Recombinant Antitoxic and Antiinflammatory Factor from the Nonvenomous Snake *Python reticulatus*: Phospholipase A_2 Inhibition and Venom Neutralizing Potential[†,‡]

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ABSTRACT: From the serum of the nonvenomous snake *Python reticulatus*, a new phospholipase A_2 (PLA_2) inhibitor termed phospholipase inhibitor from python (PIP) was purified by sequential chromatography and cloned to elucidate its primary structure and fundamental biochemical characteristics. A cDNA clone encoding PIP was isolated from the liver total RNA by reverse transcriptase−polymerase chain reaction (RT-PCR). It contained a 603 bp open reading frame that encoded a 19-residue signal sequence and a 182-residue protein. PIP showed about 60% sequence homology with those PLA_2 inhibitors having a urokinase-type plasminogen activator receptor-like domain structure. PIP was also functionally expressed as a fusion protein in *Escherichia coli* to explore its potential therapeutic significance. The recombinant PIP was shown to be identical to the native form in chromatographic behavior and biochemical characteristics. Both the native and recombinant PIP appear to exist as a hexamer of 23-kDa subunits having an apparent molecular mass of ∼140 kDa. PIP showed ability to bind to the major PLA_2 toxin (daboiatoxin, DbTx) of *Daboia russelli siamensis* at 1−2-fold molar excess of inhibitor to toxin. It exhibited broad spectra in neutralizing the toxicity of various snake venoms and toxins and inhibited the formation of edema in mice. Our data demonstrate the venom neutralizing potential of the recombinant PIP and suggest that the proline-rich hydrophobic core region may play a role in binding to PLA_2.

Since phospholipase A_2 (PLA_2)[1] is known to be implicated in lethal snake envenomations and is also involved in a variety of biological processes including eicosanoid production and inflammation, efforts have been focused on endogenous inhibitory proteins of snakes and mammals to identify good leads for development of a potent and efficacious inhibitor of PLA_2s (*1, 2*).

To date, about 13 blood-derived PLA_2 inhibitors (PLIs) have been identified from the two major families of venomous snakes (Elapidae and Viperidae); most of them have been isolated mainly from the blood plasma of snakes belonging to the subfamily Crotalinae. They include PLIα, PLI*β*, and PLI*γ* from *Agkistrodon blomhoffii siniticus* plasma (*3*), PLI I −V from *Trimeresurus flavoviridis* plasma (*4, 5*), and another PLI named *Crotalus* neutralizing factor (CNF) or crotoxin inhibitor from *Crotalus* serum (CICS) from the plasma of *Crotalus durissus terrificus* (*6, 7*). Three PLIs have also been purified from the plasma of Elapidae snakes, *Naja naja kaouthia, Laticauda semifasciata,* and *Notechis ater* (*8*−*10*), while a PLI named *Bothrops asper* mytoxin inhibitor protein (BaMIP) has been isolated from the plasma of the Viperinae snake *Bothrops asper* (*11*).

On the basis of the amino acid sequence and specificity, those PLIs from snake plasma can best be grouped into three different classes: one having a carbohydrate recognition domain (CRD) of C-type lectins that specifically inhibits group II acidic PLA_2s from Crotalinae venom, and another having a urokinase-type plasminogen activator receptor- (u-PAR-) like domain structure that exhibits broad inhibition spectrum toward all three groups of venom PLA_2s (*3, 12*), while the third class includes in its structure a leucine-rich α_2-glycoprotein- (LRG-) like domain that might be responsible for the specific binding to basic PLA_2 (*13*). Before submission of this article, we have disclosed elsewhere the existence and identification of a PLI from the serum of the nonvenomous snake *Python reticulatus* (*2, 14*). Recently, a PLI has also been identified in the serum of another nonvenomous snake, *Elaphe quadrivirgata* (*15*). In this study, a new PLA_2 inhibitor termed PIP, which we have

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[‡] The cDNA sequence reported in this paper has been submitted to GenBank under Accession Number AF 232771.

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[1] Abbreviations: PIP, phospholipase inhibitor from python; DbTx, daboiatoxin; PLA_2, phospholipase A_2; PLI, phospholipase A_2 inhibitor; FPLC and HPLC, fast and high-performance liquid chromatography, respectively; RP-HPLC, reverse-phase high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcriptase; PCR, polymerase chain reaction; IPTG, isopropyl *β*-D-thiogalactoside; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PVDF, poly(vinylidene difluoride); kDa, kilodalton; bp, base pair. Amino acids are indicated by the standard one-letter abbreviations.

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purified from the serum of the nonvenomous snake *P. reticulatus* has been cloned, sequenced, and functionally expressed in *Escherichia coli* to elucidate the primary structure, fundamental biochemical characteristics, and the potential therapeutic significance of the inhibitor as an antidote for snake envenomation and PLA_2-related inflammatory conditions.

EXPERIMENTAL PROCEDURES

Materials. Liver and blood were collected from the *Python reticulatus* snake, obtained from the Singapore Zoological Gardens. Swiss albino mice were from the Laboratory Animal Centre, National University of Singapore (NUS). *Daboia russelli siamensis* venom and monospecific antivenom were obtained from the Myanmar Pharmaceutical Corporation. Daboiatoxin and rabbit polyclonal anti-daboiatoxin serum were prepared as described previously (*16*, *17*). All other venoms and PLA_2s were from the collections of Venom and Toxin Research Program, NUS, or purchased from Sigma. UNO Q1 and UNO Q6 columns, molecular weight standards, ready gels, and electrophoresis- grade reagents for PAGE were from Bio-Rad; Superdex 75 and 200 and Sephasil C18 columns were from Pharmacia (Sweden). *Bam*HI, *Hin*dIII, *Sal*I, *Eco*RI, and *Taq* DNA polymerase were from Amersham International Inc. (U.K.); T4 DNA ligase and T4 DNA ligase buffer were from New England Biolabs Inc.; pT7Blue(R) vector and NovaBlue *E. coli* competent cells were from Novagen; QIA*express* pQE-30 expression vector and Ni−NTA−agarose were from Qiagen. Marathon cDNA amplification kit was from Clontech Laboratories, Inc., and DNA sequencing reagents were purchased from Perkin-Elmer Applied Biosystems Inc. Oligonucleotides were custom-synthesized in the Biotechnology Processing Centre (BTC), NUS. Bis(sulfosuccinimido)suberate was from Pierce; antiinflammatory peptide 2 was from Sigma; and all other chemicals (of the highest grade available) were either from Boehringer Mannheim or Sigma.

Purification of PIP. Crude python serum (800 mg of protein) was brought to 45% saturation with a saturated solution of $(NH_4)_2SO_4$ (706 g/L). Following dialysis against 25 mM Tris-HCl (pH 8.2), the resulting immunoglobulin-free fraction (100 mg) was fractionated on a Bio-Rad UNO Q-1 column (7 × 35 mm; flow rate 2 mL/min) with a gradient of 0−0.5 M NaCl in 25 mM Tris-HCl (pH 8.2) buffer. The fraction exhibiting the strongest inhibitory activity against the PLA_2 activity and lethal toxicity of *D. r. siamensis* venom was lyophilized, reconstituted in 25 mM Tris-HCl (pH 8.2) buffer containing 8 M urea (2 mg/mL), and then chromatographed on a HiLoad 16/60 Superdex 75 column (Pharmacia) at a flow rate of 0.5 mL/min. Urea (8 M) in 25 mM Tris-HCl, pH 8.2, was used for preequilibrating the column and in the subsequent elution step. Protein concentration was determined by Bio-Rad dye-based assay (*17*). For N-terminal sequencing, PIP was passed through a RP-HPLC C18 Sephasil (5 μm SC 2.1/10) column with a linear gradient of 0−60% acetonitrile in 0.1% trifluoroacetic acid, at a flow rate of 0.3 mL/min.

N-Terminal Amino Acid Sequencing. S-Pyridylethylated derivative of PIP was desalted on a C18 Sephasil RP-HPLC column with a gradient of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile, and N-terminal sequencing was carried out in an Applied Biosystems model 477A protein sequencer equipped with a 120A PTH analyzer.

Electrophoresis and Glycoprotein Detection. Native PAGE, and SDS−PAGE under reducing conditions, were carried out with the Mini-Protean II electrophoresis cell (Bio-Rad) on Tris-Glycine 10% and 12% ready gels. Proteins were visualized with Coomassie Blue. PIP immobilized on nitrocellulose membrane after SDS−PAGE was labeled with biotin and washed several times, and glycoprotein detection was performed with streptavidin−alkaline phosphatase and color-detecting reagents provided in the Bio-Rad immunoblot glycoprotein detection kit.

Cloning and Nucleotide Sequencing. Total RNA was isolated from the frozen liver tissue of *P. reticulatus* by a modified guanidinium isothiocynate extraction method (*19*) and analyzed by denaturing formaldehyde agarose gel electrophoresis (*20*). A Marathon cDNA amplification kit (Clontech) was used to obtain an uncloned library of adapter-ligated double-stranded cDNA, which was amplified by the polymerase chain reaction in a Perkin-Elmer Cetus thermal cycler (model 480) for 30 cycles (94 °C for 1 min; 50 °C for 1 min; 72 °C for 2 min; 72 °C for 10 min/cycle). GSP1 primer (5′-GACAAGTGCGAGATCTGCCACGG-3′) and an adaptor primer AP1 from the kit were used as the forward and reverse primers, respectively, in the above PCR. On the basis of N-terminal amino acid sequence of PIP, GSP1 primer was designed and synthesized at BTC, NUS. The 800 bp purified PCR product was ligated with pT7Blue(R) vector (Novagen) and the recombinant plasmids were transformed into NovaBlue competent *E. coli* cells (Novagen) by heat shock. The transformants were selected on an LB-Amp (50 μg/mL) plate supplemented with IPTG and X-gal. Following mini plasmid preparation (*21*), putative recombinant plasmids were subjected to Sanger dideoxy DNA sequencing (*22*) on an Automated DNA Sequencer (Applied Biosystems, Model 373A) as described previously (*23*). On the basis of these sequence data, GSP-2 reverse primer (5′-ACACTCCATCCCAAACATGTATGTC-3′) was designed, custom-synthesized, and used along with an adapter primer AP 1 to amplify the adapter-ligated ds cDNA by PCR. The 360 bp PCR product was then subcloned and sequenced. On the basis of the sequence data generated from the two cloned fragments, the complete nucleotide sequence of PIP cDNA was established and the amino acid sequence was deduced.

Expression and Purification of PIP. The cDNA sequence encoding the protein sequence to be expressed was amplified by PCR and cloned into the QIAexpress pQE-30 expression vector to produce a 6× His-tagged fusion protein in NovaBlue *E. coli* competent cells. PCR primers with restriction enzyme sites flanking the PIP coding region were designed from the complete nucleotide sequence of PIP. Oligonucleotide primers used were forward, 5′-GGTGGATCCGACAAATGTGAAATT-3′, and reverse, 5′-ATTAAGCTTTCATTAATCAGAGGATG-3′. A *Hin*dIII site was included in the 3′ PCR primer, while the 5′ primer incorporated a *Bam*HI site. The reconstructed 600 bp DNA fragment was cleaved with restriction endonucleases *Bam*HI and *Hin*dIII, gel-purified, and ligated to similarly prepared pQE-30 plasmid DNA. The ligated product was then transformed into competent *E. coli* cells. The resulting clone PIP-pQE-30 was analyzed by restriction digestion and screened for the presence of PIP sequence by PCR. Clones

carrying inserts were grown at 37 °C in LB-Amp medium until $A_{600} \sim 0.65$ and then induced with IPTG (1 mM final concentration) at 37 °C, harvested after 4 h, and resuspended in lysis buffer [50 mM Tris-HCl (pH 8.5), 10 mM 2-mercaptoethanol, and 1 mM PMSF], and the cells were lysed under denaturing conditions in 8 M urea by gentle vortexing and sonication, followed by centrifugation (10000*g*, 30 min, 4 °C) and collection of cleared cell lysates for affinity purification on a column packed with 2 mL Ni−NTA−agarose (Qiagen). During affinity purification, unbound proteins were eluted from the Ni−NTA column with buffer A [20 mM Tris-HCl (pH 8.5), 100 mM KCl, 20 mM imidazole, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol], and following two successive washes, respectively with buffer A and buffer B [20 mM Tris (pH 8.5), 1 M KCl, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol], the recombinant protein was eluted with a stepwise gradient (100, 150, 200, 400, and 800 mM imidazole) in buffer C (buffer A without imidazole). Renaturing of the eluted protein was achieved by dilution in Tris buffer (pH 7.5) containing 100 mM KCl, and denaturants were removed slowly by ultrafiltration through a Centricon plus 80 membrane (Millipore). The recombinant PIP was purified further by UNOQ6 chromatography and RP-HPLC (Sephasil C18), before analysis by N-terminal sequencing to confirm the amino acid sequence of the N-terminal end.

Molecular Mass Determination. To determine the molecular mass of the oligomeric native and recombinant PIP, gel-filtration chromatography of both forms of PIP was performed on a Superdex 200 (HR 10/30) column using Sigma molecular weight markers kit (MW-GF-200). Subunit molecular mass of PIP was determined by SDS−PAGE as described previously, with broad-range SDS−PAGE (Bio-Rad) markers for calibration. The molecular mass was then estimated relative to the calibration curve derived from the respective standards. Mass analysis of the native PIP was performed by electrospray ionization mass spectrometry on a Perkin-Elmer Sciex API 300 LC/MS/MS system.

Chemical Cross-Linking. Aliquots (25 μL) of native or recombinant PIP (50 μg/mL) in 0.05 M HEPES buffer (pH 7.5) were incubated with varying concentrations (0−5 mM) of bis(sulfosuccinimido)suberate (Pierce), for 60 min at 37 °C (*23*). After dilution and boiling with Laemmli SDS−PAGE sample buffer containing 0.2 M dithiothreitol, the cross-linked samples were analyzed by SDS−PAGE and Coomassie staining.

Binding of Daboiatoxin to PIP. Interaction of PIP with DbTx was demonstrated by PAGE in 10% acrylamide gels under nondenaturing conditions. Different molar ratios of native PIP to DbTx (0:1, 0.5:1; 1:1; 2:1, and 1:0) were prepared in a total volume of 20 μL of Tris-HCl buffer (25 mM, pH 8.2) and incubated at 37 °C for 1 h. Aliquots (10 μL) were then applied to a 10% polyacrylamide gel and submitted to nondenaturing PAGE, and the protein bands were visualized by Coomassie staining. In addition, the formation of PIP−DbTx complex was analyzed by electrotransferring the complex on PVDF membranes (Bio-Rad) after PAGE, and the complex was visualized by use of polyclonal antibodies directed against DbTx.

Anti-PLA_2 Activity in Vitro Assay. PLA_2 activity was measured by an acidimetric method (*25*) with egg yolk as substrate. Inhibitory activity during the course of purification was monitored by the residual PLA_2 activity of *D. r. siamensis* venom after incubation of 10 μg of crude venom with various concentrations of the column fractions at 37 °C for 1 h. Inhibitory activity of the purified PIP was evaluated by preincubating at 37 °C for 1 h a fixed concentration of a PLA_2 toxin, DbTx (0.33 μM), with various concentrations of PIP (0−2.8 μM) at 0.5:1, 1:1, 2:1, 4:1, and 8:1 molar ratios of PIP to DbTx and measuring the residual PLA_2 activity afterward. In addition, the effect of the recombinant PIP on catalytic activity of various PLA_2 toxins was also assessed by measuring their residual PLA_2 activity after incubation (37 °C; 1 h) of known amounts of PLA_2s from different sources with varying concentrations of PIP.

Anti-Lethal Activity in Vivo Assay. The protective activity of recombinant PIP was determined by injecting Swiss albino mice (20−22 g) i.p. with $2LD_{50}$ doses of the venom or PLA_2 toxins of either *D. r. siamensis* or other species of snakes, previously incubated at 37 °C for 1 h with PIP. Protective activity was assessed on the basis of survival of animals recorded 24 h postinjection. Venom or PLA_2 toxin of each snake species was injected alone as a positive control, and saline or PIP was injected as a negative control.

Inhibition of Mouse Footpad Edema. Swiss albino mice (20−25 g) in groups of three were injected subplantarly into the left paw with *D. r. siamensis* venom (5 μg), DbTx (1.5 μg), or bee venom PLA_2 (1 μg), either alone or after preincubation with various inhibitors (i.e., recombinant PIP, antiinflammatory peptide 2, monospecific antivenom, or DbTx antisera), in a total volume of 25 μL of sterile solution. Hind limbs from animals sacrificed at 45 min postinjection were removed at the ankle joint and weighed individually. The increase in weight (milligrams) due to edema was calculated by subtracting the weight of each saline-injected right hind limb. Inhibitory effect was assessed by comparing the paw edema of animals receiving venom or PLA_2 preincubated with inhibitor to those receiving venom or PLA_2 alone.

RESULTS

A variety of animal sera were screened for inhibition of PLA_2 activity toward the venom and toxin of *D. r. siamensis*. Out of nine serum samples examined, *P. reticulatus* serum was the only one found to have strong inhibitory activity toward this PLA_2 with a potency superior to that of the specific neutralizing antibody (*2*). Hence, an attempt was made to purify this PLI on the basis of its specific inhibitory activity toward the *D. r. siamensis* PLA_2.

Purification of PIP. Commencing with $(NH_4)_2SO_4$ precipitation of the crude serum followed by fractionation of immunoglobulin-free supernatant solution on UNO Q-1 column, a distinct protein peak was eluted at 0.25 M NaCl gradient that exhibited strong antilethal activity and PLA_2 inhibitory activity toward *D. r. siamensis* venom (Figure 1a). Since further fractionation under denaturing conditions considerably improved the purification process, the active fraction from UNO Q-1 was chromatographed in 8 M urea, on a HiLoad 16/60 Superdex 75 column, to recover the purified PIP in the third fraction of the eluate (Figure 1b). Once desalted and freed of urea, this fraction was found to be responsible for the antitoxic property toward the venom

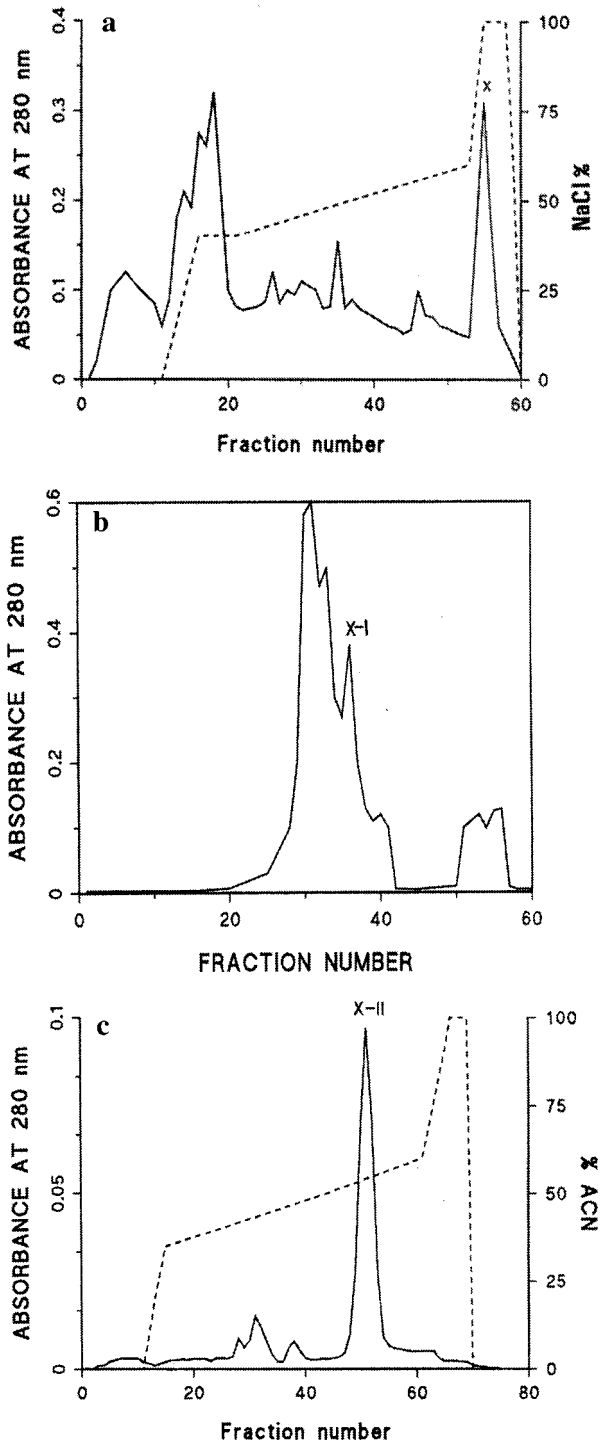

FIGURE 1: Purification of PIP. (a) Ion-exchange chromatography of $(NH_4)_2SO_4$-precipitated serum fraction on UNO Q1 column eluted with 0−0.5 M NaCl gradient in 25 mM Tris-HCl buffer (pH 8.2). Flow rate, 2 mL/min; fractions, 2 mL; (—), absorbance at 280 nm; (- - -) NaCl gradient; (×), active fraction peak. (b) Gel-filtration chromatography of peak X from panel a on HiLoad 16/60 Superdex 75 column eluted with 8 M urea in 25 mM Tris-HCl buffer, pH 8.2. Flow rate, 0.5 mL/min; fractions, 1.5 mL; (X-I), PIP peak. (c) RP-HPLC of peak X-I from panel b on C18 Sephasil column, eluted with a gradient of 0−60% of 80% acetonitrile/0.1% trifluoroacetic acid. (X-II), final purified product used for N-terminal sequencing. Flow rate, 0.3 mL/min; fractions, 0.3 mL; (—) absorbance at 280 nm; (- - -) acetonitrile gradient.

and the PLA_2 toxin (DbTx) of *D. r. siamensis*. About 2 mg of the purified PIP was finally obtained from 800 mg total protein of *P. reticulatus* crude serum. When PIP was

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ATTCAACATATCCAATCCAGCTCTGATCTTTACCAGAGAAGACATCTTGAGCC  53

ATGAAATCCCTACAGACCATTTGCCTTCTTTTCATTTTTATAGCTAGAGGAACCTCT 110
 M  K  S  L  Q  T  I  C  L  L  F  I  F  I  A  R  G  T  S  (19)
GACAAATGTGAAATTTGTCATGGCTTTGGAGATGACTGTGATGGTTATCAGGAGGAA 167
 D  K  C  E  I  C  H  G  F  G  D  D  C  D  G  Y  Q  E  E  (38)
TGTCCCTCTCCAGAAGACCGATGTGGCAAGATTCTGATAGACATCGCATTAGCACCA 224
 C  P  S  P  E  D  R  C  G  K  I  L  I  D  I  A  L  A  P  (57)
GTTTCATTCAGAGCCACGCATAAGAATTGTTTCTCATCCAGCATCTGTAAACTTGGC 281
 V  S  F  R  A  T  H  K  N  C  F  S  S  S  I  C  K  L  G  (76)
CGTGTTGACATACATGTTTGGGATGGAGTGTATATAAGAGGAAGAACAAATTGCTGT 338
 R  V  D  I  H  V  W  D  G  V  Y  I  R  G  R  T  N  C  C  (95)
GATAATGATCAGTGTGAAGACCAACCACTTCCTGGATTGCCCCTCTCCCTCCAGAAT 395
 D  N  D  Q  C  E  D  Q  P  L  P  G  L  P  L  S  L  Q  N  (114)
GGGCTCTATTGTCCTGGTGCTTTTGGTATTTTTACCGAGGACAGCACTGAACATGAA 452
 G  L  Y  C  P  G  A  F  G  I  F  T  E  D  S  T  E  H  E  (133)
GTTAAATGCAGAGGAACTGAAACTATGTGCCTTGATCTTGTGGGATACAGACAAGAA 509
 V  K  C  R  G  T  E  T  M  C  L  D  L  V  G  Y  R  Q  E  (152)
AGTTATGCTGGAAACATCACTTATAATATCAAAGGCTGTGTTTCTTCCTGTCCCTTG 566
 S  Y  A  G  N  I  T  Y  N  I  K  G  C  V  S  S  C  P  L  (171)
GTAACTTTGAGTGAAAGAGGTCATGAAGGACGCAAAAATGATCTGAAGAAGGTTGAA 623
 V  T  L  S  E  R  G  H  E  G  R  K  N  D  L  K  K  V  E  (190)
TGTAGGGAAGCCTTGAAACCTGCATCCTCTGATTAATACTGGAATCATTCTGGAATC 680
 C  R  E  A  L  K  P  A  S  S  D  end                      (201)
TGAATGTCTTCACCAGGTAGAACCTGCCTCATCAGAATGACTCTGAATGGAAACTTA 737

CATTTTTAAGTTGTGGCTCTTCCTGCTGATTAATTTTTAAAAATTAAAAAAAAAGCA 794

AATAAAAGAAGTCAAAGTGAAT-  poly (A) tail                     816
Poly (A) signal
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FIGURE 2: cDNA sequence and deduced protein sequence of PIP. Numbers on the extreme right indicate the nucleotide sequence (upper, in boldface type) and the deduced amino acid sequence (lower, in parentheses). ORF sequence is boxed (open box, leader sequence; solid box, nucleotide sequence of the matured PIP). The single-letter code beneath the second nucleotide of the appropriate codon indicates the deduced amino acid sequence of the ORF. Polyadenylation signal is underlined.

subjected to RP-HPLC, a single protein peak was detected (Figure 1c), suggesting only one species of subunit molecule.

Cloning and Sequencing of PIP cDNA. Two fragments of 800 and 360 bp were amplified from the adapter-ligated ds cDNA. On the basis of sequence data generated from those two cloned fragments, the complete nucleotide sequence of PIP cDNA was established and is shown in Figure 2, together with its deduced amino acid sequence. The full-length cDNA of PIP was 816 bp with a 5′-UTR of 53 bp, followed by an open reading frame (ORF) region of 603 bp. The ORF predicts a 201 amino acid polypeptide precursor for PIP, which includes a signal peptide of 19 amino acids in length and a peptide containing 182 amino acids with an N-terminal sequence identical to that obtained from direct amino acid sequence analysis of the native PIP.

Functional Expression of PIP cDNA. The cDNA subcloned pQE-30 was expressed as 6× His-tagged fusion protein in *E. coli*. Analysis by SDS−PAGE of cell extracts from induced cells showed the increased expression of a band with molecular mass ∼23 kDa, when compared with that of extracts from the uninduced cultures. A total yield of approximately 6 mg of PIP/L of culture was obtained from Ni−NTA column purification of the cell extracts. Further purification of the affinity-purified product on RP-HPLC gave a single peak with the same retention time as the native PIP. The electrophoretic migration of the 23-kDa monomer subunits was almost indistinguishable between the native and recombinant PIP on SDS−PAGE (data not shown). The N-terminal sequence determined up to the first 18 amino acid residues of the recombinant PIP showed identical sequence to that of the native PIP.

Characterization. A single Coomassie blue positive band observed on native PAGE and SDS−PAGE suggests that native PIP is homogeneous and has only one type of subunit. RP-HPLC of PIP also gives only a single peak. A positive

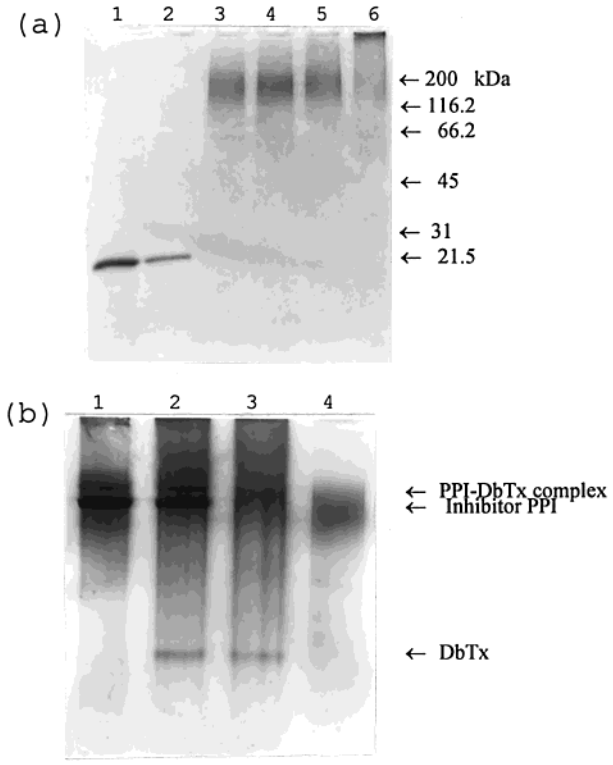

FIGURE 3: (a) Chemical cross-linking: aliquots of native PIP after cross-linking with varying concentrations of bis(sulfosuccinimido)suberate (lanes 1−6, 0, 0.25, 0.5, 1.0, 2.5, and 5.0 mM cross-linker). The samples were analyzed on SDS−12% PAGE gel, followed by Coomassie staining. (b) Analysis of a DbTx−PIP complex by native PAGE: PIP and DbTx in 20 μL final volume were incubated for 1 h at 37 °C and subjected to native PAGE in 10% acrylamide gels, and the protein bands were visualized by Coomassie blue staining (lanes 1−3, PIP and DbTx incubated at 2:1, 1:1, and 0.5:1 molar ratios; lane 4, PIP alone). A new band with a reduced mobility formed near the top of PIP band at a unimolar ratio of PIP:DbTx represents the PIP−DbTx complex.

signal obtained with the Bio-Rad immunoblot kit indicates that native PIP is a glycoprotein (data not shown). The native PIP has an apparent molecular mass of about 140 kDa, as determined by Superdex 200 gel filtration chromatography (data not shown), and its subunit molecular mass was estimated to be 23.5 kDa by SDS−PAGE under reducing conditions. This value conforms to the mass value of 23 264 determined for the RP-HPLC purified native PIP subunit by mass spectrometry. Thus the native PIP appears to exist as a hexamer of 23-kDa subunits, and as shown in Figure 3a, the subunits could be cross-linked to form an oligomeric molecular aggregate of about 140 kDa, indicating that native PIP is composed presumably of six homogeneous 23-kDa subunits. The apparent molecular mass of the recombinant PIP as estimated from its migration on SDS−PAGE was 22.5 kDa. Based on the amino acid sequence of the mature PIP (Figure 2), the molecular mass of the nonglycosylated PIP was calculated to be 20 095 Da. The molecular mass of the recombinant PPI with the attached N-terminal amino acid residues, as determined by means of mass spectrometry, was 21 035 Da, the mass of which is in accordance with that expected from a fusion protein.

Interaction of PIP with Daboiatoxin. Following incubation of native PIP and DbTx in various molar ratios at 37 °C for 1 h, an extra protein band with a reduced mobility when compared to that of PIP alone was detected by native PAGE

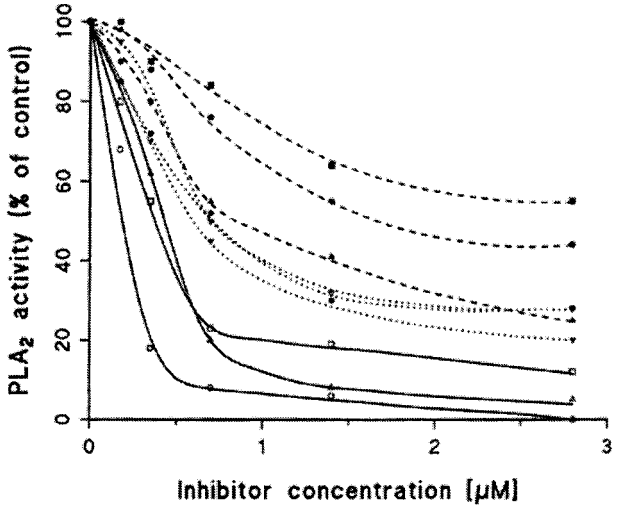

FIGURE 4: Inhibition of PLA_2 enzyme activity by recombinant PIP. Enzyme activity of PLA_2 toxins was measured acidimetrically in the absence and presence of various concentrations of the inhibitor PIP. DbTx (○), PL-VIII$_a$ (△), PL-V (□), ammodytoxin A (▽), taipoxin (●), β-bungarotoxin (▲), *Enhydrina schistosa* PLA_2 (■), crotoxin B (▼), mojave toxin B (*).

(Figure 3b). This extra band, which might represent the complex, was formed only when the PIP to DbTx ratio was 1:1 and above, but not at 0.5:1, indicating that at least 1−2-fold molar excess of the inhibitor to DbTx was required to form a complex. When the PIP−DbTx complex was electrotransferred on PVDF membrane and visualized with polyclonal antibodies directed against DbTx, interaction of PIP with DbTx was evident by the appearance of a visible band after immunodetection (data not shown).

Inhibition of PLA_2 Catalytic Activity. The inhibition profiles displayed by the recombinant PIP toward the enzyme activity of PLA_2 toxins are shown in Figure 4. On the basis of the apparent molecular masses of PIP (140 kDa) and DbTx (15 kDa), almost complete inhibition (90%) was achieved at a unimolar ratio of PIP (0.35 μM) to DbTx (0.33 μM). The enzyme activity of VRV PL-V and VRV PL-VIII$_a$, the two PLA_2 toxins from *D. r. russelli* and *D. r. pulchella*, respectively, were also strongly inhibited, whereas the other group II toxic PLA_2s such as the basic subunits of mojave toxin and crotoxin, as well as ammodytoxin A, were enzymatically less sensitive to inhibition by PIP. Group I toxic PLA_2s, on the other hand, were the least sensitive among the three groups of PLA_2 toxins examined.

Toxin Neutralizing Activity. The lethal potency of DbTx also was completely inhibited with the same molar ratio (1:1) of the recombinant PIP to DbTx, while that of the less toxic PL-V and PL-VIII$_a$ were completely neutralized with only one-tenth the amount of PIP required to neutralize the lethality of DbTx. In contrast, the lethal toxicity of other group II PLA_2 toxins could be neutralized only with a 2-fold molar excess of PIP, while for group I toxins, up to 5-fold molar excess of PIP was required to neutralize the lethality. The recombinant PIP is also an effective inhibitor toward the group III PLA_2 from the venom of the bee *Apis melifera* (Table 1).

Venom Neutralizing Activity. The recombinant PIP neutralized the lethal toxic effects of various venoms from the three major families of venomous snakes to varying degrees. Complete (100%) neutralization was achieved with 100 μg

Table 1. Neutralization of the Lethal Effects of PLA_2 Toxins by Recombinant PIP[a]

PLA_2 toxin	dose of toxin (μg/mouse)$_{i.p}$	molar ratio (inhibitor/toxin)	survival/total	protection (%)
	Group I			
taipoxin (*Oxyuranus s. scutellatus*)	10	5	2/4	50
β-bungarotoxin (*Bungarus multicinctus*)	5	5	2/4	50
Enhydrina schistosa PLA_2	5	5	2/4	50
	Group II			
daboiatoxin (*Daboia r. siamensis*)	10	1	4/4	100
VRV−PL-V (*D. r. russelli*)	50	0.10	4/4	100
VRV−PL-VIII$_a$ (*D. r. pulchella*)	100	0.15	4/4	100
ammodytoxin A (*Vipera a. ammodytes*)	5	2	3/4	75
crotoxin AB(*Crotalus d. terrificus*)	10	4	3/4	75
crotoxin B (*Crotalus d. terrificus*)	20	2	3/4	75
mojave toxin B (*Crotalus s. scutulatus*)	15	2	3/4	75
	Group III			
Apis melifera (bee venom) PLA_2	1	2	3/4	75

[a] Swiss albino mice (20−25 g) were injected i.p. with 0.2 mL of saline containing predetermined lethal ($\sim 2LD_{50}$ i.p.) doses of various toxic PLA_2s previously incubated with the indicated molar concentrations of the inhibitor PIP for 30 min at 37 °C. Survival was recorded after 24 h.

Table 2. Effect of Inhibitors on PLA_2-Induced Mouse Foot-Pad Edema[a]

treatment	paw edema (mg)[b]	% inhibition (mean)
Daboia r. siamensis venom (5 μg)	116 ± 16	0
+PIP (50 μg)	62 ± 2.2	46.5
+PIP (100 μg)	29 ± 0.8	74.6
+AIP-2 (50 μg)	79 ± 6.1	31.9
+*D. r. siamensis* antivenom (150 μg)	106 ± 2.5	ns[b]
daboiatoxin PLA_2 (1.5 μg)	167 ± 12	0
+PIP (50 μg)	18 ± 6.0	89.4
+PIP (100 μg)	13 ± 0.4	92.1
+daboiatoxin antiserum (800 μg)	99 ± 5.8	14.0
bee venom (*Apis melifera*) PLA_2 (1 μg)	87 ± 3.8	0
+PIP (50 μg)	55 ± 0.5	36.8
+PIP (100 μg)	19 ± 0.3	78.2
+AIP-2 (50 μg)	49 ± 0.2	43.7
PIP alone (100 μg)	10 ± 5.2	ns
AIP-2 alone (50 μg)	7.7 ± 3.4	ns

[a] Mice were injected subplantarly into the left paw with the indicated amounts of venom/toxins, with or without the recombinant PIP or other inhibitors. The increase in weight (milligrams) due to edema was calculated by subtracting the weight of each saline-injected right hind limb. Inhibition was assessed by comparing the paw edema of animals receiving PLA_2 plus inhibitor to those receiving PLA_2 alone. [b] Mean ± SD ($n = 3$). [b] Not significant.

of PIP toward the lethal toxicity of the venoms from *D. russelli* subspecies *D. r. siamensis*, *D. r. pulchella*, and *D. r. russelli*. The same amount of PIP (100 μg) could also neutralize up to 75% of the lethal activity of the venom of the Crotalinae snake *Agkistrodon halys blomhoffii*, but the activities of the venoms of Elapidae snakes, *N. n. naja*, *Bungarus multicinctus*, *Pseudechis australis*, *P. textilis*, and *Oxyuranus* s. *scutellatus* were neutralized to a lesser extent (50%), even with 150 μg of recombinant PIP.

Inhibition of Edematogenic Activity. Table 2 shows the antiinflammatory effect of the recombinant PIP. Co-injection of recombinant PIP with PLA_2s from *D. r. siamensis* or bee venom resulted in significant reduction of edema formation in a dose-dependent manner with maximal suppression of the inflammatory response (75−92%) observed at a dose level of 100 μg of PIP. At a 50 μg dose, it has an antiinflammatory activity comparable to that of the Sigma antiinflammatory peptide 2. Despite the larger doses used, the specific neutralizing antibodies such as *D. r. siamensis* antivenom (150 μg) and DbTx antiserum (0.8 mg), on the other hand, had insignificant inhibitory effect on PLA_2-induced mouse footpad edema.

DISCUSSION

We report here for the first time the recombinant expression of a PLI of the nonvenomous snake *Python reticulatus* and its potential therapeutic significance for lethal snake envenomation and inflammatory conditions. The expressed nontoxic fusion protein, which is obtainable in fairly large amounts by Ni−NTA affinity purification, showed strong lethal toxin neutralizing activity and potent antiinflammatory activity. On the basis of biological properties and chromatographic behavior, the recombinant protein is representative of the native PIP. The yield of fully active recombinant PIP (6 mg) that we could produce, combined with easy purification, enabled us to further study its utility as an effective inhibitor of PLA_2, which should further aid in the understanding of the role of these PLIs in complex biological processes.

The inhibitor PIP is an oligomeric protein with a molecular mass of 140 kDa that neutralizes the lethal and toxic effects of venoms and PLA_2 toxins of major families of venomous snakes through formation of a toxin−inhibitor complex. Although native PIP is a glycoprotein, the fusion protein expressed in *E. coli* has no carbohydrate, yet the fact that it can interact with PLA_2 toxins without any posttranslational modification suggests that the carbohydrate moiety of PIP may not necessarily be essential for inhibitory activity. In fact, it was observed that the native and recombinant proteins were equally active without any significant differences noted between those two forms when we compared their inhibitory effect on PLA_2-induced mouse foot-pad edema (data not shown). On the bassis of molecular weight determination by Superdex 200 gel-filtration chromatography and chemical cross-linking experiments, native as well as its functionally equivalent recombinant PIP are thought to be homohexamers of identical monomeric 23-kDa subunits

The data from the present study show that *E. coli* expressed PIP is sensitive in inhibiting not only group II PLA_2s but also those of other groups, including Elapidae venom PLA_2s (group I) and honeybee venom PLA_2 (group III), although its inhibitory activity is less sensitive toward the latter groups of PLA_2 toxins (Figure 4). It is most sensitive in inhibiting

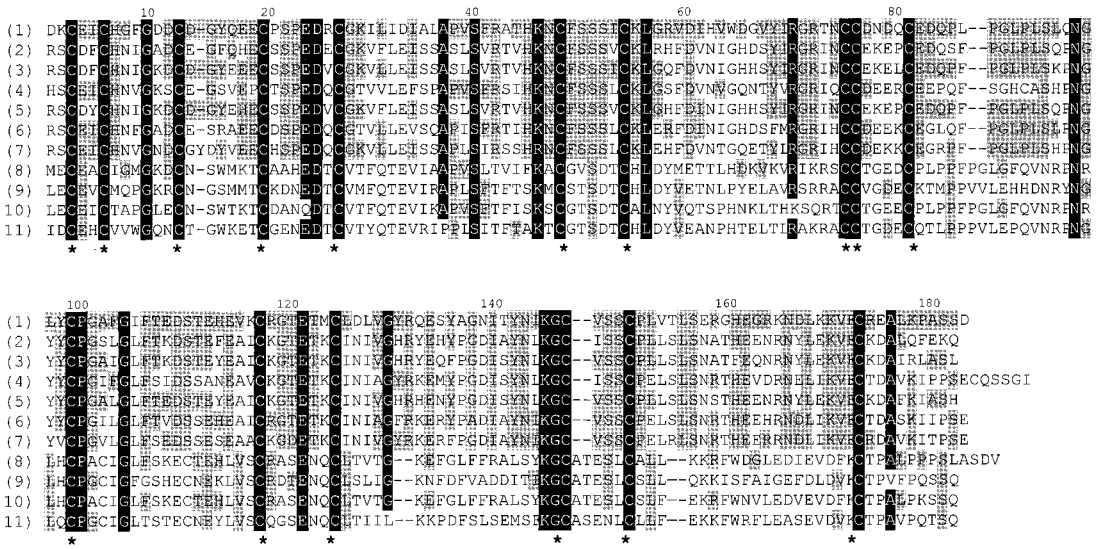

FIGURE 5: Comparison of two repeated three-finger motifs of PIP with those of other structurally related snake PLIs. (1) *Python reticulatus* PIP; (2) *T. flavoviridis* PLI−I; (3) *C. d. terrificus* CNF; (4) *N. n. kaouthia* PLI-A; (5) *A. b. siniticus* PLIγ-A; (6) *L. semifasciata* PLIγ-A; (7) *E. quadrivirgata* PLIγ-A; (8) *N. n. kaouthia* PLI-B; (9) *A. b. siniticus* PLIγ-B; (10) *L. semifasciata* PLIγ-B; (11) *E. quadrivirgata* PLIγ-B. The first (residues 1−96) and the second (residues 97−182) repeats of the three-finger motifs are shown at the top and bottom, respectively. Residues conserved in at least 10 of the 11 sequences are shown in white on black boxes. The shaded boxes indicate residues identical to those of PIP. Stars denotes half-cystine residues.

the PLA_2 toxins from different *D. russellii* subspecies, i.e., daboiatoxin of *D. r. siamensis* (*16*), VRV PL V of *D. r. russelli* (*28*), and VRV PL VIII$_a$ of *D. r. pulchella* (*29, 30*). A similar broad inhibition spectrum has also been reported for the cobra PLI, which inhibits *D. r. russelli* VRV PL V (a group II PLA_2) with a 10−100-fold higher strength than other group II PLA_2s (*12*), and for PLIγs from the plasma of *A. b. siniticus*, *L. semifasciata*, and *E. quadrivirgata* (*3, 9, 15*).

At least 1−2-fold molar excess of PIP is required to bind to DbTx, as evidenced by the presence of a new band corresponding to a PIP-DbTx complex on native PAGE, which was confirmed later by Western blotting with anti-DbTx. The facts that the enzymatic activity of DbTx is almost completely abolished and that the lethal toxicity as well as the mouse footpad edema induced by DbTx are significantly inhibited by PIP at 1:1 molar ratio to DbTx suggests the formation of the inhibitor−PLA_2 complex. Formation of such a complex has previously been shown to be responsible for the inhibition of some PLA_2 toxins (*4, 7, 31*). Presumably through multiple protein−protein contacts, snake PLIs bind to PLA_2 in the aqueous phase to form tight enzyme−inhibitor complexes that cannot bind to membranes, thereby rendering the enzyme inactive (*32*). Little is known, however, about the particular protein−protein interaction site on PLIs that is responsible for the binding to PLA_2.

A search for sequence homology reveals that PIP shows about 50−60% identity with the PLIs from the plasma of *C. d. terrificus*, *T. flavoviridis*, *N. n. kaouthia*, *A. b. siniticus*, *L. semifasciatus*, and *E. quadrivirgata*. All those PLIs, including PIP, show about 25% sequence identity in their unique pattern of cysteine residues with mammalian proteins such as urokinase plasminogen activator receptor (u-PAR), and cell surface antigens of the Ly-6 superfamily (*8*). Common to u-PAR-like PLIs, PIP is composed of two tandem repeats of a unit termed the three-finger motif, which has been found in neurotoxins (*33*). Recently, it has been shown that a segment (residue 95−181) containing one of the two three-finger motifs of PLI−I from *T. flavoviridis* plasma is capable of binding to homologous venom basic PLA_2 (*34*), implying that this region is one of the essential structural elements to recognize PLA_2s.

In the primary structure of PIP (Figure 5), we have noted a proline-rich cluster (position 85−109) on the basis of recognition of proline brackets (*35*). Alignment of PIP with structurally similar PLIs indicates that this cluster is also present in those inhibitors in the same region as in PIP but absent in any other cysteine-rich domain-containing proteins such as u-PAR and Ly-6 related proteins, and thus it might play an important functional role in the PLIs. Apart from the suggestion that this proline-rich cluster present on the PIP may play a role in the interaction with PLA_2s, the exact region necessary for the biological activity of the inhibitor is as yet unknown. Future studies should be focused on this particular region to understand the structural elements required for the PLI−PLA_2 interaction.

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