Synergistically acting PLA₂: Peptide hemorrhagic complex from Daboia russelii venom

Madhukumar Venkatesh 1, Veerabasappa Gowda*

Department of Studies in Biochemistry, Manasagangotri, University of Mysore, Mysore 570 006, Karnataka, India

A R T I C L E   I N F O
Article history:
Received 26 March 2013
Received in revised form 3 July 2013
Accepted 4 July 2013
Available online 18 July 2013

Keywords:
Neurotoxin
Russell's viper
Phospholipase A₂
Snake venom
Hemorrhagic complex
Daboia russelii

A B S T R A C T
Snake venoms are complex mixture of enzymatic and non-enzymatic proteins. Non-covalent protein–protein interaction leads to protein complexes, which bring about enhanced pharmacological injuries by their synergistic action. Here we report identification and characterization of a new Daboia russelii hemorrhagic complex I (DR–HC-I) containing phospholipase A₂ (PLA₂) and non-enzymatic peptide. DR–HC-I was isolated from the venom of D. russelii by CM-Shepadex-C25 and gel permeation chromatography. Individual components were purified and identified by RP-HPL chromatography, mass spectrometry and N-terminal amino acid sequencing. DR–HC-I complex was lethal to mice with the LD₅₀ dose of 0.7 mg/kg body weight with hemorrhagic and neurotoxic properties. DR–HC-I complex consists of non-hemorrhagic PLA₂ and neurotoxic non-enzymatic peptide. The non-enzymatic peptide quenched the intrinsic fluorescence of PLA₂ in a dose dependent manner, signifying the synergistic interaction between two proteins. PLA₂ and peptide toxin in a 5:2 M ratio induced skin hemorrhage in mice with MHD 20 mg. However, addition of ANS (1-Anilino-8-naphthalene sulfonate) to DR–HC-I complex inhibited skin hemorrhagic effect and also synergic interaction. But there was no impact on PLA₂ due to this synergistic interaction, and indirect hemolytic or plasma re-calciﬁcation activity. However, the synergistic interaction of PLA₂ and non-enzymatic peptide contributes to the enhanced venom-induced hemorrhage and toxicity of Daboia russelii venom.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Daboia russelii (Russell's viper) is one of the most perilous snakes that cause morbidity and mortality in Indian subcontinent. Its venom consists of components that are responsible for coagulopathy, necrosis, renal failure, neurotoxicity, myotoxicity, cardiotoxicity, convulsions, hypotensive, anticoagulation, inflammation, hemorrhage etc. Among these, hemorrhage on the site of bite and unhealed wounds of the victim were severe and major manifestation of envenomation, though renal failure is considered as the primary cause of death (Kularatne et al., 2011) (Warrell, 1989). Recent studies and clinical observations have indicated higher case fatalities and more severe hemorrhagic manifestations in D. russelii envenoming in Eastern India, Myanmar and in other regions (Kumar and Gowda, 2006; Mukherje et al., 2000; Senthilkumaran et al., 2012) (Warrell, 1997).

Although hemorrhage is a well-known phenomenon in the Daboia russelii bites, its mechanism of action needs to be elucidated. Several researchers attributed hemorrhagic activity to proteolysis toxins (Chakrabarty et al., 1993; Ribeiro Filho et al., 2003). However, the presence of
hemorrhagic toxins devoid of proteolysis activity have been reported (Kasturi and Gowda, 1989; Kole et al., 2000; Toom et al., 1969).

**Hemorrhagic effect of D. russelii** venom was mainly attributed to proteases and PLA2s. PLA2 hemorrhagins such as VRV-PL-VIIa (Kasturi and Gowda, 1989) and VRV-PL-VI (Vishwanath et al., 1988) were purified and characterized from Southern and Northern regional D. russelii venoms respectively. Hemorrhagic PLA2’s purified from D. russelii venom have shown organ specificity in test animals. For example, the most basic phospholipase A2 of Southern Indian venom is shown to induce lung hemorrhage (Kasturi and Gowda, 1989).

*D. russelii* venom from Western India reported to cause atrophic effects on the reproductive organs in animals (Kumar et al., 2008). A protease from the same source has been reported to cause hemorrhage in the eyes, lungs and liver (Jayanthi and Gowda, 1990). A hemorrhagin, isolated from Eastern Indian region has been reported to induce severe lung and liver hemorrhage and dermal hemorrhage (Chakrabarty et al., 1993; Chen et al., 2008). A non proteolytic toxin has been reported to induce intense intestinal hemorrhage and to a lesser extent skeletal muscle hemorrhage in mice (Kole et al., 2000).

Although, most of the snake venom research has been focused on individual toxin and their action, and very interesting synergistically acting toxin complexes have been reported. Snake venom PLA2s participates in most of the snake venom protein complexes. In many cases it interacts with other proteins which are either PLA2s or derived from PLA2 precursors or peptides. Some of the dimeric PLA2–PLA2 complexes include Ceruleotoxin from *Bungarus fasciatus* (Bon and Saliou, 1983), Vaspin from *Vipera aspis* (Jan et al., 2002). Besides these, synergistically acting PLA2 have been isolated and characterized from viperidae venoms. For example, neurotoxic complexes such as ‘Crotoxin’ (Slotta and Primosigh, 1951) ‘Vipoxin’ (Tchorbanov et al., 1978) and ‘Mojave toxin’ (Cate and Bieber, 1978) are composed of acidic and basic subunits, which interact synergistically to enhance the toxicity in vivo.

In this study, we report the identification and purification of a potent hemorrhagic and neurotoxic complex from the Eastern regional *D. russelii* venom, consisting of PLA2 and non-enzymatic peptide (DNTx-II). It has been designated as *D. russelii* hemorrhagic complex-I (DR–HC-I). Further, individual components of hemorrhagic complex were characterized and their synergism was demonstrated using spectrofluorometer and *in vivo* studies.

**2. Materials and methods**

**2.1. Materials**

CM-Sephadex C-25 and Sephadex G-50 were purchased from Sigma (St. Louis, MO, USA). Para-bromophenacyl bromide, ANS (1-anilino-8-naphthalene sulfonate) and low range molecular weight protein markers were purchased from Sigma chemicals, St. Louis, USA. [14C] Oleic acid was obtained from Perkin Elmer Life Sciences Inc., USA. Fatty acid free BSA (bovine serum albumin) was obtained from PAA Laboratories GmbH, Austria. Scintillation cocktail was obtained from Packard Biosciences BV, The Netherlands. All other chemicals and reagents of analytical grade were purchased from SRL chemicals, Mysore, India.

**2.2. Venom**

Lyophilized *D. russelii* venom of Eastern region was purchased from Hindustan Snake Park, Burdwan district of Kolkata, Kolkata.

**2.3. Animals**

Swiss male, Wister mice weighing ~ 20 g and male frogs (*Rana hexadactyla*) were procured from Central Animal House, Department of Studies in Zoology, University of Mysore, Manasagangotri, India. Animal care and handling were conducted in accordance with the CPCSEA office guidelines, University of Mysore division, Mysore, India.

**2.4. CM-Sephadex C-25 ion-exchange chromatography**

The venom (200 mg dry weight) dissolved in 4 ml of 0.02 M phosphate buffer (pH 7.0), was loaded on to CM-Sephadex C-25 column (1.5 × 104 cm) equilibrated with 0.02 M phosphate buffer (pH 7.0). Stepwise elution was carried out with phosphate buffer of various molarities and pH as indicated in Fig. 1A. Flow-rate was adjusted to 25 ml/h and 2.5 ml fractions were collected. Protein elution was monitored at 280 nm using UV–VIS 1601 Shimadzu spectrophotometer.

**2.5. Sephadex G-50 gel-permeation chromatography**

The pooled fractions of 9th peak from CM-Sephadex C-25 column were concentrated, and subjected to gel-permeation chromatography on Sephadex G-50 column (1.0 × 50 cm). The column was pre-equilibrated with 0.5 M NaCl. The protein was eluted with 0.5 M NaCl, with flow rate of 1.5 ml/6 min. The protein elution was monitored as explained earlier using Shimadzu spectrophotometer.

**2.6. Reversed phase high performance liquid chromatography (RP-HPLC)**

The fractions of peak A and B from Sephadex G-50 gel permeation chromatography were subjected to RP-HPLC on a Vyde C18 (250 × 4.6 mm, 10 μm particle size, 300 Å pore size) column in a Shimadzu LC-10AVP system with dual wavelength detector. The column was equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted using a linear gradient of 70% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The elution was carried out using a 0–100% gradient of solvent B (70% acetonitrile) against solvent A (0.2% TFA).

**2.7. Electrophoresis**

The native gel electrophoresis was carried out at pH 4.3 using β-alanine-acetic acid buffer with methyl green as tracking dye (Davis, 1964). Electrophoresis was carried out
at a room temperature for three and half hours with a constant current of 90 V. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was carried out according to the method of Laemmli (Laemmli, 1970) on 12.5% polyacrylamide gel containing 0.1% SDS. The bands were visualized by staining with Coomassie brilliant blue R-250.

2.8. Molecular weight determination by MALD-TOF MS

Molecular weight of the PLA2 and DNTx-II were determined by MALDI-TOF MS. The matrix used for both the samples was sinapinic acid. Linear spectra were obtained in the positive ion mode on a Reflex IV MALDI-TOF mass spectrometer (Bruker, Daltonics, Bremen, Germany). The M + H and M + 2H signals of horse heart myoglobin were used for external calibration of the spectra.

2.9. N-terminal amino acid sequencing

Sequencing was carried out using an applied Bio system Precise Edman Sequencer. The chemical process employed by the protein sequencer to determine the amino acid sequence was derived from the degradation method developed by Edman. N-terminal sequence homology was analyzed using the NCBI-BLAST database search. Website: http://www.ncbi.nlm.nih.gov/BLAST/.

2.10. PLA2 assay

PLA2 activity was assayed with [14C] oleate-labeled autoclaved Escherichia coli cells as substrate according to the method of Vishwanath et al. (1993). The reaction mixture contained 100 mM Tris–HCl, pH 8.0; 5.0 mM Ca2+ and 3.15 × 10⁹ autoclaved E. coli cells (corresponding to 10,000 CPM and 60 nmole of lipid phosphorous). The reaction components were mixed in the following order: Buffer, Ca²⁺, water and enzyme source (5–20 µg), and incubated at 37 °C for 60 min. The reaction was initiated by adding labeled E. coli cells. The reaction was terminated by adding, 100 µl of 2 M HCl and 100 µl of fatty acid free BSA (100 mg/ml). Then the tubes were vortex mixed and centrifuged at 20,000 g for 5 min. An aliquot (140 µl) of the supernatant

Fig. 1. Daboia russelii hemorrhagic complex-I purification. (A) CM-Sephadex-C-25 column (1.5 × 104 cm) was pre-equilibrated with 0.02 M-phosphate of buffer pH 7.0; fractionation was carried out as described in materials and methods. (B) The peak 9 (7 mg protein) from CM-Sephadex C-25 column was loaded into Sephadex G-50 gel-permeation chromatography column (1.5 × 115 cm) pre-equilibrated with saline. 1.5 ml fractions were collected and protein elution monitored as described in material and methods. (C) Electrophoresis under acidic condition at pH 4.3 using β-alanine-acetic acid buffer with methyl green as tracking dye.
containing released [14C] oleic acid was mixed with scintillation cocktail and counted in a Hewlett Packard Liquid Scintillation Analyzer TRI – CARB 2100 TR.

2.11. Pharmacological properties

2.11.1. Determination of LD₅₀

LD₅₀ values for the DR–HC-I, purified PLA₂ and DNTx-II were calculated according to the mathematical scheme adopted by Meier and Theakston (1986). The toxins were injected (i.p) separately into groups of 12 mice with doses ranging from 1 to 10 mg/kg body weight. Survival time of each animal was recorded for 24 h.

2.11.2. Hemolytic activity

Indirect/direct hemolytic activities were assayed as described by Boman and Kaletta (1957). The substrate for direct lytic activity was prepared by suspending 1 ml of packed fresh human RBC in 9 ml of saline. Whereas, the substrate for indirect hemolytic activity was prepared by suspending 1 ml of packed fresh human RBC and 1 ml of fresh hens egg yolk in eight ml of PBS (phosphate buffer saline). To one ml of the suspension 20–50 μg venom samples DR–HC-I, PLA₂ or DNTx-II were added and incubated for 45 min at 37 °C and the reaction was stopped by adding 9 ml of ice-cold PBS. The positive control received 20 μg of venom alone. The suspension was centrifuged at 2000 rpm for 20 min, and then the released hemoglobin was spectrophotometrically read at 530 nm.

2.11.3. Re-calcification time assay

Anticoagulant activity was determined according to the modified method of Condrea et al. (1981). Platelet poor plasma was prepared by diluting whole blood in the ratio nine parts blood to 1 part 0.13 M Tri-sodium citrate. The mixture was centrifuged twice at 2500 g for 15 min. The platelet poor plasma was aliquot and stored at −70 °C until next use. To 300 μl of platelet poor plasma 5 μg–60 μg DR–HC-I, PLA₂ and DNTx-II in 0.01 M Tris–HCl buffer (pH 7.4) were added. The mixture was incubated for 60 s at 37 °C. Clot formation was initiated by the addition of 30 μl of 0.25 M CaCl₂ and the time interval required for the clot formation was recorded.

2.11.4. Hemorrhagic activity

Hemorrhagic activity was determined as described by Kondo et al. (1960). For positive control, groups of three mice were injected (s.c) separately with the 10 μg of venom alone. And DR–HC-I, PLA₂ and DNTx-II were injected separately. After three hours, mice were anesthetized by anesthetic diethyl ether and sacrificed. Dorsal surface of the skin was removed for observation of Hemorrhagic spot. Various concentrations of DR–HC-I complex/PLA₂/DNTx-II (0–50 μg) were diluted in saline, and was injected intradermal (fixed volume, 100 μl). Control sites were injected with saline buffer alone. After three hours, the mice were euthanized and the dorsal skin was removed and hemorrhagic spot on the inner surface of the skin was measured. The minimum hemorrhagic dose (MHD) was defined as the amount of venom or enzyme capable of producing a hemorrhagic spot of 5 mm in diameter.

2.11.5. Edema inducing activity

Induction of edema was determined as described by Vishwanath et al. (1988). Groups of five mice were injected in the right footpad with different concentrations of DR–HC-I, PLA₂ and DNTx-II in 10 μl saline. Animals receiving only 10 μl of saline served as controls. After 45 min the animals were euthanized by cervical dislocation and the legs were removed at the ankle joints and weighed individually. The edema ratio was calculated using the formula: Edema ratio = weight of edematous leg/weight of normal leg × 100.

2.11.6. Neurotoxicity

The experimental conditions were set up as described by Harvey et al. (1984). Sciatic nerve gastrocnemius muscle preparation was isolated from the male frogs (R. hexadactyla) weighing 18–20 g. The isolated nerve muscle preparation was mounted in 10 ml organ bath containing Krebs solution of the following composition in milli-moles: NaCl, 4.7; MgSO₄, 1.2; KHPO₄, 1.2; CaCl₂, 2.5; NaHCO₃ and glucose, 11.1. Indirect twitches were evoked by electrical stimulation of the nerve by supra maximal voltage (0.2 ms, 0.1 Hz). DR–HC-I, PLA₂ and DNTx-II (0.5–20 μg/ml) were left in contact with the nerve preparation for 1 h or until the twitch response abolished.

2.11.7. Effect of PLA₂–DNTx-II interaction on pharmacological properties of PLA₂

The mixture containing the PLA₂ and DNTx-II in the molar ratio of 5:2 was tested for the PLA₂ activity, hemolytic, edema inducing, and hemorrhagic effects, as explained in their respective methods.

2.12. Fluorimetric monitoring of PLA₂ and peptide interaction

An intrinsic fluorescence emission spectrum of PLA₂ and DNTx-II was recorded using Shimadzu RF 5000 spectrofluorophotometer. The PLA₂ was incubated with 20 mM phosphate buffer, pH 7.5 at different concentrations of DNTx-II in a total volume of 3 ml. The mixture was incubated at 37 °C for 15 min before recording the fluorescence emission spectra. The excitation was fixed at 280 nm in all the fluorescence emission studies.

2.13. Effect of (ANS) 1-anilino-8-naphthalene sulfonate on PLA₂

1-Anilino-8-naphthalene sulfonate is an anion, conventionally considered to bind to pre-existing hydrophobic (nonpolar) surfaces of proteins. ANS binding to PLA₂ was followed by spectrofluorescence emission. A 2 ml solution contained 4 nmole of PLA₂ in 20 mM phosphate buffer, pH 7.5 was incubated with different molar ratios of ANS. Fluorescence emission spectra of PLA₂ alone and mixture of PLA₂ and ANS was recorded in the range of 400–600 nm after excitation at 375 nm. Further, DNTx-II was added to the ANS saturated PLA₂ and emission spectra was recorded as described above.

2.14. Statistical analysis

All numerical results were analyzed using the GraphPad Prism software (San Diego, CA, USA) and are expressed as the mean ± standard error (S.E.).
3. Results

3.1. Purification and characterization of DR–HC–I

D. russelii venom, upon fractionation on cation exchange chromatography column gave 18 peak profiles (Fig. 1A). Peak 9 eluted in 0.09 M phosphate buffer pH 7.3, accounted for 3.5% of the total venom protein loaded. It released radio labeled fatty acid from E. coli cells containing phospholipids specifically labeled at Sn-2 position with [C14] oleate, indicating the presence of PLA2.

Lyophilized peak 9 fractions (7 mg of protein) on gel-permeation chromatography (Sephadex G-50), resolved into two major peaks A, B and a minor shoulder, in the presence of 0.5 mM Nacl (Fig. 1B). The peak A possesses PLA2, indirect hemolytic, anticoagulant and edema inducing activities (Table 1), while peak B was devoid of any enzymatic activity. Whereas, a minor shoulder was negative for protein contents, in protein estimation assay and also it was devoid of any toxic effect. Polyacrylamide gel electrophoresis (PAGE) was carried out in acidic conditions that facilitate the movement of basic proteins. PLA2 and non-enzymatic peptide (DNTx-II) gave single band each. But the mobility of PLA2 and the DNTx-II differ from each other (Fig. 1C). PLA2 and DNTx-II on RP-HPLC gave a single sharp symmetric peak with retention time of 49.012 and 40.00 min respectively (Figs. 2A and 3A), which confirms the homogeneity of the proteins. The molecular weight of PLA2 and DNTx-II was found to be 13,432.756 and 6745.9, respectively (Figs. 2B and 3B), determined by MS-MALDI.

3.2. Pharmacological characterization of complex and individual components

Peak 9 (Fig. 1A) induced skin hemorrhage in mice with an MHD of 30 μg and edematous hemorrhage in foot pad, hence designated as DR–HC-I. It was lethal to mice with LD50 dose of 0.7 mg/kg body weight. Animals injected with the toxin showed neurotoxic symptoms such as respiratory distress, hind limb paralysis, lacrimation, convulsions and profuse urination. DR–HC-I also possessed direct hemolytic and plasma re-califications activities (Table 1).

The PLA2 (peak A, from Fig. 1B) alone was toxic to mice with LD50 (i.p.) dose of 7.4 mg/kg body weight. The animals did not show any signs/symptoms of neurotoxicity. The Daboia neurotoxin-II (DNTx-II) (peak B, from Fig. 1B) showed neurotoxic symptoms with LD50 of 0.24 mg/kg body weight (Table 1). The post-mortem examination revealed neither bleeding in the peritoneal cavity nor any visible damage to vital organs such as liver, lungs and kidney. Further, histological studies confirmed that there were no effects on vital organs (data not shown).

The PLA2 alone induced non-hemorrhagic edema in the footpads of mice. Edematous ratio 145 ± 5 reached maximum in 45 min after injection. Additionally, PLA2 alone showed indirect hemolytic and Plasma re-calification activity (Table 1). Whereas, DNTx-II did not have any of these activities.

The isolated nerve-muscle preparation in Krebs solution evoked muscle contraction that was well maintained during a control period of 60–80 min. DR–HC-I and its individual components decreased the frog gastrocnemius muscle twitch height in a time dependent manner on indirect stimulation (Fig. 5). DR–HC-I and DNTx-II, at 10 μg/ml, inhibited the indirectly stimulated twitch response in a time dependent manner (Fig. 5).

3.3. Protein–protein interaction

PLA2 and DNTx-II exhibited the intrinsic fluorescence emission (Fig. 6A). Both of them showed maximum emission intensity between 350 and 360 nm at a concentration of 0.5 mg/3 ml (Fig. 6A). This confirms the presence of tryptophan in PLA2 and DNTx-II. The DNTx-II quenched the intrinsic fluorescence of PLA2 in a dose dependent manner. At a molar ratio of 5:2 (PLA2: DNTx II) 50% decrease in the relative intrinsic fluorescence of PLA2 was observed (Fig. 6B). When PLA2 was treated with different molar concentrations of ANS, the fluorescence emission intensity of ANS increased and it reached the saturation point at PLA2: ANS concentration of 1:80 μmol (Fig. 7).

The individual components of DR–HC-I did not exhibit hemorrhage at the site of injection (Fig. 4C and D). However the mixture of PLA2 and DNTx-II in the molar ratio of 5:2 induced hemorrhage at the site of injection with MHD of 20 μg. In the presence of ANS they did not induce hemorrhage at the site of injection (Fig. 4E).

4. Discussion

Snake venoms are complex mixtures of enzymatic and non-enzymatic proteins/peptides. Most of the snake venom research has been focused on purification and characterization of abundant or highly toxic components from the venom. However, relatively less interest has been paid to understand the synergistic effect on pharmacological properties of the venoms. The synergism have important role in elevating the debilitating effect of the venom.
Among the Southern, Western, Northern and Eastern regional *Daboia russellii* venoms, Eastern regional venom was found to be the most toxic with the LD50 of 1.1 mg/kg body weight (Kumar and Gowda, 2006). On the other hand, the toxic principles such as PLA2s, responsible for the toxicity of Southern regional venom are absent in Eastern regional venom, but exhibited high toxicity. This may be due to the consequence of synergistically acting complex and non-enzymatic toxic peptides (Kumar and Gowda, 2006; Venkatesh et al., 2013).

A hemorrhagic complex-I was isolated from Eastern regional *D. russellii* venom by single step purification using CM-Sephadex C-25 ion-exchange column chromatography. The complex (DR–HC-I) showed very potent skin hemorrhagic activity with a MHD of 30 μg (Fig. 4) and a LD50 value of 0.7 mg/kg body weight. DR–HC-I possesses PLA2 activity. It was resolved into 2 peaks on G-50, peak A had PLA2 activity and peak B was devoid of any enzymatic activity. The molecular weight of the PLA2 and DNTx-II was found to be 13,432.756 and 6745.9 respectively (Table 1). Therefore, peak-A represents a typical PLA2. From the molecular properties peak-B appears to be a peptide toxin.

By comparing N-terminal sequence of PLA2 with those available in data banks, it was found that the sequences was
homologous to known Viperidae PLA$_2$ (Gopalan et al., 2007; Tsai et al., 1996) (Table 2). On the other hand, the N-terminal amino acid sequence of the DNTx-II peptide showed 100% homology with cytotoxin/cardiotoxin from _Naja naja atra_, upon blast search (Table 3). The peptide post synaptic neurotoxins belong to a family of short and long chain alpha-neurotoxins. Short alpha-neurotoxins consists of 60–62 amino acid residues and include 4 disulfide bridges, whereas long alpha-neurotoxins have 66–75 residues and 5 disulfides. Four disulfide bonds are conserved in all family members (Endo and Tamiya, 1991).

The peak-A and peak-B in native PAGE moved to different distances and their relative mobility varied suggesting that they are distinctly different proteins. The DNTx-II found to be more basic compared to PLA$_2$. The homogeneity of the PLA$_2$ was confirmed by its appearance as a single peak in RP-HPLC. The hemorrhagic complex PLA$_2$ appears to be quite distinct from the other PLA$_2$s isolated from Southern and Northern regional Russell’s viper venom. Among the PLA$_2$s isolated from the Indian Russell’s viper venom, VRV-PL-V is most toxic with the LD$_{50}$ 1.8 mg/kg body weight. It possesses neurotoxicity, myotoxicity, and high anticoagulant activity. This is followed by VRV-PL-VIIIa with mild neurotoxicity, myotoxicity and lung hemorrhagic activity. In contrast, the hemorrhagic complex PLA$_2$ is least toxic and it did not induce any signs and

---

**Fig. 3.** RP-HPLC and molecular weight analysis of DNTx-II toxin. (A) Peak-B was applied on a Vydac C$_{18}$ (250 × 4.6 mm, 10 µm particle size, 300 Å pore size) column in a Shimadzu LC-10AVP system with dual wavelength detector. The column was equilibrated with 0.1% Trifluoroacetic acid (TFA). They were eluted using a 0–100% gradient of solvent B (70% acetonitrile) against solvent A (0.1% TFA). (B) Peak-B linear spectra were obtained in the positive ion mode on a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The matrix used for the sample was sinapinic acid. (C) SDS-PAGE of DNTx-II under reducing condition.
symptoms of neurotoxicity when injected in to animals and also it tested negative for neurotoxicity on frog sciatic gastrocnemius muscle preparations. The Eastern PLA₂ alone did not induce myotoxicity and also MED is low compared to VRV-PL-V and VRV-PL-VIIIa (Table 1).

4.1. Synergistic interaction of PLA₂ and DNTx-II

Both DNTx-II and PLA₂ consists of tryptophan, which is confirmed by fluorescence emission spectrum. Since they are found to act synergistically, we determined the DNTx-II ability to interact with PLA₂ by fluorescence spectroscopy. The change in intrinsic fluorescence reflects conformational change in protein due to substrate or ligand interaction (Fawzy et al., 1988). Fluorescence emission intensity of PLA₂ was quenched upon the addition of non-enzymatic peptide in a concentration dependent manner. At a molar ratio of 5:2 PLA₂: DNTx-II, the quenching was maximum (Fig. 6B), suggesting that DNTx-II interacts with the PLA₂ to form a complex.

In the snake venom complexes, the role of the non-enzymatic component is believed to be that of a protective ‘chaperone’ (Choumet et al., 1993; Kini, 2003). The non-enzymatic component in the complexes are known to perform facilitator function. They enable the Phospholipases to selectively attach to the appropriate membrane. At 5:2 M ratio it was noticed that the complex acts as a different toxic molecule and induces hemorrhage at the site of injection. On the other hand, either the PLA₂ or DNTx-II individually did not induce hemorrhagic effect (Fig. 4). This suggests that the DNTx-II contributes significantly towards the enhancement of hemorrhagic activity. Hence, the hemorrhagic complex appears to undergo synergistic interaction in the molar ratio of 5:2. The reconstituted complex in 5:2 M ratio is more toxic than the native one (PLA₂ and DNTx-II present in 2.6:1.2 M ratio). In the presence of ANS, the enhancement of the hemorrhagic activity and lethal potency are abolished (Fig. 4E). ANS is an anion, which binds specifically to hydrophobic site on the surface of the PLA₂ molecule and prevents the non-covalent interaction between the PLA₂ and DNTx-II.

In the case of neurotoxin complexes such as Crotoxin, Vipoxin and Mojave toxin isolated from C. durissus terrificus (Hendon and Fraenkel-Conrat, 1971; Slotta and Primosigh, 1951), Vipera ammodytes ammodytes (Tchornov et al., 1978) and Crotalus scutulatus scutulatus (Cate and Bieber,
1978) venoms, respectively are composed of basic phospholipase associated with an acidic non-enzymatic component. And also peptides inhibited the enzymatic activity of phospholipase in vitro. On the other hand, in hemorrhagic complex, both non-enzymatic peptide and PLA2 are basic in nature, which is confirmed by their movement in acidic PAGE and also their elution behavior in ion exchange chromatography. They differ from each other in their relative mobility under acidic condition. Interestingly, unlike above-mentioned complexes, the DNTx-II doesn’t inhibit the enzymatic activity of D. russelii venom PLA2.

In conclusion, hemorrhage is one of the main pathological manifestation upon D. russelii bites, but hemorrhagic components alone are less toxic. Based on the findings, we conclude that, “the toxicity and pharmacological effects of D. russelii venom is due to the synergistic effect of toxic peptides”.

**Ethical statement**

The authors warrant that the manuscript “Purification and Characterization of Synergistically Acting Phospholipase A2 and Non-enzymatic Peptide Complex from D. russelii Venom” is an original work, it has not been published before and it is not submitted for publication anywhere. All animal experiments, animal care and handling were conducted in accordance with the CPCSEA office guidelines, University of Mysore division, Mysore, India. It contains no defamatory or other unlawful statements, and it does not infringe on the rights of others. This paper reflects our own research and analysis and does so in a truthful and complete manner. All authors have contributed significantly to the execution, analysis and writing of the study reported and all co-authors have agreed to submit the manuscript to Toxicon.

**Acknowledgments**

Madhukumar Venkatesh gratefully acknowledges Senior Research Fellowship award from the Lady TATA Memorial Trust, Mumbai, India.
Conflict of interest statement

The authors have no conflict of interest to declare.

References


Tchorkbanov, B., Grishin, E., Aleksiev, B., Ovchinnikov, Y., 1978. A neurotoxic complex from the venom of the Bulgarian viper (Vipera ammodytes ammodytes) and partial amino acid sequence of the toxic phospholipase A2. Toxicon 16, 37–44.


