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Isolation and Amino Acid Sequence of a Phospholipase A_2 Inhibitor from the Blood Plasma of the Sea Krait, *Laticauda semifasciata*[1]

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A phospholipase A_2 (PLA_2) inhibitor was purified from the blood plasma of a sea krait, *Laticauda semifasciata*, by sequential chromatography on Sephadex G-200, Mono Q, and Phenyl Sepharose columns. The purified inhibitor was found to be the same type as the PLA_2 inhibitors, named PLIγ, that had been purified from the blood plasma of the Thai cobra *Naja naja kaouthia* [Ohkura *et al.* (1994) *Biochem. Biophys. Res. Commun.* 200, 784-788] and Chinese mamushi *Agkistrodon blomhoffii siniticus* [Ohkura *et al.* (1997) *Biochem. J.* 325, 527-531]. Like other PLIγs, the *L. semifasciata* inhibitor (*Ls*PLIγ) inhibited equally all of the PLA_2s investigated including Elapid venom PLA_2s (group I), Crotalid and Viperid venom PLA_2s (group II), and honeybee PLA_2 (group III). The *Ls*PLIγ was a 100-kDa glycoprotein composed of two distinct subunits, *Ls*PLIγ-A and *Ls*PLIγ-B, with an approximate molar ratio of 2:1. The amino acid sequences of the two subunits were determined by alignment of the peptides obtained by lysyl endopeptidase, endoproteinase Asp-N, and staphylococcal V8 protease digestions. *Ls*PLIγ-A and *Ls*PLIγ-B were composed of 182 and 181 amino acid residues, respectively; and the former subunit was a glycoprotein containing one asparagine-linked sugar chain at the position 157. The sequences of *Ls*PLIγ-A and *Ls*PLIγ-B showed 65 and 74% homology, respectively, to those of the corresponding subunits of *N. naja kaouthia* PLIγ, and had two tandem patterns of cysteine residues, characteristic of the urokinase-type plasminogen activator receptor (uPAR) and members of the Ly-6 superfamily.

Key words: amino acid sequence, phospholipase A_2, phospholipase A_2 inhibitor, plasma, snake venom.

Phospholipase A_2 [EC 3.1.1.4] (PLA_2) catalyzes the hydrolysis of the acyl-ester bond at the *sn*-2 position of 1,2-diacyl-*sn*-phosphoglycerides. It plays major roles in a variety of biological processes such as digestion, membrane phospholipid metabolism, inflammatory reactions, and eicosanoid synthesis. The enzymes can generally be divided into the 14-kDa secretory PLA_2s (sPLA_2s) and the 85-kDa cytosolic PLA_2s (cPLA_2s). Snake venoms and pancreatic tissues are good sources of sPLA_2s. Snake venom PLA_2s are classified into two groups, I and II, according to the differences in the polypeptide-chain length and the intramolecular-disulfide bridges (*1*). The venom of the *Elapidae* snakes contains group-I enzymes; and that of *Viperidae* snakes, group-II enzymes.

Venomous snakes have antitoxic proteins in their blood plasma to protect themselves against their own venom toxins (*2, 3*). PLA_2 inhibitory protein is thought to be one of these protective proteins. We reported earlier the existence of three distinct types of PLA_2 inhibitory proteins (PLIα, PLIβ, and PLIγ) in the blood plasma of venomous snakes (*4*). We isolated PLIαs from the plasma of *Crotalinae* snakes, habu *Trimeresurus flavoviridis* (*5*), and Chinese mamushi *Agkistrodon blomhoffii siniticus* (*6*), and determined their primary structures (*6, 7*). PLIα specifically suppressed the group-II acidic PLA_2s from their own venom (*8*). The inhibitor was a 75-kDa glycoprotein having a trimeric structure of 20-kDa subunits. The amino acid sequence of the subunit showed a significant homology to pulmonary surfactant apoprotein and mannose-binding protein, having one carbohydrate-recognition domain (CRD) like that in calcium-dependent (C-type) lectins (*6, 7*). Recently, a similar inhibitor, named BaMIP, was purified from the plasma of *Bothrops asper* and was found to neutralize the biological activities of group-II basic PLA_2 myotoxin (*9*).

PLIβ was purified from the plasma of *A. blomhoffii siniticus* (*4*). The inhibitor was a selective inhibitor toward the group-II basic PLA_2 purified from the venom of *Crotalinae* snakes. It was found to be a 160-kDa glycoprotein and a homotrimer of 50-kDa subunits. Recently, the amino acid sequence of the subunit was found to contain

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Abbreviations: *Abs*PLIγ, *Agkistrodon blomhoffii siniticus* PLIγ; *Ls*PLIγ, *Laticauda semifasciata* PLIγ; *Nnk*PLIγ, *Naja naja kaouthia* PLIγ; PE-, *S*-pyridylethylated; PLA_2, phospholipase A_2; PLI, phospholipase A_2 inhibitor; PLIγ-A, A subunit of PLIγ; PLIγ-B, B subunit of PLIγ; TMAP-, *S*-3-(trimethylated amino) propylated.

leucine-rich repeats and to have 33% sequence identity with that of human leucine-rich α_2-glycoprotein (*10*).

PLIγs were purified from the blood plasma of Thai cobra (*Naja naja kaouthia*) (*11*) and *A. blomhoffii siniticus* (*4*). These inhibitors were characterized by broad inhibition spectra. They were glycoproteins with an apparent molecular mass of 90-100 kDa, consisting of 25- and 20-kDa subunits (designated as PLIγ-A and PLIγ-B, respectively, in the present study). We have already determined the complete amino acid sequences of the two subunits of *N. naja kaouthia* PLIγ (*Nnk*PLIγ) and showed that each subunit has two tandem patterns of cysteine residues like those found in urokinase-type plasminogen activator receptor (uPAR) and Ly-6 related proteins, such as Ly-6A/E, Ly-6C, ThB, and CD59 (*12*).

In order to generalize the occurrence of these inhibitors in the venomous snakes and to show the difference in their distribution between *Elapidae* and *Viperidae* snakes, we purified a PLA_2 inhibitor from the blood plasma of the sea krait *Laticauda semifasciata* and determined the complete amino acid sequences of its two subunits.

EXPERIMENTAL PROCEDURES

Materials—The blood plasma of *L. semifasciata* was collected at the Japanese Snake Institute. The plasma was dialyzed against 4 mM Tris-HCl buffer (pH 7.8) and lyophilized. PLA_2s from various sources were purified or purchased as described previously (*8*). *L. semifasciata* PLA_2, named PLA-I, was purified as described (*13*). *A. blomhoffii siniticus* PLIγ (*Abs*PLIγ) and *N. naja kaouthia* PLIγ (*Nnk*PLIγ) were also purified as described (*4, 11*).

Purification of the PLA_2 Inhibitor (LsPLIγ)—The lyophilized plasma (about 0.5 g) was dissolved in 10 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and 2 mM EDTA. After centrifugation, the supernatant was fractionated on a Sephadex G-200 column (3.3×90 cm) (Amersham Pharmacia Biotech, Uppsala, Sweden) that had been equilibrated with the same solvent. The fractions that inhibited PLA_2 activity were pooled and applied to a Mono Q HR 10/10 column (Amersham Pharmacia Biotech) equilibrated with the same solvent. After washing of the column, the adsorbed proteins were eluted with the same buffer containing a linear concentration gradient of NaCl from 0.1 to 0.4 M. The inhibitor fractions were dialyzed against 0.1 M Tris-HCl buffer (pH 7.5) containing 0.5 M $(NH_4)_2SO_4$, then loaded onto a Hi-Trap Phenyl Sepharose 6 FF column (low sub) (Amersham Pharmacia Biotech) equilibrated with the same buffer. The inhibitor protein was eluted with the same buffer containing a linear decreasing concentration gradient of $(NH_4)_2SO_4$ from 0.5 to 0 M. The inhibitor fractions eluted from the column were pooled and dialyzed against 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl, then further purified by passage through a Mono Q HR5/5 column equilibrated with the same solvent. The adsorbed proteins were eluted with the same buffer containing a linear concentration gradient of NaCl from 0.1 to 0.3 M.

A Superose 12 HR 10/30 column (Amersham Pharmacia Biotech) was used to determine the molecular weight of the purified inhibitor. About 20 μg of the inhibitor was applied to the column equilibrated 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 weight markers used were γ-globulin (150 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa).

Electrophoresis—Isoelectrofocusing-gel electrophoresis and SDS-polyacrylamide-gel electrophoresis were carried out with a Phast System apparatus (Amersham Pharmacia Biotech) using Phast Gel IEF 3-9 and Phast Gel Homogeneous 20, respectively. After the electrophoresis, protein bands were visualized by staining with Coomassie Blue.

PLA_2 Assays—PLA_2 was assayed fluorometrically with 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphorylcholine (10-py-PC, Molecular Probes, Eugene, OR, USA) used as a substrate according to the method of Radvanyi *et al.* (*14*). During the course of purification, the inhibitory activity was monitored by a slight modification of the method described previously (*6*). Briefly, a sample solution (20 μl) and a *L. semifasciata* PLA_2 (PLAI) solution (20 μl) were added to 1 ml of 50 mM HEPES buffer (pH 7.5) containing 10 mM $CaCl_2$ and 0.1% bovine serum albumin in a plastic cell. The reaction was started by the addition of the substrate, and the initial increase in the fluorescence intensity at 398 nm (excitation at 345 nm) was measured.

To determine the inhibition constants (K_i), we measured the enzymatic activities of various PLA_2s as described previously (*8*) in the presence of various concentrations of *Ls*PLIγ (10^{-11} to 10^{-5} M).

Deglycosylation of LsPLIγ—*Ls*PLIγ was deglycosylated by PNGase F (New England Biolabs, Beverly, MA, USA) digestion. About 100 μg of PLIγ was incubated with 25,000 U of PNGase F at 37°C for 24 or 48 h as described (*15*). Deglycosylation of PLIγ was ascertained by the decrease in the apparent molecular mass on SDS-PAGE. Deglycosylated *Ls*PLIγ thus obtained was assayed to determine its inhibitory activity as described above.

Reduction and Alkylation of the Two Subunits, LsPLIγ-A and LsPLIγ-B—The purified *Ls*PLIγ was subjected to reversed phase HPLC on a Cosmosil 5C4 AR-300 column (Nacalai Tesque, Kyoto). Two subunits, *Ls*PLIγ-A and *Ls*PLIγ-B, were separately eluted with a linear concentration gradient of acetonitrile from 24 to 48% containing 0.1% trifluoroacetic acid.

S-Pyridylethylated (PE-) derivatives of *Ls*PLIγ-A and B were prepared essentially according to Cavins and Friedman (*16*). Each subunit (200 μg) was dissolved in 300 μl of 0.5 M Tris-HCl buffer (pH 8.0) containing 6 M guanidine-HCl and 10 mM EDTA. After the addition of dithiothreitol and incubation for 3.5 h at 50°C, 5 μl of 4-vinylpyridine was added. After 5 h at room temperature, the solution was desalted on a NAP-5 column (Amersham Pharmacia Biotech) that had been equilibrated with 3% acetic acid.

The *S*-3-(trimethylated amino) propylated (TMAP-) derivative of *Ls*PLIγ-B was prepared according to Okazaki *et al.* (*17*). *Ls*PLIγ-B (200 μg) was dissolved in 250 μl of 0.5 M Tris-HCl buffer (pH 8.6) containing 8 M Urea and 5 mM EDTA. After the addition of 5 mg dithiothreitol and incubation for 5 h at 50°C, 6 mg of 3-bromopropyltrimethylammonium bromide (Sigma, St. Louis, USA) was added, and the reaction mixture was further incubated for 2.5 h at 40°C. The reaction mixture was then dialyzed against 0.5 M Tris-HCl buffer (pH 9.5).

Fragmentation of PE-LsPLIγ-A, PE-LsPLIγ-B, and

TMAP-LsPLIγ-B—PE-*Ls*PLIγ-A was digested with endoproteinase Asp-N (Boehringer Mannheim, Mannheim, Germany). Endoproteinase Asp-N digestion was carried out in 50 mM Tris-HCl buffer containing 2 M urea at 37°C for 24 h with an enzyme/substrate weight ratio of 1:300. PE-*Ls*PLIγ-A and TMAP-*Ls*PLIγ-B were cleaved with lysyl endopeptidase (Wako Pure Chemical, Osaka). Lysyl endopeptidase digestion was carried out in 0.5 M Tris-HCl buffer (pH 9.0) containing 4 M urea at 37°C for 8 h with an enzyme/substrate weight ratio of 1:200. PE-*Ls*PLIγ-B was cleaved with staphylococcal protease. Staphylococcal protease digestion was performed in 50 mM ammonium acetate (pH 4.0) at 37°C for 24 h with an enzyme/substrate weight ratio of 1:25. The peptides thus obtained were separated by reversed phase HPLC on a Vydac C4 column (The Separations Group, Hesperia, CA, USA) with 0.1% trifluoroacetic acid containing a linear gradient of acetonitrile from 0 to 48%.

Amino Acid Sequence Analysis—Amino acid sequences of PE-*Ls*PLIγ-A, PE-*Ls*PLIγ-B, and their fragments were determined by use of an Applied Biosystems model 477A protein/peptide sequencer equipped with a phenylthiohydantoin (PTH) analyzer (model 120A).

Amino Acid Analysis—Proteins and peptides were hydrolyzed with a mixture of 5.7 N HCl and 0.2% (w/v) phenol vapor in tubes sealed under vacuum at 110°C for 24 h. After evaporation, the hydrolysates were analyzed by means of an amino acid analyzer (Hitachi model L-8500).

Peptide Nomenclature—AK- and AD-refer, respectively, to lysyl endopeptidase and endoproteinase Asp-N peptides of PE-*Ls*PLIγ-A. BK- and BE-refer to lysyl endopeptidase of TMAP-*Ls*PLIγ-B and staphylococcal protease peptides of PE-*Ls*PLIγ-B, respectively.

RESULTS

Purification and Fundamental Properties of LsPLIγ—Figure 1 shows the separation profile of *L. semifasciata* plasma on Sephadex G-200. Inhibitory activity against *L. semifasciata* venom PLA_2 was found in fractions No. 72 to 88, corresponding to a 100-kDa protein. This fraction was then subjected to Mono Q HR 10/10 column chromatography, and the PLA_2 inhibitory activity was recovered in the fraction eluted with the buffer containing 0.2 M NaCl (Fig. 2). As shown in Fig. 3, this fraction was further fractionated by hydrophobic interaction chromatography using a Hi-Trap Phenyl Sepharose column. The inhibitor was eluted with the buffer containing 0 M $(NH_4)_2SO_4$. For removal of a small amount of contaminant proteins, the inhibitor fraction was further rechromatographed on the Mono Q column. The inhibitor was eluted from the Mono Q column as a single peak, and the resultant fraction was used as a purified preparation. Finally, about 1 mg of the purified inhibitor was obtained from 0.5 g of the lyophilized plasma. This inhibitor preparation showed a single protein band corresponding to an isoelectric point of 4.4 on an isoelectrofocusing gel (data not shown). As shown in Fig. 4, SDS-PAGE of the inhibitor gave two protein bands corresponding to approximate molecular masses of 25 and 20 kDa. As shown in Fig. 5, these subunits could be separated by reversed-phase HPLC. SDS-PAGE of the fractions containing peaks eluted at 29 and 38 min showed that the former peak contained the 25-kDa subunit, and the latter peak, the 20-kDa subunit. In the present study, the 25- and 20-kDa subunits were designated as A and B, respectively. The molar ratio of A and B subunits was estimated to be 2:1 from the peak areas of the chromatogram in Fig. 5. Table I shows the amino acid compositions of A and B subunits. Both subunits contained a very high amount of cysteine residues. Glucosamine was detected by the amino acid analysis of the A subunit, but it was not detected in the B subunit. Therefore, the former subunit was thought to be a glycoprotein. Since the above fundamental properties are typical of PLIγ, the purified inhibitor was regarded as *L. semifasciata* PLIγ (*Ls*PLIγ). Figure 4 also shows the comparison of SDS-PAGE of *Ls*PLIγ with those of *A. blomhoffii siniticus* PLIγ and *N. naja kaouthia* PLIγ. The subunit compositions of these PLIγs were essentially identical to each other, containing two subunits of 25 and 20 kDa, although the molecular masses of cobra PLIγ were reported to be 31 and 25 kDa in our previous study (*11*).

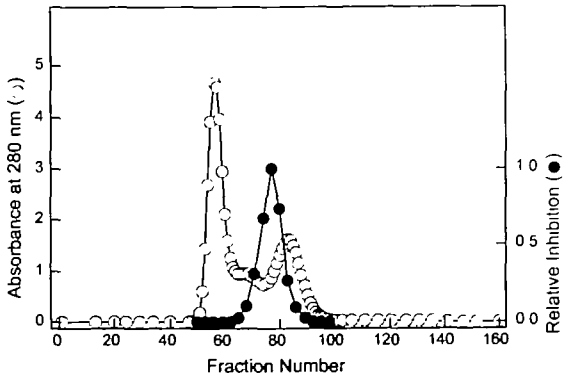


Fig. 1. **Gel filtration of the blood plasma of *L. semifasciata* on a Sephadex G-200 column.** The lyophilized plasma was dissolved in 0.1 M Tris-HCl buffer (pH 7.5) containing 2 mM EDTA and 0.1 M NaCl, and applied to a Sephadex G-200 column equilibrated with the same buffer. Each fraction contained 10 ml of the solution. ○, absorbance at 280 nm; ●, relative inhibition of *L. semifasciata* venom PLA_2.

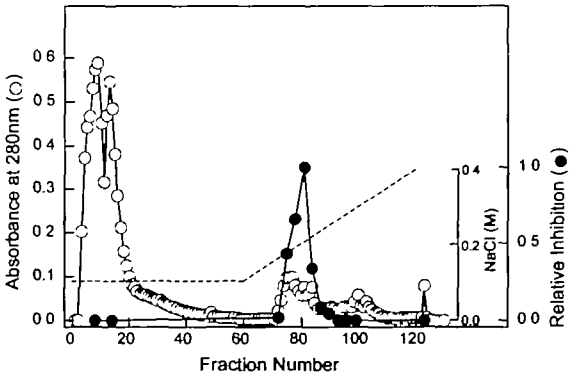


Fig. 2. **Mono Q column chromatography of the sample obtained from gel filtration.** The sample was applied to a Mono Q HR 10/10 column equilibrated with 0.1 M Tris-HCl buffer (pH 7.5) containing 2 mM EDTA and 0.1 M NaCl. The column was washed with the same buffer, and the adsorbed proteins were eluted with the same buffer containing a linear concentration gradient of NaCl from 0.1 to 0.4 M. ○, absorbance at 280 nm; ●, relative inhibition of *L. semifasciata* venom PLA_2.

This discrepancy in the apparent molecular masses for cobra PLIγ was due to the difference in the protocols used for SDS-PAGE. When SDS-PAGE of the other two PLIγs was performed according to the method of Laemmli (*18*), observed molecular masses of A and B subunits increased to 30 and 25 kDa, respectively (data not shown).

Amino Acid Sequence of LsPLIγ-A—The sequence studies on the A subunit of *Ls*PLIγ (*Ls*PLIγ-A) are summarized in Fig. 6a. The N-terminal sequence of PE-*Ls*PLIγ-A was determined by use of the sequencer up to residue 30. The subunit was digested with lysyl endopeptidase or endoproteinase Asp-N, and the resultant respective peptides were separated by reversed-phase HPLC into 9 fragments (AK-1 to AK-9) or 12 fragments (AD-1 to AD-12). By overlapping the determined sequences of the peptides, the PE-*Ls*PLIγ-A was completely sequenced. However, residue 157 of *Ls*PLIγ-A could not be detected by the sequencer. From the amino acid analysis of AK-7 and AD-10, which indicate the presence of glucosamine in these peptides, and from the sequence studies on these peptides, which suggest the presence of the Asn-X-Thr (Ser) sequence, a signal for *N*-linked glycosylation, the residue 157 of *Ls*PLIγ-A was suggested to be an asparagine linked to a glycosidic chain. *Ls*PLIγ-A was found to be composed of 182 amino acid residue with one *N*-glycosylated residue, Asn-157; and its molecular weight was calculated to be 20,284 exclusive of carbohydrate.

Amino Acid Sequence of LsPLIγ-B—The sequence studies on the B subunit of *Ls*PLIγ (*Ls*PLIγ-B) are summarized in Fig. 6b. The N-terminal sequence of PE-*Ls*PLIγ-B was determined by use of the sequencer up to residue 50. The subunit was digested with staphylococcal protease, and the peptides were separated by reversed-phase HPLC into nine fragments (BE-1 to BE-9). Since PE-*Ls*PLIγ-B was hardly soluble at neutral pH values, *Ls*PLIγ-B was alkylated with 3-bromopropyltrimethylammonium bromide to obtain the *S*-3-(trimethylated amino) propylated (TMAP-) *Ls*PLIγ-B, which could be

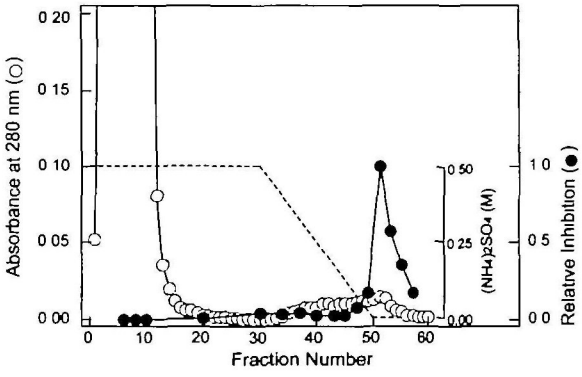


Fig. 3. **HiTrap Phenyl Sepharose column chromatography of the sample obtained from Mono Q column chromatography.** The sample, dialyzed against 0.1 M Tris-HCl buffer (pH 7.5) containing 0.5 M $(NH_4)_2SO_4$, was applied to a HiTrap Phenyl Sepharose 6 FF (low sub) column equilibrated with the same buffer. The column was washed with the same buffer, and the adsorbed proteins were eluted with the same buffer containing a decreasing linear concentration gradient of $(NH_4)_2SO_4$ from 0.5 to 0 M. ○, absorbance at 280 nm; ●, relative inhibition of *L. semifasciata* venom PLA_2.

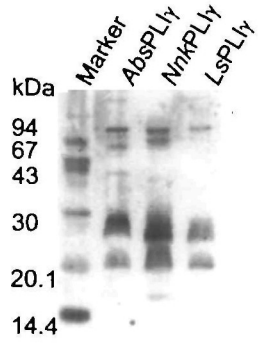


Fig. 4. **SDS-PAGE of the purified PLIγ.** *Abs*PLIγ, PLIγ from *A. blomhoffii siniticus* (*4*). *Nnk*PLIγ, PLIγ from *N. naja kaouthia* (*11*). *Ls*PLIγ, PLIγ purified from *L. semifasciata* (present study). The molecular masses (in kDa) of the markers are indicated.

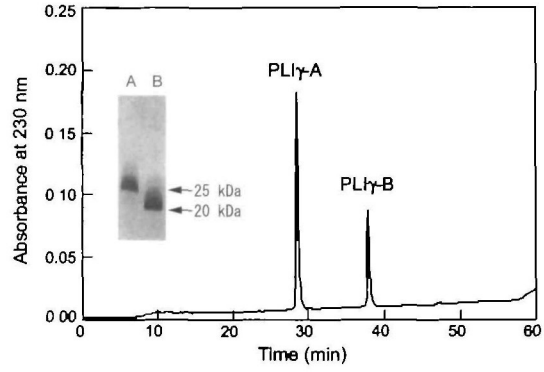


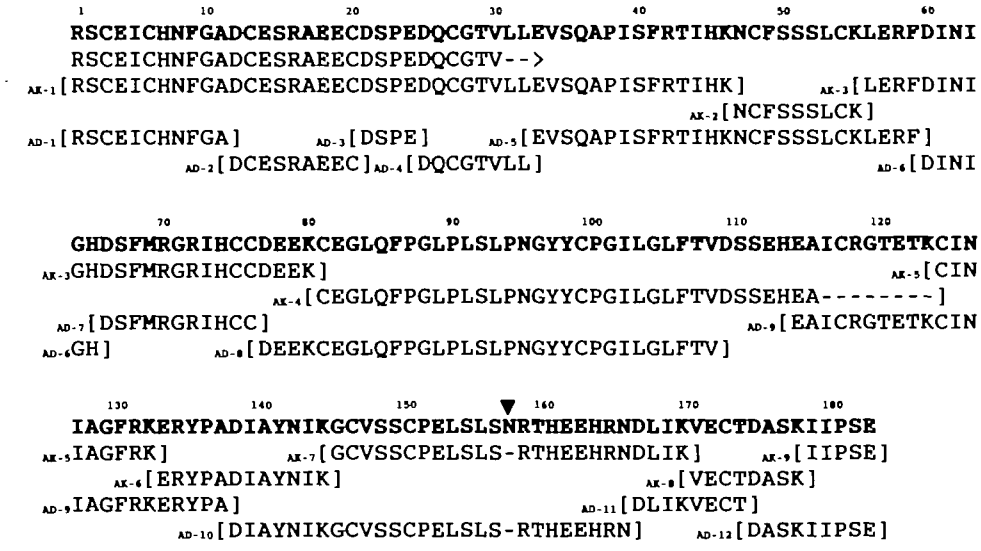
Fig. 5. **Separation of the two subunits of *Ls*PLIγ by reversed-phase HPLC.** The sample was applied to a Cosmosil 5C4 AR-300 column that had been equilibrated with 0.1% trifluoroacetic acid and eluted with the solvent containing a linear concentration gradient of acetonitrile from 24 to 48%. The inset shows the SDS-PAGE of the separated subunits.

TABLE I. **Amino acid compositions of the two subunits, *Ls*PLIγ-A and *Ls*PLIγ-B.** Values in parentheses were taken from the sequences.

Amino acid	*Ls*PLIγ-A	*Ls*PLIγ-B
Asp	16.7 (18)	14.4 (14)
Thr	6.18 (7)	17.8 (19)
Ser	15.3 (18)	14.2 (15)
Glu	20.8 (22)	22.5 (22)
Pro	9.03 (9)	11.5 (11)
Gly	11.8 (12)	10.8 (10)
Ala	7.86 (8)	7.51 (7)
Val	4.66 (5)	9.76 (10)
Met	0.98 (1)	0.00 (0)
Ile	11.8 (15)	4.60 (5)
Leu	12.5 (13)	15.3 (15)
Tyr	3.56 (4)	2.09 (2)
Phe	7.66 (8)	9.78 (10)
Lys	7.39 (8)	13.2 (13)
His	6.46 (7)	4.52 (4)
Arg	10.3 (11)	4.50 (4)
Cys[a]	14.4 (16)	15.8 (18)
Trp[b]	N.D. (0)	N.D. (2)
$GlcNH_2$[c]	+	
Total	(182)	(181)

[a]Cys was determined as pyridylethylcysteine. [b]Trp was not determined (N.D.). [c]Glucosamine was detected by amino acid analysis (+).

(a) *Ls*PLI-A



(b) *Ls*PLI-B

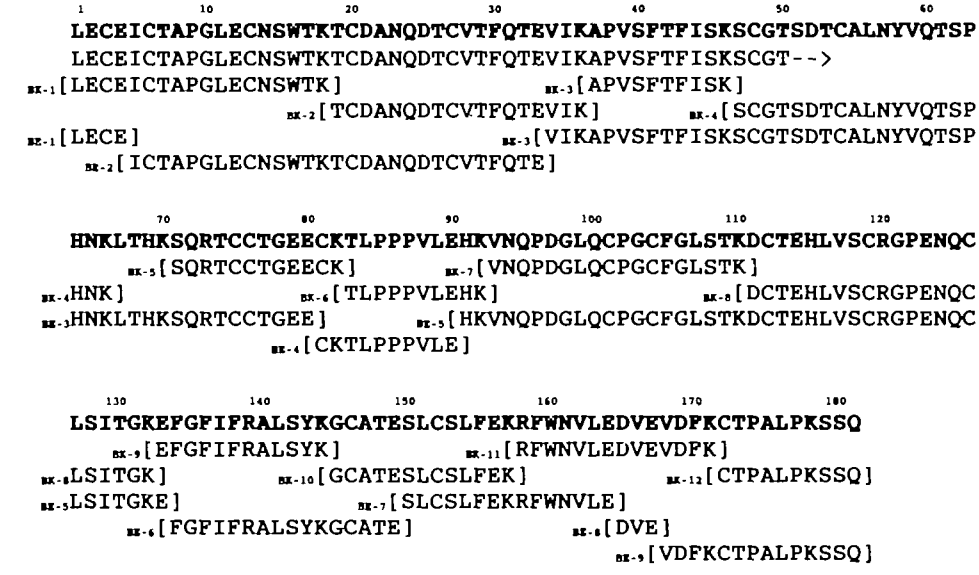


Fig. 6. **Summary of the sequence studies on *Ls*PLIγ-A (a) and *Ls*PLIγ-B (b).** Amino acid residues are given in single-letter code. Dashes indicate unidentified residues. The *N*-linked sugar chain is shown by ▼. AK- and AD-refer, respectively, to lysyl endopeptidase and endoproteinase Asp-N peptides of PE-*Ls*PLIγ-A. BK- and BE-refer to lysyl endopeptidase peptides of TMAP-*Ls*PLIγ-B and staphylococcal protease peptides of PE-*Ls*PLIγ-B, respectively.

digested with lysyl endopeptidase. The lysyl endopeptidase digests were separated by HPLC, and all the peptides obtained (BK-1 to BK-12) were completely sequenced. Finally, *Ls*PLIγ-B was found to be composed of 181 amino acid residues, giving a molecular weight of 19,921.

Inhibition Specificity of LsPLIγ toward Various PLA_2s—Inhibition by *Ls*PLIγ of the enzymatic activities of various venom PLA_2s was investigated (Fig. 7). *Ls*PLIγ strongly inhibited its own venom PLA_2 and all other PLA_2s used in the present study, including PLA_2s of groups I, II, and III. Since the enzyme concentrations used were sufficiently low (10^{-12} to 10^{-11}), the observed IC_{50} values could be regarded as the apparent inhibition constants, K_i^{app}. Table II summarizes the K_i^{app} values of *Ls*PLIγ against various PLA_2s. Unlike PLIα and PLIβ, *Ls*PLIγ showed a broad inhibition spectrum, and the K_i^{app} values were comparable to those of *Nnk*PLIγ and *Abs*PLIγ previously determined (*4, 8*).

Effect of Deglycosylation on Inhibitory Activity—To elucidate whether the carbohydrate chain of the *Ls*PLIγ-A subunit participates in the inhibitory activity, we deglycosylated PLIγ with PNGase F. After the PNGase F digestion, the apparent molecular mass of *Ls*PLIγ-A was reduced from 25 to 20 kDa on SDS-PAGE, whereas that of *Ls*PLIγ-B remained unchanged (Fig. 8). Although the *Ls*PLIγ was completely deglycosylated by incubation with PNGase F for 24 h, its inhibitory activity was unchanged, indicating no significant involvement of the carbohydrate chains of PLIγ in its inhibitory activity.

DISCUSSION

In the present study, we isolated a PLA_2 inhibitory protein corresponding to PLIγ from the plasma of the sea krait,

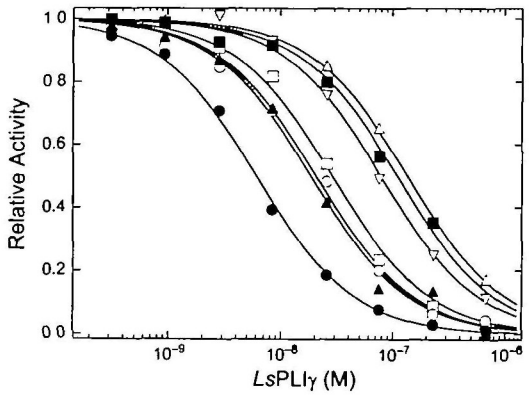


Fig. 7. **Inhibition by *Ls*PLIγ of the enzymatic activities of PLA$_2$s from various sources.** The PLA$_2$ activity was measured fluorometrically with 10-py-PC as a substrate in the presence of various concentrations of the inhibitor. ●, *L. semifasciata* PLA-I; ○, *T. flavoviridis* acidic PLA$_2$; ▲, *N. naja atra* PLA$_2$; △, *A. blomhoffii siniticus* basic PLA$_2$; ▽, *A. blomhoffii siniticus* acidic PLA$_2$; ■, *A. blomhoffii siniticus* neutral PLA$_2$; □, *N. naja kaouthia* CM-II.

TABLE II. **Apparent inhibition constants, K_i^{app}, of *Ls*PLIγ for various groups of PLA$_2$.** Group-I PLA$_2$s used in the present study were purified from the venoms of *L. semifasciata*, *Pseudechis australis*, *Naja naja kaouthia*, and *Naja naja atra*. Group-II PLA$_2$s were from the venoms of *Trimeresurus flavoviridis*, *Agkistrodon blomhoffii siniticus*, *Agkistrodon halys blomhoffii*, and *Vipera russelli russelli*. The group-III enzyme was from the honeybee, *Apis melifera*.

PLA$_2$		K_i^{app} (nM)
Group-I PLA$_2$		
L. semifasciata	PLA-I	6.14
P. australis	Pa-12A	19.5
N. naja kaouthia	CM-II	29.2
N. naja atra	PLA$_2$	18.8
Group-II PLA$_2$		
T. flavovoridis	Acidic PLA$_2$	20.8
	PL-X	76.4
A. blomhoffii siniticus	Acidic PLA$_2$	79.4
	Neutral PLA$_2$	124
	Basic PLA$_2$	138
A. halys blomhoffii	Acidic PLA$_2$	35.1
	Neutral PLA$_2$	42.3
	Basic PLA$_2$	310
V. russelli russelli	PLA$_2$-III	103
	PLA$_2$-V	71.0
Group-III PLA$_2$		
A. melifera	PLA$_2$	30.5

Laticauda semifasciata. Since no other PLA$_2$ inhibitory activities were found during the purification procedures shown in Figs. 1, 2, and 3, the serum of *L. semifasciata* seemed to contain only one type of the inhibitor, *i.e.*, that corresponding to PLIγ. Even when *A. blomhoffii siniticus* acidic PLA$_2$ was used to monitor the inhibitory activity during the course of the purification, no other fractions showing the inhibitory activity were obtained (data not shown). PLIγ seems to be indispensable to *Elapidae* snakes such as *N. naja kaouthia* and *L. semifasciata*, whereas other inhibitors, PLIα and PLIβ, which inhibit specifically group-II PLA$_2$s, are not so, since *Elapidae* snake venom contains only group-I PLA$_2$s, and no group-II PLA$_2$s (*19*).

As can be seen in Figs. 4 and 5, *L. semifasciata* PLIγ (*Ls*PLIγ) was composed of two subunits, PLIγ-A and PLIγ-B, with an approximate molar ratio of 2:1. Since the

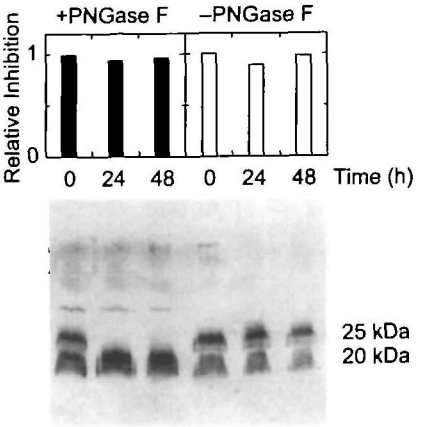


Fig. 8. **Effect of PNGase F treatment of *Ls*PLIγ on the PLA$_2$ inhibitory activity.** *Ls*PLIγ was incubated in the presence or absence of PNGase F at 37°C. After the indicated time intervals, the samples were tested for the inhibitory activity (upper) and the molecular weight by SDS-PAGE (lower). The inhibitory activity was expressed as relative inhibition of the initial velocity of the hydrolysis of 10-py-PC catalyzed by *A. blomhoffii siniticus* PLA$_2$.

subunit compositions were also retained in *A. blomhoffii siniticus* PLIγ (*Abs*PLIγ) and *N. naja kaouthia* PLIγ (*Nnk*PLIγ), both subunits may be responsible for the binding to and inhibition of PLA$_2$. On the contrary, there have also been reports of PLIγ-like inhibitors consisting of a single component. *Crotalus* neutralizing factor (CNF), a PLA$_2$ inhibitor purified from the plasma of the South American rattlesnake (*Crotalus durissus terrificus*), has been reported to be an oligomeric aggregate of only one component, which corresponds to PLIγ-A (*20*). Likewise, PLI-I was isolated from the serum of the habu (*Trimeresurus flavoviridis*), and the sequence corresponded to that of one subunit of PLIγ, PLIγ-A, and the other subunit was not identified (*21*). In the case of CNF, SDS-PAGE of the final active preparation (called CNF$_2$ in the original paper) showed an additional 20-kDa protein band; and further purification of CNF$_2$ by reversed-phase HPLC caused the loss of its inhibitory activity (*22*). Therefore, it is likely that the 20-kDa protein in the CNF$_2$ preparation was not a contaminant protein but was the other subunit of PLIγ corresponding to PLIγ-B. In the case of the *T. flavoviridis* inhibitor, we have purified PLIγ from the plasma of this snake by the same methods as described in the present study, and found that *T. flavoviridis* PLIγ was also composed of two subunits, PLIγ-A (PLI-I) and PLIγ-B, just like other PLIγs (data not shown). Therefore, all the venomous snakes, including *Elapidae* and *Crotalinae*, are likely to contain PLIγ in their sera, which is generally composed of two subunits, PLIγ-A and PLIγ-B, as one of the neutralizing factors against their venom PLA$_2$s.

PLIγ-A was found to be a major component of PLIγ and a glycoprotein with one *N*-linked oligosaccharide chain. Treatment of *Ls*PLIγ with PNGase F, which releases *N*-linked oligosaccharides from glycoproteins, resulted in a reduction of the apparent molecular mass of PLIγ-A from 25 to 20 kDa in SDS-PAGE (Fig. 8). The observed molecular mass of the deglycosylated PLIγ-A was consistent with that calculated from the amino acid sequence of the subunit determined in the present study. However, the inhibitory

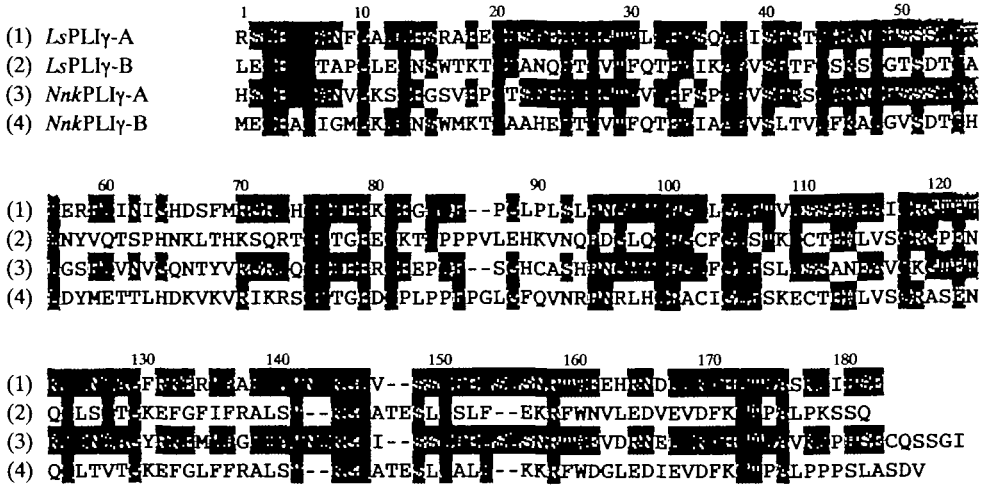


Fig. 9. **Comparison of the amino acid sequences of the two subunits of *L. semifasciata* PLIγ, *Ls*PLIγ-A and *Ls*PLIγ-B, with those of *N. naja kaouthia* PLIγ, *Nnk*PLIγ-A and *Nnk*PLIγ-B.** The stippled boxes indicate the amino acids identical to those of *Ls*PLIγ-A. The Protein Identification Resource (PIR) database accession numbers of *Nnk*PLIγ-A and *Nnk*PLIγ-B are JC2393 and JC2394, respectively.

activity of the PLIγ was not affected by the treatment with PNGase F, suggesting that no *N*-linked oligosaccharides of PLIγ-A are involved in the interaction between PLIγ and PLA_2.

We have already reported that PLIα specifically inhibited the group-II acidic PLA_2s and that PLIβ selectively inhibited only group-II basic PLA_2s from *Crotalinae* venom (*4, 8*). On the other hand, PLIγ showed a broad inhibition spectrum and inhibited all groups of $sPLA_2$s. As shown in Fig. 7, the inhibition spectrum of *Ls*PLIγ was broad, similar to that of *Nnk*PLIγ and *Abs*PLIγ. Like other PLIγs, the *Ls*PLIγ inhibited all the groups of $sPLA_2$s with K_i values from 10^{-9} to 10^{-7} M (Table II). Therefore, the broad inhibition spectrum against $sPLA_2$s can be considered as a common feature of PLIγ. This broad inhibition spectrum of PLIγs may be explained by the interaction of PLIγ with a common structural element of the PLA_2 molecule, perhaps the calcium-binding loop, which is conserved among all groups of PLA_2s.

In Fig. 9, the amino acid sequences of the two subunits of *L. semifasciata* PLIγ, *Ls*PLIγ-A, and *Ls*PLIγ-B, are compared with those of *N. naja kaouthia* PLIγ (*12*). The *Ls*PLIγ-A had 73.6% sequence identity with the 31-kDa subunit of *N. naja kaouthia* PLIγ (designated as *Nnk*PLIγ-A in the present study), and the *Ls*PLIγ-B had 64.6% sequence identity with the 25-kDa subunit (designated as *Nnk*PLIγ-B), although there is 32.2% sequence identity between *Ls*PLIγ-A and *Ls*PLIγ-B. The *N*-linked glycosylation site at Asn-157 was also conserved between *Ls*PLIγ-A and *Nnk*PLIγ-A. Therefore, it is apparent from these data that a gene duplication of the PLIγ leading to PLIγ-A and PLIγ-B subunits, occurred before the divergence of these snakes.

Like those of *Nnk*PLIγ, the respective subunits of *Ls*PLIγ contain two intramolecular repeats of cysteine-rich domains, which are commonly found in a structurally related family of proteins including urokinase-type plasminogen activator receptor (uPAR), CD59, Ly-6, and snake venom neurotoxins (*12*). Most of these members contain a single cysteine-rich domain, except for uPAR, which contains three intramolecular repeats of the cysteine-rich domain (*23*). Recently, a novel protein named RoBo-1, which was selectively expressed in rat bone and growth plate cartilage, was reported to have sequence homology with PLIγ (*24*). RoBo-1 contains two internal repeats of the cysteine-rich domain, similar to PLIγ. Although the function of RoBo-1 remains to be elucidated, it might be that RoBo-1 can inhibit PLA_2 and thus be a mammalian PLIγ. Besides having a self-defense role in venomous snakes, PLIγ might have other important physiological roles in regulating local PLA_2 activity.

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