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Biological characteristics of the *Bungarus candidus* venom due to geographical variation

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The objective of this study was to compare the biological characteristics of the venom of the snake *Bungarus candidus* from three different localities of Thailand (wild-caught groups) and from a captive-born group in aspects of the lethal toxicity, the enzymatic activities, and the molecular weight of protein components. All venom samples exhibited no significant differences in lethal toxicity and the enzymatic activities of phospholipase A_2, protease, phosphodiesterase and hyaluronidase. Significant differences between the wild-caught groups and the captive-born group exhibited in the activities of acetylcholinesterase, L-amino acid oxidase and phosphomonoesterase. Among the wild-caught groups, the enzymatic activities revealed significant differences in acetylcholinesterase, phosphomonoesterase and hyaluronidase. All *B. candidus* venom groups exhibited high lethal toxicity, high activities of phospholipase A2, acetylcholinesterase and L-amino acid oxidase, moderate activities of hyaluronidase and phosphomonoesterase and low activities of phosphodiesterase and protease. The protein components revealed variation in SDS-PAGE pattern at the molecular weight in all groups in the range of 7.1 – 41.3 kDa.

Key words: Snake venom, *Bungarus candidus*, biological characteristics, geographical variation, Thailand.

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; Salazar et al., 2007; Shashidharamurthy et al., 2002; Shashidharamurthy and Kemparaju, 2007; Tsai et al., 2003), season of venom collection (Monteiro et al., 1998; Magro et al., 2001), diet (Tan et al., 1992, 1993a, 1993b), sexes (Menezes et al., 2006; Furtado 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 A2 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 (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.

The Malayan krait (*Bungarus candidus* Linnaeus, 1758) is a prominent neurotoxic Elapid snake, which is commonly found in Southeast Asia including Thailand (Cox,

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1991 and Chanhome et al., 1998). Based upon the studies of its 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 (Kanchanapongkul, 2002; Pochanugool et al., 1997; Dixon and Harris, 1999). 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). It was, therefore, the purpose of the present study 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

Snake and venom collection

Fifteen *B. candidus* snakes were used for study. Snakes were collected from eastern, northeastern and southern parts of Thailand including captive born snakes, which were categorized into 4 groups. Group 1, three snakes were collected from Chantaburi and Chonburi representing the eastern population (BC-E); group 2, three snakes were collected from Nakhon Ratchasima representing the northeastern population (BC-NE); group 3, four snakes were collected from Nakhon Si Thammarat representing the southern population (BC-S), and group 4, five snakes of captive born snakes (BC-CB) from snake husbandry unit, snake farm of Queen Saovabha Memorial Institute (QSMI) (Table 1).

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°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.

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 snake were recorded. Animal care and procedures used for venom extraction were in accordance with guidelines of the Animal Ethic Committee of QSMI. The experiment protocol was approved by the institutional ethic committee for using experimental animal (QSMI) in accordance with the guideline of the National Research Council of Thailand.

Determination of lethal toxicity (LD_{50})

Swiss albino mice (18-20 g) were obtained from laboratory animal unit of QSMI. The lethal toxicity of the venom 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 quarantine period and experiments. The control group was performed using normal saline solution. The endpoint of lethality of the mice was determined after 24 h observation. The LD_{50} was calculated by the method of Reed-Muench (1938) and 95% confidence limits by the method of Pizzi (1950).

Determinations of enzymatic activities

All reagents for enzymatic determinations in venom were analytical grade. The *B. 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.

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. A series of 2-fold diluted venom solutions (5 µl) was introduced to the plate, and then the cross diameter of hemolytic zones were measured after 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.

Acetylcholinesterase activity

Acetylcholinesterase activity was determined using the diagnostic kit “BTC Sigma” based on the use of the butyrylthiocholine as sub-

Table 1. Characteristics of *Bungarus candidus* snakes and their venoms 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 (91.0 - 106.0)	112.50 ± 9.85 (101.5 - 120.5)	233.33 ± 62.12 (195.0 - 305.0)	3 (3M)	67.97 ± 24.98 (29.6 - 144.1)	18.93 ± 7.48 (7.7 - 40.7)	27.90 ± 3.94 (22.71 – 37.02)	0.032 ± 0.013 (0.011 – 0.074)
BC-NE	Northeast	106.00 ± 19.31 (85.0 - 123.0)	118.50 ± 23.57 (93.0 - 139.5)	398.33 ± 215.02 (185.0 - 615.0)	3 (2M 1F)	116.90 ± 69.07 (27.1 - 255.8)	30.45 ± 17.18 (7.6 - 67.7)	26.59 ± 1.79 (23.24 – 30.21)	0.026 ± 0.009 (0.010 – 0.042)
BC-S	South	101.00 ± 6.68 (91.0 - 105.0)	117.0 ± 2.83 (113.0 - 119.0)	237.50 ± 51.23 (165.0 - 285.0)	4 (3M 1F)	60.41 ± 36.68 (5.8 - 133.5)	15.85 ± 9.09 (1.7 - 34.9)	27.53 ± 4.55 (23.35 – 40.48)	0.023 ± 0.012 (0.004 – 0.047)
BC-CB	Captive-born	90.60 ± 6.58 (83.0 - 99.0)	101.98 ± 7.52 (93.0 - 112.0)	203.00 ± 48.04 (145.0 - 260.0)	5 (4M 1F)	51.95 ± 23.52 (7.9 - 86.2)	13.60 ± 6.71 (2.3 - 29.6)	27.15 ± 6.87 (18.29 – 48.09)	0.023 ± 0.010 (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; BW = body weight; 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).

strate. The mixture of 500 µl of substrate and 5 µl of venom sample was analyzed according to the manufacturer's instruction by monitoring the increase of enzyme reaction at the appropriate time intervals in absorbance at 405 nm. One unit of enzyme activity was arbitrarily defined as the difference at 405 nm / min / mg.

Protease activity

Protease or proteolytic activity was determined by mean of the hide powder azure hydrolyzing activity (Omori-Satoh et al., 1995). 10 mg of hide powder azure was suspended in 1 ml of 30 mM borax buffer (pH 9.0) and was incubated with 100 µl of venom sample at 37℃ for 1 h. The reaction was stopped with 100 µl of 100 mM EDTA, and then centrifuged at 3000 rpm for 10 min. 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.

Alkaline phosphomonoesterase activity

Alkaline phosphomonoesterase activity was determined by a modified method (Lo et al., 1966; Tan and Tan, 1988). 100 µl of venom sample 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℃ for 30 min and then added 2 ml of 200 mM sodium hydroxide and placed for 20 min. The absorbance of the mixture was read at 440 nm. One unit of enzyme activity was defined as the amount of enzyme caused the increase of 0.001 absorbance unit per min.

Phosphodiesterase activity

Phosphodiesterase activity was determined by a modified method (Lo et al., 1966; Tan and Tan, 1988). 100 µl of venom sample was added to the 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 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 min.

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 was incubated for 3 min at room temperature and then added 100 µl of venom sample. The mixture was read in absorbance at 426 nm. One unit of enzyme activity was defined as the amount of venom caused the increment of 0.001 absorbance unit per min.

Hyaluronidase activity

Hyaluronidase activity of the venom was determined with the modified method (Xu et al*.,* 1982; Tan and Tan, 1988).

The substrates containing 200 mM acetate buffer (pH 5.0), 150 mM sodium chloride, and 200 µg hyaluronic acid mixed with 100 µl of the venom sample was incubated at 37℃ for 1 h. Reaction was terminated by the addition of 2 ml of 2.5% cetyltrimethylammonium bromide in 2% sodium hydroxide solution and placed for 30 min at room temperature. The enzyme activity was expressed as National Formulary Unit per milligram (NFU/mg) at the absorbance of 400 nm.

Table 2. 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 [a]	0.064 ± 0.015 [a]	0.061 ± 0.013 [a]	0.070 ± 0.024 [a]
HLD (µg)	0.12 ± 0.11 [a]	0.04 ± 0.03 [a]	0.05 ± 0.02 [a]	0.07 ± 0.02 [a]
ACE (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 [a]	0.029 ± 0.019 [a]	0.054 ± 0.025 [a]	0.057 ± 0.010 [a]
PME (Unit / min / mg)	372 ± 257 [a]	1385 ± 1207 [b]	385 ± 173 [a]	303 ± 164 [a]
PDE (Unit / min / mg)	19 ± 5 [a]	21 ± 14 [a]	23 ± 6 [a]	17 ± 3 [a]
LAO (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 / g of mouse); 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; ACE = Acetylcholinesterase activity (unit / min / mg); PRO = Proteolytic activity (unit/mg); PME = Alkaline phosphomonoesterase activity (unit / min / mg); PDE = Phosphodiesterase activity (unit / min / mg); LAO = L-amino acid oxidase activity (unit / min / mg); HYA = Hyaluronidase activity (NFU); NFU = National Formulary Unit.

Determination of molecular weight of protein components

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). The running buffer (10x) diluted to 1x at the voltage of 30 V for 25 min, 100 V for 90 min and 150 V for 90 min using Hoefer power supply PS500 XT. The Kaleidoscope prestained standard (BioRad) ranging from 7 – 210 kDa was used as standard markers. Gels were stained with 0.2% Coomassie blue (CBB R-250).

Statistic 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

Characteristics of *Bungarus candidus* snakes and their venoms

The characteristics of snakes for the snake size, sexes, the body weight, the weights of liquid (fresh) and dry venoms (lyophilized) are presented in Table 1. BC-NE group possessed highest body weight ranged from 185.0 - 615.0 gm and was correlated with weight of liquid venom (range 27.1- 255.8 mg/snake) and weight of dry venom (range 7.6 - 67.7 mg/snake) when compared with other groups. 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 (26.6-27.9%) and no association with body weight. The sizes of snakes in all groups were recorded for the snout-vent length (range 83 - 125 cm) and the total length (range 93 - 139 cm).

The lethal toxicity and enzymatic activities

In comparison of the mean values of the lethal toxicity and enzymatic activities among groups of *Bungarus candidus* snakes are presented in Table 2. The lethal toxicity of the venoms showed no significant differences among groups of snakes. The enzymatic activities of *B. candidus* venoms 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* venoms, 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).

Determinations of the molecular weight of protein components

Patterns of SDS-PAGE under non-reducing condition of each venom are presented in Figure 1. The overall marked dense protein bands were quantitative different in

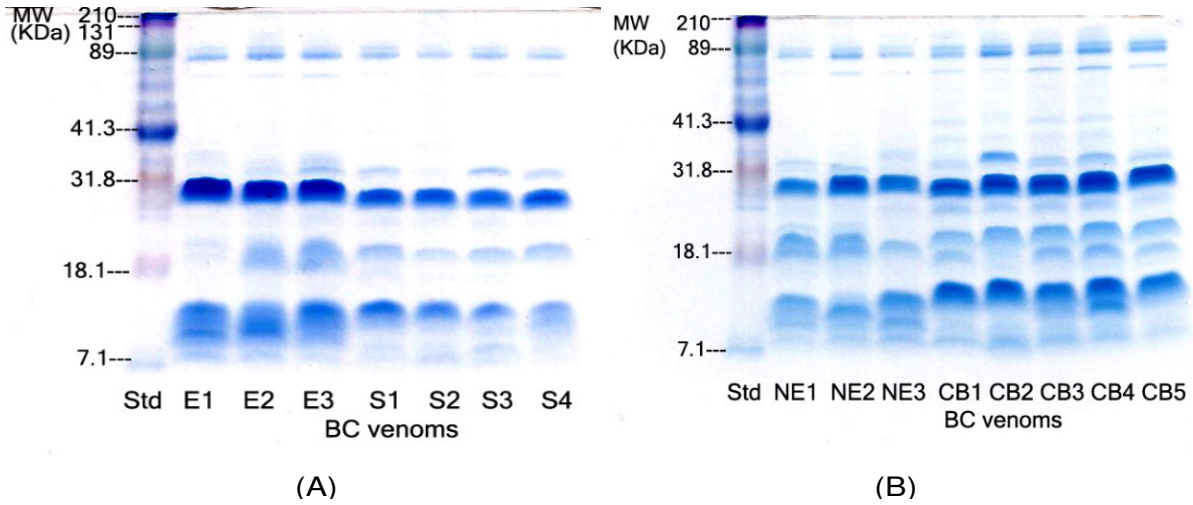

Figure 1. SDS-PAGE pattern of *Bungarus candidus* venom under non-reducing condition. Twelve microgram of venom solution (1mg/ml) from eastern (E), northeastern (NE), southern (S) regions and captive-born (CB) groups were applied to each lane for electrophoresis. Kaleidoscope prestained standard (lane 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 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.

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.

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 (*B. caeruleus, B. multicinctus and B. fasciatus*) (Tan and Ponnudurai, 1990). The enzymatic activities in the venom have been classified to the high activities of phospholipase A_2, acetylcholine-esterase, L-amino acid oxidase and hyaluronidase, the moderate activity of alkaline phosphomonoesterase and the low activities of phosphodiesterase and protease. However, our 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; Salazar et al., 2007; Shashidharamurthy et al., 2002; Shashidharamurthy and Kemparaju, 2007; Tsai et al., 2003). In the present study, no differences in lethal toxicity of *B. candidus* venoms were apparent among groups of snakes from different locations (Table 2). However, the present findings reveal that the lethal toxicity of BC-S venom (0.061 µg/g mouse) is more potent 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. The different results of these parameters suggest that other factors may account for the lower lethal toxicity of the pooled venom.

The class of β-bungarotoxin family has been charac-terized to more than 16 isoforms. These toxin isoforms show a wide range of lethal potency either in the PLA_2 activity-dependent or the PLA_2 activity-independent neurotoxic effects (Chu et al., 1995). Therefore, the differences in term of the response to lethal toxicity indicate that the toxicity is not solely due to the activity of phospholipase A_2, although the phospholipase activity has been shown to play an important role in the action of the presynaptic β-bungarotoxin, which commomly found in the venom of *Bungarus* snakes (Abe et al., 1977). 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 diffe-

rence 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 et al., 2003).

In the present study, 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.

Determination for the profile of protein bands of all venom groups by SDS-PAGE (Figure. 1) showed the quantitative differences of molecular weight of protein bands ranging 7.1 to 41.3 kDa. The distinction of the protein bands at the molecular weight from 18.1 – 41.3 kDa were pronounced in captive-born group as compared with the other three snake venom groups. The captive born snakes were fed on mice with their food preference which may be the cause of these differences. The dense protein bands at the molecular weight approximately 22 – 25 kDa in the present study may be comparable to three major lethal toxins isolated from *B. candidus* venom, which have been reported earlier (Khow et al., 2003). Analysis of their N-terminal amino acid sequences of these three major lethal toxins have been shown to be isoforms of presynaptically β-bungarotoxin. The isoforms of □-bungarotoxin are the main portion of *Bungarus* venoms that have a similar primary structure with two subunits linked by disulfide bond. A neutral polypeptide PLA_2 subunit (A-chain) and a basic polypeptide non-PLA_2 subunit (B-chain) have a molecular weight of 13 and 7.5 kDa, respectively (Chu et al., 1995; Rowan, 2001). The combination of these two subunits has the molecular weights closely to the range of 22 - 25 kDa in the present protein band profiles. However, an analysis of the isoforms of β-bungarotoxin could not make a conclusion. The studies of Tsai et al. (2002) and Yanoshita et al. (2006) by cloning and sequencing of the venom glands of *B. candidus* revealed that about 20 PLA_2 encoded the β-bungarotoxin A-chains, which some of these PLA_2 have a total of 125 amino acid residues. Its structure (61%) and molecular weight (13,982 Da) are identical to the myotoxic/cardiotoxic PLA_2 from *Ophiophagus hannah* venom and the hemorrhagic/myotoxic PLA_2 from *Notechis s. scutatus* venom (Tsai et al., 2002).

In addition, the difference in quantitative of the marked dense bands in the region of molecular weight of 7.1 to 18.1 kDa are probably compounded of α-bungarotoxin (Kuch et al., 2003), bucandin (Torres et al., 2001), candoxin (Nirthanan et al., 2002; Paaventhan et al., 2003) and bucain (Watanabe et al., 2002) isolated from the venom of *Bungarus candidus* originally from the other Southeast Asia countries. The postsynaptic toxin with the molecular weight 7983.75 Da purified from the venom of *B. candidus* from Java has been demonstrated to be identical to α-bungarotoxin isolated from *B. multicinctus* (Kuch et al., 2003). In this regard, it would support the phylogenetic study on many kraits that revealed close relationship between these two species (Slowinski, 1994). Bucain is a three-finger toxin with the molecular weight range 6000 – 8000 Da. Candoxin (MW 7334.6 Da) and bucandin (MW 7275.4 Da) are also three-finger toxins consisting of a single polypeptide chain of 62 – 68 amino acid residues with five disulfide bonds. Candoxin is a potent antagonist of muscle (αβγδ) but poorly reversible antagonist of neuronal α7 nicotinic receptors (Nirthanan et al., 2002), while bucandin has been shown non-lethal in mice when intraperitoneal and intravenous injection in dose up to 50 mg/kg (Nirthanan et al., 2003). It has been shown that candoxin produces postjunctional neuromuscular blockade with rapid and complete reversal either by washing or by the addition of anticholinesterase (Nirthanan et al., 2003). In the present study, the high activity of acetylcholinesterase and L-amino acid oxidase of *B. candidus* venom may comparable with the range of

molecular weights presented in SDS-PAGE profile. The molecular weight of L-amino acid oxidase in the snake venom has been demonstrated in range of 50 – 70 kDa in SDS-PAGE assay both under reducing and non reducing conditions (Du and Clemetson, 2002), whereas acetylcholinesterase is approximately 65 - 70 kDa (Anderson and Dufton, 1998).

Conclusion

The results of the present findings suggest that the lethal toxicity of the venom would depend on heterogeneous components in the venom by which enzymatic activities induce processes in different ways with showing complexity in the mechanism. The results of the present study about the differences in the amount of toxins and enzymes contained in individual venom of the same snake species from different geographical areas will be used for manipulation of possible clinical treatment in using antivenom effectively, including the improvement of antivenom production targeting in the future. Although the present study is needed to clearly identify the toxin purification or venom fractions of these venoms as well as evaluate their physiological effects in the experimental animal.

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