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RESEARCH ARTICLES

Cholinesterase Inhibitory Activities of the Scorpion *Mesobuthus gibbosus* (Buthidae) Venom Peptides

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Cholinesterase Inhibitory Activities of the Scorpion Mesobuthus gibbosus (Buthidae) Venom Peptides

Summary : *Scorpion venoms contain a group of neurotoxins which have been shown to interact with ionic channels in excitable membranes and contain some peptides, enzymes, amines, and proteases with various effects. In the present study, crude venom of* Mesobuthus gibbosus *(Buthidae), a scorpion found all over Anatolia except on Black Sea shore, was isolated and purified by the Sephadex G-50 gel filtration and high pressure liquid chromatographic (HPLC) separation. Two of the five fractions, obtained from the Sephadex G-50 filtration and detected as lethal on mice and* Musca domestica *larvae in in vivo toxicity tests were independently subjected to the HPLC separation. Only one of seven fractions obtained from the HPLC separation of the fraction 5 was found to be extremely lethal. Sodium dodecyl sulfate polyacrylamide gel electrophoretic (SDS-PAGE) analysis of the crude venom and its chromatographic fractions demonstrated that crude venom consisted of peptides with molecular weights of 6,500-210,000 Da while its neurotoxic fraction appeared as a single band of 28,000 Da. Two bands of 6,200 and 22,000 Da were determined in SDS-PAGE, respectively, suggesting that it might consist of two chains attached by a disulfide bridge. Crude venom and its neurotoxic fractions obtained from the Sephadex G-50 gel fitration and HPLC separation significantly and specifically inhibited acetylcholinesterase (AChE) in human erythrocytes with the apparent* K_i *values of 0.90±0.022 , 0.86±0.017 and 0.78±0.018 mg venom protein/ml, respectively, in a reversible and non-competitive manner. None of the fractions inhibited the butyrylcholinesterase (BChE) of human plasma and erythrocytes. Kinetic data indicated that cholinesterase inhibitory peptides of* Mesobuthus gibbosus *venom might interact with the enzyme at an alternative binding region possibly close to the peripheral site in the catalytic gorge of the enzyme molecule.*

Keywords: Mesobuthus gibbosus, *Buthidae, Acetylcholinesterase, Butyrylcholinesterase, Inhibition.*

Mesobuthus gibbosus *(Buthidae) Akrep Venom Peptidlerinin Kolinesteraz İnhibitör Aktiviteleri*

Özet: *Akrep venomları, sinir hücrelerinin uyarılabilir zarlarındaki iyon kanalları ile etkileşebilen bir grup nörotoksinin yanısıra çeşitli etkilere sahip peptid, enzim, amin ve proteazları içermektedirler. Bu çalışmada , Karadeniz sahili dışında tüm Anadolu'da yaygın olarak bulunan* Mesobuthus gibbosus *akrebinin venomu izole edilip Sephadex G-50 jel filtrasyonu ve Yüksek basınçlı Sıvı Kromatografisi (HPLC) yöntemleriyle saflaştırıldı. Sephadex G-50 jel filtrasyonundan elde edilen 5 fraksiyondan ikisi in vivo toksisite testlerinde fare ve* Musca domestica *larvaları üzerinde letal etki gösterdi. Letal fraksiyon 5'in HPLC ayırımı sonucu elde edilen 7 fraksiyondan yalnızca birinde letal etki gözlendi. Ham venomun sodyum dodesil sülfat polakrilamid jel elektroforezi (SDS-PAGE) ile analizi, venomun molekül ağırlıkları 6,500-210,000 Da arasında değişen peptidlerden oluştuğunu gösterirken letal fraksiyon SDS-PAGE'de 28,000 Da molekül ağırlığında tek bir peptid olarak belirdi. Redükleyici koşullarda aynı fraksiyonun 6,200 ve 22,000 Da molekül ağırlıklarında iki band halinde gözlenmesi, molekülün disülfit köprüsü ile tutunmuş iki peptid zincirinden oluşmuş olabileceğini düşündürdü. Ham venom ve venomun jel filtrasyon ve HPLC ayırımı sonucu elde edilen nörotoksik fraksiyonlarının insan eritrosit asetilkolinesteraz (AChE) enzimini tersinir ve nonkompetitif olarak inhibe ettiği gözlendi ve* K_i *değerleri sırasıyla 0.90±0.022, 0.86±0.017 ve 0.78±0.018 mg venom proteini/ml olarak hesaplandı. Ham venom ve nörotoksik fraksiyonlarının insan plazma ve eritrosit butirilkolinesteraz enzimini inhibe etmediği görüldü. Kinetik veriler ışığında,* Mesobuthus gibbosus *venomundan elde edilen asetilkolinesteraz inhibitörünün enzimin aktif merkezinin dışında ve büyük olasılıkla bu merkezin periferal kısmına yakın bir bağlanma bölgesine bağlandığı öne sürüldü.*

Anahtar kelimeler: Mesobuthus gibbosus, *Buthidae, Asetilkolinesteraz, Butirilkolinesteraz, İnhibisyon.*

INTRODUCTION

Scorpions, the largest arachnids, are among the oldest animals on the earth, and are represented by 1500 distinct species[1]. Scorpion venoms are a rich source of neurotoxic peptides which affect the Na^+,

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K^+, Cl^+ and Ca^{2+} channels of excitable membranes as well as contain proteolytic enzymes, protease inhibitors, amine oxidases and biological amines[2-7]. Although about 100,000 different peptides are surmised to exist in all the scorpion species, only a small number of these polypeptide toxins, about 200 distinct polypeptides from 30 different species, have been described and published to date[8]. Scorpion venom toxins produce systemic responses such as hypertension or hypotension, hypothermia, tachycardia, tachypnea, sialorhoea, myocarditis, high white blood count, pancreatitis, hyperglysemia, hyperamylasemia, anxiety, neurotoxicity, coagulation disorders, increase in release of catecholamines, bradykinin, prostaglandins, interleukin-1 (IL1), interleukin-6 (IL6), nitric oxide (NO), α-1 antitrypsin, tumor necrosis factor-δ (TNF-δ), and cause some respiratory problems following envenomation[9-14].

Mesobuthus gibbosus (Buthidae), is distributed across the western side of Anatolia and throughout Anatolia (Turkey) except for the Black Sea shore line in northern Turkey[15,16] . It poses a real epidemiological problem in Turkey, in terms of fatal stings, especially among children. No data has been found about the biological active peptides of *Mesobuthus gibbosus*.

Acetylcholinesterase (AChE, EC.3.1.1.7) is an important regulatory enzyme that plays a critical role in cholinergic transmission by hydrolyzing the excitatory transmitter acetylcholine (ACh). AChE belongs to the cholinesterase family which also includes butyrylcholinesterase (BChE, EC.3.1.1.8) which hydrolyzes a variety of xenobiotics. These distinct enzymes possess differences in their tissue distribution, kinetic properties, specificity for substrates and selective inhibitors[17,18]. The catalytic center of AChE was traditionally considered as composed of an esterase subsite and an anionic subsite. The esterase subsite contains the active serine residue which is the target of irreversible organophosphorous and carbamate inhibitors, while the anionic subsite is responsible for the correct positioning of the substrate in the active site, which is the target of positively charged reversible inhibitors. "Anionic peripheral" site, which is about 2 nm from the catalytic sites is the target of snake venom toxins and strong reversible bis-quarternary inhibitors[19,20].

Previous reports have indicated that spider, snake and some scorpion venoms contain significant amounts of AChE and BChE activities which have been shown to have strong immunological similarities and to possess very similar enzymatic properties[21-24]. However, there is little data in the literature have been detected about the AChE or BChE inhibitory activities of the these venoms[25].

The aim of the present study was to isolate and purify the neurotoxic peptides of *Mesobuthus gibbosus* (Buthidae) and to investigate the in vitro cholinesterase inhibitory activities of the crude venom and its peptides on the AChE and BChE enzymes of human plasma and erythrocytes.

MATERIALS AND METHODS

Source of venom

Scorpion venom *Mesobuthus gibbosus* was obtained from captive scorpions by electric stimulation of the posterior abdomen of scorpion specimens collected in the area of south-west Anatolia (Manisa).

Crude venom was dissolved in water and centrifuged at 15,000 rpm for 20 mins. Mucous debris was kept at the bottom of the tubes and the soluble phase of the venom as supernatant was lyophilized and stored at –20 °C until used.

Sephadex G-50 gel filtration of the crude venom

Crude venom was applied to the Sephadex G-50 column (41 x 1.2 cm) which was equilibrated with 20 mM ammonium acetate buffer, pH 4.7, at a flow rate of 6 ml/h following the determination of its protein content by measuring the absorbance at 280 nm. Tubes containing 1 ml eluents were pooled according to the absorbance at 280 nm. Fractions (numbered as 1 to 5) were independently lyophilized and kept at -20°C. Fractions detected to be lethal on mice and *Musca domestica* larvae were subjected to the further purification by high pressure liquid chromatographic separation.

HPLC Purification

Venom samples were injected into RP-C18 analytical HPLC column (4.6 mm internal diameter x 250 mm length), an online 5 mm peptide trap (Nucleosil 100) connected to Shimadzu LC-10 ADVP, SPD-10 AVVP detector. A gradient was formed with the following conditions: with a linear gradient from solution A (0.1% TFA in water) to 50% solvent B (0.1 %TFA in acetonitrile) in 60 mins at a flow rate of 1 ml/mins (column pressure: 1300 psi). Fractions corresponding to the peaks which appeared were collected manually into tubes by following the UV trace. The absorbance at 214 nm was plotted as a function of time. The retention times for each peak are indicated above. The chromatographic analyses and columns were run at 25°C.

Bioassays

Mice were purchased from Hacettepe University Animal Housing Facility. Purified and collected fractions were tested for in vivo toxicity on 20±2 g C57/BL6 mice. Mice were anesthetized with diethylether, injected in the left cerebral ventricle with 1ml of sample dissolved in a solution of bovine serum albumin (BSA) (0.25 mg/ml in 0.9% NaCl) and placed in glass jars for observation. Control animals injected with BSA did not show any symptoms when recovering from anesthesia. The following signs of toxicity were assessed: excitability, salivation, trembling of the legs and body, jerking of the limbs, loss of ability to walk and death. Appearance of symptoms was noted continuously during the first hour of injection and was monitored at regular intervals for 24 h or until death. "Non-toxic" indicted those mice in which the injection did not cause symptoms of intoxications, similar to injections of saline. "Toxic" indicated demonstration of any of the symptoms mentioned above. "Lethal" indicated death after demonstration of some or all of the above symptoms.

These fractions were also tested on *Musca domestica* larvae, which were obtained from Hacettepe University, Department of Biology, Ecology Section. Five larvae were used per fraction. A volume of 1 ml was injected into the abdominal segment. Observation of contraction and/or paralysis and death was noted at 5 mins, 15 mins, 60 mins and 24 h post-injection.

The maintenance of the animals and the protocols used in this study were approved by the Ethics Committee of Laboratory Animals in Hacettepe University (68-1, 2001).

PAGE and SDS-PAGE analysis of the crude venom and its chromatographic fractions

Polyacrylamide gel electrophoretic (PAGE) and sodium dodecylsulfate polyacrylamide gel electrophoretic (SDS-PAGE) analyses of the crude venom and its chromatographic fractions were determined according to the method of Laemmli[26] under reducing or non-reducing conditions. Polyacrylamide gel electrophoresis was performed in 10% resolving gel prepared in 1.5 M Tris-HCl buffer, pH 8.8, and in 3.9% stacking gel prepared in Tris-HCl buffer, pH 6.8 . In each run 10 ml of sample was taken from the lyophilized stocks and diluted as 1:1 with the sample buffer consisting of 0.5 M Tris, pH 6.8, glyserol and 0.05% Bromophenol Blue. 10 µl of diluted sample and protein molecular weight standard marker were then loaded into the wells. Electrophoresis was performed at a current voltage of 200 V for 45 mins at room temperature in Bio-Rad Mini-Protean® II Dual Slab Cell System.

Polyacrylamide gel in the presence of 15% SDS was used to estimate molecular weights of the peptides and also as a criterion of purity. SDS-PAGE was carried out in 15% separating gel and in 3.9% stacking gel. In reducing conditions, samples diluted in sample buffer which contained 2-mercaptoethanol were heated at 95°C for 4 mins before being loaded into the wells. After electrophoresis, gels were stained in 0.1% Coomassie Blue R-250 prepared in 40% methanol and 10% acetic acid for 1h. Gels were destained in 10% acetic acid and 40% methanol for 1-3 h to visualize the protein bands.

Molecular weights of the samples were determined

with the aid of the calibration curve obtained by plotting log molecular weight versus relative mobility for a group of SDS-PAGE standard proteins.

Preparation of the human plasma and erythrocyte lysates

Sodium citrated peripheral blood was obtained from the Blood Bank of Hacettepe University Hospital. Plasma was prepared by centrifuging the blood at 1400 x g for 10 mins.

Erythrocyte lysates were prepared from the human peripheral blood according to the method of Ceballos-Picot[27]. In short, erythrocyte pellets were obtained from 100 ml of blood by centrifuging at 500 x g for 15 mins at room temperature following the determination of hemoglobin content of the blood sample. The plasma and buffy coat were removed, and the erythrocytes were washed twice in 100 ml of saline and stored at –80°C until used. Lysed erythrocytes were prepared by freezing and thawing twice and by addition of three volumes of ice-cold distilled water. Hemoglobin contents of the lysates were determined by the standard cyanomethemoglobin method[28]. Hemoglobin contents of the lysates were standardized as 0.12-0.14 mg/ml.

Determination of cholinesterase activities

AChE and BChE activities of human plasma and erythrocyte lysates were determined by the method of Ellman[29] with some modifications.

For the determination of AChE activity, hydrolysis rates were measured at various acetylthiocholine concentrations in 2 ml assay medium (0.02-0.60 mM) with 100 mM phosphate buffer, pH 7.5, and 1 mM dithiobisnitrobenzoic acid (DTNB) at 25°C. 100 µl human plasma or erythrocyte lysate (5-20 µg protein) was added to the reaction mixture and preincubated for 3 mins. The total cholinesterase activity of the plasma was determined in absence and presence of ethopropazine hydrochloride (specific BChE inhibitor) in order to determine the specific AChE or BChE activities by using their specific substrates. 10 µl of 0.1% quinidine sulfate was added to the medium to inhibit the plasma esterase artifacts when erythrocyte lysates were used as enzyme source. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm. The extinction coefficient of the dianion was 1.36 x 10^4 $M^{-1}cm^{-1}$. One Ellman unit was defined as the amount of enzyme producing an absorbance increase of 1 unit at 412 nm (Shimatzu UV 160-A) at 25°C, in 1 min., in 1 ml of medium.

For BChE activity measurement, butyrylthiocholine was used as substrate (0.02-0.60 mM).

Incubation of human plasma and erythrocyte lysates with the crude venom and its chromatographic fractions

Plasma and erythrocyte lysates at mean protein content of 5-20 µg/ml were treated with the crude venom and its chromatographic fractions in the concentration ranges of 0.10-1.00 mg venom protein/ml for 60 mins at 37°C before adding to the assay medium.

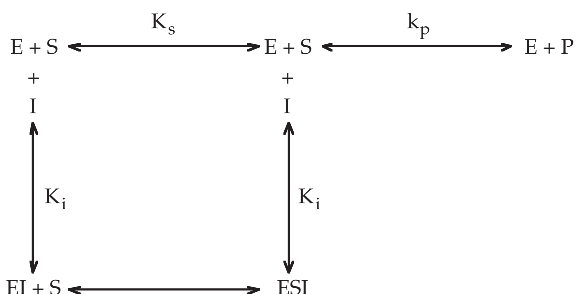
Reversibility of the inhibiton of plasma and erythrocyte cholinesterases by the venom and its chromatographic fractions was assessed by dilution. Enzyme samples (10 times the final concentration) were incubated for 60 mins at 37°C with different concentrations of the inhibitors. The samples were then diluted 10 times into the assay mixture. A parallel experiment was carried out where the enzyme (10 times the final concentration) was incubated for 60 mins at 37°C with an equivalent amount of water. The samples were then diluted 10 times into the assay mixture containing the same final concentrations of the inhibitors. Both sets were assayed for AChE and BChE activities.

Analysis of the kinetic data

The kinetics of the interaction of venom and its peptides with cholinesterases were determined by using the Lineweaver-Burk double reciprocal plot, by plotting 1/v against 1/s analyzed over a range of substrate concentrations (0.02-0.60 mM)[30]. Inhibition constants were obtained according to the scheme

previously decribed[31]. The reciprocal form of the velocity equation for the scheme below was expressed by the following equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)$$



K_i values were calculated from replots of reciprocal plot slopes or Y-intercepts versus inhibitor concentrations. In pure noncompetitive inhibition, the slope of the reciprocal plot in the presence of inhibitor is a linear function of [I]. Kinetic data was analyzed using Systat (version 5.0) package program.

RESULTS AND DISCUSSION

Chromatographic separation of *Mesobuthus gibbosus* soluble venom in the Sephadex G-50 column gave reproducible results. The value for the total protein recovery in the column was found as 89.36% (Table 1). Sephadex G-50 gel chromatography of the crude venom gave 5 fractions as indicated by numbers 1-5 in Figure 1. Only fractions 4 and 5 were found to be lethal on mice and Musca domestica larvae. These fractions were subjected to PAGE and SDS-PAGE analysis (Figures 2a and 2b). Crude venom gave 16 bands in PAGE and 19 bands in SDS-

Table 1. Protein contents and recoveries of the crude venom of Mesobuthus gibbosus and its chromatographic fractions.

Column	Fraction	Protein content mg/ml	Recovery (%)
Sephadex G-50	Soluble venom	49.00	100
	Fraction 1	4.50	6.56
	Fraction 2	7.17	13.72
	Fraction 3	13.45	30.50
	Fraction 4	10.60	22.25
	Fraction 5	8.00	16.33
Protein recovered		43.72	89.36

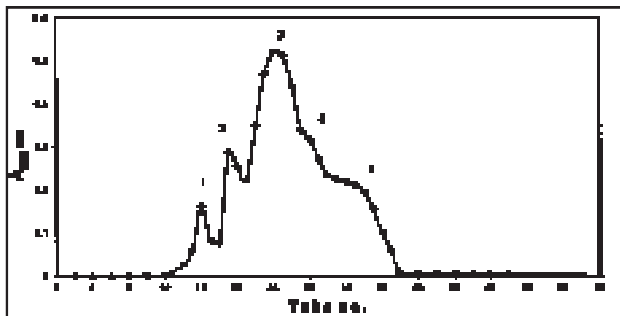


Figure 1. Sephadex G-50 filtration of crude venom of *Mesobuthus gibbosus.* The soluble venom (49 mg protein/ml) was applied to the column (41x 1.2 cm) which was equilibrated with 20 mM ammonium acetate buffer, pH 4.7, and eluted with the same buffer at a flow rate of 6 ml/h. Fraction volume was 1 ml.

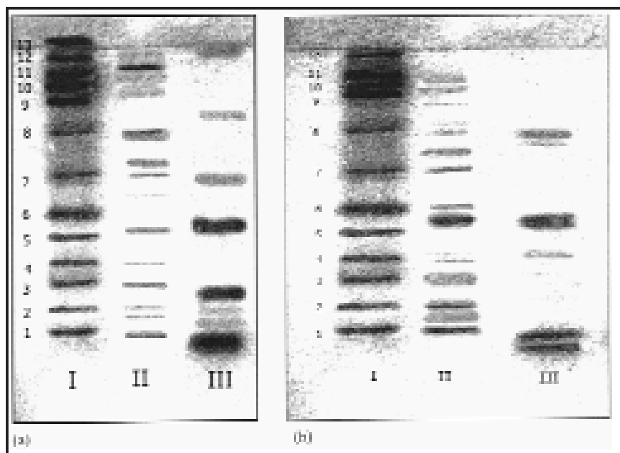


Figure 2a. Polyacrylamide gel electrophoretic (PAGE) pattern of the crude venom of *Mesobuthus gibbosus* and its chromatographic fractions. Lane I corresponds to the molecular weights of the known proteins ; Lane II corresponds to the crude venom; and Lane III corresponds to the fraction 5 obtained from the Sephadex G-50 filtration of the venom. In Lane I: 1:aprotinin 6.5 kDa; 2:α-lactalbumin 14.2 kDa; 3:trypsin inhibitor 20 kDa; 4:trypsinogen 24 kDa; 5:carbonic anhydrase 29 kDa; 6:glyceraldehyde-3-phosphate dehydrogenase 36 kDa; 7:ovalbumin 45 kDa; 8:glutamic dehydrogenase 55 kDa; 9:albumin 66 kDa; 10:fructose-6-phosphate kinase 84 kDa; 11:phosphorylase b 97 kDa; 12:β-galactosidase 116 kDa; 13:myosin 205 kDa.

Figure 2b. Sodium dodecylsulfate polyacrylamide gel electrophoretic (SDS-PAGE) pattern of the crude venom of *Mesobuthus gibbosus* and its chromatographic fractions. Lane I corresponds to the molecular weights of the known proteins ; Lane II corresponds to the crude venom; and Lane III corresponds the fraction 5 obtained from the Sephadex G-50 filtration of the venom. Lane I: 1:aprotinin 6.5 kDa; 2:α-lactalbumin 14.2 kDa; 3:trypsin inhibitor 20 kDa; 4:trypsinogen 24 kDa; 5:carbonic anhydrase 29 kDa; 6:glyceraldehyde-3-phosphate dehydrogenase 36 kDa; 7:ovalbumin 45 kDa; 8:glutamic dehydrogenase 55 kDa; 9:albumin 66 kDa; 10:fructose-6-phosphate kinase 84 kDa; 11:phosphorylase b 97 kDa; 12:β-galactosidase 116 kDa; 13:myosin 205 kDa.

PAGE with molecular weights of 6,500-200,000 Da in the absence of reducing agent. When crude venom was treated with β-mercaptoethanol, some of the bands collapsed to give faster bands in SDS-PAGE (Figures 2a and 2b, Lanes II). Fraction 4 was found to consist of 15 peptides with molecular weights of 6,500-97,000 Da (data was not shown) while fraction 5 consisted of 6 peptides with molecular weights of 6,000-55,000 Da in PAGE (Figure 2a, Lane III). SDS-PAGE of fraction 5 showed 10 bands, respectively, in the absence of reducing agent (Figure 2b, Lane III). Although fractions 4 and 5 appeared as the main sources of the toxic venom peptides of

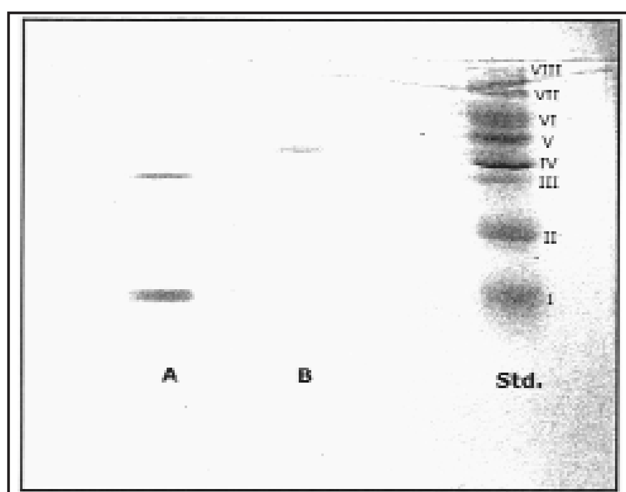


Figure 3. Sodium dodecylsulfate polyacrylamide gel electrophoretic (SDS-PAGE) pattern of the toxic fraction obtained from HPLC separation. Toxic fraction which was applied to the C-18 column was previously obtained as the toxic fraction 5 from the Sephadex G-50 filtration of the crude venom of *Mesobuthus gibbosus*. Lane std. corresponds to the molecular weights of the known low molecular weight proteins shown below. Lane A corresponds to the peptides with the treatment of the sample with mercaptoethanol; and Lane B corresponds to the the toxic peptide without treatment with mercaptoethanol; I. Aprotinin, mw 6,500 Da; II. α-Lactalbumin, mw 14,200 Da; III. Trypsin inhibitor, soybean, mw 20,000 Da; IV. Trypsinogen, bovine pancreas, mw 24,000 Da; V. Carbonic anhydrase, bovine erythrocytes mw 29,000 Da; VI. Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle, mw 36,000 Da ; VII. Ovalbumin, chicken egg, mw 45,000 Da; VIII. Albumin, bovine serum, mw 66,000 Da.

Mesobuthus gibbosus, fraction 5 was suspected to contain some ion-channel blockers or cholinesterase inhibitor peptides since it has been previously reported that scorpion venoms contain short-chain toxins (molecular weights ranging between 3,500 and 7,800 Da) which act by blocking various voltage-dependent K^+ channels[32,33]. Considering the fact that material appeared to be chromatographically pure does not necessarily mean that it is homogeneous and two different peptide entities might elute together or a major absorbance peak might contain more than one component, fraction 4 and 5 were independently applied to the C 18 HPLC column to obtain a further separation of their peptides. Fraction 4 gave 13 peaks, but none of them was found to be toxic on mice or on *Musca domestica* larvae. Seven peaks were obtained from the HPLC separation of the fraction 5 (Figure 4) . The single peak which was indicated with an asterix (Fraction 5*) was found to be extremely lethal on mice and *Musca domestica* larvae.

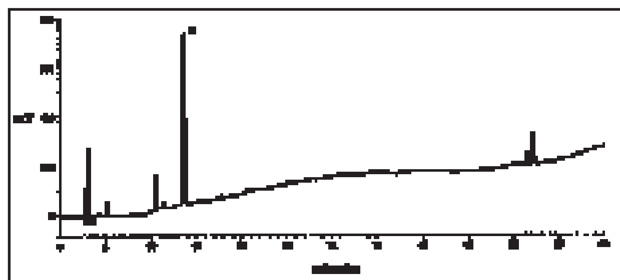


Figure 4. Venom sample was injected into RP-C18 Analytical HPLC column (4.6 x 250 mm), an online 5 m peptide trap (Nucleosil 100) connected to Shimadzu LC-10 ADVP, SPD-10 AVVP detector. A linear gradient from solution A (0.1% TFA in water) to 50% solvent B (0.1% TFA in acetonitrile) for 60 mins was obtained at 25°C. Flow rate was 1 ml/min (column pressure: 1300 psi). Fractions corresponding to the peaks which appeared were collected manually. The absorbance at 214 nm is plotted as a function of time. The retention times for each peak are indicated above.

Table 2 presents the biological activities of the crude venom and its chromatographic fractions. The results showed that crude venom and its peptide indicated as HPLC fraction 5* were significantly lethal both on mice and larvae, suggested that scorpion *Mesobuthus gibbosus* venom should be considered asmedically important.

SDS-PAGE pattern of fraction 5* (Figure 4) gave a single band of 28,000 Da in SDS-PAGE under non-reducing conditions (Figure 3, Lane B). Two bands were obtained with 6,200 and 22,000 Da after the

Table 2. Biological activity profiles of the crude venom and its fractions.

Fractions	Mice [a]	*M. domestica* larvae [b]
1. Crude venom	L	L
2. Sephadex G-50 gel filtration		
Fraction 1	NT	NT
Fraction 2	NT	NT
Fraction 3	NT	NT
Fraction 4	L	L
Fraction 5	L	L
3. HPLC separation		
Fraction 1	NT	NT
Fraction 2	NT	NT
Fraction 3	NT	NT
Fraction 4	NT	NT
Fraction 5*	L	L
Fraction 6	NT	NT
Fraction 7	NT	NT

[a] i.c.v. injection; 1.0 µl / mice
[b] postabdominal injection; 1.0 µl / 30 mg body weight
Toxic (T): Injected animals showing signs of poisoning recovered within 24 hours post-injection; Lethal (L): Injected animals dead; NT: Non-toxic.

treatment of the fraction 5* with β-mercaptoethanol (Figure 3, Lane A), suggesting that the pure toxic peptide seemed to consist of at least two chains possibly attached by disulfide bridges. However, amino acid composition of the corresponding peptide should be determined to clarify its actual molecular structure.

Determination of the biochemical properties and mode of action of the venom peptides has been of significant interest in recent years since scorpion neurotoxins with similar sequences but different binding properties can be used as powerful tools probing the structural aspects of toxin-target interactions[34]. The data indicating some neurotoxic effects of the scorpion venoms such as sensory, motor, cardiological and respiratory difficulties, antiepileptic and neurodepressant effects as well as local swelling, pain, inflammation and paralysis[35,36], together with the findings about the existence of some neurologic amines and proteases[37] in these venoms prompt us to investigate the cholinesterase inhibitory activities of the biologically active peptides of *Mesobuthus gibbosus.*

Anti-cholinesterase activities of crude venom and its chromatographic fractions were determined in human plasma and erythrocytes. K_m and V_{max} values of plasma total cholinesterase were found as 77.51±2.90 µM and 83.30±3.05 µmol/h/mg, respectively, when acetylthiocholine was used as substrate. Since it has been previously suggested that human plasma contained only BChE (3 mg/L) which is capable of hydrolyzing ACh[38], we determined the plasma cholinesterase activity in the presence of ethopropazine hydrochloride (spesific BChE inhibitor) to detect the remaining cholinesterase activity if presents. No cholinesterase activity was found in human plasma in the presence of ethopropazine indicating that the detected cholinesterase activity of the plasma originated from BchE which uses acetylcholine as substrate.

K_m and V_{max} values of the erythrocyte AChE activities were found as 130.56±7.25 µM and 200.10±6.52 µmol/h/mg, respectively, when ACh was used as substrate. BChE activity was found as 122.21±3.98 µM and 240±3.36 µmol/h/mg, respectively, in the human erythrocytes.

Crude venom of *Mesobuthus gibbosus,* fraction 5 eluted from Sephadex G-50 column and the fraction 5* obtained from HPLC separation of fraction 5 inhibited cholinesterase activities of human erythrocytes. Neither crude venom nor its chromatographic fractions inhibited BChE of human plasma. Data suggested that the venom peptide corresponding to the fraction 5* eluted from the C18 column was mainly responsible for the specific AChE inhibitory activity of the *Mesobuthus gibbosus* on human erythrocyte AChE.

K_i values obtained from the reciprocal plots for the inhibition of AChE by the crude venom and its peptides are shown in Table 3. Since the K_m value was unchanged and the V_{max} was decreased, it was suggested that the inhibition of human AChE with the venom peptides was noncompetitive[31] (Figure 5). Reversibility assays showed that the inhibition is completely reversible. Since replots of $slope_{1/s}$ vs $1/V_{max}$ were found to be linear, the inhibition was reported to be pure noncompetitive (Figure 5, inbox).

Table 3. Kinetic data for the inhibition of human erythrocyte AChE with *Mesobuthus gibbosus* venom peptides.

Inhibitor	K_i value for the inhibition of the AChE activity in human erythrocytes (mg venom protein/ml)*
Crude venom	0.90±0.022
Fraction 5 obtained from the Sephadex G-50 gel filtration	0.86±0.017
Fraction 5* obtained from the HPLC separation	0.78±0.018

* Values represent the mean±SD of six separate experiments

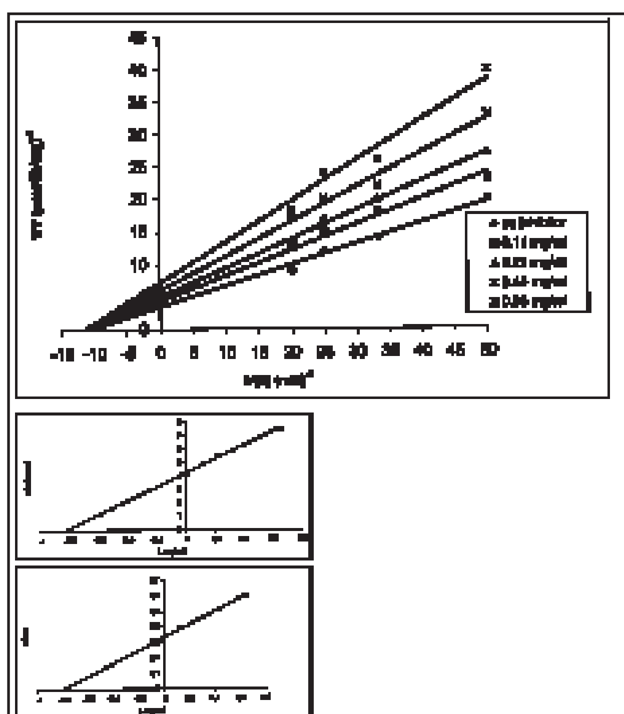


Figure 5. Kinetic analysis of human erythrocyte AChE inhibition by the venom fraction 5* in human plasma after 60 mins of incubation with the enzyme. The graph shows double-reciprocal plots of the AChE experiments in the absence and presence of the inhibitor. Replots of slopes and Y-intercepts vs [I] are shown in insets.

AChE is one of most efficient enzymes known with hydrolysis of its natural substrate reaching diffusion-controlled limits due to its three-dimensional structure. The X-ray crystal structure of AChE revealed that substrate molecules had to penetrate into a long and narrow gorge of 20 Å deep to be hydrolyzed. This gorge contained a peripheral site at the surface and an acylation site at the base where the substrate acyl group was transferred to residue Ser^{200}. Some ligands bind specifically to acylation or to the peripheral site, and ternary complexes with different ligands bound to each site can be formed. Ligands specific for the peripheral site include snake venom neurotoxin fasciculin (Fas) which was previously reported to be a potent AChE inhibitor[39,40]. Studies on the AChE inhibitory activity of Fas and molecular modelling of the AChE/Fas complex indicated that hydrophobic residues of Fas molecule could have contact with AChE by preventing substrate entry to the catalytic site or by causing some alterations in conformation of the active site[40]. Our data showing the apparent reversible and non-competitive AChE inhibitory behavior of neurotoxic peptide of scorpion *Mesobuthus gibbosus* venom suggested that this peptide might interact with the enzyme at a region close to the peripheral site of the enzyme molecule which could prevent the proper positioning of the catalytic center. Lack of BChE inhibitory activity of the *Mesobuthus gibbosus* venom or its peptides might be a result of the differences in the catalytic course of these enzymes as well as the different dimensions and the microenvironment of their active site gorges.

The present study indicated that scorpion *Mesobuthus gibbosus* venom contained peptides with specific cholinesterase inhibitory activities which might be responsible for some of the neurotoxic effects of the venom on animals and humans. However, more information about the interaction type between the peptides and the active/binding sites of AChE and further studies on the determination of the molecular structure of the inhibitor peptides are needed to clarify the mechanism of the inhibition.

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