Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1702 (2004) 111–119

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Comparative proteomics and subtyping of venom phospholipases A_2 and disintegrins of *Protobothrops* pit vipers

Inn-Ho Tsai*, Yi-Hsuan Chen, Ying-Ming Wang

Institute of Biological Chemistry, Academia Sinica, and Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan

Received 28 May 2004; received in revised form 23 July 2004; accepted 16 August 2004
Availble online 26 August 2004

Abstract

To explore the venom diversity and systematics of pit vipers under the genus *Protobothrops*, the venom phospholipases A_2 (PLA_2s) of *P. mangshanensis*, *P. elegans* and *P. tokarensis* were purified and characterized for the first time. The results were compared with the corresponding venom data of other co-generic species including *P. mucrosquamatus*, *P. flavoviridis* and *P. jerdonii*. Based on sequence features at the N-terminal regions, we identified five PLA_2 subtypes, i.e., the Asp49-PLA_2s with N6, E6 or R6 substitution and the Lys49-PLA_2. However, not all subtypes were expressed in each of the species. Venom N6-PLA_2s from *P. mangshanensis* and *P. tokarensis* venom were weakly neurotoxic toward chick biventer cervicis tissue preparations. The venoms of *P. tokarensis* and *P. flavoviridis* contained identical PLA_2 isoforms. In most *Protobothrop* disintegrins, sequences flanking the RGD-motif are conserved. Phylogenetic analyses based on amino acid sequences of both families of the acidic PLA_2s and the disintegrins clarify that these species could belong to a monophyletic group.

Keywords: Pit viper venom; Phospholipase A_2; Disintegrin; Anticoagulant; Neurotoxin; *Protobothrops*; *Trimeresurus*

1. Introduction

Phospholipase A_2 (PLA_2, EC 3.1.1.4) catalyzes the hydrolysis of the 2-acyl ester bonds of phosphoglycerides to produce lysophosphoglycerides and fatty acids. By gene duplication and fast evolution [1,2], PLA_2s of pit viper venoms have been diversified into several distinct structural subtypes [3,4] and thus serve different functional roles. They all consist of 122 amino acid residues and share the same structural scaffold. On the other hand, venom disintegrins are short (less than 9 kDa) polypeptides with RGD or KGD exposed-loop for specific binding to a variety of glycoprotein receptors on cell membrane. Both PLA_2s and disintegrins are common components of pit viper venoms.

Protobothrops is a genus of Asian pit vipers, comprised of about 12 terrestrial species. The distribution covers mainly China, Taiwan, Ryukyu (southwestern Japan), and they are responsible for significant portions of snakebites in these areas. *P. mucrosquamatus* inhabits northern India through southern China to Taiwan. *P. jerdonii* inhabits northern Indochina and southern China, and *P. mangshanensis* is an endemic species of Hunan province of China. Among the species of East Asian Islands, *P. flavoviridis* inhabits several islands of central Ryukyu, while *P. tokarensis* inhabits only the Tokaren islands of central Ryukyu, and *P. elegans* inhabits Yaeyama islands of southern Ryukyu [5].

A phylogenetic tree constructed from snake mitochondria DNA sequences suggested that *Protobothrops* is a monophyletic genus distinctive from the arboreal *Trimeresurus* (sensu stricto) and *Tropidolaemus*, and the terrestrial *Ovophis* [6–9]. These four genera were formerly grouped under *Trimeresurus* (sensu lateral). The evolution of pit vipers on

Abbreviations: HPLC, high performance liquid chromatography; dPPC, dipalmitoyl glycerophosphocholine; PLA_2, phospholipase A_2; Pel, *P. elegans*; Pfl, *P. flavoviridis*; Pma, *P. mangshanensis*; Pmu, *P. mucrosquamatus*; Pto, *P. tokarensis*; Ook, *Ovophis okinavensis*

* Corresponding author. Fax: +886 2 23635038.
E-mail address: bc201@gate.sinica.edu.tw (I.-H. Tsai).

1570-9639/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2004.08.006

East Asian Islands has been studied to address the question of whether the degree of genetic divergence reflects the history of isolation on the Ryukyu Archipelago; however, some controversy still remains [5]. Intrageneric venom variations of *Protobothrops* species also remain to be studied.

Among venom PLA_2s of various *Protobothrops* species, four from *P. mucrosquamatus* [10–12], four from *P. flavoviridis* [13,14] and at least one from *P. jerdonii* [15] have been fully sequenced. Amino acid sequences of venom disintegrins of several *Protobothrops* are also available [16,17]. In the present study, novel PLA_2s from several less studied *Protobothrops* were purified and characterized, their N-terminal sequences and masses were compared with the data of other co-generic species. Systematics of these species among the Old World and the New World pit vipers were examined based on the subtypes and molecular phylogeny of the venom PLA_2s and disintegrins.

2. Materials and methods

2.1. Venoms and other materials

Venoms of *P. mucrosquamatus*, *P. flavoviridis*, *P. tokarensis* and *P. elegans* were purchased from Latoxan Co. (Valence, France). Venom of *P. mangshanensis* is a gift from Prof. Shu, Yu-Yen (Kuangxi Medical University, China).

2.2. Purification and characterization of venom PLA_2s

Venom powder (5–15 mg) was dissolved in 0.1 or 0.2 ml of reagent-grade water. After repeated centrifugations at 12,000×*g* for 5 min each time, aliquots of 100 μl were injected into a pre-equilibrated gel-filtration column (Superdex 75, HR10/30) on a Pharmacia FPLC system (Amersham Biosciences). The column was eluted at 1.0 ml/min with 0.1 M ammonium acetate (pH 6.2) at room temperature of 23–26 °C. Fractions with PLA_2 activities were collected separately and freeze-dried. They were further purified by reversed-phase HPLC with a C8 column (4.5×250 mm, Vydac Co., USA). Purified PLA_2s were dried in a vacuum-centrifuge device (Labconco, USA). Their molecular masses were determined by electrospray ionization mass spectrometry on a mass spectrometer (API-100; Perkin Elmer, Foster City, USA). Protein sequences were determined by a gas-phase amino acid sequencer coupled with a phenylthiohydantoin amino acid analyzer (model 120A; Perkin Elmer) [18].

2.3. Enzymatic activities and other functional assays

Concentration of PLA_2 in solution was determined by reading the absorbance at 280 nm and assuming a molar absorption coefficient of 1.5 at 1.0 mg/ml of the protein. The hydrolytic activities of PLA_2s towards mixed micelles of L-dipalmitoyl phosphatidylcholine (dPPC, Avanti polar lipid, USA) and sodium deoxycholate or Triton X-100 (Sigma) were assayed at pH 7.4 and 37 °C on a pH-stat apparatus (Radiometer, Copenhagen) [3]. Neurotoxicity was assayed with chicken biventer cervicis neuromuscular tissue [19].

The effect of venom PLA_2s on blood coagulation was studied by activated partial thromboplastin time (APTT) with a Hemostasis Analyzer (model KC1, Sigma Diagnostics, USA) as described previously [20].

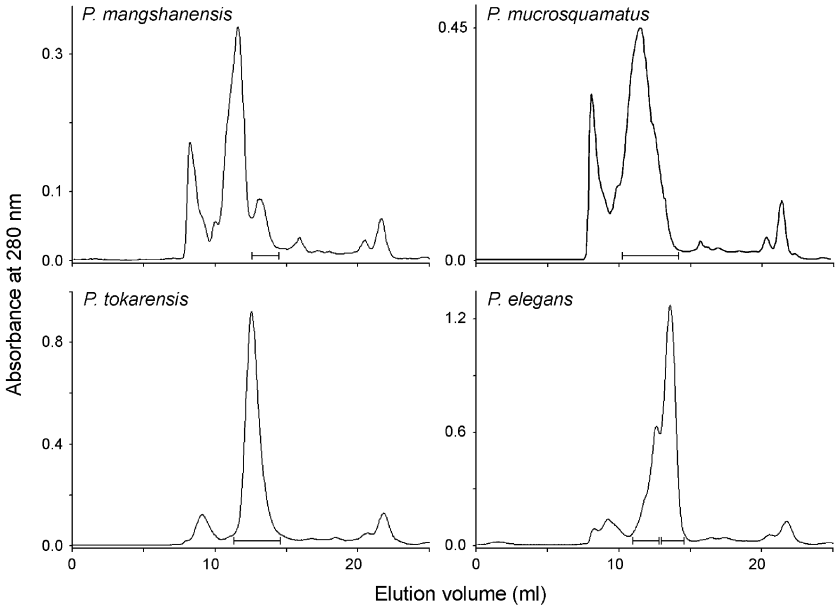

Fig. 1. Separation of PLA_2s from crude venom by gel filtration. Dissolved venom was eluted at a flow rate of 1.0 ml/min at room temperature (25 °C) on a FPLC system with a Superdex G75 (HR 10/30) column in equilibration with 0.1 M ammonium acetate (pH 6.2). Fractions containing PLA_2s (indicated by bars) were pooled for further purification.

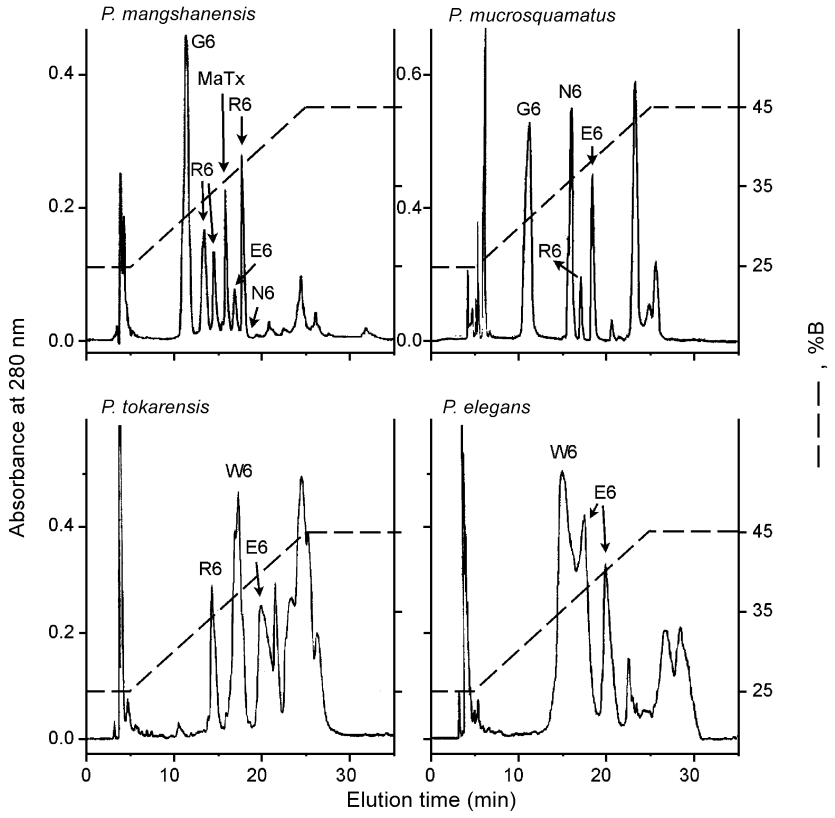

Fig. 2. Purification of PLA_2s by reversed-phase HPLC. Lyophilized fractions from Fig. 1 were redissolved and fractionated on a Vydac C_8-HPLC column with a gradient of B solvent (acetonitrile, dashed lines). Venom PLA_2s were purified and confirmed by ESI-MS and pH-stat enzyme assay. Annotations of the PLA_2s were the same as those in Tables 1 and 2, except MaTx denoted mangshantoxin.

2.4. Phylogenetic analysis

Sequences closely related to the major acidic PLA_2s from *P. mucrosquamatus* and *P. flavoviridis* were selected by BLASTp search [21]. Partial sequences of three novel acidic PLA_2s (Table 2) were also included in the data set for the phylogenetic analysis. The disintegrin sequences of related species were also collected [16,17,22]. Amino acid sequence alignment was made using PILEUP program. Cladograms were constructed based on these sequences by neighbor-joining algorithm using PHYLIP program [23], and the degree of confidence for the internal branch was determined by bootstrap methods [24].

Table 1
Molecular weights and partial sequences of the basic PLA_2 of *Protobothrops* venoms

PLA_2[a]	Venom species	Mol. wt.±1	N-terminal sequences[b]
K49-PLA_2			
*Pmu-K49	*P. mucrosquamatus*	13667	SLIELGKMIFQETG-KNPVKNYGLYGCNCG
Pma-K49-like	*P. mangshanensis*	13640	·V·····V·····-·····
Pel-K49-like	*P. elegans*	13701	·····W··V·····-·EA·····
Pto-K49-like	*P. tokarensis*	13752	··VQ·W·····-·EAA·····
*Tf-BP-I/II	*P. flavoviridis*	13753	··VQ·W·····-·EAA·····
N6D49-PLA_2			
*Trimucrotoxin	*P. mucrosquamatus*	13902	NLLQFNKMIKIMTK-KNAIPFYSSYGCYCG
Mangshantoxin	*P. mangshanensis*	13902	·····-·····
Pma-N6	*P. mangshanensis*	14055	·····-·····LF·····
Pto-N6	*P. tokarensis*	14033	·····-··GF···T·····
*Tf-PLA-N	*P. flavoviridis*	14033	·····-··GF···T·····

[a] Asterisk denotes the PLA_2 whose full amino acid sequences has been solved.
[b] One letter amino acid codes are used, residues identical to the first line are marked with a dot. Gap at residue 15 is introduced to follow the common numbering system for the enzymes [25].

3. Results

3.1. Purification, characterization and subtyping of PLA_2s

Proteins from the venoms of *P. mucrosquamatus*, *P. tokarensis*, *P. elegans* and *P. mangshanensis* were separated by gel-filtration column (Fig. 1). About 10% of the protein in the largest venom peak of the *P. mangshanensis* elution-profile contained acidic E6-PLA_2 (possibly homodimers), most of the PLA_2s were in the peak (corresponding to 12–14 kDa proteins) that follows. Fractions containing PLA_2s were further purified by HPLC, and they were eluted from the column in the following order: basic R6-PLA_2, W6/G6-PLA_2 (probably K49 PLA_2), N6-PLA_2, acidic R6-PLA_2 and finally the E6-PLA_2 (Fig. 2). After the N-terminal sequence was determined, each of the PLA_2 was annotated based on the abbreviated species name (e.g., Pma for *P. mangshanensis*) and its apparent structural subtype judged by their N-terminal sequences. Orthologous PLA_2s of these four venom species and those of *P. flavoviridis* [13] and *P. jerdonii* [15] were grouped together (Tables 1 and 2). *Protobothrops* PLA_2s could apparently be classified into five subtypes, i.e., K49, N6, acidic and basic R6, and E6-PLA_2s.

Each subtype could be easily identified based on the common structural features at residues 1–29, e.g., the substitutions L5, G6 or W6, Q11 and N28 were characteristic for K49-PLA_2s (or R49-PLA_2s [10]), while the substitutions N6, I11, Y28 and D49 were characteristic for N6-PLA_2s (Table 1). Acidic R6-PLA_2s usually contained E7 and E11 while basic R6-PLA_2s contained K7 and/or K11; and E6-PLA_2s were acidic enzymes with E6 and K11 substitutions (Table 2). Notably, although the residue at position 49 of some of the PLA_2s is not known, whether they belong to Asp49 or not is predicted from homology to others with full sequence solved (Tables 1 and 2). Members of the same subtypes apparently shared more than 76% sequence identity, while the identities across the subtypes are less than 60% [3].

Table 3
Enzymatic and anticoagulating activities of the *Protobothrops* D49-PLA_2s

PLA_2[a]	Specific activity (μmol/mg/min), toward dPPC micelles with		Anticoagulating activity
	Deoxycholate	Triton X-100	ED (μg/ml)[b]
Pma-R6-I (K7)	8±1	n.d.	0.38
Pma-R6-II (D7)	376±27	113±12	0.73
Pto-R6 (K7)	238±11	436±35	0.36
Mangshantoxin (S24)	521±15	155±4	0.70
Pto-N6 (S24)	698±38	417±24	0.64
Pma-N6 (F24)	71±3	33±1	5.1
Pma-R6-III (E7)	659±25	368±19	5.2
Pmu-PL-III (R6E7)	690±26	228±17	2.4
Pmu-PLA-I (E6)	739±32	130±10	>40
Pma-E6	563±12	63±2	6.9
Pel-E6a	761±24	82±2	21
Pel-E6b	827±10	153±10	>40
Pto-E6	1428±12	941±31	37

[a] Special amino acid substitution is shown in parentheses.
[b] Effective dose of the PLA_2 to prolong coagulation time from 30 s (of the control) to 45 s, as interpolated from the dose–effect curve.

Table 2
Molecular data of PLA_2s with E6 or R6 substitution from *Protobothrops* venoms

PLA_2[a]	Venom species	Mol. wt.±1	N-terminal sequences[b]
Acidic R6			
*Pmu-PLA-III	*P. mucrosquamatus*	13973	NLWQFREMIKEATG-KEPLTTYLFYACYCG
Pma-R6-III	*P. mangshanensis*	13922	·····-·····
*Jerdoxin	*P. jerdonii*	13855	H·····-·····
Basic R6			
Pma-R6-I	*P. mangshanensis*	13873	NLLQFRKMIKKMTG-KEPILSYATYGCNCG
Pma-R6-II	*P. mangshanensis*	13845	·····D·····-····V···F···Y··
Pto-R6	*P. tokarensis*	14020	H·····-····V···F···Y··
*Tf-PLA-B	*P. flavoviridis*	14039	H·····-····V···F···Y··
*Tf-PLA-B′ /X′ /Y	*P. flavoviridis*	13949	H·····-····V···F···Y··
E6			
*Pmu-PL-I	*P. mucrosquamatus*	13601	NLWQFENMIMKVAK-KSGILSYSAYGCYCG
Pma-E6	*P. mangshanensis*	13561	G·····-·····
Pel-E6a	*P. elegans*	13588	G·····-·····
Pel-E6b	*P. elegans*	13571	G·····-·····
Pto-E6	*P. tokarensis*	13764	G·····I··V··-·····
*Tfl-PLA1a	*P. flavoviridis*	13764	G·····I··V··-·····
*Tfl-PLA1b	*P. flavoviridis*	13925	H·M·····K··TG-R···WW·GS·····
*Ts-A6	*T. stejnegeri*	13939	H·M·····K··TG-R···WW·GS·····
*Ook-E6	*O. okinavensis*	13786	H·M···TL··I··G-R··VWW·GS·····

[a] Asterisk denotes the PLA_2 whose full amino acid sequences has been solved.
[b] One letter amino acid codes are used, residues identical to the first line are marked with a dot. Gap at residue 15 is introduced to follow the common numbering system for the enzymes [25].

Table 4
Neurotoxicity of N6-PLA_2 toward the chick biventer cervicis tissue

N6-PLA_2 toxin	Dose, μg/ml	Time for 90% inhibition of the twitch, min	
Trimucrotoxin	2.0	38±2	(n=4)[a]
	1.0	76±13	(n=5)
	0.3	106±2	(n=3)
Mangshantoxin	1.0	77±3	(n=2)
Pma-N6	2.0	>240	(n=2)
Pto-N6	3.0	205±5	(n=2)
Tf-PLA-N[b]	3.0	230	(n=3)

[a] Numbers of experiments are shown in parentheses.
[b] Data taken from Ref. [13].

3.2. Assay and functional study

The in vitro enzymatic activity of purified PLA_2 was determined at 37 °C by pH-stat using micellar lecithin substrates (Table 3). Enzymatic activities of all the K49-PLA_2s (not shown) and some basic R6-PLA_2s (e.g., Pma-R6-II) were hardly detectable. The hydrolytic activities of the PLA_2s for dPPC in the negatively charged (deoxycholate) micelles were between two- and threefold higher than those in the neutral (Triton X-100) micelles, except for the basic R6-PLA_2s (e.g., Pto-R6) which had higher specificities toward Triton X-100 micelles.

In agreement with previous data [20], the K49-PLA_2s are relatively poor anticoagulants (not shown). Anticoagulating activities of the D49-PLA_2s are shown in the last column of Table 3. We found that basic R6-PLA_2s and most N6-PLA_2s, e.g., Pma-R6-II, trimucrotoxin, mangshantoxin and Pto-N6, are strong anticoagulants, but acidic R6-PLA_2s and E6-PLA_2s are not.

All the N6-PLA_2s are basic enzymes [25]. Among them, trimucrotoxin has been shown to be neurotoxic [10] and myotoxic [26], and Tf-PLA-N is weakly neurotoxic [13]. We further discovered that the neurotoxicity of Pto-N6 toward chick tissue was as weak as that of Tf-PLA-N and Pma-N6 was hardly neurotoxic (Table 4). In addition, the toxicity of Pto-N6 was not significantly increased by the addition of crotoxin A (the acidic subunit of crotoxin [25]) (Table 4).

3.3. Molecular phylogeny of venom proteins

Based on the protein sequences determined or deduced from gene sequences, phylogenetic trees were constructed for medium-sized disintegrins [22] (Fig. 3) and acidic E6-PLA_2s (Fig. 4). Kistrin, the disintegrin of *Calloslasma rhodostoma* venom was assigned as an out-group for the disintegrin tree. The trees revealed the relationship between the venom proteins of *Protobothrops* and those of other related species with high bootstrap values or confidence.

4. Discussion

Like *Bothrops* venoms, most of the *Protobothrops* venoms contain K49-PLA_2s. The gel-filtration patterns of *Protobothrops* venoms are usually similar to one another (Fig. 1) but distinct from those of other genera [20,25]. We have previously suggested that four venom PLA_2s subtypes (E6, N6, R6, and K49) have been diverged and evolved in parallel in the present-days pit vipers [3,11]. Recently, another report including a tree/cladogram of full sequences of the Asian crotalid venom PLA_2s also revealed the presence of the same four subtypes [13,27]. The acidic R6-PLA_2s are further separated from the basic R6-PLA_2s in our investigation (Table 2). Each PLA_2 subtype possibly plays special functional roles.

The acidic R6-PLA_2s have been found so far only in *Protobothrops* venoms, while the E6- and K49-PLA_2s are

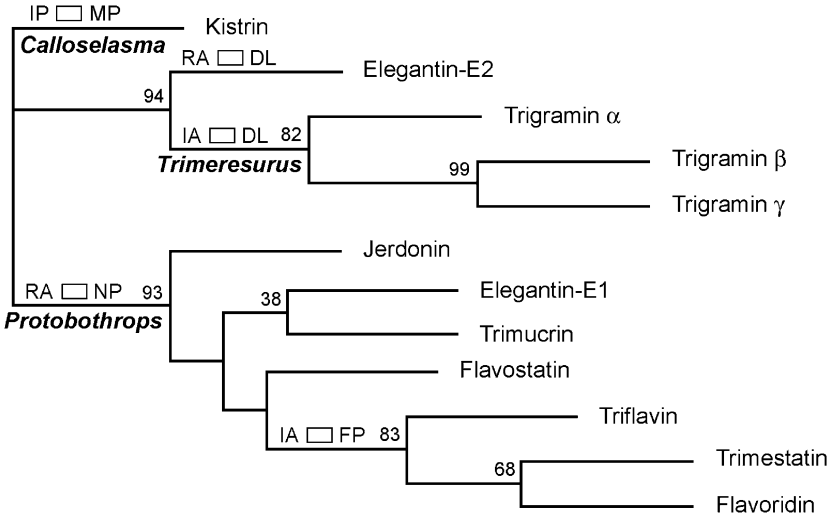

Fig. 3. Phylogenetic tree showing structural relationships of disintegrins from *Protobothrops* venoms. The sequence data set includes: trimucrin (X77089, GenBank accession number), jerdonin [16], elegantin E1 and E2 [38], trimestatin [41], and other medium-sized disintegrins of related species [22]. Kistrin is assigned as an out-group, and values on branching points are calculated bootstrap values. Specific sequence flanking the RGD motif (represented by a rectangle) is also shown.

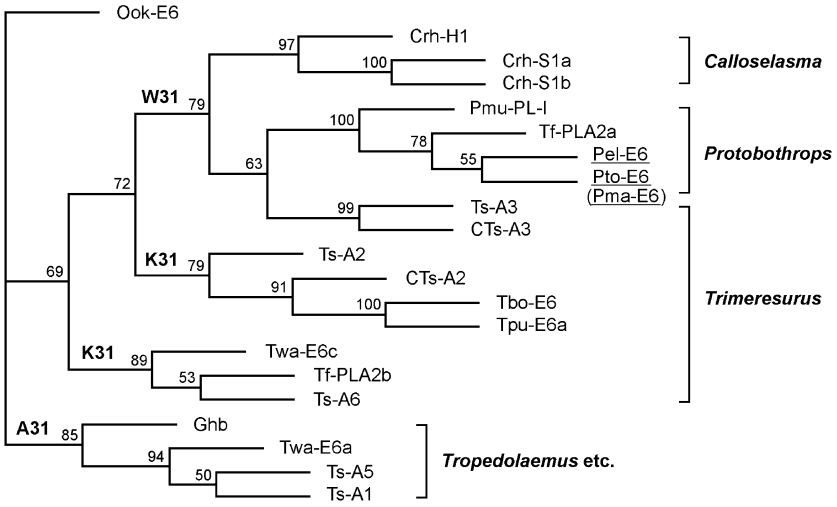

Fig. 4. Phylogenetic tree of venom E6-PLA_2s from *Protobothrops* and related species. Full amino acid sequences of the acidic PLA_2s form *P. mucrosquamatus* (X77088, GenBank accession number) and *P. flavoviridis* (D10724, D10725 GenBank accession numbers) were included in addition to those previously used [20]. Partial sequences (N-terminal residues 1–30) were underlined and the sequence of Pma-E6 is identical to that of Pel-E6. Ook-E6a (P00625, GenBank accession number) from *O. okinavesis* venom is the out-group. Values are calculated bootstrap values, and the amino acid residue-31 characteristic for each E6-PLA_2 cluster is also shown on the node.

rather common pit viper venom components [28] and basic R6- and N6-PLA_2 are also present in other genera of pit vipers [20,25]. Judging from the phylogeography of pit vipers and the content of their venom, it appears that the major PLA_2s subtypes diverged possibly before the branching of most if not all the *Protobothrops* species. Then, minor diversity of the venom also occurred during separation, speciation, and adaptation of the species.

The K49-PLA_2s of pit viper venom are basic proteins with extremely low lipolytic activity but strong myotoxic and edema-inducing activities. Their highly basic C-terminal regions have been known to be related to the toxicity [29,30]. The N6-PLA_2s exhibit neuro-myotoxicities and rather high lipolytic activities, and their structure–activity relationships have been studied extensively [10,25]. They are lethal for mice at 1.2–2.0 mg/g [10,13]. Tf-PLA-N also exhibited strong cytotoxicity to cancer cells such as HL-60 [27]. Moreover, most of the N6-PLA_2s and basic R6-PLA_2s of *Protobothrops* venoms caused prolonged blood coagulation time (Table 3).

Many of the acidic venom PLA_2s are conceived as platelet aggregation inhibitors [20]. The Glu6 and some aromatic amino acids at regions 20, 21, and 113–119 have been shown to be important for the anti-platelet activity of the E6-PLA_2s [31]. In addition, the venom acidic R6-PLA_2s of Tmu-PLA-III [11] and *P. jerdonii* venom [32] were shown to inhibit platelet aggregation. It is interesting that all the acidic R6-PLA_2s contain acidic residues at positions 7 and 11, while both positions are usually basic in the basic R6-PLA_2s (Table 1). Residues responsible for inhibitory activities of the acidic R6-PLA_2s are not clear but Lys7, 10, 11 and 16 at the N-terminal helix are probably involved in the anticoagulating effect of basic R6-PLA_2s since previous mutations of basic residues at all positions 7, 10 and 16 to Glu7 reduced the anticoagulating activities of human group II PLA_2 [33]; these surface exposed sites has been implicated in the anticoagulating action of another viperid basic R6-PLA_2 [34].

Notably, the data presented in Table 3 revealed that basic R6-PLA_2s (e.g., Pma-R6-I and Pto-R6) had much lower enzymatic activities than the acidic R6-PLA_2s (e.g., Pma-R6-III). Previous report also showed that the basic R6-PLA_2s of *P. flavoviridis* had weak lipolytic activities toward egg-yolk emulsion [14]. In corroboration, the anticoagulating mechanism of some basic group II PLA_2s is not related with their enzymatic activities [33].

Although two extra PLA_2 pseudogenes have been cloned [27], *Ovophis okinavensis* venom contains only one acidic PLA_2 (Ook-E6) [35], which is structurally rather different from those of *Protobothrops* venoms (Table 2). Moreover, we found that both *Ovophis monticola* and *Ovophis gracilis* venoms express only E6-PLA_2 (Tsai, et al., unpublished). Thus, our results demonstrate that co-generic viperid species usually express the same conserved set of venom PLA_2 subtypes. Previous species trees support the monophyletic nature of *Protobothrops*, which is at relatively root position among the crotalid snakes [6,9]. Unlike *Protobothrops*, other genera of pit vipers usually contain less or only an incomplete set of the venom PLA_2s subtypes [3,20]. Thus, it seems likely that certain venom PLA_2 subtypes could be lost before or during evolution of the viperid genus.

Notably, *P. mangshanensis* expresses a rather complete set of venom PLA_2 subtypes although each is not in large quantity, and it is the only *Protobothrops* venom species found to contain both the acidic and the basic R6-PLA_2s and the N6-PLA_2s of either S24 or F24 substitution (Tables 1 and 2). Previously, we reported the cloning of both S24 and F24 N6-PLA_2s from the venom glands of a *Sistrurus*

species while other rattlers' venoms express only one of them or none [25]. The close evolutionary relationships between *Protobothrops* and the New World rattlesnakes are thus further supported.

In spite of the genus-specific expression of venom subtypes, venom contents may be complicated by geographic variations and differential expression as a result of adaptation to their feeding habits or ecology [20,36]. For examples, *P. jerdonii* venom appears to lack E6- and N6-PLA_2s [31], some populations of *P. flavoviridis* do not express N6-PLA_2s [13], and some Taiwanese populations of *P. mucrosquamatus* do not express acidic R6-PLA_2s [11], which are expressed in the venoms of continental *Protobothrops* species but absent from those of the three Ryukyu species (Table 2). Inter-island sequence diversities of the basic R6-PLA_2s have been reported for *P. flavoviridis* venoms [14], Tf-PLA-N also revealed some geographic variations [13,27]. An extraordinary diversity of the disintegrins of *P. flavoviridis* (Fig. 3) also suggests its rich inter-island biodiversities.

Judging from the N-terminal sequences, the venom PLA_2s of three continental *Protobothrops* species (*P. mucrosquamatus*, *P. mangshanensis* and *P. jerdonii*) are close to each other than to those from the three Ryukyu species (*P. flavoviridis, P. tokarensis* and *P. elegans*) (Tables 1 and 2). However, the E6-PLA_2s sequences (Table 2) of southern Ryukyu *P. elegans* are rather similar to those of *P. mucrosquamatus* and *P. mangshanensis*. It is conceived that central Ryukyu was insulated in the late Pliocene (1.8–2 my) [5]. Its fauna probably diverged from the ancestral or allied species from China and Taiwan at relatively early stages of evolution. In contrast, the identical PLA_2s in *P. flavoviridis* and *P. tokarensis* venoms suggests that the separation of their inhabiting islands and subsequent speciation are relatively recent events [5].

Because of the small size and stability, disintegrins are as good as venom PLA_2s for studying the biosystematics of venom species. Cladogram for the medium-size disintegrins commonly found in *Protobothrops* venom has been constructed based on their amino acid sequences (Fig. 3). The tree shows that southern Ryukyu *P. elegans* is similar to *P. mucrosquamatus*, while the central Ryukyu *P. flavoviridis* is not. Previous species trees based on mtDNA sequences [5] or snake morphologies [37] also showed the sisterhood of *P. elegans* with *P. mucrosquamatus*, and "*flavoviridis* and *tokarensis* clade" with *P. jerdonii*. Three variants of trigramin (the disintegrins from the bamboo tree viper *T. stejnegeri*) were also included in the phylogenetic tree (Fig. 3), and trigraminβ is identical to albolabrin of *T. albolabris* [22]. *Protobothrops* disintegrins are separated from *Trimeresurus* disintegrins in this cladogram.

Moreover, amino acid residues flanking the RGD motif are rather conserved among the *Protobothrops* disintegrins (Fig. 3). Most of them conserved the sequences RA**RGD**NP, which is the binding site for the integrins $\alpha IIb\beta_3$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ receptors [38,39]. This is different from those of the arboreal *Trimeresurus* containing IA**RGD**DL. However, *P. elegans* venom contains additional disintegrin variant with RA**RGD**DL while *P. flavoviridis* contains additional variant with the sequences IA**RGD**FP. Previous finding showed that large hydrophobic side chains at the position X of the RGDX motif are favorable for high-affinity interactions with human platelet $\alpha IIb\beta_3$ receptor [38]. The position right after X was also found to be important for the disintegrin binding and specificity [40,41]. Notably, the sequences of the RGD-imbedding loop in *Protobothrops* disintegrins (Fig. 3) and those in the rattlesnake disintegrins (e.g., molossin, viridian, basilicin and cereberin [38]) are highly similar.

In the cladogram for acidic E6-PLA_2 (Fig. 4), Tf-PL-Ib (a non-expressing E6-PLA_2 from *P. flavoviridis* venom gland) is looped out. The topology of the venom tree agrees, in general, with snake taxonomy except that *T. stejnegeri* has exceptionally high diversity of the E6-PLA_2 variants [20]. Interestingly, each of the E6-PLA_2 cluster shares a distinct residue 31 (W31, K31 or A31, respectively) (Fig. 4). This residue at the entrance of substrate of the enzyme is one of the important interface recognition sites regulating its catalytic specificity [42].

In conclusion, by comparing HPLC profiles, and N-terminal sequences, we found that the venom PLA_2s of *Protobothrops* are genus-specific, and evolved with five paralogous subtypes (Tables 1 and 2). Both venom trees of the acidic PLA_2s and the disintegrins support the monophyletic nature of *Protobothrops* and its distinctness from other genera including *Trimeresurus* (sensu stricto) and *Ovophis*. We found that *P. mangshanensis* retains many venom PLA_2s typical of *Protobothrops*; whether it belongs to a separated genus is questionable [5]. The venom of *P. tokarensis* is almost identical to those of *P. flavoviridis*. The fact that *P. flavoviridis* differs from *P. mucrosquamatus* and *P. jerdonii* possibly resulted from either its especially fast evolution [27] or the hybridization of *P. flavoviridis* with other species in ancient time as revealed by its extra E6-PLA_2 and disintegrin variants in the venom (Figs. 3 and 4).

Acknowledgments

This study was supported by research grants from the Administration of Education (grant 89-B-FA01-1-4) and National Science Council (grant NSC91-2311-B001-114) of Taiwan, R. O. C. We thank Prof. Yu-Yan Su for the gift of *P. mangshanensis* venom.

References

[1] F. Gubensek, D. Kordis, Venom phospholipases A_2 genes and their molecular evolution, in: R.M. Kini (Ed.), Venom Phospholipase A_2 Enzyme: Structure, Function and Mechanism, J. Wiley and Sons, London, 1997, pp. 73–95.

[2] K. Nakashima, T. Ogawa, N. Oda, M. Hattori, Y. Sakaki, H. Kihara, M. Ohno, Accelerated evolution of *Trimeresurus flavoviridis* venom gland phospholipase A2 isozymes, Proc. Natl. Acad. Sci. U. S. A. 90 (1995) 5964–5968.
[3] I.H. Tsai, Phospholipases A_2 from Asian snake venom, J. Toxicol., Toxin Rev. 16 (1997) 79–113.
[4] B.R. Francis, J. Meng, I.I. Kaiser, Classification of snake venom group II phospholipases A_2 according to amino acid sequences, in: G.S. Bailey (Ed.), Enzymes from Snake Venom, Alaken, Colorado, USA, 1998, pp. 503–544.
[5] M.C. Tu, H.Y. Wang, M.P. Tsai, M. Toda, W.J. Lee, F.J. Zhang, H. Ota, Phylogeny, taxonomy, and biogeography of the oriental pitvipers of the genus *Trimeresurus* (Reptilia: Viperidae: Crotalinae): a molecular perspective, Zool. Sci. 17 (2000) 1147–1157.
[6] C.L. Parkinson, Molecular systematics and biogeographic history of pitviper as determined by mitochondrial and ribosomal DNA sequences, Copeia 1999 (1999) 576–586.
[7] S. Creer, A. Malhotra, R.S. Thorpe, Assessing the phylogenetic utility of four mitochondrial genes and a nuclear intron in the Asian pit viper genus, *Trimeresurus*: separate, simultaneous and conditional data combination analyses, Mol. Biol. Evol. 20 (2003) 1240–1251.
[8] A. Malhotra, R.S. Thorpe, A phylogeny of the *Trimeresurus* group of pit vipers: new evidence from a mitochondrial gene tree, Mol. Phylogenet. Evol. 16 (2000) 199–211.
[9] F. Klaus, D.G. Mink, W.M. Brown, Crotalin intergeneric relationships based on mitochondrial DNA sequence data, Copeia 1996 (1996) 763–773.
[10] I.H. Tsai, P.J. Lu, Y.M. Wang, C.L. Ho, L.L. Liaw, Molecular cloning and characterization of a neurotoxic phospholipase A_2 from the venom of Taiwan habu (*T. mucrosquamatus*), Biochem. J. 311 (1995) 895–900.
[11] I.H. Tsai, Y.H. Chen, Y.M. Wang, M.Y. Liau, P.J. Lu, Differential expression and geographic variation of the venom phospholipases A_2 of *Calloselasma rhodostoma* and *Trimeresurus mucrosquamatus*, Arch. Biochem. Biophys. 387 (2001) 257–264.
[12] C.S. Liu, J.M. Chen, C.H. Chang, S.W. Chen, C.M. Teng, I.H. Tsai, The amino acid sequence and properties of an edema-inducing Lys-49 phospholipase A_2 homolog from the venom of *Trimeresurus mucrosquamatus*, Biochim. Biophys. Acta 1077 (1991) 362–370.
[13] T. Chijiwa, S. Hamai, S. Tsubouchi, T. Ogawa, M. Deshimaru, N. Oda-Ueda, S. Hattori, H. Kihara, S. Tsunasawa, M. Ohno, Interisland mutation of a novel phospholipase A_2 from *Trimeresurus flavoviridis* venom and evolution of Crotalinae group II phospholipases A_2, J. Mol. Evol. 57 (2003) 546–554.
[14] T. Chijiwa, Y. Yamaguchi, T. Ogawa, M. Deshimaru, I. Nobuhisa, K. Nakashima, N. Oda-Ueda, Y. Fukumaki, S. Hattori, M. Ohno, Interisland evolution of *Trimeresurus flavoviridis* venom phospholipase A_2 isozymes, J. Mol. Evol. 56 (2003) 286–293.
[15] Q.M. Lu, Y. Jin, J.F. Wei, W.Y. Wang, Y.L. Xiong, Biochemical and biological properties of *Trimeresurus jerdonii* venom and characterization of a platelet aggregation-inhibiting acidic phospholipase A_2, J. Nat. Toxins 11 (2002) 25–33.
[16] X.D. Zhou, Y. Jin, R.Q. Chen, Q.M. Lu, J.B. Wu, W.Y. Wang, Y.L. Xiong, Purification, cloning and biological characterization of a novel disintegrin from *Trimeresurus jerdonii* venom, Toxicon 43 (2004) 69–75.
[17] I.H. Tsai, Y.M. Wang, Y.H. Lee, Characterization of a cDNA encoding the precursor of platelet aggregation inhibition and metalloproteinase from *Trimeresurus mucrosquamatus* venom, Biochim. Biophys. Acta 1200 (1994) 337–340.
[18] M.W. Hunkapiller, L.E. Hood, Analysis of phenylthiohydantoins by ultrasensitive gradient high-performance liquid chromatography, Methods Enzymol. 91 (1983) 486–493.
[19] B.L. Ginsborg, J. Warriner, The isolated chick biventer cervicis nerve-muscle preparation, Br. J. Pharmacol. 15 (1960) 410–441.
[20] I.H. Tsai, Y.M. Wang, Y.H. Chen, T.S. Tsai, M.C. Tu, Venom phospholipases A_2 of bamboo viper (*Trimeresurus stejnegeri*): molecular characterization, geographic variations and evidence of multiple ancestries, Biochem. J. 377 (2004) 215–223.
[21] S. McGinnis, T.L. Madden, BLAST: at the core of a powerful and diverse set of sequence analysis tools, Nucleic Acids Res. 32 (2004) W20–W25.
[22] J.J. Calvete, M.P. Moreno-Murciano, R.D.G. Theakston, D.G. Kisiel, C. Marcinkiewicz, Snake venom disintegrins: novel dimeric disintegrins and structural diversification by disulfide bond engineering, Biochem. J. 372 (2003) 725–734.
[23] J. Felsenstein (1992) PHYLIP: the PHYLogeny Inference Package, version 3.573. Computer program distributed by the U. of Washington, Dept. of Genetics, Seattle, U.S.A.
[24] J. Felsenstein, Confidence limits on phylogenies: an approach using the bootstrap, Evolution 39 (1985) 783–791.
[25] Y.H. Chen, Y.M. Wang, M.J. Hseu, I.H. Tsai, Molecular evolution and structure–activity relationships of crotoxin-B like asparagin 6 phospholipases A_2 in pit viper venoms, Biochem. J. 381 (2004) 25–34.
[26] L.N. Chen, C.S. Liu, C.C. Chang, Isolation and characterization of a toxic phospholipase A2 from the venom of the Taiwan habu (*Trimeresurus mucrosquamatus*), Biotechnol. Appl. Biochem. 19 (1994) 61–73.
[27] M. Ohno, T. Chijiwa, N. Oda-Ueda, T. Ogawa, S. Hattori, Molecular evolution of myotoxic phospholipases A_2 from snake venom, Toxicon 42 (2003) 841–854.
[28] M. Wang, Y.F. Liew, K.Y. Chang, I.H. Tsai, Purification and characterization of the venom phospholipases A_2 from Asian monotypic Crotalinae snakes, J. Nat. Toxins 8 (1999) 331–340.
[29] L. Chioato, A.H.C. de Oiveira, R. Ruller, J.M. Sa', R.J. Ward, Distinct sites for myotoxic and membrane-damaging activities in the C-terminal region of a Lys49-phospholipase A_2, Biochem. J. 366 (2002) 971–976.
[30] B. Lomonte, E. Moreno, A. Tarkowski, L.A. Hanson, M. Maccarana, Neutralizing interaction between heparins and myotoxin II, a lysine 49 phospholipase A2 from *Bothrops asper* snake venom. Identification of a heparin-binding and cytolytic toxin region by the use of synthetic peptides and molecular modeling, J. Biol. Chem. 269 (1994) 29867–29873.
[31] X.L. Liu, X.F. Wu, Y.C. Chou, Identification of key residues responsible for enzymatic and platelet-aggregation-inhibiting activities of acidic phospholipase A_2s from *Agkistrodon halys* Pallas, J. Nat. Toxins 10 (2001) 43–55.
[32] Q.M. Lu, Y. Jin, J.F. Wei, D.S. Li, S.W. Zhu, W.Y. Wang, Y.L. Xiong, Characterization and cloning of a novel phospholipase A_2 from the venom of *Trimeresurus jerdonii* snake, Toxicon 40 (2002) 1313–1319.
[33] C.M. Mounier, P. Luchetta, C. Lecut, R.S Koduri, G. Faure, G. Lambeau, E. Valentin, A. Singer, F. Ghomashchi, S. Béguin, M.H. Gelb, C. Bon, Basic residues of human group II phospholipase A_2 are important for binding to factor Xa and prothrombinase inhibition comparison with other mammalian secreted phospholipases A_2, Eur. J. Biochem. 267 (2000) 4960–4969.
[34] K.H. Zhao, Z.J. Lin, S.Y. Song, Y.C. Zhou, Structure of a basic phospholipase A_2 from *Agkistrodon halys* Pallas at 2.13 Å resolution, Acta Crystallogr., D 54 (1998) 510–521.
[35] F.J. Joubert, T. Haylett, Snake venoms. Purification, some properties and amino acid sequence of a phospholipase A2 (DE-I) from *Trimeresurus okinavensis* (Hime-habu) venom, Hoppe Seyler Z. Physiol. Chem. 362 (1981) 997–1006.
[36] J.C. Daltry, W. Wuster, R.S. Thorpe, The role of ecology in determining venom variation in Malayan pitviper, *C. rhodostoma*, in: R.S. Thorpe, W. Wuster, A. Malhotra (Eds.), Venomous Snakes, Clarendon Press, Oxford, U.K, 1997, pp. 155–172.
[37] B.H. Brattstrom, Evolution of the pit vipers, Trans. S. Diego Soc. Nat. Hist. 13 (1964) 185–268.
[38] A. Scaloni, E.D. Matino, N. Miraglia, A. Pelagalli, R.D. Morte, N. Staiano, P. Pucci, Amino acid sequence and molecular modelling of

glycoprotein IIb–IIIa and fibronectin receptor iso-antagonists from *Trimeresurus elegans* venom, Biochem. J. 319 (1996) 775–782.

[39] L.W. Lee, H.C. Peng, W.C. Ko, W.C. Hung, C.H. Su, C.H. Lin, T.F. Huang, M.H. Yen, J.R. Sheu, Triflavin potentiates the antiplatelet activity of platelet activating factor receptor antagonist on activated neutrophil-induced platelet aggregation, Eur. J. Pharmacol. 364 (1999) 239–246.

[40] C.P. Chang, J.C. Chang, H.H. Chang, W.J. Tsai, S.J. Lo, Positional importance of Pro53 adjacent to the Arg^{49}–Gly^{50}–Asp^{51} sequence of rhodostomin in binding to integrin αIIbβ3, Biochem. J. 357 (2001) 57–64.

[41] Y. Fujii, D. Okuda, Z. Fujimoto, K. Horii, T. Morita, H. Mizuno, Crystal Structure of trimestatin, a disintegrin containing a cell adhesion recognition motif RGD, J. Mol. Biol. 332 (2003) 1115–1122.

[42] O.P. Kuipers, J. Kerver, J. van Meersbergen, R. Vis, R. Dijkman, H.M. Verheij, G.H. de Haas, Influence of size and polarity of residue 31 in porcine pancreatic phospholipase A_2 on catalytic properties, Protein Eng. 3 (1990) 599–603.