Archives of Biochemistry and Biophysics
Vol. 387, No. 2, March 15, pp. 257–264, 2001
doi:10.1006/abbi.2000.2229, available online at http://www.idealibrary.com on IDEAL®

# Differential Expression and Geographic Variation of the Venom Phospholipases $A_2$ of *Calloselasma rhodostoma* and *Trimeresurus mucrosquamatus*[1]

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Received July 11, 2000, and in revised form November 17, 2000; published online February 9, 2001

**To investigate the geographic variations in venoms of two medically important pitvipers, we have purified and characterized the phospholipases $A_2$ ($PLA_2$s) from the pooled venoms of *Calloselasma rhodostoma* from Malaysia, Thailand, Indonesia, and Vietnam, as well as the individual venom of *Trimeresurus mucrosquamatus* collected from both North and South Taiwan. Enzymatic and pharmacological activities of the purified $PLA_2$s were also investigated. The complete amino acid sequences of the purified $PLA_2$s were determined by sequencing the corresponding cDNAs from the venom gland and shown to be consistent with their molecular weight data and the N-terminal sequences. All the geographic venom samples of *C. rhodostoma* contain a major noncatalytic basic $PLA_2$-homolog and two or three acidic $PLA_2$s in different proportions. These acidic $PLA_2$s contain Glu6-substitutions and show distinct inhibiting specificities toward the platelets from human and rabbit. We also found that the *T. mucrosquamatus* venoms from North Taiwan but not those from South Taiwan contain an Arg6-$PLA_2$ designated as TmPL-III. Its amino acid sequence is reported for the first time. This enzyme is structurally almost identical to the low- or nonexpressed Arg6-$PLA_2$ from *C. rhodostoma* venom gland, and thus appears to be a regressing venom component in both of the Asian pitvipers.** 

***Key Words: Trimeresurus mucrosquamatus; Calloselasma rhodostoma;* snake venom; phospholipase $A_2$; cloning; complete sequence; platelet aggregation.**

*Calloselasma rhodostome* (Malayan pitviper) is widely distributed in Southeast Asia and is the most common cause of snakebites in Malaysia and Thailand (1, 2), while *Trimeresurus mucrosquamatus* is distributed in Taiwan, China, Vietnam, Burma, Northern Bangladesh, and Assam (3). Both species are ground dwelling and primitive pitvipers. The venom of *C. rhodostoma* has been reported (2) to contain moderate to low levels of phospholipases $A_2$ ($PLA_2$s[3]; E.C. 3.1.1.4.). We found that *T. mucrosquamatus* venom contains relatively abundant $PLA_2$s (4). Snake venom $PLA_2$s usually exist in multiple isoforms of ≥50% sequence homology, which exert specific pharmacological effects such as neurotoxicity, myotoxicity, haemolytic activity, edema-induction, antiplatelet, or anticoagulating activity (5).

Recently, we have cloned and sequenced the cDNAs encoding the $PLA_2$s from the venom gland of a single specimen of *C. rhodostoma* (Thailand origin) (6) and that of *T. mucrosquamatus* (South Taiwan) (4, 7). Interestingly, the respective sequences of the Lys49- and the Arg6-$PLA_2$s in these two venom species were found to be almost identical (6). On the other hand, electrophoretic analyses on the venoms from about 200 individual geographic samples of *C. rhodostoma* revealed that the venom variations can be correlated with the diet or feeding ecology (8, 9). This is so far the only venomous snake whose diets and geographic variations have been investigated in details. We are thus

[1] The novel cDNA sequence of TmPL-III has been deposited to Genbank under Accession No. X77646.

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[3] Abbreviations used: CRV, *Callosellasma rhodostoma* venom; ESI-MS, electrospray ionization mass; FPLC, fast protein liquid chromatography; PCR, polymerase chain reaction; $PLA_2$, phospholipase $A_2$; PRP, platelet rich plasma; RP-HPLC, reversed phase high performance liquid chromatography; TFA, trifluoroacetic acid; Tm, *Trimeresurus mucrosquamatus*.

0003-9861/01 $35.00

prompted to compare the venom $PLA_2$ expression patterns of these two species from different regions and try to relate the intraspecies variation of venom proteins possibly to the snake feeding ecology.

Herein, we purified and characterized the $PLA_2$s from the pooled venom samples of *C. rhodostoma* from five geographic regions, as well as 19 individual venom samples of *T. mucrosquamatus* collected from South and North Taiwan. The identification of venom $PLA_2$ was made according to results of ESI-MS and automatic sequencing. Our results suggest that the major causes of geographic variations in both venom species are associated with differential expression of existing mRNAs of venom proteins. Moreover, we show that the functional specificities of certain venom proteins are likely to be related with the feeding ecology of the snake.

## MATERIALS AND METHODS

*Venoms and other materials.* Venom powder of *C. rhodostoma* from Vietnam, Thailand, and West-Java origins were purchased from Kentucky reptile Zoo (U.S.A.), Latoxan Co. (France), and Venom Supplies pty Ltd. (W. Australia), respectively. *C. rhodostoma* venom from Malaysia and origin-unidentified sample from the Hong Kong Zoo were gifts from Professors D. A. Warrel and R. D. G. Theakston (U.K.). *T. mucrosquamatus* venoms were also collected from nine and ten specimen of individual snakes from North and South Taiwan, respectively. Trifluoroacetic acid (TFA), acetonitrile were purchased from Merck. ADP, apyrase, and sodium citrate were obtained from Sigma. Other reagents and biochemical used are as those described previously (4, 7, 10).

*Cloning and sequencing of $PLA_2$s.* The cDNA library of *T. mucrosquamatus* venom gland was prepared as described (7). In order to amplify the cDNA encoding venom $PLA_2$, PCR was conducted using cDNA synthesized from the venom gland mRNA as the template. A pair of mixed-base oligonucleotide primers (21 and 18 residues) was designed based on the highly conserved cDNA regions from other group-II $PLA_2$s (6).

The PCR was performed with SuperTaq DNA polymerase (11). A 0.4-kb DNA fragment was specifically amplified. After being treated with polynucleotide kinase, it was inserted into the pGEM-T vector (Promega) and then transformed into *Escherichia coli* strain JM109. While transformants were picked-up and specific cDNA clones were selected. Both strands of the cDNA were sequenced by the dideoxynucleotide method (12) using a sequencing kit (Sequenase Version 2.0, U.S. Biochemical). A total of four $PLA_2$-cDNAs were cloned from venom glands of *T. mucrosquamatus* (4, 7) and 10 were cloned from that of *C. rhodostoma* (6).

*Purification of $PLA_2$s.* About 15 mg of the *C. rhodostoma* venom were dissolved in up to 0.3 ml of water. After centrifugation at 12,000-rpm aliquots of 100 $\mu$l were injected into a gel filtration column (Superdex G75, HR10/30) on a FPLC system (Pharmacia). The column was preequilibrated with 0.1 M ammonium acetate (pH 6.5) at room temperature, and the sample was eluted at 1 ml/min. Fractions corresponding to proteins of 14 and 28 kDa were separately collected and lyophilized. The proteins were further purified by RP-HPLC on a Vydac $C_8$ column or Chemcosorb $C_{18}$ column (4.5 $\times$ 250 mm). Similarly, 10–15 $\mu$l of fresh venom collected from individual *T. mucrosquamatus* specimen were diluted with 120 $\mu$l 0.07% TFA and centrifuged. The supernatant was filtrated through a 0.45 $\mu$ mini-filter before injected on the HPLC column. The elution gradient was generated by a solvent delivery system (140B, Applied Biosystem–Perkin–Elmer) using both A-solvent (0.07% TFA) and B-solvent (0.07% TFA in $CH_3CN$).

*Protein determination and enzyme assay.* Concentration of $PLA_2$ was determined by the absorbance at 280 nm and assuming an extinction coefficient of 1.5 at 1.0 mg/ml. The chromatographic procedures did not affect the $PLA_2$ activities. The hydrolysis toward mixed micelles of dipalmitoyl phosphatidylcholine with either deoxycholate or Triton X-100 was measured on a pH-stat apparatus at pH 7.4 and 37°C (7). Assay of neurotoxicity using a neuromuscular preparation of 6–10 days old chick was performed as previously described (7).

*Molecular mass and N-terminal sequences.* Purified $PLA_2$s from HPLC were dried in a vacuum-centrifuge device (Labconco). Their molecular weights were determined by electrospray ionization mass spectrometry (ESI-MS) on a PE-Sciex API100 mass analyzer (Perkin–Elmer). The N-terminal amino acid sequences of $PLA_2$s were determined by an automatic gas-phase sequencer (model 477A, Applied Biosystems) coupled with an on-line phenylthio-hydantion amino acid analyzer using the Normal-program.

*Preparation of platelets and the aggregation assay.* Blood was collected from rabbit and healthy human donor and mixed with 3.8% sodium citrate (9:1, v/v). The blood was centrifuged at 130*g* for 15 min at room temperature to prepare the platelet-rich plasma (PRP). Platelet aggregation was measured by an aggregometer (Payton, module 600B, Canada). The PRP was kept at 37°C and aliquots (0.45 ml) were preincubated with the $PLA_2$ for 2 min in a siliconized cylindrical glass cuvette under constant stirring. The aggregation was initiated by the addition of 10 $\mu$M ADP and followed for a period of 5 min. The $IC_{50}$ value of a $PLA_2$, defined as the concentration to inhibit 50% of the aggregation of PRP caused by 10 $\mu$M ADP, was determined from the dose-dependence curve (13).

## RESULTS AND DISCUSSION

### *Cloning and Predicted Amino Acid Sequences of TmPL-III*

We have reported the sequences of three distinct $PLA_2$s from *T. mucrosquamatus* venom, namely the acidic $PLA_2$-I (4), a basic neurotoxic trimucrotoxin (7), and a Lys49-$PLA_2$ (10). By cDNA cloning and sequencing that use the venom gland of a southern Taiwan specimen, we have now solved the protein sequence of the fourth one, an acidic $PLA_2$ designated as TmPL-III (Fig. 1). This $PLA_2$ contains all the conserved Cys and the active site residues including the essential $Ca^{2+}$ binding loop (14, 15). The calculated molecular weight (13,974, assuming seven disulfide bonds) of TmPL-III is also consistent with that determined by ESI-MS of the $PLA_2$ purified (peak 7, Fig. 2). The signal-peptide is 16 residues-long and highly similar to those in the cDNA of other of pitviper $PLA_2$s (4, 6, 7).

### *Geographic Variation of TmPL-III in the Venom*

Separated by a river and inhabiting in ranges less than 300 km apart, the southern and the northern *T. mucrosquamatus* in Taiwan are not identical in their skin patterns, e.g., there is an extra dark spot on cheek of the southern snakes. The gel filtration patterns of

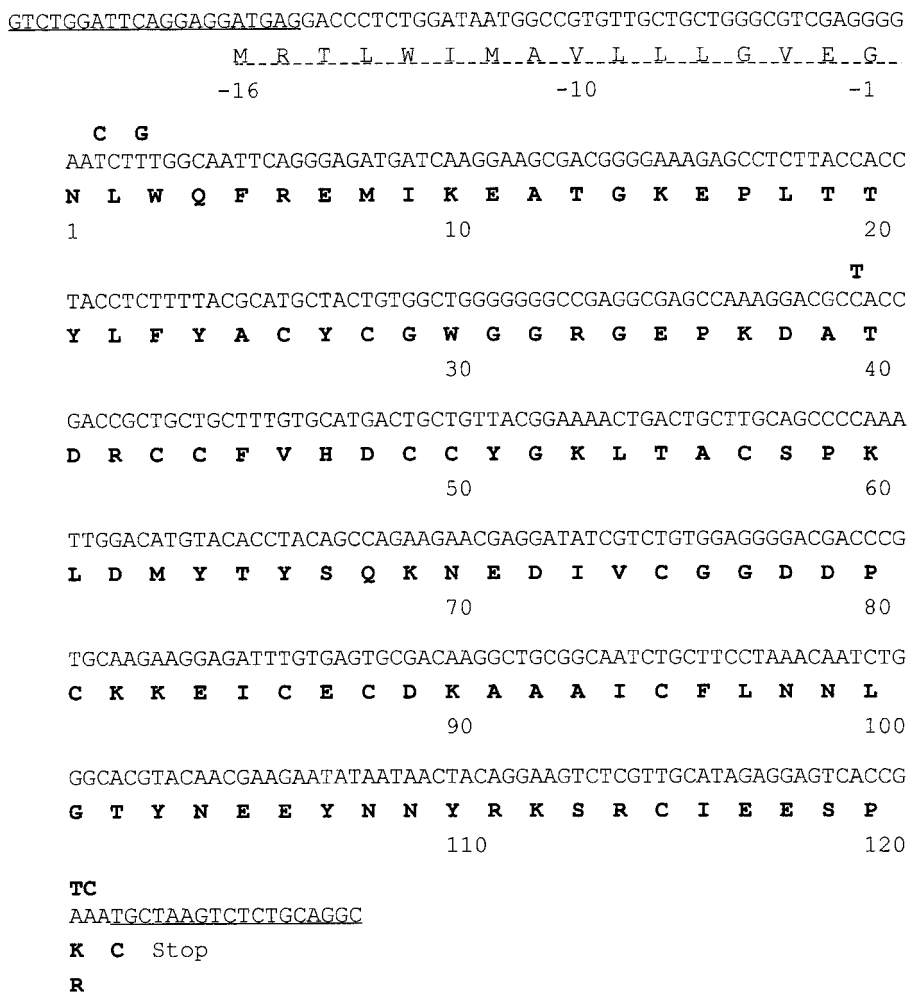


**FIG. 1.** The cDNA sequence and deduced amino acid sequence of TmPL-III. One-letter codes of amino acids are used and the numbering is shown below the sequences. PCR primers were underlined and the signal peptide was dash-underlined. The nucleotide sequences of CRV-R6 $PLA_2$s differ from that of TmPL-III by 5 bases (shown in bold letter above the sequence) and result in the Lys121Arg substitution.

the geographic venom samples of *T. mucrosquamateus* are very similar but their HPLC elution patterns reveal geographic differences (Fig. 2). We found that three of the four $PLA_2$ isoforms are present at high abundance in all the Taiwanese habu venoms analyzed whereas TmPL-III is present in the northern but missing in the southern habu venom. A venom sample of *T. mucrosquamatus* from Hunan, China, also showed similar HPLC pattern of $PLA_2$s as those from the northern Taiwan samples (data not shown). By integrating the area under their UV-absorbing peaks, the average yields of the *T. mucrosquamatu* $PLA_2$s were calculated. For the $PLA_2$s with K49, N6, R6, or E6 substitution, the yields are about 10, 7, 2, and 3% of the total proteins in the northern habu venoms, while the corresponding $PLA_2$ yields were 10, 10, 0–0.3, and 3% in those of the southern venoms analyzed (Fig. 2), respectively.

*Structure and Function of TmPL-III*

TmPL-III showed very high catalytic activities toward the mixed micelles of 3 mM dipalmitoyl phosphatidylcholine with sodium deoxycholate or Triton X-100, the rates were 690 ± 26 and 228 ± 17 μmol/min/mg, respectively. Nonlethal at a dose of 3 μg/g body weight of the mice (i.p.), it showed antiplatelet activity but very low neurotoxicity toward chick biventer cervicis tissue (7). Addition of 8 μg/ml TmPL-III to the tissue bath during the electrophysiological assay, the twitch of chicken tissue was 90% inhibited in 163 ± 15 min and 100% inhibited in 263 ± 21 min (average of four experiments). Thus, the toxicity is 110-folds weaker than trimucrotoxin (7), and the acidic subunit of crotoxin could not increase its toxicity. TmPL-III contains Arg6 and its calculated p*I* value is 5.3. However, Arg6-$PLA_2$s from the venoms of the ge-

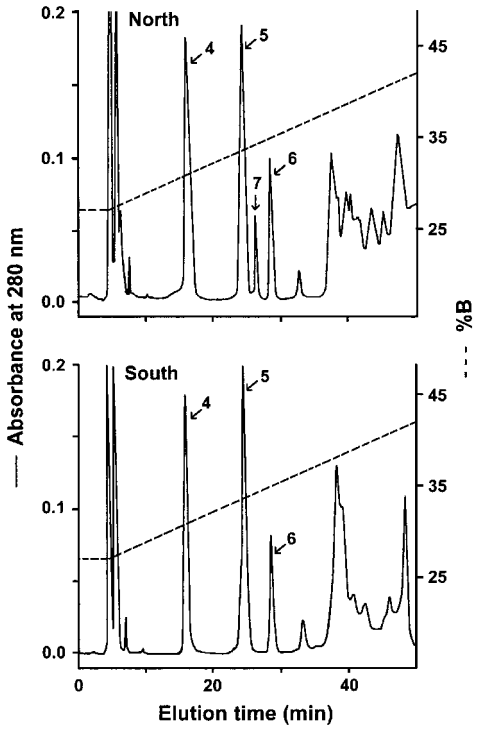


**FIG. 2.** Purification of $PLA_2$s of *T. mucrosquamatus* venoms by HPLC. The habu enzymes from (A) North Taiwan and (B) South Taiwan were purified by a Chemcosorb RP-HPLC column (ODS-H, 5 $\mu$m, 1 × 25 cm). Solvent A was 0.07% TFA in $H_2O$, solvent B was 0.07% TFA in $CH_3CN$, and the elution was by a linear gradient (27–42%) over 45 min at a flow rate of 1 ml/min. Peaks 4–7 were identified as the $PLA_2$ isoforms with K49, N6, E6, and R6 substitutions, respectively.

nus *Gloydius* (formerly the Old World *Agkistrodon*) (14) and the tree viper *T. stejnergeri* are usually much more basic (p*I* 9.2) (16). How would the differences affect their functions remain to be investigated further.

### *Common Arg6-$PLA_2$ and Lys49-$PLA_2$ Genes of Two Asian Venoms Species*

Molecular cloning and cDNA sequencing are very helpful to determine the complete sequences of venom $PLA_2$s and also to find the un-translated $PLA_2$ mRNAs. Recent phylogenetic analyses based on mitochondrial DNA sequences suggest that *T. mucrosquamatus* and *C. rhodostoma* are only loosely related (18), but some of their venom $PLA_2$s (mRNA) share surprising similarities. Two $PLA_2$s, designated as CRV-R6a and CRV-R6b, cloned from the venom gland of *C. rhodostoma* (6) are structurally identical to TmPL-III except a semi-conserved substitution at residue 121 (Fig. 1). Their signal peptide sequences are also identical. However, both CRV-R6-$PLA_2$s are not translated into venom proteins, a situation similar to the un-translated TmPL-III in the southern Taiwan habu.

Polymorphic mRNAs for venom $PLA_2$s (19) and other venom proteins (20, 21) have been suggested to result from gene-duplication and accelerated evolution to acquire new functions or new specificities to preys. Venom components may be synergistic to each other and important for catching certain preys thus their mRNA tend to be retained for possible future use. However, the low-expressing Arg6-$PLA_2$ in both pitviper venoms is more likely examples of the regressing venom component. The presence of low level of certain $PLA_2$ mRNAs in the venom glands possibly bears adaptation importance or is beneficial to snake survival.

### *Geographic Variations of C. rhodostoma Venom*

Geographic variations in the electrophoretic pattern of crude venoms, diets, and mitochondrial DNAs of

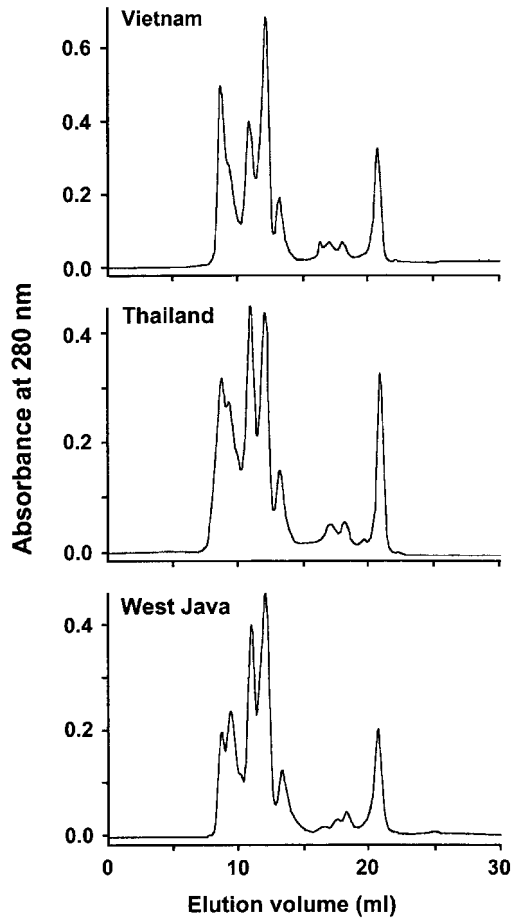


**FIG. 3.** Gel-filtration of geographic venom samples of *C. rhodostoma*. About 10 mg of the pooled crude venoms were dissolved and fractionated on a Superdex G-75 column (1 cm × 31 cm, Pharmacia, Sweden) in 0.1 M ammonium acetate, pH 6.5. Fractions of 0.5 ml were collected at a flow rate of 1 ml per min. Pooled fraction I and II contained proteins of 26 ± 2 kDa and 14 kDa, respectively. The gel-filtration profile of the Thailand venom sample (6) was almost identical to that of the Malaysia venom.

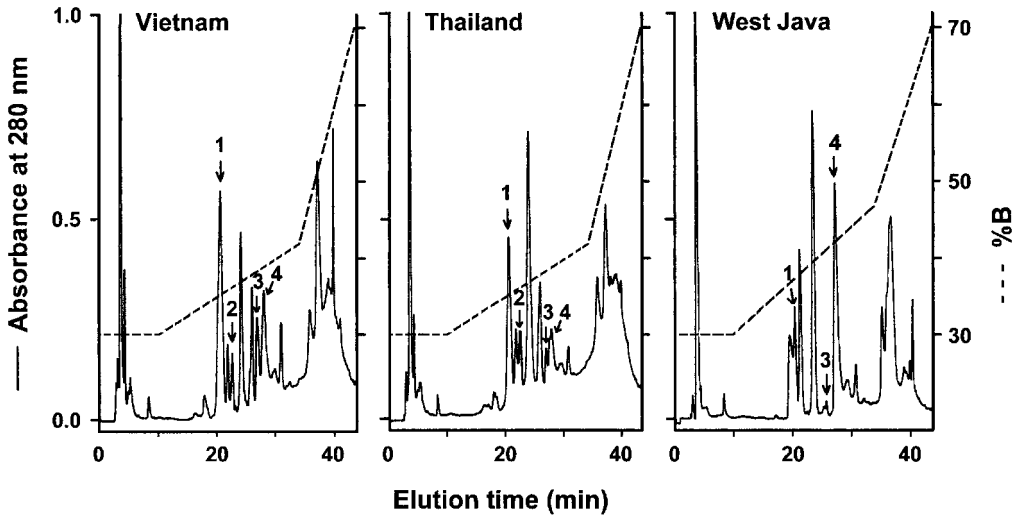


**FIG. 4.** Purification of the *C. rhodostoma* venom $PLA_2$s by direct HPLC of the venom. About 1.5 mg of the *C. rhodostoma* venom from each region was dissolved in 0.1% TFA (in $H_2O$) and purified after centrifugation by reversed-phase HPLC on a C8 silica gel column. Peaks 1–4 denote the CRV-$PLA_2$ W6, H1E6, S1E6a, and S1E6b, respectively.

more than 200 *C. rhodostoma* samples have been studied (8, 9). Variations of the venoms have been attributed to difference in snake diet since genetic difference could not be detected. After characterization of the purified *C. rhodostoma* $PLA_2$s, we found that the antiplatelet acidic $PLA_2$s contribute to the systemic bleeding symptom, while the nonhydrolytic basic $PLA_2$-homolog is responsible for edema and myonecrosis followed the snakebite (6). We now address the questions whether the proportional changes of venom $PLA_2$s are related to the observed geographic variations of the snake and whether functional specificities of the venom $PLA_2$s may possibly correlate with their prey types.

Although all the geographic samples of *C. rhodostoma* venom we analyzed show very similar gel-filtration pattern (Fig. 3), their RP-HPLC patterns revealed differences (Fig. 4). The basic $PLA_2$-homolog CRV-W6D49 (6) was purified from the 14-kDa fractions, while two or three acidic $PLA_2$s were purified from the 28-kDa fractions in variable proportions, depending on the venom locality. The N-terminal sequences and the masses of the purified $PLA_2$s derived from ESI-MS analyses are consistent with those deduced from the corresponding cDNA sequences we recently published (6). The elution condition (% B) and the molecular weight determined are: W6 (36%, 13,674), H1E6 (37%, 13,904), S1E6a (38%, 14,071), and S1E6b (39%, 14,052), respectively. Their relative contents in the venoms were also estimated from the area under the UV-absorbance peak (Table I). Notably, the venom samples from Indonesia (West Java) do not contain the CRV-H1E6 in contrast to those from Vietnam and Thailand. CRV-S1E6a is more abundantly expressed in the Vietnam samples (and the sample from Hong Kong Zoo) than those from the other regions. The content of CRV-S1E6b is relatively higher in the Malaysia and Java samples.

**TABLE I**

Summary of $PLA_2$s in Different Geographic Samples of *C. rhodostoma* Venom

| Venom origin | $PLA_2$ variants | Relative abundance (%)[a] |
|---|---|---|
| Unidentified (Hong Kong Zoo) | W6 | 56 |
| | H1E6 | ≤4 |
| | S1E6a | 15 |
| | S1E6b | 25 |
| Vietnam | W6 | 55 |
| | H1E6 | 8 |
| | S1E6a | 17 |
| | S1E6b | 20 |
| Thailand | W6 | 63 |
| | H1E6 | 14 |
| | S1E6a | 6 |
| | S1E6b | 17 |
| Malaysia | W6 | 59 |
| | H1E6 | ≤5 |
| | S1E6a | 9 |
| | S1E6b | 27 |
| West Java | W6 | 41 |
| | S1E6a | 5 |
| | S1E6b | 54 |

[a] Relative amount of the $PLA_2$ was calculated from area under the absorbance peak at 280 nm, and the total area of purified $PLA_2$ peaks in each sample was taken as 100%.

**TABLE II**

Enzymatic Activities of the Acidic $PLA_2$s of *C. rhodostoma* Venom

| $PLA_2$ | Enzyme activity ($\mu$mol/min/mg)[a] | |
|---|---|---|
| | DPPC + deoxycholate | DPPC + Triton X-100 |
| H1E6 | 584 ± 9 | 291 ± 26 |
| S1E6a | 245 ± 27 | 83 ± 4 |
| S1E6b | 269 | 82 ± 6 |

[a] The initial rate was measured by pH-stat method, using mixed micelles of 3 mM L-dipalmitoyl glycero-3-phosphocholine (DPPC) with either detergents in the presence of 10 mM $CaCl_2$ and 0.1 M NaCl at pH 7.4 and 37°C. Data shown are the average results of two independent assays.

It was pointed out that the sources of intraspecies variations of pooled venoms include size/age or sex of the snakes, season, and geographic regions covered in the venom collection (22). However, collecting venoms from a broad geographic area and analyzing venoms by precise subcategories are laborious, expensive, and hazardous (23). The *C. rhodostoma* venoms we have studied are representative samples of the gross areas or Nations. Apparently, differential expression of venom $PLA_2$ messengers, possibly under ecological induction, may contribute to the observed geographic variations of venom proteins. Quantitative variations in the proportion of venom $PLA_2$s were also reported for Okinawa *T. flavoviridis* (24) and Mexican *Crotalus r. ruber* (25). In some other cases, qualitative changes in venom protein sequences were found to be responsible for the venom geographic variations (26–28).

### *Comparison of the Acidic $PLA_2$s of C. rhodostoma Venom*

The hydrolytic activities of the $PLA_2$s toward two types of micellar substrates are listed in Table II. The activities toward the zweitter-ionic micelles (with Triton X-100) are apparently lower than toward the cationic micelles (with deoxycholate). CRV-H1E6 has 2–3-fold higher enzymatic activity than CRV-S1E6a and b which have similar activities and specificities toward the lecithin substrates.

Amino acid sequences of the acidic CRV-E6-$PLA_2$s are aligned with the most similar sequences of other $PLA_2$s in Fig. 5. Notably, CRV-E6 $PLA_2$s are structurally more similar to the E6-$PLA_2$s from American pitvi-

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                    1         10        20        30        40        50        60
                    |         |         |         |         |         |         |
1. CRV-S1E6a        SLVQFETMIMKLAK-RSGFFWYSFYGCYCGWGGHGLPQDPTDRCCFVHDCCYGKVT---NC-----
2. CRV-S1E6b        .......L.....-.....................................---.-.-----
3. CRV-H1E6         H.L.....I.MT.-QT.L.S............R.....................---.-.-----
4. C. adamanteus    .......L...V..-...LL...A..........R...A............A.---.-.-----
5. D. acutus        ..I....L...VV.-K..M....A..........R...A..................---G.-----
6. A. p. piscivorus D.M....L...I..-...M....A.........Q.R...A..................---G.-----
7. T. mucrosquamatus N.W...N....V..-K..ILS..A.........R.T.K.A..................---G.-----
8. A. halys pallas  ..I....L...V..-K..M....N.........Q.R...A..................---G.-----

                    70        80        90        100       110       120       130       %identity
                    |         |         |         |         |         |         |
1.  NPKTATYSYTEENDGIVCGG-DDPCKKQVCECDRVAAMCFRDNKDTYDSDKYWKLPPQKC-QEDPEPC          100
2.  .....................-.....................................-.......          99
3.  D..A.A....I..G.....-.......I.....A........L...NYA....FSAKD.-..ESD..          78
4.  ....VS.T.S...GE.....-.....GT.I....KA..I.....IPS...-N...LF..KD.-RQE....      77
5.  D..MDS.T.S...GD.....-.....REI........V.....L...N..T..RY.TKN.-..E.D..          76
6.  D..LDS.T.SV..GDV....-NN....EI.....A..I......V....-N...RF...N..-K.ES...      75
7.  ...LGK.T.SS..GD.I....-.G...-E......A..I.....L....RKT...Y.ASN.-....S...      73
8.  D..MDV..FS...GD.....-.......EI.....A..I.....LN..NDK...AFGAKN.P..ES...      72
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**FIG. 5.** Multiple sequence alignment of representative pitviper venom $PLA_2$s containing a Glu6. *C. rhodostoma* venom $PLA_2$s are abbreviated with CRV. The sequence data for CRV-H1E6, CRV-S1E6a, and CRV-S1E6b have been deposited in the GenBank database with the accession numbers AF104067, AF104068, and AF104069, respectively. Refer to (35, 36) for other sequences. Single-letter codes of amino acids are used. The numbering system follows that of Renetseder *et al.* (17). Residues identical to those in the top line are denoted with dots, gaps are marked with hyphens.

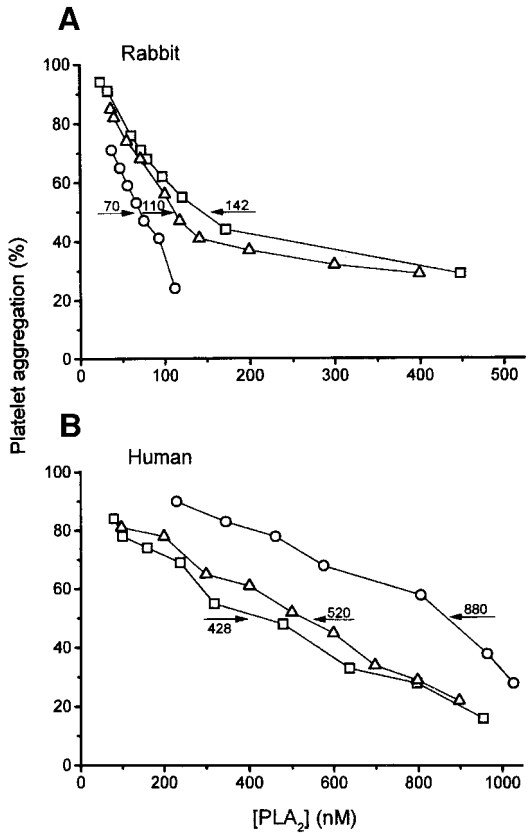


**FIG. 6.** Dose-dependent inhibition of platelet aggregation by CRV-H1E6 (○), CRV-S1E6a (□), and CRV-S1E6b (△). The ADP-induced platelet aggregation was studied with PRP from (A) rabbit and (B) human. Values of $IC_{50}$ (nM) are shown after arrows, and data points are the average from two or three experiments.

per venoms (sequences 4 and 5, Fig. 5) as compared with other Asian E6-$PLA_2$s. According to previous crystallographic study on a venom E6-$PLA_2$ (29), the negative-charged surface region involving acidic residues 6 and 115 and aromatic residues 20, 21, 113, and 119 of the $PLA_2$s have been postulated to be essential for the antiplatelet activity. These residues are mostly conserved in the CRV-E6-$PLA_2$s. The enzyme mechanism has been related with a change in the cytoskeleton structure and signal-transduction (30), and the lysophospholipid produced may also play a vital part (31).

The platelet-rich-plasma (PRP) from human and rabbit were used to assay the inhibition specificities of the CRV-E6-$PLA_2$s in ADP-induced platelet aggregation (Fig. 6). Although CRV-H1E6 has about threefold higher hydrolytic activity than CRV-S1E6a or b (Table II), CRV-H1E6 is a weaker inhibitor ($IC_{50}$ 880 nM) than CRV-S1E6 ($IC_{50}$ 428 or 520 nM) toward the human platelets but a much stronger inhibitor toward the rabbit platelets ($IC_{50}$ 70 nM) (Fig. 6). It is speculated that the lower activity of CRV-H1E6 than CRV-S1E6 for human platelets is due to the substitutions of W21S, D114N (29), and N67D (26). CRV-S1E6a differs from CRV-S1E6b only by a single substitution (Fig. 5). The single change of Met8 to Leu8 in CRV-S1E6 may increase its antiplatelet potency toward the rabbit platelets but decrease that toward the human platelets by about 25% (Fig. 6). It is known that the platelets from different mammals may have different susceptibilities toward antiplatelet agents (13). Thus, expression of the venom antiplatelet $PLA_2$s possibly has been evolved to cope with the feeding ecology of the snakes (8, 9, 24). Interestingly, the venoms of many other pitvipers, e.g., *T. flavoviridis* (32), *T. stejnergeri* (33), and *Agkistrodon p. picivorus* (34), also contain two or more acidic E6-$PLA_2$s. Before a final conclusion on structure–activity relationships of the antiplatelet $PLA_2$s can be reached, the inhibitions of the platelets from more species (including reptiles and amphibians) by various $PLA_2$ isoforms or mutants need to be studied in details.

## *Conclusion*

This study demonstrates the convenient use of HPLC and ESI-MS to identify geographic and intraspecies variations of venom proteins. In five geographic venom samples of *C. rhodostoma,* the variations were likely attributed to the acidic $PLA_2$s expressed in different proportions. We also showed that these $PLA_2$s have different inhibition specificities toward the platelets from human and rabbit and presumably may distinguish platelets from various prey species. A novel venom $PLA_2$ (TmPL-III) was purified and completely sequenced and identified as a regressing protein, which is missing in the *T. mucrosquamatus* venom of southern Taiwan, and is transcribed but not translated in the venom glands of *C. rhodostoma.* Therefore, the geographic variations in snake venom proteins are usually quantitative rather than qualitative, and may result from differential expression of the venom mRNAs.

## ACKNOWLEDGMENTS

We thank Dr. D. A. Warrell and Dr. R. G. D. Theakston for generous gifts of the venom of *C. rhodostoma* from Malaysia, Thailand, and Hong Kong Zoo, and Professor W. Y. Wang of Kunming Zoology Research Institution for the venom sample of *T. mucrosquamatus* (Hunan Province, China). This study was supported by grants from Academia Sinica, and National Science Council of Taiwan, ROC (Grant 89-B-FA01-1-4).

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