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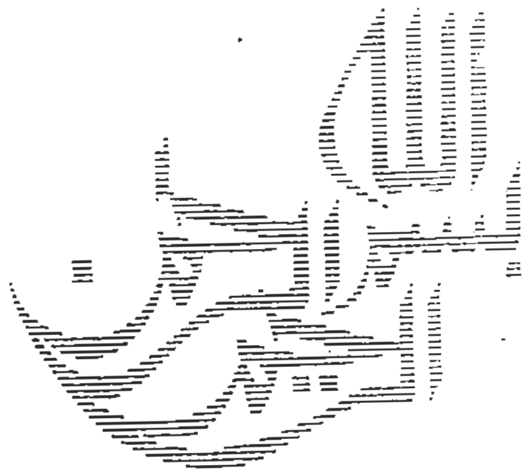

In the Name of Allah,
the Beneficent, the Merciful

TO,

my mother and
brother whom I love most.

AMINO ACID SEQUENCE OF MAJOR NEUROTOXINS FROM *BUTHUS SINDICUS* (SCORPION) VENOM

THESIS SUBMITTED IN FULFILMENT

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SUMMARY

The present study on the venom of *Buthus sindicus* was undertaken to characterize scorpion venom proteins. The separation of scorpion venom components was carried out by various Chromatographic techniques. The pure proteins/peptides were subjected to enzymatic degradation and separated on HPLC Vydac C-18 column. The amino acid compositions of proteins/peptides were determined after hydrolysis.

Scorpion venom were characterized on the basis of homology and biological activity. The major components of the venom consist of a novel peptide, insect toxin and neurotoxin of 28, 35 and 65 amino acid residues respectively. These were sequenced by manual DABITC method and gas phase sequencing.

The fatty acids of scorpion body were also determined by GCMS. LD_{50} were checked on *Rana tigrina* muscles and, on flies (*Musca domestica*) and cockroaches (*Blattella germanica*). The results identify the proteins BuS I and BuS II as a member of "four disulfide core proteins" and extend suggestions of relationship with various peptides involved in binding or interacting functions.

1.0 INTRODUCTION

1.1 SCORPION

Scorpions are the most venomous arachnids [1]. They are regarded as the oldest terrestrial arthropods as their fossils could be found in the Silurian period [2]. Very little has changed in their general morphological features and today they are regarded as "living fossils". They are largely found, at present in the tropical, subtropical and warm temperate parts of the world [3]. Although often thought of as desert dwellers, they are abundantly found in arid and semi-arid zones, living at altitudes that range from sea level to 5000 meters [4].

1.2 CLASSIFICATION

Scorpions are nocturnal animals [5], remaining in holes and crevices during the day, hiding under wood, stone and in the debris. They are broadly divided, on the basis of their habitates into three categories: (i) burrowing (Psammophilous or Pelophilous), (ii) rock dwelling (Lithophilous) and (iii) arboreal [4]. Buthus and Heterometrus belong to the burrowing scorpion category. They live in small pit like burrows [6]. Buthid burrows are difficult to locate, as the burrows are very small in diameter and not easily traceable [7].

Some Buthoid species live in colonies. Their members are very active

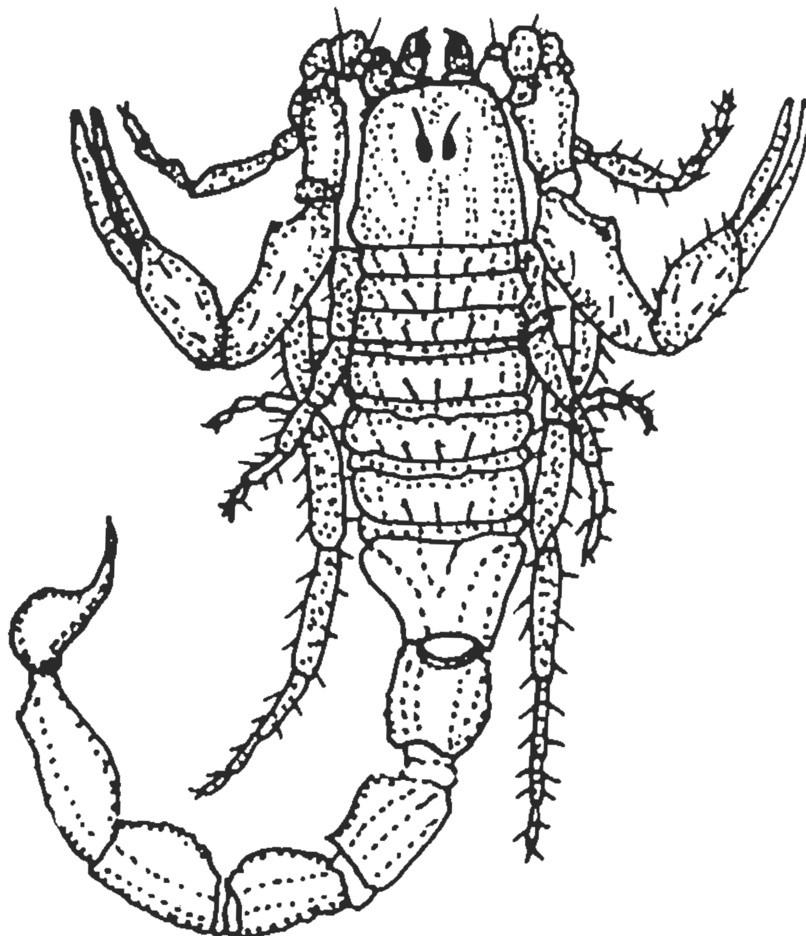
and retreat quickly, if disturbed. Most scorpions avoid water, some however, live among plants near the sea-shore, moving about in the intertidal zone and able to remain under tidal water for a short time. Scorpions, being inhabitants of warm climates, become sluggish in cold weather, but can withstand freezing temperature for several weeks. Some scorpions, like Panidus palamnaeus are forest dwellers, while others e.g. Buthus androctonus and Buthus sindicus are inhabitants of dry and desert regions. Many scorpion species require humid environment and live in tropical rain forests [4].

1.3 ORDER

The order Scorpionida [8], comprises six or seven families, of which Buthidae [7], is the most important having more than 600 species [9,10]. It include Buthus sindicus and Buthus occitanus, the common yellow scorpions of Pakistan and the Mediterranean region, and Androctonus australis, the fat-tailed scorpion of North Africa, while the species of Centrurus and Tityus are neotropical.

1.4 EXTERNAL FEATURES

Different species of scorpions differ from one another in size, colour, distribution and morphological features [11], but the general morphology is more or less similar. A scorpion has a long narrow



SCORPION (BUTHUS SINDICUS)

body, which is dorso-ventrally flattened [12]. The size varies from species to species. The average size of Buthus sindicus is about 5 cm in length. The colour is variable, usually corresponding with the habitat of the animal; which is generally blackish dorsally and slightly light coloured ventrally. The species, living in tropical jungles are of shining black colour, whereas the species Buthus sindicus, found in the Sind province (Pakistan), is pale yellow in colour, having much darker dorsal than the ventral surface. The elongated body is divided into two major regions [12]. (i) Prosoma, (ii) abdomen, the terminal part of which in the living animal is habitually carried over the back constituting the tail, at the end of which the sting is situated. The carapace bears a pair of large eyes about its middle and several pairs of smaller eyes on the antero-lateral margin. The mouth is situated on the anterior end of prosoma towards the ventral side between the cephalic appendages. On its ventral aspect, a lobe which overhangs it in front is the labrum. On each side of the mouth is a three jointed appendages, the chelicera, which is terminated by a chela. Behind these are the very large pincer-claws. Following the pincer-claws are four pairs of walking legs, the basal segments of the first two pairs of walking legs are modified so as to perform, to some extent, the function of jaws [12,13].

1.5 SENSORY ORGANS

Scorpions have three types of sense organs, (i) sensillae, (ii) pectines

and (iii) eyes. (i) Sensillae are sensory setae and hairs, which are generally much longer on the legs and tail. The sensory setae are tactile organs of scorpion. Each of the seta is connected to a nerve fibre. The hairs are extremely sensitive to touch. (ii) Pectines are a pair of modified appendages, which are attached to the sternum of second segment of mesosoma and joined to each other in the middle. They are sensitive, either to touch or smell. (iii) Eyes in scorpion consists of four pairs; a pair of median eyes and three pairs of lateral eyes. Median eyes of scorpion are intermediate between compound and simple eye of insects. Both lateral and median eyes are sensitive to light but are incapable of forming images, whereas lateral eyes are like simple eye of insects [3].

1.6 POISON GLAND

There are two poison glands, one on each side of the middle line of the telson, which is a post-anal sclerite. Each gland has strong compressor muscles from the mesial and dorsal sides. When these muscles contract. the poisonous secretion is ejected from the gland. A duct from each gland runs along the aculeus and opens separately just before its extreme end. The venom apparatus of the scorpion consists of a balbous vesicle drawn out to a sharp, curved stinger. The extent of the muscular contraction and consequently the amount of venom ejected seems to be under the control of the animal [14].

The venom is ejected through an opening near the tip of the stinger. The venom is a tasteless, odourless and faintly acidic viscous fluid. The first drop is always clear and colourless and dries to a transparent vitreous pellicle which appears faintly yellowish. The next two or three drops are clear and opalescent and the rest is white and opaque. The venom, if dried in vacuum, forms a white amorphous brittle mass which breaks into minute irridescent flakes.

1.7 POISONING SYMPTOMS

The symptoms caused by scorpion poison of the less virulent type consist mostly of sudden sharp pain, followed by numbness of the limbs and local swelling [15,16]. It causes severe local irritation, which results in redness and burning pain radiating from the site. It is generally accompanied with headache, giddiness, nausea, profuse perspiration and muscular cramps, and may be followed by unconsciousness. Though the symptoms generally manifest themselves within 24-48 hours, neurological effects may persist upto one week. Children are worst affected and may die from pulmonary oedema, whereas the mortality in adults is negligible [17]. The poison has a haemolytic action, destroying red blood corpuscles and also contains adenosine tri-phosphate in large quantities.

1.8 MODE OF ACTION OF SCORPION-TOXINS

The venom of the scorpion from the family Buthidae is mainly composed of basic peptides. Some of these have been identified as neurotoxins [18]. The scorpion venom acts on exposed fibers or on muscles directly or through motor nerves and causes neuromuscular intoxication. The venom interact with nerve tissue at the exposed presynaptic terminals at the neuromuscular junction. The resulting muscular twitching and fibrillation is attributed to the release of a transmitter substance. Scorpion neurotoxins depolarize the excitable membranes resulting in an increase in the sodium permeability of the resting membrane, thereby reducing the rate and amount of sodium inactivation [19]. The administration of the venom of Mexican scorpions, in mammals, showed spontaneous muscular twitches and fibrillations due to an effect on the motor neurons from the spinal cord or a direct effect on neuromuscular junctions [20-22]. The slow contractory effect of scorpion venom on the smooth muscle is due to the activation of para-sympathetic post-ganglionic nerves [23].

The mode of action of the venom *Leiurus quinquestriatus* quinquestriatus and that of *Buthus tamulus* have been studied on isolated frog fiber and squid giant axon preparation respectively [24]. These studies have shown that under voltage clamp condition, it provoke

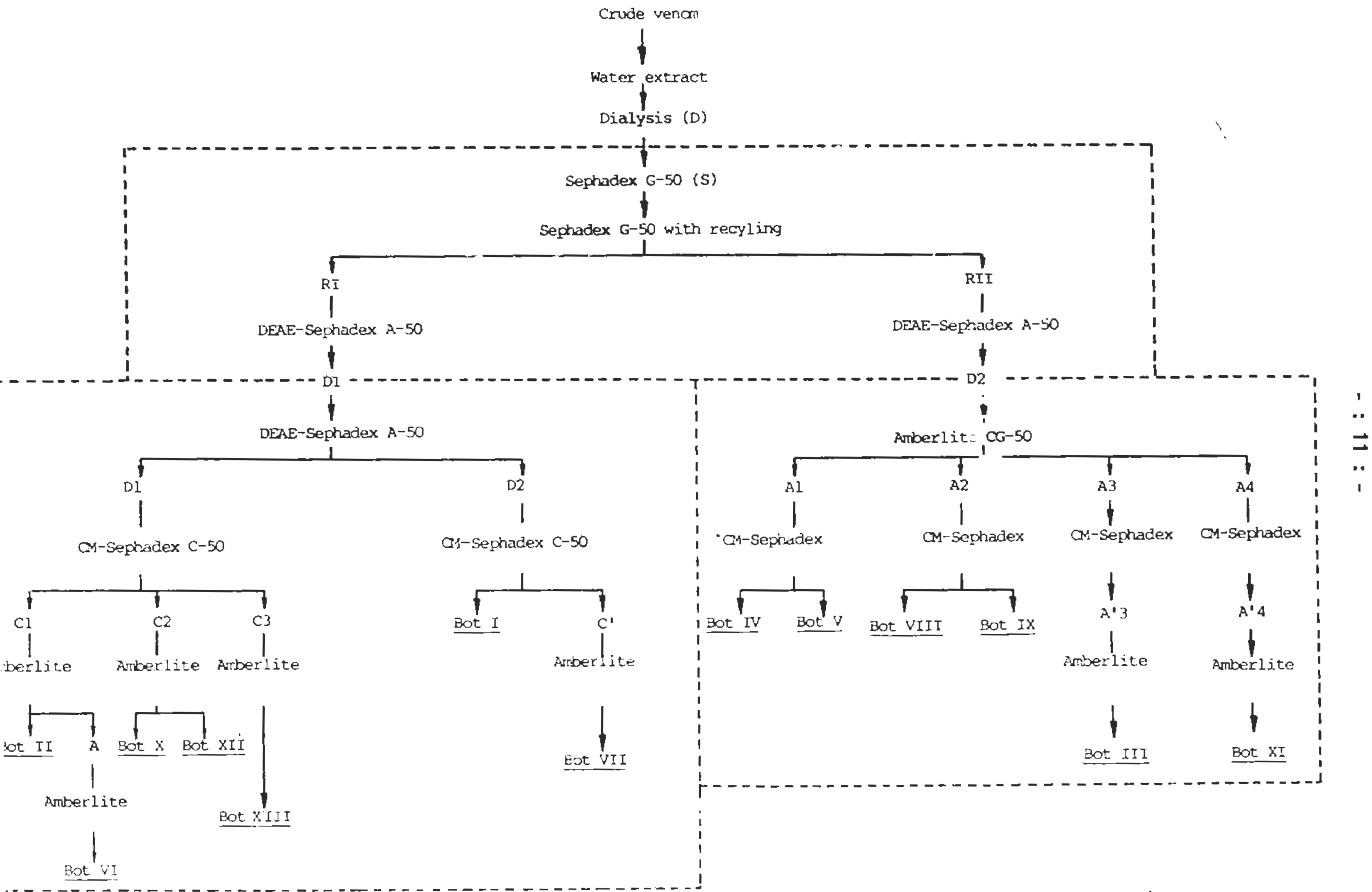
inhibition of the sodium ion current inactivation and suppression of the K^+ currents. Calcium ions were found to antagonize the change of membrane properties brought about by scorpion venom [25]. The purified venom of Androctonus australis Hector affects both the closing of the Na^+ channels, prolongation of the action potential with a typical marked plateau, retardation and partial blockage of sodium inward current inactivation and all opening of the K^+ channels (decrease the steady-state potassium current). The neuromuscular junction is highly sensitive to the action of scorpion venom [26-30] and it has been shown that intact nerve trunks are relatively resistant [21,31,32]. The scorpion venom also effect respiratory system [28,33] and the homeostatic mechanism of the body.

The pharmacological effects of scorpion venom on heart is reported to be bradycardia (cholinergic phenomenon), followed by tachycardia (adrenergic phenomenon) [34-37]. Although the sympathetic post-ganglionic nerves are initially affected by the venom, actylcholine is mainly responsible for both bradycardia and tachycardia [38].

1.9 PURIFICATION OF SCORPION VENOM

Several methods have been employed for the purification of venom proteins for the structural studies. The crude venom of scorpion is extracted in water and centrifuged. It is purified by gel filtration

Fig. 1.1: Purification of scorpion venom (Buthus occitanus tanetanus).



column and recycling on Sephadex G-50, followed by one or two ion exchange columns of a variety of packing material; such as carboxymethylcellulose, Amberlite IRC-50 or CG-50, DEAE Sephadex A-50 or Bio-Rex-70, (Fig. 1.1) [25,39-43].

1.10 STRUCTURE OF VENOM PROTEINS

1.10.1 Primary Structure

Primary structure of scorpion venom protein provide information about its toxicity as well [44-46].

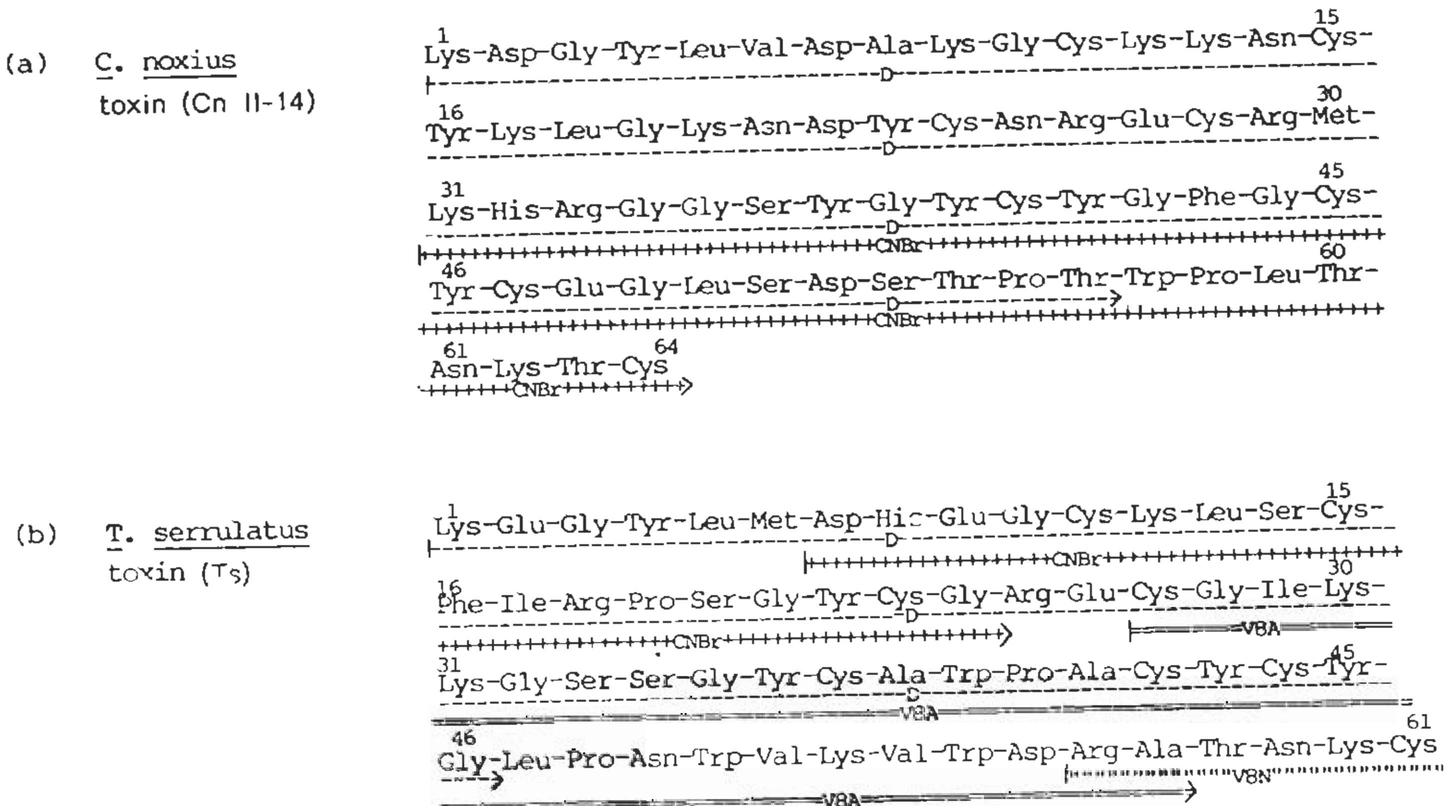
The toxins are classified on the basis of similarities in the primary structure. The sequences were aligned with respect to the position of half cystine residues, (Fig. 1.2) [47,48]. These venom proteins are composed of 28-73 amino acid residues in a single chain [25,45,49-52]. Most of the toxins have 60-65 amino acids [53]. However in a chactoid species [54], small neurotoxins consisting of 28-36 residues have been characterized [49,55], but their sequences have not been determined. Complete primary structure of toxins II-14 from the Mexican scorpion <u>Centruroides noxius</u> Hoffmann and toxins Y from the Brazilian scorpion <u>Tityus serrulatus</u> Lutz and Mello have been reported by L.D. Possani et al. [56]. Amino acid sequence of residue 7-24 was carried out by liquid phase sequencer upto 55 residue

Fig. 1.2: Primary Structure of different scorpion's toxin.

Toxin	Sequence (1 10 20 30 40 50 60 70)	Reference
AaH1	KRDGYIVYPNN-CVYHCVPP-----CDGLCKKN--GGSSGSCSFLVPSGLACWCKDLPDNVPIK---DTSRK-CT	25
AaH1'	KRDGYIVYPNN-CVYHCIPP-----CDGLCKKN--GGSSGSCSFLVPSGLACWCKDLPDNVPIK---DTSRK-CT	25
AaH1"	KRDGYIVYPNN-CVYHCVPP-----CDGLCKKN--GGSSGSCSFLVPSGLACWCKDLPDNVPIK---DTSRK-CTR	25
AaH III	VRDGYIVNSKN-CVYHCVPP-----CDGLCKKN--GAKSGSCGFLIPSGLACWCVALPDNVPIK---DPSYK-CHS	25
AaH II	VKDGYIVDDVN-CTYFC---GRNAYCNEECTKL--KGESGYCQWASPYGNACYCYKLPDHVRTK---GPGR--CH	25
Bot III	VKDGYIVDDRN-CTYFC---GRNAYCNEECTKL--KGESGYCQWASPYGNACYCYKVPDHVRTK---GPGR--CN	57
Bot XI	LKDGYIVDDRN-CTYFC---GTNAYCNEECVKL--KGESGYCQWVGRYGNACWCYKLPDHVRTV---QAGR--CRS	58
Lqq V	LKDGYIVDDKN-CTFFC---GRNAYCNDECKKK--GGESGYCQEASPYGNACWCYKLPDRVSIK---EKGR--CN	25
Amm V	LKDGYIIDDLN-CTFFC---GRNAYCDDECKKK--GGFSGYCQWASPYGNACWCYKLPDRVSIK---EKGR--CN	25
Be M10	VRDGYIADDKD-CAYFC---GRNAYCDEECKK---GAESGKCWYAGQYGNACWCYKLPDWVPIK-QKVSGK--CN	59
Lqq IV	GVRDAYIADDKN-CVYTC---GSNSYCNTECTKD--GAESGYCQWLGKYGNACWCIKLPDKVPIR---IPGK--CR	60
Bot 1	GRDAYIAQPEN-CVYEC---AQNSYCNDLCTKN--GATSGYCQWLGKYGNACWCKDLPDNVPIR---IPGK--CHF	57
Bot II	GRDAYIAQPEN-CVYEC---AKNSYCNDLCTKN--GAKSGYCQWLGRWGNACYCIDLPDKVPIR---IEGK--CHF	57
Bom III	GRDGYIAQPEN-CVYHCF-PGS-SGCDTLCKEK--GATSGHCGFLPGSGVACWCDNLPNKVPIV---VGGEK-CH	57
Css II	KEGYLVSKSTGCKYECLKLGDNDYCLRECKQQYGKSSGGYC-----YAFACWCTHLYEQAVVW--PLPNKT-CN	25
CsE I	KDGYLVEK-TGCKKTCYKLGENDFCNRECKWKHIGGSYGYC-----YGFFCYCEGLPDSTQTW--PLPNK--CT	62
CsE VI	KEGYLVKKSDGCKYDCFWLGKNEHCNTECKAKNQGGSYGYC-----YAFACWCEGLPESTPTY--PLPNK--CS	61
CsE V2	KEGYLVNKSTGCKYGCLKLGENEGCNKECKAKNQGGSYGYC-----YAFACWCEGLPESTPTY--PLPNK--CSS	61
CsE V3	KEGYLVKKSDGCKYGCLKLGENEGCNTECKAKNQGGSYGYC-----YAFACWCEGLPESTPTY--PLPNKS-C	61
AaH1T	KKNGYAVDSS-GKAPECL-L--SNYCNNQCTKVH-YADKGYCCLLS-----CYCFGLNDDKKVLEISDTRKSYCDTTIN	52

AaH Androctonus australis Hector; **Bot,** Buthus occitanus tunetanus; **Lqq,** Leiurus quinquestriatus quinquestriatus; **Amm,** Androctonus mauretanicus mauretanicus; **Be,** Buthus eupeus, **Bom,** Buthus occitanus mauretanicus; **Css,** Centruroides suffusus suffusus; **CsE,** Centruroides sculpturatus Ewing.

Fig. 1.3: Primary Structure of toxin Cn II-14 from C. noxius and toxin Ts from T. serrulatus.



(a) Toxin Cn II-14 from C. noxius. Residues were determined by sequential Edman degradation directly (D) and after cleavage with CNBr. (b) Toxin Ts γ from T. serrulatus. Residues were determined as in (a) and also by proteinase V8 cleavage at pH 4.0 (V8A) and pH 7.8 (V8N).

peptides, 7-61 obtained by CNBr cleavage of native toxin γ. Primary structures of scorpion toxins are shown in fig. 1.3 [52,57-62].

1.10.2 Secondary And Tertiary Structures

A particular amino acid sequence can favour one of several possible types of molecular organization to form secondary and tertiary structures [63]. The secondary and tertiary structures are determined by ORD (optical rotatory dispersion), CD (circular dichroism), X-ray diffraction and NMR (nuclear magnetic resonance) techniques. These techniques have permitted the determination of the main structural features of the scorpion toxins. It is generally believed that all scorpion toxins constitute a family of homologous polypeptides. The stabilization of toxin structure and function is dependent on the presence of several disulfide bridges [64-66].

1.11 STRUCTURE FUNCTION RELATIONSHIP

Three groups of neurotoxins are present in scorpions and all of them are capable of effecting sodium channels. Tetrodotoxin and saxitoxin block the transmembrane current of sodium ions and appear to bind with the ion-conducting subunit of the membrane. While the other group of toxins called alkaloid toxins : batrochotoxin, veratidine, aconitine, gray anotoxins activate the sodium channels and change

their ion selectivity. The neurotoxins which interact with the channel subunit, transport the sodium ions. Several subgroups of mammalian toxins were analyzed structurally, immunologically and functionally [67].

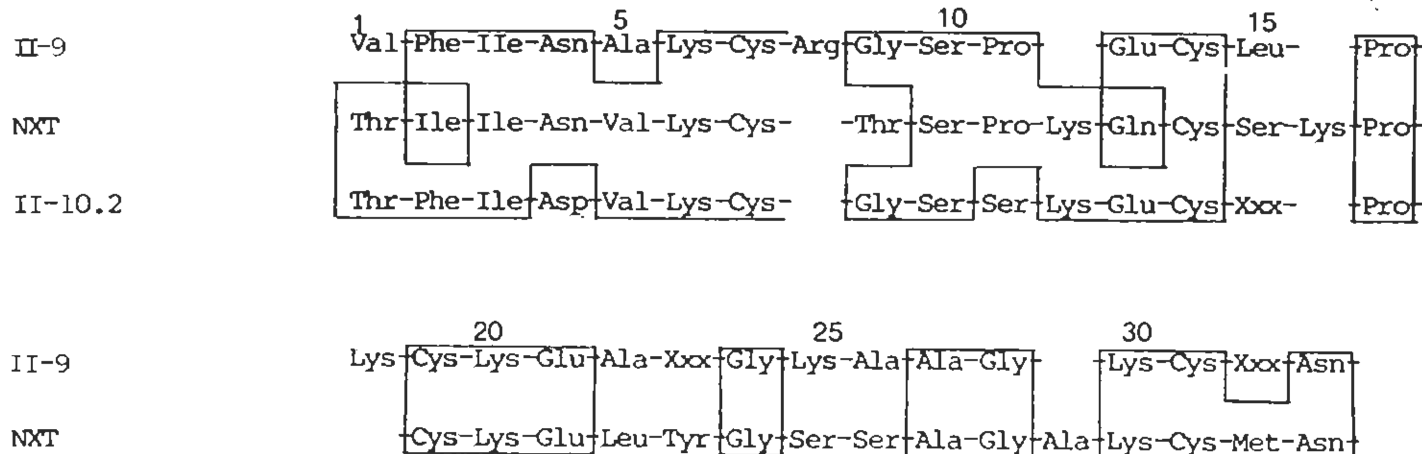
The pharmacological activity of crude venom from Buthus tamulus and its purified neurotoxins has been determined and both of them found to be biologically active. These toxins cause inhibition of Na^+ current inactivation and suppression of the K^+ current. These experiment were done on the isolated nerve fibre of the toad. Results of these experiments showed that toxins belong to the α-type. Studies on primary structure showed that at the 59th position arginine is present in Buthus occatinus tanetanus I & II, while arginine is replaced by valine in Buthus occatinus mardochei III. It is also suggested that lysine on position 58 is essential for the biological activity of the three most potent α-toxins [68,69], namely A. australis Hector I & II and Leiurus quinquestriatus quinquestriatus V. The replacement of lysine 58 by valine in the sequence of Buthus occatinus tunetanus XI results in complete loss of biological activity [69,70]. Methylation and reduction of one disulfide bridge coupled with acetylation of lysine and tyrosine residues of this toxin abolishes both toxin and antigenic activities. Modification of the tryptophan at position 38 of toxin II does not inactivate the toxin guanidination. Citraconylation of toxin II and toxin III from A. australis leads to complete loss

of toxicity, which is restored upon decitraconylation. Among the lysines of the molecules, the penultimate in the primary structure (lysine 56) of toxin II seems to be very important for toxicity [71]. Modifications such as substitutions, deletions or insertions of amino acids in the corresponding primary structures produce inactivation of the toxins or at least modulation of the function of these toxins at the molecular level. In fig. 1.4 the N-terminal sequence of three toxins are compared. The biological action of these toxins are very similar, they decreased the K^+ channel permeability reversibly and independent of polarization, without effecting the Na^+ channel properties [72,73].

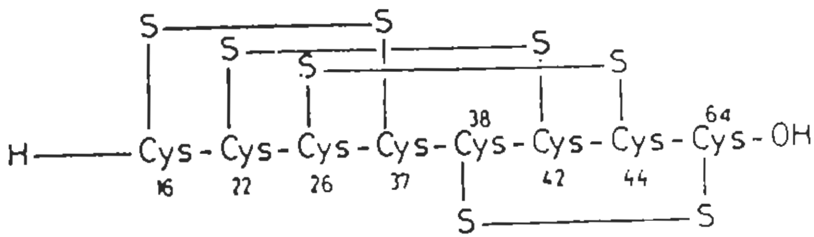
Four disulfide bridges are present in both mammal toxin II and insect toxin from the venom of Androctonus australis Hector (Fig. 1.5). Out of these four disulfide bridges, three are in homologous positions. While in the fourth, one of the two half cystines at position 12 in mammals toxin II, shifts to position 38 in the insect toxin. In mammal's toxins, two disulfide bridges have also been found in similar position of toxin I of the Androctonus australis Hector [74].

The disulfide bridges are conserved in all scorpion neurotoxins active on mammals. Therefore, it is believed that the change of position of one half-cystine residue in the insect toxin could induce conformational changes in the structure of the protein and this in turn effect

Fig. 1.4: Comparison of K^+ channel-blocking toxins.



The N-terminal sequence of toxin II.10.2 and noxius toxin (NTX) from C. noxius are compared with the N-terminal amino acid sequence of toxin II-9A from T. serrulatus. Cysteines have been aligned, and gaps are introduced accordingly. Residues that are invariant in the toxins are boxed. Positions labeled with X mean uncertainty in the sequence; for toxin II-10.2, position 15 is either tryptophan or leucine ; for T. serrulatus II-9A, position 23 is either isoleucine or phenylalanine, and position 32 is either valine or methionine [73].



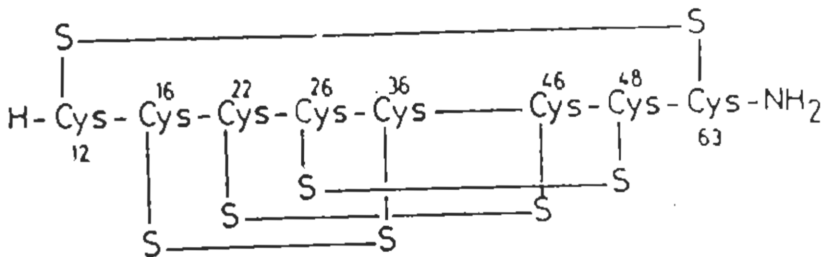


Fig. 1.5: Comparison of the position of the disulfide bridges in the insect toxin (top) and the toxin II (bottom) from the scorpion Androctonus australis Hector.
Darbon, H., Zlotkin, E., Kopeyan, C., Rietschoten, J.V., and Rochat H. (1982), **Int. J. Peptide Protein** Res., 20., 320-330.

the toxicity of the toxins.

1.12 BIOLOGICAL ACTIVITIES OF SCORPION VENOM

The neurotoxic activity is attributed to its $\frac{1}{4}$th N-terminal amino acid sequence [46]. It is also reported that toxicity of pure component is reduced many fold and that carrier proteins are necessary for their action [75-78]. The mechanism by which scorpion venom produces toxic effects like hyperexcitability and spastic paralysis has been elucidated in a number of physiological studies [79-84].

1.13 OBJECTIVE OF THE PRESENT STUDIES

Much work has been carried out on the scorpion venom of different species, however there is little data available on the class Buthidae. The present study, on the primary structure of venom of *Buthus sindicus*, is undertaken to determine the protein/peptide nature of its venom and to find out its relationship with other venom sequences.

This work includes isolation of venom components, separation of the polypeptide chains, determination of the primary structure and comparison with known sequences of other species. Attempts have been made to understand the nature of activities associated with various protein/peptide fractions.

REFERENCE SECTION

2.0 MATERIALS AND METHODS

2.1 SAMPLE COLLECTION AND IDENTIFICATION

Scorpions (Buthus sindicus) about 5000 were collected from the Karachi (Sind) region and were identified at the Department of Zoology, University of Karachi.

2.2 EXTRACTION OF VENOM

The venom was obtained by electrical stimulation of telson (1250 v. DC) [15]. The healthy scorpion ejected about 1-2 µl of venom. The venom collected was diluted with de-ionized water (1:10). The extract was centrifuged (HITACHI Model SCP-85H) to remove the muco protein at 12000g for 20 minutes, the supernatant was freeze dried and stored at -30°C.

2.3 PURIFICATION OF PROTEINS

2.3.1 Reverse Phase High Performance Liquid Chromatography (RP-HPLC) For Separation Of Crude Venom

The lyophilized crude scorpion venom was purified by RP-HPLC [85,86], where the separation was achieved according to the hydrophobicity of the components in the sample. The HPLC system used was JASCO Model Twinckle pump with GP-A40 Gradient programmer

and Recorder RC-250 (Tokyo Japan). A column of Vydac C-18 (15x0.46 cm) was used in 0.1% TFA with a linear gradient of 0.1% TFA in acetonitrile [87].

2.4 ELECTROPHORESIS

2.4.1 Polyacrylamide DISC -GEL Electrophoresis

Disc electrophoresis deals with the discontinuous separating systems, using buffers of different pH values and composition. Different pore size gels of polyacrylamide were used to create discontinuous voltage and pH [88,89].

The crude venom samples were analyzed by electrophoresis on polyacrylamide gel. Desired gels were prepared by mixing the following solutions in different ratios.

Solution A:	36.3 g Tris-(hydroxymethyl)-amino methane, 48 ml 1N HCl and 0.46 ml N,N,N,N-tetramethylethylenediamine, made upto 100 ml with distilled water.
Solution B:	25 g Acrylamide and 0.4 g N,N-methylene bisacrylamide, made upto 100 ml with distilled water.

Solution C: 0.14 g Ammonium per-oxo-disulfate in 50 ml distilled water.

Mixing Ratio For Gel (10%)

GEL SOLUTIONS

Solution A	2.0 ml
Solution B	6.4 ml
Solution C	2.0 ml
Water	5.6 ml
Total Volume	16.0 ml

Electrode buffers

Upper: Tris 5.16 g and glycine 3.48 g pH 8.8, made upto 1 litre with distilled water.

Lower: Tris 14.5 g adjusted to pH 8.0 with HCl, volume made upto, 1 litre with distilled water.

Running Conditions: 7 mA Current per tube.

Staining solution: 1% Amido black 10B in 7% acetic acid for 10 minutes.

Destaining Solution: 7% Acetic acid was used for destaining the gels.

2.4.2 Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Purity and molecular mass of each purified protein was determined by SDS-PAGE according to Laemmeli [90], 10-20 µg protein required for SDS-PAGE was denatured by heating it in the sample buffer for few minutes in boiling water bath before applying on the gel. The gel was prepared as described below:

Solutions and buffers for preparing gels

Solution A:	38.84 g Acrylamide and 1.06 g bisacrylamide in 100 ml distilled water.
Solution B:	Tris-HCl 1.5 M, pH 8.8.
Solution C:	Tris-HCl 0.5 M, pH 6.8.
Solution D:	Ammonium persulphate 0.08g in 100 ml distilled water.
Solution E:	N,N,N,N-Tetramethylethylenediamine (TEMED) 2% v/v in distilled water.
Solution F:	Sodium dodecylsulphate (SDS) 1% w/v in distilled water.

Composition Of Separation And Stacking Gels

Solution	Separation gel (15%) (ml)	Stacking gel (3.2%) (ml)
A	7.5	1.0
B	5.0	-
C	-	2.0
D	2.0	1.0
E	3.0	3.0
F	2.0	0.4
H_2O	0.5	0.6
Total	20.0	8.0

Sample treatment buffer: Tris-HCl 0.6 M, pH 6.8 containing 1% SDS and 1% 2-mercaptoethanol.

Electrode buffer: Tris-glycine 0.05 M, pH 8.5 containing 0.1% SDS.

Running conditions: 150 Volts (constant) for 2-4 hrs.

Staining solution: 0.2% Coomassie brilliant blue R-250 in 7% acetic acid, 5% methanol in distilled water.

Destaining solution: 7% Acetic acid and 5% methanol in distilled water.

2.5 MODIFICATION OF VENOM PROTEINS

2.5.1 Carboxymethylation Of Proteins

The proteins were dissolved in 0.4M Tris-HCl buffer pH 8.15, containing 6M guanidine hydrochloride and 2mM EDTA, reduced with dithiothreitol (0.6M/mole of cysteine residues) incubated at 37°C under nitrogen.

After 2 hours, it was treated with the neutral ^{14}C iodoacetic acid (specific activity 2400 cpm/nM) and incubated at 37°C for another 2 hours under nitrogen in dark [91].

To ensure complete carboxymethylation, the ^{14}C-carboxymethylated protein was again treated with 10 fold molar excess of reagents (using unlabelled iodoacetic acid). After completion of the reaction, the excess reagents were removed by HPLC using TSK 2000 column, the pooled material was lyophilized.

2.5.2 Oxidation With Performic Acid

The protein samples prior to hydrolysis were oxidized, using performic acid, following the procedure of Hirs (1956) [92]. For the preparation of performic acid, pre-cooled (0°C) formic acid and hydrogen peroxide

were mixed in the ratio of 9:1 (v/v) and left for 4 hours at $0^{o}C$. A known quantity of the protein sample was mixed with performic acid in a ratio of 1:40 (w/v) and left over night at $0^{o}C$. The treated material was lyophilized.

2.6 ENZYMATIC CLEAVAGE

The carboxymethylated protein was dissolved in 1 ml of 20 mM ammonium bicarbonate buffer (adjusted at pH 8.0) followed by addition of TPCK-trypsin (stock solution 5 mg enzyme/ml of 10^{-3} M HCl) in a w/w ratio of 1:100 (enzyme : substrate). The mixture was incubated at $37^{o}C$ for 4 hours. After 2 hours of incubation, a second addition of enzyme was made to give a w/w ratio of 1:50 (enzyme : substrate) and was further incubated. The contents were lyophilized after digestion [93].

2.7 TRYPTIC PEPTIDE SEPARATION BY RP-HPLC

The separation of tryptic peptides were carried out on reverse phase HPLC [94-96], using Vydac C-18 column in 0.1% TFA with a linear gradient of 0.1% TFA in acetonitrile. Eluent was monitored at 214 nm, fractions were collected manually.

2.8 AMINO ACID ANALYSIS

Amino acid analysis was performed on an automatic amino acid

analyzer LC 6001 (Biotronik West Germany). 1-10 nmoles of native or modified (carboxymethylated) protein/peptide was hydrolyzed with 6N HCl containing 0.5% phenol at 110°C for 20 hours under vacuum [97-100]. After hydrolysis, samples were evaporated and taken in the sodium citrate buffer pH 2.2.

2.9 AMINO ACID SEQUENCING OF PROTEINS AND PEPTIDES

The sequence analysis of protein/peptide was carried out by manual as well as automatic methods. Each of the methods used is described below:

2.9.1 Manual Sequence Analysis Of Proteins And Peptides By Double Coupling DABITC Method

The proteins/peptides (2-10 nmole) were sequenced by DABITC/PITC double coupling method [101].

The DABTH-amino acids were identified as described by Chang et al.[102] and Edman et al.[103].

The protein/peptide (2-10 nmole) was dissolved in 80 μl of 50% pyridine in a clean 1.2x10 cm screw capped pyrex tube. The first coupling reaction was carried out by adding 40% DABITC solution (0.1 mg/40 μl). The tube was flushed with N_2 and incubated at 50°C for 45 min. After the first coupling, 10 μl of PITC (2nd coupling

agent) was added and coupling reaction was carried out for 30 min at 50°C. After the reaction, the excess reagents and by-products were removed by washing, after vortexing and centrifugation, with 500 µl x 3 volumes of heptane/ethyl acetate (3:1). The organic phase separated by centrifugation and discarded. The mixture was evaporated in high vacuum and cleavage carried out with concentrated TFA for 15 min at 50°C, after that the sample was evaporated and dissolved in 50 µl of wate . The extraction of DABTZ amino acids and PTZ-amino acid was performed by mixing with two-portions of 50 µl butyl acetate. The cleaved amino acids being highly hydrophobic was extracted in butyl acetate layer by centrifugation and contents were transfered to an eppendorf tube. The water phase was evaporated and taken to next cycle while butyl acetate phase was evaporated to dryness with nitrogen and to it 50 µl of 50% aqueous TFA was added, for conversion of DABTZ amino acid into DABTH derivatives, by incubation at 50°C for 50 min. After which the sample was dried, dissolved in 5 µl of ethanol and an aliquot taken for TLC identification. In case of labelled proteins/ peptides, an aliquot was taken for monitoring the release of radioactivity.

The red spots for DABTH amino acids and other by-product spots were employed for identification, according to their position with reference to the standard (blue spot).

2.9.2 Automatic Sequence Analysis

The intact proteins/peptides, obtained after cleavage, were sequenced by the liquid phase sequencer [104] (Beckman 890C or Beckman 890D) and the peptides, with less quantities, by a gas-phase sequencer [105] (Applied Biosystems 470A).

In automatic method, the coupling and the cleavage reaction was carried out by the sequencer and the conversion performed manually. 1 M Quodral (N,N,N',N' tetrakis (2-hydroxypropyl)-ethylenediamine was used with phenylisothiocyanate for the coupling step followed by the cleavage with hepta fluorobutyric acid. In the first step double coupling and cleavage was employed followed by single coupling and cleavage in the later step.

The peptides (10-80 nmoles) were applied to a liquid phase sequencer in 30% acetic acid after pre-cycling (3-5 step) with glycine-polybrene [106].

On gas phase sequencer, the protein/peptides (1-2 n moles) was applied to the filter precycled with biobrene. The PTH-amino acids were identified by reverse phase HPLC as described [107] and in case of liquid phase sequencer, degradations by thin layer chromatography [108] and HPLC.

2.10 ANALYSIS OF SCORPION FATTY ACIDS BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY (GCMS)

About 400 scorpions after collecting the venom, were exhaustively extracted with hexane in a Soxhlet apparatus for two days. The hexane soluble extract was shaken with 90% aqueous methanol to remove the steroidal compounds. The hexane phase was washed with water, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue [109-111] thus obtained was refluxed with 5% methanolic KOH and shaked with ethyl acetate to remove the unsaponifiable matter. The lower alkaline phase was acidified and free fatty acids were extracted with ethyl acetate. After usual work up, the residue was esterified with methanol in presence of H_2SO_4. Methyl esters of scorpion fatty acids were analyzed on GCMS, recorded on MAT 112 S mass spectrometer coupled with Varian 3700 gas chromatograph connected to DEC PDP 11/34 computer system. 2 µl sample was injected in a SE 30 capillary glass column having a length of 30 meters and temperature range of 60-250°C at a rate of change of 8°C per minute. Helium was used as carrier gas with a flow rate of 1 ml/min EI energy, emission current and ion source temperature of the mass spectrometer were 80 ev, 0.7 mA and 260°C respectively.

2.11 BIOLOGICAL ACTIVITIES OF THE VENOM

2.11.1 Determination Of Neurotoxicity By Physiograph

The crude venom and its HPLC separated fractions were evaluated for its electrophysiological behaviour on indirectly elicited contraction of muscle. All experiments were carried out in vitro on isolated gastrocnemius sciatic preparation of Rana tigrina.

Recording of maximum isometric twitches of freshly dissected nerve muscle preparation was fixed horizontally in the perspex muscle chamber isometrically. The muscle and nerve were immersed in 150 ml Ringer's solution containing 10 mM NaCl, 0.1 mM KCl, 0.12 mM $NaHCO_3$ and 0.09 mM $CaCl_2$. The maximum isometric twitch and resting length of muscle were determined according to Winchester [112]. The experimental twitches were recorded on physiograph-FOUR A (Nacro Biosystem, USA), at a paper speed of 10 cm/sec and supramaximal voltage of 100V for 5 msec duration giving a singlet stimulus. The buffer solution of the chamber was changed with Ringer's solution containing 0.036 mg/ml of the crude venom and isometric twitches were recorded for its toxicity measurements as above at intervals of 2, 5 and 10 minutes.

Similarly the HPLC separated fractions were evaluated for activity at intervals of 2, 5, 10, 20, 30, 40, 50 and 60 minutes. Separate preparations were used for each fraction and each of these also served as the control.

These recordings were assayed for height of twitch, contraction time and half relaxation time for crude venom and separated fractions.

2.11.2 Determination Of Toxicity By Injection Method

The toxicity of the cruce venom was checked by "injection method" [113-118]. 0.05% solution of crude venom, in distilled water, was injected in the abdomen of adult house flies (Musca domestica). For this treatment, micro applicator and a micro syringe was used and different doses were given such as 0.01, 0.25, 0.5, 0.75 and 1.0 µl. At the same time a control batch was also kept for the determination of environmental effects.

The treated and untreated flies were kept under chemneys and provided food. After 24 hours of the treatment, the number of dead flies were noted to find out the toxicity of the given venom.

In the same way, adult German cockroaches (Blattella germanica), were also treated with the crude venom. 0.25, 0.5 And 1.0 µl of

0.05% solution of crude venom in distilled water was injected and the insects were observed under chemneys. After 24 hours, mortality percentage was noted by counting the number of dead insects.

3.0 RESULTS

3.1 VENOM

The venom from each scorpion ranged from 1 µl to 2 µl. A total of 5000 scorpion's venom was collected during this study.

3.2 ELECTROPHORESIS

3.2.1 Disc Gel Electrophoresis

Electrophoresis pattern of the crude venom is shown in fig. 3.1. It shows to contain 6 major and 5 minor proteins.

3.3 SEPARATION OF SCORPION VENOM PROTEINS ON RP-HPLC

The crude venom was separated on Vydac C-18 column using 0.1% TFA acetonitrile gradient. A total of six major and several minor peaks were obtained (Fig. 3.2). The purity of the separated peaks were checked on SDS-PAG electrophoresis. Peak BuS I, II, V, VI and VII were found to be pure, whereas BuS IV contained two bands. The other peaks were also collected but no further study could be undertaken due to minute quantities.

3.4 MOLECULAR MASS OF THE SEPARATED PEAKS BY SDS-PAGE

Molecular mass of all the peaks obtained from the RP-HPLC-Vydac

Fig. 3.1: Disc electrophoresis of Buthus sindicus scorpion venom on polyacrylamide gel.

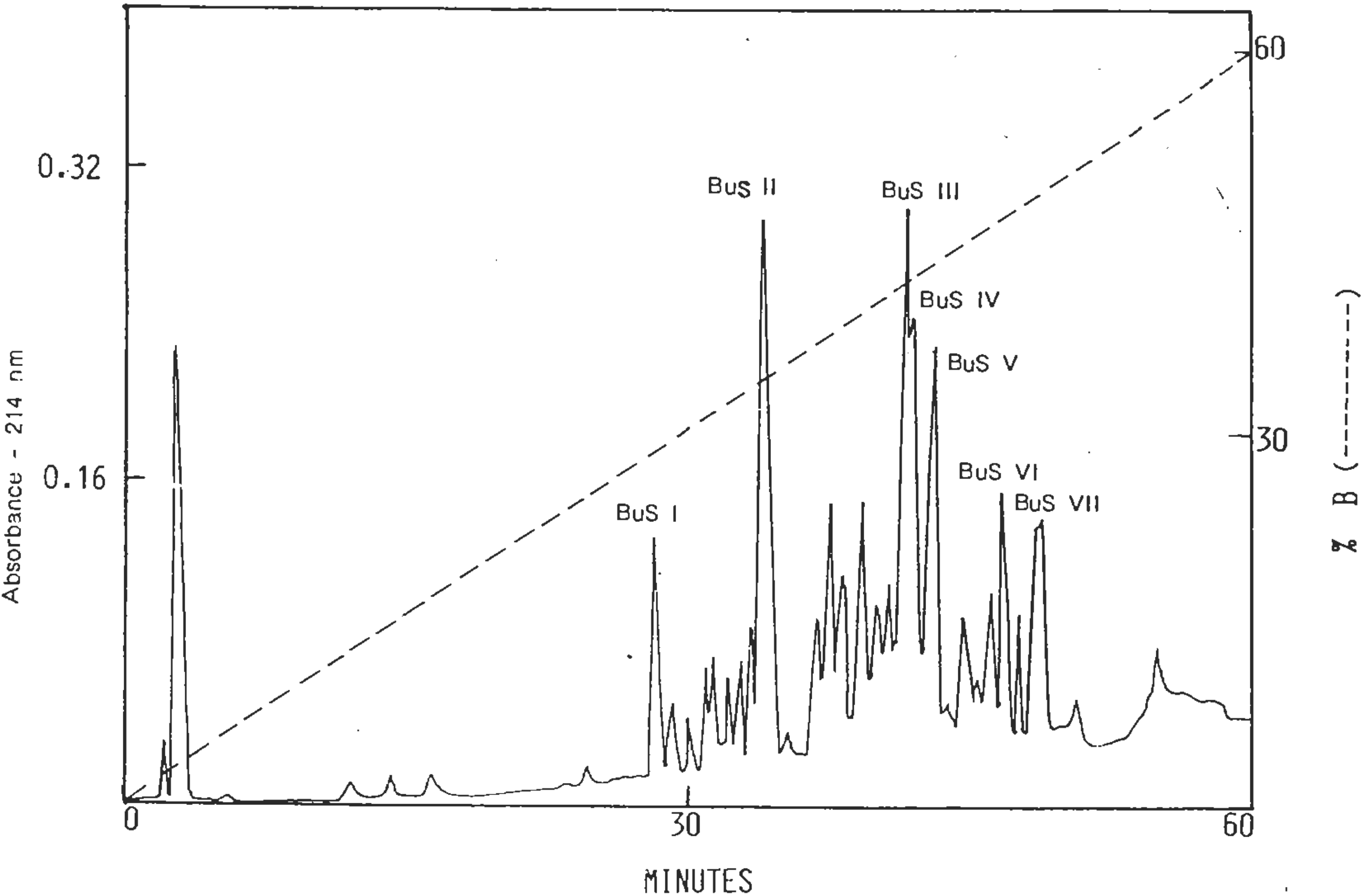
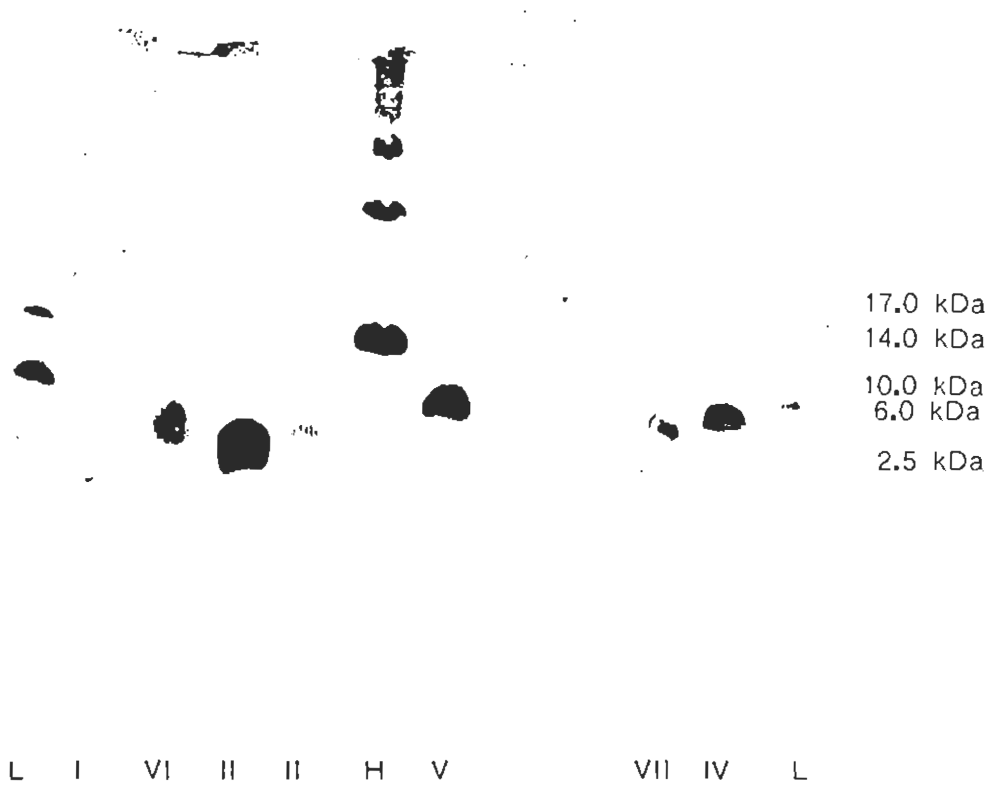


Fig. 3.2: Separation of scorpion venom proteins on a Vydac C-18 column. The sample was eluted in 0.1% TFA with a gradient of 0.1% TFA in acetonitrile. Fractions were pooled as indicated by its name and are mentioned as such in the text.

Fig. 3.3: SDS-PAGE of peaks from reverse phase HPLC of scorpion venom on 15% gel. (L) represent low molecular mass standard, (H) represent high molecular mass standard. The position for each standard is mentioned in the figure.



C-18 column were determined on SDS-PAGE (15% gel concentration), fig. 3.3, and is shown in table-1.

Table-1

Peak No.	Mol.Wt.
BuS I	2.8 kDa
BuS II	3.5 kDa
BuS III	Not identified
BuS IV	6.5, 14 kDa
BuS V	6 kDa
BuS VI	6.6 kDa
BuS VII	6.6 kDa

3.5 CHARACTERIZATION OF PEPTIDES

3.5.1 Studies On BuS I

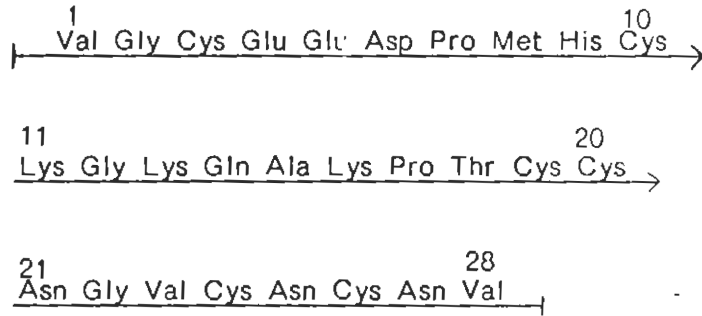
3.5.1.1 Amino Acid Analysis

BuS I, a peptide of molecular mass 2.8 kDa, was found to be pure. Carboxymethylated peptide was acid hydrolysed. Table-2 shows the amino acid composition of the hydrolysate of carboxymethylated derived peptide.

Table-2: Amino acid composition of short peptide BuS I.

Amino acid	No. of residues
Cys (Cm)	6.0 (6)
Asx	4.2 (4)
Thr	1.0 (1)
Glx	3.0 (3)
Pro	2.0 (2)
Gly	3.0 (3)
Ala	1.0 (1)
Val	2.8 (3)
Met	1.2 (1)
Lys	2.9 (3)
His	0.9 (1)

Fig. 3.4: Primary structure of the peptide BuS I determined by direct sequence analysis of the intact carboxymethylated peptide.



3.5.1.2 Amino Acid Sequence Determination

Amino acid sequence of the peptide BuS I was determined both manually and on gas phase sequencer. The sequence is shown in fig. 3.4.

3.5.2 Studies On BuS II

3.5.2.1 Amino Acid Analysis

The amino acid composition of the BuS II is shown in table-3.

3.5.2.2 Separation Of Tryptic Peptides

Tryptic peptides were separated by HPLC on Vydac C-18 column. The separation profile is shown in fig. 3.5.

3.5.2.3 Amino Acid Sequence Determination Of Tryptic Peptides

Pure peptides from tryptic digest were sequenced according to DABITC and the results are as under:

TAble-3: Amino acid composition of ^{14}C carboxymethylated BuS II.

Amino acid	No. of residues
Cys (Cm)	7.7 (8)
Asx	2.1 (2)
Thr	2.5 (3)
Ser	1.0 (1)
Glx	2.0 (2)
Pro	2.8 (3)
Gly	4.8 (5)
Ala	0.9 (1)
Met	0.9 (1)
Leu	1.0 (1)
Tyr	0.9 (1)
Phe	1.0 (1)
Lys	5.0 (5)
Arg	1.0 (1)

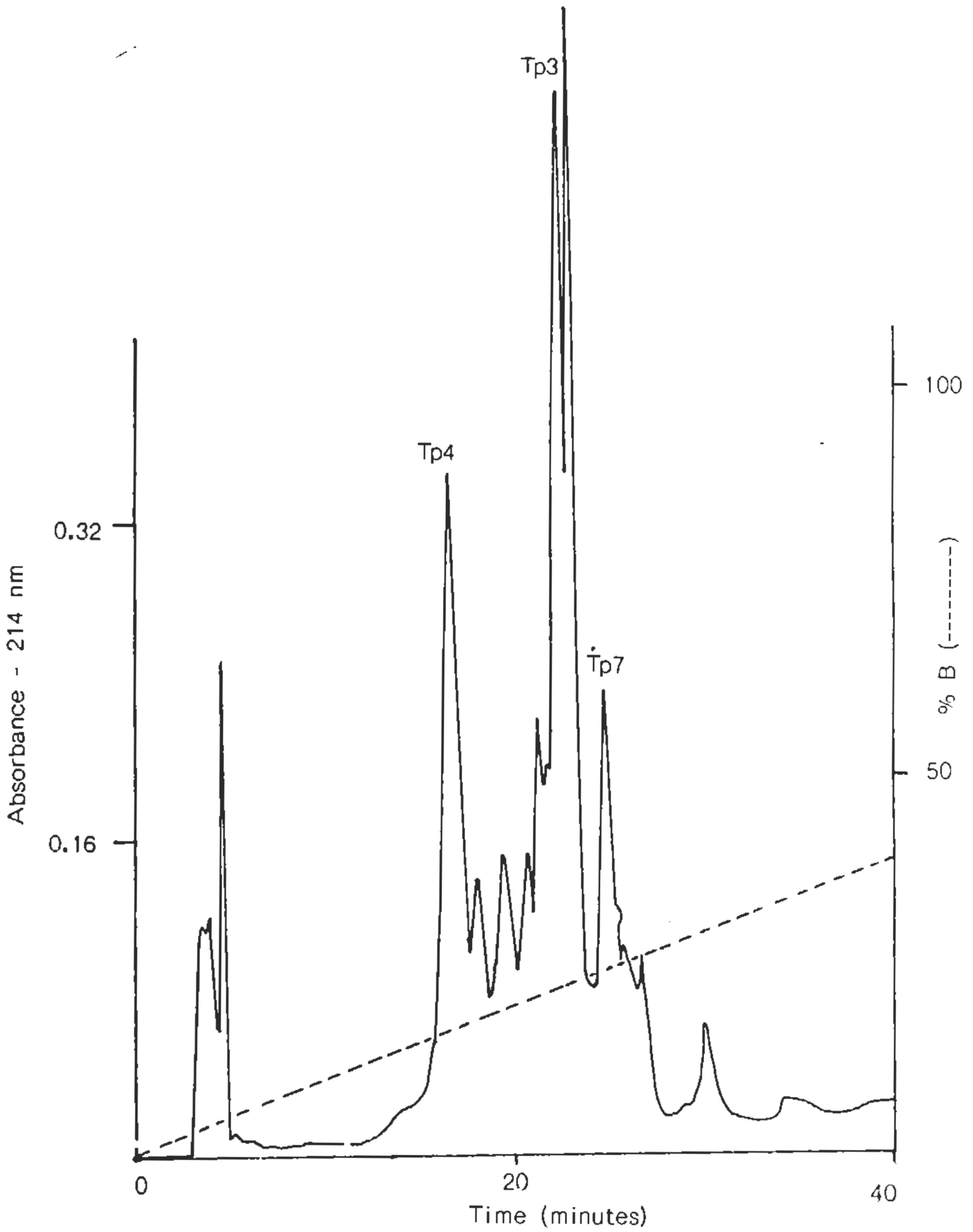
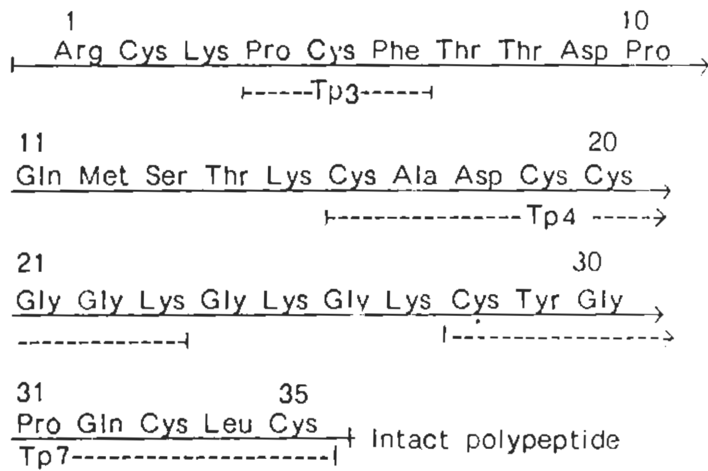


Fig. 3.5: Purification of tryptic peptides BuS II by reverse phase HPLC on a Vydac C-18. Eluted with 0.1% TFA with a gradient of acetonitrile.

Fig. 3.6: Complete primary structure of characterized scorpion venom BuS II. The structure was deduced by direct sequence analysis of the intact carboxymethylated protein chain. Broken lines represent tryptic peptides sequence.



Peak No.	Residue No.	Sequence
BusII TP3	4-6	Pro-Cys-Phe
BuSII TP4	16-23	Cys-Ala-Asp-Cys-Cys-Gly-Gly-Lys
BuSII TP7	28-35	Cys-Tyr-Gly-Pro-Gln-Cys-Leu-Cys

The manual N-terminal sequence showed the presence of single spot corresponding to DABTH Arg. The complete amino acid sequence was established on liquid phase sequencer by degrading the intact carboxy-methylated polypeptide and also with alignment of the tryptic peptides (Fig. 3.6).

3.5.3 Studies On BuS III

The BuS III was not obtained in the pure form as revealed by the sequence analysis upto 12 residues. Further studies could not be carried out on this peptide due to insufficient quantity of the material.

3.5..4 Studies On BuS IV

3.5.4.1 Purification Of BuS IV

The BuS IV from RP-HPLC-Vydac C-18, consisting of two polypeptides of molecular mass 6.5 kDa and 14 kDa (section 3.4) was further

chromatographed on TSK-SW-2000 column separated in two peaks BuS IVa and BuS IVb (Fig. 3.7). The purity of these peaks were confirmed by SDS-PAGE (Fig. 3.8) and N-terminal sequence of BuS IVb was carried out using DABITC method.

3.5.4.2 Amino Acid Composition

The amino acid composition of BuS IVb is given in table-4. The protein has 65 residues with 8 cysteine.

3.5.4.3 Separation Of Tryptic Peptides Of BuS IVb

The elution pattern of tryptic peptides on RP-HPLC is shown in fig. 3.9 and their amino acid compositions are given in table-5.

3.5.4.4 Amino Acid Sequence Determination

The complete primary structure was derived by sequence of intact carboxymethylated BuS IVb and with the sequence of trypsin cleaved peptides, the sequence deduced is shown in fig. 3.10.

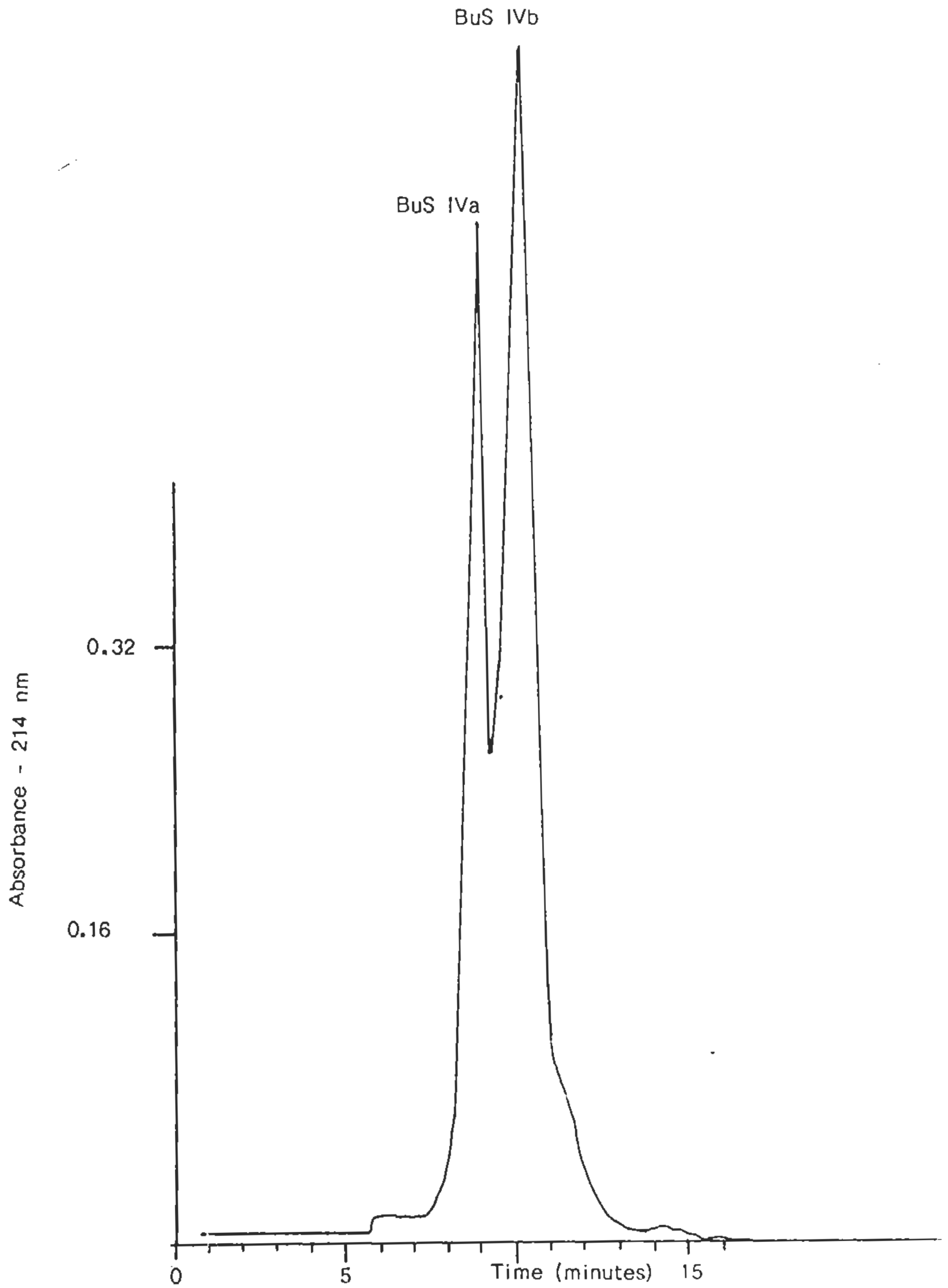


Fig. 3.7: Rechromatography of BuS IV on a TSK-SW-2000. Eluted with 0.1% TFA at a flow rate of 1 ml/min.

Fig. 3.8: SDS-PAG electrophoresis of peak BuS IVa and BuS IVb on 15% gel, (M) represent high molecular weight marker. The position of each marker is mentioned in the figure.

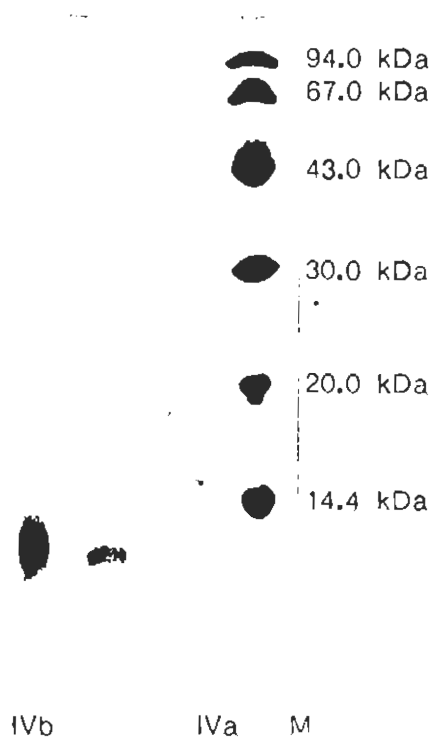


Table-4: Amino acid composition of intact carboxymethylated ^{14}C neurotoxin BuS IVb.

Amino acid	No. of residues
Cys(Cm)	7.8 (8)
Asx	8.4 (8)
Thr	2.8 (3)
Ser	2.6 (3)
Glu	2.8 (3)
Pro	2.5 (3)
Gly	6.9 (7)
Ala	3.7 (4)
Val	2.6 (3)
Ile	3.6 (4)
Leu	1.7 (2)
Tyr	3.8 (4)
His	0.9 (1)
Trp	2.7 (3)
Lys	5.8 (6)
Arg	3.0 (3)

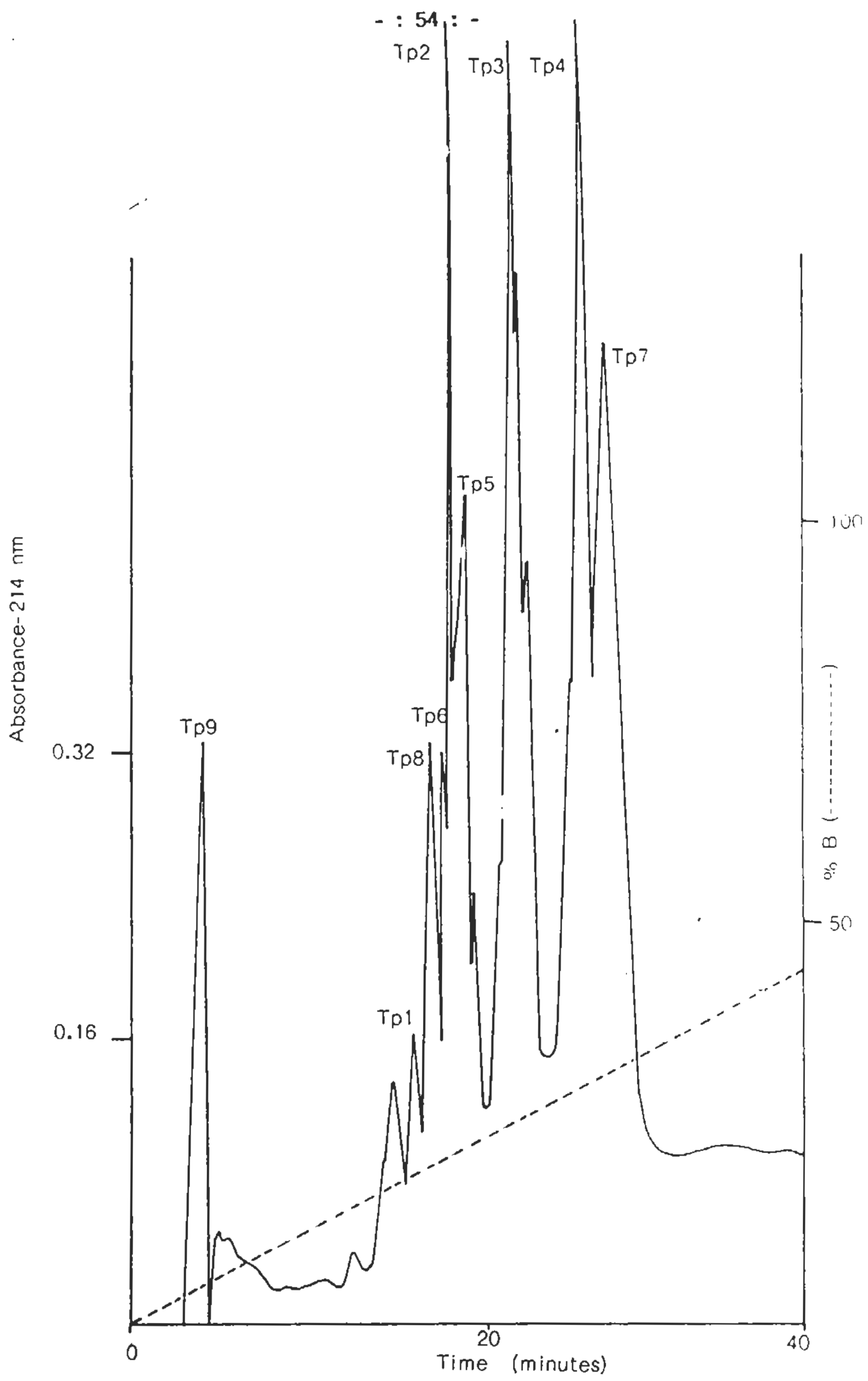


Fig. 3.9: Purification of tryptic peptides BuS IVb by reverse phase HPLC on a Vydac C-18. Eluted with 0.1% TFA with a gradient of acetonitrile.

Table-5: Amino acid composition of neurotoxin BuS IVb tryptic peptides.

Amino acid	Pos. 30-42 Tp4	Pos. 12-29 Tp3	Pos. 1-3 Tp1	Pos. 43-51 Tp5	Pos. 4-11 Tp2	Pos. 56-59 Tp7	Pos. 64-65 Tp9	Pos. 60-63 Tp8	Pos. 52-55 Tp6
Cys (Cm)	1.0 (1)	3.7 (4)	-	2.0 (2)	-	-	1.0 (1)	-	-
Asx	1.0 (1)	2.8 (3)	-	-	2.7 (3)	-	-	-	1.0 (1)
Thr	-	3.0 (3)	-	-	-	-	-	-	-
Ser	1.0 (1)	1.9 (2)	-	-	-	-	-	-	-
Glu	1.8 (2)	0.8 (1)	-	-	-	-	-	-	-
Pro	-	-	-	-	-	0.6 (1)	-	0.7 (1)	1.0 (1)
Gly	2.8 (3)	1.0 (1)	1.0 (1)	0.9 (1)	-	-	-	1.0 (1)	-
Ala	1.0 (1)	-	-	1.0 (1)	1.8 (2)	-	-	-	-
Val	-	1.0 (1)	0.7 (1)	-	-	1.0 (1)	-	-	-
Ile	-	-	-	0.8 (1).	0.9 (1)	0.8 (1)	-	1.0 (1)	-
Leu	1.0 (1)	-	-	-	-	-	-	-	0.9 (1)
Tyr	-	1.6 (2)	-	1.0 (1)	1.0 (1)	-	-	-	-
His	1.0 (1)	-	-	-	-	-	-	-	-
Trp	1.0 (1)	-	-	1.7 (2)	-	-	-	-	-
Lys	1.0 (1)	1.0 (1)	-	1.0 (1)	1.0 (1)	-	-	1.0 (1)	0.8 (1)
Arg	-	-	1.0 (1)	-	-	1.0 (1)	1.0 (1)	-	-
Total	13	18	3	9	8	4	2	4	4

Fig. 3.10: Primary structure of scorpion BuS IVb. Solid line represent those parts which were determined by direct automatic sequanator and broken lines represent those parts which were determined by tryptic peptides.

```
Gly Val Arg Asp Ala Tyr Ile Ala Asp Asp
|-------------------------------------->

Lys Asn Cys Val Tyr Thr Cys Gly Ser Asn
--------------------------------------->

Ser Tyr Cys Asn Thr Glu Cys Thr Lys Asp
--------------------------------------->
                                   |-Tp4--

Gly Ala Glu Ser Gly His Cys Glu Trp Leu
-------------------TP4----------------------->

Gly Lys Tyr Gly Trp Ala Cys Trp Cys Ile
-------| |-------------------Tp5----------->

Lys Leu Pro Asp Lys Val Pro Ile Arg Ile
--| |---------Tp6----| |--------Tp7---| |->

Pro Gly Lys Cys Arg
-----Tp8-----| |--Tp9---|
```

3.5.5 Studies On BuS V

3.5.5.1 Amino Acid Analysis Of BuS V

The amino acid composition of the acid hydrolyzate of the peptide is presented in table-6.

3.5.5.2 Separation Of Tryptic Peptides

The BuS V was cleaved with trypsin and the peptides separated by RP-HPLC , using Vydac C-18 column (Fig. 3.11). Nearly all the major peptides were analyzed (Table-7).

3.5.5.3 Amino Acid Sequence Determination

The sequence of peptide BuS V was determined manually by DABITC method upto 18 residues (Fig. 3.12).

3.5.6 Studies On BuS VI

3.5.6.1 Amino Acid Analysis

The total amino acid composition of purified BuS VI was determined after hydrolysis with 6M HCl for 24 hours at 110°C. The composition of this peptide is given in table-8.

Table-6: Amino acid composition of carboxymethylated BuS V

Amino acid	No. of residues
Cys (Cm)	7.8 (8)
Asx	12.6 (13)
Thr	3.7 (4)
Ser	4.8 (5)
Glu	1.0 (1)
Pro	0.8 (1)
Gly	4.7 (5)
Ala	1.0 (1)
Val	1.9 (2)
Met	0.9 (1)
Leu	1.8 (2)
Tyr	5.6 (6)
His	1.7 (2)
Trp	1.0 (1)
Lys	9.7 (10)
Arg	1.8 (2)

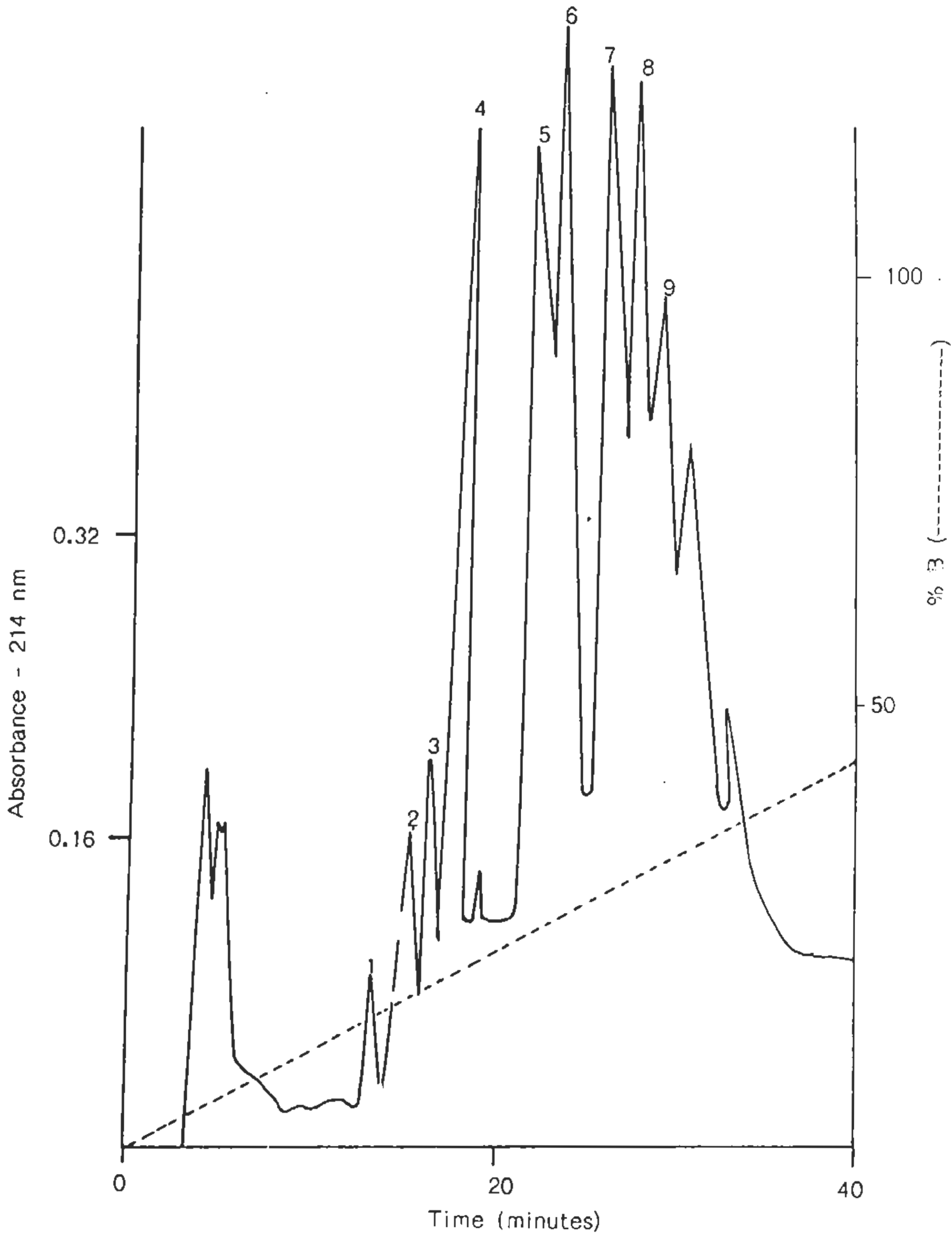


Fig: 3.11 : Purification of tryptic peptide of BuS V by reverse phase HPLC. Condition as described in section 2.7.

Table-7: Amino acid composition of tryptic peptides purified from BuS V.

Amino acid	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8	Peak 9
Cys (Cm)	0.9 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.8 (2)	1.0 (1)	1.0 (1)	-
Asx	3.9 (4)	-	2.6 (3)	1.0 (1)	-	2.8 (3)	-	1.0 (1)
Thr	0.7 (1)	-	-	-	-	0.7 (1)	-	1.0 (1)
Ser	-	1.7 (2)	1.0 (1)	-	-	-	-	1.6 (2)
Glu	-	-	-	-	1.0 (1)	-	-	-
Pro	-	-	-	-	-	1.0 (1)	-	-
Gly	-	1.0 (1)	1.0 (1)	-	1.7 (2)	-	1.0 (1)	-
Ala	-	-	1.0 (1)	-	-	-	-	-
Val	-	-	1.7 (2)	-	-	-	-	-
Leu	-	-	-	-	-	1.0 (1)	-	-
Tyr	0.9 (1)	1.0 (1)	0.6 (1)	0.6 (1)	1.0 (1)	-	0.8 (1)	-
His	-	0.6 (1)	-	-	-	-	0.6 (1)	-
Trp	-	-	-	-	-	-	-	0.8 (1)
Lys	-	1.0 (1)	0.7 (1)	1.0 (1)	-	-	1.0 (1)	0.7 (1)
Arg	0.8 (1)	-	-	-	1.0 (1)	-	-	-
Total	8	7	11	4	7	7	5	6

Fig. 3.12: Partial amino acid sequence of scorpion venom BuS V upto 18 steps by manual degradation according to DABITC method.

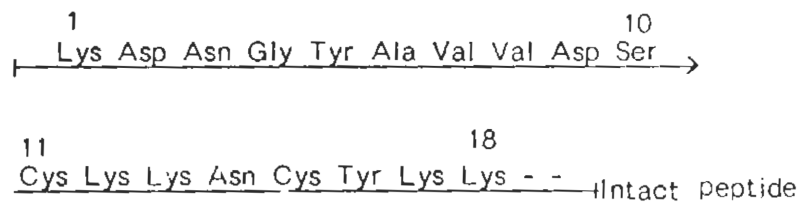


Table-8: Amino acid composition of BuS VI of scorpion venom.

Amino acids	No. of residues
Asx	7.9 (8)
Thr	2.8 (3)
Ser	3.1 (3)
Glu	3.0 (3)
Pro	2.6 (3)
Gly	6.5 (6)
Ala	2.6 (3)
Cys	7.7 (8)
Val	2.6 (3)
Ile	2.7 (3)
Leu	3.2 (3)
Tyr	4.0 (4)
Phe	1.7 (2)
His	5.7 (6)
Lys	5.4 (5)
Arg	1.7 (2)

Fig. 3.13: Partial characterization of BuS VI upto 9 residues by DABITC method.

1 9
Asp-Gly-Tyr-Ileu-Val-Asp-Asp-Val-Asn---

3.5.6.2 Amino Acid Sequence Determination

The N-terminal sequence analysis of BuS VI was followed upto 9 residues as shown in fig. 3.13.

3.5.7 Studies On BuS VII

3.5.7.1 Amino Acid Analysis

The amino acid composition of BuS VII is shown in table-9.

3.5.7.2 Amino Acid Sequence Determination

Amino acid sequence of BuS VII was determined manually upto 9 residues. The sequence is shown in fig. 3.14.

3.6 BIOLOGICAL ACTIVITIES OF THE VENOM

3.6.1 Determination Of Neurotoxicity

The toxic effect of crude venom and three fractions from HPLC Vydac C-18 column (BuS IV, VI, VII) were carried out on gastrocnemius sciatic preparation from *Rana tigrina*. These show the toxic effect produced

Table-9: Amino acid composition of intact BuS VII scorpion venom after the hydrolysis at 110^{0}C.

Amino acids	No. of residues
Asx	7.7 (8)
Thr	3.2 (3)
Ser	2.6 (3)
Glu	3.0 (3)
Pro	2.4 (2)
Gly	10.4 (10)
Ala	2.5 (2)
Cys	7.6 (8)
Val	2.6 (3)
Ile	2.2 (2)
Leu	3.3 (3)
Tyr	4.6 (5)
Phe	1.3 (1)
His	5.9 (5)
Lys	4.0 (4)
Arg	2.9 (3)

Fig. 3.14: Partial characterization of BuS VII upto nine residues by DABITC method.

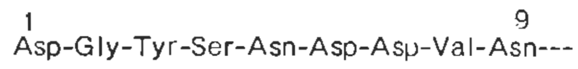


Table-10: Neurotoxicity of <u>Buthus sindicus</u>, crude venom and its fractions (IVb, VI and VII) from Vydac C-18 HPLC separated fractions.

Sample	Control twitch	Duration								
		2	4	5	10	20	30	40	50	60
<u>Crude</u>										
T.H.	1.03	↑23%	↑1.07%	-	-	-	-	-	-	-
C.T.	0.02	↓2f	↓2f	-	-	-	-	-	-	-
½ R.T.	0.03	↑17f	↑18f	-	-	-	-	-	-	-
<u>BuS IVb</u>										
(11 Ce 5)										
T.H.	1.2	↑ 66.6%	-	↑66.6%	↑50.0%	↑50.0%	↑ 41.66%	↑ 33.33%	-	-
C.T.	0.04	N	-	↓1.3f	↓1.3f	↓1.3f	↓1.3f	↓1.3f	↓1.3f	↑ 1.25f
½ R.T.	0.01	N	-	↑ 2f	N	N	N	↑ 2f	↑ 2.5f	↑ 2.5f
<u>BuS VI</u>										
(15 Cd-5)										
T.H.	4.4	↑ 6.8%	↓15.9%		↓22.72%	↓31.8%	↓ 52.27%	↓ 52.27%	↓ 79.54%	-
C.T.	0.01	N	-	↑ 1.5f	↑ 2f	↑3f	↑ 4f	↑ 5f	↑ 3f	-
½ R.T.	0.01	N	-	↑ 1.5f	↑ 2f	↑ 3f	↑ 3f	↑ 4f	↑ 15f	-
<u>Bus VII</u>										
(24 Cd-5)										
T.H.	1.3	↑ 46.2%	-	↑ 46.2%	↑46.2%	↑ 46.2%	↓ 23.1%	↓ 53.8%	↓ 69.2%	-
C.T.	0.02	↓ 2f	-	↓ 2f	↓2f	N	N	↑ 1.5f	↑ 1.5f	-
½ R.T.	0.025	N	-	N	↑ 1.2f	↑ 1.2f	↑ 2f	↑ 1.2f	↑ 1.2f	-

T.H. = Twitch height
C.T. = Contraction time
½ R.T. = ½ Relaxation time
f = Fold

↑ = Increase
↓ = Decrease
N = Normal

on the nerve muscle preparation as shown in table-10.

3.6.2 Determination Of Toxicity By Injection Method

Toxicity of the crude extract of scorpion venom with a wide range of doses against adult flies and cockroaches was found to be regular.

For regression curve, Abbot's formula [119] was used and graph were plotted from it on log log graph paper (Fig. 3.15, 3.16). To determine the mortality rate of the compounds, the data was statistically analyzed.

By plotting [113-116] an average value on log log graph paper, the LD_{50} of crude venom against Musca domestica was found 0.9 µg/fly, while in the case of Blattella germanica it was found 4.5 µg/cockroach.

3.7 SCORPION FATTY ACID DETERMINATION BY GCMS

GCMS spectra showed 14 molecular ion peaks, 11 of which were saturated, 2 mono-unsaturated and 1-di-unsaturated methyl esters. These results and characteristic mass fragments observed in mass spectra are given in table-11.

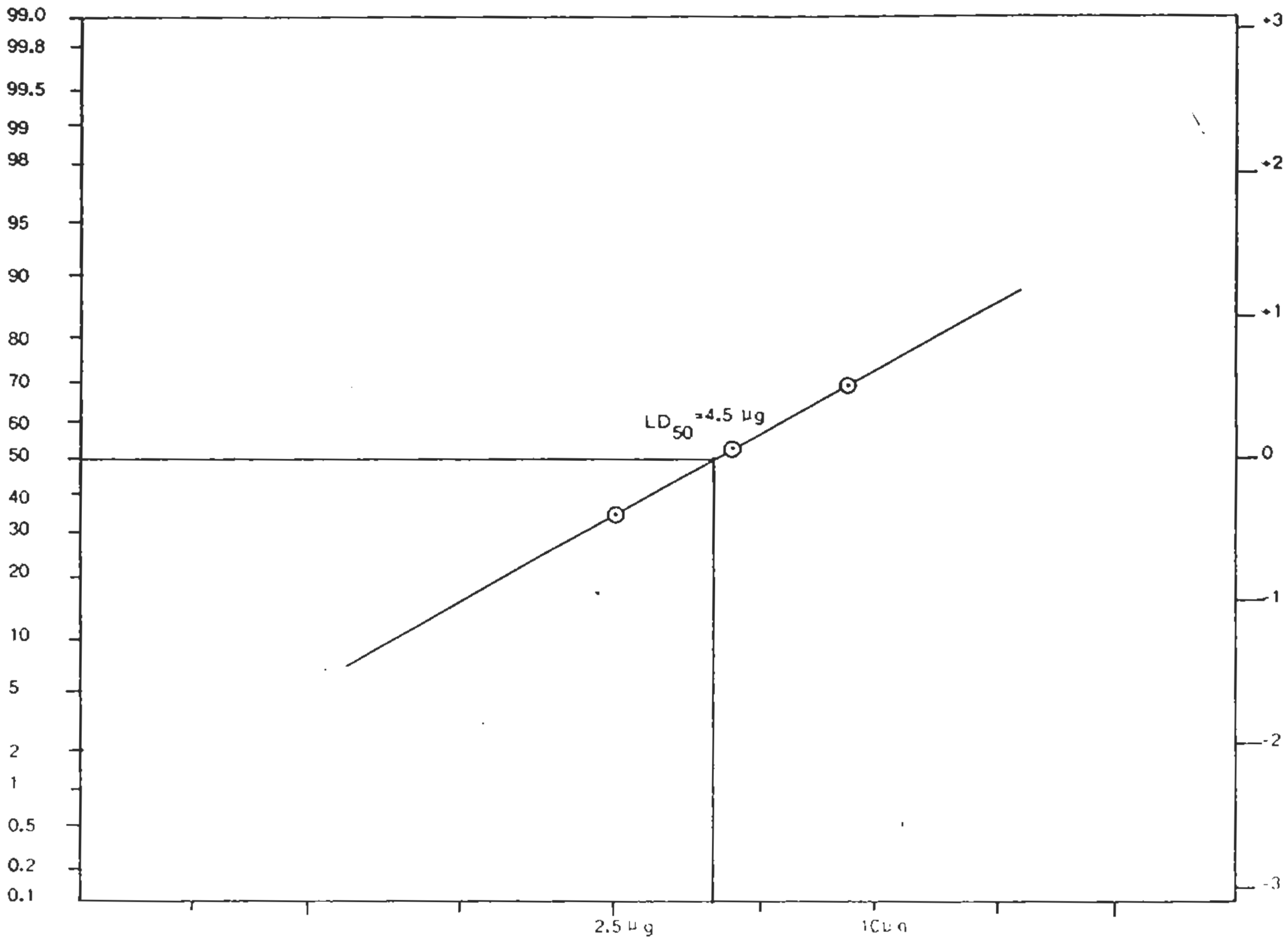


Fig. 3.15: Determination of toxicity on Blatella germanica by injection method.

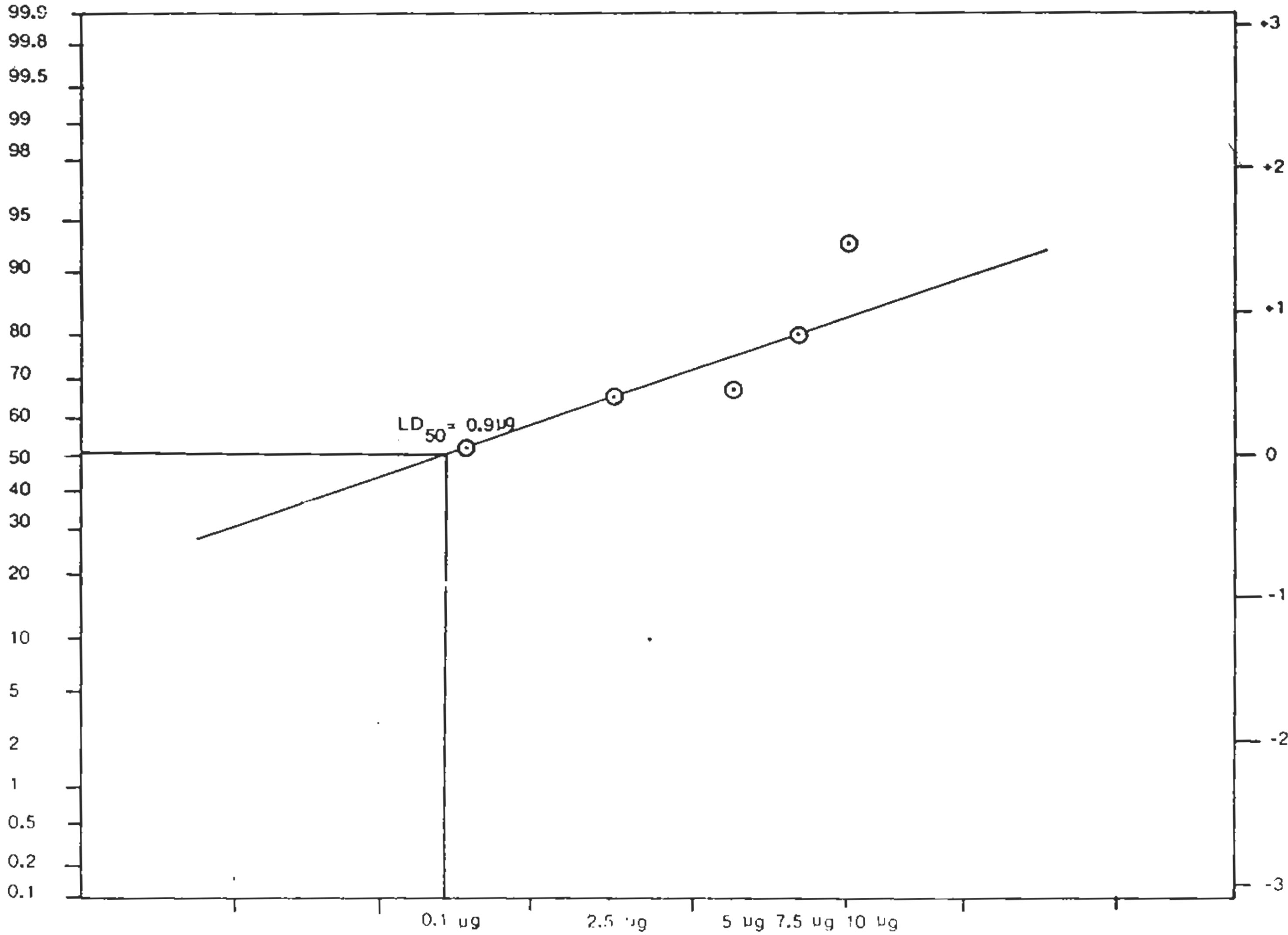


Fig. 3.16: Determination of toxicity on Musca domestica by injection method.

Table-11: Mass spectral data for the fatty acids ester of total lipid contents of Buthus sindicus, m/z (rel. intes. %).

M^+	$M^+ - 31$	$M^+ - 43$	$M^+ - 59$	$M^+ - 74$	Fatty Acid
130 (8)	99(10)	87(25)	71(50)	-	Hexanoic
158(7)	127(6)	115(22)	99(18)	-	Octanoic
186(6)	155(6)	143(13)	127(8)	-	Decanoic
214(12)	183(8)	171(5)	155(10)	-	Dodecanoic
242(6)	211(9)	199(6)	183(10)	-	Tetradecanoic
270(10)	239(6)	227(18)	211(12)	-	Hexadecanoic
294(5)	263(8)	-	-	220(5)	Octadecadienoic
296(21)	265(10)	-	-	222(9)	Octadecenoic
298(10)	267(12)	255(9)	239(6)	-	Octadecanoic
326(14)	295(3)	283(6)	267(5)	-	Eicosanoic
352(2)	221(5)	-	-	278(4)	Docosenoic
354(4)	323(10)	311(1)	295(3)	-	Docosanoic
396(2)	365(5)	353(4)	337(1)	-	Pentacosanoic
424(1)	393(4)	381(2)	365(1)	-	Heptacosanoic

4.0 DISCUSSION

4.1 CHARACTERIZATION OF NOVEL SHORT PEPTIDE BuS I

The amino acid composition (Table-2, Section-3.5.1.1) shows the presence of six cysteine residues. The amino acid sequences (Fig. 3.4, Section--3.5.1.2) of complete peptide containing 28 amino acids shows that these cysteine residues are spread all along the chain which is in conformative with other peptides isolated from various venoms, except that this peptide has 6 cysteine instead of 8 cysteine. The peptide does not show significant homology with other venom peptides isolated from the Buthus species. It has, however, some sequence homology with a known insect toxin (Fig. 4.1) from Buthus sindicus. On comparison, it shows 7/28 identical resioues. These homologies are largely restricted to cysteine residues which can be aligned by allowing number of gaps (Amm, Be and BuS II). These homologies show that the peptide is novel and has not been reported from Buthus species or any other scorpion species.

4.1.1 Superficial Similarities With Other Proteins

With the characterization of this peptide, it could be judged that several types of fragments which occur in the venom of Buthoid scorpion may have relation to various other proteins.

The similarities in the sequence of scorpion's short peptide with the

Fig. 4.1: Comparison of BuS I peptide of Buthus sindicus with other short peptides [Buthus sindicus BuS II, (insect toxin) Androctonous mauretanicus mauretanicus, Buthus eupeus] isolated from various species. Identical residues are boxed.

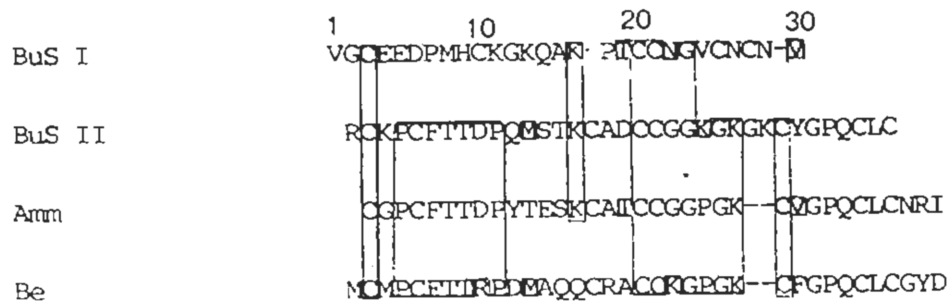
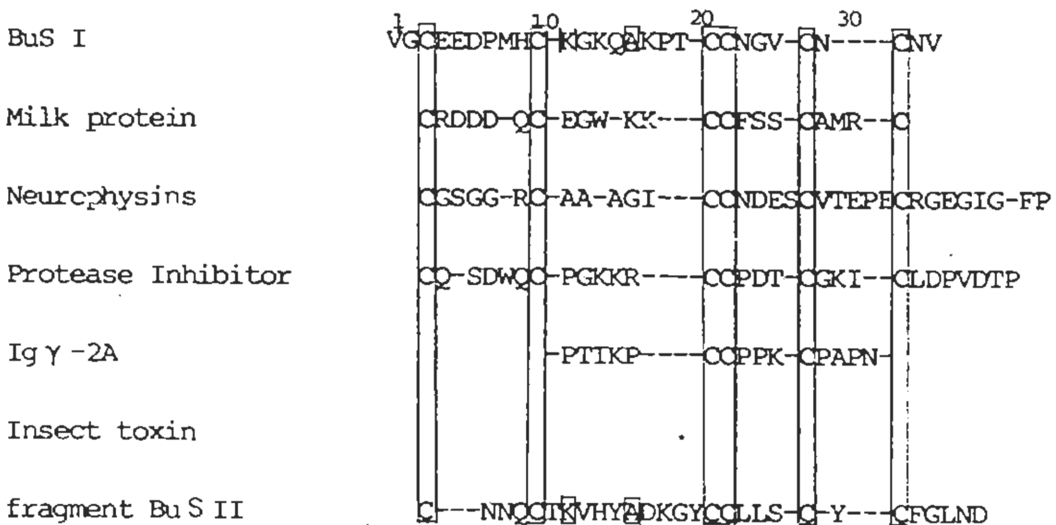


Fig. 4.2: Comparison of BuS I short peptide with various other proteins (Milk whey acidic protein, neurophysins, Red sea turtle protease inhibitor, Ig γ -2A (mouse MOPC-173), Insect toxin fragments.



family of "four disulphide core proteins" (including neurophysins, toxin, agglutinins, several other proteins and protease inhibitor) indicate divergent evolution of these proteins, which may have a common progenitor and has duplicated to produce different proteins with structural similarities but variable functions. Some of these proteins have a rather longer core between the cysteine residues. In fig. 4.2, it is noticeable that BuS I is related to neurophysins; neurophysins act as carrier proteins for neurophyseal hormones (oxytoxin and vasopressin); [120] therefore, it can be assumed that scorpion protein may have a role in binding with receptors. There is a structural relationship between scorpion protein and protease inhibitor.

4.2 IDENTIFICATION OF INSECT TOXIN LIKE PEPTIDE BuS II

The complete sequence (Fig. 4.3) consists of 35 residues with 8 cysteines corresponding to four disulphide bridges. These types of peptides usually possess saturated disulphides to maintain their structural properties [65].

4.2.1 Comparison With Other Scorpion Species

This peptide, on the basis of its primary sequences is related to two other characterized scorpion venom proteins [25,121]. The sequence

Fig. 4.3: Comparison of BuS II (Buthus sindicus II) structure with that of other identical peptide of different scorpion species (Buthus eupeus, Androctonous moretanicus moretanicus) [25,121]. Gaps are indicated by dashes. Identical residues of the three proteins are boxed.

```
        1       10        20        30
BuS II  RCKPCFTTDPQMSRKCADCCGGKGKGKCYGPQCLC
Be      MCMPCFTTRPDMAQQCRACCKGRGK--CGGPQCLCGYD
Amm      CGPCFTTDPYTESKCATCCGGRGK--CVGPQCLCNRI
```

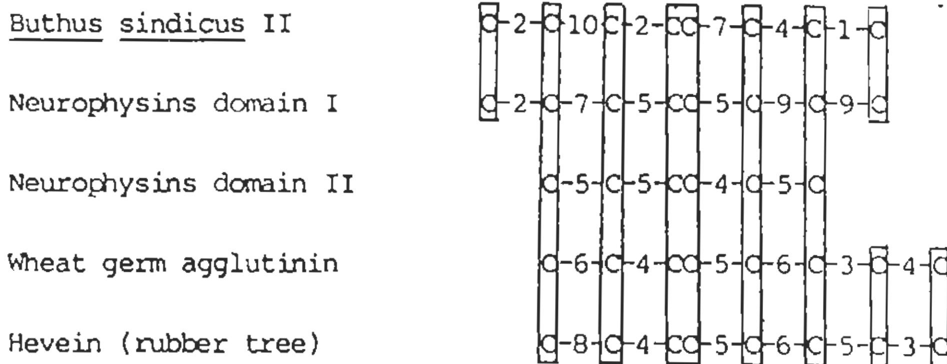
is compared by aligning the cysteine residues, the gaps are introduced at position 26 and 27 in the two sequences. One of these proteins belongs to a closely related species, i.e. Buthus eupeus (Central Asian scorpion) and has been identified as insect toxin. It shows conservation of 21/38 residues (55% identity), with the present structure, while the protein from a different species (last entry; Fig. 4.3) shows similarity in 24 out of 38 positions (63% identity). These variations among closely related species and between another species of a different genus suggest that the genes for these proteins have rapidly evolved. However conservation of cysteine residues indicate identical conformation.

4.2.2 Relationship To Four Disulphide Core Proteins

The "four disulphide core proteins" family has been shown to possess wheat germ agglutinins, neurophysins and hevein proteins [122], fig 4.4. It has been observed that the protein characterized from scorpion (Buthus sindicus) venom and its homologues have a similar distribution of cysteine residues (Fig. 4.4) as encountered in "four disulphide core proteins" [122,123]. On the basis of the observations made in cysteine pattern for the scorpion protein, it is assumed that the scorpion toxin may have closely related conformations like agglutanins.

As shown in fig. 4.4, 7 out of 8 cysteines can be aligned with the

Fig. 4.4: Alignment of cysteine position in (BuS II) scorpion insect toxin and among various proteins of the "four disulphide core proteins" (Neurophysins domain I, Neurophysins domain II, Wheat germ agglutinin, Hevein (rubber tree)) [123]. Numbers above cysteines represent the position in the intact chain and those between them show the original number of residues present between two consecutive cysteine residues. Numbering for cysteine positions in scorpion proteins is according to fig. 4.3.



position in other members of the "four disulphide core" family. It has been noticed that cysteines in scorpion proteins are closely related to either of the member of this group. However similarities at the primary structure level are negligible, which suggest that all these proteins have identical conformations but different residue requirements for their biological activity. Studies on modification of different residues would help to understand the role of various conserved residues in this type of toxin molecule. Further studies to establish the structures of such toxins from other species would help in establishing the evolutionary connections of these proteins.

4.3 CHARACTERIZATION OF NEUROTOXIN BuS IVb

The primary structure of peptide BuS IVb, determined by sequence analysis of the intact protein and peptides obtained by enzymatic cleavage, show that it consists of 65 residues. BuS IVb peptide chain, when compared on the basis of their homology with other Buthus species, was characterized as a neurotoxin. The neurotoxicity of this peptide was also confirmed by testing the biological activity on Rana tigrina muscle.

4.3.1 Comparison With Other Species

The primary structure of scorpion venom Buthus sindicus protein, BuS

Fig. 4.5: Comparison of amino acid sequence of BuS IVb with 17 different scorpion venoms (Buthus occitanus tunetanus I, II, III, VIII, XI, Buthus occitanus Paris I, II, Leiurus quinquestriatus quinquestriatus neurotoxin IV, Buthus eupeus, Buthus occitanus mauretanicus I, III, P1, IV, VI, North American scorpion neurotoxin I, II, III). Gaps are introduced to get the maximum homology. Star represents incomplete sequences. X represents unidentified residue.

```
                       1        10        20        30        40        50        60        70
BuS IVb               GVRDAYIADDK-NCVYTCGS---NSYCNTECTKD-GAESG-HCEWLGKYGWACWCIK-LP-DKVPIRIPGK---CR
BoT I                  GRDAYIAQPE-NCVYECAQ---NSYCNDLCTKQ-GATSG-YCDWLGKYGNACWC-KDLP-DNVPIRIPGK---CHF
BoT II                 GRDAYIAQPE-NCVYECAK---NSYCNDLCTKN-GAKSG-YCQWLGRWGNACYC-IDLP-DKVPIRIEGK---CHF
BoT III                VRDGYIVDDR-NCTYFCGR---NAYCNEECNKLKG-ESG-YCQWASPYGNACYCYK-VP-DHVRTKGP---GRCN-
*BoT VIII              VRDAYIAQNY-NCVYTCFK---NDYCNDICTKN-GAXXG-YC...
*BoT XI                LKDGYIVDDR-NCTYFCGT---NAYCNEECVKLKG-E...
*BOP I                 GRGVYIADIA-NCAY...
*BOP II                GRDAYIADDX-NCAYXCAL---XXYCN...
Lqq.N.T.4             GVRDAYIADDK-NCVTICGS---NSYCNTECTKD-GAESG-YCQWLGKYGNACWCIK-LP-DKVPIRIPGK---CR
Be M10                 VRDGYIADDKD-CAYFCGR---NAYCDEECKKG-GAESG-KCWYAGQYGNACWCYK-LP-DWVPIKQKVSGK-CN
N.A.S. neurotoxin I    K-DGYLVEKTG-CKKTCYKLGEADFCNREC-KWKHIG-GSY-GYCY--GFGCYCEG-LP-DSTQTWPLPN-K-CT
N.A.S. neurotixon II   KE-GYLVNKSTGCLKL-GENEGNK-C--EC-KAKNQ--GGS-YCYC-YAFECWCEG-LP-ESTPTYPLPN-K-CSS
Bom III                GRDGYIAQPE-NCVYHCFP-GSSG-CDTLC-KEKGATSG-HCGFLPGSGVACWCDNLPNNK-VPIVV-GGEK-CN
*Bom P1                GRDAYIAQPE-NCVYECAK--SS-YCNXLC...
*Bom I                 GRDCYIAQPE-NCVYHCFP-GSHG-CDTLC-KEKGATSG-XCGFLPGXXVA...
*Bom IV                GRDAYIAQPE-NCVYECAK-NS--YCNDLCTKX-GATSG-YCXW...
*Bom VI                VRDAYIAQPD-NCVYHCAK-D-N-YCNDLCT...
N.A.S. neurotoxin III  KEG-YLVKKSDGCKYGCLKLGENEGCDTECKAKNQGGSYGYCTAFACW---CWCEGLPESTPTYPLPN---KSC
```

IVb, when compared with previously deduced sequence of other species (Fig. 4.5) [BoT 1 [57], BoT II [57], BoT III [57], Leiurus quinquestrialus quinquestrialus neurotoxin IV [6C], BeM10 [59], North American scorpion neurotoxin II [61], Bom III [57] and North American scorpion neurotoxin III [61]] shows nine invariant amino acids i.e. 14% homology. The similar positions are 6, 14, 18, 27, 31, 43, 53, 55 and 75. All the cysteine are aligned with other species. The sequence of (Buthus sindicus) BuS IVb compared with Leiurus quinquestriatus quinquestriatus sequence shows highest homology i.e. 94% with BoT I it is 72% homology. Comparison between BuS IVb and BoT II, BeM10 and BoT III, BoM III, North American scorpion neurotoxin I, II and III, the noted homology is 66%, 62%, 55%, 48%, 27%, 26% and 23% respectively.

The presence of 75% non polar amino acids between position 46-56, which are invariant in most of these neurotoxins, suggests that this fragment might play a role in interaction with its receptor.

4.4 PARTIAL SEQUENCE OF TOXIN BuS V

BuS V was separated by RP-HPLC and molecular weight determined to be 6kDa. The protein contains cysteine (12.5%), glycine (7.8%) and lysine (15.6%), while isoleucine is absent. The amino acid composition of carboxymethylated peptide is given in section 3.5.5.1, table-6.

Fig. 4.6: Comparison of amino acid sequences of BuS V with venom peptide of C. noxius (Cn II-14) and T. serrulatus. Gaps are introduced to get the maximum homology.

```
BuS V      KD-NGYAVVDS--CKKNCYKK
C-II-14    KD--GYLV-DAKGCKKNCYKLGKNDYC...
T. Serr.   K-E-GYLM-DHEGCKLSCFIRPSG-YC...
```

4.4.1 Comparison With Other Species Toxins

Sequence was determined manually by DABITC method upto 18 residues. The partial sequence of the BuS V was compared with other species toxins i.e. Mexican scorpion toxin Cn II-14 and Tityus serralatus (Brazilian). The Buthus sindicus and Mexican scorpion have greater homology (68%) than Brazilian scorpion (36% ; Fig. 4.6). Toxin CnII14 is a long polypeptide toxin from Centruroides noxius venom, but the BuS V is partially sequenced till 18 residues and by their N-terminal homology it is identified as toxin. Gaps in the alignment are present in order to maximize the homology, positions 1, 5, 6, 10, 14, 15 and 18 have common residues in these three toxins, the result is, the conservation of the cysteine residues, which possibly help in maintaining the structure of toxin, while deletions and mutations show the evolutionary changes. However complete sequences will assist to understand the evolutionary pattern of the molecule.

4.5 N-TERMINAL PARTIAL SEQUENCE OF NEUROTOXINS (BuS VI, BuS VII)

These neurotoxins were purified by RP-HPLC and homogeneity checked on SDS-PAGE, which shows their molecular weight in the range of 6.6kDa.

Fig. 4.7: Amino acid sequences of neurotoxins from Buthus sindicus (BuS VI and VII). Comparison is made with five different species (A) N-terminal sequences upto 9 residues (B) Alignment after introducing gaps. Dots represent incomplete sequences.

Fig. A

Bus VI	DGYIVDDVN...
BuS VII	DGYSNDDVN...
BoT III	VKDGYIVDDRN...
BoT XI	LKDGYIVDDRN...
Lqq NT4	GVRDAYIADDKN...
BeM10	GVRDAYIADDYN...
BuS IVb	GVRDAYIADDKN...

Fig. B

BuS VI	DGYI-VDDVN...
BuS VII	DGY-SNDDVN...
BoT III	VKDGYI-VDDRN...
Bot XI	LKDGYI-VDDRN...
Lqq NT4	GVRDAYI-ADDKN...
BeM10	GVRDAYI-ADDYN...
BuS IVb	GVRDAYI-ADDKN...

Their purity was also checked by Edman degradation (DABITC) method, each gave a single derivative of pure peptides.

The composition of these neurotoxins are given in section 3.5.6.1 and 3.5.7.1, table-8 and 9, in which Cys, Asp, Gly, Tyr, His and Lys are present in high quantity. The amino acids composition of both neurotoxins show certain differences.

The N-terminal amino acid sequences were determined by the DABITC method upto nine steps. On the basis of homology and physiograph, it is characterized as neurotoxins. The alignment of BuS VI and BuS VII revealed that both neurotoxins have high degree of homologies i.e. 7/9 residues to be invariant (Fig. 4.7). Comparison of BoT III with that of BoT XI shows 10/11 residue to be invariant. Sequence of neurotoxin L.q.q, BeM10 and BuS IVb revealed 11/12 residues are found to be identical. Comparison of the sequences of these peptides with BuS VI and BuS VII show some heterogeneity. Maximum degree of homology was obtained, when all these neurotoxins were aligned, after introducing certain gaps as presented in fig. 4.7A and B.

4.6 BIOLOGICAL ACTIVITIES OF THE VENOM

4.6.1 Determination Of Neurotoxicity By Physiograph

The crude venom produced postsynaptic effect in concentration of

0.36/ml, as is evident from 107% increase in twitch amplitude and about 18 fold greater relaxation period within 5 minutes of exposure.

The three toxins (IVb, VI, VII) fractions showed increase in twitch height after two minutes which was transient in fraction BuS IVb and prolonged in BuS VI and BuS VII. Increase in C.T. and 1/2 R.T. of the first two fraction were also noted.

The site of action of neurotoxin is the neural-membrane where Na^+ and K^+ imbalance cause prolongation of action potential. The effect of toxin on Na^+ channels causes release of neuro-transmitter [25]. The prolongation of falling phase of twitch with a typical marked plateau observed in our present studies might be due to closing of this channels i.e. retardation and partial blockage of Na^+ inward current inactivation and possibly the opening of K^+ channel causing a slow trend of inward passage and curtailing the steady state of K^+ current.

The crude venom and its active fractions, as in other mamalian toxins, is likely to interact with a single receptor site in axonal membrane as has been reported by Rathmyer et al. [78] for purified toxins from scorpion A. australis.

4.6.2 Determination Of Toxicity By Injection Method

In the light of these results it is evident that the crude scorpion venom is highly effective against Musca domestica adults; and less effective against Blattella germanica adults, a wide difference has been calculated among these two insects against the same compound, in similar concentration and similar doses under the same environmental conditions.

4.7 SCORPION FATTY ACID DETERMINATION BY GCMS

GCMS studies in hibernating and post-hibernating animals provide an insight in the physiological behaviour and lipid-energy relationship. The diagnostic peaks observed in case of saturated fatty acid esters were molecular ion peaks (M^+), M^+-31 resulting from the loss of methoxy group, M^+-43 and M^+-59 arising from the loss of methoxy group and two hydrocarbon units ($2CH_2$). In methyl ester of mono-unsaturated and di-unsaturated fatty acid, the diagnostic mass fragments were M^+-31 and M^+-74 [109] (Section 3.7 Table-11).

5.0 REFERENCES

1. Tikader, B.K. (1973), **Bull. Natn. Inst. Sci. India**, 45, 260-276.

2. Jordan, E.L. and Verma, P.S. (1976), **Invertebrate Zoology**, Chand S. and Company Limited, India.

3. Parker, T.J. and Haswell, W.A. (1960), **A Text Book of Zoology**, Macmillan and Company Limited, New York.

4. **The Fauna of India Scorpions Scorpionida : Arachnida** (1983), vol. **III**, Tikader, B.K. and Bastawade, D.B., Eds., Zoological of India, Calcutta.

5. Thorell, T. (1876), **On the Classification of Scorpions**, Ann. Magnat. **Hist.**, 17, 1-15.

6. Tilak, R. (1970), **Sci. Cult.**, 36, 174-176.

7. Vachon, M. (1948), **Etudes sur les Scorpions I**, 26, 24-90, Arch Insti. Pasteur, Alger.

8. Stahnke, H.L. (1974), **Bio. Science**, 24, 339.

9. Simon, E. (1880), **Annls. Soc. Ent., Fr, Ser.**, 10, 377-398.

10. Simon, E. (1882), **Annali. Mus. Civ. Stor. Nat. Giacomo Doria**, 18, 207-260.

11. Tembe, V.V. and Awati, P.R. (1942), **J. Univ. Bombay**, 11, 54-76.

12. Stahnke, H.L. (1970), **Ent. News.** 81, 25-32.

13. **Larousse Encyclopaedia of Animal Life** (1976), Bertin, L., Ed., Hamlyn Publishing Group Limited, U.S.A.

14. J.M. Arena, M.D. (1979), **Poisoning Toxicology Symptoms Treatment**, Thomas, C.C., Ed., Springfield, Illinois, U.S.A.

15. El-Asmar, M.F., Ibrahim, S.A. and Rabie, F. (1972), **Toxicon**, 10, 73-77.

16. Parkih, C.K. (1982), **Parikh's Textbook of Medical Jurisprudence and Toxicology**, Medical Publications, Colaba, Bombay, India.

17. Husain, S.S. (1981), **A Textbook of Medical Jurisprudence and Toxicology**, Caravan Book House, Lahore, Pakistan.

18. Possani, L.D., Fletcher, Jr., P.L., Alagon, A.B.C., Alagon, A.C. and Julia, J.Z. (1980), **Toxicon**, 18, 175-183.

19. Katz, B. (1966), **Nerve, Muscle and Synapse,** p. 167, McGraw Hill, London.

20. Del Pozo, E.C. (1956), **Venoms,** p. 123, Buckley, E.E. and Porges, W., Eds., Washington, D.C., A.A.A.S.

21. Del Pozo, E.C. and Anguiano, L.G. (1947), **Revta Inst. Salubr. Enferm. Trop. Mex., 5,** 227.

22. Del Pozo, E.C. and Derbez, J. (1949), **Revta Inst. Salubr. Enferm. Trop. Mex., 10,** 203.

23. Diniz, C.R., Gomez, M.V., Antonio, A. and Corrado, A.P. (1966), **Mems. Inst. Butantan. Simp. Internac, 33,** 453.

24. Narahashi, T. (1972), **Fed. Proc., 31,** 1124-1132.

25. Rochat, H., Bernard, P. and Couraud, F. (1979), Advances in Cytopharmacology, **3,** 325-334.

26. Tafuri, W.L., Maria, T.A., Freire-Maia, L. and Cunha Melo, J.R. (1971), **Toxicon, 9,** 427-428.

27. Gomez, M.V., Dai, M.E.M. and Diniz, C.R. (1973), **J. Neurochem., 20,** 1051-1061.

28. Ismail, M., Osman, O.H. and El-Asmar, M.F. (1973), Toxicon, 11, 15-20.

29. Diniz, C.R., Piminta, A.F., Netto, J.C., Pompola, S., Gomez, M.V. and Bohm, G.M. (1974), **Experientia, 30,** 1304-1305.

30. Bohm, G.M., Pompolo, S., Diniz, C.R., Gomez, M.V., Pimenta, A.F. and Netto, J.C. (1974), **Toxicon, 12,** 509-511.

31. Adam, K.R. and Weiss, C. (1959), Z. Tropenmed. Parasit, 10, 334.

32. Parnas, I. and Russell, F.E. (1967), **Animal Toxins,** p. 401, Russell, F.E. and Saunders, P.R., Eds., Oxford, Pergamon Press.

33. Freire-Maia, L., Azevedo, A.D. and Costa, Val., V.P. (1973), **Toxicon,** 11, 255-257.

34. Yarom, R. and Gueron, M. (1971), **Pathol. Microbiol.,** 35, 114-116.

35. Gueron, M. and Yarom, R. (1970), Chest, 57, 156-162.

36. Ismail, M., Osman, O.H., Ibrahim, S.A. and El-Asmar, M.F. (1972), **E. Afr. Med. J.**, 49, 273-281.

37. Cheymol, J., Bourillet, F., Roch-Arveilier, M. and Heckle, J. (1974), **Toxicon**, 12, 241-248.

38. Corrado, A.P., Antonio, A. and Diniz, C.R. (1966), **Mems. Inst. Butantan Simp. Internac.**, 33, 957.

39. Hagag, M., Tu, A.T. and El-Asmar, F. (1983), **Arch. Biochem. Biophys.**, 220, 459-466.

40. Miranda, F., Rochat, H., Rochat, C. and Lissitzky, S. (1966), **Toxicon**, 4, 145-152.

41. Possani, L.D., Dent, M.A.R., Martin, B.M., Maelicke, A. and Svendsen I. (1981), **Carlsberg Res. Commun.**, 46, 207-214.

42. Possani, L.D., Martin, B.M., Mochca-Morales, J. and Svendsen, I. (1981), **Carlsberg Res. Commun.**, 46, 195-205.

43. Rochat, C., Rochat, H., Miranda, F. and Lissitzky, S. (1967), **Biochemistry**, 6, 578-585.

44. Miranda, F. and Lissitzky, S. (1961), **Nature** (Lond.), 190, 443-444.

45. Miranda, F., Kupeyan, C., Rochat, H., Rochat, C. and Lissitzky, S. (1970), **Eur. J. Biochem.**, 16, 514-523.

46. Rochat, H., Rochat, C., Kupeyan, C., Miranda, F. and Lissitzky, S. (1970), **FEBS Lett.**, 10, 349-351.

47. Fitch, W.M. and Margoliash, E. (1967), **Science**, 155, 279-284.

48. Rochat, H. (1978), **Scorpion Toxins, Third Int. Symp. Cytopharmacol.**, July, Venice.

49. Lazarovici, P., Yanai, P., Pelhate, M. and Zlotkin, E. (1982), **J. Biol. Chem.**, 257, 8397-8404.

50. Zlotkin, E., Miranda, F. and Rochat, H. (1978), **Handbook of Experimental Pharmacology**, vol. 48, pp. 317-369, Bettin, S., Ed., Springer Verlag, Berlin.

51. Zhdanova, L.N., Adamovich, T.B., Nasimov, I.V., Grishin, E.V. and Ovehinnikov, Y.A. (1977), **Bioorg. Khim.**, 3, 485-493.

52. Darbon, E., Rochat, H., Kupeyan, C. and Reitschoten, J.V. (1982), **Toxicon**, 20, 64.

53. Possani, L.D. (1984), **Handbook of Natural Toxins.**, vol. **2**, pp. 513-550. Tu, A., Ed., Marcel Dekker Inc. New York.

54. Goyffon, M. and Kovoor, J. (1978), **Handbook of Experimental Pharmacology-Arthropod Venoms**, vol. **48**, pp. 395-418, Springer--Verlag, Berlin.

55. Lazarovici, P. and Zlotkin, E. (1982), **Comp. Biochem. Physiol., 71C**, 177-181.

56. Possani, L.D., Martin, B.M., Svendsen, Ib., Rode, G.S. and Erickson, B.W. (1985), **Biochem. J., 229**, 739-750.

57. Vargas, O., Gregoire, J., Martin, M.F., Bechis, G. and Rochat, H. (1982), **Toxicon**, 20, 79.

58. Sampieri, F. and Habersetzer-Rochat, C., **Personal Communication.**

59. Grishin, E.V., Soldatova, L.N., Shakhparonov, M.I. and Kazakov, V.K. (1980), **Bioorganic Chem., 6**, 714-723.

60. Kupeyan, C., Martinez, G. and Rochat, H. (1982), **Toxicon. 20**, 71.

61. Babin, D.R., Watt, D.D., Goos, S.M. and Mlejnek, R.V. (1975), **Arch. Biochem. Biophys., 166**, 125-134.

62. Babin, D.R., Watt, D.D., Goos, S.M. and Mlejnek, R.V. (1974), **Arch. Biochem. Biophys., 164**, 694-706.

63. Dickerson, R.E. and Geis, I. (1969), **The Structure and Action of Proteins.**, Harper and Row, New York.

64. Johnson, C.K. (1965), ORTEP, A Fortran Thermal Ellipsoid Plot Program for crystal structure Illustration, Report ORNL-3794, Oak Ridge National Laboratory, Oak Ridge, TN.

65. Fontecilla-Camps, J.C., Almassy, R.J., Suddath, F.L., Watt, D.D. and Bugg, C.E. (1980), **Proc. Nat. Acad. Sci.**, U.S.A., 77, 6496-6500.

66. Possani, L.D., Steinmetz, W.E., Dent, M.A.R., Alagon, A.C. and Wuthrich, K. (1981), **Biochim. Biophys. Acta., 669**, 183-192.

67. Delori, P., Van Rietschoten, J. and Rochat, H. (1981), **Toxicon, 19**, 393-407.

68. Darbon, H., Jover, E., Couraud, F. and Rochat, H. (1983), **Int. J. Peptide Proteins Res., 22**, 179-186.

69. Darbon, H., Sampieri, F., El-Ayeb, M. and Rochat, H. (1985), **Proc. 16th FEBS Mett. B.**, 423-429.

70. Martin, M.F. and Rochat, H. (1984), **Toxicon, 22**, 279-291.

71. Sampieri, F. and Habersetzer-Rochat, C. (1978), **Biochim. Biophys. Acta, 535**, 100-109.

72. Carbone, E., Wanke, E., Prestipino, G., Possani, L.D. and Maelicke, A. (1982), **Nature, 296**, 90-91.

73. Possani, L.D., Martin, B.M. and Sevendsen, I. (1982), **Carlsberg Res. Commun., 47**, 285-289.

74. Couraud, F., Jover, E., Dubois, J.M. and Rochat, H. (1981), **Proceedings of the 4th European Symposium on Animal, Plant and Microbial Toxins, Marseille**, June, 1981.

75. Rochat, C., Rochat, H., Mirarda, F. and Lissitzky, S. (1967), **Biochem. J., 6**, 578-585.

76. Romey, G., Abita, J.P., Chicherportiche, R., Rochat, H. and Lazdunski, M. (1976), **Biochim. Biophys. Acta, 448**, 607-619.

77. Warnick, J.E., Albuquerque, E.X. and Diniz, C.R. (1976), **J. Pharmacol. Expt. Therap., 198**, 155-167.

78. Rathmayer, W., Walter and Zlotkin, E. (1977), **Comp. Biochem. Physiol., 56c**, 35-39.

79. Kupeyan, C., Martinez, G., Lissitzky, S., Miranda, F. and Rochat, H. (1974), **Eur. J. Biochem., 47**, 483-489.

80. Adam, K.R., Schmidt, H., Stampfli, R. and Weiss, C. (1966), **Br. J. Pharmacol., 26**, 666-667.

81. Koppenhofer, E. and Schmidt, H. (1968), **Pflugers Arch., 303**, 133-149.

82. Parnas, I., Avgar, D. and Shulov, A. (1970), **Toxicon**, 8, 67-79.

83. Narahashi, T., Shapiro, B.I., Deguchi, T., Scuka, M. and Wang, C.M. (1972), **Am. J. Physiol., 222**, 850-857.

84. Cahalan, M.D. (1975), J. Physiol., 244, 511-534.

85. Petrides, P.E., Jones, R.T. and Bohlen, P. (1980), Anal. Biochem., 105, 383.

86. Congote, L.F., Bennett, H.P.J. and Solomon, S. (1979), Biochem. Biophys. Res. Commun., 89, 851.

87. Fazal, A., Shafqat, J., Beg, O.U. and Zaidi, Z.H. (1988), J. Chem. Soc., Pak., 10, (in press).

88. Ornstein, L. (1964), Ann. N.Y. Acad. Sci., 121, 321.

89. Davis, B.J. (1964), Ann. N.Y. Acad. Sci., 121, 404.

90. Laemmli, U.K. (1979), Nature, 227, 680-685.

91. Crestfield, A.M. Moore, S. and Stein, W.H. (1963), J. Biol. Chem., 238, 622-627.

92. Hirs, C.H.W., Stein, W.H. and Moore, S. (1956), J. Biol. Chem., 221, 151-159.

93. Hirs, C.H.W. (1967), Methods in Enzymol., 11, 218-220.

94. Bennett, H.P.J., Browne, C.A. and Solomon, S. (1980), J. Liq. Chromatogr., 3, 1353-1365.

95. Hearn, M.T.W. and Hancock, W.S. (1979), Biol. Biomedical Application of Liquid Chromatography, 243-271, Hawk, G.L., Ed., Marcel Dekker, New York.

96. Schroeder, W.A., Shelton, J.B., Shelton, J.R. and Powars, D. (1979), Journal of Chromatography, 174, 385-392.

97. Moore, S. and Stein, W.H. (1948), Ann. N.Y. Acad. Sci., 49, 265.

98. Spackman, D.M., Stein, W.H. and Moore, S. (1958), Analyt. Chem., 30, 1185.

99. Hamilton, P.B. and Anderson, R.A. (1959), Analyt. Chem., 31, 1564.

100. Peterson, E.A. and Sober, H.A. (1959), Analyt. Chem., 31, 857.

101. Von Bahr-Lindstrom, H., Hempel, J. and Jornvall, H. (1982), J. Prot. Chem., 1, 257-262.

102. Chang, J.Y., Brauer, D. and Wittman-Liebold, B. (1978), FEBS Lett., 93, 205-214.

103. Edman, P. and Begg, G. (1967), **Eur. J. Biochem.**, 1, 80-91.

104. Hewick, R.M., Hunkapiller, M.W., Hood, L.E. and Dreyer, W.J. (1981), **J. Biol. Chem.**, **256**, 7990-7997.

105. Hunkapiller, M.W., Hewick, R.M., Dreyer, W.J. and Hood, L.E. (1983), **Methods Enzymol.**, **91**, 399-413.

106. Jornvall, H. and Philipson, L. (1980), **Eur. J. Biochem.**, **104**, 237-247.

107. Zimmerman, C.L., Apella, E. and Pisano, J.J. (1977), Anal. **Biochem.**, 77, 569-573.

108. Inagami, T. and Marakami, K. (1972), **Anal. Biochem.**, 47, 501-504.

109. Iqbal, M. (1967), **M.Sc. Thesis**, Department of Biochemistry, University of Karachi.

110. Stenhagen, E. (1964), **Z. Anal. Chem.**, **205**, 109.

111. Rhyage, R. and Stenhagen, E., (1963), **Mass Spectrometry in Organic Ions**, p. 399, Mclafferty, F.W., Ed., Academic Press, N.Y.

112. Winshester, B. (1969), **M.Sc. Thesis**, Department of Physiology, University of Alberta, Edmonton, Alberta, Canada.

113. Barlow, F. and Hadaway, A.B. (1975), PANS., **21**, 233-238.

114. MacCuaig, R.D. and Sawyer, K.F. (1957), **Bull. Entomol. Res.**, **48**, 435-445.

115. Quraishi, M.S. and Thorsteinson, A.J. (1967), **J. Econ. Entomol.**, **60**, 84-88.

116. Seidel, E. (1971), **Z. Ang. Zool.**, **58**, 385-440.

117. Watts, W.S. (1970), **Proc. Ind. Study Conf.** Lond., 407-411.

118. Yeates, M.N.D.B. (1973), **Miscellaneous** Report No. 13, CGRP, London, 1-10.

119. Abbot, W.S. (1925), **J. Econ. Ent.**, **18**, 265-267.

120. Acher, R., **Evolution and Tumor Pathology** of the **Neuroendocrine System** (1984), Falker, S., Hakanson, R. and Sundler, F., Ed., pp. 181-201, Elsevier, Amsterdam.

121. Zhdanova, L.N., Adamovich, T.B., Nazimov, I.V., Grishen, E.V. and Ovchinikov, Y.A. (1978), **Sov. J. Bioorg. Chem.**, 3, 366-372.

122. Drenth, J. (1981), **J. Biol. Chem., 256,** 2601-2602.

123. Drenth, J., Low, B.W., Richardson, J.S. and Wright, C.S. (1980), **J. Biol. Chem., 255,** 2652-2655.