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Venom phospholipases of Russell's vipers from Myanmar and eastern India—Cloning, characterization and phylogeographic analysis ☆

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Abstract

Venoms of Russell's vipers (genus *Daboia*) are known for their deadly coagulopathic and other effects. We herein studied various isoforms of venom phospholipases A_2 (PLAs) from two *Daboia* species at their geographic boundary. From Myanmar *Daboia siamensis* venom (designated as DsM), four PLAs (designated DsM-aI, aI', aII' and bI') were purified, and the cDNAs encoding two acidic (DsM-aI and aII) and two basic PLAs (DsM-bI and S1) were also cloned from its venom-glands. DsM-S1 is identical to the major venom PLA of southern India *Daboia russelii*, but the protein is absent from the venom. Additionally, four PLAs (designated DrK-aI, aII, bI and bII) were cloned from cDNA obtained from venom glands of a Kolkata *D. russelii*, and the PLAs were purified from the pooled venom (designated as DrK). The acidic DrK-aI is the most neurotoxic and lethal among these PLAs; DsM-aI which differs from DrK-aI by only the Phe2 substitution shows greatly reduced enzymatic activity and lethality. Both acidic PLAs do not form dimeric complex with basic PLAs in the same venoms. DsM-bI' is neurotoxic and lethal but its orthologous DrK-bI (97% identical to DsM-bI') is a much weaker toxin. Given the fact that most of the orthologous PLAs of DrK and DsM share 97–100% sequence identity, *Daboia* vipers of Myanmar and Kolkata must be closely related. Molecular phylogenetic analyses on 30 venom PLAs of Eurasian vipers' revealed co-evolution of five subtypes of venom PLAs in both *Daboia* and *Vipera* genera. Our results shed light on the intra- and inter-species variations and structure–function relationships of viperid venom PLAs.

Keywords: Venom phospholipase A_2; Cloning and sequencing; Phylogenetic analysis; Geographic variation; Russell's viper (*Daboia siamensis, Daboia russelii*)

1. Introduction

Russell's vipers (Viperinae of the genus *Daboia*) are important causes of morbidity and mortality in South and Southeast Asia. Two species of *Daboia*, *Daboia russelii* [1] and *Daboia siamensis* [2], have been identified. *D. russelii* is mainly distributed in the Indian sub-continent while *D. siamensis* is discontinuously spread over a wide area from Myanmar to Taiwan and south to Indonesia. Clinical effects and antigenicities of *Daboia* venoms vary throughout these different geographic ranges [2–8]. Envenoming by *D. siamensis* poses a remarkably severe problem especially in Myanmar where it has been the country's 5th leading single cause of death [3–5]. Clinical observations also indicated higher case fatalities and more severe hemorrhagic manifestations in *Daboia* envenoming in Myanmar and eastern India than in other regions [2,6–9].

Snake venom is the richest source of secreted phospholipases A_2 (PLA; EC 3.1.1.4), the Ca^{+2}-dependent enzymes that hydrolyzes the 2-acyl ester of phosphoacylglyceride [9,10]. Paralogous PLA variants in viperid venoms have been derived from gene duplication and rapidly evolved to acquire functional diversity [11–13]. Up to 65% of the venom proteins of *Daboia* from Taiwan, Thailand, southern India and Pakistan

Abbreviations: DsM, *Daboia siamensis* (Myanmar); DrK, *Daboia russelii* (Kolkata); dPPC, L-dipalmitoyl phosphatidylcholine; PLA, Phospholipase A_2; HPLC, high performance liquid chromatography

☆ GenBank accession numbers for the novel nucleotide sequences of venom PLAs are DQ090654-7 for those from *D. siamensis* (Myanmar), and DQ090658-61 for those from *D. russelii* (Kolkata).

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are PLAs, many of them have been cloned or fully sequenced [14–16]. Notably, most of the lethal or neurotoxic PLAs found in snake venom are basic proteins, but two acidic PLAs of high lethal potency have recently been purified from *Daboia* venoms of eastern India [9] and Myanmar [17,18]. Since their complete sequences were not solved, the structure–activity relationships of both PLA-toxins were difficult to understand.

The aim of the present study is to resolve structures and functions of novel venom PLAs from *Daboia* subspecies and better understand their geographic variations. We thus purified, characterized and cloned venom PLA isoforms of *Daboia* vipers from Myanmar (i.e. Burma) and Kolkata (eastern India), at the boundary between *D. russelii* and *D. siamensis* ranges. The results were compared with the venom PLA data of *Daboia* from adjacent areas, including Pakistan, southern India, Sri Lanka, Thailand, and Taiwan [16–19]. By phylogenetic analyses and sequence alignments of 30 Viperinae venom PLAs, the classification and structure–function relationships of these PLAs are discussed.

2. Materials and methods

2.1. Venoms and other materials

D. siamensis venom samples from southern Myanmar were kindly given by Prof. R.D.G. Theakston, Liverpool School of Tropical Medicine (UK) while another pooled sample from northern Myanmar was obtained from Prof. Y. Y. Shu of Kuangxi Medical University, China. Meanwhile, live specimens of *Daboia* were also caught near Yangon (Myanmar) and Kolkata (eastern India), respectively. Fresh venom glands were dissected immediately from each snake after they were euthanized 48–60 h after venom collection. The glands were preserved for several weeks in the RNAlater solution (Ambion, USA) prior to RNA extraction.

Modifying enzymes, restriction enzymes and the pGEM-T vector were purchased from Promega Corp. (Madison, USA). Synthetic L-dipalmitoyl phosphatidylcholine (dPPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Triton X-100, sodium deoxycholate, acetylcholine, and other chemicals were of reagent grade from either Merck (Darmstadt, Germany) or Sigma (St. Louis, USA).

2.2. Purification of venom PLA

Crude *Daboia* venom was dissolved in 0.1 M ammonium acetate buffer (pH 6.5) and then fractionated on a FPCL equipped with a Superdex G75 column (HR10/30, Pharmacia) pre-equilibrated with the same buffer, at room temperature. The PLA_2-containing factions were pooled and freeze-dried. The venom proteins were further purified by reversed-phase HPLC using a C_{18} column (4.5×250 mm, 10 μ, Vydac) equilibrated with 0.07% aqueous trifluoroacetic acid (TFA, solvent A), and eluted with a linear gradient of 20–45% CH_3CN containing 0.07% trifluoroacetic acid (solvent B). Each peak was dried in a vacuum-centrifuge device (Labconco, USA). Concentrations of crude venom and PLA in solutions were determined spectrophotometrically at 280 nm, assuming an extinction coefficient of 1.5 at 1.0 mg/ml [14] and method of Bradford [20].

2.3. Protein sequences and masses

Purity of venom protein was assessed by SDS-PAGE and *N*-terminal sequencing. The *N*-terminal sequence of each purified protein was determined by an Applied Biosystems amino acid sequencer (Model Procise 492) [21]. Its molecular weight was analyzed by QSTAR XL nano-ESI mass spectrometer System (Applied Biosystems, Foster City, USA).

2.4. Cloning and sequencing

One *Daboia* specimen from each region was euthanized 2 days after venom extraction, at which point the venom glands were removed immediately for RNA extraction. The mRNA was prepared and the cDNA synthesis kits (Stratagene, USA) was used, as previously described [14]. Using venom gland cDNA as a template, the PCR [22] was conducted using SuperTaq DNA Polymerase with a pair of pairs of mixed-base oligonucleotide primers (sense primer 1: TCTGGATT-SAGGAGGATGA GG; antisense primer 2: GCCTGCAGRACTTAGCA), that were specifically designed based on conserved 3′ and 5′ untranslated regions of several homologous cDNA encoding other viperid venom PLAs [12,14]. In addition, a sense primer 3 (GCGGAGATGATCGTNAARATG) based on the amino acid sequence AEMIVK was used in conjunction with primer 2 in an attempt to specifically amplify Drk-aI' and DsM-aI'.

After treatment with polynucleotide kinase, the amplified DNA fragment was inserted into the pGEM-T easy vector (Promega Biotech, Wisconsin, USA). It was then transformed into *Escherichia coli* strain JM 109. Only the white transformants were picked-up and the cDNA clones were selected by restriction enzyme gel pattern. The DNA sequencing System model 373A and the Taq-Dye-Deoxy terminator cycle sequencing kit (PE Applied Biosystems, USA) were used to determine the cDNA sequences by dideoxynucleotide method [23]. Full amino acid sequences of venom PLAs were deduced from the nucleotide sequences of cDNA.

2.5. Enzymatic activities and pharmacological effects

PLA_2 activities were measured by pH-stat titration method at 37 °C on a pH-stat apparatus (Radiometer RTS 822, Copenhagen, Denmark). Released fatty acids, from 3 mM L-dipalmitoyl phosphatidylcholine (dPPC) mixed either with an equal concentration of deoxycholate or two fold concentration of Triton X-100 in 0.10 M NaCl, were titrated at pH 7.4 with 6 mM NaOH. The initial rate was recorded for more than five min and corrected for non-enzymatic spontaneous rate. Specific activity was expressed as μmol of dPPC hydrolyzed per min per mg of the enzyme. In addition, 1.0% (w/w) fresh egg yolk in 0.15 M NaCl was used as substrate for comparing PLA activities by pH-stat titration methods.

Induction of edema by venom PLA on hind paw of anaesthetized Wistar rats (150–160 g body mass) was monitored up to 5 h by a plethysmometer, as described previously [12]. To test the lethal effects of crude venom and purified PLAs, ICR mice of 30–35 g body weight were used [9,24]. The venom proteins were dissolved in sterile PBS and centrifuged at 10,000×*g* to remove insoluble materials before injected to the mice. The medium lethal dose (LD_{50}) was estimated by intraperitoneal (i.p.) injection of graded doses of the protein to the mice. Effects of venom proteins on four mice in each group were examined; the lethality was estimated as the dosage-range which was able to kill about 50% of the test animals within 24 h.

2.6. Phylogenetic analyses

Amino acid sequences of venom PLAs from various species under Viperinae subfamily were retrieved by Blast search [25] and aligned along with those solved in the present study by AlignX in VectorNTI (Invitrogen, USA) program. Cladograms were constructed based on these sequences by neighbor-joining algorithm using program PHYLIP [26]; degree of confidence for the internal linage of the tree was determined by bootstrap methods [27].

3. Results

3.1. Purification and biochemical characterization

Patterns of gel filtration of the crude venoms by Superdex G75 column on a FPLC system were shown in Fig. 1. The PLA-containing fractions were lyophilized and further purified by reversed-phase HPLC (not shown). Four venom PLAs could be

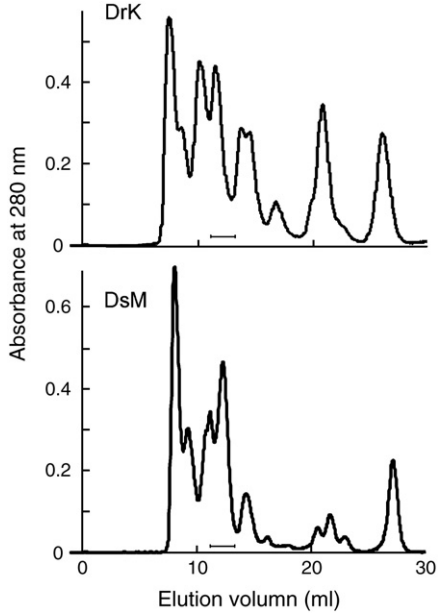


Fig. 1. Gel filtration of the crude venom of DrK and DsM. Crude venom was dissolved and injected into a Superdex G75 (HR10/30) column on a FPLC system. The elution was carried out with the equilibration buffer, 0.1 M ammonium acetate (pH 6.4), at a flow rate of 1.0 ml/min. Fractions of 1.0 ml were collected. The PLA-containing fractions (shown by bars) were pooled.

purified and identified from each of DrK and DsM (northern Myanmar). Their protein masses, N-terminal sequences and enzyme activities were also determined, as listed in Table 1. The PLA isoforms were designated according to their pI values deduced from the protein sequence (a, for acidic; b for basic), as well as the N-terminal sequence homology. In addition, small amount of the crude venom was fractionated by HPLC directly (Fig. 2) to confirm the PLA-profiles obtained by the two-step procedure.

The acidic DrK-aI is the most neurotoxic and lethal among these PLAs, similar to a previous report [9]. Notably, DrK-aI showed strong preference for the anionic substrate over the zwitterionic substrate (Table 1). This is consistent with previous findings for other venom presynaptic neurotoxins, including β-bungarotoxin, crotoxin [28] and ammodytoxin [29]. Notably, several PLA isoforms of DsM and DrK bear a Phe2 substitution which was seldom observed in other venom PLAs, and they all showed much lower catalytic activities than the PLAs containing Leu2 when both types of micellar substrates were used *in vitro* (Table 1). When 1.0% (w/w) solution of egg yolk was used as substrate, DrK-aI had a low specific activity of 23 μmol/min/mg at 37 °C, while the activities of the Phe2-PLAs (DsM-aI and Drk-aI') were too low to be measured.

Since cDNA had been obtained from venom glands of a *D. siamensis* specimen collected near Yangon (southern Myanmar), PLAs were also purified from several venom batches of *D. siamensis* specimens, which had been collected in the same area (supplied by Prof. R.D.G. Theakston). HPLC profiles, masses, and N-terminal sequences of the PLA isoforms in these samples suggested that the PLAs are present in different proportions and possibly show some individual variations. Each of the venom sample contains four or five PLA-variants, mass of the major PLAs have been determined to be 13607, 13810, 13830, and 14084 Da, respectively. Thus, DsM-PLA variant with mass of 13830 appears to be present in *Daboia* venom of southern Myanmar and DrK but not that of northern Myanmar (Table 2).

3.2. cDNA cloning and protein sequence alignments

PCR has been carried out using specifically designed primers for venom PLAs with the venom gland cDNAs as templates [12,14]. After the cDNAs were amplified and cloned, their nucleotide sequences were determined to predict the PLA protein sequences and pI values. Based on sequencing more than 45 selected PLA clones for each species, sequences of four distinct Asp49 PLAs, respectively, could be deduced from the cDNA sequences of both DrK and DsM (Table 2). Their complete amino acid sequences were then aligned with those of most related venom PLAs obtained by BlastP search [25]. Three putative categories of the Asp49-PLAs from Viperinae venoms (i.e. acidic Asn1, basic Asn1, and Ser1) were aligned in Fig. 3A, B and C, respectively.

Most of the cDNA deduced sequences (Table 2) could match those of the purified PLA isoforms (Table 1), except that the masses of purified DsM-aII' (13810) and DsM-bI' (14084) do not match DsM-aII (13830) and DsM-bI (14055) predicted from the cDNA sequences, respectively, though their N-terminal sequences compromised. Further PCR experiments using primers 3 and 2, followed by cloning and sequencing of another 20 cDNAs, failed to produce any new clones encoding DsM-aI'. It lays the possibility that the two PLA pairs, DsM-aII and aII', DsM-bI and bI', are allelic proteins from different individual snakes or geographic samples.

3.3. Lethal and edematous effects

DrK and DsM appear to evolved functionally different PLAs, in spite of the fact that their PLA sequences are very

Table 1
Characterization of purified venom PLAs from DrK and DsM venoms

Venom PLA	% content (w/w)	Mass, Da	Specific activities [a] (μmol/mg/min)		N-terminal sequence determined
			deoxycholate	Triton X-100	
DrK-aI	17	13573	1127±58	196±15	NLFQFAEMIVK
DrK-aI'	7	13812	24±1	5.8	NFFQFAEMIVK
DrK-bI	6	14077	2352±224	614±36	NLFQFARMINQ
DrK-bII	0.2	13704	1150±22	42±0.9	NLLQFGRMINQ
DsM-aI	12	13607	10	6.2	NFFQFAEMIVK
DsM-aI'	4	13812	29±2	7	NFFQFAEMIVK
DsM-aII'	7	13810	1538±25	320±4	NLYQFGEMINQKT
DsM-bI'	12	14084	1131±26	622±26	NLFQFARLIDA

Masses were determined by ESI-MS spectrometry.
[a] Enzymatic hydrolysis of dPPC were measured in the presence of deoxycholate or Triton X-100 with 10 mM $CaCl_2$ at 37 °C. Values shown are median±S.E. of results from three independent experiments, and underlined values are averages of two experimental results.

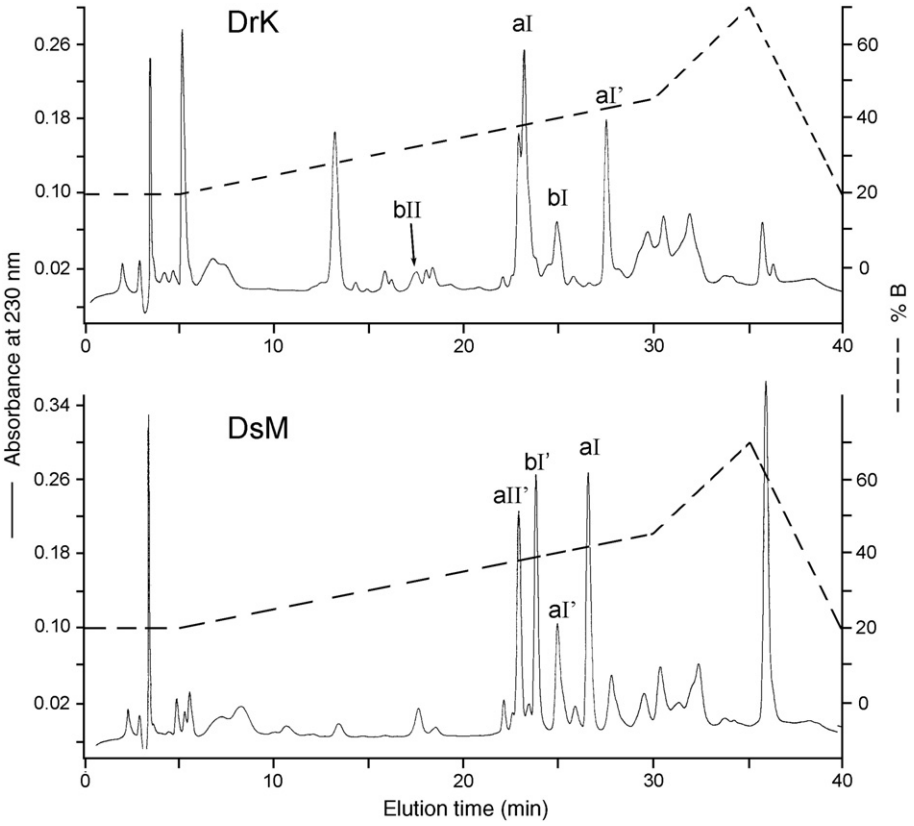


Fig. 2. Purification of PLAs by reversed-phase HPLC. Solubilized crude venoms of DrK and DsM in solvent A were fractionated on a C_{18}-Vydac HPLC column. The PLA-containing peak was identified by ESI-MS and N-terminal sequencing and annotated (as shown in Table 1).

similar. Purified DrK-aI at doses between 0.06 and 0.10 μg/g was lethal to about half of the mice in 24 h. In contrast, DsM-aI, which differed from DrK-aI by only residue 2, had very low enzymatic activity (Table 1) and was not so toxic to mice (LD_{50}>3.0 μg/g). All the mice injected with DsM-aI survived in our experiments but were hypo-locomotive the first few hours. We also compared the edema-inducing effects of DsM-aI and DrK-aI on rat hind paws. Both PLAs at the dose of 10 μg effectively induced edema but DrK-aI had a stronger and faster effect than DsM-aI. Relative swelling of paw was peaked in 4 or 5 h after the injection, and reached 38.5% and 30.3% for DrK-aI and DsM-aI, respectively (Fig. 4).

While toxicity of DsM-aI was weak, the basic DsM-bI' in the same venom was more neurotoxic and lethal and its toxicity was not affected or enhanced by the presence of DsM-aI (Table 3). Notably, DrK-bI has good enzyme activity but it is not as lethal as DsM-bI' to mice although both PLAs share about 97% sequence identity (Fig. 3B).

Table 2
cDNA data for venom PLAs of DrK and DsM

PLA cloned	Calculated mass (Da)	pI	No. of clones	(Signal peptide) and residues 1–10
DrK-aI	13573	4.6	3	(MRTLWIVAVCLIGVEG) NLFQFAEMIV
DrK-aII[a]	13830	4.9	4	(MRTLWIVAVCLIGVEG) NLYQFGEMIN
DrK-bI	14076	8.7	5	(MRTLWIVAMCLIGVEG) NLFQFARMIN
DrK-bII	13704	8.4	3	(MRTLWIVAVCLIGVEG) NLLQFGRMIN
DsM-aI	13607	4.6	11	(MRTLWIMAVCLIGVEG) NFFQFAEMIV
DsM-aII[a]	13830	4.9	2	(MRTLWIVAVCLIGVEG) NLYQFGEMIN
DsM-bI	14055	8.7	2	(MRTLWIVAMCLIGVEG) NLFQFARLID
DsM-S1[a]	13625	8.4	2	(MRTLWIVAVCLIGVEG) SLLEFGKMIL

Isoelectric point (pI) and molecular mass were predicted from the deduced protein sequence.
[a] cDNA deduced proteins failed to match those listed in Table 1.

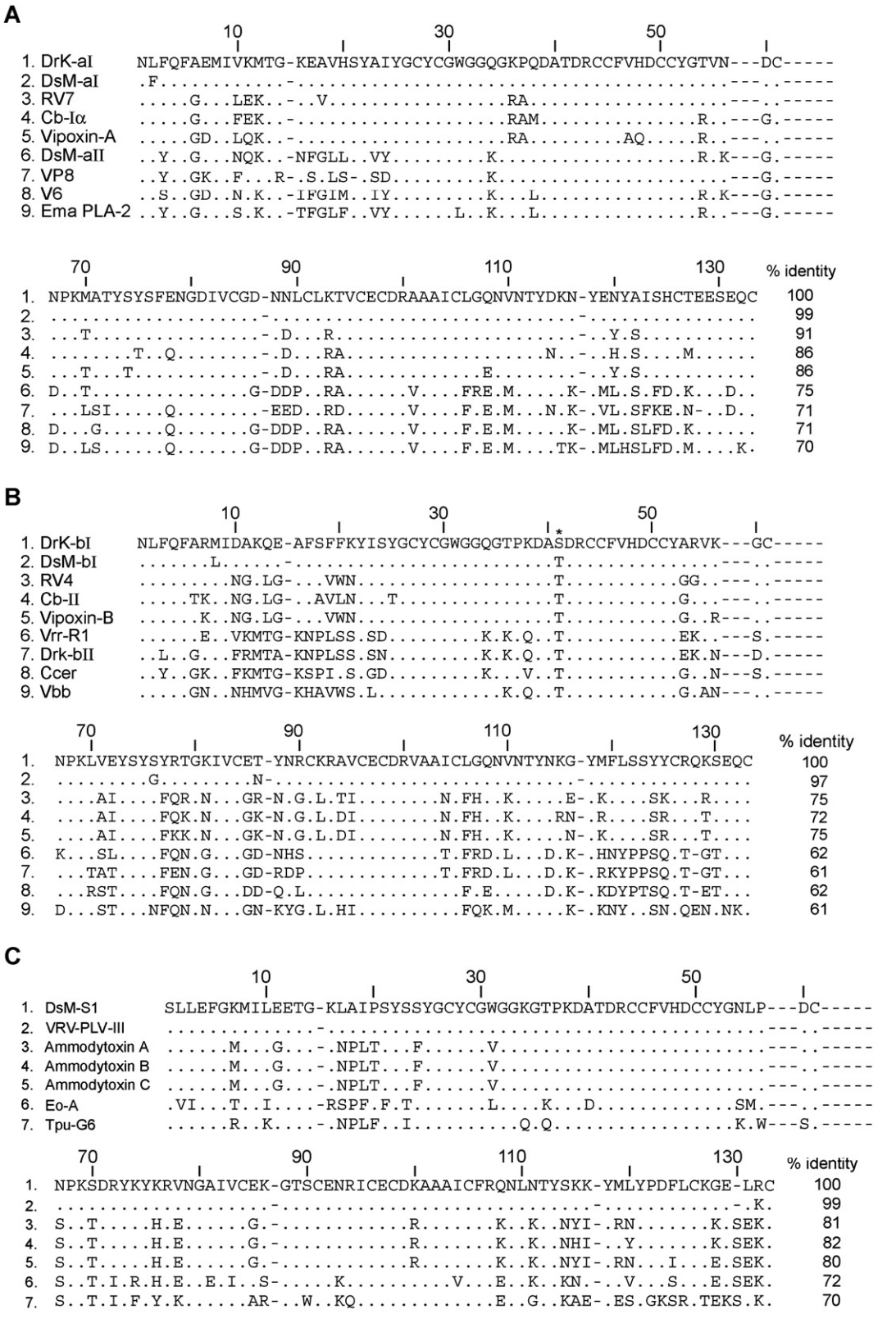
3.4. Phylogenetic analyses

Cladogram was established based on the amino acid sequences of venom PLAs from selected Eurasia Viperinae including most *Vipera* and *Daboia*. A basic venom G6D49-PLA from *Trimeresurus puniceus* (an Indonesia pitviper) was used as the out-group (Fig. 5). Remarkably, this robust tree of Viperinae venom Asp49-PLAs shows that the PLAs with Asn at the *N*-terminus (designated as N1) form a distinct cluster separated from those with Ser at the *N*-terminus (designated as S1). In the N1-PLA cluster, at least four subtypes of PLAs with distinct *N*-terminal sequences are selectively present in various

Vipera and *Daboia* venom species (Fig. 5), including two acidic subtypes with either F3 or Y3 (or T/S 3) substitution, and a highly basic subtype containing A11K12 and a less basic subtype containing K11M12.

4. Discussion

Contents of venom PLAs in DsM and DrK (28–38% of the venom proteins by weight) are lower than those in *Daboia*



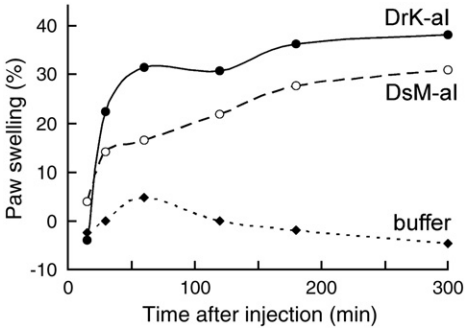


Fig. 4. Oedema-inducing activities of DrK-aI and DsM-aI on rat paw. Time course of the swelling of rat hind-paw was followed after injection of 10 μg of the PLA dissolved in 100 μl PBS. Volume of the paw was measured by a plethysmometer, % paw swelling (relative to the volume measured 1 min after the injection) was the average from results of two independent experiments.

Table 3
Lethal potencies of crude venom and purified PLAs on ICR mice

Venom or PLA	Lethality, (i.p.) μg/g mouse body weight
DrK-aI	$0.06<LD_{50}<0.13$
DsM-aI	$LD_{50}>3.0$
DrK-bI	$LD_{50}>3.0$
DsM-bI'	$0.13<LD_{50}<0.20$
DsM-aI+DsM-bI' (1:1)	$0.40<LD_{50}<0.50$
DrK soluble venom	$1.0<LD_{50}<1.33$
DsM soluble venom	$0.3<LD_{50}<0.4$

venoms from Thailand, China, Taiwan and Pakistan (55–65% of the total proteins). Various effects elicited by the *Daboia* PLAs have been reported, including neurotoxic [14,17], cytotoxic, myotoxic [16], hypotensive, and/or anticoagulating [30], and some PLAs may show more than one of the effects. In the present study, four PLAs were purified from each of DrK and DsM, and eight novel venom PLAs have been cloned and fully sequenced (Table 2, Fig. 3). PLAs of all geographic venom samples of *Daboia* are found to contain Asp49, and many of the DrK and DsM PLAs contain rather unique Ala6 substitution.

We previously reported the *N*-terminal sequences of five PLAs purified from *D. siamensis* (formerly *Vipera r. siamensis*) venom purchased from Miami Serpentarium (Florida, USA) [19]. However, our later analyses of *Daboia* venom obtained from Thai Red-Cross Society, Bangkok revealed that it contained only the RV4/RV7 heterodimeric PLA (unpublished results), just like Taiwan *D. siamensis* venom [14]. This was supported by another cloning study using Thai *D. siamensis* venom glands [15]. In fact, the *N*-terminal sequences of three minor PLAs (S1-2, S1-1 and S3) in previous report [19] are identical to those of DsM-aI, DsM-aII and DsM-bI, respectively (Table 1). Thus, the *D. siamensis* venom from Miami Serpentarium was heterogeneous and possibly contained 20% of the venom from Myanmar.

Results in Tables 1 and 2 are helpful for the identification and matching of purified PLAs with their cDNA clones. However, masses of purified DsM-aI' and of DsM-bI' cannot exactly match those of DsM-aI and of DsM-bI (deduced from the cDNA data). The discrepancies could be attributed to the fact that we used venom glands of *Daboia* specimen from southern Myanmar for cDNA cloning, but the pooled northern Myanmar *Daboia* venom was used for protein analyses. We suspect that the PLA pairs, DsM-aI and aI', DsM-bI and bI', are probably allelic proteins from different geographic samples. However, possibilities that we did not obtain all the transcripts of PLA from DsM venom glands, or that some of purified PLAs have undergone post-translational modification or processing, could not be ruled out. In addition, Blast-search helped to detect similar data recently reported by other researchers after our data have been deposited. For examples, *D. r. siamensis* PLA-III (AY303800) and PLA-II (AY286006) appear to be identical to DsM-aI and DsM-bI, while *D. r. russelii* PLA-I (DQ365974) and PLA-II (DQ365975) are identical to DrK-aI and DrK-bI, respectively. Another clone (AY256974) in the databank was possibly a hybrid of DsM-aII and aI, and the other (DQ365977) appeared to be a hybrid of DrK-aI and bII, they possibly derived from artifacts of PCR experiments.

Envenoming by both DsM and DrK was reported to be more hemorrhagic than by *Daboia* specimens from other geographic areas [3–5,7,8], which is attributed to special expression of haemorrhagic metalloproteinases in both DsM and DrK (results to be published). Except for the absence of a DrK-bII homolog in DsM (Table 1), all the deduced DsM-PLA sequences are ≥97% similar or identical to their orthologous PLAs in DrK (Fig. 3), and a PLA-isoform identical to DsM-aI is also present in low content in DrK. These venom similarities suggest that *Daboia* vipers from Myanmar and eastern India are closely related species and constitute a special lineage of the *Daboia* populations. Results of previous studies on western India *Daboia* venom (supplied by Haffkine Institute, Mumbai) also showed that an acidic PLA (FrIII-3, pI 4.2) had most potent neuromuscular blocking action among the five PLAs purified [31]. However, venom differences between the eastern and western India *D. russelii* have been reported [7,8].

Morphological differences between the *D. siamensis* populations of Thailand and Myanmar [32] have been reported, and heterologous antivenoms in four times greater dosages were found to be necessary to neutralize toxic effects of each venom [33]. The fact that DsM is more similar to DrK than to the *D. siamensis* venoms from Thailand, China and Taiwan might be explained by the notion that dispersal of *Daboia* "out of India" probably was

Fig. 3. Alignments of the amino acid sequences of Asp49 PLAs from Viperinae venom: (A) acidic N1-PLAs; (B) basic N1-PLAs; (C) S1-PLAs. Representative sequences were retrieved from BlastP search [25]. Single-letter codes of amino acids and the numbering system of Renetseder et al. [51] are used. Residues identical to those in the top line are denoted with dots, gaps are marked with hyphens. PLAs and the species (as the original taxonomic designation submitted) and their GenBank or SwissProt accession numbers are: *C. cerastes* Ccer, P21789; *Vipera russelii* (southern India) VRV-PL VIII, P59071; *Vipera r. russelii* (Pakistan) Vrr-R1, P81458; *Vipera russelii formosensis* RV-4 and RV-7, Q02471 and P31100; *E. mafocmahonii* Ema-PLA 2, P24294; *Echis ocellatus* Eo-A, P59171; *Pseudocerastes persicus* Cb-I, AAB36097, Cb-II, AAB36096; *T. puniceus* Tpu-G6D49, AAR14167; *Vipera a. ammodytes* Ammodytin I2, P34180, Ammodytoxin-A, B and C, P00626, P14424 and P11407; *Vipera a. meridionalis* Vipoxin-A, P04084, Vipoxin-B, P14420; *Vipera aspis zinnikeri* Vaspin-An, AF548351; *Vipera b. berus* V6, AY159811; *Vipera palaestinae* VP8 AAC78084.

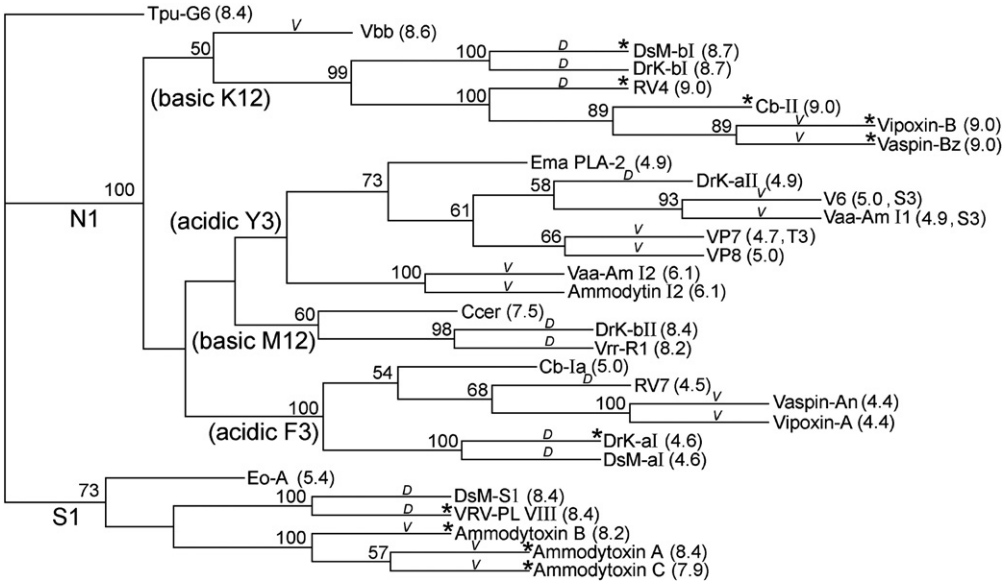


Fig. 5. Phylogenetic tree based on amino acid sequences of Viperinae venom Asp49 PLAs. The dataset includes full sequences of 30 PLAs from Viperinae venom, and a pitviper PLA (Tpu-G6D49) was used as out-group. Their pI and special N-terminal substitutions were shown in parentheses. Neurotoxic PLAs or PLA-subunits are marked with asterisks. Species names and accession numbers are: *Pseudocerastes persicus* Cb-II, AAB36096; *Vipera a. aspis* Ammodytins, Vaa-Am I1, AY159807 and Vaa-Am I2, AY158637; *Vipera aspis zinnikeri* Vaspin-Bz, AY158635, *Vipera b. berus* Vbb, P31854; *Vipera palaestinae* VP7, AAC78085. Values at the nodes indicate the percentage of 1000 bootstrap replicates. "*D* or *V* " above branches denote origin of the PLA from *Daboia* or *Vipera* venom, respectively.

hampered by the high mountain ranging from the eastern border of Myanmar. This bio-geographical distribution has also been proposed for frog and some other vertebrate groups [34].

Based on the mitochondria DNA analyses [35,36], the genus *Daboia* is closely related to European and western Asian *Vipera* and *Macrovipera*. Moreover, both *Daboia* and *Vipera* are specifically equipped with venom procoagulating components, i.e. Factor-X and Factor-V activators [37,38]. It is interesting that their venom powder can be either white or yellow color in appearance [39], and the white venoms were less necrotic or hemorrhagic than the yellow ones [6]. Five subtypes of venom Asp49 PLA genes have been found for geographic samples of *Vipera a. aspis* [40,41] and possibly other *Vipera* [42]. The phylogenetic tree of PLAs in Fig. 5 unravels not only the close relationship between the two genera *Daboia* and *Vipera* but also parallel evolution of their venom PLA paralogs. It has been suggested that gene duplications followed by accelerated evolution of surface functional domains have resulted in venom PLA diversity [13,43]. Venom components may be differentially expressed according to geological separation [44], adaptation to prey-ecology [45] or other factors [2].

A lethal acidic PLA designated as PFIIc' was previously purified from eastern Indian *Daboia* venom [9]. DrK-aI probably is PFIIc' because they share identical *N*-terminal sequences and high content in the venom (15% by weight), and are highly lethal to mice. In contrast, the most lethal PLA of DsM is the basic DsM-bI', which is present in equal abundance as DsM-aI (Table 3). Notably, enzymatic activity and toxicity of DsM-bI' were not affected by the addition of equal concentration of DsM-aI (Table 3), thus DsM-aI does not play a chaperon role. The venom content of DrK-bI is much lower than that of DrK-aI (Fig. 2), they possibly do not associate into dimers either. It is found that DrK-bI is much less lethal than DsM-bI or bI' (Table 3) although they differed by only four residues (Fig. 3B). Since Thr41 has been conserved in all the neurotoxic or myotoxic PLAs listed in Fig. 3 as well as crotoxin B and agistrodontoxin [11] while Ser41 is usually present in less toxic PLAs and crotoxin A, it is speculated that the substitution of Thr41 might be one of the reasons behind the lower toxicity of DrK-bI relative to DsM-bI or bI'.

In both pancreatic and snake venom PLAs, Leu2 is highly conserved [46], and probably is important for binding of the substrates fatty acyl chain. Mutagenesis of Leu2 to Trp2 in pancreatic PLA resulted in 33-folds decrease of the enzymatic rate [47]. DsM-aI differs from DrK-aI only at residue 2, the 40–45 folds reduction in the catalytic rates and the lethality of DsM-aI relative to DrK-aI thus can be solely attributed to its Phe2-substitution (Table 1). The hydrolytic products of neurotoxic PLAs are suggested to be crucial for their presynaptic toxicities [48]. Mutants of PLA-neurotoxins with lower catalytic activities than the native form usually showed decreased toxicities [29,49]. The *N*-terminal structures of PLA have also been known to be critical for the neurotoxicities [49,50]. Thus, single amino acid substitution at strategic position of a venom PLA toxin may greatly affect its biological functions.

Having an identical *N*-terminal sequence to that of daboiatoxin previously purified from Myanmar *D. siamensis* venom [17,18], DsM-aI is much less lethal than daboiatoxin (Table 3). This discrepancy and the reason why the viper expresses such inactivated DsM-aI remain puzzling. Although DsM-aI showed significant edematous effect (Fig. 4), its other functions remain to be investigated. Moreover, the sequence of

DrK-aI is 91% identical to that of RV7 from Taiwanese *Daboia siamensis* venom (Fig. 3A). The substitutions of about ten amino acid residues in RV7, including E7K, T70M and D89N, Y120N, could transform this non-lethal chaperone-like subunit [14] to a highly lethal and neurotoxic DrK-aI, or vise versa. These naturally occurring isoforms with minor structural differences but great functional variations thus provide a new platform for further investigation of structure-function relationships of venom PLAs.

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