J. TOXICOL.—TOXIN REVIEWS, 16(3), 79–113 (1997)

PHOSPHOLIPASES A_2 OF ASIAN SNAKE VENOMS

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ABSTRACT

This review up-dated the structural and functional information of various phospholipase A_2 (PLA_2) isoforms purified from Asian snake venoms. A phylogenic tree of group I PLA_2s was constructed herein based on many recently resolved amino acid sequences of the venom enzymes. It was found that PLA_2s of Asian elapid venoms are structurally different from those of sea-snake/Australian elapid venoms, and are usually associated with cardiovascular effect, although exceptions such as β-bungarotoxins do exist. Two types of venom PLA_2s appear to be present in the venom of Asiatic viperinae such as *Daboia* and *Echis*, one has a N-terminal residue Asn and the other has the residue Ser. In the venom of Asiatic crotalinae, up to four subgroups of PLA_2 isoforms are present and each of them is characterized by a distinct substitution at residue 6 (Glu, Asn or Arg) or residue 49 (Asp or Lys) in their sequences. The venom PLA_2s in each of the subgroup show more or less functional similarity specific for the subgroup: the Glu6-PLA_2s are usually antiplatelet,

the Asn6-PLA_2s are neurotoxic and/or myotoxic and many Arg6-PLA_2s are anticoagulating, while the Lys49-PLA_2s are myotoxic and edema-inducing. Mechanisms for the pharmacological actions of venom PLA_2s have been discussed, including neurotoxicity, myotoxicity, antiplatelet activity, anticoagulating activity, heparin-binding, protein-acylation and deacylation. Conclusions derived from many recent studies on pancreatic PLA_2 by method of protein engineering render valuable information about the structure-activity relationship of the secretory PLA_2 superfamily. Site-directed-mutagenesis methods coupled with relevant and dissecting functional assays are essential for understanding the structure-activity relationship of snake venom PLA_2s with special function or toxicity.

1. INTRODUCTION

Asia is the habitat of various species of venomous snakes including Elapidae, Hydrophilidae and Viperidae. According to statistics, the rate of snake bite and envenomation in Asia has been the highest among the world [1]. The characterization of snake venom components is important because a suitable medical treatment depends on a better understanding of the site and mode of action of the venom components. Phospholipases A_2 (PLA_2s, EC 3.1.1.4) are a group of enzymes that catalyze the Ca^{2+}-dependent hydrolysis of the 2-acyl ester bond in 3-*sn*-phospholipid. Secreted forms of the enzyme are abundant in the mammalian pancreas and in snake and bee venoms. Amino acid sequences of many PLA_2s have been determined, with most being about 120 amino acids long and having 14 Cys residues forming seven disulfide bonds. Overall these proteins are closely related (>45% identity), with key residues that are required for catalysis and structure to be conserved (for review, see references [2-8]). PLA_2s are classified into two groups based on their Cys positions. PLA_2s in group I are found in venom of the elapid and hydrophid

snake families and in the mammalian pancreas, and group II are from the viperid snake venoms and mammalian nonpancreatic sources. Interestingly, PLA_2 isoforms of diverse physiological functions exist in the same venom source and they usually synergize with other venom components to display special pharmacological effects. This mini-review would update the recent progress in purification, structural analysis, toxicology and reaction mechanism of venom PLA_2s from important species of Asia snakes. Since the 14 kDa PLA_2 family has been the subject of many comprehensive reviews [2, 5], I will focus on recent findings about molecular and mechanistic analysis related with the PLA_2s from Asian snake venom, and examine roles of the PLA_2 isoforms in the context of toxinology of the whole venom and the phylogenic relationship between the PLA_2s.

2. STRUCTURE OF PLA_2 ISOFORMS FROM SNAKE VENOM

a. Elapidae and Hydrophiidae

Highly homologous PLA_2s from Asian elapid venoms were purified and characterized. More than 15 PLA_2 sequences from cobra and ringhal venoms were known [35]. Recently, the cDNAs encoding three PLA_2 isoforms in *Naja naja sputatix* [9] and two PLA_2 isoforms in *Naja naja atra* [10, 11] were cloned and sequenced.

Two acidic PLA_2s with pI of 3.8 and 3.9 [12] and one with pI of 5.2 [13] were purified from King cobra (*Ophiophagus hannah*) venom. The amino acid sequence of the latter PLA_2 was completed [13, 27] and found to be similar to that of PLA_2 III of *Bungarus fasciatus* venom and those of cobra PLA_2s.

It was found that as many as eight PLA_2 isoforms exist in the venom of southeast-Asia golden krait *Bungarus fasciatus*, including four catalytically active PLA_2s (III, Vb-1, X-1, X-2), three less active Pro30 PLA_2s (Va, Vb-2 and VI), and one inactive Ala49 PLA_2 (i.e. fraction I). Interestingly, fractions V

and VI together count for about 70% of the total mass of the crude venom. The complete amino acid sequences of these PLA_2 were all solved [14, 15].

Highly neurotoxic venom of *Bungarus multicinctus* contains the unique pre-synaptic neurotoxin, β-bungarotoxin, in addition to α-bungarotoxin, the post-synaptic neurotoxins whose homologous toxins are commonly present in all the Elapidae and Hydrophiidae venoms. There are more than 10 isoforms of β-bungarotoxin present in the venom and each is a covalent heterodimer of PLA_2 (A chain, 14 kDa) and a homolog of Kunitz-type protease inhibitor (B chain, 7 kDa) [16]. Six variants of the A chain of β-bungarotoxin have been identified and at least four of their cDNA sequences were analyzed [17-20].

Therefore, PLA_2s in the venom of *Bungarus* species are much diversified as compared with cobra venom PLA_2s. Interestingly, the venom PLA_2s of *B. fasciatus* are not presynaptically neurotoxic but cardiotoxic [21] and structurally very different from those of *B. multicinctus*. This is in accord with the finding that their venom components other than PLA_2 are also different: for example, procoagulating factor X activator was found in venoms of *B. fasciatus* [22] and king cobra [23] but not in that of *B. multicinctus*. The acidic PLA_2 from king cobra venom was found to damage both heart and skeletal muscle[24] and also inhibit platelet aggregation [27]. Venom of sea-snakes usually contains strong post-synaptic neurotoxins and myotoxic PLA_2s. The primary structures of PLA_2s from hydrophiid snake venom were solved for the following species: *Enhydrina schistosa* [25], *Laticauda colubrina* [26], *Laticauda laticaudata* [28], *Laticauda semifasciatus* [29] and *Aipysurus laevis* [30]. Some of the myotoxic sea-snake PLA_2s are also neurotoxic [25, 26]. Results of phylogenic analysis of representative PLA_2s from elapid and hydrophilid venoms (Fig. 1) suggest that sea-snake PLA_2s are structurally more related with those of Australian snake venoms than with those from venoms of cobra, ringhal or krait, i.e. Asian and African *Elapidae*. Similar conclusions could also be drawn from the phylogenic tree contructed previously from

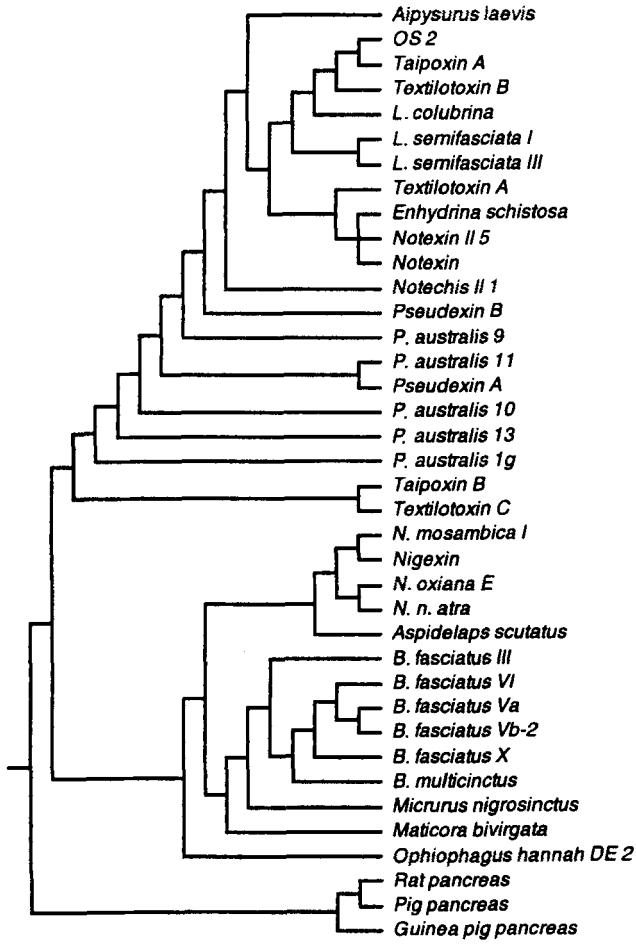

Fig. 1. Phylogenic tree of selected group I venom PLA_2s based on the amino acid sequences. The sequences were extracted from the Swiss-Port data bank. The tree was constructed using program PAUP [156], and using pancreatic PLA_2 as the outgroup.

another set of sequences [102]. β-Bungarotoxins are highly specialized and phylogenically branch-off from other elapid PLA_2s [102].

Although the best known feature of envenoming by elapid and hydrophilid snakes is neurotoxicity, the cobra venom PLA_2s are non-

neurotoxic in clinical symptoms. Having a conserved structure with sequence identity of about 85%, they show cardiovascular effects either alone or in synergy with the cytotoxin (cardiotoxin) in the same venom both *in vivo* and *in vitro* [31-35]. The acidic and neutral cobra PLA_2s are more active than the basic cobra PLA_2s in causing cardiac stimulation and transient hypotension [36]. The highly active cobra PLA_2s also cause hyperkaliemia in the blood of victims due to membrane leakage and cell lysis [37]. It has been speculated that the non-neurotoxic and monomeric PLA_2 in *B. multicinctus* venom may cause a sharp fall in arterial blood pressure [37].

The three-dimensional structures of a single-chained PLA_2 from Taiwan cobra venom [38] and of notexin, an Australian elapid PLA_2 neurotoxin [39, 40] were solved by X-ray crystallography at 2 Å. The crystal structure of β_2-bungarotoxin was also solved, showing a occluded substrate-binding surface and reduced hydrophobicity of the PLA_2 subunit [41].

b. Subfamily Viperinae

The medically important viperinae snakes in Asia are *Daboia russelli* (Russell's viper), *Echis carinatus* [42], and *Cerastes*. The primary structures of PLA_2 isoforms from these venoms were not characterized until recently. *Daboia russelli* has been classified into at least four subspecies: *Daboia russelli formosensis* (Taiwan), *Daboia russelli pulchella* (Sri Lanka and southern India), *Daboia russelli russelli* (northern India and Pakistan), and *Daboia russelli siamensis* (China and south-east Asia). The geographic variations of their bite-symptoms are remarkable and their antivenins usually show poor cross-neutralization. Venoms of four Russell's viper subspecies were compared in terms of their HPLC profiles and partial amino acid sequences of their PLA_2s. A potent, heterodimeric PLA_2 neurotoxin (designated as Russtoxin) was found in all the viper venoms analyzed except that of *D. r. pulchella* [43]. The PLA_2s of *D. russelli* (southern India)

previously studied by Gowda et al. [44, 45] appear to be structurally the same as those of *D. r. pulchella* (Sri Lanka), while the Russtoxins from *D. r. russelli* (Pakistan) and *D. r. siamensis* (Thailand) resemble that from *D. r. formosensis* [43]. Moreover, the published N-terminal amino acid sequences of the venom PLA_2s of *D. r. siamensis* from Fujian (China) [46] and from Burma [47] are also similar to that of *D. r. formosensis*. However, PLA_2s of *D. r. siamensis* from these three regions show some variations in their N-terminal sequences (Table 1). The structural and functional data of the venom PLA_2s provide evidence for the presence of two major types of Russell's vipers. The species *D. r. formosensis*, *D. r siamensis* (Fujian, Thailand, and Burma) and *D. r. russelli* (Pakistan) represent one type whose venom contains hypotensive and neurotoxic PLA_2s having an Asn residue at the N-terminus [43, 48], while *D. r. pulchella* (southern India and Sri Lanka) represents the other type whose venom contains myonecrotic PLA_2s [49] with a N-terminal Ser residue. This finding is consistent with the reported antivenom differences between the Sri Lankan and the northern or western Indian subspecies [50] and also in accord with the report that two distinct groups of *Daboia russelli* population was found by means of multivariate morphometrics [51].

The complete sequences for heterodimeric neurotoxic PLA_2 (F4-F7) from the venom of *D. r. formosensis* were deduced from the cDNA sequences of both subunits. They are 92% identical to the vipoxin/inhibitor pair from the venom of Bulgarian *Vipera ammodytes* [48]. Structures of both subunits of the heterodimeric PLA_2 toxin from *Pseudocerastes fildi* (false horned viper, Israel) were found to be over 90% identical to those of the F4-F7 and the vipoxin/ inhibitor pairs [52]. Moreover, similar heterodimer PLA_2 toxins were found in the venoms of western-Asian species including *Vipera aspis* [53] and *Vipera palaestinae* [54]. The acidic subunit or charperon of the toxins plays the role of protecting the basic subunit against non-specific binding and thereby increases the probability of basic subunit reaching the neuromuscular junction or the

Table 1. The N-terminal amino acid sequences of PLA_2s from the venom of *D. russelli* subspecies and *Vipera aspis*

									*								*									*							
R1	N	L	F	Q	F	A	E	M	I	V	K	M	T	G	K	N	P	L	-	S	Y	S	D	Y	G	C	Y	C	G	W	G	G	K	G	K	P	Q	D
S1-2																																			
S1-1				Y			G	R		F	R			A									N											
									*								*									*							
F4(RV-4)	N	L	F	Q	F	A	R	M	I	N	G	K	L	G	A	F	S	V	W	N	Y	I	S	Y	G	C	Y	C	G	W	G	G	Q	G	T	P	K	D
R2																																			
S2																																			
S3										D	A		Q	E				F		K														
DRS (Fujian)											K							F																
V.aspis PLI B							L																												
									*								*									*							
F7(RV-7)	N	L	F	Q	F	G	E	M	I	L	E	K	T	G	K	E	V	V	H	S	Y	A	I	Y	G	C	Y	C	G	W	G	G	Q	G	R	A	Q	D
R3																																			
S4																																			
DbTx(Burma)		F								V	K	M					A																	
V.aspis PLI A							D				Q		Y				A																	
									*								*									*							
P1	S	L	L	E	F	G	K	M	I	L	E	E	T	G	K	L	A	I	P	S	Y	S	S	Y	G	C	Y	C	G	W	G	G	K	G	T	P	K	D
P2-1							M											V		F														
P2-2																		V		F														
P3							M											V		F			D											
VRV-PL-VIIIa																																			

Asterisks denote numbering by each 10 residues. For PLA_2 in each subgroups (separated by dashed lines), only the sequence of the first PLA_2 and residues different from those of the first are shown. Abbreviations of PLA_2s are the same as [43] with R stands for *D. r. russelli*, F for *D. r. formosensis*, S for *D. r. siamensis* (Thai), and P for *D. r. pulchella* (Sri Lanka), DRS (Fujian) for *D. r. siamensis* from Fujian, China [46]. DbTx for daboiatoxin from *D. r. siamensis* (Burma) [47].

endothelium membrane causing neurotoxicity or hypotension (Tsai *et al.* to be published). Recently, synaptosomal binding of ^{125}I-labelled daboiatoxin from Burmese *Daboia r. siamensis* was studied [55]. The toxin binding could not be antagonized by the myotoxic PLA_2s from south Indian *D. russelli* (i.e. *D. r. Pulchella*) venom. However, it remains to be checked whether Burmese daboiatoxin present in the venom as a monomer or heterodimer.

Besides the heterodimeric toxic PLA_2s, monomeric PLA_2s of low lethal potency were identified and sequenced for the venoms of Pakistan *Cerastes cerastes* [56], west-Pakistan *Eristocophis macmahoni* [57], and those of *D. r. siamensis* and *D. r. russelli* (e.g. R1, S1-1, S1-2 in Table 1) [43, 58]. These homologous PLA_2s appear to play a hypotensive or antiplatelet role in rats.

Venom of *Echis carinatus sochureki* (north India and Pakistan) contains a Ser49 PLA_2 which was recently sequenced and characterized [59]. The substitution of Ca^{2+}-binding Asp49 with a Ser49 in this enzyme does not abolish its enzymatic activity and this basic PLA_2 could induce platelet aggregation. Previously, partial sequences of two PLA_2 from the venom of Kenyan *Echis pyramidum leakeyi* (formerly *E. c. leakeyi*) were reported [60] and one of the sequence is very similar to that of the *E. c. sochureki* Ser49 PLA_2, while the other is similar to that of the Asp49 PLA_2 from Pakistan vipers [56, 57]. The Croatia/ Slovenija *Vipera ammodytes* venom also contain Ser49 myotoxic PLA_2 (i.e. Ammodytin L) but without detectable enzyme activity [61]. A weakly basic PLA_2 was purified from Indian *E. carinatus* venom but the sequence was not reported [62].

c. Subfamily Crotalinae

The generic name of some of the Asiatic *Agkistrodon* species has been changed or remains controversial [42] e.g. the monotypic *Calloselasma rhodostoma* and *Deinagkistrodon acutus* have been renamed. *Agkistrodon halys* Pallas is designated as *A. blomhoffii brevicaudus* now. The green species

of *Trimeresurus* have given rise to considerable confusion because of the great similarity between different forms. *T. gramineus* has been mistakenly used to name *Trimeresurus stejnegeri* (Taiwan) by toxinologists in reporting PLA_2 and other components of this venom [42, 70, 71].

The venom PLA_2 isoforms from the following species of Asian pit vipers have been characterized and sequenced: *T. flavoviridis* [63-65], *T. okinavensis* [63], *T. mucrosquamatus* [66-69], *T. stejnegeri* (*T. gramineus*) [70, 71], *A. b. brevicaudus* [72-74], *A. h. blomhoffii* [75-77], *Deinagkistrodon acutus* [78-80]. My classification of these PLA_2 according to similarities in their structures and pharmacological properties resulted in four subgroups of the crotalid venom PLA_2 (Fig. 2-5). Each subgroup has a distinct residue 6 or 49. Sequence identities within a subgroup are usually >70% while between the subgroups are usually <55%. PLA_2s in the Glu6 subgroup (Fig. 2) are inhibitors of platelet aggregation [78, 81], those in the Arg6 subgroup (Fig. 3) are strong anticoagulant [82], direct-hemolytic [77] and bactericidal when associated with mammalian bactericidal/ permeability-increasing protein [75, 83-85]. PLA_2s in the Asn6 subgroup(Fig. 4) are neurotoxic or/and myotoxic [67, 86-89], and those in the Lys49 subgroup (Fig. 5) possess myotoxic [65, 71, 91], edema-inducing [68] and membrane depolarizing activities [65, 92]. The enzymatic activities of Lys-49 PLA_2s are usually hardly detectable or lower than those of the Asp-49 enzymes [89, 93]; however, the hydrolytic activity of the Lys49 PLA_2s of *T. flavoviridis* [94] and of other venoms [91] could be demonstrated.

Three dimensional structures of crotalid PLA_2s have been investigated by X-ray diffraction on the crystals derived from the acidic dimeric PLA_2 of *T. flavoviridis* [95], the acidic monomeric PLA_2 of *A. h. blomhoffi* venom [96], the acidic PLA_2 of *A. b. brevicaudus* venom [97], and the Lys49 PLA_2 from *A. p. piscivorus* venom [98] whose sequence is highly similar to those of the Lys49 PLA_2s of other Asian pit-vipers (Fig. 5). X-ray crystallographic structures of the recombinant wild-type and mutants of a cationic Asp49 PLA_2

Fig. 2. Comparison of amino acid sequences of *Crotalinae* phospholipases A_2 with Glu 6 substitution. Single-letter codes of amino acids were used. The numbering system follows that of Renetseder *et al.* [90]. Residue identical to that in the top line is denoted with a dot; gaps are marked with hyphens. Abbreviations and references: Abb, Ahb, App, DAV are *Agkistrodon blomhoffii brevicaudus, Agkistrodon halys blomhoffi, Agkistrodon piscivorus piscivorus* and *Deinagkistrodon acutus* [79], respectively; TMV, TFV, TGV are venom of *Trimeresurus flavoviridis* [64], *Trimeresurus mucrosquamatus* [66], and *Trimeresurus gramineus* or *stejnegeri* [70], respectively.

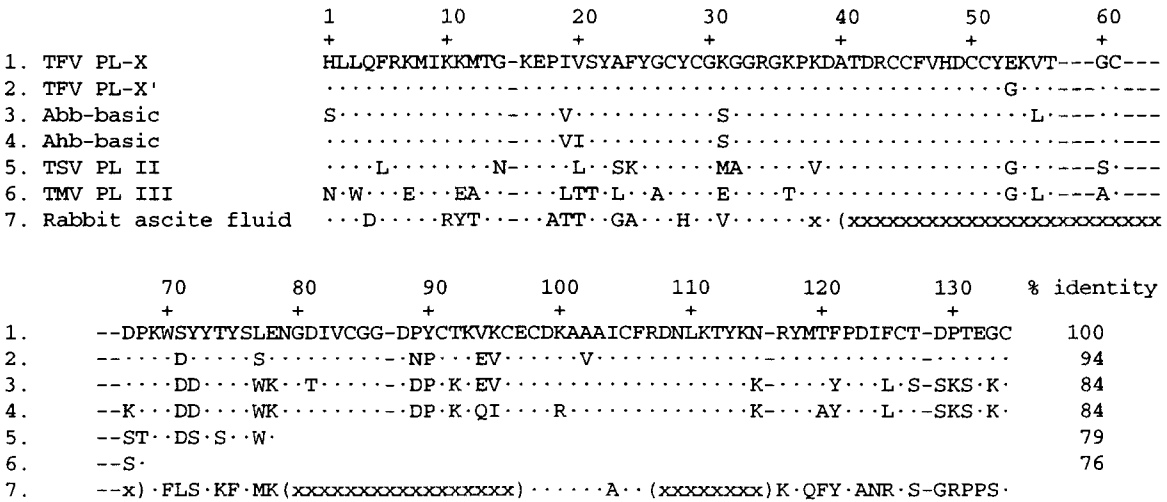

Fig. 3. Comparison of amino acid sequences of *Crotalinae* PLA_2 with an Arg 6. Single-letter codes of amino acids were used. The numbering system follows that of Renetseder *et al.* [90]. Residue identical to that in the top line was denoted with a dot, gaps are marked with hyphens, "x" denotes unknown residue. Abbreviations are as those in Fig. 2. References for sequences: 1 and 2 [63, 64], 3-5 [74-76], 6 [69] and 7 [85].

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                          1         10        20        30        40        50        60
                          +         +         +         +         +         +         +
1. DAV toxin              HLLQFNKMIKIMTR-KNAFPFYTSYGCYCGWGGRGWPKDATDSCCFVHDCCYGKLT---GC---
2. Trimucrotoxin          N···········K-···I···S·········Q·K·····R·············D·---
3. Crotoxin B1            ·········FE···-···I···AF··········R·····R···········A---K·---
4. Crotoxin B2            S·········FE···-···V···AF·········Q·R·····R···········A---K·---
5. Agkistrodotoxin        N·········EE·G-···I···AF·········Q·K···G··R·········R·V---N·---
6. B. jararaca-TxII       D·W··GQ··LKE·G-··LP··Y··T·········Q·Q·····R·············---N·---
7. B. asper PLA III       S·IE·A···LEE·K-RLP··Y··T·········Q·Q·····R···········S---N·---
8. C. v. viridis toxin    N·········M··K-·····

      70        80        90        100       110       120       130       % identity
      +         +         +         +         +         +         +
1. --SPKWDIYPYSWKTGVIICGE-GTPCEKEICECDRAAAVCLGENLRTYKTK-YMFYPDFLC-KKPSKQC       100
2. -----S···S·····I·····---E···K···········H·····KR-·········-TD··EK·        84
3. --NT·····L·S·Y·T··K---W··EQ·····V··E··RRS·S···YG-·····SR··RG··ET·        75
4. --NT·····R··L·S·Y·T··K---W·KEQ·····V··E··RRS·S···NE-·····SR··RE··ET·     72
5. --NT·S·T·S··L·E·Y·T··K---N··EQ·····V··E·FRR··D··NNG-····R·SK·-TET·EE·     67
6. --K··T·R·S··REN·····-·····Q·····K·····FR·····KR---A···V··-···AEK·         74
7. --K··T·R·S··R·S·····-·····Q·····K·····FR·····KR---A···L··-···AEK·         73
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Fig. 4. Comparison of amino acid sequences of *Crotalinae* phospholipases A_2 with a neutral residue 6 (or Asn 6). Single-letter codes of amino acids were used. The numbering system follows that of Renetseder *et al.* [90]. Residue identical to that in the top line is denoted with a dot; gaps are marked with hyphens. References for the sequences are 1-5 [79, 86], 6, 7 [88] and 8 [89].

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                                1         10        20        30        40        50
                                +         +         +         +         +         +
1. D. acutus K49                SLIELGKMIFQETG-KNPVKNYGLYGCNCGVGNRGKPVDATDRCCFVHKCCYKKVT---
2. T. mucrosquamatus K49        ·····-·····-·····-·····-·····-·····-·····-·····-·····---
3. T. flavoviridis BPI          ··VQ·W·····-·EAA·····R····K····S··Y·····-·····---
4. T. gramineus PLA-V           ·V·····-·····ATS·····P·G·R··K·····Y·····L·----
5. B. asper-II                  ··F·····L·····-·A·S··A·····LG····K·····Y·····L·----
6. B. jararacussu-I             ··F·····L·····-·A·S··A·····LG····K·····Y·····L·----
7. A. p. piscivorus K49         ·VL·····L·····-AITS··S·····W·H··Q·K·····L·----
8. A. c. laticinctus MTX        ··L·····L·····-AITS··S·····W·H··Q·K·····L·----

   60        70        80        90        100       110       120       130    identity
   +         +         +         +         +         +         +         +
1. GC-----DPKKDRYSYSWENKAIVCGEKNPPCLKQVCECDKAVAICLRENLGTYDKK-HRVTVKFLC-KAPESC    100
2. ·····-·····-·····-·····-·····-·····-·····-·····-·····-·····-·····-·····-·····    100
3. ·····-·····M·S·····K·····-·····-·····-·····-·····N···-YTIYP·PF···-KADT·     81
4. D·····-·····I·····-·····V·····-·····D·····-·····EM·····-·····F····D·····-KKINL·LF·K·TS·Q·     78
5. ·····-·····N·····-·····KD·T·····N·····-·····EL·····-·····-·····N···-Y·YRL·PF···-KADP·     78
6. ·····-·····N·····-·····KD·T·····N·S-····EL·····-·····-·····N··N···-Y·YYL·P····-KADA·     78
7. D·····-·····NH·T·····-·····K····I·E·····-·····EM·····-·····D··N···-YKAYF·LK···-K·DT·     74
8. D·····-·····NH·T·····-·····K····I·E·····-·····EM·····-·····D··N···-YKAYF··K···-K··T·     77
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Fig. 5. Comparison of amino acid sequences of *Crotalinae* PLA_2 with Lys 49 substitution. Single-letter codes of amino acids were used. The numbering system follows that of Renetseder *et al.* [90]. Residue identical to that in the top line is denoted with a dot; gaps are marked with hyphens. References for the sequences are [71, 80, 91].

from *A. p. piscivorus* venom were investigated and the crucial roles of its residues 7 and 10 in interfacial binding were shown [99]. These crystallographic studies confirmed the conservation of core area conformation of the PLA_2 molecules and variations in surface residues and loops. Notebly, results from recent NMR studies [100, 101] on the pancreatic PLA_2 have demonstrated that the N-terminal residues 1-3 of the secretory 14 kDa PLA_2 in solution are not ordered and the enzyme is activated upon binding to the aggregated substrate. The molecular dynamics were not revealed by previous crystallographic data.

3. EVOLUTION OF VENOM PLA_2

Study on the evolution and the phylogeny of venom PLA_2 at the molecular level started several years ago [102]. The group I and group II PLA_2s are known to have evolved separately. Five cDNA and six genes encoding the venom gland PLA_2s of *T. flavoviridis* were sequenced [64], so were the PLA_2 genes from *T. gramineus* [103], those of Mojave toxin (*C. s. scutulatus*) [104], and those from *Vipera a. ammodytes* [105]. The data confirmed a positive Darwinian evolution of these genes. Interestingly, four and five exons were found in *Crotalinae* and *Viperinae* PLA_2 genes, respectively. The first intron was retained in the mRNA of *Crotalinae* PLA_2 in contrast to that of *Viperinae* PLA_2, possibly due to change in secondary structure of the first exon of *Crotalinae* PLA_2 gene [105]. The protein-coding regions are much more diversified than the 5' and 3' untranslated regions (UTRs) and the introns except for the signal peptide domain. The numbers of nucleotide substitutions per site for the UTRs and the introns were approximately one-quarter of the numbers of nucleotide substitutions per synonymous site for the protein-coding regions. However, the UTRs and the introns of venom PLA_2 genes have evolved at similar rates to those of non-venomous genes. Apparently, gene

duplication and accelerated evolution in the protein coding regions is universal in PLA_2 genes of *Crotalinae* and *Viperinae* venom. The venom genes have been evolving under adaptive pressure to acquire new physiological activities [64, 88, 103, 106].

It has been shown that possibly four group II PLA_2 genes which map to the same chromosome are present in mammalian genome [107], and it is likely that snakes also have several group II PLA_2 genes expressed in non-venomous tissues, and venom PLA_2 isoforms may be derived from more than one non-venomous PLA_2 gene.

Examination of nucleotide sequences for five group I PLA_2 cDNAs from four genera of elapids [108] also revealed high similarity of the non-coding region and more variability in the coding regions; but the conserved sequences from the elapids have no nucleotide sequence similarity to corresponding regions in viperid PLA_2 genes. Furthermore, when nucleotide sequences for cDNA clones of two metalloproteases from *Agkistrodon contortrix laticinctus* [109] and those from *Echis pyramidum leakeyi* [110] were compared, there appears to be high conservation of noncoding DNA sequences for the metalloprotease genes. Thus, most of the venom protein genes probably undergo a coordinated and fast evolution to ensure the production of an effective venom to meet the requirment of snakes for food and defense.

4. BINDING PROTEIN AND ACTION MECHANISM

It has been difficult to define the pharmacological effect of a venom PLA_2 *in vivo* and *in vitro*. Endeavours in mechanistic studies of venom PLA_2s have been fruitful but many questions remain to be answered.

a. M-type receptor

This 180 kDa membrane protein, identified to be present in mammlian tissues, binds tightly to some non-neurotoxic venom PLA_2s, pancreatic PLA_2

and the group II inflammatory-type PLA_2 [111-113]. The receptor gene has been cloned and expressed [111, 114] and was found to have endocytic property [115]. Significance of the receptor in the regulation of PLA_2 action remains to be elucidated.

b. Neurotoxic PLA_2s and binding proteins

High affinity and specific receptor for presynaptically toxic β-bungarotoxin has been identified to be a voltage dependent K^+-channel [116-118]. The binding proteins (or its subunit) on synaptic membrane for crotoxin and taipoxin have been found to be the norepinephrine transporter [119]. The receptors for ammodytoxin A and C on the bovine brain were identified, too. However, ammodytoxins could not inhibit all the high affinity binding of crotoxin, suggesting that receptors for crotoxin and ammodytoxin are not identical [120, 121]. The hypotensive PLA_2s from Indian *Daboia r. russelli* venom bind receptors rather differently from the receptors mentioned above [122-124]. Recently, synaptic binding protein of daboiatoxin, the myotoxic and neurotoxic PLA_2 from Burmese *Daboia russelli* venom, has been identified to be a 100 kDa protein with two subunits of 25 kDa and 75 kDa [55]. There was no competition by ammodytoxin or crotoxin for the receptor. It remains to be clarified whether the heterodimeric PLA_2 toxins from various *D. russelli* venoms [43,52] have similar binding sites as daboiatoxin or not. The diversified binding sites for presynaptic PLA_2 toxins have been reviewed recently by Tzeng [125].

We and others have shown that the N-terminal region and regions 76-81 and 119-125 of PLA_2 neutrotoxins are involved in their receptor binding or neurotoxicity [48, 67, 126, 127]. The cellular effects of the PLA toxins on the nerve-terminal have been reviewed in a recent paper [128]. The detailed mechanism after receptor binding and the role of phospholipid hydrolysis in the blockade of neurotransmitter release by the toxins are not clear.

c. Anticoagulating activity

Basic motif with four or five Arg/Lys in strongly anticoagulant PLA_2 from *Naja nigricollis* venom [82] is involved in its inhibition of prothrombin complex [129]. The anticoagulating human group II PLA_2 inhibited coagulation factor Xa or its interaction with factor Va also by a basic region at residuses 51-62 [130]. However, specific sites in other anticoagulating PLA_2s from viperid venoms, which are involved in a possibly similar mechanism, remains to be identified.

d. Antiplatelet activity

Venom PLA_2 interferes with hemostasis by either anticoagulant or antiplatelet effects. The inhibition of platelet aggregation by PLA_2s may be mediated by the generation of lysophospholipid [131, 132] and by resultant change in cytoskeleton and hence the loss of release reaction of platelets [27]. However, the specificity for platelet membrane appears to be a prerequisite of the antiplatelet activity of venom PLA_2. Recently, a structural feature responsible for this activity was proposed based on the 3-dimensional structure of the antiplatelet PLA_2 from *A. b. brevicaudus* (ie. *A. h.* Pallas) venom. A unique aromatic patch (residues 20, 21, 113 and 119) surrounded by two acidic residues (Glu 6 and Asp 115) on one face of the PLA_2 molecule was postulated to be implied in the recognition of platelet membrane [97].

e. Myonecrotic action and myotoxicity

In accord with their phylogeny relation (Fig. 1) hydrophiid and Australian elapid venom PLA_2s cause systemic myotoxicity and myoglobinuria in common while viperid basic PLA_2s with or without neurotoxicity and the Lys49 PLA_2s produce local myonecrosis [24]. The damaged muscle showed dilatation of sarcoplasmic reticulum, vascuolation, then disruption and hypercontraction of the fibres, and inflammatory reaction. Influx of Ca^{2+} into sarcoplasma and activation of proteases appear to play a vital role after

membrane damaging by PLA_2s [24, 133]. PLA_2 myotoxins showed differential specificity for different types of skeletal muscle [134]. The Lys49 PLA_2s are cytotoxic, edema-inducing and disrupt membrane by poorly understood Ca^{2+}-independent mechanism. Their edema-inducing activity involves degranuation of mast cell and PMN [68].

f. Binding to sulfated proteoglycans

A cluster of three or more basic residues is a potential motif in protein for its binding to heparin or other sulfated proteoglycans [135]. This motif is present in some Lys49 PLA_2 sequences and possibly also exists in the folded conformation of some Asp49 PLA_2s. It was well documented that some venom PLA_2s bind heparin [136-138], and so does the human group II PLA_2 [138, 139]. The binding not only serves for anchoring or concentrating the PLA_2 to specific cells but also modulates its enzyme activity.

g. Protein deacylation and autocatalytic acylation of PLA_2

It has been shown that PLA_2 may hydrolyze thioester bond of long chain acyl-CoA and show protein-deacylase activity for acyl-carrier protein [140]. It is likely that venom PLA_2 may hydrolyse some acyl-protein anchored on cell membranes although no such case has been reported. A PLA_2 may undergo autocatalyzed acylation of specific Lys residues to certain extend in the presence of phospholipid substrate [141]. On the other hand, Lys 49 and Ser 49 PLA_2s are able to undergo acylation spontaneously with free fatty acids [142]. The acylated PLA_2s usually became more hydrophobic and tightly bound to membrane or catalytically more active.

5. CONCLUSION AND PERSPECTIVES

Due to their abundance in snake venoms, thermal and acid stability, and relative ease of purification, PLA_2s are among the best studied enzymes.

Advances in technology including HPLC, mass spectrometry, polymerase chain reaction and nucleotide sequencing have facilitated the determination of amino acid sequences of more venom PLA_2s. Secondary and tertiary structures of PLA_2s were elucidated by X-ray crystallography, NMR and other methods [3, 143]. Although previous works, mainly chemical modification studies [144], have accumulated rich data, the structure-activity relationship of venom PLA_2 remain to be reinvestigated for conclusion by incorporating new methods such as *in vitro* mutagenesis.

The enzymatic activity of PLA_2 found *in vitro* may not well reflect that *in vivo*; and bilayer, monomeric substractes need to be tested in addition to micellar substrates with different electrostatic properties or phospholipid substrates with different head-groups. For functional studies, relevant and diversified assays are ususlly needed to study the pharmacological effects of a venom PLA_2. It may be essential to dissect the PLA_2 action into steps including receptor targeting and binding, interfacial binding and activation, active site catalysis, kinetic specificity, etc. Independent analysis of each step is necessary to make a conclusion about the structure-activity relationship of the PLA_2 action. Besides, species specificity [23] and tissue specificity [134] in toxin assays are important considerations. These are challenges for the researchers of venom PLA_2 toxins. It would not be surprising to find that conformational change occurs upon binding of a venom PLA_2 to its binding protein or specific target, since conformational dynamics of group I PLA_2s at the N-terminal and 53-72 regions upon interfacial binding [98, 145] or ligand binding [39] have been well documented.

In the past few years, site-directed-mutagenesis studies of pancreatic PLA_2s have shed considerable light on the role of its active-site residues [93, 146, 147], the N-terminal [145, 148] and the C-terminal regions [149], the disulfide bonds [150] and its interficial residues [97, 145, 148, 151, 153]. The results are important and fundamental for understanding the structure-activity

relationship of the 14 kDa PLA_2 family. However, venom PLA_2s have evolved from nontoxic enzymes to those with special toxicity or tissue specificity. More protein-engineering studies on their functional domains are apparently required. Interfacial-binding mutants of a cationic PLA_2 from the venom of *A. p. piscivorus* have recently been studied and the results showed that the critical charged groups involved in interfacial adsorption for this groups II PLA_2s is very different from those for pancreatic PLA_2 [99, 154]. It is likely that interfacial residues of each subgroup of venom PLA_2s are characteristic. The interfacial binding surface of the bee venom PLA_2 was shown to be part of its neuronal receptor-recognition domain by site-directed-mutagenesis [155]. There is clearly much scope for future research on the structure-activity relationship of venom PLA_2 isoforms with such complexicity and functional diversity.

Acknowledgement: I thank the National Science Council of Taiwan and Academia Sinica for grant supports, and Dr. T. Y. Chiang at Department of Biology, Cheng-Kung University for preparing phylogenic tree of PLA_2. The secretarial helps of Dr. Yuh-Ling Chen and Miss S. H. Rao are greatly appreciated.

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