Structural Analysis of Two Isoforms of Subunit B in Phospholipase A_2 Inhibitor PLIγ from the Serum of Habu, *Trimeresurus flavoviridis*

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Abstract

Two distinct types of phospholipase A_2 inhibitors (PLIs), PLIα and PLIγ, are present in the serum of Habu (*Trimeresurus flavoviridis*). It is expected that, like other PLIγs, *T. flavoviridis* PLIγ may contain two homologous subunits, A- and B-chains (also called α- and β-chains). Five proteins, termed PLI-I to PLI-V, were identified as the constituting subunits of two types of PLIs. Based on the primary structures and the molecular sizes, PLI-I was assigned to be the subunit A of *T. flavoviridis* PLIγ. In order to prove that PLI-II and PLI-III are the subunit B, we have purified these proteins from the same serum, and determined the complete amino acid sequences by peptide analysis. The cDNA encoding PLI-II was also cloned from a cDNA library of the *T. flavoviridis* liver. The respective nucleotide sequence encoded 19-residue signal sequences, followed by 181-residue proteins. Both PLI-II and PLI-III were glycosylated at Asn^{14}, and showed a high homology to several B-chains of PLIγ identified in other snakes. Gel filtration analysis showed that all the PLIs exist in the *T. flavoviridis* serum as the high molecular forms of more than 60 kDa, and PLI-II and PLI-III also associate to form the corresponding homodimers.

Key words: amino acid sequence, cDNA cloning, phospholipase A_2 inhibitor, snake serum, *Trimeresurus flavoviridis*

INTRODUCTION

Phospholipase A_2 (EC 3.1.1.4) is the major component of most snake venoms [1], and the detailed insight into the structure and mechanism of these enzymes is available [2]. It catalyzes the hydrolysis of phospholipids at its *sn*-2 position. Snake venom PLA_2 induces several pharmacological effects including presynaptic neurotoxicity, myotoxicity and cardiotoxicity as well as anticoagulation, hemolysis, hemorrhage, edema-induction and platelet-aggregation inhibition [3–5]. Habu (*T. flavoviridis*) inhabiting in the southwestern islands of Japan contains several PLA_2s and the related

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Abbreviations: BPI, basic protein I; BPII, basic protein II; MALDI, matrix-assisted laser desorption ionization; PLA2, phospholipase A2; PLI, phospholipase A2 inhibitor; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TOF, time-of-flight; UTR, untranslated region.

proteins such as acidic [Asp^{49}]-PLA_2s (PLA2 and PLA-N), a basic [Asp^{49}]-PLA_2 (PLA-B), and basic proteins I and II (BPI and BPII) [5–8].

Venomous snakes have PLA_2 inhibitors (PLIs), proteins capable of inhibiting snake venom PLA_2s, in their blood sera [9]. The role of these proteins is postulated to protect themselves from the leakage of their own venomous PLA_2s into their circulatory system. Many PLIs have been isolated from various snake sera and their primary structures have been determined [10–14]. PLIs can be divided into three groups (PLIα, PLIβ, and PLIγ) based on their structural characteristics. PLIαs are 75–100-kDa glycoproteins composed of three or four subunits and have the carbohydrate-recognition domain of Ca^{2+}-dependent (C-type) lectins [12,16]. PLIβ is a 160-kDa glycoprotein composed of three identical 50 kDa subunits with leucine-rich repeats [17]. PLIγs are oligomeric proteins consisting of glycosylated subunit A and non-glycosylated subunit B [12–14,18]. The subunit B of *Elaphe quadrivirgata* PLIγ, however, contains a suger chain at residue 12 [19]. The primary structure of both subunits has dual three-finger motifs and is related to the Ly-6 superfamily [20].

Five proteins named PLI-I to PLI-V in *T. flavoviridis* serum have already been identified by affinity chromatography [21]. PLI-I corresponds to a subunit A of PLIγ, and the cDNA has already been cloned [21]. PLI-IV and PLI-V are the member of α type PLIs, and identical to PLI-A and PLI-B [10], respectively. The structures of PLI-II and PLI-III have not yet known. In the present study, we have isolated two PLA_2 inhibitors, PLI-II and PLI-III, from *T. flavoviridis* serum, and determined their complete amino acid sequences by peptide sequencing as well as by cDNA cloning of PLI-II. We also discuss the subunit structure of *T. flavoviridis* PLIγ.

MATERIALS AND METHODS

Materials: The blood of *T. flavoviridis* in the Amami Oshima islands was collected by decapitation. The serum was separated by centrifugation and stored at −20℃. Ethanol precipitation was carried out as described [22]. All other reagents were purchased from Wako Pure Chem. (Osaka).

Electrophoresis: SDS–PAGE was carried out on 12.5% gels by Laemmli's method [23], or on 10% polyacrylamide gels as described by Schagger and von Jagow [24]. Bovine serum albumin (67,000), ovalbumin (46,000), carbonic anhydrase (30,000), and chymotrypsinogen (24,000) were used as molecular weight markers. Prestained XL-ladder marker kit (Apro science) was also used. After running the gels under a constant current, they were stained with 0.1% Coomassie brilliant blue R-250 and destained with 10% acetic acid.

Mass spectrometric analysis: Mass spectrum was measured on a Voyager DE-STR matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) mass spectrometer (PerSeptive Biosystems). Sample was dissolved in 0.1% trifluoroacetic acid –50% acetonitrile containing α-cyano-4-hydroxycinnamic acid (10 mg/ml) as the matrix, and 2-μl aliquots were analyzed. Spectrum was calibrated by the molecular mass of apomyoglobin.

Reverse-phase HPLC: Preparative reverse-phase HPLC was performed on a μBondashere 5μ-C8-300Å column (1.9×15 cm, Waters), and the proteins were eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA) at a flow rate of 5 ml/min.

Protein sequencing: Protein was reduced

and *S*-pyridylethylated (PE) according to Friedman *et al.* [25] The PE-protein (500 μg each) was digested at 37°C with bovine trypsin (E/S＝1:50) for 24 h in 20 mM Tris–HCl (pH 9.0), Lys-C (E/S＝1:100) for 5 h in 2 M urea-20 mM Tris–HCl (pH 9.0), α-chymotrypsin (E/S＝1:50) for 16 h in 1 M urea–50 mM NH_4HCO_3, Asp-N (E/S＝1:100) for 16 h in 50 mM Tris–HCl (pH 8.0), V8 protease (E/S＝1:30) for 16 h, or cleaved at room temperature by CNBr (100 equivalents) for 21 h in 70% HCOOH. The digests were lyophilized, dissolved in 0.1% TFA, and fractionated by reverse-phase HPLC on a YMC-Pack ODS column in 0.1% TFA with an appropriate gradient of acetonitrile. The amino acid sequences of proteins were determined by an automatic protein sequencing system PPSQ 21 (Shimadzu).

Analytical gel filtration: A sample was dissolved in 0.2 M NaCl-50 mM phosphate buffer (pH 7.0), and put on a TSKgel G3000SW column (0.75×30 cm, Tosoh). Elution was carried out by the same buffer at a flow rate of 1.0 ml/min. Elution was monitored at 280 nm and the peak area was determined by 807-IT integrator (Jasco). Molecular weight was calibrated by the retention times of bovine serum albumin (67,000), ovalbumin (46,000), and soybean trypsin inhibitor (21,500).

Synthesis of partial fragment of PLI cDNA. Total RNA was extracted from 0.5 g of *T. flavoviridis* liver by acid guanidinium-phenol-chloroform (AGPC) method, and reverse transcribed to synthesize cDNA first strands using adaptor-linked Oligo(dT) primer (5'-GGCCACGCGTCGACTAGTAC-$(dT)_{17}$-3'). cDNA obtained was used as the template for 3'-RACE (rapid amplification of cDNA ends) reaction. Synthetic oligonucleotide, *PLI-II_NS* (5'-CCNGGNGARACNTGYAAYGGNACNATGATG-3') and 3'-Adp (5'-GGCCACGCGTCGACTAGTAC-3'), were used for PCR amplification. *PLI-II_NS* primer was designed upon N-terminal amino acid sequence of PLI-II, and 3'-Adp corresponded to the adaptor sequence within adaptor-linked oligo(dT) primer. Amplification product was once subcloned into plasmid vector, and its nucleotide sequence was determined. As the result, the sequence was confirmed to be the cDNA partial fragment for PLI-II. PLI-II cDNA fragment was radiolabeled with [α-^{32}P]-dCTP (3000 Ci/mmol) using random primer DNA labeling kit (Takara Bio) and used for hybridization screening of cDNA library.

Construction of T. flavoviridis liver cDNA library: T. flavoviridis liver cDNA library was constructed using Creator SMART cDNA Library Construction kit (BD Biosciences) according to manufacturers' instruction. Briefly, cDNA first strand was synthesized using 1 μg of total RNA, followed by five cycles of PCR for non-specific enrichment of full-length cDNAs. The cDNA fragments were then ligated to pDNR-LIB vector. When the plasmid clones were used to transform *E. coli* JM109, resulting library contained 2.0×10^6 independent clones.

Cloning and sequence determination of cDNA encoding PLI-II: Clones (9.6×10^4) from unamplified cDNA library were plated on LB agar plates and bacterial colonies were transferred onto Hybond-NX membranes (GE Healthcare Bio-Science) and fixed by UV irradiation. The resulted replica membranes were prehybridized in Church's hybridization solution at 65℃ for 30 min and then hybridized with radiolabeled PLI-II cDNA overnight at 50℃ in Church's hybridization solution. Membranes were finally washed twice for 15 min at 50℃ with 1× SSC, 0.1% SDS and hybridization signals were visualized using

BioImage Analyzer (Fuji Film). Accordingly, 14 bacterial colonies were isolated, cultured and their plasmids were purified by standard Alkali-SDS method. Nucleotide sequences of cDNA inserts were determined using ABI PRISM 377 DNA Sequencing System (Applied Biosystems).

RESULTS

Purification of phospholipase A_2 inhibitors: Ethanol fraction $E_{2.0}$ of the *T. flavoviridis* serum was prepared as described previously [22], and subjected to reverse-phase HPLC using a C8 column. Among several peaks observed in Fig. 1A, five peaks (I−V) contained the PLI-related proteins as analyzed by SDS-PAGE and the N-terminal sequencing. Since the protein in peak III was not homogeneous on SDS-PAGE (Fig. 1B), it was further purified by gel filtration using a Sephacryl S-200HR column (data not shown). From the N-terminal sequence analysis, peaks I, IV, and V were assigned to be PLI-I [21], PLI-A, and PLI-B [10], respectively. We assumed that peaks II and III corresponded to PLI-II and PLI-III because of their molecular mass as well as the relative retention time on reverse-phase HPLC [21]. The apparent molecular masses of the purified PLI-I, PLI-II, and PLI-III were estimated to be 24, 24, and 25 kDa by SDS-PAGE, respectively (Fig. 1C). The exact masses were determined by MALDI–TOF mass spectrometry to be 22,653.9, 22,339.0, and 22,491.0 for PLI-I, PLI-II, and PLI-III, respectively.

Sequence analysis of PLI-II and PLI-III: The N-terminal sequences of *S*-pyridylethylated PLI-II and PLI-III were directly determined and shown in Fig. 2. Both proteins had a common sequence of XXCXXC, where X is any amino acid residue other than Cys. This agreed with the structural

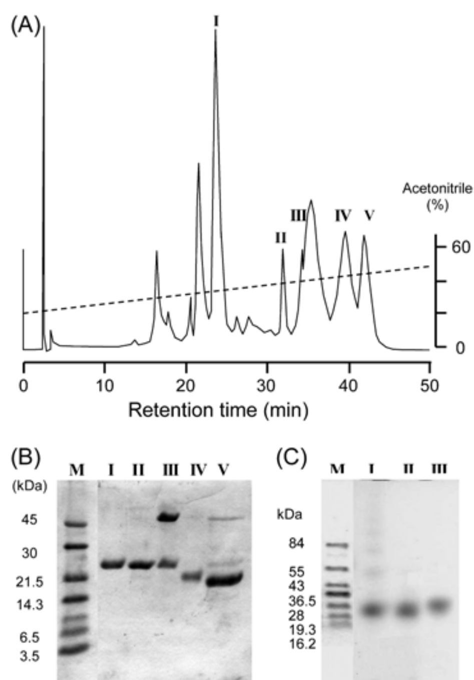

Fig. 1. (A) Purification of PLA_2 inhibitors by reverse-phase HPLC on a μBondashere 5μ-C8-300Å column (1.9×15 cm). (B) SDS-PAGE analysis of proteins in peak I–V in Fig. 1A. 12.5% gel was used. M, marker proteins. (C) SDS-PAGE analysis of purified proteins on a 10% gel. M, prestained XL-ladder marker; I, PLI-I; II, PLI-II; III, PLI-III.

feature of N-terminals in all the PLIγs. The complete amino acid sequences of two inhibitors were determined on the basis of the results of sequence analysis of peptide fragments generated by several enzymatic and chemical digestions. The sequence of PLI-II consisting of 181 amino acids was determined by the combination of BrCN-cleavage and tryptic digestion (Fig. 2A). A residue 14 could not be determined by Edman degradation, suggesting that it might be the Asn residue having a carbohydrate chain.

The entire primary structure of PLI-III

was also determined similarly by several enzymatic digestions using Lys-C, chymotrypsin, and V8 protease. The arrangement of the fragments and the complete amino acid sequence are summarized in Fig. 2B. PLI-III was a very similar protein to PLI-II having 181 residues. It may also have a sugar chain at residue 14.

Cloning of cDNA encoding PLI-II: T. flavoviridis liver cDNA was synthesized using an adaptor-linked oligo(dT) primer. Partial cDNA fragment for PLI-II was then amplified by PCR using the oligonucleotide *PLI-IIa_NS* that was originally designed based on N-terminal amino acid sequence of PLI-II (Fig. 2). As a result, a DNA fragment of the expected size (approx. 0.7 kb) was obtained. The nucleotide sequence was confirmed to be the partial fragment of cDNA in accord with the amino acid sequences of PLI-II. The cDNA library was screened with PLI-II DNA fragment as a probe. Six positive clones were obtained from 9.6×10^4 plaques by washing in high stringency (0.2 ×SSC containing 0.1% SDS at 65℃). Fig. 3 shows the nucleotide sequence of full-length cDNA for PLI-II. It consisted of 920 bp, including a 118-bp 5'-untranslated region (UTR), 603-bp open reading frame encoding 200 amino acids including a potential signal peptide of 19 amino acids, and 202-bp 3'-UTR. The nucleotide sequence in the mature protein-coding region of PLI-II showed 53.9% identity to PLI-I [21]. Amino acid sequence deduced from the nucleotide sequence of PLI-II cDNA was agreed well to the protein sequence as cited in Fig. 2.

Comparison of amino acid sequences of PLIs: The amino acid sequences of *T. flavoviridis* PLI-II and PLI-III were compared with those of several subunit proteins that constitute PLIγs from the Elapidae, Hydrophidae, Boidae, and Colubridae families (Fig. 4). Sequences of subunits A and B are separately shown and the conserved residues are also marked separately. Nev-

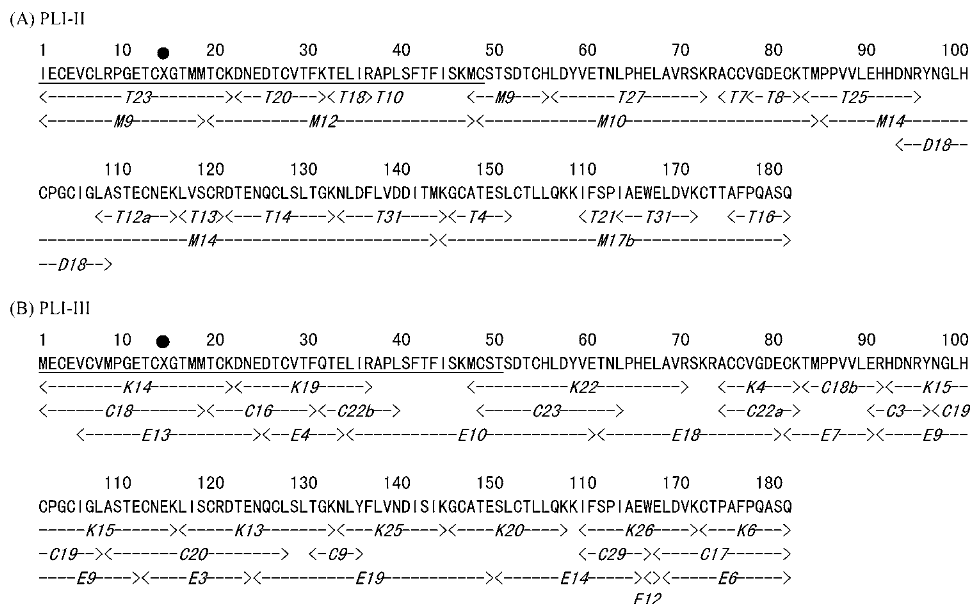

Fig. 2. Sequence determination of *T. flavoviridis* phospholipase A_2 inhibitors. Sugar-attached Asn residues are marked by closed circles. Sequences determined directly from the intact proteins are underlined.

ertheless, most of the Cys residues are located at the same positions in both subunits except Cys[105] and Cys[113] of subunit B. The *T. flavoviridis* PLI-I has the common features of PLIγ-subunit A: the presence of highly conserved 16 cysteine residues and N-linked carbohydrate site at Asn[157] (the position 158 in the sequence (1) in Fig. 4A). It showed the highest similarity to *Agkistrodon blomhoffii siniticus* PLIγ-subunit A (87% identity; the sequence (2) in Fig. 4A) [26].

On the other hand, the *T. flavoviridis* PLI-II and PLI-III were highly homologous (more than 53% identities) to other subunits B of PLIγs (Fig. 4B). In addition, all the cysteine residues were invariable. A sugar chain is attached at residues 14 of *T. flavoviridis* PLIγ-B (PLI-II and PLI-III) as well as at residue 12 of *E. quadrivirgata* PLIγ-B [19]. Although *G. blomhoffii siniticus* PLIγ-B has a putative glycosylation site at residue 14, the protein was not glycosylated [17]. Other proteins have no sugar chain.

Oligomer formation of PLIs: Analytical gel filtration using a TSKgel G3000SW column showed that PLI-I, PLI-II, and PLI-III were eluted at 9.07, 8.84, and 9.13 min which corresponded to the molecular masses of 25,

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  1 TGCCCATTGAATGATAGCATCTGGTACTCTCTGAGAAAAATTGCTGGCTGCTGCTTCTTG
 61 AACGTCTACGTATTTTTAATCCAGATTGACCTGGGCATCAATCACCTTGTTCTCCACCAT
                                                             M
121 GAAGTCCCTCTTATTCTGCTGCCTCTTGGGCATTTTCTTAGCTACAGGCATGTGTATTGA
      K  S  L  L  F  C  C  L  L  G  I  F  L  A  T  G  M  C  I  E
181 GTGTGAAGTTTGTTTGAGGCCTGGTGAGACTTGCAACGGTACCATGATGACCTGTAAAGA
      C  E  V  C  L  R  P  G  E  T  C  N  G  T  M  M  T  C  K  D
241 TAATGAAGATACTTGCGTGACGTTTAAAACTGAACTGATAAGAGCTCCTCTGTCCTTCAC
      N  E  D  T  C  V  T  F  K  T  E  L  I  R  A  P  L  S  F  T
301 TTTCATTTCAAAAATGTGCAGCACGTCTGACACCTGCCATCTTGACTACGTGGAGACAAA
      F  I  S  K  M  C  S  T  S  D  T  C  H  L  D  Y  V  E  T  N
361 TCTACCACATGAACTGGCAGTGAGGTCCAAAAGAGCGTGCTGTGTTGGGGATGAATGTAA
      L  P  H  E  L  A  V  R  S  K  R  A  C  C  V  G  D  E  C  K
421 AACTATGCCGCCTGTTGTGCTTGAACATCACGACAATCGTTACAATGGACTCCATTGTCC
      T  M  P  P  V  V  L  E  H  H  D  N  R  Y  N  G  L  H  C  P
481 TGGATGCATTGGACTCGCCTCAACTGAATGCAATGAAAAACTGGTTTCCTGCCGGGATAC
      G  C  I  G  L  A  S  T  E  C  N  E  K  L  V  S  C  R  D  T
541 TGAAAACCAGTGTTTGTCTCTAACTGGGAAGAACCTAGATTTCTTGGTCGATGATATAAC
      E  N  Q  C  L  S  L  T  G  K  N  L  D  F  L  V  D  D  I  T
601 TATGAAAGGATGCGCTACTGAGAGTTTGTGCACTTTACTTCAGAAGAAGATCTTCTCACC
      M  K  G  C  A  T  E  S  L  C  T  L  L  Q  K  K  I  F  S  P
661 TATAGCGGAATGGGAACTAGATGTCAAATGTACTACAGCTTTCCCACAGGCTTCCCAGTG
      I  A  E  W  E  L  D  V  K  C  T  T  A  F  P  Q  A  S  Q  *
721 ATGTATAACTGCACGTCAAGGTCATCGCTTCCACCTTACTTATGCTTAGGAGCCAACATG
781 GCTGATTTTGTTTTAGTCAGCTTTCAGCAGAATTCTTGTTCTTCTGGATGTTTCCCACAA
841 TGAATGTGAAAACTAACTGAACCATGGTTCAATCAATAAAGCAGTTACTTTAAAAAGAAA
896 AAAAAAAAAAAAAAA
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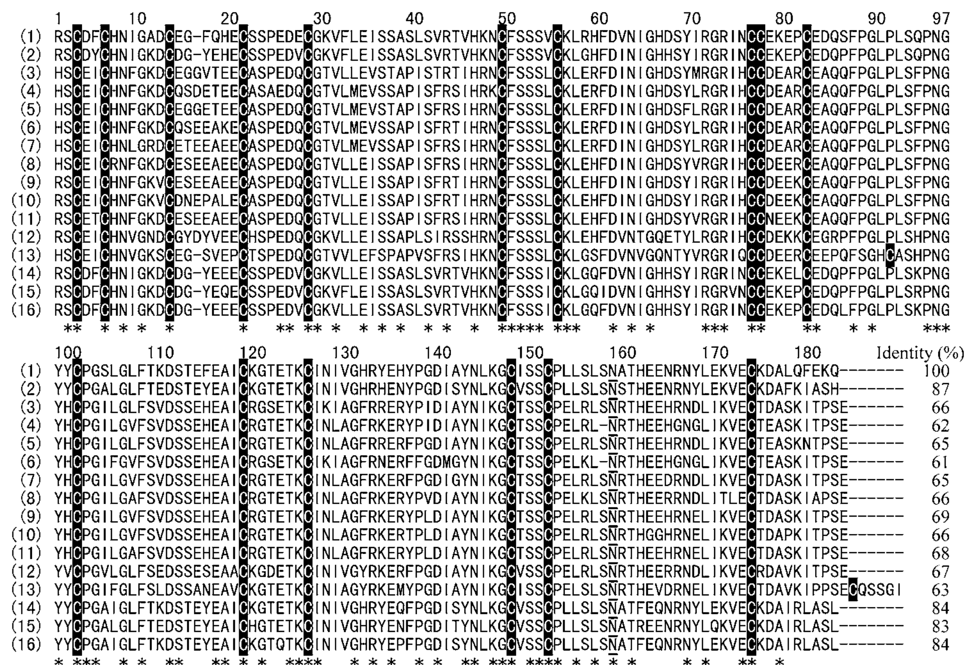
Fig. 3. Nucleotide sequence of PLI-II. Deduced amino acid sequence is cited below the nucleotide sequence. Start and stop codons are boxed. Polyadenylation signal is underlined.



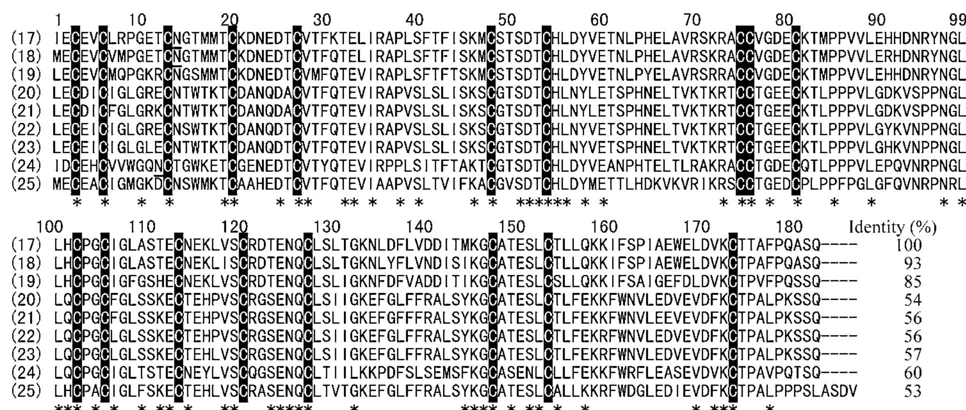


Fig. 4. Comparison of the amino acid sequences of γ-type phospholipase A$_2$ inhibitors. (A) Subunits A, (1) [accession No. BAA24503] *T. flavoviridis* PLI-I (subunit A), (2) [BAA86970] *Gloydius blomhoffii siniticus* subunit A, (3) [AAF23778] *Notechis ater* α-subunit 1, (4) [AAF23779] *Notechis ater* α-subunit 2, (5) [AAF23780] *Notechis ater* α-subunit 3, (6) [CAB56616] *Notechis scutatus* α-subunit ii, (7) [CAB56617] *Notechis scutatus* α-subunit iii, (8) [AAF23781] *Oxyuranus scutellatus* α-subunit 1, (9) [AAF23782] *Oxyuranus scutellatus* α-subunit 2, (10) [AAF23783] *Pseudonaja textilis* α-subunit, (11) [AAF23785] *Oxyuranus microlepidotus* α-subunit 2, (12) [BAA83078] *Elaphe quadrivirgata* subunit A, (13) [AAB32582] *Naja kaouthia* 31 kDa subunit, (14) [AAA19162] *Crotalus durissus terrificus* CNF, (15) [AAR04437] *Lachesis muta muta* α-subunit 1, and (16) [AAR04438] *Lachesis muta muta* α-subunit 2. (B) Subunits B, (17) *T. flavoviridis* subunit B1 (PLI-II), (18) *T. flavoviridis* subunit B2 (PLI-III), (19) [BAA86971] *Gloydius blomhoffii siniticus* subunit B, (20) [AAF21051] *Pseudonaja textilis* β-subunit 2, (21) [AAF21050] *Pseudonaja textilis* β-subunit 1, (22) [AAF21047] *Oxyuranus scutellatus* subunit B, (23) [AAF21046] *Notechis ater* subunit B, (24) [BAA83079] *Elaphe quadrivirgata* subunit B, (25) [AAB32583] *Naja kaouthia* 25 kDa subunit. Cysteine residues are indicated in white-on-black type. Conserved residues in each of subunit A and B are marked by asterisks. Carbohydrate-attached Asn residues are underlined.

30, and 24 kDa, respectively, and also gave an additional peak corresponding to the molecular mass of 100, 56, or 50 kDa, respectively (data not shown). These results indicate that PLI-I is likely to exist as an oligomer, and PLI-II and PLI-III prefer to form the homodimers.

When *T. flavoviridis* serum was analyzed by gel filtration under the same conditions, four protein peaks were eluted at 5.00, 6.64, 7.54, and 11.68 min (Fig. 5A). The last peak contained no protein. To examine the presence of PLIs in these peaks on gel filtration, effluents from the column were divided into five fractions (F1 to F5) as shown in Fig. 5A, and the fractions were subjected directly to an analytical reverse-phase HPLC. As shown in Fig. 5B, no monomeric PLI was found in fraction F5. Most of the PLIs were detected in fractions F3, and a small amount in F2 and F4 as well. The major components of *T. flavoviridis* serum proteins in these fractions are summarized in Table 1. The apparent molecular-mass range of F3 was a range from 65 to 130 kDa. This result clearly indicates that all of the PLIs are not monomeric but exist in the high-molecular-mass form in the serum as previously reported [9].

DISCUSSION

Two groups of PLIs have been identified in the serum of *T. flavoviridis*, PLIα and PLIγ [21]. PLIγ have the two repeats of a unit termed the three-finger motif [20], which has also been found in urokinase-type plasminogen activator receptor [27], Ly-6 [28], CD59 [29] and neurotoxins [30]. In the present study, we have purified five proteins (PLI-I to PLI-V) from the serum of *T. flavoviridis*. Though the fraction III contained two proteins (Fig. 1B), a pure PLI-III was obtained after a contaminating 47-kDa protein was removed by gel filtration. This 47-kDa protein was identified to be a novel HSF-like protein (unpublished data). Analytical gel filtration showed that the resultant each of PLI-I, PLI-II, and PLI-III gave an additional peak in the molecular mass range from 50 to 100 kDa being attributable to its dimer or oligomer.

Sequence analysis showed that PLI-IV and PLI-V corresponded to PLI-A and PLI-B [10], respectively, and are known to

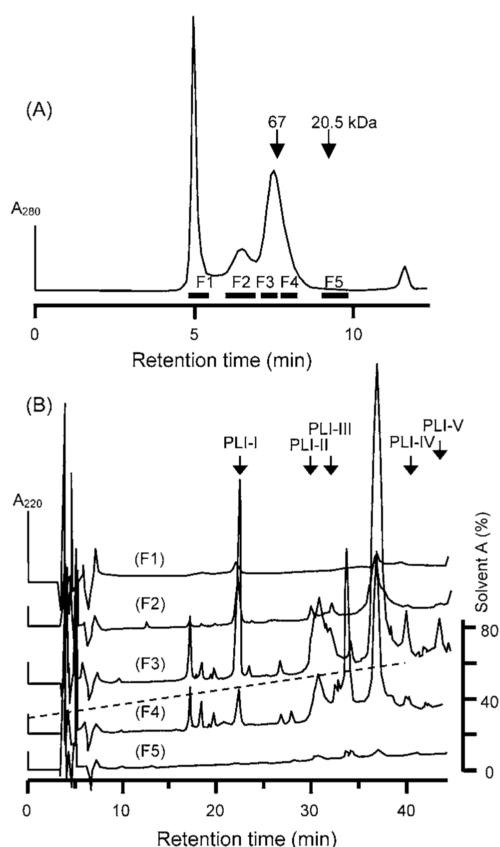


Fig. 5. Analysis of molecular sizes of PLIs. (A) Analytical gel filtration of *T. flavoviridis* serum on a TSKgel G3000SW column (0.75×30 cm) in 0.2 M NaCl–50 mM phosphate buffer (pH 7.0). (B) Reverse-phase HPLC of gel filtration fractions on a SepaxBio-C8 column (0.46×25 cm). Arrows show the retention times of several PLIs.

Table 1. Characterization of fractions of *T. flavoviridis* serum on an analytical gel filtration.

Fraction	Molecular size (kDa)[a)]	Components[b)]
F1	>500	High molecular mass proteins
F2	130–340	Globulins
F3	65–130	Serum albumin, PLI-I, PLI-II, PLI-III, PLI-A, PLI-B, HSF
F4	30–65	serotriflin, SSP-1 to SSP-5
F5	18–30	Low molecular mass compounds

a) The column was calibrated by thyroglobin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), bovine serum albumin (67 kDa), ovalbumin (46 kDa), soybean Kunitz trypsin inhibitor (20.5 kDa), and SSP-1 (1.0 kDa).
b) HSF, habu serum factor [22]: SSP, small serum protein.

be the subunits of α-type PLIs. Other proteins, PLI-I to PLI-III, were assigned to the subunits of PLIγ. According to Nobuhisa *et al.* [21], PLI-I is a major component of inhibitory proteins against three basic PLA_2 isozymes in *T. flavoviridis* venom, that is, a basic PLA-B, BPI and BPII [7,8]. PLI α is known to bind mainly to PLA2 (a major $[Asp^{49}]$ PLA_2 in the venom) [6]. Although PLI-II and PLI-III are thought to be the components of hetrooligomeric PLIγ [21], their structures were not yet elucidated. Therefore, we have determined their complete amino acid sequences by peptide analysis as well as the cDNA cloning.

As the results, PLI-II and PLI-III have been proved to be the isoforms of *T. flavoviridis* PLIγ-B since they showed a high homology to the subunits B from several other snakes. However, we have no evidence that PLI-I and PLI-II/III can associate to form a high molecular-mass heterooligomer in the blood plasma of *T. flavoviridis*. Moreover, as far as we know, the organization of *T. flavoviridis* PLIγ has not been reported. Though the molar ratio of 25- to 20-kDa subunits was reported to approximately 2 in *A. blomhoffii siniticus* PLIγ [17], the contents of two subunits in the *T. flavoviridis* serum are greatly different and seem unexplainable by the formation of 2:1 heterooligomer. Considering that three subunits of *T. flavoviridis* PLIγ were detected in a wide range of the serum fractions (F2–F4 in Fig. 5) on the analytical gel filtration, most of PLIγ may be composed of PLI-I alone and a part of PLI-I may exist as the heterooligomer with the subunits B.

When various amounts of the purified PLI-II or PLI-III were mixed with a fixed amount of PLI-I and the mixtures were then analyzed by the analytical gel filtration, any new peak corresponding to the complex could not be observed (data not shown). Because our PLIs were purified by reverse-phase HPLC under the acidic conditions, they could be significantly damaged. When PLI-I was purified under the mild conditions, it gave a single peak at approximately 120 kDa and no peak of 25 kDa was detected (data not shown).

Some snakes have the isoforms of either subunit A (α-chain), B (β-chain), or both (Fig. 4). For example, *Oxyuranus scutellatus*, *Notechis ater*, and *N. scutatus* have several isoforms of subunit A, and *O. microlepidotus*, *Pseudonaja textilis*, and *T. flavoviridis* possess the isoforms of subunit B [32]. The isoforms are found for both subunits of *O. microlepidotus* PLIγ (Sekuloski *et al.*, unpublished data). This enables to expand the repertoires of the specificity by the combination of many isoforms, and seems to the results of diversification of

PLIγ genes to adapt the rapid evolution of the target enzyme, PLA_2 [33].

Recently, the nucleotide sequence of the cDNA encoding *T. flavoviridis* PLI-II was uploaded by So *et al.* [34] on the DDBJ sequence data bank: accession No. BAF75726. Three nucleotide was different from our sequence: $A^{323}TGCCT^{328}$ instead of our sequence $A^{323}CGTCT^{328}$. This results in the change of the primary structure of PLI-II from Thr^{50}-Ser^{51} to Met^{50}-Pro^{51}. These nucleotide substitution may reflect the intraspecies diversification, or represent the similar gene on a different allele.

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