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Enzymatic and biochemical characterization of *Bungarus sindanus* snake venom acetylcholinesterase

Ahmed M (1), Latif N (2), Khan RA (1), Ahmad A (1), Rocha JBT (2), Mazzanti CM (2), Bagatini MD (2), Morsch VM (2), Schetinger MRC (2)

(1) Department of Biotechnology, University of Science and Technology, Bannu, Kyber Pakhtunkhwa, Pakistan; (2) Department of Biochemistry and Toxicology, Center of Natural and Exact Sciences, Federal University of Santa Maria, Santa Maria, Rio Grande do Sul State, Brazil.

Abstract: This study analyses venom from the elapid krait snake *Bungarus sindanus*, which contains a high level of acetylcholinesterase (AChE) activity. The enzyme showed optimum activity at alkaline pH (8.5) and 45°C. Krait venom AChE was inhibited by substrate. Inhibition was significantly reduced by using a high ionic strength buffer; low ionic strength buffer (10 mM PO_4 pH 7.5) inhibited the enzyme by 1. 5mM AcSCh, while high ionic strength buffer (62 mM PO_4 pH 7.5) inhibited it by 1 mM AcSCh. Venom acetylcholinesterase was also found to be thermally stable at 45°C; it only lost 5% of its activity after incubation at 45°C for 40 minutes. The Michaelis-Menten constant (Km) for acetylthiocholine iodide hydrolysis was found to be 0.068 mM. Krait venom acetylcholinesterase was also inhibited by $ZnCl_2$, $CdCl_2$, and $HgCl_2$ in a concentration-dependent manner. Due to the elevated levels of AChE with high catalytic activity and because it is more stable than any other sources, *Bungarus sindanus* venom is highly valuable for biochemical studies of this enzyme.

Key words: acetylcholinesterase, inhibition, krait, ionic strength, acetylthiocholine iodide, *Bungarus sindanus*, snake venom.

INTRODUCTION

Acetylcholinesterase is present in all vertebrates, particularly in the muscles and nervous tissues (1). Structurally and functionally acetylchloinesterase is a serine hydrolase (AChE, acetylcholine hydrolase, EC. 3.1.1.7). The enzyme is found in both synaptic and non-synaptic tissues (1-3). In synaptic tissue the main role of acetylcholinesterase is the hydrolysis of neurotransmitter acetylcholine (ACh). The catalytic activity of this enzyme is essential for normal cholinergic transmission and neuromuscular function (4).

In a non-synaptic context, AChE and BChE are expressed in early embryonic development some time before the formation of cholinergic synapsis, where its function is the formation and regulation of the acetylcholine gradient which guides the growth of nerve cells (5-7). A noteworthy amount of acetylcholinesterase is also present in blood cells, particularly in erythrocytes and lymphocytes (8, 9). The most common non-synaptic example is snake venom, in which its function is unknown (10, 11). In snake venom, acetylcholinesterase exists in all Elapidae venom except mambas (12). Recent literature indicates that acetylcholinesterase is also present in non-poisonous snake venoms (13).

The *Bungarus* genus of Elapidae is well-known due to the large amount of acetylcholinesterase, about 8 mg/g in dried venom (0.8% w/w), which is highly active (> 60,000 Elman units/mg) (10). Although highly active, it is non-toxic even when

tested at high concentrations (14). The enzyme reaction catalyzed by acetylcholinesterase is one of the most efficient reactions. Partial peptide sequence studies show that snake venom enzymes have close homology with other acetylcholinesterases, and have the same catalytic triad for substrate hydrolysis (14). The efficiency of substrate hydrolysis could be related to three-dimensional structures of the enzyme. The long narrow active site gorge is 20 Å deep, composed of two subsites for ligand interaction: an acylation site at the base of the gorge with a catalytic triad and peripheral site at its mouth, distinct from a catalytic active site (15, 16).

Previously, we found that *Bungarus sindanus* venom AChE is inhibited by commonly used pesticides and herbicides with inhibitory behavior similar to human serum BChE (17). Furthermore, krait (*Bungarus sindanus*) venom enzyme shows high sensitivity towards tacrine, commonly use in treating Alzheimer's disease (18). However snake venom acetylcholinesterase is different from other vertebrate tissues since it is present in soluble non-amphiphilic monomeric form (14). This study was designed to discover more about krait venom AChE in terms of thermal stability, substrate specificity, and substrate inhibition, and the effects of ionic buffer strength, pH, temperature, and metals.

MATERIALS AND METHODS

Materials

Acetylthiocholine iodide, butyrylthiocholine iodide, DTNB [5,5′-dithiobis(2-nitro-benzoic acid)], bovine serum albumin, and Coomassie Brilliant blue R-250 were purchased from Sigma (USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Neon Comercial LTDA (Brazil); and Tris (hydroxymethyl amino methane) from Vetec (Brazil). All other reagents used were of analytical grade.

Venom

Venom from live *Bungarus sindanus* snakes was manually milked, immediately lyophilized and stored at −20°C for future use.

Protein Determination

Protein was assayed according to Bradford (19) using bovine serum albumin as standard.

Statistical Analysis

Statistical analysis was performed using one way ANOVA, followed by post-hoc analysis (Duncan multiple range test).

Cholinesterase Assay

Cholinesterase activities were determined according to Ahmed *et al.* (20) with some modifications Rocha *et al.* (21). Hydrolysis rates (V) were measured at various acetylthiocholine (S) concentrations (0.05 to 3 mM) in 1-mL assay solutions with 10 mM phosphate buffer, pH 7.5, and 0.2 mM DTNB at 25°C. Snake venom (40 µL equivalent to 1 µg of protein) were added to the reaction mixture and pre-incubated for 30 minutes at 37°C. We did not find butyrylcholinesterase in the crude venom so we did not use ethopropazine (a specific butyrylcholinesterase inhibitor) in any of the enzyme essays. Hydrolysis was monitored by verifying the formation of the thiolate dianion of DTNB at 412 nm for 2 to 3 minutes (15 second intervals) using a using Hitachi U-2001 spectrophotometer (Tokyo, Japan). All samples were run in duplicate or triplicate.

K_m and V_{max} Determination

The kinetic parameters K_m and V_{max} were determined from Linewear and Burk (22) double reciprocal plot analyzed over a range of acetylthiocholine concentrations (0.05 to 3 mM). For comparison Hanes-Woolf, Eadie-Hofstee, and Direct Linear Wilkinson plot were used to determine K_m and V_{max} values.

Kinetic Analysis

Kinetic analysis was performed using Slide Write software.

RESULTS

Substrate specificity of snake venom acetylcholinesterase (AChE) was evaluated by testing acetylthiocholine (AcSCh) and butyrylthiocholine (BuSCh) as a substrate varying from 0.05 to 3 mM. Krait (*Bungaraus sindanus*) venom AChE only hydrolyzed acetylthiocholine (AcSCh) (Figure 1).

Substrate kinetic parameters for acetylthiocholine were calculated by double reciprocal Linweaver-Burk plot (Linweaver and Burk, 1934) resulting in K_m = 0.068mM and V_{max} = 651 µmole/min/mg protein (Figure 2). Calculated

K_m and V_{max} values by different plots are shown in Table 1. Krait venom AChE was inhibited by substrate. However, substrate inhibition varies with buffer ionic strength. We closely observed that low ionic strength buffer (10 mM PO_4; pH 7.5) inhibited the enzyme by 1.5 mM substrate while with high ionic strength buffer (62 mM PO_4; pH 7.5) venom AChE was more sensitive to substrate inhibition and was inhibited by 1mM acetylthiocholine (Figure 3).

The enzyme is pH sensitive and shows higher activity in alkaline media (pH 8.5) whilst in acid media (pH 4) the enzyme does not show its hydrolytic property (Figure 4). It does become slightly more active at 45°C for substrate hydrolysis (Figure 5). It is also thermally stable compared to electric eel acetylcholinesterase by incubating at 45°C for 40 minutes (Figure 5 – A). We found only 5% loss in venom acetylcholinesterase after 40 minutes at 45°C, which is far less than electric eel acetylcholinesterase. Electric eel AChE lost 69% of its activity after 15 minutes at 45°C compared to inhibition at 37°C. This enzyme lost 80% of its activity after incubation at 45°C for 40 minutes (Figure 5 – B). *Bungaraus sindanus* venom AChE is also inhibited by $ZnCl_2$, $CdCl_2$, and $HgCl_2$ in a concentration dependent manner (Figure 6). Inhibition of krait (*Bungarus sindanus*) venom AChE by metals was also confirmed by electrometric method using acetylcholine substrate (data not shown).

Table 1. K_m and V_{max} values from different plots

	Lineweaver-Burk	Hanes-Woolf	Eadie-Hofstee	Direct linear	Wilkinson
K_m	0.068	0.0749	0.0547	0.0576	0.0492
V_{max}	651	635	597	567	587

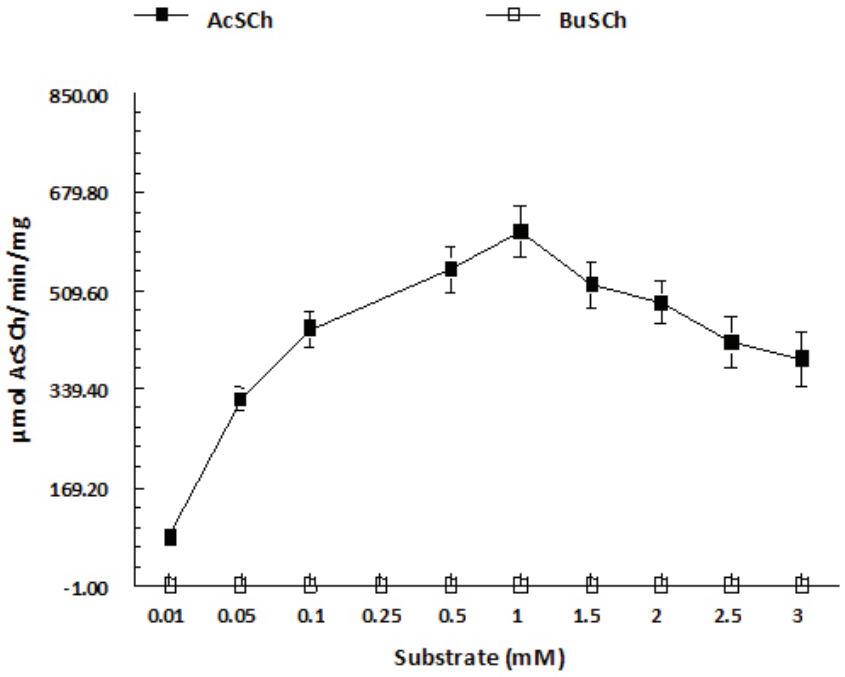

Figure 1. Substrate specificity. Hydrolysis of acetylthiocholine (AcSCh) and butyrylthiocholine (BuSCh) by krait (*Bungaraus sindanus*) venom AChE. Venom protein (1 µg) was preincubated for ten minutes in 1 mL assay solutions with 10 mM PO_4 buffer, pH 7.5, and 0.2 mM DTNB [5,5-dithiobis(2-nitrobenzoic acid)] before adding substrate at 0.05 to 3 mM. All experiments were repeated at least three times and similar results were obtained.

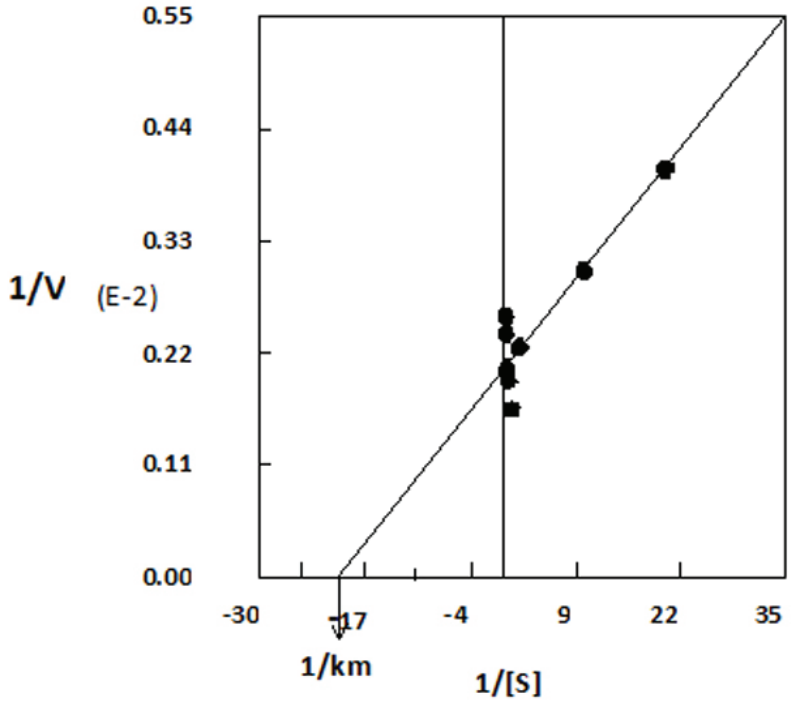

Figure 2. The Lineweaver-Burk plot, representing reciprocal of initial enzyme velocity versus AcSCh concentration for K_m determination. K_m 0.068 mM; V_{max} = 651 µmol/min/mg protein.

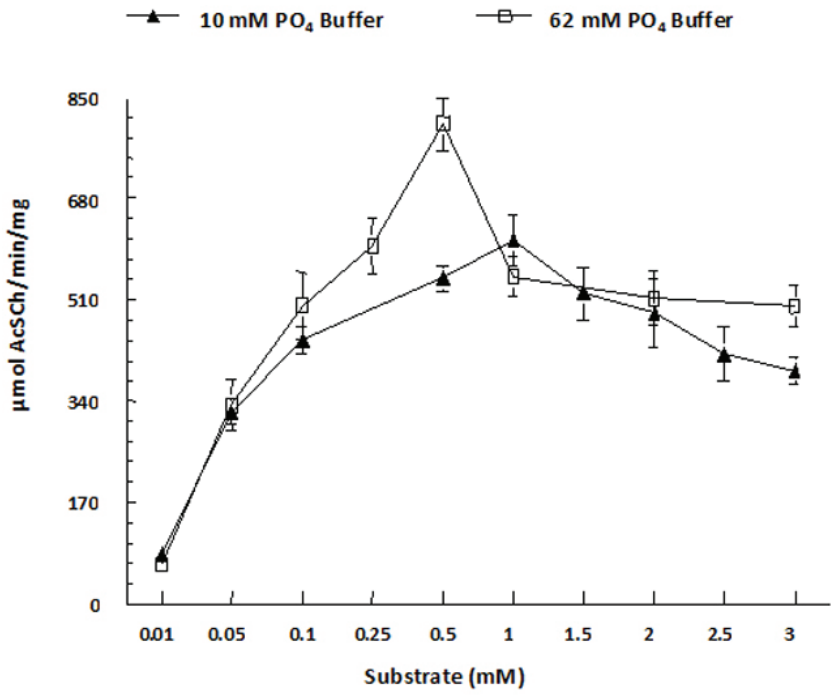

Figure 3. Reduction of enzyme substrate inhibition in high ionic strength buffer. Hydrolysis of acetylthiocholine in 10 mM PO_4, pH 7.5, buffer and 62 mM PO_4, pH 7.5, buffer by krait (*Bungaraus sindanus*) venom AChE. Reduction of substrate inhibition is clearly visible in high ionic strength buffer (62 mM PO_4, pH 7.5). Venom AChE was incubated in the media for ten minutes at 37°C before addition of different substrate concentrations (0.05-3 mM). Results are the mean of four different experiments performed in duplicate.

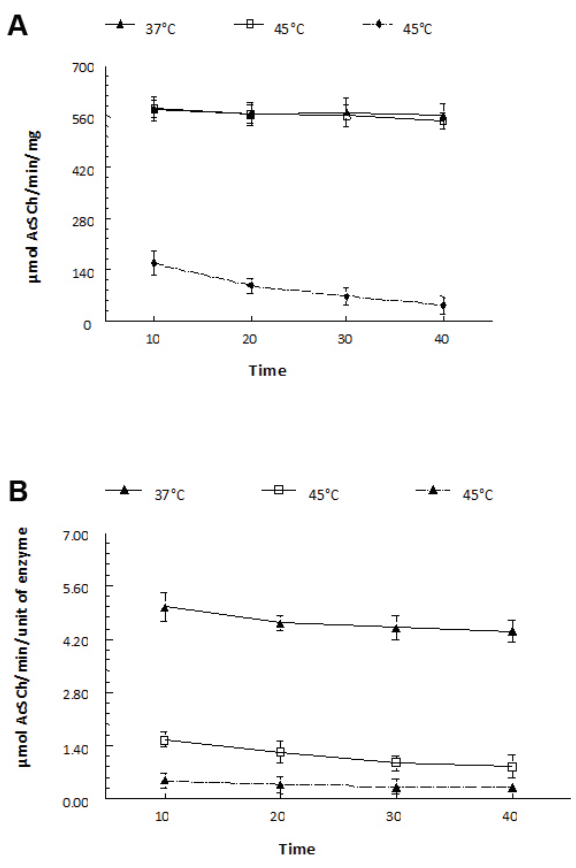

Figure 4. (**A**) The effect of pH on enzyme activity was analyzed by incubating the enzyme for ten minutes in different buffers; 10 mM sodium acetate buffer (pH 4 and 5.5), 10 mM PO_4 buffer (pH 6.5 and 7.5), 10 mM Tris-HCl buffer (pH 8.5-10.5) at 37°C before the addition of 1 mM acetylthiocholine as substrate. Results are the mean of four different experiments and similar results were obtained. The effect of temperature on the enzyme activity was analyzed by incubating the enzyme for ten minutes at different temperature; 25, 37, 45, 50, and 60°C before the addition of 0.2 mM DTNB and 1 mM acetylthiocholine as a substrate. (**B**) Results are the mean of four different experiments and similar results were obtained.

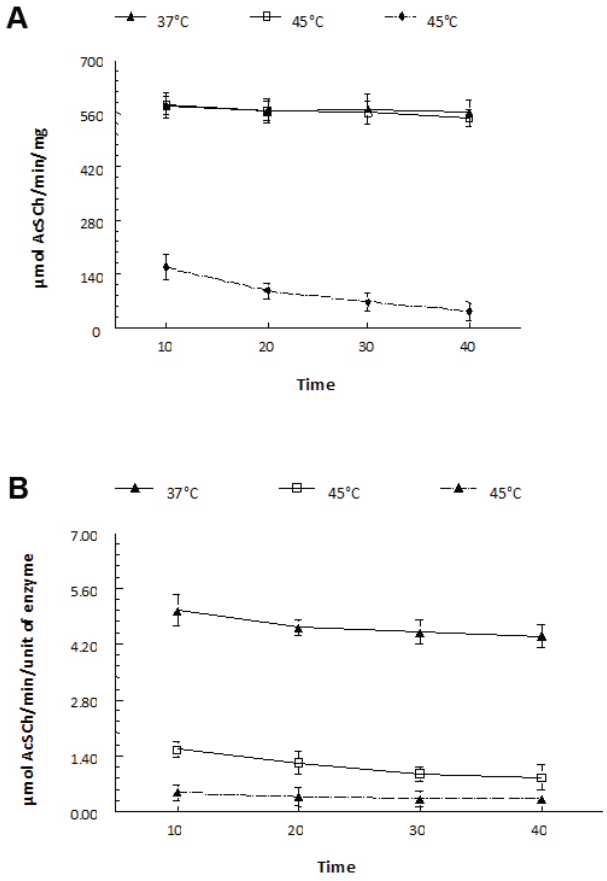

Figure 5. Comparison of thermal stability between (**A**) krait (*Bungarus sindanus*) venom AChE and (**B**) pure electric eel AChE incubated at different temperatures (37 to 50°C) and time intervals (10 to 40 minutes) before the addition of 0.2 mM DTNB and 1 mM acetylthiocholine as substrate.

DISCUSSION

In this study, we show that *Bungarus sindanus* venom has a true AChE, which presents characteristic catalytic properties, as previously reported (23). The *Bungarus* genus is the richest source of AChE in Elapidae. The optimum substrate concentration was 1 mM using a medium of 10 mM PO_4 at pH 7.5 (Figure 1). This concentration is much lower than desert cobra (*Walterinnesia aegyptia*), in which optimum activity was observed at 3 mM (24). At substrate concentrations greater than 1 mM krait venom AChE hydrolytic property was reduced (Figure 1). Similar substrate inhibition was observed with other snake venom acetylcholinesterase (10, 24, 25).

Bungararus sindanus AChE was specific for acetylthiocholine, it did not hydrolyze butyrylthiocholine (Figure 1). Snake AChE from all other genera also lacks the ability to hydrolyze butyrylthiocholine (10). The Michaelis-Menten constant (K_m) calculated from the Lineweaver-Burk plot (22) for acetylthiocholine iodide

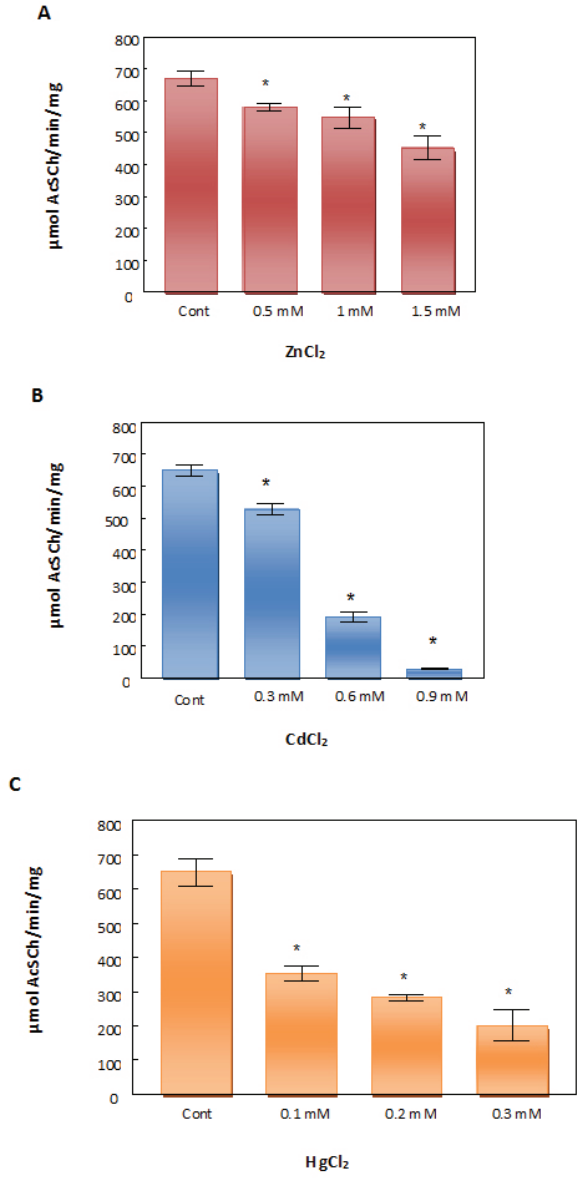

Figure 6. Inhibition of krait (*Bungarus sindanus*) venom AChE by metals. Various concentrations of (**A**) $ZnCl_2$, (**B**) $CdCl_2$, and (**C**) $HgCl_2$ were incubated at 37°C for ten minutes in 10 mM Tris-HCl buffer, pH 8.5. Reactions were started by adding 1 mM acetylcthiocholine (AcSCh) as a substrate. (**A**) $p < 0.029$ for $ZnCl_2$, (**B**) $p < 0.00028$ for $CdCl_2$, and (**C**) $p < 0.000128$ for $HgCl_2$ compared to control. Results represent the mean of four different experiments performed in duplicate.

hydrolysis was 0.068 mM with V_{max} = 651 µmol/min/mg protein (Figure 2). These values are comparatively higher by Eadie-Hofstee, direct linear, and Wilkinson plots (Table 1).

Bungarus V_{max} is very high compared to that of other Elapidae snakes. A significant reduction in krait venom acetylcholinesterase substrate inhibition by high ionic strength buffer was observed. With low ionic strength buffer (10 mM PO_4, pH 7.5), the enzyme was inhibited by 1.5 mM AcSCh while with high ionic strength buffer (62 mM PO_4, pH 7.5) it was inhibited by 1 mM AcSCh (Figure 3). It is worth noting that significant reduction in substrate inhibition with high ionic strength buffer was also mentioned by Frobert *et al.* (10). Also, the enzyme shows higher activity in high ionic strength buffer (62 mM PO_4, pH 7.5) than in low ionic strength buffer (10 mM PO_4, pH 7.5) (Figure 3).

Bungarus sindanus enzyme shows optimum activity in alkaline pH (Figure 4 – A). AChE from all other sources also shows higher activity in alkaline media (26). Optimal temperature is 45°C (Figure 4 – B), higher than desert cobra (*Walterinnesia aegyptia*) which shows its highest activity at 30°C (24). We also found that krait (*Bungarus sindanus*) venom acetylcholinesterase is thermally stable at 45°C. It only lost 5% of its activity after incubation at 45°C for 40 minutes (Figure 5 – A) while at 37°C we did not find any loss in its activity (Figure 5 – A). In comparison, electric eel AChE lost 69% of its activity after 15 minutes at 45°C compared to incubation at 37°C while after 40 minutes it lost 80% of its activity (Figure 5 – B).

Generally snake venom AChE is more stable than other sources. *Bungarus* snake venom is more stable than *Haemacatus, Ophiophagus,* and *Naja* enzymes (10). A thermal stability study of *Bungarus* AChE by capillary electrophoresis also found that venom AChE is stable under standard conditions (27). Due to its higher activity and stability, this enzyme is very suitable for industrial use. Furthermore, $ZnCl_2$, $CdCl_2$, and $HgCl_2$ (Figure 6) can also be considered snake venom AChE inhibitors; however, very high concentrations are necessary. This may be due to the absence of a free sulfhydryl group. According to Frasco *et al.* (28), when a free sulfhydryl group is absent in the enzyme (*Drosophila melonogaster* acetylcholinesterase and human serum butyrycholinesterase) inhibition by mercury will occur in the millimolar range, while in the presence of a free sulfhydryl group (*Electrophorus electricus*), the inhibition will only require a micromolar concentration (28). It is possible that the inhibitory effects of metals on venom acetylcholinesterase are due to the formation of inactive enzyme aggregate (29).

In conclusion, the *Bungarus* venom contains large amounts of AChE with the highest catalytic activity of all sources and is comparatively more stable than any other source, making it a valuable source for biochemical study.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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CORRESPONDENCE TO

Mushtaq Ahmed, Department of Biotechnology, University of Science and Technology, Bannu, Khyber Pakhtunkhwa, Pakistan. Phone: +92928633425. Email: mushtaq213@yahoo.com.

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