Purification and characterization of three distinct types of phospholipase A₂ inhibitors from the blood plasma of the Chinese mamushi, *Agkistrodon blomhoffii siniticus*

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Three distinct types of phospholipase A₂ (PLA₂) inhibitory proteins (PLI α , PLI β , and PLI γ) were isolated from the blood plasma of the Chinese mamushi, *Agkistrodon blomhoffii siniticus*. PLI α is an inhibitor that we have already purified and whose amino acid sequence we have already determined [Ohkura, Inoue, Ikeda and Hayashi (1993) J. Biochem. (Tokyo) 113, 413–419]. It inhibited selectively the group-II acidic PLA₂s from Crotalidae venom. PLI β was a 160-kDa glycoprotein having a trimeric structure composed of 50-kDa subunits. The amino acid sequence of the first 30 amino acids of the N-terminal part of the 50-kDa subunit was determined and found to have no significant homology to that of known proteins. PLI β was a selective

inhibitor against the group-II basic PLA₂s from Crotalidae venom. Some amino acid residues located in or close to the interfacial binding surface of the group-II basic PLA₂s were suggested to be involved in selective binding to PLI β . PLI γ was a 100-kDa glycoprotein containing 25-kDa and 20-kDa subunits and inhibited all of the PLA₂s investigated equally, including Elapidae venom PLA₂s (group I), Crotalidae and Viperidae venom PLA₂s (group II) and honey-bee PLA₂ (group III). From the N-terminal sequences of the two subunits, PLI γ was found to be the same type of PLI that had been purified from Thailand cobra plasma.

INTRODUCTION

Phospholipase A₂ (PLA₂) catalyses the hydrolysis of the acyl-ester bond at the *sn*-2 position of 1,2-diacyl-*sn*-phosphoglycerides. Snake venom is one of the most abundant sources of PLA₂s. Snake-venom PLA₂s are classified into two groups, I and II, according to differences in their primary structure [1]. Elapidae venoms contain group-I PLA₂s, and Crotalidae and Viperidae venoms, group-II PLA₂s. We have already purified PLA₂ inhibitory proteins (phospholipase A₂ inhibitors; PLIs) from the blood plasma of two Crotalidae snakes, *Trimeresurus flavoviridis* [2] and *Agkistrodon blomhoffii siniticus* [3], and have determined their amino acid sequences [3,4]. These inhibitors were 75-kDa glycoproteins and trimers of 20-kDa subunits having sequence homology to the carbohydrate recognition domain of C-type lectins [3,4]. These Crotalidae inhibitors inhibited specifically the group-II acidic PLA₂s present in their respective venom [5]. On the other hand, we purified another type of PLA₂ inhibitory protein from the blood plasma of an Elapidae snake, *Naja naja kaouthia* [6]. This inhibitor was a 90-kDa glycoprotein containing 30-kDa and 25-kDa subunits, each of which had two tandem patterns of cysteine residues found in urokinase-type plasminogen activator receptor and Ly-6-related proteins, such as Ly-6A/E, Ly-6C, ThB, and CD59 [7]. This Elapidae inhibitor inhibited not only the group-I PLA₂ from its own venom but also the group-I, -II and -III PLA₂s from other sources [5,6].

In the present study, we purified three types of PLA₂ inhibitory proteins (PLI α , PLI β , and PLI γ) from the blood plasma of *A. blomhoffii siniticus*. Besides the PLI α characterized previously, this plasma contained two additional PLIs, PLI β and PLI γ . The former was found to be a new type of inhibitor and the latter, the same type as the Elapidae inhibitor.

EXPERIMENTAL

Materials

The blood plasma and venom of the Chinese mamushi (*A. blomhoffii siniticus*) were collected at the Japanese Snake Institute (Yabuzuka, Gunma). PLA₂s from various sources were purified or purchased as described previously [5].

Separation of three inhibitors from *A. blomhoffii siniticus* plasma

The freeze-dried plasma was fractionated on a Sephadex G-200 column (Pharmacia LKB Biotechnology) as described previously [3]. The fractions exhibiting PLA₂ inhibitory activity were dialysed against 50 mM Tris/HCl buffer (pH 7.5) containing 2 mM EDTA and then applied to a Q-Sepharose column (Pharmacia Biotech) that had been equilibrated beforehand with the same buffer. Three fractions (fractions I, II, III) were obtained by elution with a buffer containing a linear concentration gradient of NaCl (0–0.5 M). From the flow-through fraction (fraction I), PLI α was purified by use of a Blue Sepharose CL-6B column as described previously [3]. Fractions II and III were used for the preparation of PLI β and PLI γ respectively.

Purification of PLI β from fraction II

Fraction II was dialysed against 0.1 M Tris/HCl buffer (pH 7.5) and then applied to a Hi-Trap Blue column (Pharmacia Biotech) that had been previously equilibrated with the same buffer. The column was washed with the same buffer, and the flow-through fraction was dialysed against 0.1 M Tris/HCl containing 1.0 M (NH₄)₂SO₄. The dialysed sample was then loaded on to a Hi-Trap Phenyl HP column (Pharmacia Biotech) that had been

equilibrated earlier with the same buffer, and $\text{PLI}\beta$ was eluted with a decreasing linear concentration gradient of $(\text{NH}_4)_2\text{SO}_4$ (1.0–0 M). This fraction was dialysed against 0.1 M ethanolamine/HCl buffer (pH 9.5) and further purified by Mono Q HR 5/5 column (Pharmacia Biotech) chromatography, the column having been equilibrated previously with the same buffer. $\text{PLI}\beta$ was eluted with the same buffer containing a linear concentration gradient of NaCl (0–0.5 M).

Purification of $\text{PLI}\gamma$ from fraction III

Fraction III was dialysed against 0.1 M Tris/HCl buffer (pH 7.5) containing 1.0 M $(\text{NH}_4)_2\text{SO}_4$ and then loaded on to a Hi-Trap Phenyl HP column that had been equilibrated earlier with the same buffer. $\text{PLI}\gamma$ was eluted with a decreasing linear concentration gradient of $(\text{NH}_4)_2\text{SO}_4$ (1.0–0 M). For separation of the subunits of $\text{PLI}\gamma$, the purified preparation was subjected to reversed-phase HPLC on a Cosmosil C4 column (Nacalai tesque). Two kinds of subunits were separately eluted with 0.1 % (v/v) trifluoroacetic acid containing a linear concentration gradient of acetonitrile (24–48 %).

Molecular-mass determination on a Superose 12 column

A Superose 12 HR 10/30 column (Pharmacia Biotech) was used to determine the molecular masses of the inhibitors. Approx. 50 μg of each inhibitor was applied to the column, which had been equilibrated earlier with 50 mM Hepes buffer (pH 7.5, ionic strength 0.2), and eluted at a flow rate of 0.4 ml/min. The molecular-mass markers used were γ -globulin (150 kDa), BSA (67 kDa) and ovalbumin (45 kDa).

SDS electrophoresis

SDS/PAGE was carried out with a Phast System apparatus (Pharmacia Biotech) using Phast gel Homogeneous 20. After the electrophoresis, protein bands were made visible with Coomassie Blue.

Amino acid composition and sequence analysis

The 50-kDa subunit of $\text{PLI}\beta$ and the 25- and 20-kDa subunits of $\text{PLI}\gamma$ were pyridylethylated in the presence of 6 M guanidine hydrochloride by the method described previously [8]. The amino acid compositions of the pyridylethylated subunits were determined with an amino acid analyser (Hitachi model L-8500), after the samples had been hydrolysed with 6 M HCl containing 0.2 % (w/v) phenol at 110 °C for 24 h in evacuated, sealed tubes. Amino acid sequences of the pyridylethylated subunits were determined with a sequencer (Applied Biosystems model 477A).

PLA_2 assays

PLA_2 activities were assayed fluorimetrically with 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphorylcholine (Molecular Probes), used as the substrate, according to the method of Radvanyi et al. [9] with some modifications as described previously [5]. The activities of PLA_2 s from various sources were measured in the presence of various concentrations of the inhibitors (10^{-11} – 10^{-5} M). Since the enzyme concentrations used were sufficiently low (10^{-12} – 10^{-11} M), the inhibitor concentrations that can reduce the PLA_2 activities by half may correspond to their inhibition constant, K_i .

RESULTS AND DISCUSSION

Purification of the three types of PLI s

The venom of the Chinese mamushi, *A. blomhoffii siniticus*, contains three isoenzymes of PLA_2 s (acidic, neutral and basic PLA_2) [3,5]. Figure 1 shows the inhibition of the enzymic activities of these three PLA_2 s by the blood plasma of *A. blomhoffii siniticus*. The plasma inhibited the activities of all these enzymes. Earlier we purified a PLI from the blood plasma of this snake [3].

Figure 1 Inhibition of enzymic activities of three PLA_2 s by the plasma of *A. blomhoffii siniticus*

●, Acidic PLA_2 ; ▲, neutral PLA_2 ; ■, basic PLA_2 .

Figure 2 Q-Sepharose column chromatography of the sample obtained from Sephadex G-200 column chromatography

○, Absorbance at 280 nm; ●, relative inhibition to acidic PLA_2 ; ▲, relative inhibition to neutral PLA_2 ; ■, relative inhibition to basic PLA_2 .

Figure 3 Hi-trap Phenyl column chromatography of PLI β (A) and PLI γ (B)

(A) Fraction II of the Q-Sepharose column chromatography was applied to a Hi-trap Blue column, and the resulting flow-through fraction was applied to a Hi-trap Phenyl column, equilibrated with 0.05 M Tris/HCl buffer (pH 7.5) containing 1.0 M $(\text{NH}_4)_2\text{SO}_4$. $\circ$, absorbance at 280 nm; $\blacksquare$, relative inhibition of basic PLA₂. (B) Fraction III of the Q-Sepharose column chromatography was applied to a Hi-trap Phenyl column, equilibrated with the same buffer described above. $\circ$, absorbance at 280 nm; $\blacksquare$, relative inhibition of basic PLA₂.

Since this PLI inhibited specifically the acidic PLA₂ in the venom [5], it was suggested that the plasma contained other kinds of inhibitors that could inhibit the neutral and basic PLA₂s. Therefore we investigated the inhibitory activities of various fractions of the blood plasma against all three types of PLA₂s separately during the course of the purification. Figure 2 shows the elution profile of the material after Q-Sepharose column chromatography of the sample obtained from Sephadex G-200 gel filtration of the blood plasma of *A. blomhoffii siniticus*. The inhibitory activity was separated into three fractions (fractions I, II and III). Fraction I contained an inhibitor that inhibited specifically the acidic PLA₂, whereas fraction II contained an inhibitor that inhibited specifically the basic PLA₂. The fraction III inhibitor inhibited all three PLA₂s. These inhibitors were designated as PLI α , PLI β and PLI γ respectively. Fraction I was further purified by Blue Sepharose column chromatography, and the purified PLI α was found to be the PLI that we had purified and characterized previously [3].

Fraction II was subjected to Hi-trap Blue column chromatography, and the inhibitory activity against basic PLA₂ was recovered in the flow-through fraction (results not shown). The fraction thus obtained was further purified by hydrophobic interaction chromatography on a Hi-trap Phenyl column. As can be seen in Figure 3(A), PLI β was eluted with the buffer containing 0.7 M $(\text{NH}_4)_2\text{SO}_4$. In order to remove the small amount of contaminant proteins, this fraction was further purified by Mono-Q column chromatography (results not shown). Finally, approx.

Figure 4 SDS/PAGE of purified PLI α , PLI β and PLI γ

The positions of molecular-mass standards are indicated.

2 mg of the purified PLI β was obtained from 1.0 g of the freeze-dried plasma of *A. blomhoffii siniticus*.

In a previous study, we found another inhibitory activity in the Mono-Q adsorbed fraction, besides the fraction containing PLI [3]. The former fraction in the previous study seemed to correspond to fraction III in the present study. Fraction III was further purified by Hi-trap Phenyl column chromatography. As shown in Figure 3(B), PLI γ was eluted with the buffer containing 0 M $(\text{NH}_4)_2\text{SO}_4$. The PLI γ fraction obtained in this step showed a single protein band corresponding to that of a pI of approx. 4.4 following isoelectric focusing (results not shown). Finally, approx. 3 mg of the PLI γ was obtained from 1.0 g of the freeze-dried plasma.

Fundamental properties of PLI β

The molecular mass of the purified PLI β was determined to be 160-kDa by gel filtration on a Superose 12 column (results not shown). As shown in Figure 4, SDS/PAGE of the PLI β gave a single protein band with a molecular mass of 50 kDa. When the purified PLI β was subjected to reversed-phase HPLC, only a single peak was detected (results not shown). Therefore the PLI β was thought to be a trimer of 50-kDa subunits. As shown in Table 1, the amino acid composition of the 50-kDa subunit of PLI β was somewhat different from that of PLI α . The 50-kDa subunit of PLI β contained a very large amount of leucine residues (19.2%). The detection of glucosamine by amino acid analysis of the 50-kDa subunit suggests that the PLI β is a glycoprotein. The N-terminal 30-amino-acid sequence of the pyridylethylated 50-kDa subunit of PLI β was determined to be Val-Leu-Tyr-Cys-Pro-Pro-Thr-Pro-Ala-Pro-Glu-X-Val-Thr-Glu-Phe-Val-Cys-Asn-Ser-Pro-Leu-Leu-His-Glu-Phe-Pro-Thr-Gly-Phe. No proteins having a statistically significant sequence homology with this sequence were found in the computer search of proteins in GenBank. Also, no significant sequence homology to the known PLIs, such as *T. flavoviridis* PLI-A and -B, *A. blomhoffii siniticus* PLI α , *Crotalus durissus terrificus* CNF, and 31- and 25-kDa subunits of *N. naja kaouthia* inhibitor, was found. Therefore the *A. blomhoffii siniticus* PLI β was thought to be a new type of PLI. Further sequencing of PLI β is now in progress.

Fundamental properties of PLI γ

The molecular mass of the purified PLI γ was determined to be 100 kDa by gel filtration on a Superose 12 column (results not

Table 1 Amino acid compositions of three PLA₂ inhibitors from *A. blomhoffii siniticus*

Values indicate the molar fraction of each amino acid (%). Composition of PLI α was calculated from the sequence already determined. Cys was determined as pyridylethylcysteine. Trp was not determined. Glucosamine (GlcNH₂) was detected by amino acid analysis (+). N.D., not determined.

Amino acid	Amino acid composition (%)			
	PLI α	PLI β	PLI γ	
			25-kDa subunit	20-kDa subunit
Asp	12.9	10.8	11.4	11.8
Thr	4.6	6.0	3.4	6.6
Ser	6.1	8.6	10.3	7.8
Glu	10.2	11.6	10.5	12.2
Pro	2.7	8.3	4.8	2.4
Gly	8.2	4.1	8.1	6.3
Ala	6.8	3.8	2.9	4.2
Val	7.5	3.1	4.0	4.1
Met	2.0	1.4	0.0	2.7
Ile	2.7	3.7	5.8	4.2
Leu	8.8	19.2	7.0	8.3
Tyr	2.0	1.1	5.1	2.8
Phe	6.1	4.5	3.5	4.7
Lys	6.8	4.3	5.9	6.2
His	4.1	3.9	5.5	4.0
Arg	4.1	3.6	3.2	3.8
Cys	2.7	2.0	8.5	8.1
Trp	1.4	N.D.	N.D.	N.D.
GlcNH ₂	+	+	+	–

Figure 5 Comparison of the N-terminal amino acid sequences of the subunits of PLI γ with those of CNF and cobra inhibitor

The boxes indicate identical amino acids in the respective subunits. The bold face residues indicate identical amino acids conserved in all the sequences. Literature references for the sequences are as follows: *C. durissus terrificus* CNF [10]; 31- and 25-kDa subunits of *N. naja kaouthia* [7].

shown). As shown in Figure 4, SDS/PAGE of PLI γ gave two protein bands corresponding to approximate molecular masses of 25 and 20 kDa, suggesting that the PLI γ was composed of two subunits, 25- and 20-kDa subunits. The two subunits of PLI γ could be separated by reversed-phase HPLC (results not shown). The molar ratio of 25- to 20-kDa subunits was calculated to be approx. 2 on the basis of the area of each peak monitored by the absorbance at 280 nm. Therefore 1 mol of PLI γ might be composed of 2 mol of the 25-kDa subunit and 1 mol of the 20-kDa subunit. But the precise composition of the subunits in PLI γ is still ambiguous. As shown in Table 1, the 25- and 20-kDa subunits of PLI γ contained a very large amount of cysteine residues (8.5 and 8.1 % respectively). Glucosamine was detected by amino acid analysis of the 25-kDa subunit, but not by that of the 20-kDa one. Therefore the 25-kDa subunit of PLI γ is thought to be a glycoprotein. These fundamental properties of PLI γ are very similar to those of a PLI purified from the plasma of the Thailand cobra *N. naja kaouthia* [6]. The sequences of the N-terminal 30 amino acids of the pyridylethylated 25- and 20-kDa subunits of PLI γ were determined, and these subunits were

Table 2 Binding constants ($1/K_i^{app}$) of various groups of PLA₂ for binding to PLI β and PLI γ , compared with that for PLI α

The data for PLI α are cited from Ref. [5]. Group-I PLA₂s used in the present study were purified from the venoms of *Naja naja kaouthia*, *Naja naja atra* and *Pseudechis australis*. Group-II PLA₂s were from the venoms of *Trimeresurus flavoviridis*, *A. blomhoffii siniticus*, *Agkistrodon halys blomhoffii* and *Vipera russelli russelli*. The group-III enzyme was from the honeybee, *Apis mellifera*.

		$10^{-7} \times (1/K_i^{\text{app}}) \text{ (M}^{-1}\text{)}$		
PLA ₂		PLI α	PLI β	PLI γ
Group-I PLA ₂				
<i>N. naja kaouthia</i>	CM-II	0.0176	< 0.01	5.03
<i>N. naja atra</i>	PLA ₂	< 0.01	< 0.01	1.06
<i>P. australis</i>	Pa-12A	< 0.01	< 0.01	3.17
Group-II PLA ₂				
<i>T. flavoviridis</i>	acidic PLA ₂	0.289	< 0.01	0.535
	PL-X	0.0514	3.83	2.42
<i>A. blomhoffii siniticus</i>	acidic PLA ₂	66.9	< 0.01	0.958
	neutral PLA ₂	0.0877	< 0.01	1.33
	basic PLA ₂	0.0266	134	0.935
<i>A. halys blomhoffii</i>	acidic PLA ₂	9.77	< 0.01	2.84
	neutral PLA ₂	0.0393	< 0.01	9.07
	basic PLA ₂	0.0112	88.1	6.62
<i>V. russelli russelli</i>	PLA ₂ -III	0.0281	< 0.01	0.844
	PLA ₂ -V	< 0.01	< 0.01	4.37
Group-III PLA ₂				
<i>A. mellifera</i>	PLA ₂	< 0.01	< 0.01	0.655

found to have significant homology to the 31- and 25-kDa subunits respectively of the cobra inhibitor (Figure 5). The discrepancy in the apparent molecular masses of the subunits between the PLI γ and cobra inhibitor can be interpreted in terms of the differences in the protocols used for SDS/PAGE, for when SDS/PAGE of the cobra inhibitor was carried out under the same conditions as used for PLI γ , the migrations of the two subunits were indistinguishable between the two inhibitors. The *A. blomhoffii siniticus* PLI γ is therefore thought to be the same type of inhibitor as the cobra one.

Inhibition specificity of PLI β and PLI γ against various PLA₂s

The inhibition spectra of PLI β and PLI γ were compared with that of PLI α (Table 2). As has already been described, PLI α inhibited specifically the group-II acidic PLA₂s [5]. PLI β inhibited only some group-II basic PLA₂s, such as *A. blomhoffii siniticus* basic PLA₂, *Agkistrodon halys blomhoffii* basic PLA₂ and *T. flavoviridis* PL-X, and did not inhibit other kinds of PLA₂s. The amino acid sequences of PLA₂s that were selectively inhibited by PLI β were compared with those of other PLA₂s (Figure 6). The residues His-1, Arg-6, Glu-17, Trp-70, Lys-111 and Ile-124 are conserved between *A. halys blomhoffii* basic PLA₂ and *T. flavoviridis* PL-X, but they are replaced by other residues in other PLA₂s. Among these residues, His-1, Arg-6, Glu-17 and Trp-70 are located in or close to the interfacial binding surface of the PLA₂ molecule. Therefore PLI β might recognize the specific amino acid residues located at the interfacial binding surface of the group-II basic PLA₂s, and bind to this surface to cover the catalytic site of PLA₂.

In contrast with PLI β , which showed a narrow inhibition spectrum, PLI γ exhibited a broad one. PLI γ inhibited all the groups of PLA₂s, with K_i values from 10^{-8} to 10^{-7} M. The inhibition spectrum of the cobra inhibitor was also broad, as described previously [5]. The specificity of PLI γ was somewhat lower than that of the cobra inhibitor. These broad spectra of the PLI γ and cobra inhibitors suggest that both can recognize a

Figure 6 Sequence alignment for the PLA₂s used in the present study

The boxes indicate amino acids identical between *A. halys blomhoffii* (*A. h. b.*) basic PLA₂ and *T. flaviviridis* PL-X (*T. f.* PL-X), but replaced by different residues in other PLA₂s. The common numbering system defined by Renetseder et al. is used [14]. As mentioned previously [3], the sequences of *Agkistrodon halys pallas* PLA₂s were used for those of *A. blomhoffii siniticus* (*A. b. s.*), since *A. blomhoffii siniticus* is one of the subspecies of *A. halys pallas*. Literature references for the sequences are as follows: *A. blomhoffii siniticus* acidic PLA₂ [11], neutral PLA₂ [15]; *A. halys blomhoffii* acidic PLA₂ [16], neutral PLA₂ (Y. Samejima unpublished work), basic PLA₂ [17]; *T. flaviviridis* acidic PLA₂ [18], PL-X [19]; *V. russelli russelli* (*V. r. r.*) PLA₂-III and PLA₂-V (S. Inoue, E.-P. Mo, K. Ikeda and K. Hayashi, unpublished work); *N. naja kaouthia* (*N. n. k.*) CM-II [20]; *Naja naja atra* PLA₂ [21]; and *P. australis* (*P. a.*) Pa-12A [22].

common structural element, such as the calcium-binding loop, which is conserved among all groups of PLA₂s.

Physiological implications of the presence of three different types of PLIs

The three PLA₂s from the venom of *A. blomhoffii siniticus* exhibit distinctly different pharmacological actions, i.e. the acidic enzyme inhibits platelet aggregation, the neutral enzyme (agkistrotoxin) displays presynaptic neurotoxicity and the basic enzyme is haemolytic [11]. If these PLA₂s also act on the snake with these same pharmacological effects, the leakage of the venom into the circulatory system would be fatal for the snake. However, the PLIs existing in the blood serum may function as neutralizing factors of these PLA₂s, although the pharmacological actions of these inhibitors were not investigated in the present study. The enzymic activities of the acidic and basic PLA₂s were inhibited specifically by PLI α and PLI β respectively. Some group-II PLA₂s, as in the case of the mammalian platelet-derived group-II PLA₂, might play important physiological roles in snakes, such as in inflammation responses; and thus the inhibitors might have become highly specific for the venom group-II PLA₂s.

No specific inhibitors for the neutral PLA₂ were present in the serum, although the neutral PLA₂ is the most toxic among those three PLA₂s, having an LD₅₀ value of 0.055 mg/kg in mice [11]. This may suggest that the biological target of the neutral PLA₂ has been altered during the evolution of this snake and therefore the neutral PLA₂ does not exhibit presynaptic neurotoxicity in this snake. In contrast, CNF purified from the plasma of *C. durissus terrificus* is a PLI that can neutralize the neurotoxic effects of crotoxin, a presynaptic neurotoxic PLA₂ [10,12]. Crotoxin is a heterodimer composed of a basic PLA₂ subunit

(CB) and an acidic non-enzymic subunit (CA), and CNF can replace CA of the crotoxin to form a stable complex with CB, suppressing the synergistic activity between the two subunits [10,12]. Since the neutral PLA₂ of *A. blomhoffii siniticus* (agkistrotoxin) is the single-chain non-basic PLA₂ [11], it is important to determine whether or not the neurotoxic activity of the neutral PLA₂ is suppressed by PLI γ , which inhibits its enzymic activity.

The high sequence homology among PLI γ , CNF and cobra inhibitors suggests that these inhibitors are categorized in the same family of PLIs, designated as PLI γ in the present study. PLI γ may occur in the sera of all the venomous snakes, including Elapidae and Crotalidae. The sera of many species of snakes, including non-venomous snakes, have been reported to neutralize various snake venoms [13]. The resistance of these snakes to their own venom without any strict specificity might be responsible for the broad inhibition spectrum and the wide distribution of PLI γ .

At present, the PLIs corresponding to PLI α and PLI β could not be detected in the blood plasma of the Thailand cobra (*N. naja kaouthia*), one of the Elapidae snakes whose venom does not contain group-II, but does contain group-I, PLA₂s. Therefore PLI α and PLI β may occur only in the Crotalidae and Viperidae snakes, which have group-II PLA₂s in their venom.

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