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Isolation and characterization of a novel postsynaptic/ cytotoxic neurotoxin from *Daboia russelli russelli* venom

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Abstract: A postsynaptic neurotoxin was purified from *Daboia russelli russelli* venom using gel filtration, ion-exchange chromatography and reverse-phase high-performance liquid chromatography. The N-terminal sequence, molecular mass and pharmacological activities of the neurotoxin/cytotoxin indicate that it is a short-chain neurotoxin like that found in Elapid venom. This is the first report on the presence of such a postsynaptic neurotoxin from *D. r. russelli* venom.

Abbreviations: DNTx, *Daboia* neurotoxin; EAT, Ehrlich ascites tumour cells; HPLC, high-performance liquid chromatography; MS-MALDI, mass spectrometry-matrix assisted laser desorption ionization; LD_{50}, lethal dose for 50% animals; TFA, trifluoroacetic acid; VRV PL-V, Vipera Russell venom phospholipase-V.

Venoms of the Viperidae family are characterized by the presence of hemorrhagins, myotoxins, proteases that alter the blood coagulation cascade, factors that cause edema and local tissue necrosis (1–3). The composition of the venom of the Viperidae family varies geographically in India, with the presence of small molecular mass proteins in the east (2), edema-inducing factors in the west and a presynaptic neurotoxin that has PLA_2 activity in the south (4,5). The neurotoxins *per se* are subdivided into two groups with respect to their mode of action; the presynaptic and postsynaptic neurotoxins. Presynaptic neurotoxins bind to the presynaptic membrane and bring about the inhibition of neurotransmitter release (6). Postsynaptic neurotoxins bind to acetylcholine receptors and inhibit impulse formation (7). Elapid and Hydrophid venoms are characterized by the

presence of postsynaptic neurotoxins. These are further subdivided into two types, based on their molecular mass, the short-chain (60–63 amino acids) and long-chain (70–74 amino acids) neurotoxins (8–11) with molecular masses of ≈7000 and 8000 Da, respectively (12). There have been reports on the presence of postsynaptic neurotoxins in false horned viper (13) and pit viper (14) but the existence of a postsynaptic neurotoxin has never been reported in *Daboia russelli russelli* venom.

In our laboratory, we isolated a postsynaptic neurotoxin that comprises ≈10% of the total protein of the whole venom from *D. r. russelli*. We report the purification, characterization, N-terminal analysis and biological activity of this postsynaptic neurotoxin.

Materials and Methods

Sephadex G-75, G-50 and CM-Sephadex C-25 (4.5 mEq/g) were all purchased from Pharmacia, Fine Chemicals (Uppsala, Sweden). Acetylcholine was from Sigma (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade. Lyophilized *D. r. russelli* venom was purchased from Hindustan Park, Calcutta, India.

Sephadex G-75 column chromatography

One hundred milligrams (by protein estimation) of the crude venom was dissolved in 2 mL of 0.05 M phosphate buffer pH 7.0 and loaded onto a G-75 column (0.75×70 cm) pre-equilibrated with the above buffer. Two-milliliter fractions were collected and the protein profile was monitored in a UV-VIS 1601 Shimadzu spectrophotometer.

CM-Sephadex C-25 column chromatography

Peak III of the G-75 column was concentrated and loaded onto a CM-Sephadex C-25 column (0.75×60 cm) that had been pre-equilibrated with 0.05 M phosphate buffer pH 7.0, and was eluted stepwise with phosphate buffers of various molarities and pH values as indicated in Fig. 2. Fractions (2.5 mL) were collected and the protein profile monitored using a UV-VIS 1601 Shimadzu spectrophotometer.

Sephadex G-50 column chromatography

Peak IV, which eluted at 0.15 M phosphate buffer on the CM-Sephadex C-25 column was lyophilized and dissolved in 2 mL of saline and loaded onto a Sephadex G-50 column (0.75×60 cm) pre-equilibrated in saline, 2-mL fractions were collected and the protein profile monitored as before.

Reverse-phase HPLC

The homogeneity of the protein peak (B) from the G-50 column was checked by passing the protein through an RP HPLC Vydac C_{18} column. The peptide was eluted using a 0–100% gradient of solvent B (70% acetonitrile) against solvent A (0.1% TFA).

Molecular mass determination by MS-MALDI

The molecular mass of the peptide was determined by mass spectrometry in a Kratos PC-Kompact MALDI 4 in the positive ionization mode, Linear high, Power: 45.

N-Terminal sequencing

N-Terminal sequencing of the peptide was carried out in a fully automated Shimadzu protein sequencer, PSQ-1 system. Peptide purity was determined by SAS-PAGE.

Determination of LD_{50}

LD_{50} values for the purified peptide and various venom fractions were calculated according to the mathematical scheme adopted by Meir & Theakston (16). The dose injected intraperitoneally varied from 0.1 to 1 mg/kg body weight for the purified peptide and from 1 to 10 mg/kg body weight for the other fractions. The survival time of each animal was recorded up to 24 h.

Frog nerve muscle preparation

Sciatic nerve gastrocnemus muscle from *Rana hexadactyla* was dissected and mounted in physiological Ringer's solution. The experimental conditions were as described by Harvey *et al*. (17). The preparation was mounted in an organ bath of 10–15 mL with a resting tension of 0.5 g applied to each preparation. The nerve was stimulated at 1-min intervals either by a single impulse (1 ms duration, supramaximal voltage) or by submaximal concentrations of acetylcholine.

Cytotoxicity assay

Cell viability was assayed as described by Chwetzoff *et al*. (18), using Ehrlich ascites tumour cells (EAT) grown in the

peritoneal cavity of Swiss albino mice. EAT cells were suspended in Tyrode Ringer buffer (5×10^6 cells in final volume of 1 mL) and incubated with various concentrations of this purified peptide for 15 min. One hundred microliters of Trypan Blue solution (1% in saline) were then added. The stained cells (not viable) and the unstained cells (viable) were counted using a hemocytometer.

Determination of myotoxicity

Myotoxicity was determined according to the method of King (19) and Hughes (20).

Results

Sephadex G-75 column chromatography

Venom from *D. r. russelli* yielded four peaks on passing through the G-75 column. The elution profile indicated that the low molecular mass proteins (the III peak) constituted a major part of the whole venom (Fig. 1).

CM-Sephadex C-25 column chromatography

Peak III of the G-75 column on fractionation on the CM-Sephadex C-25 column resolved into a major peak and three minor peaks. The elution profile of the G-75 column indicates that all these peaks contain peptides of low molecular mass (Fig. 2).

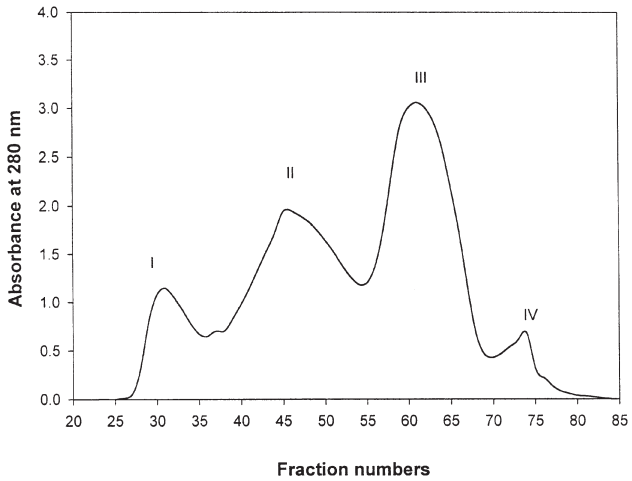

Figure 1. Sephadex G-75 column chromatography of *Daboia russelli russelli* (whole venom). The column (0.75×70 cm) was equilibrated with 0.05 M phosphate buffer, pH 7.0. Fractionation was carried out as described in Materials and Methods.

G-50 column chromatography

The major peak was cleared of impurities by passing through a G-50 column. On storage (4°C) the peptide formed a fine precipitate. This was centrifuged and the supernatant loaded onto a G-50 column. A small peak (A) appeared immediately after the void volume, followed by a single major peak, peak B (Fig. 3). This major peak was subjected to HPLC.

High-performance liquid chromatography

The peptide eluted with a retention time of 32 min as a single sharp peak (Fig. 4). This peak was concentrated and used for N-terminal analysis and molecular mass determinations.

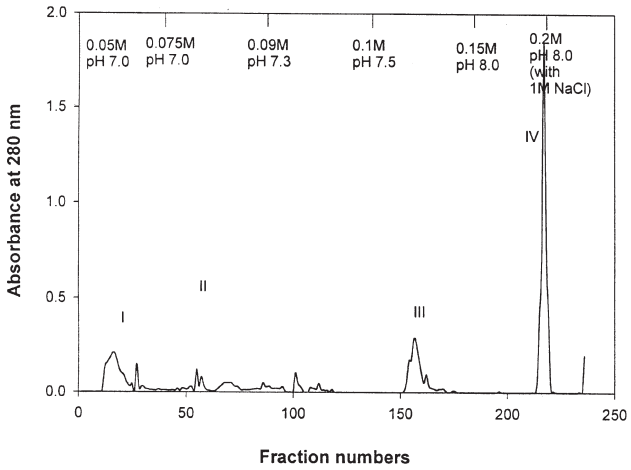

Figure 2. CM-Sephadex C-25 column chromatography of peak III from the G-75 column. Fractionation was carried out as described in Materials and Methods.

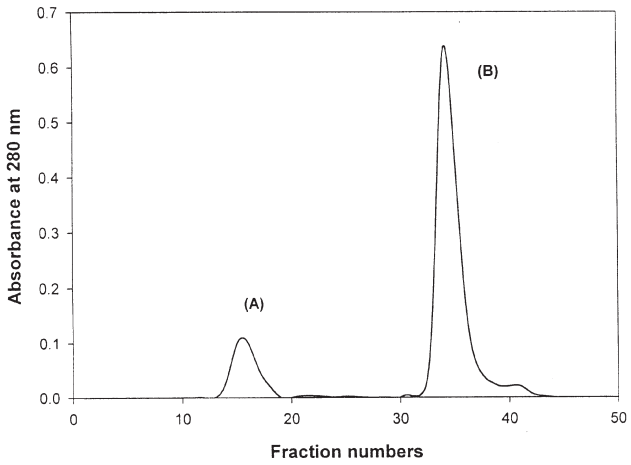

Figure 3. Sephadex G-50 column chromatography of peptide peak. The column (0.75×70 cm) was equilibrated with saline. Fractionation was carried out as described in Materials and Methods.

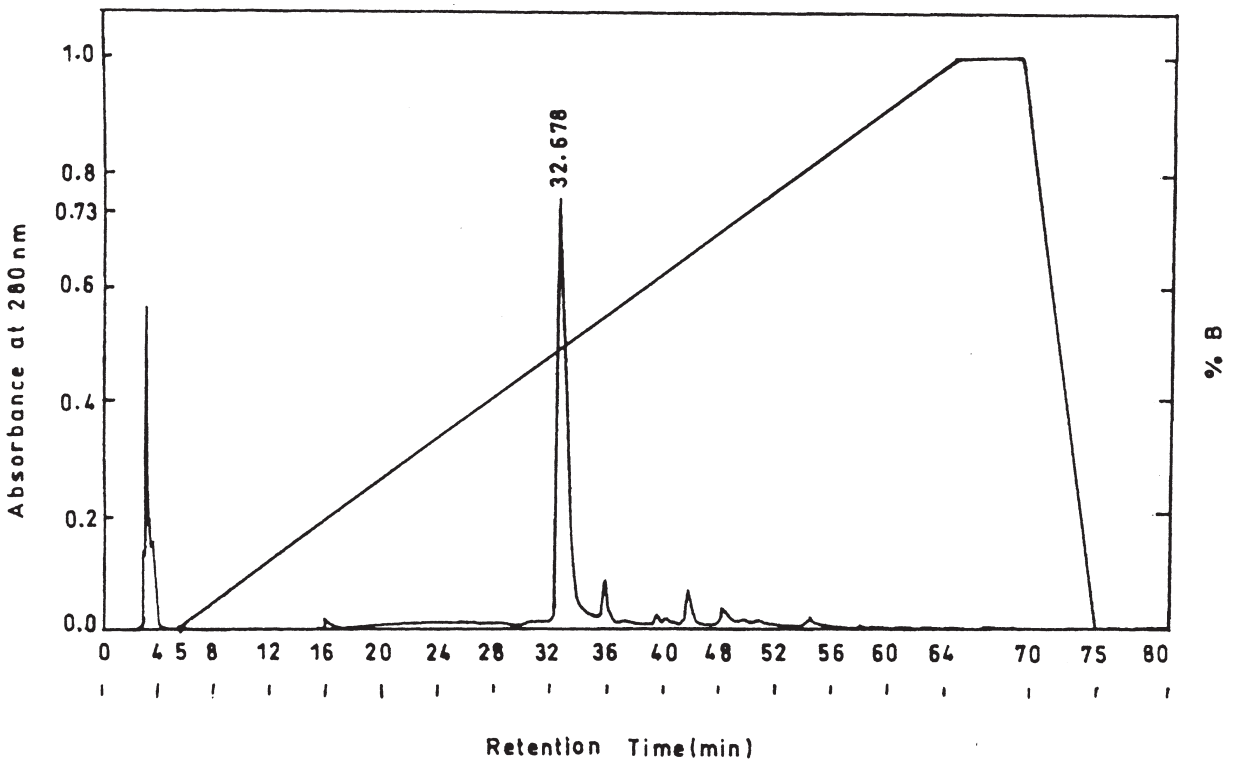

Figure 4. RP–HPLC fractionation of *Daboia* neurotoxin I (DNTx I) on a Vydac C_{18} column at a 0–100% gradient of B (70% acetonitrile) in the presence of A (0.1% trifluoroacetic acid). Flow rate, 1 mL/min.

Molecular mass determination by MS-MALDI

Molecular mass determination using the MS-MALDI technique gave a value of 6675 Da (Fig. 5).

N-Terminal sequence

N-Terminal sequence analysis of the peptide toxin gave the following sequence.

N-Terminal sequence: L E C N K L Q P I A S K

Effect of the peptide fraction on frog nerve–muscle preparation

The peptide toxin decreased the twitch height in a dose-dependent manner on indirect stimulation. Inhibition of the twitch was almost total at a concentration of 4 μg/mL, the mode of inhibition of the twitch was similar to that effected by α toxin, a postsynaptic neurotoxin isolated from *Naja naja* venom (Fig. 7).

At a neurotoxin concentration of 2.5 μg/mL, the twitch response was not restored on addition of a submaximal concentration of acetylcholine (Fig. 6), whereas in the preparation treated with a presynaptic neurotoxin (VRV PL-V) the twitch response was restored to the level observed when preparations were treated independently with acetylcholine.

Determination of LD_{50}

The lethal dose of various fractions is given in Table 1. The specific toxicity increased with purification of the low molecular mass peptides.

Determination of cytotoxicity

The cell viability assay showed that the neurotoxin was cytotoxic to EAT cells in a dose-dependent manner (Fig. 8).

Determination of myotoxicity

The peptide did not show any myotoxic activity.

Discussion

Snake venoms of the Elapid and Hydrophid families are known to contain short-chain, three-finger postsynaptic neurotoxins that contribute largely to the lethality of the

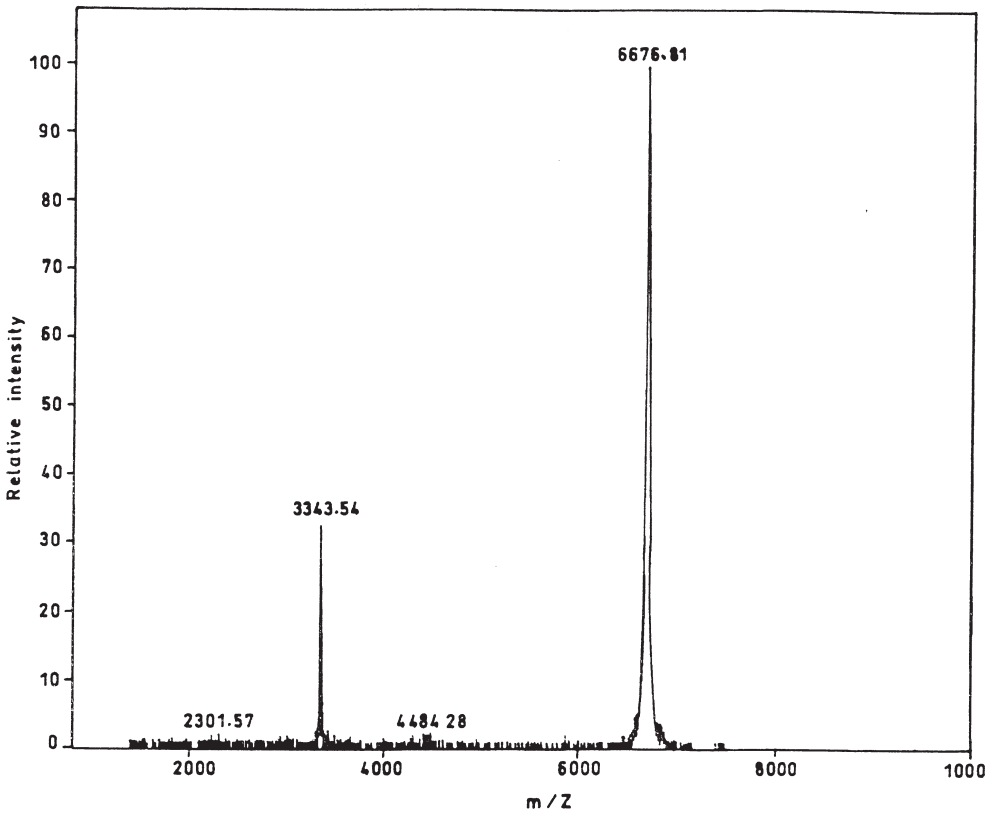

Figure 5. MS-MALDI: molecular mass determination by MS-MALDI in a Kratos PC-Kompact 4 instrument.

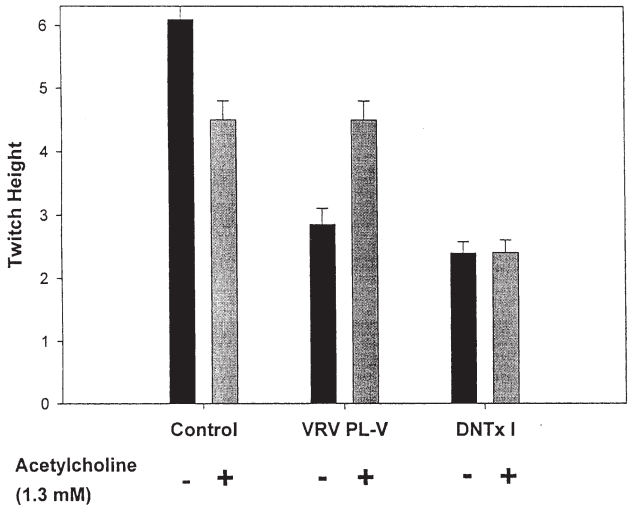

Figure 6. Effects of VRV PL-V (20 μg/mL) and DNTx I (2.5 μg/mL). On indirect stimulation and in the presence of acetylcholine (1.3 mM). Nerve–muscle preparations were incubated with the respective toxins for 15 min. Columns represent the means of determinations made on six preparations. Bars represent SEM.

***Table 1.* Lethal potency of the crude venom (CV), different fractions (peaks I, II, III) of G-75 chromatography of *Daboia russelli russelli* venom and the purified DNTx I peptide**

		Peak			
	CV	I	II	III	DNTx I
LD_{50} (ip) (mg/kg body wt.)	1.3 (0.3)	2 (2.85)	>6.0	0.71 (0.15)	0.39 (0.1)

Values expressed as mean (SD) (n=6).

crude venom (21). We isolated a similar short-chain neurotoxin from the venom of the snake *D. r. russelli* a member of the Viperidae family. The venom of snakes belonging to the same Viperidae family but a different subfamily, such as Crotolidae, are known to contain α neurotoxin-like peptides that are postsynaptic neurotoxins (13,14). Their molecular mass and mode of action suggest that these are short-chain three-fingered neurotoxins. Also, antibodies raised against Viper venom have been reported to show cross-reactivity with Elapid venom (22).

The fractionation profile of gel filtration shows that low molecular mass proteins form a major portion of the *D. r. russelli* venom (Fig. 1). On ion-exchange chromatography, this fraction gave a single prominent peak *Daboia* Neurotoxin 1 (DNTx I) (Fig. 2). The homogeneity of the peak was confirmed by SDS–PAGE (data not represented) and RP–HPLC. The toxin induced neurotoxic symptoms such

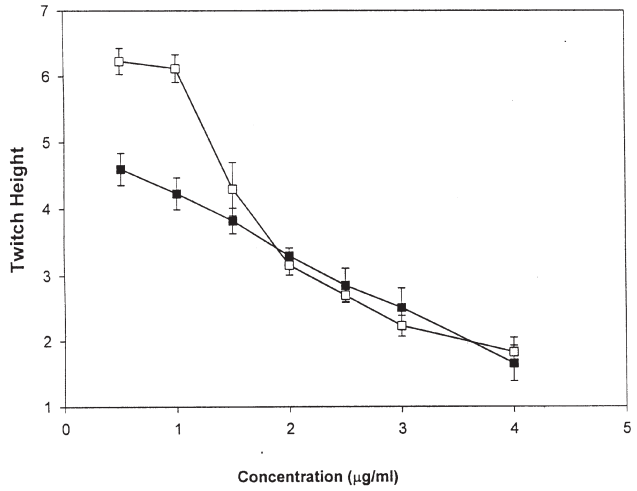

Figure 7. Dose-response curve for DNTx I (□). α Neurotoxin from Naja naja venom (■). Points represent the mean for six determinations. Bars indicate SEM.

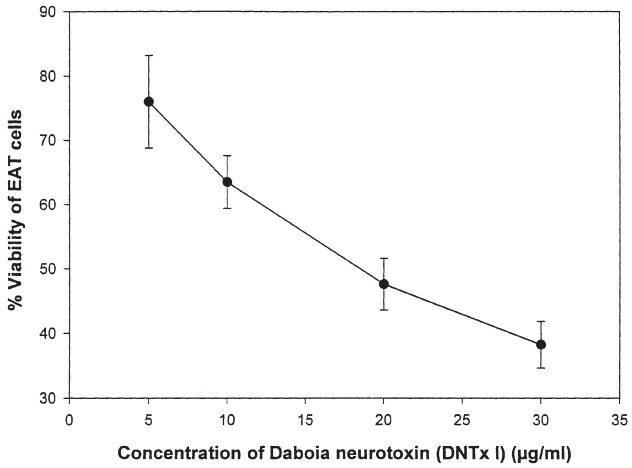

Figure 8. Effect of concentration of DNTx I on the viability of EAT cells. The values plotted are the means of six determinations. Bars indicate SEM.

as severe respiratory distress, violent convulsions and hind limb paralysis before death. These are the typical symptoms caused by α neurotoxins.

DNTx I forms almost 10% of the crude venom protein. The peptide appears to be the major component contributing to the toxicity of the *D. r. russelli* venom as the other chromatographic fractions did not show high toxicity. This is in accordance with the findings that, in general, neurotoxins are mainly responsible for the lethality of venoms. This peptide is devoid of any phospholipase activity, has a molecular mass of 6700 Da (Fig. 5) and is highly toxic. The high toxicity of Elapid venoms (≈1 mg/kg body weight) is due to the neurotoxins present in them which could have presynaptic and postsynaptic action. The LD_{50} of *D. r. russelli* venom suggested the presence of highly toxic neurotoxins in the venom, in contrast to the LD_{50} of *Vipera russelli* venoms of southern and western region of India which varies between 4 and 5 mg/kg body weight (2).

On pharmacological characterization of the toxin it was observed that the muscle twitch inhibition induced by DNTx I was not lifted on addition of acetylcholine (Fig. 6), in contrast to the preparation treated with a presynaptic neurotoxin, in which twitch inhibition was lifted on addition of acetylcholine. The inhibition pattern was like that of α neurotoxin from cobra venom (Fig. 7).

N-Terminal sequence analysis did not show significant homology with short-chain neurotoxins. However, the peptide showed 80% homology with that of a cytotoxin/cardiotoxin from *Naja naja atra* on BLAST search.

DNTx I was also cytotoxic, as it decreased the viability of EAT cells in dose-dependent manner (Fig. 8). Therefore,

a) L E C N K L Q P I A S K
b) L K C N K L V P L F Y K
c) L E C H N Q Q S S Q P P
d) L E C H N Q Q S S Q T P
e) L E C H N Q Q S S Q P P
f) L K C H K L V P F L S K

Figure 9. Alignment chart for homologous snake venom polypeptides. (A) Neurotoxic peptide (DNTx I) from *Daboia russelli russelli* venom. (B) Cardiotoxin from *Naja naja atra* [23]. (C) α Neurotoxin from *Naja nigricollis* [24]. (D) Cobrotoxin from *Naja naja atra* [25]. (E) *Hemachatus hemachatus* IV [26]. (F) Non-neurotoxic polypeptide from *Hemachatus hemachatus* [27].

the peptide appears to possess both cytotoxicity and neurotoxicity. Comparision of the LD_{50} value of DNTx I (0.39 mg/kg body weight) with that of postsynaptic neurotoxins (<0.2 mg/kg body weight) (28) and cytotoxins (>1 mg/kg body weight) (29) also supports the dual mode of action of DNTx I. Hence, we suggest that this peptide has two different binding sites. One specific to the acetylcholine receptor in the neuromuscular junction and the other which is nonspecific and results in cytotoxicity. These dual binding sites could be the reason for the reduced lethality of DNTx I compared with the other postsynaptic neurotoxins.

The molecular mass of 6700 Da, the pharmacological effects and the N-terminal homology with that of short-chain neurotoxins all indicate that DNTx I is a short-chain postsynaptic neurotoxic/cytotoxic peptide.

In summary, we report for the first time the isolation and characterization of a short-chain postsynaptic neurotoxin with cytotoxic property from *D. r. russelli* venom.

The discovery of such a short-chain neurotoxin from Viperid venom shows that the phylogenetic intra-relationship of the Elapid and Hydrophid family through the short/long-chain neurotoxins may also be extended to the Viperid family. Further investigation of such neurotoxins from Viperid venom may throw light on the origin and development of these secretions.

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