venom. Since the resolution of the RP-HPLC studied here was quite high compared to that of other chromatographies e.g., ion exchange, it may be possible to quantitate the abundance of the lethal toxin(s) for comparative purposes. The conditions used for RP-HPLC described here may also be useful for efficient isolation of the toxins of these snakes. Therefore antivenom production would require pooled venoms from various geographical areas as it cannot be focused in any specific components of the venom.

CHAPTER VI

IN VITRO STUDIES FOR THE EFFECTS OF *BUNGARUS CANDIDUS* VENOM ON HEMOLYTIC AND NEUROTOXIC APPEARANCES

INTRODUCTION

Venomous snakes in Family Elapidae comprise a large group of different species such as cobras, kraits, coral snakes, and death adders. Snake venoms are complex mixtures of polypeptides and enzymes with diverse actions on prey and human victims. The Elapid snake venoms are mainly consisting of neurotoxins which cause death within 24–48 hours in untreated victims. The secreted phospholipases A_2 are the most common enzyme found in various snake venoms isolated from the snakes in Families Elapidae, Viperidae, and Crotalidae. The venom PLA_2s possess a digestive function and a wide array of pharmacological actions such as antiplatelet, anticoagulant, hemolytic, neurotoxic, myotoxic, edema-inducing, hemorrhagic, cytolytic, cardiotoxic, and muscarinic inhibitor activities (Harris, 1991; Kini, 2003). PLA_2 enzymes hydrolyze glycerophospholipids at the sn-2 position of the glycerol backbone releasing lysophospholipids and fatty acids (Lambeau and Lazdunski, 1999). Purified PLA_2 from Ringhals cobra venom hydrolyzed the phospholipids of the intact red cell membrane in the presence of Ca^{2+} (Condrea et al., 1970). In addition, hemolysis of washed human red blood cells by snake venoms in the presence of albumin occurs as a consequence of the spitting of membrane phospholipids (Gul, Khara, and Smith, 1974). Interactions between membrane proteins and phospholipids treated by phospholipases can alter the mosaic of membrane components by a change in osmotic resistance (Ruitenbeek, Edixhoven, and Scholte, 1979). Therefore red blood cells have become more fragile. Snake venom PLA_2s mostly express their pharmacological effects on their own, however some exhibit the full potency of their pharmacological effects only when they form a complex, for example β-bungarotoxin (Kini, 2003).

β-Bungarotoxin (β-BuTx) is well known as presynaptic neurotoxin that constitutes the main portion of *Bungarus* snake venoms (the kraits). β-BuTx is

composed of two subunits linked by a disulfide bond. One is a PLA_2 subunit which exhibits remarkable structural similarity to other vertebrate enzymes and the other is a non- PLA_2 which is homologous to proteinase inhibitors and dendrotoxins (Bon, 1997; Kini, 2003). In this respect, β-BuTx is a PLA_2 neurotoxin potentially in blocking nicotinic acetylcholine transmission at the presynaptically neuromuscular junction. According to the complexity of snake venom components, PLA_2 may somehow interact with the other venom proteins, such as cardiotoxin (Condrea et al. 1970). Snake venom cardiotoxins, also known as cytotoxins or membrane toxins, are generally regarded as low molecular weight polypeptides that cause cardiac arrest, muscle contracture, membrane depolarization and hemolysis (Chang, 1979; Condrea, 1979; Harvey, Marshall, and Karlsson, 1982; Fletcher et al., 1991). Cardiotoxin-like PLA_2 found in *Bungarus fasciatus* venom possessed two mechanisms of hemolysis; the first was independent of PLA_2 activity and the second appeared to be dependent on PLA_2 activity at high Ca^{2+} concentration and high pH (≥ 8.5) (Jiang, Fletcher, and Smith, 1989). Small amount of PLA_2 can enhance the ability of cardiotoxins to lyse red blood cells, but does not markedly increase the ability of cardiotoxins to depolarize and cause the contracture of skeletal muscle (Harvey, Hider, and Khader, 1983).

The red blood cell or erythrocyte is a highly dynamic functional unit although relatively simple when compared with other somatic cells. Several physiologic factors including venom can alter the membrane permeability. The method, namely osmotic fragility index is known to use to estimate the stability and functionality of membrane integrity of red blood cells during exposure to membrane destabilizing agents. If the *B. candidus* venom has biological components of red blood cell haemolysis, the study will establish the contributions of the action of *B. candidus* venom on cell injury. Therefore, the aim of this study was thus carried out to evaluate the effects of *B. candidus* venom on, i) mean corpuscular volume and the osmotic fragility of the rabbit red blood cells, and ii) its neurotoxic effect in mouse phrenic nerve diaphragm preparation.

MATERIALS AND METHODS

The experiments in Chapter VI were performed in *in vitro* studies. Two experiments were carried out; the first (Experiment 6.1) to study the effect of *B. candidus venom* on mean corpuscular volume and the osmotic fragility of the rabbit red blood cells, the second (Experiment 6. 2) on neurotoxic effect.

Experiment 6.1. Effects of *Bungarus candidus* venom on mean corpuscular volume and osmotic fragility test of red blood cells

6.1.1 Blood collection

Blood samples were drawn from ear veins of New Zealand white rabbit (weight 3.0–3.5 kg) and collected into either heparinized or EDTA tubes as an anticoagulant.

6.1.2 Experimental protocol

Determination of mean corpuscular volume of red blood cells were carried out. *B. candidus* crude venom (0.7 and 2.0 µg/50 µl of 0.9% saline solution) was added into 1 ml of heparinized or EDTA rabbit whole blood. The control blood was added 50 µl of normal saline solution (NSS-treated whole blood). The *B. candidus* venom-treated whole blood was gently mixed and held at 37^{o}C for 30 minutes. Mean corpuscular volume and packed cell volume were measured (Fig. 6-1).

Determination of osmotic fragility of the Bungarus candidus venom-treated red blood cells was carried out based on the modified method of Dacie and Lewis (1984). Briefly, 0.02 ml of the venom-treated whole blood and the NSS-treated whole blood was added into the glass tubes in varying concentrations of 5 ml buffer saline (0.1–0.9 %) and in distilled water. The tubes were gently mixed and incubated at room temperature for 30 minutes. Then the samples were centrifuged at 2,000 rpm for 5 minutes (Figure 6-1). Supernatants were collected for measurement of plasma hemoglobin by the method of Simmons (1968) with the procedure listed in Chapter III. Hemolysis or plasma hemoglobin was expressed as percentage of the absorbance in distilled water. The conventional osmotic fragility curve was drawn by plotting the percentages of hemolysis or plasma hemoglobin against decreasing saline concentrations. The saline concentration at which 50% of the red blood cells lysed was used to express the results as the mean corpuscular fragility (MCF; g%). The

stability of red blood cells was evaluated as percentage of the quotient of the difference between the MCF value of the test and control samples (Chikezei et al., 2009).

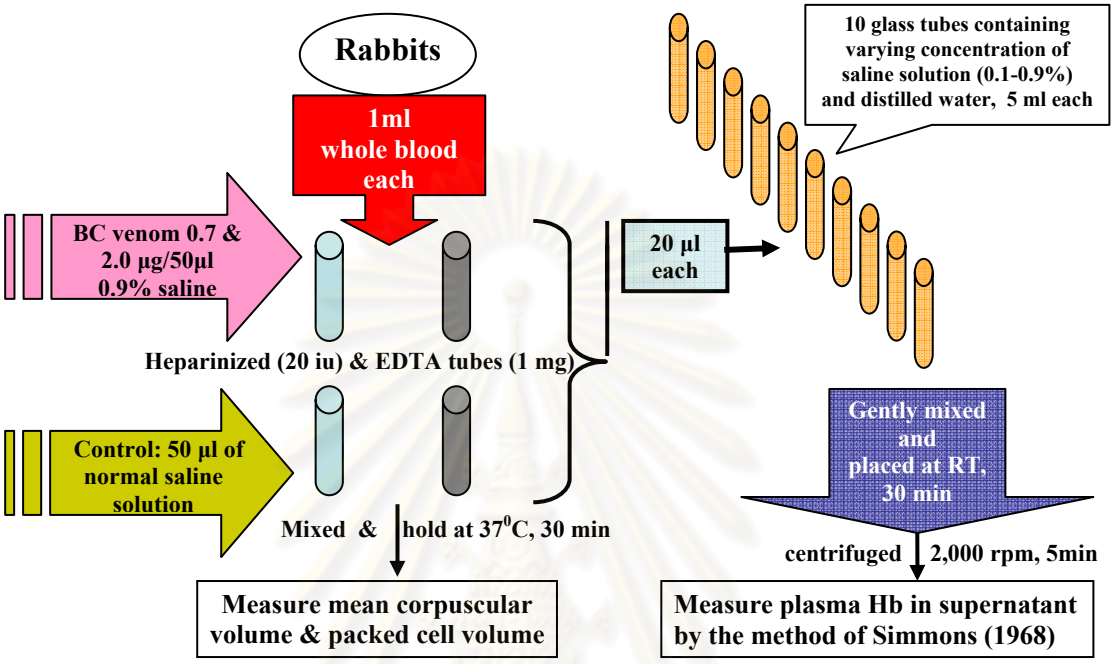


Figure 6-1 Schematic diagram of osmotic fragility test of rabbit red blood cells.

Experiment 6.2. Neurotoxic effect of *Bungarus candidus* in mouse phrenic nerve diaphragm preparations

Mouse phrenic nerve-diaphragm preparation (PNDp)

The phrenic nerve-diaphragm preparation was obtained from mice sacrificed by cervical dislocation (Nirthanan et al., 2003). The hemi-diaphragm associated with phrenic nerve was isolated and mounted under a tension of 1 g in a vertical chamber of organ bath containing 35 ml of Krebs solution (pH 7.4) aerated with 95% O_2 and 5% CO_2 at 37^{O}C. Krebs solution was prepared according to the detail in Chapter III. The indirect stimulation to the phrenic nerve (0.2 Hz, 0.2 ms, 1–10 V) was applied via stimulating electrode (Suction electrode, A-M Systems, Inc.) connected to a stimulator (Grass S48). The direct stimulation to the muscle was achieved by electrical stimulation (0.2 Hz, 0.2 ms, 10-30 V). Isometric twitch tension of muscle was recorded via a force displacement transducer coupled to Polygraph recorder

(Grass Model 79) (Figure 6-2). The PNDps were stabilized for at least 20 minutes before the experiments which were carried out as follow:

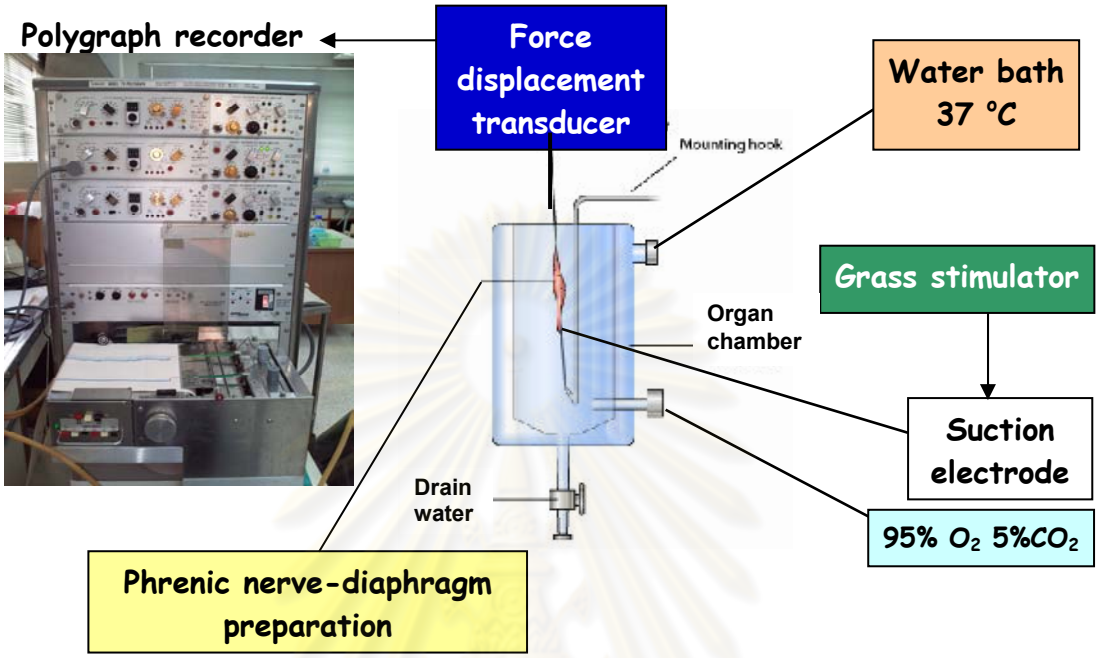


Figure 6-2 Schematic diagram of mouse phrenic nerve-diaphragm preparation.

6.2.1 Neurotoxicity study: the application of venom alone

The experiments were done to compare the time-course induced neurotoxic effect of all *B. candidus* venom groups (from Chapter IV: the wild-caught groups: BC-NE, BC-S and BC-E and a captive born group: BC-CB) at the various concentration of 1, 3.5 and 7.0 µg/ml of Krebs solution.

6.2.2 Neutralization studies

The monospecific *B. candidus* antivenom (Lot no. BC4701) was obtained from Queen Saovabha Memorial Institute. The experiments were undertaken to investigate the neutralizing capability of antivenom (BC-AV) to the neurotoxicity of *B. candidus* venom (BC-V). The proportion used for neutralization was represented by the weight of venom per volume of antivenom (w/v).

6.2.2.1 The addition of antivenom at t_0

BC-AV (17 µl/ml of Krebs solution) was applied promptly with the venom at the concentration of 3.5 µg/ml. BC-NE and BC-S venom groups were tested.

6.2.2.2 The addition of antivenom at t_{50}

BC-AV (17 µl/ml of Krebs solution) was applied after the venom produced 50% inhibition of twitch tension (t_{50}). All venom groups were tested in each experiment at the concentration of 3.5 µg/ml of Krebs solution.

6.2.2.3 The pre-incubation experiment

The mixture of BC-NE and BC-AV was incubated at 37 ^{O}C for 30 minutes prior to the addition into the organ bath. The ratio of BC-V: BC-AV was 1 µg: 4.8 µl (w/v).

6.2.3 Substituting strontium (Sr^{2+}) in Krebs solution

Strontium chloride ($SrCl_2$) was substituted for calcium chloride ($CaCl_2$) on an equimolar basis in Krebs solution. The preparation was repeatedly washed in this solution prior to add the venom. The BC-NE venom (3.5 µg/ml) was applied to PNDps in the organ bath containing Sr^{2+} substitute Ca^{2+} Krebs solution 35 ml.

6.2.4 The control experiments were done using Krebs solution alone or Krebs solution plus antivenom.

The patterns of indirect and direct stimulation to PNDps in all experiments were followed the diagram in Chapter III.

Statistical analysis

The results of hemolytic experiments were expressed as the mean ± SD, and differences were compared using pair *t*-test. All values of neurotoxic experiments were expressed as mean ± SEM, and differences were compared using ANOVA followed by Newman-Keuls test with $p<0.05$ indicating significance.

RESULTS

Hemolytic effect of B. candidus venom on mean corpuscle volume and osmotic fragility of the rabbit red blood cells

The conventional osmotic fragility curve was drawn by plotting the percentage of hemolysis or plasma hemoglobin against decreasing saline concentrations (Figure 6-3). The mean corpuscular fragility (MCF) due to *B. candidus* venom treated on heparinized and EDTA blood were significantly different ($p<0.05$) at the concentrations of 0.73–0.74% and 0.48% NaCl, respectively. Venom-treated heparinized blood exhibited membrane destabilization of red blood cells (60.10 ± 2.59% at low venom dose and 61.86 ± 2.40% at high venom dose), whereas venom-treated EDTA blood was more stabilized on the red blood cell membrane (2.04 ± 0.51% at low venom dose and 2.47 ± 1.57% at high venom dose) (Table 6-1). Packed cell volume (PCV) and mean corpuscle volume (MCV) were increased in envenomed blood using heparin as anticoagulant during treatments at low or high doses of venom after incubation for 15- 60 minute. No effects were apparent in envenomed blood using EDTA as anticoagulant throughout 60 minutes of incubation (Table 6-2).

Table 6-1 Osmotic fragility of rabbit red blood cells treated by *Bungarus candidus* venom.

	Treatment	Heparinized whole blood	EDTA whole blood
MCF (g%)	Control	0.46 ± 0.01	0.49 ± 0.02
	LD	0.73 ± 0.00*	0.48 ± 0.01
	HD	0.74 ± 0.00*	0.48 ± 0.01
% Stability	LD	60.10 ± 2.59(d)	2.04 ± 0.51(s)
	HD	61.86 ± 2.40(d)	2.47 ± 1.57(s)

All values are presented as mean ± SD; n = 3-4 experiments. Mean values indicated with superscripts are significantly different from control by paired *t*-test (*p<0.05). MCF = mean corpuscular fragility; the saline concentration at which 50% of the red blood cells were lysed. Control = untreated venom whole blood.

$$\% \text{ Stability} = \frac{\text{MCFtest} - \text{MCFcontrol}}{\text{MCFcontrol}} \times 100$$

LD = low dose of *B. candidus* venom, 0.7 µg/ml blood.

HD = high dose of *B. candidus* venom, 2.0 µg/ml blood.

d = percentage of membrane destabilization

s = percentage of membrane stabilization

Table 6-2 Changes in packed cell volume and mean corpuscle value of whole blood treated by *Bungarus candidus* venom.

Variable	PCV (%)		MCV (μM^3)	
	Heparinized whole blood	EDTA whole blood	Heparinized whole blood	EDTA whole blood
Control	37.3 ± 1.6	36.7 ± 4.3	86.1 ± 12.2	76.7 ± 20.3
LD 15	38.4 ± 1.8	36.8 ± 3.5	92.6 ± 13.8	77.1 ± 20.3
HD 15	38.7 ± 1.6	36.2 ± 4.3	94.6 ± 15.1	77.5 ± 20.8
LD 30	38.5 ± 1.7	36.1 ± 3.6	93.1 ± 15.2	76.6 ± 20.8
HD 30	38.4 ± 1.4	36.4 ± 4.3	95.0 ± 14.7	76.6 ± 21.5
LD 45	38.9 ± 1.7	36.0 ± 4.1	94.5 ± 14.1	76.2 ± 21.4
HD 45	38.3 ± 1.6	36.1 ± 4.1	94.6 ± 14.4	76.5 ± 21.9
LD 60	39.5 ± 1.3	35.8 ± 4.1	94.6 ± 13.9	75.5 ± 21.3
HD 60	40.8 ± 3.1	36.0 ± 4.0	94.4 ± 14.0	76.3 ± 22.4

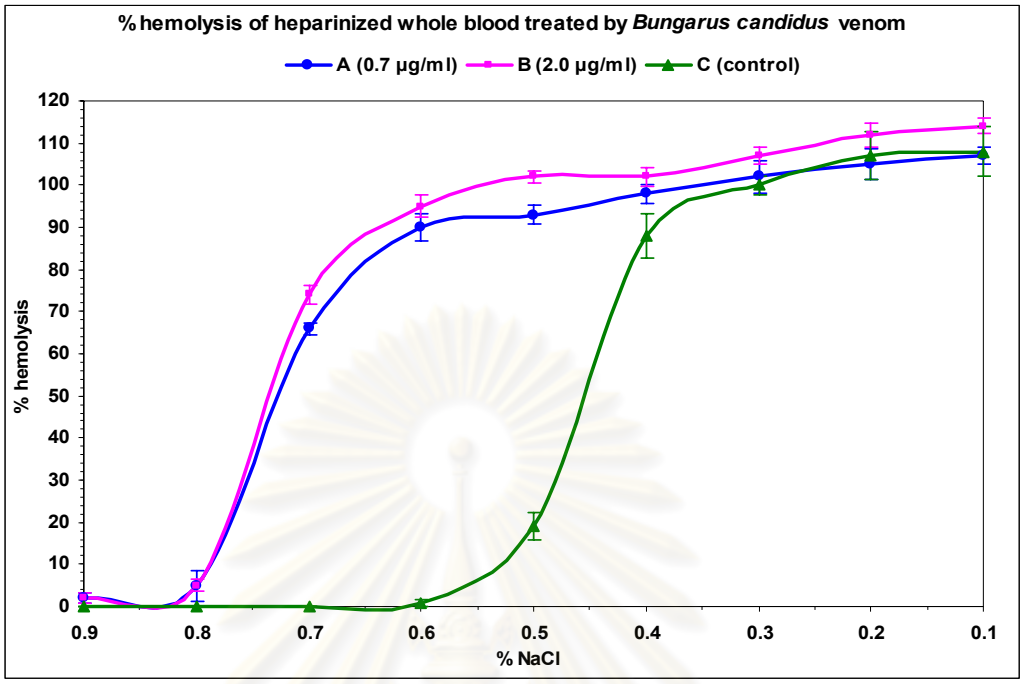
All values are presented as mean ± SD; n = 3; Control = untreated venom whole blood.

LD 15,…, LD 60 = treated with *Bungarus candidus* venom 0.7 µg/ml blood.

HD 15,…, HD 60 = treated with *Bungarus candidus* venom 2.0 µg/ml blood.

PCV = packed cell volume; MCV = Mean corpuscular volume.

A.



B.

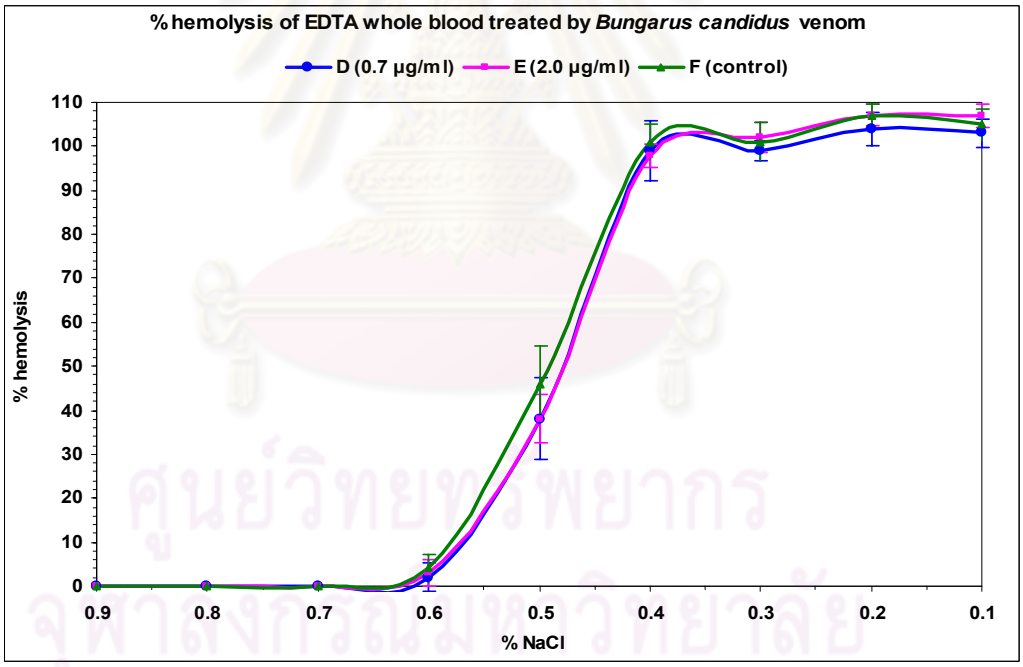


Figure 6-3 The conventional osmotic fragility curve was drawn by plotting the percentage of hemolysis or plasma hemoglobin against decreasing saline concentrations. *Panel A:* heparinized whole blood treated by *B. candidus* venom at the concentration of 0.7 and 2.0 µg/ml. *Panel B:* EDTA whole blood treated by *B. candidus* venom at the concentration of 0.7 and 2.0 µg/ml. The points represent the mean ± SD of 3-4 experiments each.

Neurotoxic effect of B. candidus in mouse phrenic nerve diaphragm preparations

Neurotoxicity studies: the application of venom alone

The time-course to produce 25, 50, 75 and 90% of twitch tension blockade from baseline control was determined in mouse phrenic nerve diaphragm preparation experimented by the various concentrations of *B. candidus* of all venom groups are presented in Table 6-3. Time-course of inhibited indirectly evoked twitches by *B. candidus* venom of all groups at the concentrations 3.5 and 7 µg/ml showed significant differences among groups particularly the quick response of BC-NE venom (Figure 6-4 A and B). BC-NE and BC-S venom groups were compared their neurotoxicity with three different doses of venom. At a concentration of 1.0 µg/ml, 90% blockade occurred in 57.06 ± 3.26 and 55.45 ± 3.49 min for BC-NE and BC-S venom groups, respectively (Figure 6-5 A and B). Direct stimulation was not affected by the venom of all doses. On the other hand, the repeated washing of the preparations was ineffective in reversing the venom-induced blockade.

Neutralization of neurotoxicity of Bungarus candidus venom (BC-V) by B. candidus antivenom (BC-AV)

BC-AV (17 µl/ml) reversed 50% blockade produced by each venom at different levels (Figure 6-6). The venom-induced blockade was partially reversed from 52.40 ± 2.06 to 70.20 ± 8.54 % twitch tension remained and 49.60 ± 4.96 to 60.20 ± 8.72 % twitch tension remained for BC-S and BC-CB venom groups, respectively. Whereas BC-NE and BC-E venom groups showed no significant effect to antivenom as the twitch blockade was still progressed at the time of 80 min (Table 6-4).

When applied BC-AV (17 µl/ml) immediately with the BC-V (3.5 µg/ml) into the PND preparation, the antivenom effectively neutralized the neurotoxic effect of BC-S and BC-NE venoms from 100% of the baseline control to 93.83% and 97.33% evoked twitch remained, respectively, during 60 min of each experiment (Figure 6-7). In the pre-incubation experiment, BC-AV neutralized the neurotoxic effect of BC-NE venom from 100% of the baseline control to 89.60% evoked twitch remained at the end of 60 min (Table 6-5).

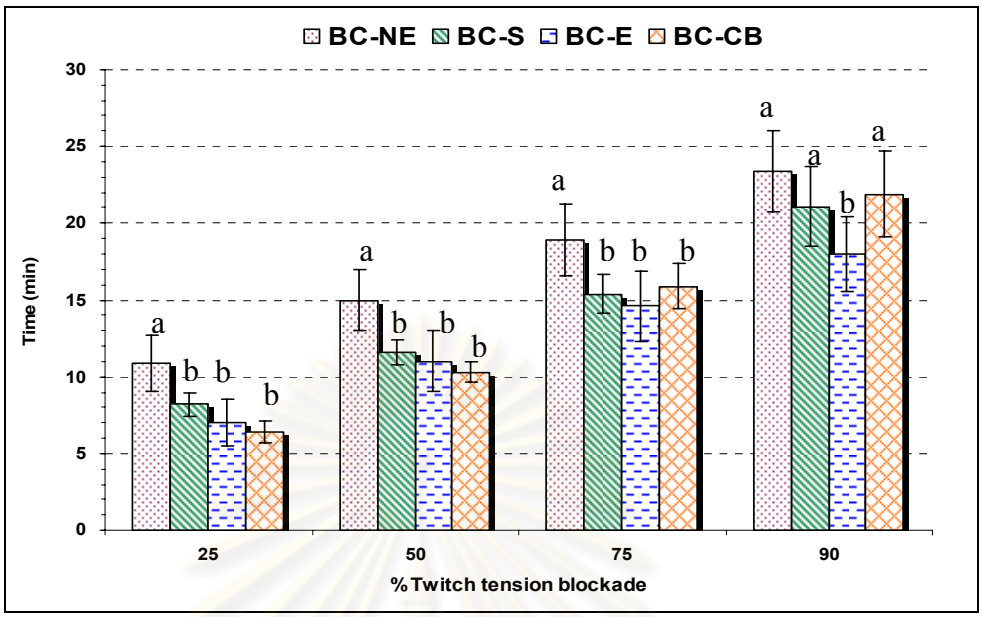
Substituting strontium (Sr^{2+}) in Krebs solution

The effect of BC-NE venom in Sr^{2+} substitute Ca^{2+} Krebs solution is summarized in Table 6-8 and Figure 6-9. The venom-induced blockade was incomplete when compared with the venom in normal Krebs solution, which the twitch tension was remained to 61.00 ± 13.18% of the baseline control at 60 min of experiment.

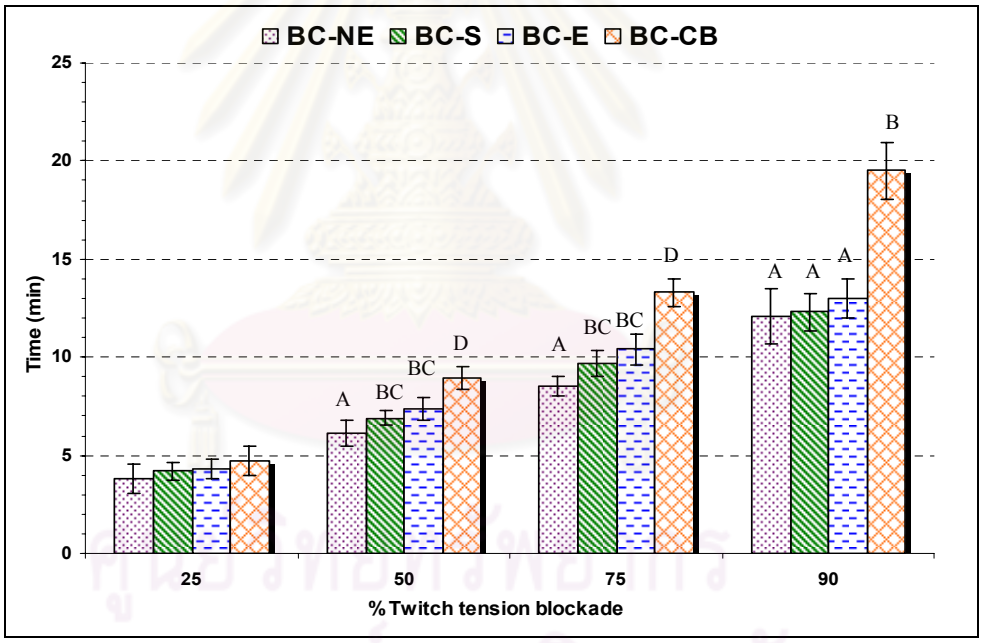
Table 6-3 The time-course for percent twitch tension blockade in mouse phrenic nerve-diaphragm preparation experimented by the various concentrations of *Bungarus candidus* venom from the different parts of Thailand (BC-NE, BC-S and BC-E) and a captive born group (BC-CB).

Venom group	Venom dose (µg/ml)	Time for % twitch tension blockade (min)			
		25%	50%	75%	90%
BC-NE	**7.0**	**3.75 ± 0.76**	**6.05 ± 0.67^{A}**	**8.50 ± 0.49^{A}**	**12.12 ± 1.43^{A}**
	3.5	**10.85 ± 1.83^{a}**	**14.95 ± 2.01^{a}**	**18.89 ± 2.37^{a}**	**23.39 ± 2.68^{a}**
	1.0	21.20 ± 2.64	30.60 ± 3.00	43.65 ± 2.64	57.06 ± 3.26
BC-S	**7.0**	**4.20 ± 0.45**	**6.91 ± 0.36^{BC}**	**9.68 ± 0.67^{BC}**	**12.26 ± 0.98^{A}**
	3.5	**8.17 ± 0.76^{b}**	**11.60 ± 0.80^{b}**	**15.40 ± 1.25^{b}**	**21.14 ± 2.55^{a}**
	1.0	24.55 ± 1.30	33.75 ± 1.92	43.50 ± 2.59	55.45 ± 3.49
BC-E	**7.0**	**4.25 ± 0.49**	**7.37 ± 0.58^{BC}**	**10.35 ±0.80^{BC}**	**12.95 ± 0.98^{A}**
	3.5	**7.00 ± 1.52^{b}**	**11.04 ± 1.97^{b}**	**14.63 ± 2.28^{b}**	**17.97 ± 2.46^{b}**
BC-CB	**7.0**	**4.68 ± 0.76**	**8.88 ± 0.58^{D}**	**13.27 ± 0.72^{D}**	**19.50 ± 1.48^{B}**
	3.5	**6.35 ± 0.67^{b}**	**10.34 ± 0.67^{b}**	**15.87 ± 1.48^{b}**	**21.90 ± 2.82^{a}**

The results are the mean ± SEM of 5 experiments for each group. Mean values within the column of the same venom dose indicated with different superscripts ($^{A, B, C, D,}$ or $^{a, b, c, d}$) are significantly different by Newman-Kuels test at $p<0.05$.



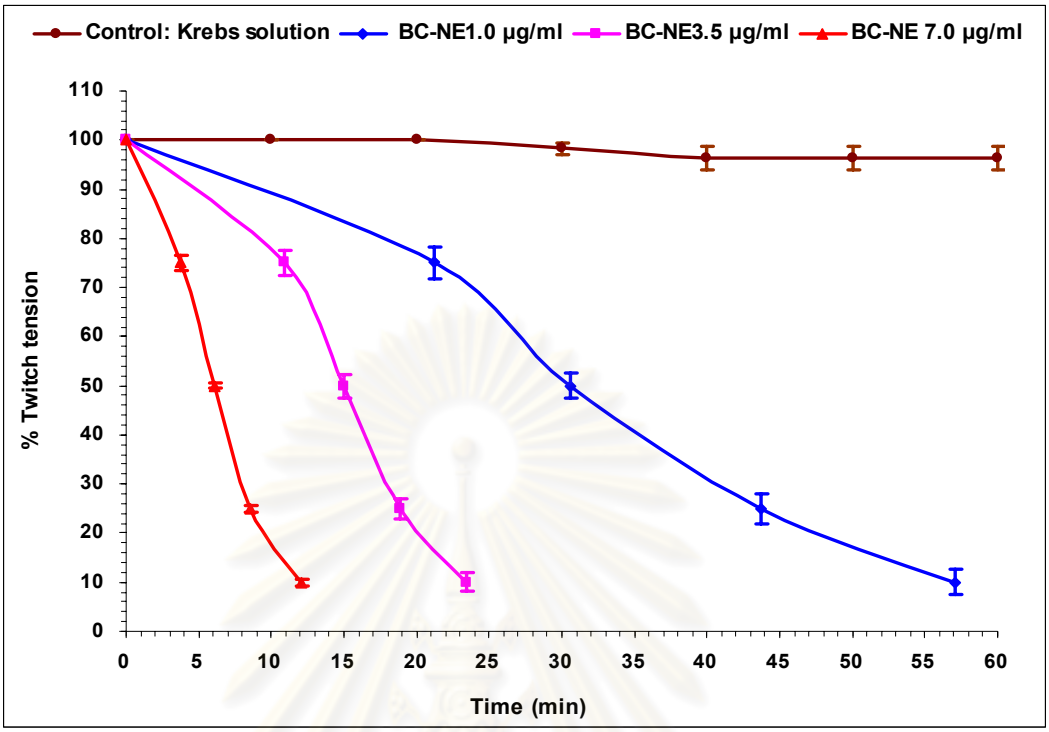
A.



B.

Figure 6-4 Comparison the time-course of percent twitch tension blockade from baseline control of mouse phrenic nerve-diaphragm preparation experimented by *B. candidus* venom from three different parts of Thailand (BC-E, BC-NE and BC-S) and a captive born group (BC-CB) at a concentration of of 3.5 µg/ml *(Panel A)* and 7.0 µg/ml *(Panel B)* of Krebs solution. The columns are the mean ± SEM of 5 experiments for each group. Different superscripts indicated significantly different by Newman-Keuls test ($^{A, B, C, D,}$ or ^{a,b,c,d} $p<0.05$).

A.



B.

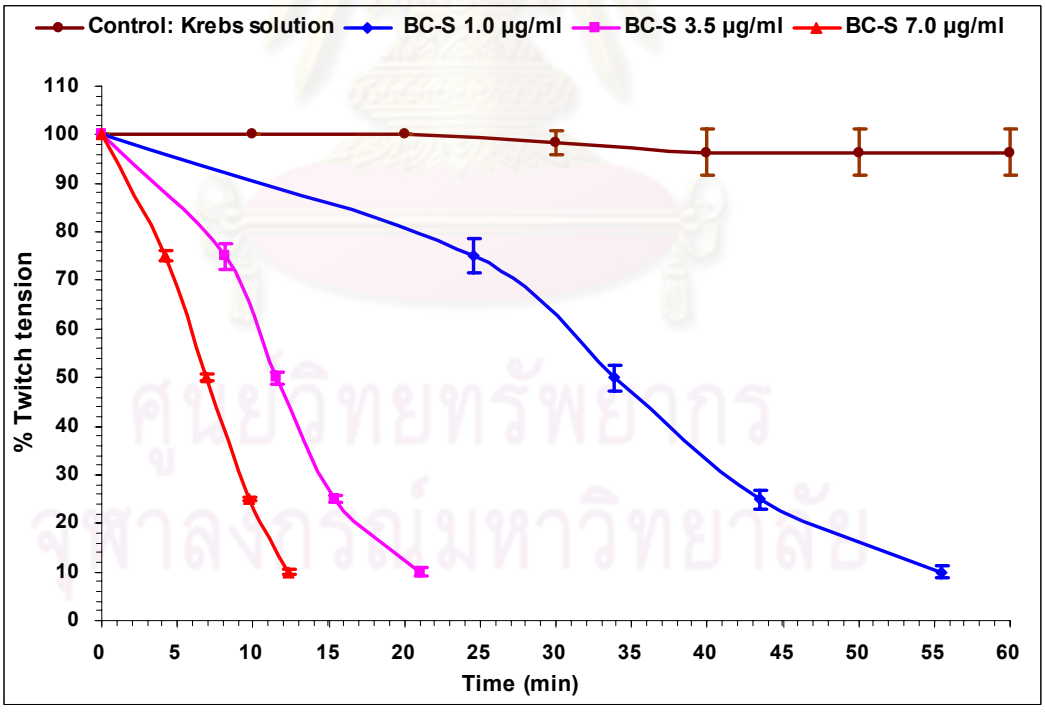


Figure 6-5 The effect of *B. candidus* venom, *Panel A:* from northeastern Thailand (BC-NE), and *Panel B:* from southern Thailand (BC-S), at the various concentrations (1.0, 3.5 and 7.0 µg/ml) on indirectly evoked twitch tensions in mouse phrenic nerve-diaphragm preparation. The points represent the mean ± SEM of 5 experiments each.

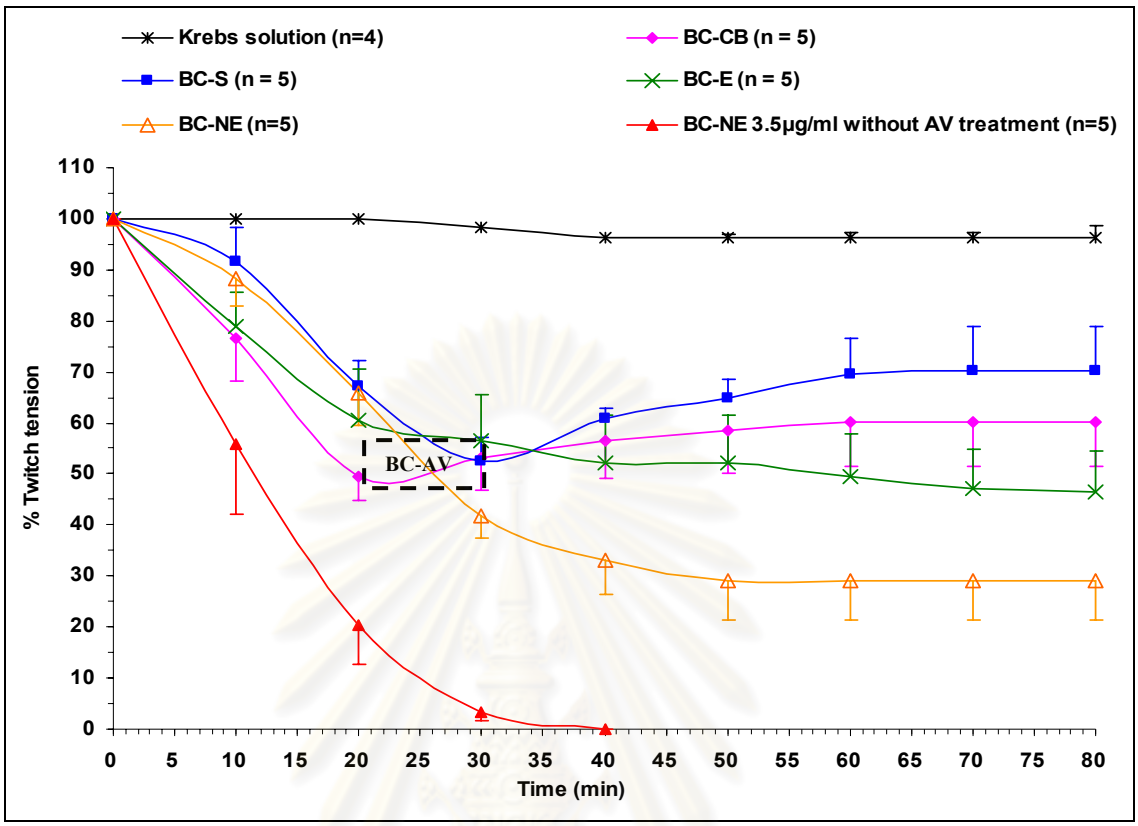


Figure 6-6 Comparison of percent twitch tension remained from baseline control of mouse phrenic nerve-diaphragm preparation experimented by *B. candidus* venom from three different parts of Thailand (BC-NE, BC-S and BC-E) and a captive born group (BC-CB) at a concentration of 3.5 µg/ml Krebs solution which treated by monospecific *B. candidus* antivenom at about 50% blockade occurred (black rectangular). The points represent the mean ± SEM of 5 experiments each.

Table 6-4 Comparison of percent twitch tension remained from baseline control of mouse phrenic nerve-diaphragm preparation experimented by *Bungarus candidus* venom from three different parts of Thailand (BC-NE, BC-S and BC-E) and a captive born group (BC-CB) at a concentration of 3.5 μg/ml of Krebs solution which treated by monospecific *B. candidus* antivenom at about 50% blockade occurred (at time 20–30 minutes). The results are expressed as the mean ± SEM of 5 experiments each.

Time after venom Infusion (min)	Percent twitch tension remained or reversed after BC-AV treatment at 50% twitch blockade occurred				
	BC-NE (n=5)	BC-S (n = 5)	BC-E (n = 5)	BC-CB (n = 5)	Krebs solution(n=4)
10	88.40 ± 5.41	91.60 ± 6.57	78.80 ± 6.80	76.40 ± 8.14	100.00 ± 0.0
20	65.80 ± 6.31	67.20 ± 5.01	60.40 ± 10.24	49.60 ± 4.96	100.00 ± 0.0
30	41.80 ± 4.29	52.40 ± 2.06	56.40 ± 9.21	53.00 ± 6.31	98.25 ± 0.49
40	33.00 ± 6.48	60.80 ± 7.02	52.00 ± 9.57	56.60 ± 7.33	96.25 ± 0.98
50	29.20 ± 7.74	64.80 ± 7.51	52.00 ± 9.57	58.60 ± 8.59	96.25 ± 0.98
60	29.20 ± 7.74	69.40 ± 8.63	49.60 ± 8.36	60.20 ± 8.72	96.25 ± 0.98
70	29.20 ± 7.74	70.20 ± 8.54	47.00 ± 7.69	60.20 ± 8.72	96.25 ± 0.98
80	29.20 ± 7.74	70.20 ± 8.54	46.60 ± 7.78	60.20 ± 8.72	96.25 ± 0.98

Table 6-5 Comparison of percent twitch tension remained from baseline control of mouse phrenic nerve-diaphragm preparation experimented by *Bungarus candidus* venom from northeastern (BC-NE) and southern (BC-S) Thailand at a concentration of 3.5 µg/ml of Krebs solution which treated by monospecific *B. candidus* antivenom at zero time (BC-S / BC-AV_{imm} and BC-NE / BC-AV_{imm}) or pre-incubated the mixture of venom and antivenom for 30 minutes (BC-NE + BC-AV_{30min}) before infusion into the organ bath. The results are expressed as the mean ± SEM.

Time after venom treatment (min)	**Percent twitch tension remained**					
	BC-AV_{alone} (n = 4)	**BC-S / BC-AV_{imm} (n = 6)**	**BC-NE / BC-AV_{imm} (n = 6)**	**BC-NE + BC-AV_{30min} (n = 5)**	**BC-NE in substituted Sr^{2+} solution (n=4)**	**Substituted Sr^{2+} solution (n=3)**
10	100.00 ± 0.00	100.17 ± 2.40	97.83 ± 1.51	97.40 ± 2.53	74.50 ± 12.19	90.33 ± 3.42
20	100.00 ± 0.00	98.00 ± 4.00	92.83 ± 3.51	93.20 ± 4.74	61.00 ± 12.34	84.67 ± 5.56
30	97.75 ± 1.84	98.67 ± 4.82	95.83 ± 2.90	89.60 ± 3.84	55.50 ± 13.21	79.67 ± 7.42
40	97.75 ± 1.84	95.50 ± 4.70	96.67 ± 2.16	89.60 ± 3.84	53.25 ± 12.94	78.00 ± 8.29
50	96.00 ± 1.59	93.83 ± 3.92	97.33 ± 1.67	89.60 ± 3.84	62.75 ± 13.23	76.00 ± 8.64
60	95.25 ± 1.51	93.83 ± 3.92	97.33 ± 1.67	89.60 ± 3.84	61.00 ± 13.18	76.00 ± 8.64

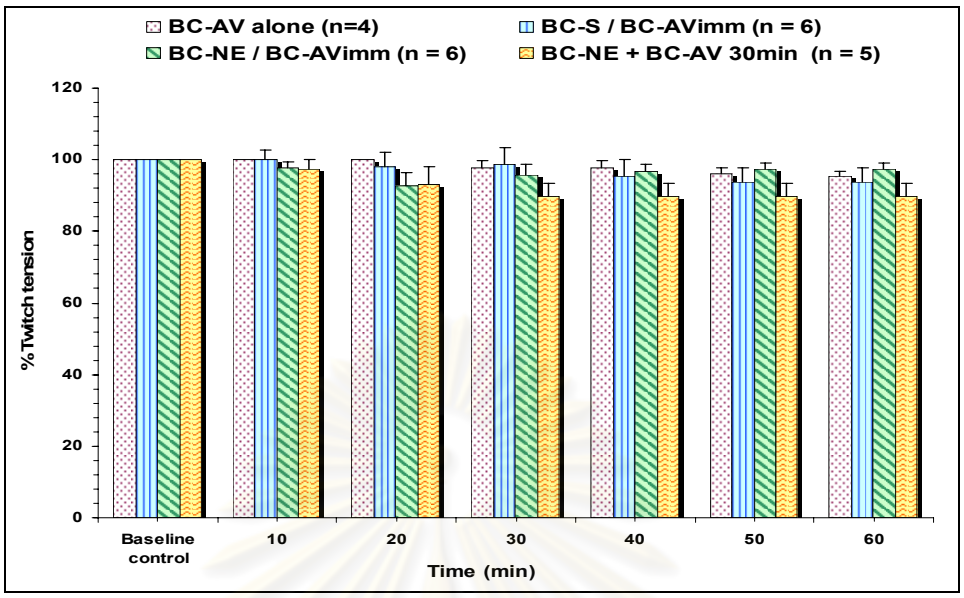


Figure 6-7 Comparison of percent twitch tension remained from baseline control of mouse phrenic nerve-diaphragm preparation experimented by *B. candidus* venom from northeastern (BC-NE) and southern (BC-S) Thailand at a concentration of 3.5 µg/ml of Krebs solution which treated by monospecific *B. candidus* antivenom at zero time (BC-S / $BC\text{-}AV_{imm}$ and BC-NE / $BC\text{-}AV_{imm}$) or pre-incubated the mixture of venom and antivenom (1 µg : 4.8 µl) for 30 minutes (BC-NE + $BC\text{-}AV_{30min}$) before infusion into the organ bath. The results are expressed as the mean ± SEM.

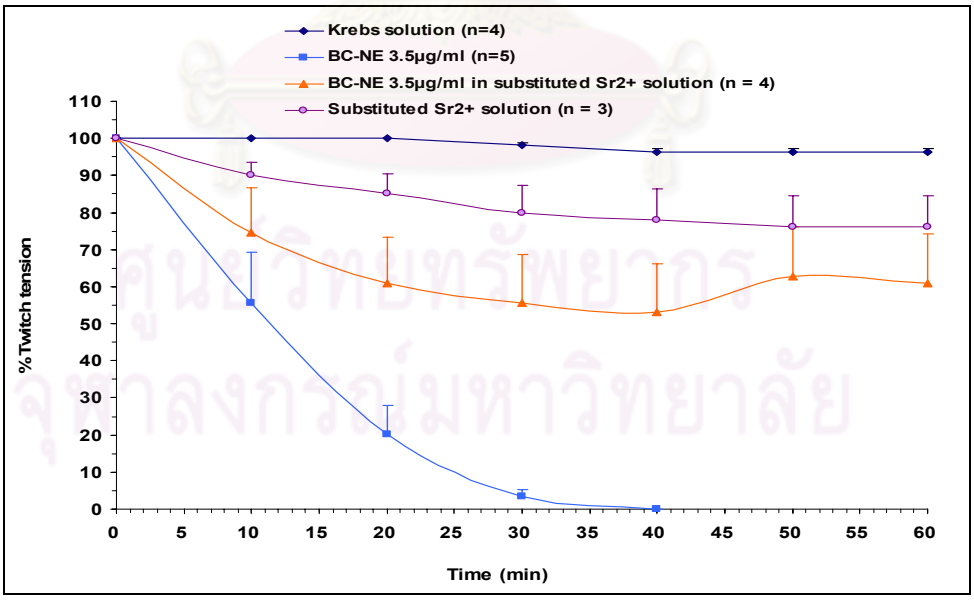


Figure 6-8 Percent twitch tension remained from baseline control of mouse phrenic nerve-diaphragm preparation experimented by *B. candidus* venom from northeastern Thailand (BC-NE) at a concentration of 3.5 µg/ml infused into the organ bath containing normal Krebs solution (blue line), in comparison with Sr^{2+} substitute Ca^{2+} buffer solution (orange line). The results are expressed as the mean ± SEM.

DISCUSSION

In *vitro* hemolytic effect of *B. candidus* venom examined the osmotic fragility of rabbit red blood cells as represented by the MCF value showed the significant difference between venom-treated heparinized blood (0.73–0.74% NaCl) and venom-treated EDTA blood (0.48% NaCl). It indicates that red blood cells in the venom-treated heparinized blood are more susceptible than that of in the venom-treated EDTA blood to osmotic lysis. Increase MCV of red blood cell would be attributed to cellular toxicity induced by the venom (Table 6-2). Although, previous studies reported that rabbit red blood cells are more prone to hypotonic lysis (MCV = 67 ± 4; MCF = 0.465 ± 0.010) than human red blood cells (MVC = 90 ± 5; MCF = 0.445 ± 0.005) (Vaysse, Pilardeua, and Garnier, 1986). The present study of red blood cell osmotic fragility index revealed that certain pathologic factors in the venom have influence on cellular permeability (Sackey, 1999). This is evident in the high value of hematocrit coincided with high percentage of membrane destabilization in venom-treated heparinized blood. Trend in hemolytic effect might occur with higher dose of venom than that used in the present experiment. A significant increase in osmotic resistance of erythrocytes coincided with no changes in MCV obtained from envenomed blood samples stored in EDTA compared to heparinized blood. It indicates that phospholipase activity in venom was not activated by calcium ion, which was chelated in envenomed blood samples stored in EDTA (Bournazos et al., 2008). The requirement of Ca^{2+} for activation most of PLA_2 enzymes for in the cell has been noted (Bonventre, 1990). Phospholipase A_2 (PLA_2s) catalyse the hydrolysis of the *sn*-2 ester bond of phospholipids resulting in lysophospholipids and free fatty acid which are the precursors of eicosanoids such as prostaglandins, thromboxanes, leukotrienes and lipoxins (Dunn and Broady, 2001). PLA_2 in *B. candidus* venom may play a role in hydrolysis of phospholipids in red blood cell membrane. A number of studies reported that several snake PLA_2 activated the rat mast cells to induce histamine release leading to microvascular leakage and inflammatory cell accumulation (Versani and Pearce, 1997; Teixeira et al., 2003; Wei et al., 2009).

The capacity of red blood cells to withstand osmotic stress was defined as its osmotic fragility index (Oyewale and Ajibade, 1990), which is useful to ascertain the

stability and function of red blood cell membrane (Krogmeiger, Mao, and Bergen, 1993). Thus, changes in the phospholipids distribution in red blood cells may explain some hemolytic processes observed in human and animal pathology. Enzymatic hydrolysis by PLA_2 alters the chemical integrity of phospholipid in red blood cell membrane. The split products of PLA_2 hydrolysis, e.g. lysophospholipids and free fatty acid, remain in the membrane bilayer if cells are suspended in saline buffer. This may account for the swelling of the PLA-treated red blood cells without significant hemolysis (Vaysse et al., 1986). At this point, it is proven that PLA_2 from *B. candidus* venom is employed as calcium-dependent activity. Cardiotoxin-like PLA_2 found in *B. fasciatus* venom possesses two mechanism of hemolysis, the first is similar to the cardiotoxin that is independent of PLA_2 activity and the second is related to PLA_2 activity which is only evident at high Ca^{2+} concentration (Jiang et al., 1989).

In *in vitro* studies for neurotoxic effect were examined in mouse phrenic nerve diaphragm preparation. All *B. candidus* venom groups produced inhibition of indirect twitches at all venom concentrations tested, of which time-course of percent twitch tension blockade is the dose-dependent. All wild-caught *B. candidus* venom groups were significantly more potent than a captive-born venom group at 7 µg/ml, when compared the time-course in inhibited indirectly evoked twitches at 50–90% blockade (Table 6-3, Figure 6-4 B). BC-NE venom group were significantly different to the other three venom groups at the venom concentrations 3.5 and 7 µg/ml. For the studies of neutralization, the neurotoxicity of *B. candidus* venom was effectively attenuated in the same level by administration of antivenom promptly (t_0) with venom or by pre-incubation of venom and antivenom (1:4.8 w/v). In pre-incubation study, it might be the outstanding venom from the venom-antivenom forming complex that acted on nerve-muscle preparation, indicating to non-competitive binding of venom and antivenom at the neuromuscular receptors. The venom: antivenom ratios for the neutralization of neurotoxicity were chosen based on the prescription of antivenom (1 ml neutralizes 0.6 mg of Malayan krait venom). But the ratios in the prescription did not provide effective neutralization (data not shown), therefore the amount of antivenom was increased properly in the present experiments. BC-AV (17µl), administered at t_{50}, produced reversal of the twitch blockade caused by BC-V as indicated by the percentage from the point received antivenom in the following rank

order: BC-S (+33.97%); BC-CB (+13.58%); BC-E (-17.38%) and BC-NE (-30.14%). Differences in the rate of reversal of the neurotoxicity produced by venoms from geographical variation, after addition of antivenom indicate to two possibilities: i) the differences in venom components of each venom, and ii) the diverse range of venom used for antivenom production is limited (Chetty et al., 2004; Beghini et al., 2005; Gutiérrez et al., 2009).

Many of PLA_2s are the potentially toxic fractions of the elapid snake venoms, such as the kraits. *B. candidus* venom consists of pre- and post-synaptic neurotoxins (Abe et al., 1977; Kuhn et al., 2000; Torres et al., 2001; Tsai et al., 2002; Khow et al., 2003; Kuch et al., 2003). The post-synaptic α-bungarotoxins bind to the α-subunit of nicotinic cholinoreceptor at the neuromuscular junction and cause the failure of neurotransmission. The neuromuscular paralysis caused by the post-synaptic toxins is reversed by appropriated antivenoms and anticholinesterases (Watt et al., 1986; Gatineau et al., 1988). β-BuTx, the main portion of *Bungarus* snake venoms, are the neurotoxic phospholipases acting on presynaptic nerve terminal. β-BuTx is composed of two subunits linked by a disulfide bond, one is a PLA_2 subunit and the other is a non- PLA_2 (Bon, 1997; Kini, 2003). The exposure of β-BuTx on isolated nerve-muscle preparations caused a triphasic change of neuromuscular transmission failure (Su and Chang, 1984), which an initial reduction phase of spontaneous acetylcholine release, followed by a second prolong phase of facilitated release, and then by a third progressive decline phase of spontaneous neurotransmission. β-BuTx caused the facilitation on spontaneous release of neurotransmitter release (Liou et al., 2004) by triggering the liberation of Ca^{2+} from internal stores (Liou et al., 2006). In present study, the effect of *B. candidus* venom in Sr^{2+} substitute Ca^{2+} Krebs solution partially inhibited twitch tension. Though Ca^{2+} plays an important role in the fast facilitation of neurotransmitter but Sr^{2+} can enter the presynaptic terminal through the Ca^{2+} channel and trigger the release of Ca^{2+} from the internal stores such as endoplasmic reticulum (Nachshen and Blaustein, 1982; Tanabe and Morota, 1999). Moreover the PLA_2 activity of β-BuTx in *B. candidus* venom might be negligible and only post-synaptic neurotoxin affected on nerve-muscle preparation. β-BuTx requires Ca^{2+} for its phospholipase activity, and fails to show significant phospholipase activity after

substitution of Sr^{2+} for Ca^{2+} (Strong et al., 1977). The inhibition of toxin is presumably due to binding to the presynaptic nerve membrane at or close to the sites of transmitter release. The PLA_2 activity of β-BuTx would act on the membrane and cause the transient increase in transmitter release and eventual endplate denervation (Abe and Miledi, 1978). The repeated washing of the nerve-muscle preparation in the present experiment was ineffective in reversing the venom-induced blockade. This might be the effect of β-BuTx which reported to cause the depletion of synaptic vesicles and the degeneration of the nerve terminal and intramuscular axons (Dixon and Harris, 1999; Prasarnpun et al., 2004).

CHAPTER VII

IN *VIVO* STUDY:

EFFECTS OF *Bungarus candidus* VENOM

ON CARDIOVASCULAR FUNCTION AND RENAL HEMODYNAMICS

INTRODUCTION

Snake envenomation is the principle medical problem in rural tropical and subtropical regions with more than 85,000 deaths and 150,000 permanent sequelae each year (Warrell, 2007). In Myanmar and Sri Lanka, the mortality rate from snakebite of 5-15/100,000 population have been annually reported (Lalloo, 2005). *Bungarus candidus* Linnaeus, 1758 (Malayan krait) is an important venomous snake distributed widely throughout Southeast Asia. Its venom posseses a powerful neurotoxin causing severe neurotoxicity (Warrell et al., 1983; Pochanugool et al., 1997; 1998). The first clinical report of 5 cases envenoming by *B. candidus* from eastern Thailand and northwestern Malaysia was described (Warrell et al., 1983), with one patient responded to the treatment by Haffkine's polyvalent antivenom against the venoms of *Bungarus caeruleus, Naja naja, Echis carinatus* and *Vipera russeli* (presently known as *Daboia russelii*). In Thailand, the survey of 15 provincial hospitals in 1980, *B. candidus* was responsible for 13 of 46 cases of fatal snake bite which respiratory failure is the major cause of death (Looareesuwan et al., 1988). Gradual recovery has been observed in neurotoxic snakebite following respiratory support and other supportive treatment without antivenom administration (Pochanugool et al., 1997; Kanchanapongkul, 2002). The decreased parasympathetic activity in 3 victims envenoming by *B. candidus* from northeastern Thailand manifested by mydriasis, prolong hypertension and tachycardia has been described (Laothong and Sitprija, 2001). Three of four cases of *B. candidus* bite in northeastern Thailand recovered after receiving the specific *B. candidus* antivenom. Response to specific antivenom depends on the time between the onset of signs and symptoms of

neurotoxicity and the first dose of antivenom after snake bite (Leeprasert and Kaojarern, 2007).

Snake venom is a complex of proteins and peptides which may induce local injury, neurological, hematological, cardiovascular and renal function disturbance. *B. candidus* venom contains enzymes (e.g. phospholipaseA_2, hyaluronidase, acetylcholinesterase, and L-amino acid oxidase), toxins (e.g. beta-bungarotoxin and alpha-bungarotoxin) and several peptides (Bon and Saliou, 1983; Tan et al., 1989; Tsai et al., 2002; Khow et al., 2003; Nirthanan et al., 2002; Kuch et al., 2003; Chanhome et al., 2009). The major lethal fraction has phospholipase (Tan et al., 1989). The recovery of victims requires the proper artificial ventilation and prolonged periods in intensive care (Pochanugool et al., 1997; Dixon and Harris, 1999; Kanchanapongkul, 2002). Recovery in different victims is inconsistent. Variations in the results of these observations would indicate heterogeneous components in the venom. Neurotoxicity is well known for envenoming by *B. candidus*. The neurotoxic symptoms after snake bite can be progressive upon arrival to the hospitals, without previous critical signs. Acute renal failure is a frequent complication observed in victims of viper bites (Burdmann et al, 1993). The hypotensive effect of the *B. candidus* venom with secondary renal function change has been described in a victim envenomed by *B. candidus* in northeastern Thailand despite monospecific *B. candidus* antivenom administration (Wirat Leeprasert, 2008 per. comm.). Few data are available concerning sequential changes of renal function produced by *B. candidus* venom, although many studies have reported the occurrence of lethal acute renal failure after snakebites (Chugh et al., 1984; Chugh, 1989; de Silva et al., 1994: Schneemann et al., 2004; Sitprija, 2006). Hemodynamic alterations are factors accounted for the pathogenesis of renal failure (Sitprija and Chaiyabutr, 1999).

Human autopsy was performed in the deaths envenomed by common krait (*Bungarus caeruleus*) bite in Sri Lanka. In the acute cases died within 24 hours, one showed the histological changes of acute renal tubular necrosis and pan lobular microvesicular fatty changes in the liver, and the other showed petichial haemorrhages in the myocardium, renal cortex and adrenal gland macroscopically. One of the late deaths revealed congestion of kidneys with cortical haemorrhage microscopically (Kularatne and Ratnatunge, 2001). Inoculation of the *B. caeruleus*

crude venom in rat model experiments, the histopathological changes were found hemorrhage, multifocal areas of myocardial fiber necrosis and constriction of blood vessels in the heart; congestion of the vessels, hemorrhage and necrosis of proximal tubules in the kidney; congestion and hemorrhage in the liver (Kiran et al., 2004; Mirajkar et al., 2005). *B. candidus* venom have mainly studied on its components, sequence of amino acid, toxic and biochemical properties, very little has been studied on cardiovascular function and renal hemodynamics. It is therefore interesting in studying the effect of *B. candidus* venom in the animal model to investigate the alterations in cardiovascular function and renal hemodynamics. Such informations from animal experiments envenomed by *B. candidus* venom are hopefully used to explain the systemic effects in human envenomation that may be overlooked by physicians.

MATERIALS AND METHODS

Animal preparation

Twelve adult male New Zealand white rabbits weighing 2.5–3.5 kg were used. The animals were deprived of food but not of water for 12 hours prior to the study. On the day of experiments, the animal was anesthetized with pentobarbital sodium (25mg/kg) by intravenous injection, and supplemental doses were given to maintain anesthesia throughout the experiment. The animals were tracheotomized, and an endotracheal tube was inserted to free the airway. The carotid artery was catheterized with polyethylene tubes for recording cardiac output, arterial blood pressure and heart rate (Grass Polygraph Model 79 E, Grass Instrument CO, USA) and for blood sample collections. The jugular vein was catheterized with polyethylene tubes for fluid infusion. A left flank incision was made via a retroperitoneal approach, and the left ureter was cannulated with a polyvinyl catheter for urine collection and for renal clearance studies.

The experimental protocol

7.1 Determination of the minimal lethal dose of Bungarus candidus venom in rabbit

Before the experiment, the minimal lethal doses of *B. candidus* venom were determined in twelve anesthetized rabbits. A single dose of the lyophilized crude *B. candidus* venom dissolved in 0.9% saline solution (1 mg/ml) was infused intravenously at the dose of 75 μg/kg in 6 rabbits, and 150 μg/kg in 6 rabbits. The general condition was measured for heart rate and arterial blood pressure.

7.2 The effects of Bungarus candidus venom on cardiovascular function and renal hemodynamics

Twelve rabbits were divided into 3 groups of four animals each. Animals in group 1 were received normal saline as the control group. Animals in group 2 were received the *B. candidus* snakes venom from the northeast (BC-NE) and group 3 were received the *B. candidus* snakes venom from the south (BC-S). The experimental study in each group was divided into pretreatment and treatment periods. Both pretreatment and treatment period, measurements of renal and cardiovascular functions were performed.

7.2.1 Measurements of renal plasma flow and glomerular filtration rate were performed using the standard techniques (Smith, 1962) with the modification. For treatment period, the rabbits were administered with the *B. candidus* crude venom dissolved in 0.9% saline solution (1 mg/ml) at the dose of 50 μg/kg intravenously. All changes of parameters were observed initial post venom injection and recorded in every 30 min. interval after envenomation. Urine samples were collected at 10 minute interval.

7.2.2 Measurement of cardiac output was performed by dye dilution technique (Chaiyabutr et al., 1980) using Evan blue (T-1824). The concentration of dye in the plasma was determined by spectrophotometry at the absorbance of 620 nm. The sum of all concentration was calculated for cardiac output (CO) using the standard formula (Burton, 1965).

The procedures of experimental protocol are presented in Chapter III.

Chemical analysis

Blood and urine samples were determined inulin, PAH, packed cell volume, osmolality, sodium, potassium and chloride. Inulin clearance and PAH clearance were performed for the glomerular filtration rate (GFR) and effective renal plasma flow (ERPF), respectively. Osmolality was determined by the freezing-point depression method (The Advanced^{TM} Osmometer Model 3D3). Measurement of sodium and potassium were made by flame photometry (Clinical Flame Photometer 410C), chloride with a Chloride Analyzer 925. Plasma lactate dehydrogenase (LDH) and creatine phosphokinase (CK) were determined by immunoturbidimetric assay using COBAS INTEGRA 800 (Roche Diagnostics GmbH, Manheim) and measured the increase in absorbance at 340 nm. Plasma calcium concentrations were determined using Cresolphthalein Complexone method (Gitelman, 1967).

Calculation

Renal blood flow (RBF) was calculated from ERPF and packed cell volume. Total peripheral resistance (TPR) and renal vascular resistance (RVR) were calculated from mean arterial blood pressure (MAP). Filtration fraction was obtained by dividing GFR by ERPF. Cardiac output (CO) and renal blood flow (RBF) using the standard formula (Burton, 1965). See derived calculation in Chapter III.

Statistics

All data are expressed as mean ± standard deviation. The results were analyzed by analysis of variance (ANOVA), post hoc tests for comparisons of each value of post-treatment periods against the value of pretreatment period using Bonferroni *t*-test. Comparisons of mean values among groups of studies were performed using the Duncan's test. The significant differences among treatments were determined at $p < 0.05$.

RESULTS

Determination of the minimal lethal dose of Bungarus candidus venom in rabbit

The dose of *B. candidus* venom used for determination of the minimal lethal dose in rabbit was modified from the LD_{50} dose in mouse at 75 µg/kg. It was found that one hundred percent of rabbits died within 167 minutes, when they were envenomed with *B. candidus* venom solution at a dosage of 150 µg/kg by intravenous injection. 83% died within 5–153 minutes in animals given 75 µg/kg of venom. Therefore the dose of 50 µg/kg of *B. candidus* venom was selected to use in further experiments to observe its effects on cardiovascular function and renal hemodynamics.

The effects of Bungarus candidus venom on cardiovascular function and renal hemodynamics

The cardiovascular function and renal hemodynamics were observed and recorded the alterations of all parameters for 150 minutes.

Observation in the cardiovascular system.

Data in Table 7-1 shows the changes in heart rate (HR), pulse pressure (PP), and mean arterial pressure (MAP) throughout 150 minutes after intravenous injection with the *B. candidus* at the doses of 75 and 150 µg/ml. HR and MAP in both envenomed animal groups were fallen off within 2 minutes after venom infusion. The same changes in cardiovascular responses occurred within 2 minutes after venom infusion in the animal groups receiving the venom dose of 50 µg/ml (Table 7-2). A marked decrease in MAP occurred within 2 – 5 min. after envenomation, afterwards gradually returned closely to baseline values (Figure 7-1). There were stepwise decreases in HR and cardiac output (CO) (Figure 7-2). In comparison, HR and MAP of animal group 2 (BC-NE venom group) was immediately dropped than that of animal group 3 (BC-S venom group). The significant decreases of respiratory rate (RR) revealed at 150 minutes after envenomation in both envenomed animal groups. Packed cell volume (PCV) values significantly decreased after 60 minutes in both envenomed animal groups (Table 7-3). The slightly increase in total peripheral resistance (TPR) was apparent, whereas the left ventricular work (LVW) was

decreased (Table 7-4) after envenomation. The alterations in pulse pressure and stroke volume after envenomations were no significant in both envenomed animal groups.

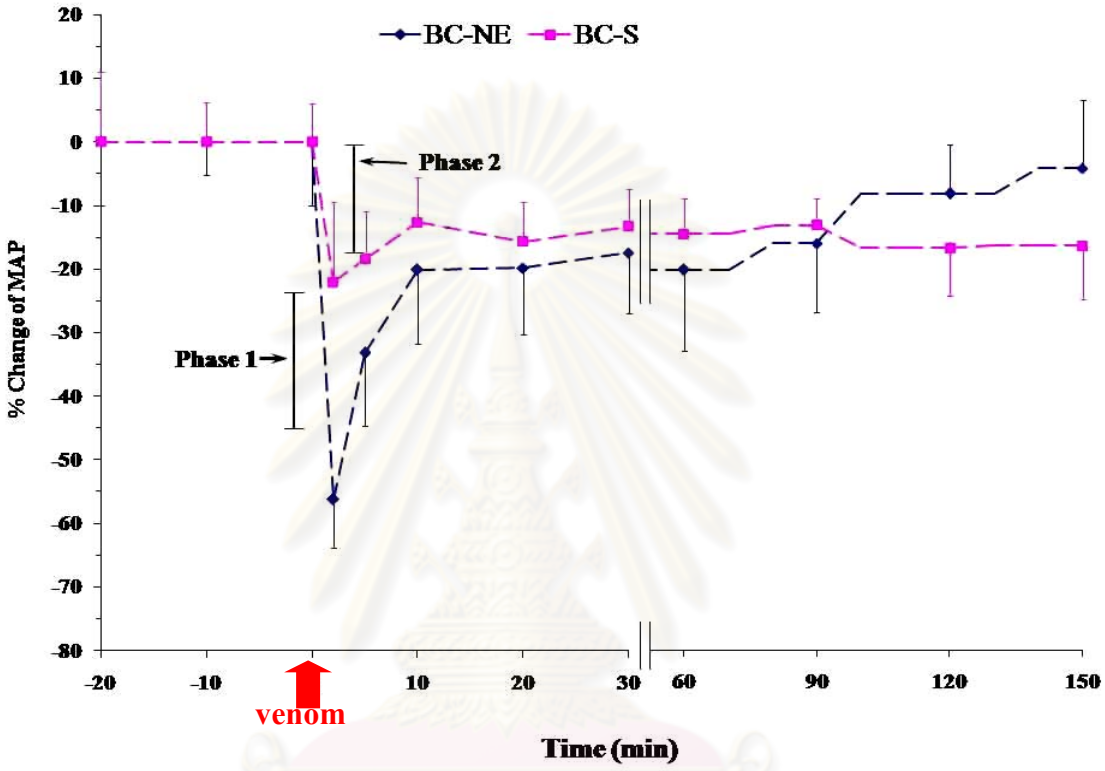


Figure 7-1 Time-course of the percent change in mean arterial pressure (MAP) of rabbits envenomed by *B. candidus* venom from northeastern (BC-NE) and southern (BC-S) Thailand. The values are mean ± SD (n = 4).

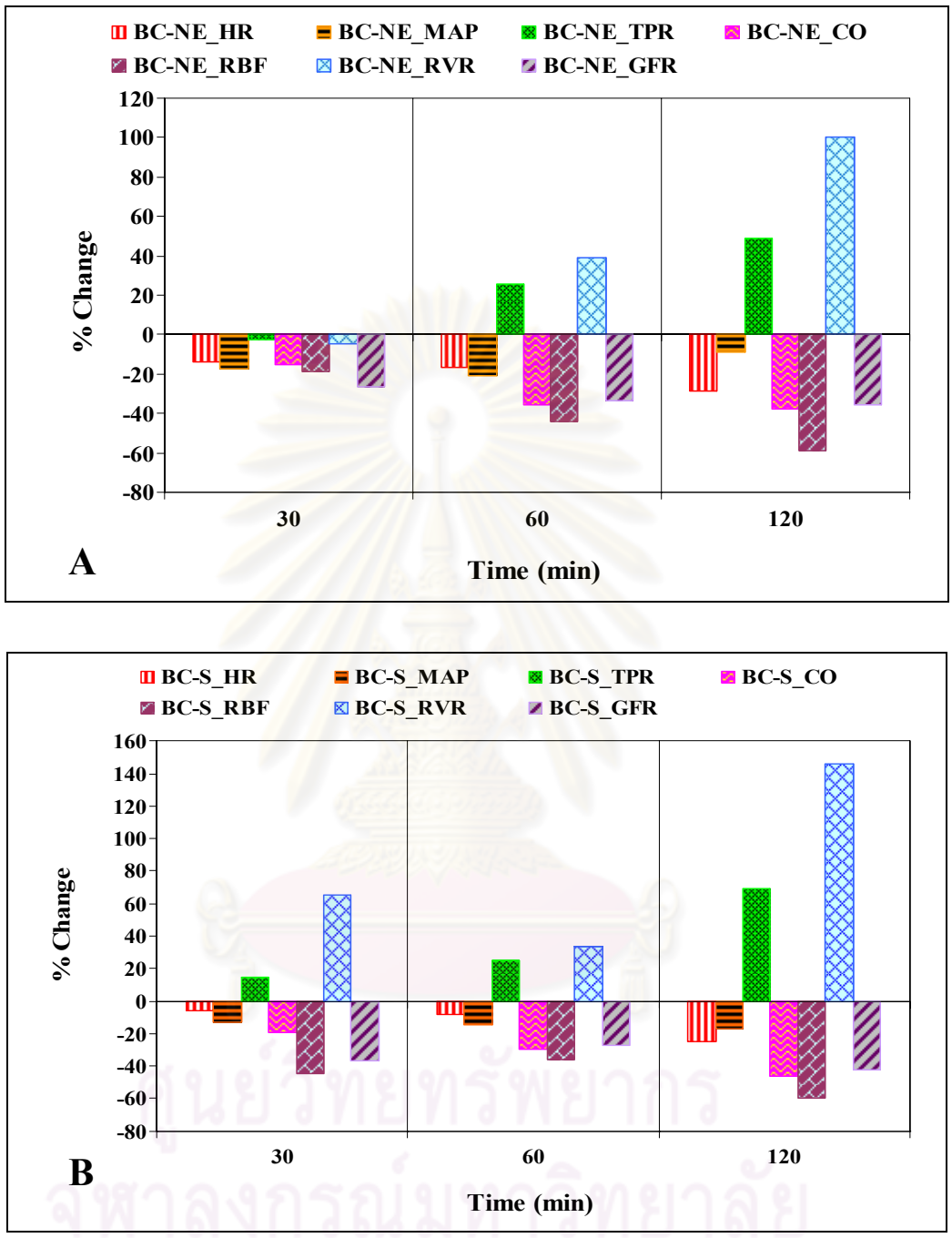


Figure 7-2 Time-course of the percent changes in cardiovascular function and renal hemodynamics in rabbits after experimental envenoming by *B. candidus* venom from northeastern (panel A) and southern (panel B) Thailand. HR = heart rate; MAP = mean arterial pressure; TPR = total peripheral resistance; CO = cardiac output; RBF = renal blood flow; RVR = renal vascular resistance; GFR = glomerular filtration rate.

Observation in the renal hemodynamics

The renal hemodynamics was summarized in Table 7-5. Glomerular filtration rate (GFR) significantly decreased at 150 min in both venom groups. In animal group 2, effective renal plasma flow (ERPF) and effective renal blood flow (ERBF) significantly decreased at 60-150 min, while filtration fraction (FF) significantly increased at 150 min. Renal fraction (RF) revealed a reduction, and renal vascular resistance (RVR) increased stepwise throughout the experimental period of 150 min in both envenomed animal groups (Figure 7-2). The rate of urine flow markedly decreased at 150 min both envenomed animal groups.

In both envenomed animal groups, there were no differences in the plasma concentration of sodium (P_{Na+}) and chloride (P_{Cl-}) as compared to the pre-treatment values. The plasma concentration of potassium (P_{K+}) trended to increase after envenomation. Urinary excretion (E) of Na^+, K^+ and Cl^- showed stepwise decreases, while no alteration in fractional excretion (FE) of these electrolytes after venom infusion (Table 7-6 and 7-7). Osmolal and water clearance were decreased without alteration of both plasma and urine osmolarity. Creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) levels in plasma increased at 30 – 120 min after envenomation (Table 7-8). CPK level (U/L) changed from 272.25 ± 56.65 U/L at the baseline level to 464.50 ± 198.14; 445.25 ± 168.97; 382.75 ± 160.00 and 344.00 ± 195.75 U/L at 30, 60, 90 and 120 min, respectively. LDH changed from 124.75 ± 83.62 U/L at the baseline level to 159.25 ± 94.56; 172.25 ± 108.16; 135.75 ± 68.01 and 149.25 ± 73.99 U/L at 30, 60, 90 and 120 min, respectively. Plasma calcium levels significantly decreased from 8.7 ± 1.1 mg % at the pre-treatment period to 6.5 ± 1.4 mg% at 120 min post-treatment of venom (Table 7-8).

DISCUSSION

The aim of the present study was to determine whether *B. candidus* venom plays any effects to changes in the general circulation and renal functions. The *B. candidus* venom probably affects almost all cells or tissues and their biological characteristics properties have been determined by various biologically active components (Chanhome et al., 2009). Nephrotoxicity has been observed in myotoxic

and hematotoxic snakebites (Chugh et al., 1984; Chugh, 1989; de Silva et al., 1994: Schneemann et al., 2004; Sitprija and Chaiyabutr, 1999; Sitprija, 2006). Effects of snake venom may be directly or indirectly promoted by the release of heterogeneous active biologically substances.

The present studies show that an immediate decrease in systemic arterial blood pressure and HR (Figure 7-1) were apparent after intravenous injection of *B. candidus* venoms (BCV). The reduction of arterial blood pressure persisted for a short duration and then gradually improved to approach the control level within 30 min. The persistency hypotensive effect of BCV was accompanied by an increase in total peripheral vascular resistance. This is in contrast to hemodynamics effects of Russell's viper venom in which hypotension is due to decreased systemic vascular resistance (Tungthanathanich et al., 1986). The presence of phospholipase A in biological components has been reported in *B. candidus* venoms (Tan et al., 1989; Chanhome et al., 2009). PLA_2 is known to play a major role in release of arachidonic acid which is the substrate for the synthesis of several lipid mediators of inflammation, such as prostaglandins, prostacyclin, thromboxane and leukotrienes (Teixeira et al., 2003). The enzymatic action of phospholipase on membrane phospholipid can change membrane permeability. Therefore, phospholipases can cause increased vascular permeability, edema and hypotension. Phospholipase A in venoms would be a factor for hypotension after envenomation by release of thromboxane A_2. It might cause pulmonary vasoconstriction leading to restriction of blood return to the heart and decreased cardiac output (Huang, 1984).

The decreased HR and sustained hypotension from BCV was not thought to be cholinergic in origin, since atropinization in experimental rabbits did not prevent initial hypotension after intravenous injection of BCV (unpublished data). Thus, a general manifestation of a direct toxic effect of venom on cardiovascular function, especially the reduction of heart rate in the initial hypotensive response, could not be excluded. Hypotension is attributed to decreased cardiac output and systemic vasodilatation, which may occur secondary to the release of vasoactive mediators including nitric oxide (NO), prostaglandin E2, kinins and histamine after envenomation. Decreased heart rate persisted despite the compensatory mechanism which activates the sympathetic nervous system. Thus, the cardiovascular responses

may be a central effects. The fall in blood pressure and heart rate may be due to a direct inhibitory effect of the snake venom via the blood-brain barrier acting on the medullary cardiovascular center, and reducing the tonic discharge of impulses to the sympathetic vasoconstrictor fibers and to the cardioaccelerator nerves thus producing hypotension and bradycardia (Telang, Lutunya, and Njoroge, 1976). The systemic hypotension accompanying the decrease in respiration in BCV treated animals may be secondary to hypoxaemia resulting from respiratory failure (Tibballs et al., 2003).

In this study decreased cardiac output was a striking finding and importantly accounted for hypotension. Decreased heart rate and hypotension persisted despite the compensatory mechanism though activation of the sympathetic nervous system. Persistent decrease in cardiac output and heart rate despite hypotension suggest direct cardiac effect of the venom. It has been reported that Bucain, isolated from the venom of *B. candidus*, which is a three-fingered α-neurotoxin in structure is similar to cardiotoxin (Watanabe et al., 2002; Murakami et al., 2009). In the present results, the reduction of plasma calcium level after envenomation may be a direct toxic effect of venom on the membrane calcium channels (Lalloo et al., 1997). In this respect, the venom could have a calcium channel blocking effect which worth further investigation. The alteration in the homeostasis of Ca^{2+}, including K^{+} and Na^{+} concentrations in cardiac myocytes, may affect cardiac contractility leading to heart failure. Cardiotoxin of cobra venom has been shown to increase membrane permeability by causing pore formation, thus allowing Ca^{2+} influx and involving in Ca^{2+} binding and transport in the heart (Cher et al., 2005). β-cardiotoxin purified from the venom of *Ophiophagus hannah* acted directly on the cardiac tissue in a dose-dependent decrease in heart rate without affecting contractility have been noted (Rajagopalan et al., 2007). It is known that the reduction of CO and blood pressure would contribute to vasoconstriction and an increase in systemic vascular resistance via an activation of sympathetic tone releasing catecholamine as a compensatory mechanism (Hall and Hodge, 1971). In the present findings, the reduction of CO and blood pressure from the effect of snake venom, may be related to many enzymes in venom such as kininogenases, phospholipases and hydrolases of arginine ester, which may involve in these processes. Hemodynamic side-effects such as vasodilatation with resultant hypotension are due to the release of mediators including biological

amines such as histamine, serotonin or prostaglandins. They might be expected to occur during envenomation. Release of histamine relating to phospholipase concentrations has been noted (Hyslop and De Nucci, 1993). The consequences of hypotensive effects on reductions in RBF and GFR coinciding with an increase in RVR were apparent after envenomation in present findings.

The present findings reveal that the *B. candidus* venom could affect both general circulation and renal hemodynamics, which resulted in a reduction of renal fraction (ERBF/CO). The magnitude of an increase in renal vascular resistance appeared to be more than an increase in total peripheral resistance throughout the experimental periods, thereby leading to reductions of GFR and ERPF after envenomation. A disproportionate decrease in ERPF and GFR resulted in significant increase in filtration fraction after envenomation. In the present experiments, the urinary sodium excretion decreased, while fractional sodium excretion was not reduced in the venom-treated group when compared to the control group. Urinary sodium excretion depends on the relationship between GFR and sodium reabsorption. Therefore, the low rate of urine flow would be in part due to the decrease in the filtered load of electrolytes after envenomation. The present results demonstrate that BCV decreased the renal fraction while RVR increased in the kidney. The renal fraction tended to decrease, but not significantly. Thus BCV had prominent effect on the kidney vasculature. The increments of plasma CPK and K^+ levels after envenomation in this study might suggest rhabdomyolysis which may be an effect of toxin in *Bungarus* venom (Theakston et al., 1990). In this regard, rhabdomyolysis needs to be proved by reliable methods of detection myoglobin in urine which unfortunately was not done in the present experiment. Moreover the further experiment should be performed in an appropriate model for determination of rhabdomyolysis. The CPK and LDH levels were slightly increased in the BCV-treated animals. These finding probably showed a nonspecific toxic effect of the venom on muscle cells. Decreases in RBF and GFR were encountered, and may be due to myoglobinuria caused by damaged skeletal muscles (Ayer et al., 1971; Campion, Arias, and Carter, 1972). The elevation of myoglobinuria affecting kidney functions after BCV injection needs to be further investigated. It can conclude that BCV did not

direct affect to cause an acute nephrotoxicity in the experimental rabbit, which differed from that described in Russell's viper venom (Chaiyabutr and Sitprija, 1999).

CONCLUSION

The present experiment is the first demonstration of the action of *B. candidus* venom on the kidney function. Changes in renal function in envenomed animals are related to systemic hemodynamics. A sustained fall in CO after envenomation would be associated with the reduction in HR. Hypotension was attributed to decreased CO due to the effect of the venom on cardiac muscle. A similar effect to calcium blocking agents was postulated. Sustained hypotension would contribute to reduction of RBF which results in decrease in GFR throughout the 150 min post venom injection. The present findings revealed that the *B. candidus* venom could affect both general circulation and renal hemodynamics. The magnitude of an increase in RVR appeared to be more than an increase in TPR throughout the experimental periods, thereby leading to reductions of GFR and ERPF after envenomation. A further reduction of renal function might be expected to appear if animals were observed for a longer period of time.

Table 7-1. Changes in the heart rate, pulse pressure and mean arterial pressure in rabbits after intravenous injection with *Bungarus candidus* venom at the concentration of 75 and 150 µg/kg.

Venom dose	75 µg/kg (n = 3)			150 µg/kg (n = 4)		
Parameters / Time(min)	HR (beats / min)	PP (mmHg)	MAP (mmHg)	HR (beats / min)	PP (mmHg)	MAP (mmHg)
Pre-treatment	200 ± 22.7	29 ± 9.8	93 ± 16.9	204 ± 24.4	28 ± 8.0	96 ± 9.3
2	174 ± 57.2	25 ± 17.8	56 ± 29.9*	163 ± 40.4	23 ± 18.9	42 ± 27.9*
5	162 ± 59.9	23 ± 14.6	59 ± 35.1	163 ± 32.5	24 ± 11.1	46 ± 23.4*
10	181 ± 27.2*	25 ± 13.0	68 ± 25.9*	186 ± 23.1*	30 ± 13.5	75 ± 15.2*
20	179 ± 27.9*	28 ± 11.9	70 ± 18.6*	184 ± 17.2*	23 ± 8.7	91 ± 8.9
30	167 ± 22.5*	32 ± 17.7	71 ± 12.0*	183 ± 22.4*	24 ± 8.3	78 ± 27.3
60	163 ± 19.9*	32 ± 17.7	77 ± 19.1	159 ± 35.1*	29 ± 11.8	75 ± 29.0
90	144 ± 31.7*	31 ± 16.8	86 ± 15.0	114 ± 19.5*	30 ± 9.3	94 ± 44.6
120	141 ± 10.6*	35 ± 14.1	102 ± 16.3	71 ± 21.2*	32 ± 9.2	108 ± 7.1
150	106 ± 28.3*	36 ± 0.7	70 ± 13.4	76 ± 11.3*	34 ± 15.6	76 ± 17.7

All values are shown as mean ± SD; Pre-treatment period = no venom injection. Mean values within the column indicated with superscripts are significantly different from pre-treatment by Bonferroni *t* - test (* $p < 0.05$). HR = heart rate; PP = pulse pressure; MAP = mean arterial pressure.

Table 7-2 Effect of intravenous injection of *Bungarus candidus* venom (50 µg/kg) on cardiovascular functions and respiration at the first 20 minutes after envenomation in rabbits.

Parameters	Group	Pre-treatment	Post-treatment (min)			
			2	5	10	20
HR	Control	235 ± 14.6	235 ± 12.5	236 ± 14.5	235 ± 17.3	235 ± 17.3
(beats / min)	BC-NE venom	208 ± 9.0	192 ± 14.7	179 ± 31.1	173 ± 35.1	177 ± 24.2
	BC-S venom	219 ± 7.7	219 ± 7.7	215 ± 12.4	208 ± 15.5	206 ± 8.5
RR	Control	45 ± 10.4	54 ± 6.9	53 ± 7.5	54 ± 6.9	53 ± 6.4
(Breaths/min)	BC-NE venom	25 ± 2.9	30 ± 12.7	26 ± 7.7	23 ± 6.2	26 ± 3.9
	BC-S venom	33 ± 6.0	33 ± 7.5	35 ± 6.6	36 ± 4.3	38 ± 4.6
PP	Control	25.0 ± 8.2	26.2 ± 8.5	27.0 ± 9.1	29.0 ± 6.7	28.7 ± 7.7
(mmHg)	BC-NE venom	28.1 ± 2.4	31.5 ± 6.6	23.2 ± 4.6	20.0 ± 7.1	26.2 ± 6.3
	BC-S venom	37.5 ± 10.4	44.5 ± 17.1	37.5 ± 15.5	31.25 ± 11.5*	36.0 ± 14.6
MAP	Control	92.0 ± 6.9	90.5 ± 13.3	84.0 ± 8.8	83.5 ± 8.6	83.2 ± 8.8
(mmHg)	BC-NE venom	105.1 ± 10.1	46.0 ± 14.6*	70.3 ± 22.5	84.0 ± 21.8	84.2 ± 21.2
	BC-S venom	90.7 ± 10.9	70.7 ± 21.6	74.0 ± 21.5	79.3 ± 15.1*	76.5 ± 10.8

All values are shown as mean ± SD; n = 4 rabbits in each group. Control group and Pre-treatment period = no venom injection. Mean values within the row indicated with superscripts are significantly different from pre-treatment by Bonferroni *t* - test (* $p < 0.05$). HR = Heart rate; RR = Respiratory rate; PP = Pulse pressure; MAP = Mean arterial pressure.

Table 7-3 Effect of intravenous injection of *Bungarus candidus* venom (50 μg/kg) on cardiovascular functions and respiration during 150 minutes after envenomation in rabbits.

Parameters	Group	Pre-treatment	Post-treatment (minutes)				
			30	60	90	120	150
HR	Control	235 ± 14.6	235 ± 17.3	235 ± 13.3	234 ± 12.0	234 ± 12.9	233 ± 11.4
(beats / min)	BC-NE venom	208 ± 9.0	180 ± 14.7*	174 ± 17.7*	162 ± 12.9*	148 ± 5.7*	122 ± 17.2*
	BC-S venom	219 ± 7.7	206 ± 9.0	200 ± 11.4	186 ± 15.5	164 ± 35.1*	132 ± 32.1*
RR	Control	45 ± 10.4	50 ± 7.5	51 ± 6.0	50 ± 7.5	50 ± 7.5	49 ± 8.6
(breaths/min)	BC-NE venom	25 ± 2.9	28 ± 6.6	30 ± 10.6	21 ± 11.9	14 ± 5.9	9 ± 1.3 *
	BC-S venom	33 ± 6.0	42 ± 7.1	44 ± 13.3	31 ± 21.0	13 ± 4.1	10 ± 5.4 *
PP	Control	25.0 ± 8.2	29.0 ± 9.0	28.3 ± 17.3	28.8 ± 14.4	28.0 ± 17.8	25.5 ± 17.2
(mmHg)	BC-NE venom	28.1 ± 2.4	26.3 ± 3.2	29.4 ± 3.2	29.4 ± 8.3	23.7 ± 9.7	23.1 ± 6.6
	BC-S venom	37.5 ± 10.4	39.4 ± 18.3	38.1 ± 16.1	36.3 ± 17.9	25.0 ± 4.6	29.4 ± 9.7
MAP	Control	92.0 ± 6.9	90.7 ± 13.4	89.5 ± 14.6	86.5 ± 14.9	85.2 ± 15.5	86.7 ± 15.4
(mmHg)	BC-NE venom	105.1 ± 10.1	86.8 ± 19.0	83.5 ± 27.0	87.9 ± 22.4	96.0 ± 23.0	100.2 ± 28.1
	BC-S venom	90.7 ± 10.9	78.7 ± 10.3	77.2 ± 10.3	78.4 ± 7.1	75.2 ± 22.1	75.5 ± 31.6
PCV	Control	36.7 ± 1.5	36.5 ± 1.3	35.5 ± 1.3	35.3 ± 1.7	34.5 ± 1.3	34.3± 1.5
(%)	BC-NE venom	35.5 ± 0.6	35.3 ± 1.0	34.5 ± 0.6	34.0 ± 0.8 *	33.8 ± 0.5 *	33.0 ± 0.8 *
	BC-S venom	35.8 ± 0.5	35.3 ± 1.0	33.8 ± 0.9 *	33.3 ± 0.50 *	32.8 ± 0.5 *	32.5 ± 0.6 *

All values are shown as mean ± SD; n = 4 rabbits in each group. Control group and Pre-treatment period = no venom injection. Mean values within the row indicated with superscripts are significantly different from pre-treatment by Bonferroni *t* - test (* $p < 0.05$). HR = Heart rate; RR = Respiratory rate; PP = Pulse pressure; MAP = Mean arterial pressure**;** PCV = Packed cell volume.

Table 7-4 Effects of intravenous injection of *Bungarus candidus* venom (50 μg/kg) on cardiovascular functions during 150 minutes after envenomation in rabbits.

Parameters	Group	Pre-treatment	Post-treatment (minutes)				
			30	60	90	120	150
CO	Control	121.7 ± 10.3	121.3 ± 20.3	122.3 ± 21.9	ND	124.75 ± 13.3	ND
(ml / min / kg)	BC-NE venom	118.2 ± 15.3	100.1 ± 25.0	76.1 ± 24.8	ND	74.00 ± 27.0	ND
	BC-S venom	103.2 ± 31.9	82.8 ± 32.4	72.7 ± 23.2	ND	55.40 ± 4.1	ND
TPR	Control	0.76 ± 0.11	0.73 ± 0.23	0.76 ± 0.24	ND	0.70 ± 0.21	ND
(mmHg/ml/min/kg)	BC-NE venom	0.90 ± 0.15	0.88 ± 0.24	1.13 ± 0.38	ND	1.34 ± 0.23	ND
	BC-S venom	0.97 ± 0.33	1.11 ± 0.37	1.21 ± 0.37	ND	1.64 ± 0.16	ND
SV	Control	0.52 ± 0.05	0.52 ± 0.15	0.52 ± 0.09	ND	0.54 ± 0.07	ND
(ml/beat/kg)	BC-NE venom	0.57 ± 0.09	0.54 ± 0.15	0.43 ± 0.11	ND	0.51 ± 0.18	ND
	BC-S venom	0.48 ± 0.25	0.40 ± 0.18	0.36 ± 0.13	ND	0.41 ± 0.15	ND
LVW	Control	160.27 ± 11.84	147.04 ± 8.03	154.80 ± 21.92	ND	150.59 ± 12.61	ND
(kgm / min)	BC-NE venom	178.85 ± 35.32	127.86 ± 56.24	96.05 ± 51.86	ND	105.50 ± 57.59	ND
	BC-S venom	136.90 ± 36.26	98.86 ± 36.77	85.46 ± 25.36	ND	72.15 ± 3.49	ND

All values are shown as mean ± SD; n = 4 rabbits in each group. Control group and Pre-treatment period = no venom injection. Mean values within the row indicated with superscripts are significantly different from pre-treatment by Bonferroni *t* - test (* $p < 0.05$). ND = Not Determined. CO = Cardiac output; TPR = Total peripheral resistance; SV = Stroke volume; LVW = Left ventricular work.

Table 7-5 Effects of intravenous injection of *Bungarus candidus* venom (50 µg/kg) on renal hemodynamics in rabbits.

Parameters	Group	Pre-treatment	Post-treatment (minutes)				
			30	60	90	120	150
Urine flow	Control	0.36 ± 0.21	0.33 ± 0.21	0.29 ± 0.20	0.30 ± 0.20	0.28 ± 0.15	0.29 ± 0.21
(ml / min)	BC-NE	0.31 ± 0.12	0.22 ± 0.15	0.27 ± 0.19	0.29 ± 0.18	0.27 ± 0.15	0.15 ± 0.12
	BC-S venom	0.41 ± 0.26	0.24 ± 0.18	0.23 ± 0.15	0.24 ± 0.19	0.19 ± 0.15	0.14 ± 0.12
GFR	Control	2.87 ± 0.83	2.81 ± 0.85	2.50 ± 0.97	2.70 ± 0.86	2.65 ± 0.81	2.71 ± 0.61
(ml / min / kg)	BC-NE	3.26 ± 0.82	2.39 ± 0.64	2.17 ± 0.40	2.15 ± 0.17	2.09 ± 0.37*	1.23 ± 0.70 *
	BC-S venom	2.78 ± 0.66	1.75 ± 0.61	2.02 ± 0.18	1.88 ± 0.57	1.60 ± 0.74	1.34 ± 0.70 *
ERPF	Control	12.17 ± 3.42	10.50 ± 1.11	9.79 ± 0.50	11.25 ± 3.32	10.47 ± 4.47	11.13 ± 3.33
(ml / min / kg)	BC-NE	13.98 ± 4.11	11.40 ± 2.99	7.94 ± 3.44*	6.84 ± 1.38*	5.86 ± 0.99*	3.11 ± 2.20 *
	BC-S venom	11.91 ± 4.95	6.60 ± 3.12	7.83 ± 2.78	6.58 ± 3.60*	4.98 ± 3.65	4.78 ± 4.09
ERBF	Control	19.26 ± 5.38	16.59 ± 1.95	15.19 ± 0.92	17.47 ± 5.52	16.02 ± 6.96	17.02 ± 5.47
(ml / min / kg)	BC-NE	21.69 ± 6.45	17.69 ± 4.71	12.10 ± 5.18*	10.39 ± 2.14*	8.85 ± 1.54*	4.65 ± 3.29 *
	BC-S venom	18.52 ± 7.69	10.20 ± 4.84	11.79 ± 4.05	9.84 ± 5.38	7.42 ± 5.46	7.10 ± 6.11
FF	Control	25.31± 5.88	26.82 ± 8.16	25.34 ± 8.97	23.92 ± 0.98	26.43 ± 6.65	24.97 ± 5.36
(%)	BC-NE	23.81 ± 3.09	21.16 ± 4.07	29.77 ± 8.70	32.10 ± 5.70	35.74 ± 2.44	45.65 ± 11.97*
	BC-S venom	26.14 ± 11.22	28.95 ± 10.86	28.30 ± 9.59	32.43 ± 11.16	38.41 ± 16.16	36.74 ± 18.14
RF	Control	16.09 ± 5.65	13.96 ± 2.90	12.83 ± 3.03	ND	13.94 ± 6.19	ND
(%)	BC-NE	18.72 ± 6.14	15.74 ± 1.70	15.82 ± 3.21	ND	9.67 ± 6.05	ND
	BC-S venom	21.43 ± 16.74	16.32 ± 10.34	15.33 ± 7.17	ND	14.13 ± 9.70	ND
RVR	Control	5.01 ± 1.16	5.20 ± 0.82	5.87 ± 0.66	5.20 ± 1.13	5.89 ± 1.96	5.25 ± 0.66
(mmHg/ml/min/kg)	BC-NE	5.39 ± 2.51	5.13 ± 1.59	7.48 ± 3.29	8.49 ± 1.86	10.80 ± 1.58	32.40 ± 20.54
	BC-S venom	5.50 ± 2.12	9.11 ± 4.38	7.33 ± 3.08	9.73 ± 4.22*	13.53 ± 6.87	14.26 ± 6.37

All values are shown as mean ± SD; n = 4 rabbits in each group. Control group and Pre-treatment period = no venom injection. Mean values within the row indicated with superscripts are significantly different from pre-treatment by Bonferroni *t* - test (* $p < 0.05$). ND = Not Determined. GFR = Glomerular filtration rate; ERPF = Effective renal plasma flow; ERBF = Effective renal blood flow; FF = Filtration fraction; RF = Renal fraction; RVR = Renal vascular resistance.

Table 7-6 Effects of intravenous injection of *Bungarus candidus* venom (50 μg/kg) on plasma and urinary electrolytes in rabbits.

Parameters	Group	Pre-treatment	Post-treatment (minutes)				
			30	60	90	120	150
P_{Na+} (mEq / L)	Control	137.25 ± 6.08	136.00 ± 5.29	136.50 ± 7.33	137.00 ± 2.71	136.25 ± 8.99	135.50 ± 1.91
	BC-NE venom	140.25 ± 2.99	141.50 ± 3.70	141.25 ± 3.86	141.50 ± 0.58	139.75 ± 6.24	141.00 ± 6.00
	BC-S venom	145.25 ± 2.06	135.50 ± 4.51	135.25 ± 1.89	142.75 ± 4.57	139.50 ± 5.74	144.25 ± 4.27
E_{Na+} (μEq / min)	Control	60.52 ± 28.30	55.85 ± 30.26	43.40 ± 28.14	42.99 ± 29.98	41.34 ± 25.90	43.12 ± 32.30
	BC-NE venom	51.68 ± 14.04	37.34 ± 24.10	38.96 ± 24.17	40.67 ± 22.66	41.95 ± 24.71	23.07 ± 17.66
	BC-S venom	60.51 ± 33.32	35.71 ± 28.06	31.47 ± 21.31	34.70 ± 27.32	27.85 ± 22.14	19.84 ± 15.88
FE_{Na+} (%)	Control	7.06 ± 3.19	4.61 ± 2.86	5.56 ± 2.78	4.84 ± 2.97	5.04 ± 3.46	3.96 ± 2.46
	BC-NE venom	8.26 ± 3.35	7.31 ± 4.47	8.12 ± 4.36	8.85 ± 4.76	9.27 ± 5.29	8.18 ± 5.29
	BC-S venom	10.34 ± 5.51	9.85 ± 7.22	8.11 ± 5.46	9.20 ± 8.05	8.39 ± 6.90	7.15 ± 5.72
P_{K+} (mEq / L)	Control	2.33 ± 0.10	2.33 ± 0.06	2.48 ± 0.22	2.33 ± 0.10	2.33 ± 0.24	2.43 ± 0.10
	BC-NE venom	2.40 ± 0.41	2.75 ± 0.25	2.68 ± 0.22	2.65 ± 0.31	3.23 ± 0.67	3.28 ± 0.29
	BC-S venom	2.35 ± 0.13	2.78 ± 0.47	2.63 ± 0.46	2.45 ± 0.47	3.03 ± 1.20	2.88 ± 0.57
E_{K+} (μEq / min)	Control	5.58 ± 2.81	5.76 ± 2.85	4.73 ± 3.58	4.94 ± 2.70	4.79 ± 2.31	5.01 ± 3.29
	BC-NE venom	2.73 ± 0.76	2.89 ± 1.29	2.53 ± 0.59	2.15 ± 0.60	2.35 ± 0.82	1.53 ± 1.01
	BC-S venom	3.16 ± 1.38	2.05 ± 0.86	2.50 ± 0.77	2.20 ± 0.66	2.06 ± 1.11	1.94 ± 1.45
FE_{K+} (%)	Control	37.40 ± 13.48	31.43 ± 20.43	32.10 ± 12.03	29.82 ± 14.23	27.61 ± 11.02	24.14 ± 11.32
	BC-NE venom	24.34 ± 5.72	28.49 ± 5.82	29.70 ± 5.89	25.32 ± 4.71	24.13 ± 8.07	24.58 ± 5.97
	BC-S venom	35.88 ± 17.51	29.32 ± 8.44	35.53 ± 18.22	34.70 ± 8.68	32.38 ± 13.81	33.05 ± 11.24

All values are shown as mean ± SD; n = 4 rabbits in each group. Control group and Pre-treatment period = no venom injection. Mean values within the row indicated with superscripts are significantly different from pre-treatment by Bonferroni *t* - test (* $p < 0.05$). E = urinary excretion; FE = fractional excretion; Na^+ = Sodium; K^+ = Potassium.

Table 7-7 Effects of intravenous injection of *Bungarus candidus* venom (50 μg/kg) on plasma and urinary electrolytes, osmolar and free water clearance in rabbits.

Parameters	Group	Pre-treatment	Post-treatment (minutes)				
			30	60	90	120	150
P_{Cl}- (mEq / L)	Control	101.50 ± 3.87	105.00 ± 2.65	103.25 ± 4.11	101.00 ± 3.56	103.50 ± 3.32	102.50 ± 2.65
	BC-NE venom	103.75 ± 2.63	107.25 ± 3.50	106.25 ± 1.89	108.50 ± 3.11	104.50 ± 3.51	105.25 ± 3.77
	BC-S venom	101.33 ± 5.69	97.00 ± 9.54	103.00 ± 3.61	100.67 ± 11.93	107.33 ± 6.66	106.67 ± 6.03
E_{Cl}- (μEq / min)	Control	58.67 ± 26.80	56.81 ± 30.88	40.96 ± 30.04	43.05 ± 30.93	41.45 ± 27.59	40.87 ± 32.10
	BC-NE venom	41.09 ± 13.16	27.36 ± 18.13	34.01 ± 21.76	32.57 ± 20.69	32.97 ± 20.19	21.42 ± 7.01
	BC-S venom	69.21 ± 10.25	39.20 ± 20.74*	35.57 ± 12.94*	37.63 ± 14.25*	28.72 ± 15.10*	19.84 ± 10.57*
FE_{Cl}- (%)	Control	9.14 ± 3.73 [a]	6.12 ± 3.94	7.36 ± 3.51	6.61 ± 4.45	6.69 ± 4.89	4.97 ± 3.30
	BC-NE venom	8.87 ± 3.84 [b]	8.66 ± 4.89	9.25 ± 5.25	9.17 ± 5.67	9.90 ± 6.39	9.71 ± 2.75
	BC-S venom	16.52 ± 1.70 [b]	14.45 ± 7.28	12.27 ± 5.36	14.06 ± 7.36	11.30 ± 7.77	9.70 ± 6.81
Uosm (mOsm)	Control	606.00 ± 213.39	533.75 ±	523.75 ± 165.87	490.25 ± 121.34	500.25 ± 139.25	511.75 ± 114.95
	BC-NE venom	514.25 ± 167.02	603.00 ±	586.50 ± 237.51	469.25 ± 97.37	468.50 ± 85.57	508.25 ± 74.89
	BC-S venom	512.50 ± 325.33	496.75 ±	517.00 ± 238.73	536.25 ± 257.62	568.75 ± 307.24	609.00 ± 337.00
Posm (mOsm)	Control	291.00 ± 3.92	292.67 ± 5.77	290.50 ± 6.86	291.00 ± 5.94	288.25 ± 5.56	289.50 ± 5.74
	BC-NE venom	286.25 ± 13.72	291.25 ± 5.25	304.50 ± 20.95	311.75 ± 20.60	299.75 ± 20.65	298.75 ± 7.18
	BC-S venom	293.25 ± 9.11	298.25 ± 7.09	297.00 ± 7.83	299.75 ± 4.27	299.75 ± 9.11	300.75 ± 11.09
Cosm (ml / min))	Control	0.69 ± 0.29	0.53 ± 0.40	0.49 ± 0.35	0.49 ± 0.35	0.46 ± 0.26	0.49 ± 0.35
	BC-NE venom	0.51 ± 0.12	0.37 ± 0.19	0.42 ± 0.17	0.39 ± 0.17	0.40 ± 0.19	0.24 ± 0.16
	BC-S venom	0.53 ± 0.23	0.33 ± 0.23	0.33 ± 0.18	0.31 ± 0.22	0.26 ± 0.18	0.20 ± 0.14
C_{H2O} (ml / min)	Control	-0.33 ± 0.14	-0.23 ± 0.17	-0.20 ± 0.16	-0.19 ± 0.16	-0.18 ± 0.13	-0.20 ± 0.15
	BC-NE venom	-0.20 ± 0.13	-0.16 ± 0.06	-0.14 ± 0.09	-0.10 ± 0.08	-0.12 ± 0.07	-0.09 ± 0.05
	BC-S venom	-0.13 ± 0.15	-0.09 ± 0.08	-0.08 ± 0.02	-0.08 ± 0.04	-0.07 ± 0.04	-0.07 ± 0.03

All values are shown as mean ± SD; n = 4 rabbits in each group. Control group and Pre-treatment period = no venom injection. Mean values within the row indicated with superscripts are significantly different from pre-treatment by Bonferroni *t* - test (*$p < 0.05$). E = urinary excretion; FE = fractional excretion; Cl^- = Chloride; Uosm = urine osmolality; Posm = plasma osmolality; Cosm = osmolal clearance; C_{H2O} = water clearance.

Table 7-8 Effects of intravenous injection of *Bungarus candidus* venom (50 μg/kg) on plasma concentrations of creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and calcium ion in rabbits.

Parameters	Pre-treatment	Post-treatment (minutes)			
		30	60	90	120
CPK (U/L) (n = 4)	272.25 ± 56.65	464.50 ± 198.14	445.25 ±168.97	382.75 ± 160.00	344.00 ± 195.75
LDH (U/L) (n = 4)	124.75 ± 83.62	159.25 ± 94.56	172.25 ±108.16	135.75 ± 68.01	149.25 ± 73.99
Calcium (mg %) (n = 8)	8.7 ± 1.1	8.4 ± 1.0	8.0 ± 1.3	7.5 ± 1.5	6.5 ± 1.4*

All values are shown as mean ± SD; Comparison of the mean values between pre- and post -treatment using Bonferroni *t* - test with superscripts are significantly different (*$p < 0.05$).

CHAPTER VIII

THE TOXINOKINETICS OF *BUNGARUS CANDIDUS* VENOM IN RABBITS

INTRODUCTION

Bites by kraits, such as *Bungarus candidus*, produce minimal local effects when compared with the cobra or viper bites (Looareesuwan et al., 1988; Ismail et al., 1996). The clinical signs with neurotoxicity after envenomation with *B. candidus* appear within 30-60 minutes or prolonging for some hours. The onset of respiratory paralysis has been reported to be 2-8 hours with the delay to 20 hours (Pochanugool, 1997; Laothong and Sitprija, 2001; Leeprasert and Kaojarern, 2007). Systemic envenomation may recur within hours or days after an initially response to antivenom therapeutics. The patients received specific *B. candidus* antivenom at 10-12 hours after envenoming developed rapid improvement of neurotoxic signs and symptoms within 10 hours-2 days, but persistent mydriasis and fixed pupils were remained in some patients for many days (Leeprasert and Kaojarern, 2007). The possible explanation for the variation in the onset, development and duration of toxicity of *B. candidus* envenoming might reflect to the differences in the amount of venom inoculated when bitten, absorption, distribution, tissue binding and elimination of venoms or some venom compositions. Few data are available on the study of venom toxinokinetics in experimental animals, although the *B. candidus* venom has been mainly studied on its compositions, sequence of amino acid, toxic and pharmacological properties (Abe et al., 1977; Tan et al., 1989; Chu et al., 1995; Dixon and Harris, 1999; Kuhn et al., 2000; Torres et al., 2001; Tsai et al., 2002; Khow et al., 2003; Kuch et al., 2003). Information of the toxinokinetics of *B. candidus* venom would give a better understanding of its envenoming syndrome in human. Therefore, the purpose of this study was to determine the disposition kinetic variable of *B. candidus* venom and its urinary excretion in urine following intravenous envenomation in rabbits. From the disposition kinetic data, the information may contribute to better insight into the patho-physiological basis after envenomation by *B. candidus* bite.

MATERIALS AND METHODS

Animal preparation

Ten healthy rabbits, weighing between 2.5-3 kg, were divided into two groups of five animals each. Animals in both groups were anesthetized with pentobarbital sodium (25 mg/kg body weight). Before the start of experiment, two catheters were inserted into the carotid artery and the jugular vein using polyethylene catheters. The urine was collected from the polyvinyl catheter which was catheterized into the ureter.

Experimental and assay procedures

Animals in group 1 and group 2 were injected intravenously with *B. candidus* venom at the dose of 50 and 150 μg/kg, respectively. Following the envenomation, blood samples (1 ml) were withdrawn from the carotid artery into heparinized tubes before and at 5, 10, 20, 30, 60, 90, 120 and 150 min. after envenomation of *B. candidus* venom. Plasma was collected after centrifugation at 2500 rpm for 25 min. and kept at -20^0C until analysis. The urine samples were collected before and at 5, 10, 20, 30, 60 and 120 min. after envenomation. The volume of urine was measured and approximately 2 ml was frozen for analysis.

Analytical procedure

Blood and urine samples were determined for the concentration of the *B. candidus* venom by enzyme linked immunosorbent assay (ELISA).

Standardization of the ELISA for the quantification of B. candidus venom

The intra- and inter-assay coefficients of variation (CV) were calculated from data obtained in independent assays. The coefficient of determination (r^2) was used to analyze the correlation between venom concentrations and absorbance in ELISA standard curves.

Determination of venom kinetic parameters

In the present experiment which *B. candidus* crude venom was injected intravenously into rabbits at the dose of 50 and 150 μg/kg. It was assumed that venom was distributed between two pools, the plasma pool and another less well-defined pool. With two-pool model, the equation describing the kinetics of venom in one of

those pools will be made up of two phases. However these two phases do not directly equate to the two pools since only the plasma pool was sampled. Therefore the parameters and equations defined in this study refer to venom kinetics in the plasma pool. Plasma venom concentration at specified time was determined by ELISA. Then the values of venom appearance (ng/ml) with time-course (minute) for each individual animal were plotted on a semi logarithmic graph paper. The log concentration-time curves obtained were biphasic, comprising a rapid initial declining phase (α) followed by a slower declining phase (β). A bi-exponential equation of this two compartment open model was fitted to each experimental data set, using the following equation:

$$Ct = Ae^{-\alpha t} + Be^{-\beta t}$$

Ct is the concentration of the venom in plasma at time *t*; *α* and *β* are distribution and elimination rate constants, respectively; *A* and *B* are the zero intercepts of the fast (distribution) and slow (elimination) phases, respectively. Venom kinetic parameters were calculated following the formulas outlined below (Krifi et al., 2005):

Area under the curve from zero to infinity: AUC = $A/\alpha + B/\beta$

Area under the mean curve from zero to infinity: AUMC = $A/\alpha^2 + B/\beta^2$

Total plasma clearance: $CL_T = D/AUC$, where *D* being the total injected venom.

Volume of the distribution at steady state: $Vd_{ss} = (D \times AUMC) / AUC^2$

Mean residence time: MRT = $AUMC / AUC$

Half-life of each compartment ($T_{1/2}$) were estimated graphically from the slopes of the least-square lines, then the respective rate constant *(α* and *β)* is 0.693 divided by $T_{1/2}$.

Statistical analysis

All results are expressed as mean ± standard deviation. Statistical significance was analyzed by unpaired *t*-test; the level of significance was set as $p < 0.05$.

RESULTS

Standardization of the ELISA for the quantification of Bungarus candidus venom

The standard curve of *B. candidus* venom in buffer used to quantify venom in plasma and urine is shown in Figure 8-1. The coefficient of determination (r^2) was 0.9951. In present assay, the parameters showed accuracy >90% and coefficients of variation <10% (Intra-CV = 1.2% and Inter-CV = 3.5%).

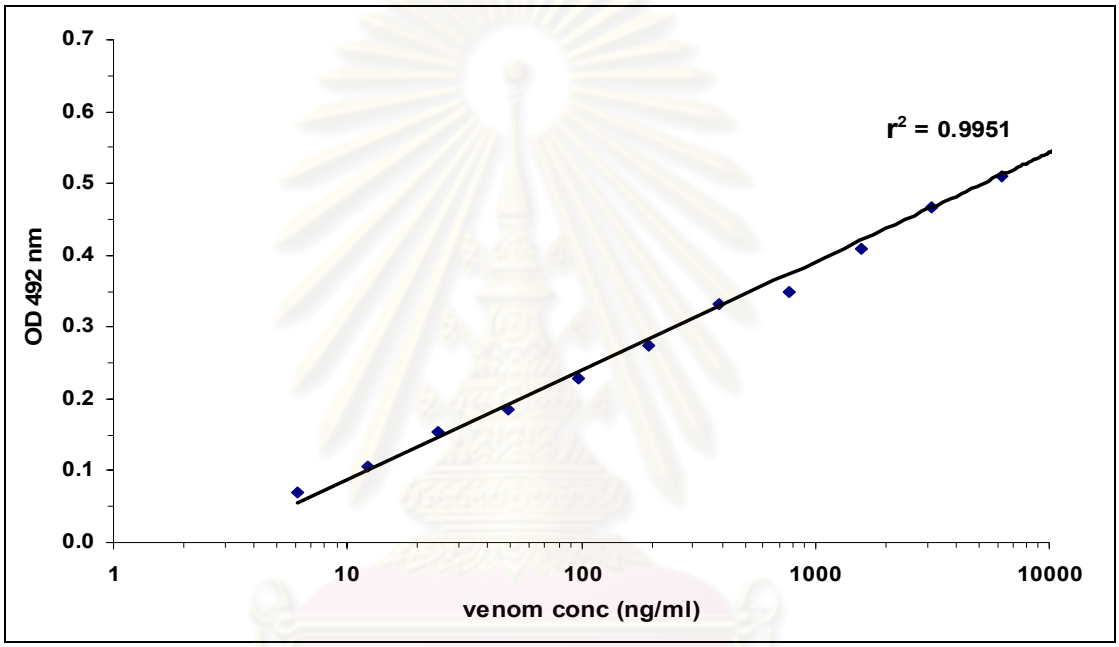


Figure 8-1 Standard curve for ELISA used to detect *Bungarus candidus* venom in plasma and urine samples. The points are mean ± SD (n = 3).

The kinetics of Bungarus candidus venom

The concentrations of *B. candidus* venom in plasma samples were determined through time by ELISA. After intravenous injection of *B. candidus* venom (50 and 150 μg/kg), the kinetic profile of venom are shown in Figure 8-2. The bicompartmental kinetics with a rapid initial decrease (α phase) corresponding to venom distribution, followed by a slow linear decrease (β phase) corresponding to venom elimination were apparent. The kinetic parameters for *B. candidus* venom in the plasma pool of envenomed rabbits are shown in Table 8-1. The kinetic parameters between two groups two groups of different doses of venom showed no significantly

differences in the rate constants of α phase (8.56 ± 1.06 and 8.47 ± 0.43 min^{-1}) and of β phase (0.40 ± 0.05 and 0.40 ± 0.06 min^{-1}); the half-life of the distribution phase ($T_{1/2\alpha}$ = 8.20 ± 1.10 and 8.20 ± 0.45 min) and the elimination phase ($T_{1/2\beta}$ = 175.00 ± 19.86 and 176.00 ± 26.79 min); the volume of distribution of α phase (Vα = 1.53 ± 0.42 and 1.40 ± 0.27 L/kg), of β phase (Vβ = 2.37 ± 0.89 and 2.77 ± 0.77 L/kg) and the volume of distribution at steady state (Vd_{ss} = 5.47 ± 1.78 and 6.31 ± 1.04 L/kg); the mean residence time of of α phase (MRTα = 11.83 ± 1.58 and 11.83 ± 0.65 min), of β phase (MRTβ = 252.53 ± 28.66 and 253.97 ± 38.65 min) and of plasma pool (MRTp = 236.32 ± 29.75 and 233.40 ± 40.92 min); total body clearance (TBC = 9.00 ± 4.21 and 10.24 ± 3.93 ml/min/kg). The significant differences revealed in the values of A (36.20 ± 11.71 and 110.00 ± 28.28 ng/ml), B (24.20 ± 8.41 and 56.40 ± 14.57 ng/ml) and the area under the curve (AUC = 6663.78 ± 2736.15 and 15763.35 ± 4473.10 ng/ml/min). The *B. candidus* venom was detected in urine at the given times between 5 – 120 min with the venom excretion rate as shown in Table 8-2.

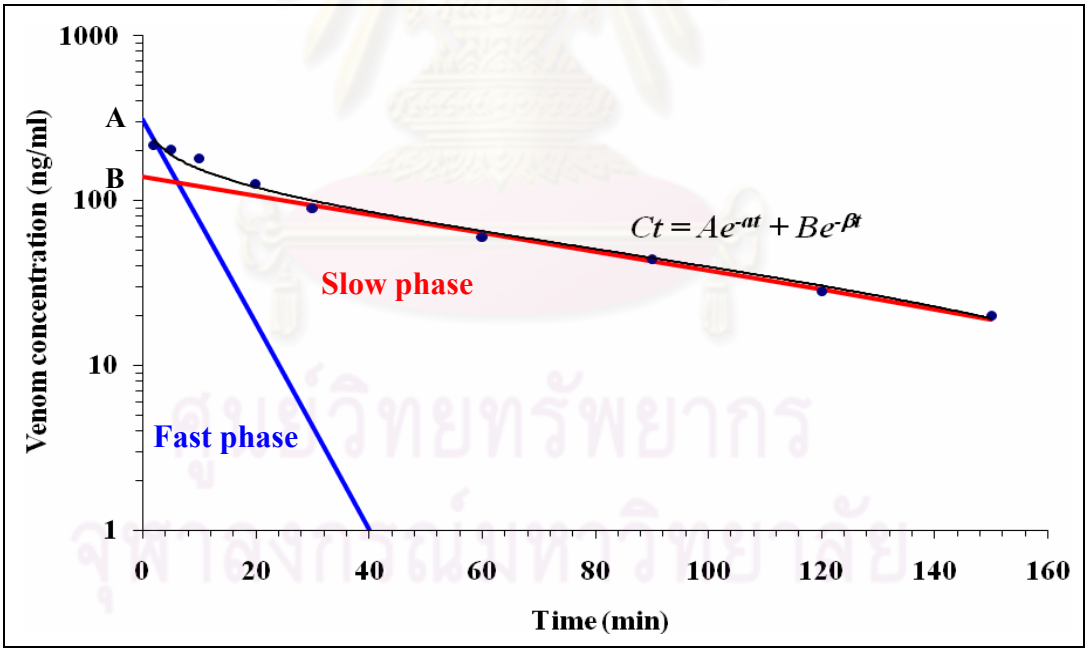


Figure 8-2 A semilogarithmic plot of levels of venom after single intravenous doses with 50 µg/kg and 150 µg/kg of *B. candidus* venom in rabbits. Data were analyzed according to two compartment open model represented by the equation $Ct = Ae^{-\alpha t} + Be^{-\beta t}$. A and B are the zero-intercepts of the fast (distribution) and slow (elimination) phases. The calculation points (•) of the distribution phase are obtained by feathering techniques.

Table 8-1 Kinetic parameters of *Bungarus candidus* venom in rabbits in which venoms were administered in a single intravenous dose of 50 μg/kg and 150 μg/kg.

	Venom dose (μg / kg)	
Kinetic parameters	50 μg / kg	150 μg / kg
A (ng/ml)	36.20 ± 11.71*	110.00 ± 28.28*
B (ng/ml)	24.20 ± 8.41*	56.40 ± 14.57*
α (min^{-1}) $x10^2$	8.56 ± 1.06	8.47 ± 0.43
β (min^{-1}) $x10^2$	0.40 ± 0.05	0.40 ± 0.06
$T_{1/2\alpha}$ (min)	8.20 ± 1.10	8.20 ± 0.45
$T_{1/2\beta}$ (min)	175.00 ± 19.86	176.00 ± 26.79
Vα (L/kg)	1.53 ± 0.42	1.40 ± 0.27
Vβ (L/kg)	2.37 ± 0.89	2.77 ± 0.77
Vd_{ss} (L/kg)	5.47 ± 1.78	6.31 ± 1.04
MRTα (min)	11.83 ± 1.58	11.83 ± 0.65
MRTβ (min)	252.53 ± 28.66	253.97 ± 38.65
MRTp (min)	236.32 ± 29.75	233.40 ± 40.92
AUC (ng/ml/min)	6663.78 ± 2736.15*	15763.35 ± 4473.10*
TBC (ml/min/kg)	9.00 ± 4.21	10.24 ± 3.93

All values are mean ± SD (n = 5 rabbits each). * $p < 0.05$, significantly difference between groups by unpaired *t*-test.; A and B = venom concentrations at the zero time intercepts of the α and β phases; α and β = the rate constants for the fast (distribution) and slow (elimination) phases, respectively. $T_{1/2\alpha}$ and $T_{1/2\beta}$ = the elimination half-lives for the α and β phases; Vd_{ss} = estimated volume of distribution at steady state; MRTα, MRTβ and MRTp = mean residence time of the α, β phases and plasma pool, respectively; AUC = area under the curve; TBC = total body clearance.

Table 8-2 The rate of excretion of *Bungarus candidus* venom (ng/min/kg) into the urine in envenomed rabbits. The values are shown as mean ± SD.

Venom dose (μg/kg)	Time after venom injection (min)						
	5	10	20	30	60	90	120
50 (n = 5)	0.40±0.35	0.66±0.40	1.21±0.71	0.91±0.50	1.76±1.03	1.22±1.05	0.73±0.53
150 (n = 3)	0.86±0.38	3.52±1.11	3.44±1.46	2.90±1.38	1.37±0.66	0.65±0.34	0.44±0.19

DISCUSSION

In the present study, the parameters and equations defined after intravenous injection of two different doses of *B. candidus* crude venom into rabbits (50 and 150 μg/kg) were assumed to venom kinetics in the plasma pool since only the venom concentration in the plasma samples were analyzed by the ELISA determination. Evaluation of the mean plasma concentration of *B. candidus* venom as a function of time of organ regulation in rabbits revealed biphasic decline of plasma venom concentration, which were fitted in two compartment open model. The disposition of *B. candidus* venom was adequately described by a bi-exponential equation, $C = 36.2e^{-8.56t} + 24.2e^{-0.40t}$ and $C = 110.0e^{-8.47t} + 56.4e^{-0.40t}$ for the venom doses 50 and 150 μg/kg, respectively. Other venoms, after intravenous envenomation has also reported to follow two compartment model in mice (Rocha et al., 2008), rat (Mello et al., 2010) and rabbit (Nakamura et al., 1995; Krifi et al, 2005). The similar low values of distribution rate constant (α) of two doses of *B. candidus* venom (8.56 ± 1.06 and 8.47 ± 0.43 min^{-1}) and volume of distribution (5.47 ± 1.78 and 6.31 ± 1.04 L/kg) indicated that the venom is slowly distributed into various body fluids and tissues. The elimination haft-life of the *B. candidus* venom was averaged 175 min which was considerably higher than that study in habutobin toxin (50.42 ± 7.89 min) in the venom of *Trimeresurus flavoviridis* (Nakamura et al., 1995). The present findings revealed marked differences in kinetic behavior of the venom as compared to other Elapid venoms such as *Naja melanoleuca, N. nivea, N. nigricollis, N. haji and*

Walterinnesia aegyptia venoms, which fitted a tri-exponential equation characteristic of a three-compartment open pharmacokinetic model after intravenous administration of venom in rabbits (Ismail et al., 1996; Ismail, Abd-Elsalam, and Al-Ahaidib, 1998). The overall elimination half-life of the venoms of these *Naja* species ranged from 15 to 29 hrs and of *W. aegyptia* was 10.8 ± 1 hrs., indicating slower elimination from the body than that of *B. candidus* venom. In accordance to the present findings, the value of the total plasma clearance of *B. candidus* venom (9.00 ± 4.21 to 10.24 ± 3.93 ml/min/kg) was higher as compared to other types of venom like *Naja* (0.09–0.18 ml/min/kg) and *W. aegyptia* (0.686 ml/min/kg) venoms. The urinary excretion during 150 min of experiment was 0.0008–0.0035% and 0.0003–0.0024% of total envenomed dose at 50 and 150 μg/kg, respectively, which was different from that reported in *Bothrops alternatus* venom experimented in rats detected in urine at 3 – 24 hrs post envenomation (Mello et al., 2010). Excretion via the renal system is the major route to eliminate the venoms or toxins (Mebs, 1978). A number of studies have reported the detection of venom or its degraded products in the urine of envenomed human and experimented animals (Hanvivatvong et al., 1987; Audebert et al., 1993 & 1994; Tanigawa et al., 1994; Mello et al., 2010). Only a few percent of venom was recovered in urine which less than 10% of the injected dose (Audebert et al., 1994; Tanigawa et al., 1994). However, the nephrotoxicity of snake venom is not always associated with its excretion via renal system (Mello et al., 2010).

Significant differences in the value of AUC and the estimated maximum concentration of venom at the zero time of α and β phases (A and B values) between two different doses of venom injection reflected to the different plasma levels of free venom antigens. Nevertheless, other kinetic parameters were shown to be the same with the two different venom doses. The toxinokinetic parameters of *B. candidus* venom indicate that the rapid distribution half-life ($T_{1/2\alpha}$ = 8.20 ± 1.10 and 8.20 ± 0.45 min) and the slow elimination half-life ($T_{1/2\beta}$ = 175.00 ± 19.86 and 176.00 ± 26.79 min) were concomitant with the great volume of distribution (5.47 ± 1.78 and 6.31 ± 1.04 L/kg) of *B. candidus* venom in the plasma pool of experimentally envenomed animals. These findings are probably consistent with the onset of clinical symptoms occurred in human envenomation, which the earliest systemic signs mainly observed upon arrival to the hospitals at 30 – 60 minutes after *B. candidus* bite (Pochanugool et

al., 1997; Laothong and Sitprija, 2001; Leeprasert and Kaojarern, 2007). It has been known that *B. candidus* venom have severe neurotoxic and systemic effects which involve the circulating of venom or other toxic components in blood stream as well as the specific tissue binding toxins especially at the neuromuscular junctions (Prasarnpun et al., 2005). The time interval of bite to death of envenoming bite by *B. candidus* in the previous report is fitted well to the mean residence time of free venom circulating in the plasma pool (MRTp) about 4 hours in the present study. Though the total body clearance of venom from the plasma pool were rapidly eliminated (9.00 ± 4.21 to 10.24 ± 3.93 ml/min/kg) which related to the recovery of venom in urine during 150 minutes of animal experiments. This is suggested that some venom might be eliminated by the renal route (Devaux et al., 2004).

ELISA quantification of *B. candidus* venom in the plasma samples is limited to detect only free venom antigens. Some of venom proteins binding to plasma proteins can mask antigenic determinants which are undetectable by ELISA determination (Rivière et al., 1997). On the other hand, some quantity of venom is distributed into the peripheral or extravascular compartments that can redistribute into the vascular space for instance. Thus the kinetic parameters in the present study are a prime value of toxinokinetic study of *B. candidus* venom in experimented animal that needs further study for a better understanding of its envenoming syndrome and to improve for a standard serum therapeutic protocol.

CHAPTER IX

GENERAL DISCUSSION

The results of initial experiments in Chapter IV have shown that *B. candidus* snakes from different localities of Thailand would be considered color variation of black and white bands in snakes from individual to individual. Though the color pattern is a non-specific variation in taxonomic study. The distinct color difference of *B. candidus* from northeastern and eastern Thailand hints to phylogenetic study. According to the venom collection using hematocrit tube attached directly to the snake fangs in this study, the liquid and dry venoms per snake were individually different and related the body weight of snakes (Figure 9-1). However the mean values of percentage of dry matter of venom or the percentage of venom yield per gram of snake body weight showed no differences among groups with narrow ranges. The percentage of dry matter of venom in this study is corresponded to the previous report that the extracted liquid venom yield was allometrically correlated with snake body mass and had mean moisture content of 70.9% (McCue, 2006). These values are useful for estimating the dosage range of antivenom treatment in case of *B. candidus* envenomation if the victim can take the snake to the physicians. The mean value of the percentage of venom yield per gram snake body weight could be indicated the approximate amount of venom injected into the victim although the snakes obtained to the hospital are varied in sizes or total lengths.

In the present study, determinations of individual *B. candidus* venom of the wild-caught and the captive-born groups were comparative studied in term of lethal toxicity, enzymatic activities, protein components, isolation and purification of the major neurotoxins (Chapter V). The present findings revealed no significant differences in lethal toxicity and the enzymatic activities of phospholipase A_2 (PLA_2), protease (PRO), phosphodiesterase (PDE) and hyaluronidase (HYA) among the wild-caught and the captive-born venom groups. In contrast to the enzymatic activities of acetylcholinesterase (AChE), alkaline phosphomonoesterase (PME), L-amino acid oxidase (LAAO) and hyaluronidase (HYA) presented the significant differences among snake venom groups. The PME activity of BC-NE venom group and the

LAAO activity of BC-S venom group were significantly higher than that of BC-CB venom group. On the other hand, the AChE activity of BC-CB venom group presented significantly higher than that of BC-E venom group. The captive-born snakes were born from the southern-origin parents and fed on mouse which differed from the wild-caught snakes that fed on the same non-venomous snakes as in the nature. Therefore, the differences in the activities of PME, LAAO and AChE between some of the wild-caught and the captive-born venom groups might be related to the food difference. Comparison of BC-S and BC-CB venom groups reveal no significant difference in all of enzymatic activities except for LAAO activity. This is postulated not only from food difference but also age-dependency of these two groups though ages of BC-S snake group were not mentioned (Figure 9-1).

The SDS-PAGE pattern under non-reducing conditions of individual *B. candidus* venom showed marked differences in the banding pattern suggesting the variation in venom protein composition. BC-S and BC-CB venom groups showed quantitative differences of protein composition at the molecular weight of 18.1–41.3 kDa. The venom protein composition of female in BC-S, BC-NE and BC-CB groups revealed no difference to male venom in the SDS-PAGE pattern and the profiles of RP-HPLC. However, a number of studies reported the controversial results in the variability of venom composition influenced by diet and sex differences (Daltry et al., 1996; 1997; Magro et al., 2001; Sanz et al., 2006; Zelanis et al., 2007). Ontogenetic shifts of diet in venomous snakes were associated with changes in venom compositions (López-Lozano et al., 2002; Mackessy, Williams, and Ashton, 2003; Saldarriaga et al., 2003).

Compositional differences between snake venoms analyzed by RP-HPLC can be employed as a taxonomy signature for unambiguous species identification independently of geographic origin and morphological characteristics (Tashima et al., 2008; Gutiérrez et al., 2009). In the present study, RP-HPLC was performed for protein detection in individual *B. candidus* venom. Thereafter the percentage of the total venom proteins of the different protein families were determined from the relation of the sum of the peak areas containing proteins from the same family to the total area of venom protein peaks in reverse phase chromatogram (Tashima et al., 2008; Calvete et al., 2009). The qualitative and quantitative differences were observed

among members of the same group. Since all the snakes were kept under the same conditions and fed with live snakes, except BC-CB snakes which were fed with mice. These subtle differences in RP-HPLC profiles were likely to be due to the genetic background. These findings are assumed for the correlation of lineage among snake groups distributed in different parts of Thailand where the geography is closely ranged such as between the northeastern and the eastern regions. Moreover the similarity of RP-HPLC profile of some BC snake in present study is corresponded to the study on venoms of the captive-bred Malayan pitviper that closely matched to those of the wild-caught snakes from the same locality as their parents. Although the captive Malayan pitviper were fed on mouse which is incongruous with the natural preys. This indicates that the dietary association is genetically based rather than environmentally induced for the short term in captivity (Daltry et al., 1997).

In vitro study for the effect of *B. candidus* venom on the osmotic fragility of rabbit red blood cells revealed that red blood cells in venom-treated heparinized blood were more susceptible to osmotic lyses than that of venom-treated EDTA blood (Chapter VI). It was also evident in the high value of packed cell volume coincided with high percentage of membrane destabilization in venom-treated heparinized blood, without the alteration in MCV obtained from venom-treated EDTA blood (Figure 9-1). It indicates that PLA activity in venom was not activated by calcium ion, which was chelated in venom-treated EDTA blood (Bournazos et al., 2008). Most of PLA_2 enzymes require Ca^{2+} for activation in the cell (Bonventre, 1990). On the other hand, EDTA is a reagent of choice as an anticoagulant using chelating metal ions e.g. Ca^{2+} including Mg^{2+} and Mn^{2+} ions (Kumar and Satchidanandam, 2000; Bournazos et al., 2008). The PLA_2 enzyme in *B. candidus* venom may play a role in hydrolysis of phospholipids in red blood cell membrane, resulting in lysophospholipids and free fatty acid which are the precursors of eicosanoids such as prostaglandins, thromboxanes, leukotrienes and lipoxins (Dunn and Broady, 2001). PLA_2 may somehow interact with the other venom proteins, such as cardiotoxin (Condrea et al. 1970), which also known as cytotoxins or membrane toxins. Snake venom cardiotoxins are generally regarded as low molecular weight polypeptides that cause cardiac arrest, muscle contracture, membrane depolarization and hemolysis (Chang, 1979; Condrea, 1979; Harvey et al., 1982; Fletcher et al., 1991). Small amount of

PLA_2 can enhance the ability of cardiotoxins to lyse red blood cells, but does not markedly increase the ability of cardiotoxins to depolarize and cause the contracture of skeletal muscle (Harvey et al., 1983). An increase in both MCV and packed cell volume in venom-treated blood revealed cytotoxicity of *B. candidus* venom.

The difference in the neurotoxicity of venom and its toxins are reported due to the use of different experimental conditions. Therefore *in vitro* neuromuscular preparations are recently used as the method of choices to determine the neurotoxic effect of crude venom and its isolated toxins (Harvey et al., 1994; Hodgson and Wikramaratana, 2002). In the present study, *in vitro* mouse phrenic nerve-diaphragm preparation showed significant difference in inhibited indirectly evoked twitches among the *B. candidus* venoms of three geographical variants and a captive-born snakes (Chapter VI) (Figure 9-1). The neurotoxic effect of *B. candidus* venom is reliably prevented either by pre-incubation with monovalent *B. candidus* antivenom or by the addition of antivenom promptly with the venom. It has been known that *B. candidus* venom has severe neurotoxic and systemic effects which involve the circulating of venom or other toxic components in blood stream as well as the specific tissue binding toxins especially at the neuromuscular junctions (Montecucco and Rossetto, 2000; Prasarnpun et al., 2005). If the antivenom given time was delayed about 20-30 min, the capacity of antivenom to neutralize the venom was inconsistency. β-Bungarotoxin (β-BuTx), the main constituent in the venoms of *Bungarus* snakes (Chu et al., 1995), is composed of chain A and chain B subunits linked by a disulfide bond. The chain A is a PLA_2 subunit exhibited remarkable structural similarity to other vertebrate enzymes, of which has the phospholipase A_2 activity to catalyze the calcium-dependent hydrolysis of the 2-acyl groups in 3-sn-phosphoglycerides. The chain B is a non-PLA_2 subunit which is homologous to proteinase inhibitors and dendrotoxins (Bon, 1997; Kini, 2003). In this respect, β-BuTx is a PLA_2 neurotoxin potentially in blocking nicotinic acetylcholine transmission at the presynaptically neuromuscular junction. However in the absence of detergents (such as deoxycholate), β-BuTx has negligible PLA_2 activity which almost no hemolytic activity by failure to digest phospholipids (Jeng, Hendon, and Fraenkel-Conrat, 1978).

In the present study of venom kinetics (Chapter VIII), the mean residence time of free venom (about 4 hours) circulating in the plasma pool is fitted well to the time interval of bite to death of envenoming bite by *B. candidus* in the previous report (Looareesuwan et al., 1988). The total body clearance of venom rapidly eliminated from the plasma pool was also related to the recovery of venom in urine within 150 min after envenomation. This is suggested that some venom might be eliminated by the renal route (Devaux et al., 2004). However the nephrotoxicity-affecting snakebite is not always associated with its venom excretion via renal system (Mello et al., 2010).

In vivo experiment of envenomed rabbits by *B. candidus* venom (Chapter VII), systemic hypotension was observed during 150 minutes of monitoring. *B. candidus* venom (50 μg/kg, i.v) produced concentration-dependent bi-phasic responses in MAP in anesthetized rabbits. This response consisted of an initial short-lasting depressor phase (2-5 minutes; phase 1) and a long-lasting hypotension phase (30-150 minutes; phase 2). Neither phase 1 nor phase 2 of the response to venom was affected by the muscarinic receptor antagonist, e.g. atropine sulphate (0.20 mg/kg, i.v.). Thus, the scavenge acetylcholine inducing hypotension by stimulating M1, M2 and M4 muscarinic receptors (Aird, 2002) was not apparent in *B. candidus* envenomation. The stepwise decreases in HR and CO, while the slightly increase in TPR was apparent, suggesting that *B. candidus* venom would contribute to vasoconstriction and an increase in systemic vascular resistance via an activation of sympathetic activity releasing catecholamine as a compensatory mechanism (Hall and Hodge, 1971). It was reported that the fall in BP and HR may be due to a direct inhibitory effect of the snake venom via the blood-brain barrier acting on the medullary cardiovascular center, and reducing the tonic discharge of impulses to the sympathetic vasoconstrictor fibers and to the cardioaccelerator nerves thus producing hypotension and bradycardia (Telang et al., 1976) (Figure 9-2). The persistent decreases in CO and HR despite hypotension may suggest direct cardiotoxicity of the venom. It has been reported that Bucain, a three-fingered α-neurotoxin in structure isolated from *B. candidus* venom, is similar to cardiotoxin (Watanabe et al., 2002; Murakami et al., 2009). The reduction of CO and BP may be related to enzymes in venom, such as kininogenases, phospholipases and hydrolases of arginine ester, which may involve in these processes. Hemodynamic side-effects such as vasodilatation with resultant

hypotension are due to the release of mediators including biological amines such as histamine, serotonin or prostaglandins. They might be expected to occur during envenomation. Release of histamine related to phospholipase concentrations has been noted (Hyslop and Nucci, 1993). PLA_2 enzyme in snake venom hydrolyses the cell membrane phospholipids resulting in lysophospholipids and free fatty acid which are the precursors of prostaglandins (Dunn and Broady, 2001). Prostaglandins generated in kidney have an important role in blood pressure regulation (Schlondorff and Aradaillou, 1986). In addition, PLA_2 is also a critical enzymatic effector of Ca^{2+} action in the cells. Ca^{2+} has been implicated as a regulatory factor in many physiological and pathophysiological processes in the renal cell. Most of the releasable cell Ca^{2+} resides in the non-mitochondrial compartments (Bonventre, 1990). Therefore the alterations in cardiovascular functions of these experimental envenomed animals may be an effect of some enzymes in *B. candidus* venom (Figure 9-2).

According to a case report of envenomation by *B. candidus* bite recently in northeastern Thailand was given monospecific *B. candidus* antivenom at 13 hours after snake bite, thereafter passed dark-colored urine 1 hour after antivenom administration (Wirat Leeprasert, 2008 per comm.). This evident was not convinced either because of an effect of *B. candidus* venom or the effect of monospecific *B. candidus* antivenom that induced rhadomyolysis-like syndrome. On the other hand, dark-colored urine in the envenomation of snakebite may have been caused by hemolysis and hemoglobinuria (Tibballs et al., 2003). However an evidence of rhabdomyolysis-like syndrome in the present study was observed by the increase of plasma CPK, LDH and K^+ levels after venom infusion into the animals (Chapter VII). These findings have been suspected for a nonspecific toxic effect of the *B. candidus* venom on muscle cell. Decreased in RBF and GFR may be due to myoglobinuria caused by damaged skeletal muscles (Ayer et al., 1971; Campion et al., 1972). Few data are available concerning sequential changes of renal function produced by *B. candidus* venom, although many studies have reported the occurrence of lethal acute renal failure after snakebites by the other venomous snakes (Burdmann et al, 1993; Chugh, 1989; de Silva et al., 1994; Schneemann et al., 2004; Sitprija, 2006). However some of case studies in human autopsy and animal experiments by *Bungarus*

caeruleus envenomation in Sri Lanka were demonstrated the histopathological changes in cardiovascular system, kidneys, liver, adrenal gland (Kularatne and Ratnatunge, 2001; Kiran et al., 2004; Mirajkar et al., 2005). The evidence of rhadomyolysis-like syndrome and increased myoglobinuria affecting kidney functions after envenomation by *B. candidus* venom needs to be further investigated. It can be concluded that *B. candidus* venom did not directly affect acute nephrotoxicity in the experimental animal. Case of *B. candidus* bite in the future should be closely observed the clinical symptoms and the detection of more laboratory parameters concerning electrolytes, enzymes, hemoglobin and myoglobin in urine of envenomed victims.

CONCLUSION

The variability in venom compositions between individuals of *Bungarus candidus* snakes give strongly supportive evident to venom composition being under genetic control (Chippaux et al., 1991). In addition, variation in the concentration of specific components between individuals is hinted to investigate the specific biological activities in envenomed victim by *B. candidus* bite. The overall findings of the *in vivo* and *in vitro* studies contribute to the effects of *B. candidus* venom on cell injury and changes of bodily functions e.g. the cardiovascular system and renal hemodynamics (Figure 9-2) which have never been reported in human envenomed by *B. candidus* bite. Intraspecific, geographic and ontogenetic variability in the venom compositions and enzymatic activities of the same snake species are of fundamental importance in snakebite pathology and therapeutics (Calvete et al., 2009; Gutiérrez et al., 2009). This is also necessary for the selection of snake from various regions to prepare the venom pools for antivenom production in different countries. Therefore, understanding the evolutionary processes affected to venom variation can employ not only for the effective development of antivenom production but also for the clinical therapeutics in the disparity of symptoms in envenomed victims.

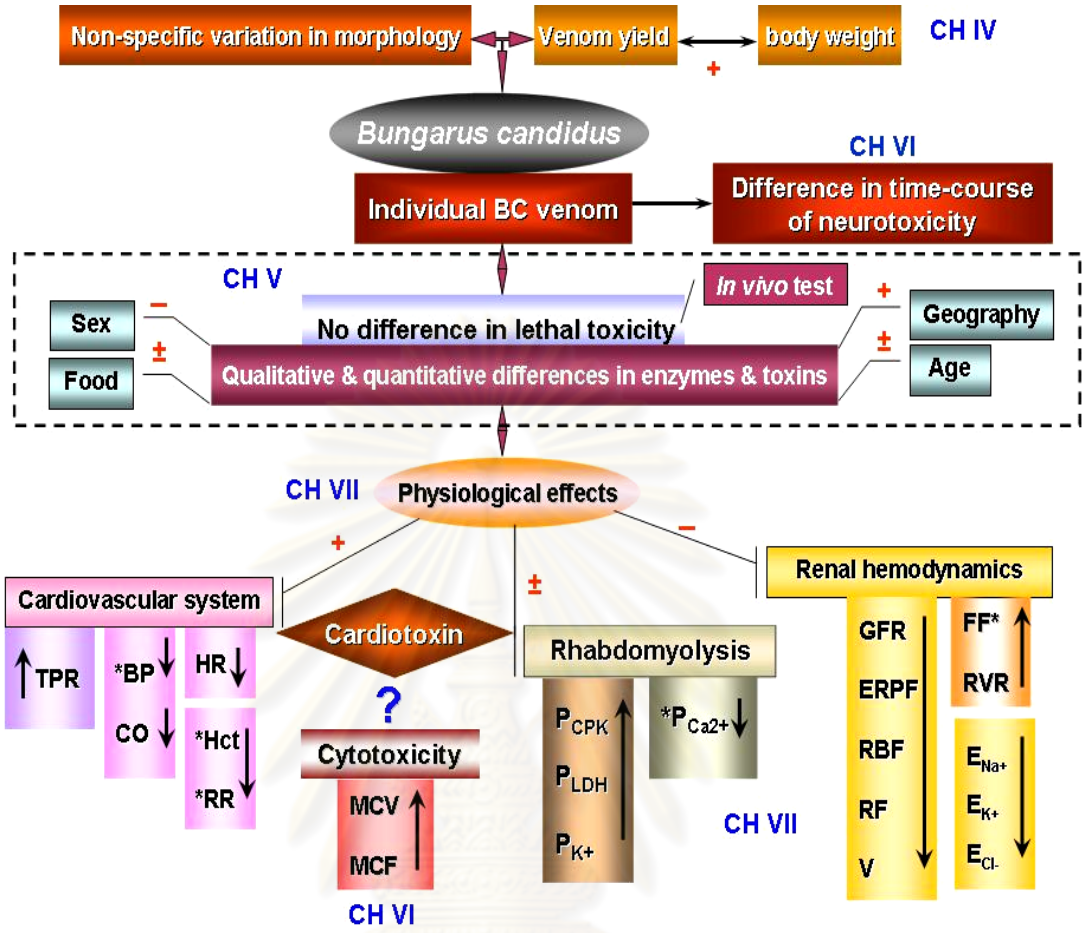


Figure 9-1 Schematic diagram represents the conclusion of studies in the biological characteristics and physiological effects of the venom of *Bungarus candidus* from different parts of Thailand and a captive-born snakes.

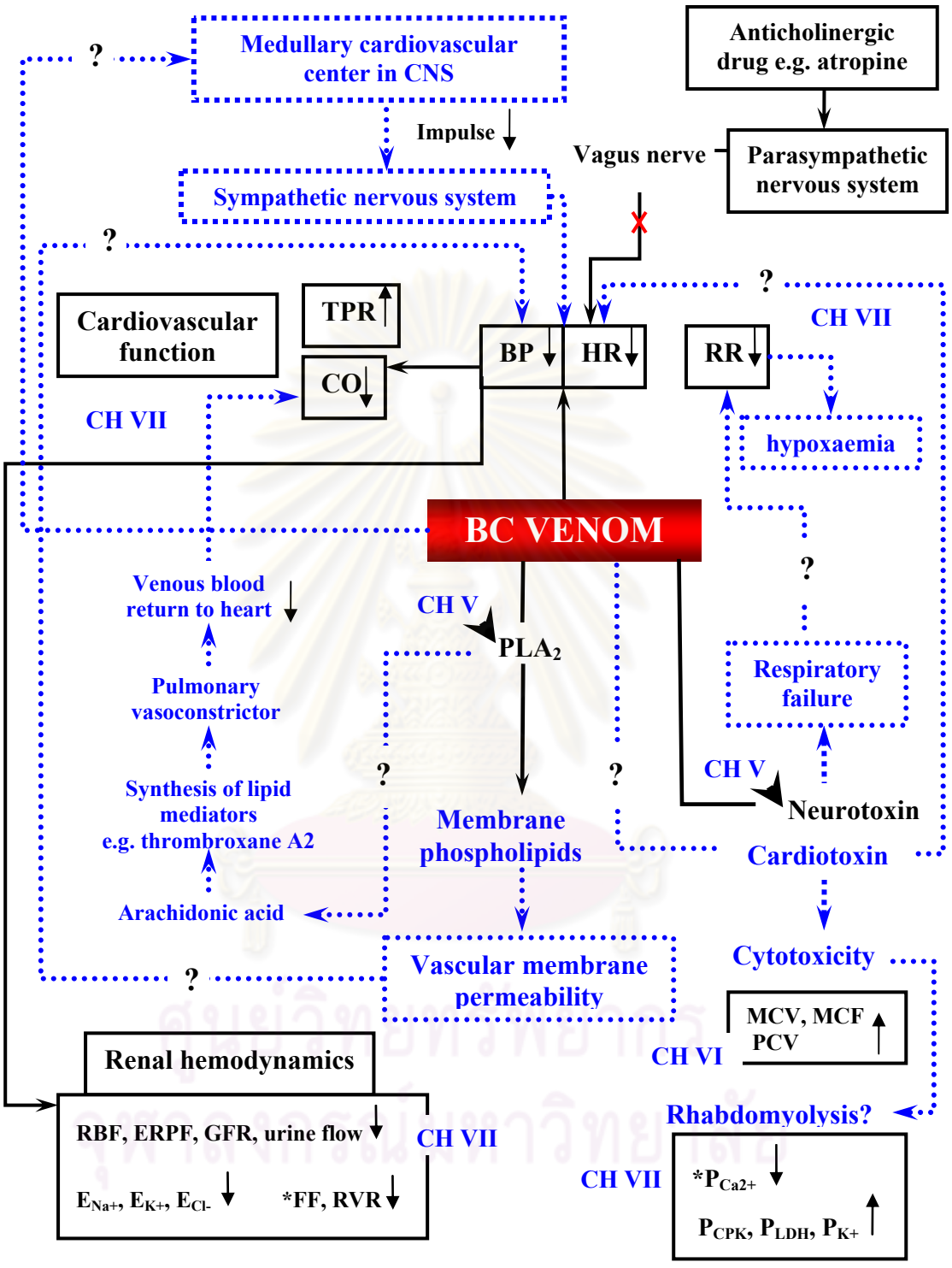


Figure 9-2 Schematic diagram represents the pathways of *Bungarus candidus* affected to bodily functions and renal hemodynamics, **the solid black lines** represent the demonstrated pathways whereas **the dot blue lines** represent the speculative pathways.

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APPENDIX

PUBLICATIONS

Chanhome, L., Khow, O., Puempunpanich, S., Sitprija, V. and Chaiyabutr, N. 2009. Biological characteristics of the *Bungarus candidus* venom due to geographical variation. J. Cell Anim. Biol. 3 (6): 93 – 100.

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