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Variations of Phospholipases A_2 in the Geographic Venom Samples of Pitvipers

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ABSTRACT

The geographic variations of phospholipases A_2 (PLAs) in the venom of four medically important pit vipers were investigated. We have studied the PLAs by HPLC-purification, cDNA cloning and sequencing, mass characterization, and functional classification. We found that: 1) Anti-platelet acidic PLA isoforms in the venoms of *Calloselasma rhodostoma* from five southeastern Asian countries, and those of the *Crotalus v. viridis* from seven American States are differentially expressed depending on locality. The variations could be attributed to their distinct specificities towards the platelets of different prey, and to possible adaptation for playing other functional roles. In contrast, structures of the myonecrotic and the edema-inducing basic PLAs in both venoms were relatively conserved. 2) A special type of the acidic anti-platelet PLA is present in the venom of some *Protobothrops* species. Its expression level is diminished in the snake of the southern or the tropical ranges. 3) The venom of Bamboo tree vipers (*Trimeresurus stejnegeri*) in Taiwan and China showed extraordinary geographic variations in their acidic and basic PLAs. The high RNA-polymorphism of their venom proteins may have been derived from interbreeding between several ancestral pit viper species. In addition, migration, isolation of different populations and rapid evolution of the venom proteins to adapt for diversified diets may have resulted in further variations in this venom species.

Key Words: Pitviper venom; Phospholipase A_2; Geographic variations of venom; Molecular cloning.

I. INTRODUCTION

The multigene snake venom families have gone through gene-duplication and accelerated evolution to generate variants with adapted functions (Kordis et al., 2002). While ontological, seasonal, and geographic variations of the venom proteins are recognized, the details and underlying causes remain to be elucidated. In order to characterize the intra-species venom variations, not only carefully pooled, but also many individual venoms should be collected from various localities, and efficient protein analyses should be performed on many samples (Chippaux et al., 1991).

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The secreted phospholipase A_2 (PLA) is a well-characterized enzyme family found in almost all viperid venoms (Danse et al., 1997; Kini, 1997). It has been known that many pit viper venoms contain distinct subtypes or isoforms of PLAs (Tsai, 1997), and show geographic variations. For examples, the Lys49–PLA homologs were absent in the venom of Okinawa *T. flavoviridis* but present in the habu venoms on other neighboring islands (Chijiwa et al., 2000); contents of Mojave-toxin-like PLA in the venom of *Crotalus s. scutatus* and *Crotalus h. horridus* were varied with different localities (Glenn et al., 1983, 1994); the proportion of two acidic venom PLAs in *C. rubber* changed from North to South in Baja Peninsula (Mexico) (Straight et al., 1992), and variations related with a form of local adaptation was found in *B. asper* venom (Sasa and Barrantes, 1998).

It is important to know the geographic variations of the venom for the purpose of antivenin manufacture, snakebite treatment and conservation management. Besides genetic background of the species, microhabitat separation and adaptation to diet or ecology may gradually result in variations in snake venom toxins (Creer et al., 2002; Daltry et al., 1998). How and why are venom PLAs varied according to its locality are the questions to be addressed at the molecular level. We have used a comparative proteomic approach coupled with cloning and complete sequencing of the venom PLAs. The primers used in our PCR experiments were reliable for cloning most of the viper venom PLAs from the fresh venom glands (Tsai et al., 2001, 2003). Other alternative primers have also been designed based on distinct sequences at the N-terminal of the PLAs of interest.

II. RESULTS

A. PLA Variations in the Venom of *Calloselasma rhodostoma* and *Crotalus v. viridis*

During a typical reversed phase-HPLC purification of the venom PLAs under $pH < 3$, the basic PLAs were eluted earlier than the acidic PLAs, which often form dimers in buffer solution of $pH > 4.2$ (Tsai et al., 2001). The acidic anti-platelet PLAs in the venoms of Malayan pit viper (*Calloselasma rhodostoma*) from four southeastern Asian Nations (Table 1) are differentially expressed depending on locality (Tsai et al., 2001). These variations may be attributed to the adaptive evolution and distinct specificities of the PLAs towards platelets of different preys (Table 2).

Another example of the geographic variation was observed for the venom PLA of the prairie rattlesnake (*Crotalus v. viridis*) from seven American States (Tsai et al., 2003). Venoms of *C.rhodostoma* and *C.v. viridis*, contain only a single form of basic PLAs playing myonecrotic and/or edema-inducing role

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Table 1. Proportion of PLAs in different geographic pooled venom samples of *C. rhodostoma*.

Venom origin	Vietnam	Thailand	Malaysia	West Java
PLA	Relative abundance (%)			
W6	55	63	59	41
H1E6	8	14	≤ 5	0
S1E6a	17	6	9	5
S1E6b	20	17	27	24

(Tsai et al., 2001, 2003), but contain several acidic PLAs with E6 substitution (Tables 1 and 3). Cvv–E6a is more potent in the inhibition of human platelets than Cvv–E6e, while the reverse is true for the inhibition of rabbit platelets. Another acidic PLA, Cvv–E6f, may cause edema in the rat paw (Tsai et al., 2003). The functions of Cvv–E6 b, c, and d are not clear and a role for the digestion of prey is suspected. Notably, Cvv–E6e and Cvv–E6f are only expressed in the southern range (e.g. Texas, New Mexico, and Arizona), while Cvv–E6b and Cvv–E6c are only found in the northern range of the rattlesnake (e.g. South Dakota, Wyoming, and Colorado). The content of the myotoxic PLA with N6 substitution increased as the range of the rattlers moved toward Southeast in accord with their adaptation to the diets consisting more small mammals. Our results on venom PLA correlates well with those derived from phylogenetic analyses of mtDNA of the *C.viridis* complex (Anaya et al., 1992).

B. PLA Variations in the Venom of *Protobothrops mucrosquamatus*

The venoms of *P. mucrosquamatus* (formerly *Trimeresurus mucrosquamatus*) in Taiwan contain three or four PLAs depending on the sample site. Only the habu snakes from northern Taiwan contain an acidic

Table 2. The inhibition potency of three venom PLAs of *C. rhodostoma* on the aggregation of rabbit and the human platelets induced by ADP.

	Rabbit platelet	Human platelet
PLA variant	IC_{50} (nM)	
H1E6	70	880
S1E6a	142	428
S1E6b	110	520

Table 3. Masses and enzymatic activities of the acidic PLAs of *C. v. viridis* venom.

PLA	Mass	Enzyme activity (μmol/min/μg enzyme)	N-terminal sequences
E6a	13467	351 ± 16	S L V Q F E T L I M K I A G R S G L L W Y S A
E6b	13660	1129 ± 20	N · · · · · L · · · · V · K · · · · · S · · ·
E6c	13817	840 ± 6	N · · · · · L · · · · V · K · · · · · S · · ·
E6d	13782	680 ± 5	· · · · · · M · · · · V · K · · · · F S · · ·
E6e	13633	1306 ± 42	N · · · · · L · · · · V · K · · · · · S · · ·
E6f	13876	518 ± 25	· · · · · · M M · I · V · K · · · · F · · G ·
N6	14200	280 ± 8	N · L · · N K M · K M M T K K N A F P F · T S

Masses were determined by ESI-MS. The initial rates toward 3 mM dipalmitoyl phosphatidylcholine and 3mM sodium deoxycholate were measured twice in the presence of 10 mM Ca^{2+} at 37°C.

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anti-platelet PLA with Arg6 substitution (designated as TmPL-III) (Tsai et al., 2001). This PLA is absent in all the ten individual venom samples from southern Taiwan we analyzed. The mRNAs encoding a PLA very similar to TmPL-III were found in both venom glands of *C. rhodostoma* (Tsai et al., 2000) and *Protobothrops jerdonii* (Lu et al., 2002) and the protein sequences are shown in Figure 1. It appears that the expression of this PLA reduced remarkably as the range of *P. mucrosquamatus* moved toward South or became tropical, and this PLA is not expressed in all the geographic venom samples of *C. rhodostoma*, a tropical species (Tsai et al., 2001).

C. PLA Variations in the Venom of *Trimeresurus stejnegeri*

Previous analyses on the mtDNA and venom proteins revealed unusually high geographic diversity in the populations of bamboo-tree viper (Creer et al., 2001–2003). Two linage of Taiwanese *T. stejnegeri* (previously named *T. gramineus*) have been identified. The smaller linage was restricted to the north and east coasts, whereas the larger linage occupied all but the northern range of the species (Creer et al., 2001). Although five PLAs (i.e. K49a, A1, A2, A5 in Table 4a and Tgr-PLA-IV) have been reported in the pooled venom of Taiwanese *T. stejnegeri* (Fukagawa et al., 1992; Nakai et al., 1995; Oda et al., 1991), we further identified about 8 novel PLAs from about 20 geographic venom samples of this species and compiled in Table 4a.

Recent results of a survey on the masses of the PLAs from 104 venom samples of Taiwanese *T. stejnegeri* collected from 38 sites identified about 22 PLA isoforms (Creer et al., 2003). By matching the PLA-mass results with our molecular data (Table 4a), the PLA-profiles for the venom samples from different localities were summarized in Table 4b. Apparently, the geographic variations are rather complicated and individual differences were also observed. In general, K49a, Ts-3 and 4 are found in the northern coast population; Ts-R6, G6, A2 and A5 are found mainly in the *T. stejnegeri* venom from southwestern Taiwan; Ts-A1 and K49a are common in the eastern population; and Ts-A1 and K49a may have evolved into Ts-A6 and K49c, respectively, in *T. stejnegeri* on the two off-shore islands (Table 4b).

In addition, we have identified about 6 distinct venom PLAs from *T. stejnegeri* collected from three southeastern Chinese provinces (data not shown). Only two of them are identical to those from Taiwanese *T. stejnegeri* venom. Thus, there are great venom differences between the Taiwanese and the Chinese *T. stejnegeri*. A cladogram or phylogentic tree, based on selected amino acidic sequences in the acidic PLAs from Asiatic viper venoms, suggested that the venom genes of *T. stejnegeri* were

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                1         10        20        30        40        50        60
                |         |         |         |         |         |         |
1.   TmPL-IIINLWQFREMIKEATG-KEPLTTYLFYACYCGWGGRGEPKDATDRCCFVHDCCYGKLT---AC-----

2.CRV-R6a    ..........-.........-.........-.........-.........-.........-.....

3.CRV-R6b    ..........-.........-.........-.........S.........-.........-.....

4.P.jerdonii H.........-.........-.........-.........-.........-.........-.....

                70        80        90        100       110       120       130
                |         |         |         |         |         |         |
1.   SPKLDMYTYSQKNEDIVCGG-DDPCKKEICECDKAAAICFLNNLGTYNEEYNNYRK-SRCIEESPKC   100

2.   ..........-.........-.........-.........-.........-.........-.....R.    99

3.   ..........-.........-.........-.........-.........-.........-.....     99

4.   .....I.S..........-GTE.E.Q.........-.........D.....K.....S.-.....     92
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Figure 1. Alignment of the amino acid sequences of the acidic PLAs with Arg-6 substitution from *P. mucrosquamatus* (Tsai et al., 2001), *C. rhodostoma* (Tsai et al., 2000) and *P. jerdonii* (Lu et al., 2002). The residues identical to those in the first line are denoted with a dot, gaps (–) are introduced to be consistent with the common numbering system for the group II PLAs.

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Table 4.

a. Inventory of the PLA variants found in the venom samples of Taiwanese *T. stejnegeri*.

PLA variant	Mass	N-terminal sequences 1 ~ 23
Ts-R6	13689	HLLQL**R**KMIKKMTNKEPILSYGK
Ts-K49a	13892	SVIEL**G**KMIFQETGKNPATSYGL
Ts-K49b	13929	GVIEL**T**KMFVQEMGKNALTSYSL
Ts-K49c	13876	SVIEL**G**KMIFQETGKNPATSYGL
Ts-G6	13805	SLLEF**G**RMIKEETGKNPLSSYIS
Ts-A1	13734	HLMQF**E**TLIMKVAGRSGVWYYGS
Ts-A2	13779	NLLQF**E**NMIRNVAGRSGIWWYSD
Ts-A3	13750	SLIQF**E**TLIMKVAKKSGMFSYSA
Ts-A4	13925	SLIQF**E**TLIMKVAKKSGMFSYSA
Ts-A5	13711	NLMQF**E**TLIMKVAGRSGVWYYGS
Ts-A6	13939	HLMQF**E**NMIKKVTGRSGIWWYGS
Ts-A7	13905	HLLQF**E**TMIIKMTKQTGLFSYSF

b. Geographic variations of PLAs in the venom of Taiwanese *T. stejnegeri* (Creer et al., 2003).

Geographic region	Sample site	Composition of PLA variants[a]
Northern Taiwan	Taipei	K49a, A3, A4, (A1)
	Taoyaun	A3, K49a, G6
Western Taiwan	Miaoli	A1, K49a, G6
	Taichung	A1 or A3, (G6, or A2)
	Nantou	A5 or A3, (A6, R6, or G6)
Southwestern Taiwan	Chiai	R6, G6, A3
	Kaohsiung	R6 or A1, G6, (A5, or K49a)
	Pingtung	A5, K49a, (K49b, A2, or A6)
Eastern Taiwan	Ilan	K49a, A1
	Hualien	A1, K49a
	Taitung	K49a, A1or A3
Pacific Islands	Green Island	A6, K49c
	Lanyu	A6, K49c

Abbreviations of the PLAs: Ts, *T. stejnegeri* venom; R6, PLA with Arg6 substitution; A1 ~ A7, the acidic PLAs. Masses were determined by ESI-MS spectrometry, and the amino acid residues 6 are bolded.

[a]PLA isoforms listed are those found in the majority of the samples from that locality, and in the order of the Mass peak intensity (by MALDI-TOF) (Creer et al., 2003). The PLAs found in only one venom or less-representative venom are listed in parentheses.

possibly resulted from combination or merger of the genes from several ancestral species. Fast evolution and adaptation to diversified diets, migration and separated populations may also contribute to the venom variations.

III. CONCLUSION

Depending on the natural history and degree of ecological diversity of the snake, the venom compositions show more or fewer geographic variations. Venom variations are obviously not restricted to the venom PLA, e.g., variations in the venom proteases of rattlesnake have been shown (Minton and Weinstein, 1986), and venom serine proteases present in *T. stejnegeri* of Taiwan apparently are different from those of China (Chang and Huang, 1995; Zhang et al., 1998). However, venom PLAs are convenient and informative windows for comparison of the variations. In the cases of *P. mucrosquamatus* and *C.rhodostoma*, variations in the venom PLAs are manifested mainly in their different proportions, while an on-and-off switch for the expression of individual PLA is common in the cases of *C.v. viridis* and *T. stejnegeri*. Usually, the acidic PLAs of the pitviper venoms have evolved with greater variations than the basic PLAs. This is probably because the functional specificities toward the preys' platelets are diversified among the acidic PLAs (Tsai et al., 2003). In contrast, the basic myotoxic, edema-inducing PLAs possibly have wider specificity and demand less structural variations. However, contents of the basic isoforms often are increased as the pit vipers move to hotter-drier localities, probably as an adaptation to diets containing more small mammals.

Our results provide clues for the fast evolution and high diversity of venom proteins of pit vipers. PCR-assisted cDNA cloning and sequencing, as well as high-throughput proteomic tools have greatly facilitated the venom research. Ecological study has also helped to correlate the venom diversity with the prey-environment. But it remains difficult to explain the venom variations in terms of the diet-ecology of the snakes. More studies on the functional roles and specificities of venom components are essential for deciphering the meaning of the venom diversity. Hopefully, clarification of the functional subtypes of the toxins may also improve their usages for medical or pharmaceutical purposes.

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