Archives of Biochemistry and Biophysics
Vol. 375, No. 2, March 15, pp. 289–303, 2000
doi:10.1006/abbi.1999.1672, available online at http://www.idealibrary.com on IDEAL®

Phospholipase A_2 with Platelet Aggregation Inhibitor Activity from *Austrelaps superbus* Venom: Protein Purification and cDNA Cloning[1]

Simran Bedi Singh,*,† Arunmozhiarasi Armugam,* R. Manjunatha Kini,† and Kandiah Jeyaseelan*,†,[2]

**Department of Biochemistry and †Bioscience Centre, National University of Singapore, 10 Medical Drive, Singapore 119260*

Received September 21, 1999, and in revised form December 20, 1999

Four phospholipase A_2 (PLA_2) enzymes (Superbins a, b, c, and d) with varying platelet aggregation inhibitor activities have been purified from *Austrelaps superbus* by a combination of gel filtration, ion-exchange, and reversed-phase high-pressure liquid chromatography. Purity and homogeneity of the superbins have been confirmed by high-performance capillary zone electrophoresis and mass spectrometry. The electron spray ionization mass spectrometry data showed that their molecular masses range from 13,140 to 13,236 Da. Each of the proteins has been found to be basic and exhibit varying degrees of PLA_2 activity. They also displayed different platelet aggregation inhibitory activities. Superbin a was found to possess the most potent inhibitory activity with an IC_{50} of 9.0 nM, whereas Superbin d was found to be least effective with an IC_{50} of 3.0 μM. Superbins b and c were moderately effective with IC_{50} values of 0.05 and 0.5 μM, respectively. The amino-terminal sequencing confirmed the identity of these superbins. cDNA cloning resulted in the identification of 17 more PLA_2 isoforms in *A. superbus* venom. It has also provided complete information on the precursor PLA_2. The precursor PLA_2 contained a 27-amino-acid signal peptide and 117- to 125-amino-acid PLA_2 (molecular mass ranging from 13,000 to 14,000 Da). Two of these PLA_2 enzymes resembled more closely (87%) Superbin a in structure. Two unique PLA_2 enzymes containing an extra pancreatic loop also have been identified among the isoforms.

Key Words: **phospholipase A_2; platelet aggregation inhibitor activity; venom; Australian elapid; *Austrelaps superbus*.**

Platelet aggregation plays an important role in platelet retraction and wound healing (1, 2), where any aberration in platelet aggregation could lead to death or debilitation. Due to their pivotal and fundamental role in hemostasis and thrombosis, inhibitors of platelet aggregation are highly sought after as drugs in the prevention/treatment of cardio- and cerebrovascular diseases and atherosclerosis and in the regulation of tumor growth in cancer. Phospholipase A_2 (PLA_2)[3] enzymes are also useful as tools in the study of molecular mechanisms of platelet aggregation and also have potential in the development of drugs against platelet aggregation (3, 4).

Phospholipase A_2 (EC 3.1.1.4) enzymes are frequently found in nature, especially in mammalian pancreas and animal venoms. They catalyze the hydrolysis of phospholipids at the *sn*-2 position of the glycerol backbone to release fatty acid and the corresponding 1-acyl lysophospholipid (5). In snake venoms, PLA_2 enzymes exhibit a wide variety of pharmacological effects (6, 7) by interfering in normal physiological processes.

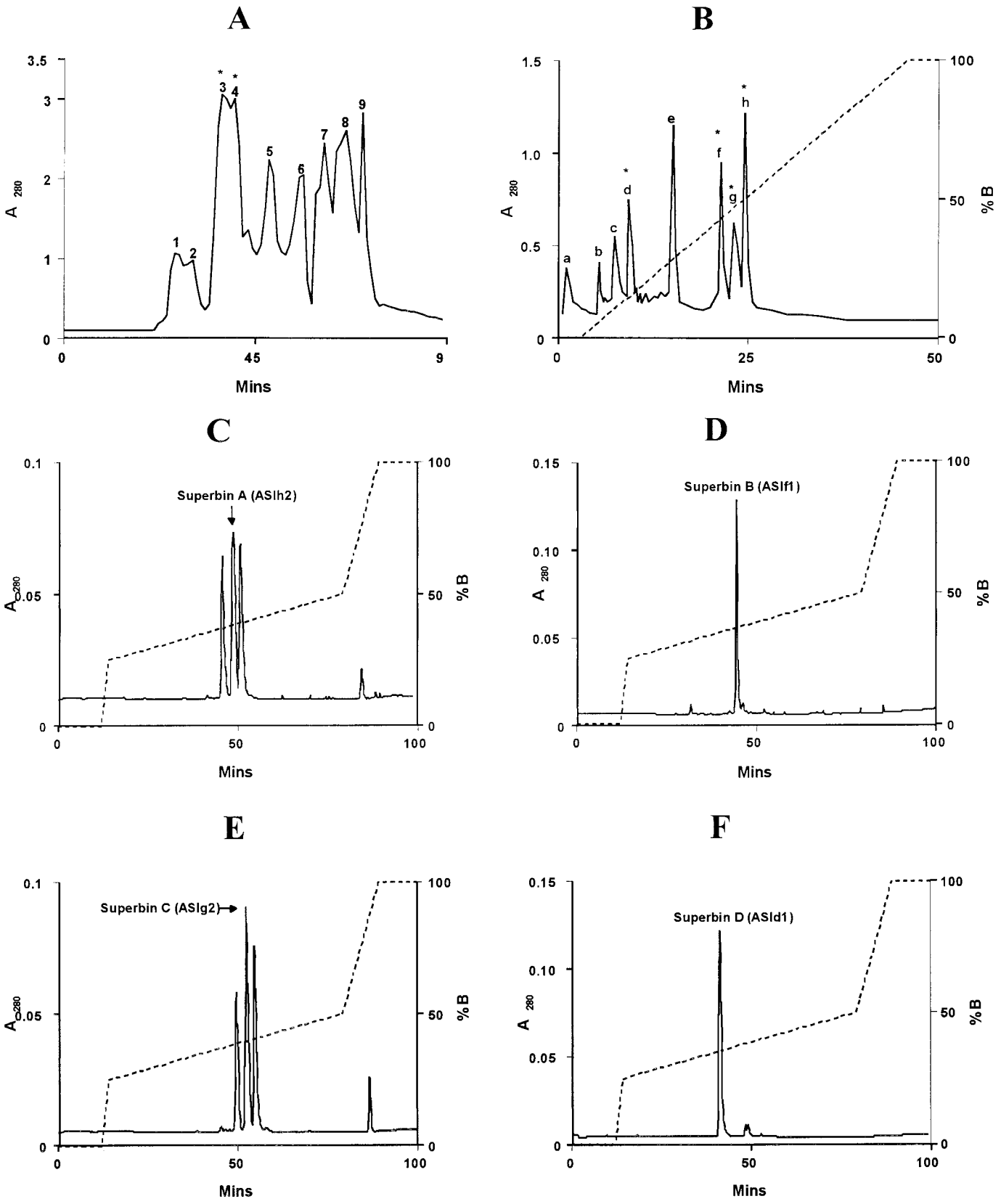
A number of PLA_2 enzymes from snake venoms that affect platelet aggregation have been purified and characterized (8–12). They are the most potent among snake venom enzymes that cause platelet aggregation. The information available on platelet aggregation inhibitors from Australian snake venoms is still very sparse, even though Australian snake venoms are

[1] The nucleotide sequences described in the report have been submitted to GenBank under Accession Nos.: AF184127 to AF184143.

[2] To whom correspondence to be addressed. Fax: (65) 7791453. E-mail: bchjeya@nus.edu.sg.

[3] Abbreviations used: PLA_2, phospholipase A_2; RT-PCR, reverse transcription polymerase chain reaction; FPLC, fast-performance liquid chromatography; TFA, trifluoroacetic acid; ESI, electron spray ionization; CZE, capillary zone electrophoresis; PC, phosphatidylcholine; PBS, phosphate-buffered saline; MuMLV, murine Moloney leukemia virus; BSA, bovine serum albumin; dNTP, deoxynucleotide triphosphate.

0003-9861/00 $35.00


A
B
C
D
E
F
Superbin A (ASIh2)
Superbin B (ASIf1)
Superbin C (ASIg2)
Superbin D (ASId1)
Mins
% B

among the most lethal in the world, containing interesting groups of protein toxins that are different from other snake venoms, presumably because of different evolutionary pressures. Platelet aggregation inhibitors that have recently been purified and characterized are from *Pseudichis papuanus* (13), *Acanthophis praelongus* (14), and *Acanthophis antarticus* (15). Also, three such PLA_2 enzymes have been reported from *Austrelaps superbus* (16, 17).

Most primary structures of PLA_2 reported so far have been obtained by protein sequencing studies. However, some sequences have also been determined via cDNA cloning and nucleotide sequencing (18–21).

In this paper, we report the purification and partial characterization of four inhibitors of platelet aggregation from *A. superbus* venom. These inhibitors are PLA_2 enzymes and have been named Superbins a, b, c, and d. Superbins a and b exhibited quite potent antiplatelet activity. We also report the cloning of cDNAs encoding 17 isoforms of PLA_2 from the same snake.

MATERIALS AND METHODS

Materials. Lyophilized crude venom and venom glands from a single *A. superbus* (Mount Gambier, South Australia) snake were purchased from Venom supplies (Tanunda, South Australia).

Superdex 75 (16 mm/60 cm) columns were purchased from Pharmacia LKB Biotechnology, (Uppsala, Sweden); UNO S1 (7 × 35 mm) cation-exchange columns and cartridges and reagents for p*I* determination were purchased from Bio-Rad (Richmond, CA); and reverse-phase C-18 columns (Nucleosil 5, 10 × 250 mm) were purchased from Phenomenex (Torrance, CA). Reagents for N-terminal sequencing were from Applied Biosystems (Foster City, CA). Collagen and ADP for platelet aggregation assay and lecithin for PLA_2 assay were purchased from Chrono-Log (Havertown, PA) and ICN Biochemicals (Irvine, CA), respectively. The chromatographic reagents were from Fisher Scientific (Springfield, NJ) and Fluka (Buchs, Switzerland).

The *Escherichia coli* strain used as host was JM 109 [*F′tra*D36 *lac*I^q Δ(*lac*Z) M15 *pro*A^+B^+I e14$^-$ (McrA$^-$) Δ(*lac–pro*AB) *thi gyr*A96 (Nal^r) *end*AI *hsd*R17($r_k^-m_k^+$) *rel*AI *sup*E44 *rec*A1] (22). pT7Blue T-vector (Novagen, U.S.A.) was used in subcloning and sequencing of reverse transcription polymerase chain reaction (RT-PCR) products.

Restriction endonucleases, T4 DNA ligase, *Taq* polymerase, and T4 DNA polymerase and deoxynucleotides were purchased from Amersham International p/c (Buckinghamshire, UK). The kits for automated DNA sequencing were obtained from Applied Biosystems (Foster City, CA). The oligonucleotide primers were synthesized and supplied by Oswel DNA service (UK) based on the conserved noncoding regions of the reported PLA_2 precursor gene (18, 19, 21).

All other analytical grade reagents and chemicals used were obtained from Sigma (St. Louis, MO).

Isolation and purification of platelet aggregation inhibitor proteins. Crude *A. superbus* snake venom (150 mg) was dissolved in 1.0 ml of deionized water and applied to a Superdex 75 (16/60) column equilibrated with 50 mM ammonium bicarbonate. Proteins were eluted with the same buffer at the flow rate of 1.5 ml/min and monitored at 280 nm. All the major peaks obtained from the elution profile were pooled and lyophilized and assayed for antiplatelet activities. Fractions that showed antiplatelet activity were then subfractioned on UNO S1 cation-exchange columns by loading 8 mg/ml deionized water, after equilibrating the column with 50 mM ammonium acetate buffer, pH 5.0. Elution was done with a linear gradient of NaCl up to 1 M in the same buffer for 40 min at 2 ml/min on a Pharmacia FPLC system. The elution was monitored at 280 nm. Platelet aggregation inhibitor activities were again monitored for all major peaks. The antiplatelet active fractions were finally chromatographed on a semipreparative reverse phase HPLC column (C-18, Nucleosil 5) equilibrated with 0.1% TFA. The bound protein was eluted with a linear gradient of acetonitrile in 0.1% TFA. Different peaks of proteins, as detected by absorbance at 280 nm, were pooled, lyophilized, and assayed for platelet aggregation inhibitor activities.

Electron spray ionization mass spectrometry (ESI-MS). Molecular masses of native proteins were determined by ESI-MS on a Perkin–Elmer Sciex API III triple-stage quadrupole instrument equipped with an Ionspray interface (Perkin–Elmer Sciex API III LC/MS/MS systems, Thornton, Canada). This technique is useful in determining accurately (0.01% error) the molecular weights of the protein (23). The ionspray and orifice voltages were 4600 and 70 V, respectively; and the interface temperature was 60°C. Nitrogen was used as the curtain gas with a flow rate of 0.6 liter/min while compressed gas was used as nebulizer gas. The samples were infused into the mass spectrometer at a flow rate of 10 μl/min.

Capillary zone electrophoresis (CZE). The purity of the protein samples was determined by capillary zone electrophoresis. CZE was performed on a Biofocus 3000 Capillary Electrophoresis System from Bio-Rad. The samples were injected under pressure mode of 10 psi/s into a coated capillary (25 μm × 24 cm). The run was carried out from positive to negative polarity mode in 0.1 M phosphate buffer (pH 2.5) at 18°C at a constant potential of 12 kV. The proteins were detected by UV absorbance of 200 nm.

Isoelectric point determination. The isoelectric point (p*I*) was determined using the above capillary electrophoresis system. The samples were pressure injected at 100 psi for 60 s into a coated capillary (25 μm × 24 cm), which was thermally controlled at 27°C. The run was carried out from positive to negative polarity mode with focusing and mobilizing voltages at 10 kV. The protein peaks were detected at 280 nm. To determine isoelectric points of pure PLA_2 (in ampholyte), BioMark synthetic p*I* markers (p*I* range: 10.4, 8.4, 7.4, 6.4, and 5.3) were also run under identical conditions. Both samples and standards were run in triplicate to determine the p*I* of the proteins.

Reduction and pyridylethylation. Pyridylethylated proteins were prepared by resuspending the samples in 100 μl of the denaturant buffer (6.0 M guanidium hydrochloride, 0.25 M Tris, 1 mM EDTA, pH 8.5) containing 0.07 M β-mercaptoethanol. The solutions were heated at 37°C for 2 h. Subsequently, a 1.5-fold molar excess (over sulfhydryl groups) of 4-vinylpyridine was added and incubated at

FIG. 1. Purification of superbins. (A) Gel filtration: Superdex 75 column, equilibrated with 0.05 M ammonium bicarbonate, was loaded with 150 mg/ml of *A. superbus* venom dissolved in 1.0 ml of deionized water. Elution was carried out at a flow rate of 1.5 ml/min at room temperature. Fractions AS3 and AS4 showed antiplatelet activity. (B) Cation-exchange chromatography: UNO S1 column, equilibrated with 0.05 M ammonium acetate buffer (pH 5.0), was loaded with 8 mg/ml ASI (AS3 and AS4) fraction. Elution was carried out at a flow rate of 2 ml/min, using a linear gradient of NaCl up to 1 M in the same buffer for 40 min. Fractions ASId, ASIf, ASIg, and ASIh exhibited antiplatelet activity. (C–F) Reverse-phase HPLC: Nucleosil (C-18) column, equilibrated with 0.1% TFA, was used to elute fractions ASId, ASIf, ASIg, and ASIh, respectively, in a linear gradient of ACN in 0.1% TFA. Fractions ASId1 (F), ASIf1 (D), ASIg2 (E), and ASIh2 (C) were found to exhibit antiplatelet activity.

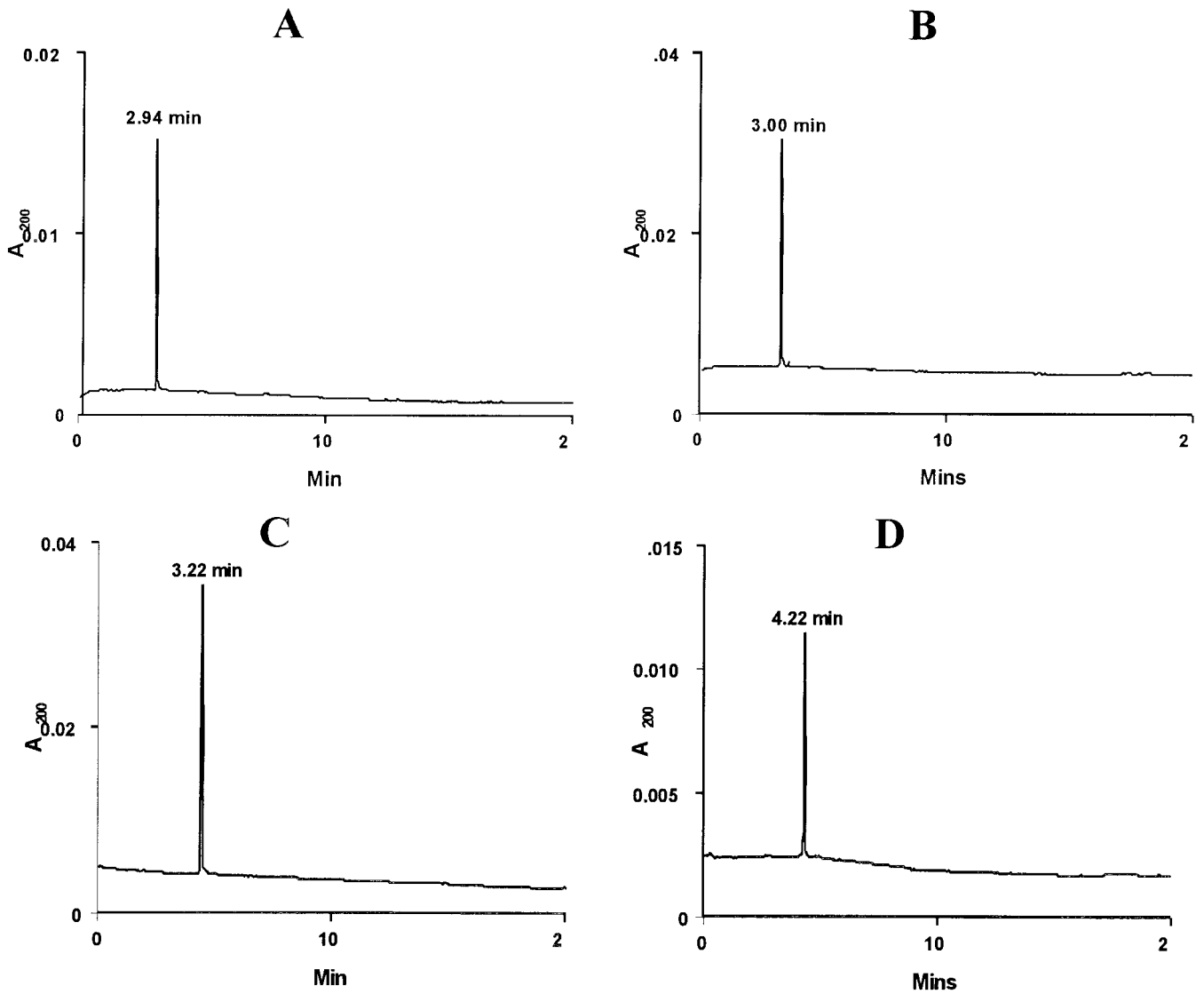


FIG. 2. (A–D) Electropherograms of superbins: CZE was performed using 0.1 M phosphate buffer (pH 2.5) in a coated capillary. Separations were conducted in positive polarity mode at 12 kV at 18°C. Sample was injected under pressure mode of 10 psi/s. (A), (B), (C), and (D) represent Superbins a, c, b, and d, respectively. (E–H) Mass spectrometry: The spectra show a series of multiply charged ions, related to the molecule. Insets show the deconvoluted mass spectrum and also confirm the purity of each of the superbins, as determined by CZE. (E), (F), (G), and (H) represent Superbins a, b, c, and d, respectively.

room temperature. After 2 h, the samples were immediately desalted by HPLC on a Sephasil C-8 reverse-phase column (SC 2.1/10; Pharmacia LKB Biotechnology).

Amino terminal sequencing. The N-terminal amino acid sequences of the pyridylethylated phospholipases were determined by automatic Edman degradation using an Applied Biosystems 477A pulsed liquid phase sequencer. Phenylthiohydantoin amino acids were identified using on-line reverse-phase HPLC on a PTH C-18 column.

Assay for phospholipase activity. PLA_2 activity was determined by following the protocol of Kawauchi *et al.* (24), with slight modification. An aqueous emulsion of 20 mM phosphatidylcholine (PC), in the presence of 10 mM Triton X-100 and 10 mM Ca^{2+}, was used as substrate. Released fatty acids were titrated at pH 8.0 with 0.02 M NaOH at room temperature with a 718 STAT Titrino (Metrohm Herisau, Switzerland). The specific activity of the enzyme was expressed as micromoles of PC hydrolyzed per minute per milligram of protein.

Assay for platelet aggregation inhibitor activity. Inhibition of platelet aggregation was determined by electrical impedance measurements in a whole-blood aggregometer (Chrono-Log Model 500) according to the method of Cardinal and Flower (25). Human blood was collected from volunteers who had not taken any medication for at least a week and had been alcohol-free for at least 4 days. The blood was citrated using 0.11 M trisodium citrate (1:9, v/v). During the studies blood was stored at room temperature and used within 4 h. Aliquots of 500 μl citrated whole blood were transferred into siliconized plastic cuvettes containing 500 μl PBS, pH 7.4 (prewarmed at 37°C), and placed into the reaction chamber, with constant stirring at 1000 rpm. After 2 min, platelet aggregation was

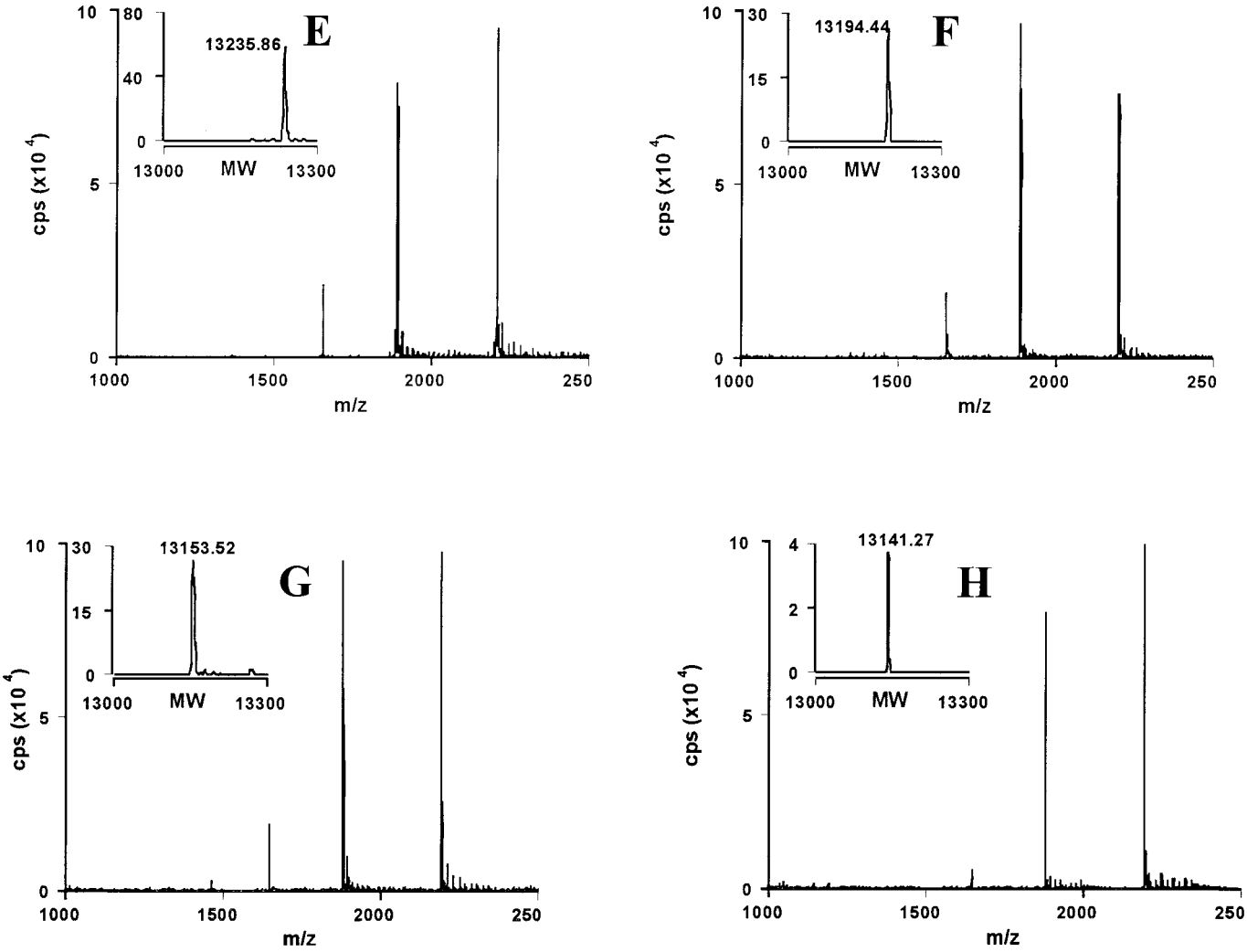


FIG. 2—*Continued*

induced by the addition of an agonist, collagen (2 μl, calf skin, Sigma), and the aggregation was monitored for 6 min. Extent of platelet aggregation was calculated as the percentage of rate of aggregation in the presence of inhibitors as compared with that in their absence.

Isolation of total RNA and RT-PCR. Preparation of total RNA from the *A. superbus* venom gland was based on the method described by Chomzynski and Sacchi (26). The integrity of the total RNA was analyzed by denaturing formaldehyde agarose gel electrophoresis (27, 28). Five micrograms of total RNA isolated from the *A. superbus* venom gland was reverse transcribed using 50 U of MuMLV reverse transcriptase, 2 μl of antisense primer (1 OD_{260}), 2 μl of reverse transcriptase buffer (100 mM Tris–HCl, pH 8.4, 250 mM KCl; 12.5 mM $MgCl_2$, 0.5 mg/ml BSA) in a total volume of 10 μl at 42°C for 1 h and the reaction was terminated at 95°C for 5 min. The entire reaction mixture was used in a 50 μl PCR. PCR was carried out on a Perkin–Elmer Cetus Thermal Cycler (Model 480) for 30 cycles, with each cycle consisting of a denaturing step (94°C for 1 min), an annealing step (50°C for 1 min), and an elongation step (72°C for 2 min) followed by a final extension step of 10 min at 72°C. The PCR mixture contained the reverse-transcribed products, dNTPs (200 μmol of each), sense and antisense primers (5 μl each, 1 OD_{260}), reaction buffer (5 μl; 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin), and 1 U of *Taq* polymerase (29). The fidelity of *Taq* polymerase has been tested as described by Jeyaseelan *et al.* (30) to eliminate the possibility of PCR-induced mutations on RT-PCR products.

Cloning, sequencing, and analysis of cDNAs. The PCR products were cloned into pT7Blue vector using the procedure described by the supplier Novagen. The ligated products were then transformed into the *E. coli* strain JM 109, and transformants were selected on a LB-Amp (50 μg/ml) plate supplemented with IPTG and X-Gal according to Sambrook *et al.* (31).

DNA sequencing was carried out on denatured, double-stranded circular DNA (32) of pT7 Blue-based templates using M13 forward and reverse primers. The sequencing reaction was carried out using the *Taq* Dyedeoxy Terminator cycle sequencing kit supplied by the manufacturer in the Perkin–Elmer Cetus Thermal Cycler. The sequencing reaction was performed using an Automated DNA sequencer (Applied Biosystems; Model 373A). Both strands of the DNA were sequenced. The DNA sequence was translated in all six frames using GenPlot (DNAstar, UK). Deduced amino acid sequences were compared with those of DNA translated in all six frames from nonredundant GenBank using the BLASTP network services at NCBI (33). The sequence homologies were obtained using ALIGN and

TABLE I

Summary of the Properties of Superbins

PLA_2 enzymes	CZE migration time (min)	Molecular mass (Da)	PLA_2 specific activity (μmol phosphatidylcholine hydrolyzed/min/mg)	p*I*	IC_{50} (μM)
Superbin a	2.94	13,235.86 ± 0.39	6.372 ± 0.080	9.33 ± 0.10	0.009
Superbin b	3.22	13,194.44 ± 0.62	266.442 ± 6.190	9.20 ± 0.09	0.050
Superbin c	3.00	13,153.52 ± 1.44	17.192 ± 0.100	9.30 ± 0.10	0.500
Superbin d	4.22	13,141.27 ± 0.52	186.045 ± 5.27	7.95 ± 0.11	3.000

AALIGN programs from DNAstar and SeqEd program (Applied Biosystems).

RESULTS

Isolation and Purification of Superbins

Superbins a, b, c, and d were purified from crude *A. superbus* venom by a three-step purification process. The initial fractionation of the venom (150 mg) on Superdex 75 gel filtration yielded nine major peaks (Fig. 1A) of which fractions AS3 and AS4 were found to have both platelet aggregation inhibitor and PLA_2 activities. These two fractions were pooled and used as fraction ASI for purification on UNO S1, a cation exchanger (Fig. 1B). This second purification step yielded eight major fractions, of which only ASId, ASIf, ASIg, and ASIh exhibited both antiplatelet and PLA_2 activities. For final purification, each of the four lyophilized peaks was subjected separately to reverse-phase HPLC (C-18 column, Nucleosil). Fractions ASId and ASIf yielded only one major peak each (Figs. 1F, 1D), whereas fractions ASIg and ASIh yielded three major peaks each (Figs. 1E, 1C). Peaks ASId1, ASIfI, ASIg2, and ASIh2 were found to exhibit both platelet aggregation inhibitor and PLA_2 activities. These peaks were renamed on the basis of their activities on platelet aggregation. ASIh2 was renamed superbin a, ASIf1 superbin b, ASIg2 superbin c, and ASId1 superbin d. The total yields of superbins a, b, c, and d were 1.5, 7, 2.5, and 6.6 mg, respectively. The additional peaks observed in Figs. 1C and 1E showed no inhibitory property on platelet aggregation and their PLA_2 activities were also rather low (3.0–5.1 μmol of phosphotidylcholine hydrolyzed/min/mg protein).

Homogeneity Determinations and MS Studies

Purity and homogeneity of the four superbins were assessed by CZE and ESI-MS. All four superbins produced single peaks on the CZE runs (Figs. 2A–2D). The migration times, though close, were distinct for the four phospholipases. Superbins a, b, and c were much closer in terms of migration time as compared with superbin d. The migration time of superbin a was found to be 2.94 min. Superbins c and b had migration times of 3.00 and 3.22 min, whereas that of superbin d was 4.22 min (Figs. 2A–2D, Table I).

MS studies were in agreement with the CZE results, reconfirming the homogeneity of the four superbins isolated. Each of the samples yielded one peak only (Figs. 2A–2D). The molecular masses of superbins a, b, c, and d were found to be 13,235.86, 13,194.44, 13,153.52, and 13,141.27 Da, respectively (Figs. 2E–2H, Table I).

pI Determinations

The synthetic markers were all eluted within the range 6–14 min on the 25 μm × 24 cm coated capillary. The calibration plot of normalized migration time versus pH produced a linear regression line. Superbin a was found to be most basic with a p*I* of 9.33. Superbins b, c, and d were also found to be basic with p*I* values of 9.20, 9.30, and 7.95, respectively (Table I).

PLA_2 Activities

PLA_2 activities were detected in all four superbins. Superbins a and c had low specific activities of 6.372 and 17.192 U/mg. In contrast, Superbins b and d had much higher specific activities of 186.045 and 266.442 U/mg, respectively (Table I).

Platelet Aggregation Inhibitor Assay

The effect of superbins on platelet aggregation was measured by using healthy human whole blood with collagen as platelet agonist. All four superbins were found to inhibit collagen-induced platelet aggregation to varying degrees and this effect appears to be dose dependent. The IC_{50} (inhibitor concentration causing 50% inhibition) values were estimated from the dose–response curves (Fig. 3).

Superbin a was found to be the most potent inhibitor of platelet aggregation, having an IC_{50} of 9.0 nM. However, the IC_{50} values of Superbins b, c, and d were relatively lower at 0.05, 0.5, and 3.0 μM, respectively (Table I).

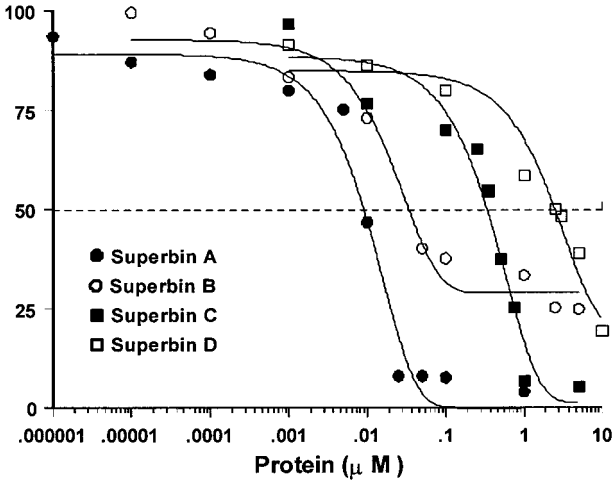


FIG. 3. Platelet aggregation curves: Different concentrations of Superbins a, b, c, and d were added to citrated blood and the effects of electrical impedance were measured in a whole-blood aggregometer using collagen as platelet agonist.

The potencies of the phospholipases seemed to correspond to the basicities of their isoelectric points; i.e., the most potent of the three phospholipases seemed to be highly basic as compared with the least potent. Similar observations were made with *Acathophis praelongus* venom (14). Also, platelet activity does not seem to correspond to PLA_2 activity (Table I).

Amino-Terminal Sequences of Superbins

To confirm the identity of superbins and to partially characterize them, amino acid sequencing of pyridylethylated proteins was carried out. The amino terminals of all four superbins were identified unequivocally by automated Edman degradation. Superbins a and b were sequenced up to 62 and 57 residues, respectively, whereas Superbins c and d were sequenced up to 46 and 48 residues (Fig. 4).

All four sequences have the same first two residues (asparagine and leucine). There appear to be two main continuous conserved regions, residues 25 to 30 (YGCYCG) and residues 35 to 45 (GTPVDELDRCC). The differences in position of histidine and proline were apparent. Histidine was observed at position 21 of all but superbin b. Also, proline was present at position 31 of superbin a (the most potent PLA_2) in addition to position 37, thus enclosing a short region between two prolines. A survey of the database of protein–protein interaction sites indicates that proline residues are found predominantly in the flanking segments of the interaction site (34), so this could be a potential protein–protein interaction site.

cDNA Cloning

Total RNA extracted from *A. superbus* snake venom gland was reverse transcribed into single-strand cDNA

Superbin a

```
1          10          20          30          40          50          60  62
NLYQFKNMIQ CANHGRRATW HYLDYGCYCG PGGLGTPVDE LDRCCKTHDD CYIEAGKKGC FP
```

Superbin b

```
1          10          20          30          40          50        57
NLYQFKNMIQ CANRGSRHWL AYADYGCYCG WGGSGTPVDE LDRCCKTHDD CYTEAGK
```

Superbin c

```
1          10          20          30          40        46
NLIQLSNMIK CAIPGSRPLL HYTDYGCYCG KGGSGTPVDE LDRCCK
```

Superbin d

```
1          10          20          30          40          48
NLVQFSNMIQ CANHGSRPTR HYVDYGCYCG WGGSGTPVDE LDRCCQTH
```

FIG. 4. Amino-terminal sequences of superbins: Two main continuous conserved regions have been underlined. All the histidine residues are shown in bold. Two closely placed prolines (Superbin a) enclosing a short segment have been italicized.

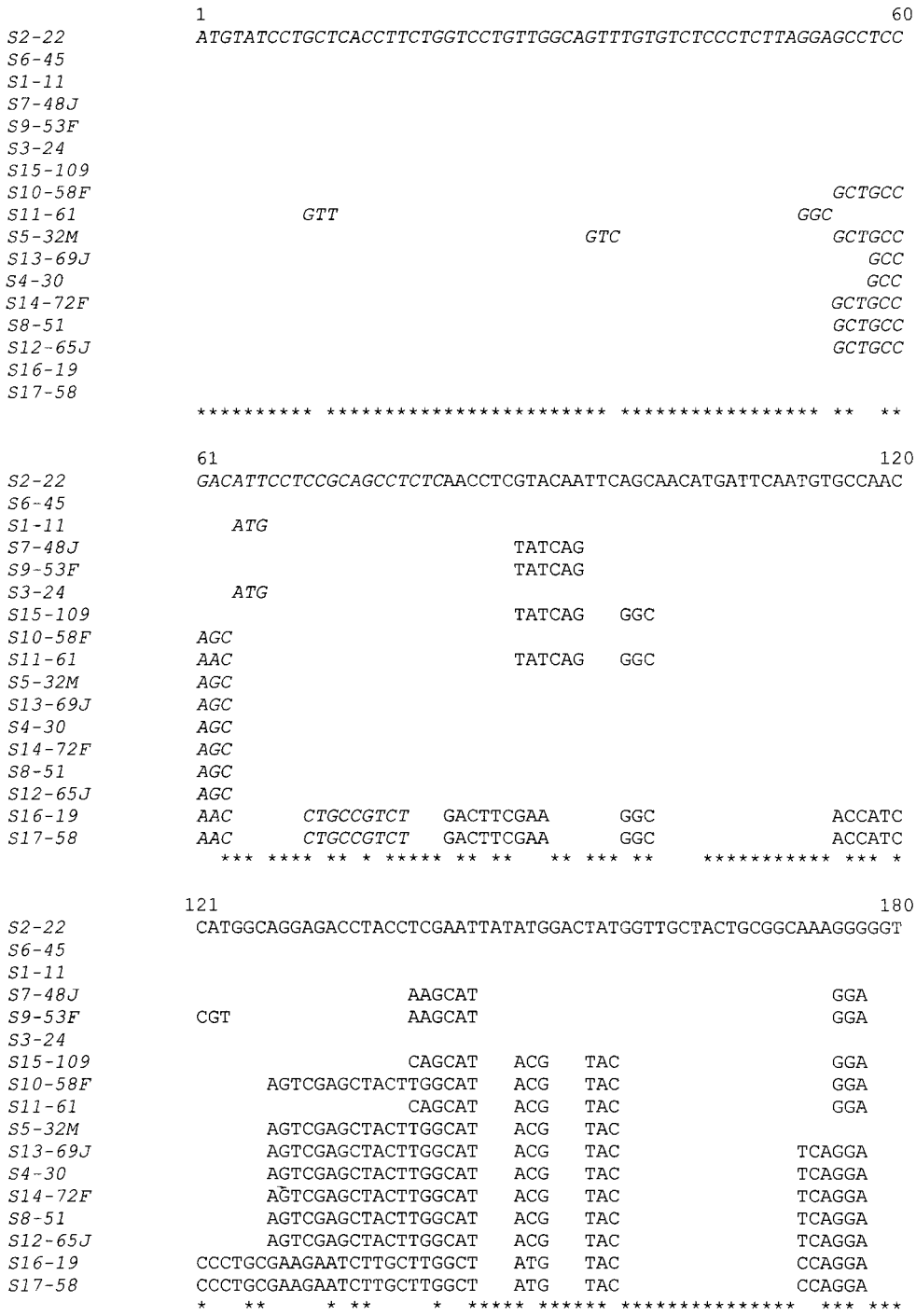


FIG. 5. Nucleotide sequences of cDNAs: Seventeen isoforms of PLA_2 enzyme in *Austrelaps superbus* were compared. The similar nucleotide bases are indicated with asterisks. The signal peptide regions are indicated in italics. Small gaps indicate alignment gaps. Blank spaces represent the conservation of nucleotide sequence (codon). The codons harboring mutations have been shown for 16 of the 17 sequences. The complete sequence of one of the clones (S2-22) has been considered as a typical sequence.

using MuMLV reverse transcriptase. Second-strand cDNA synthesis and subsequent PCR amplification were performed with *Taq* DNA polymerase, using PLA_2 gene-specific primers. The degenerate primers were (sense) 5′ TTC A(C/A)(C/T) (A/T)C(G/T) GAC AAA ATG 3′ and (antisense) 5′ (G/C)(A/T)(A/T) GC(T/C) TCT CAA ATA TCA 3′. The expected 500-bp cDNA was observed from the RT-PCR. These cDNAs were sub-

```
                181                                                                  240
S2-22           AGCGGGACACCGGTAGATGAGTTGGATAGGTGCTGCAAAATACATGACGACTGCTATGGT
S6-45
S1-11
S7-48J                          GAA
S9-53F                          GAA                 GTA
S3-24                           GCG
S15-109                                             ACA                 ACT
S10-58F                                             ACA                 ACT
S11-61                                              ACA                 ACT
S5-32M
S13-69J               CCCGTG                      CAGACT      AAC       GCT
S4-30                 CCCGTG                      CAGACT      AAC       GCT
S14-72F               CCCGTG                      CAGACT      AAC
S8-51                 CCCGTG                      CAGACT      AAC
S12-65J               CCCGTG                      CAGACT      AAC
S16-19          AGT   CCGTTA                    CGCCAGACT     AAC       GCT
S17-58          AGT   CCGTCA                      CAGACT      AAC       GCT
                ** ********    ****  ************ ** *    ****** ********  *

                241                                                                  300
S2-22           GAAGCCGAAAAAA-----GCCAGAAGTGCGCCCCCTACTGGACGTGGTA----TACTTGG
S6-45                               AAT
S1-11
S7-48J                              ATT
S9-53F                              ATT
S3-24                               ATT
S15-109               GGA           A--AGGA-TGCTACCCCAAGTTGACGTTGT       AGT
S10-58F               GGA           A--AGGA-TGCTACCCCAAGTTGACGTTGT       AGT
S11-61                GGA           A--AGGA-TGCTACCCCAAGTTGACGTTGT       AGT
S5-32M                              A--AGGA-TGCTACCCCAAGATGTCGGCGT       GATTAC
S13-69J                             A--AGGA-TGCTACCCCAAGATGTCGGCGT       GATTAC
S4-30                               A--AGGA-TGCTACCCCAAGATGTCGGCGT       GATTAC
S14-72F                             A--AGGA-TGCTACCCCAAGATGTTGGCGT       GATTAC
S8-51                               A--AGGA-TGCTACCCCAAGATGTCGGCGT       GATTAC
S12-65J                             A--AGGA-TGCTACCCCAAGATGTTGGCGT       GATTAC
S16-19             GCTGGA   CTTCCTGCATGTAAGGCCATGCTGAGTGAGCCCTACAACGACACCTAT
S17-58             GCTGGA   CTTCCTGCATGTAAGGCCATGCTGAGTGAGCCCTACAACGACACCTAT
                ***** * ****                **    *  *           *        *

                301                                                                  360
S2-22           AAATGTGGCAGCGATGGACCACA------GTGCGACGATTCAAAAACGGGGTGTCAACGT
S6-45
S1-11                                                       GAG      AGTCGA
S7-48J                                                      GAA
S9-53F                                                      GAA
S3-24                                                       GAA          AAA
S15-109                                                     GAA
S10-58F               ACTGGAAAGGCACCCAC-    --C   A---AT                 AAA
S11-61                ACTGGAAAGGCACCCAC-    --C   A---AT                 AAA
S5-32M          TAC      GAAAATGCACCCTA-    --C   AGAAATATC    AAGGAG
S13-69J         TAC      GAAAAT   CCCTA-    --C   AGAAATATC    AAGGAG
S4-30           TAC      GGC      CCCTA-    --C   AGAAATATC    AAGGAG
S14-72F         TAC      GGC      CCCTA-    --C   AGAAATATC    AAGGAG
S8-51           TAC      GGC      CCCTA-    --C   AGAAATATTAGAAAGGAG
S12-65J         TAC      GGC      CCCTA-    --C   AGA   ATC    AAGGAG
S16-19          TCATAC   TGTATTGAACGCCAACTCACC   AAC   GACAACGATGAG    AAAGCC
S17-58          TCATACAGCTGTATTGAACGCCAACTCACC   AAC   GACAACGATGAG    AAAGCC
                   *          * **          ***    **         * * **  *
```

FIG. 5—*Continued*

cloned into pT7Blue vector and the recombinant clones (maintained in *E. coli* JM 109) were confirmed by restriction with *Eco*RI and *Sal*I, followed by agarose gel electrophoresis to check for the presence of inserts. Thirty-two recombinant plasmids harboring the 500+-bp DNA were obtained.

Nucleotide Sequencing and Analysis

All positive clones have been sequenced using universal M13/pUC reverse and forward primers. Each of these sequences (nucleotide and the deduced amino acid sequence), when compared using BLAST analysis

```
                361                                                                  420
S2-22           TTTGTGTGTGATTGTGACGCCACAGCAGCCAAGTGCTTTGCCAAAGCCCCTTACAACAAG
S6-45
S1-11
S7-48J          TCT       GAA   GAT   ATA                                       GAC
S9-53F          TCT       GAA   GAT   ATA                                       GAC
S3-24           ATT             GTCGAA       GAC   TTC   GGA                   AAC
S15-109                 GAT
S10-58F         ACT       GAT                                                   AAC
S11-61          ACT       GAT                                                   AAC
S5-32M                    GTTGAA                    AGA                         GAC
S13-69J                   GTTGAA                    AGA                         GAC
S4-30                     GTCGAA                    AGA                         GAC
S14-72F                   GTCGAA                    AGA                         GAC
S8-51                  GAT   ATA                                                GAC
S12-65J         TTGTGTGTGATTGTGACG-TCGAA                                  GAC
S16-19             ATT   AAT       CGCGCA   GTCATC   TTCTCCGGA                 GAC
S17-58             ATT   AAT       CGCGCA   GTCATC   TTCTCCGGA                 GAC
                    *  **   * ***       ***** *   *****  **  ********** ** *

                421
S2-22           GAGAACTACAAT------ATAAAGACACGTTGCCAA
S6-45
S1-11                     ATCGAG
S7-48J          GCG   TGGGATATCGACACCGAG
S9-53F          GCG   TGGGATATCGACACCGAG
S3-24           GCA   TGG   ATCGACACC   AAA
S15-109                           CCT
S10-58F         AAG         ATCGACACC   AAA
S11-61          AAG         ATCGACACC   AAA
S5-32M          GCG   TGG   ATCGACACC   AAA   TGT
S13-69J         GCG   TGG   ATCGACACC   AAA   TGT
S4-30           GCG   TGG   ATCGACACC   AAA   TGT
S14-72F         GCG   TGG   ATCGACACC   AAA   TGT
S8-51           GCG   TGGGATATCGACACCGAG
S12-65J            AAAGGAGAACTACAATAT
S16-19          TCG       GATATCGGCACCATTGAACAT   AAA
S17-58          TCG       GATATCGGCACCATTGAACAT   AAA
                   **     *              ** ***  **
```

FIG. 5—*Continued*

on GenBank and SwissProt databases, confirmed the presence of PLA_2 sequences.

On comparing the 32 cDNA sequences among themselves, 17 unique clones were identified on the basis of sequences of the PLA_2 proteins. Figures 5 and 6 are comparisons of the nucleotide and the deduced amino acid sequences, respectively. This is also the first report of so many isoforms being present in a single species.

Some of the clones were found to be identical. Clone S14-72F was found to be the most abundant form (six clones). Clones S2-22 and S1-11 were found to have five clones each. Clones S13-69 J and S11-61 were the third most abundant types (two clones each); all others had one clone each.

The proteins were grouped into three groups based on the conserved regions (residues 24–37) of the protein sequences (Fig. 6). About 58.8% of the clones belonged to Group 1 and 29.4% to Group 2; Group 3 had only 11.8% clones. Based on the amino acid sequences (Fig. 6), eight different signal peptides were identified. Clone S5-32M showed the most common signal peptide (37.5%), and Clone S15-109J contained the second most common form (31.3%). The frequency of all the others was less than 10%. Despite the slight difference in these forms, the hydrophobicity of the signal peptide is maintained.

The residues that are necessary for the enzymatic activity of PLA_2, such as the catalytic network, His^{48}, Asp^{49}, Tyr^{52}, Tyr^{73} and Asp^{99}; the hydrophobic region around the enzymatic site, Leu^{2}, Phe^{5}, Tyr^{22}, Cys^{29}, Cys^{45}, Ala^{102}, Ala^{103}, and Phe^{106}; and the Ca^{2+} binding site residues, Tyr^{28}, Gly^{30} and Gly^{32} and Asp^{49} (35), are all conserved in the sequences (Fig. 6).

Clones S16-19 and S17-58 were found to have the amino acid residues (Ala^{62} to Glu^{66}) observed in pancreatic PLA_2 enzymes (36). The pancreatic loop is between two proline residues (Pro^{59} and Pro^{68}) and contains several hydrophilic amino acids.

Characterization of cDNA Clones

Based on the nucleotide and protein sequences, the molecular masses, p*I* values, and number of amino acids for each of the 17 clones (Table II) were calculated. The molecular masses were all found to be within the range 13,000 to 14,000. The smallest molec-

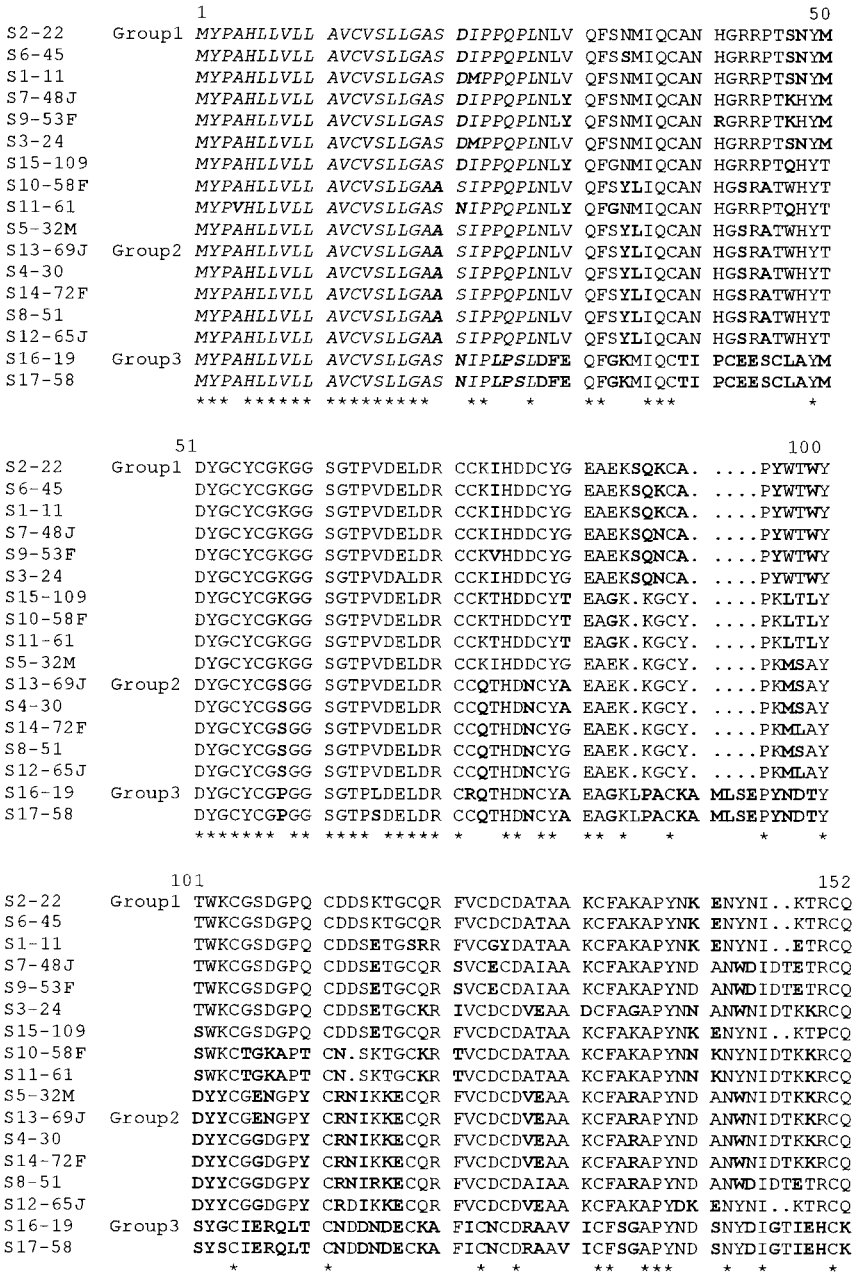


FIG. 6. Amino acid sequences of isoforms of PLA_2: The amino acid sequences were aligned using the MegaAlign program (DNASTAR). The signal peptide regions are given in italics. Asterisks show the exact consensus region. The bold residues indicate deviation from the consensus. The phospholipases are grouped on the basis of homologous residues 24 to 37 of the protein.

ular mass was 13,029.80 Da, and as expected, the largest molecular masses were those of Clones S17-58 and S16-19, which had maximum number of amino acids because of the presence of the pancreatic loop.

On observing the p*I* values of all the clones, it was found that 13 of 17 clones were acidic. The most acidic clones were those containing the pancreatic loop, S17-58 and S16-19, with p*I* values of 4.02 and 4.11, respectively. Of the four basic clones, the most basic was Clone S11-61, with a p*I* of 8.56 (Table II).

The number of amino acids varied for the clones, with a range of 117–125 (Table II). Mostly all the clones had 117–120 amino acids, except two (S17-58 and S16-19). There were two forms with 117 amino acids, 3 with 118, and 5 each with 118 and 119 amino acids.

Comparison of Clones with Superbins

All the cDNA isoforms were compared with each of the superbins, in terms of amino-terminal sequences.

TABLE II

Characterization of cDNA Clones

Clone No.	Molecular mass (Da)	p*I*	No. of amino acids
S1-11	13,336.80	6.80	118
S2-22	13,321.00	7.36	118
S3-24	13,381.90	4.84	120
S4-30	13,499.30	6.52	119
S5-32M	13,610.60	6.99	119
S6-45	13,294.00	7.36	118
S7-48J	13,572.10	4.75	120
S8-51	13,444.20	5.16	119
S9-53F	13,577.00	4.75	120
S10-58F	13,128.30	7.97	118
S11-61	13,168.30	8.56	118
S12-65J	13,290.20	6.07	117
S13-69J	13,570.30	6.52	119
S14-72F	13,511.40	6.52	119
S15-109	13,029.80	6.99	117
S16-19	13,904.00	4.11	125
S17-58	13,854.90	4.02	125

None of the cDNA clones could give a 100% match. It was observed that Clones S11-61 and S15-109 had the closest match of 87.10% each (both were identical for the first 62 residues) to Superbin a (most antiplatelet activity): 84.21% each to Superbin b; and 80.43% to Superbin c (Clones S7-48J and S9-53F also had the same homology). All other clones, except S16-19 and S17-58, were found to have a consensus of more than 74% with Superbin a and more than 73% with Superbins b and c. The clones most homologous to Superbin d (least antiplatelet activity) were S4-30, S7-48J, S12-65J, S13-69J, and S14-72F. All other clones, except S16-19 and S17-58, were found to have a consensus of more than 83%.

Clones S16-19 and S17-58 possessing the pancreatic loop were found to have the minimum consensus with each of the superbins (54–63%).

DISCUSSION

Snake venom PLA_2 enzymes, in addition to their possible role in digestion of prey, exhibit a wide variety of pharmacological effects, including effects on platelet aggregation [either initiating or inhibiting or both (10, 11, 37, 38)]. Several platelet aggregation inhibitors have been purified and characterized from various snake venoms, but the information on Australian snake venoms, with respect to platelet aggregation inhibitors is still scant. Thus, several Australian snake venoms were screened to detect antiplatelet activity. Among these the crude venom from *A. superbus* was found to have potent antiplatelet activity, and so studies were initiated to identify and characterize the component(s) responsible. Four new antiplatelet proteins (PLA_2 isoforms)—Superbins a, b, c, and d—have been isolated and purified from *A. superbus* venom by a three-step sequential purification process.

The superbins were characterized in terms of PLA_2 activities, p*I* values, molecular masses, and IC_{50} values. Each of these proteins exhibited varying degrees of PLA_2 activity when assessed for phospholipase activity by a titrimetric method using phosphatidylcholine as substrate. Superbins a and c showed low specific activities of 6.37 and 17.19 U/mg, and Superbins b and d exhibited much higher activities of 186.05 and 266.44 U/mg, respectively.

Superbins a, b, c, and d have been found to be basic, with p*I* values of 9.33, 9.2, 9.3, and 7.95, respectively. The majority of Australian elapid PLA_2 enzymes have been found to be basic proteins with 118 amino acids and seven disulfide bridges and molecular masses around 13 kDa (39). The basicity can also be observed in superbins.

The molecular masses as determined by MS are also similar to monomeric molecular masses of venom PLA_2 enzymes (39). Superbins a, b, c, and d had molecular masses of 13,235.86, 13,194.44, 13,153.52, and 13,141.27 Da, respectively. Superbin a has a molecular mass similar to that observed by Subburaju and Kini (17) for Superbin II, which was nonhomogeneous.

All four superbins inhibited platelet aggregation in human whole blood. Dose–response studies show that Superbin a is the most potent antiplatelet PLA_2, with an IC_{50} of 9 nM. Superbins b and c form the intermediate group, with IC_{50} values of 50 and 500 nM, respectively; and Superbin d exhibits the least antiplatelet activity, with an IC_{50} of 3000 nM.

PLA_2 enzymes that affect platelet aggregation have been classified into three major classes (40): Class A contains PLA_2 enzymes that induce platelet aggregation; Class B contains PLA_2 enzymes that inhibit platelet aggregation; and Class C contains PLA_2 enzymes that act as both inducer and inhibitor. Since all four isoforms of superbins inhibit platelet aggregation, they belong to Class B. Class B can be further subdivided into two subgroups: Class B1, inhibitors that are dependent on the catalytic activity of the enzymes such as PLA_2 from *A. superbus* (16, 41) and *Lachesis muta* (42); and Class B2, inhibitors that are independent of its enzyme activity, such as PLA_2 from *A. halys* venom (43). These nonenzymatic mechanisms have not yet been deciphered.

Proline residues are known to break the continuity of any secondary structure of the flanking regions (44) and protect the conformation and integrity of the interaction sites. They set the interaction sites apart by inducing kinks and bends. Thus, proline brackets facilitate protein–protein interaction (45). Superbin a, the most potent platelet aggregation inhibitor PLA_2, was found to have two closely placed prolines, enclos-

ing five residues. This site could possibly be one of the protein–protein interaction sites.

cDNA cloning has shown the existence of 17 isoforms of PLA_2 mRNAs in the venom gland of *A. superbus*. It is possible that these messages are translated into PLA_2 isoforms to be present in the venom. The presence of such multiple isoforms has also been reported for *Vipera russelli formosensis, Naja naja atra, Crotalus durissus, Naja naja sputatrix, Bungarus multicinctus,* and *Trimeresurus flavoviridis* (19, 21, 46–50).

Comparison of the nucleotide sequences of the cDNAs of the *A. superbus* PLA_2 isoforms reveals that substitution for the nucleotide occurred mostly in the first and second positions of the triplet codons. This unusually high rate of substitution in the first and second positions has also been observed by Armugam *et al.* (21). The open reading frame of the cDNA covers the entire phospholipase gene sequence, containing an 81-bp signal sequence, approximately 357-bp (not the same for all isoforms) coding sequence for mature protein, and approximately 36-bp 5′ and 3′ untranslated regions. It was also observed that there was much more base substitution in the structural gene as compared with that in the signal peptide. A similar observation was noted where the nucleotide sequence of the signal peptide coding domain of the exon from a genomic sequence from *Trimeresurus flavoviridis* PLA_2 was demonstrated to be much more conserved than the PLA_2 protein coding region (51). They suggest that the PLA_2 protein coding regions of the gene evolved with a greater substitution rate. The signal peptide coding domain was reported to be in a separate exon, the first exon. This exon is known to show a low rate of variation as compared with the other exons coding for mature protein. Krizaj *et al.* (52) proposed that a higher mutational rate of the protein coding region, together with gene duplications, finally would result in a broad spectrum of snake PLA_2 species and their homologs with different biological activities that can be found in a single snake species.

The building block of a typical signal peptide comprises a basic N-terminal region, a central core hydrophobic region, and a more polar C terminal. A helix-breaking residue (Pro/Gly) normally occurs before the cleavage site and ends with an uncharged amino acid residue (53). The deduced amino acid sequences for the signal peptides were compared to identify these sites. Each of the PLA_2 had a 27-amino-acid residue signal peptide, beginning with a methionine residue, followed by neutral and polar tyrosines and then neutral and hydrophobic prolines. The next neutral and hydrophobic residue was alanine for all but one clone (S11-61) where the replacement was with another neutral and hydrophobic residue, valine. A basic histidine residue and a number of highly hydrophobic residues followed this. A cysteine residue occurs at the midregion of the hydrophobic core. Four to five helix breakers (Pro/Gly) are also found along the hydrophobic region.

The C-terminal pentapeptide of the signal peptides contains two or three proline residues, which is similar to those of Group I snakes, suggesting that *A. superbus* PLA_2 isoenzymes indeed belong to Group I. Similar observations have been reported for venom PLA_2 enzymes from Elapidae and Hydrophidae (21, 49, 54, 55).

Austrelaps superbus PLA_2 enzymes consist of 117–120 amino acid residues. Only two PLA_2 enzymes had 125 amino acids each because of the presence of the pancreatic loop. On the basis of sequences, the molecular masses and p*I* values were also estimated. All of the PLA_2 isoforms determined by cDNA cloning had molecular masses ranging from 13 to 14 kDa, which are similar to monomeric molecular masses of venom PLA_2 enzymes (39). However, approximately, 76% of the clones have been found to be acidic, thus making these isoforms distinct from most of the Australian snake venom PLA_2 enzymes, which are known to be basic (39, 54, 56).

The primary structure of all these isoforms exhibited characteristic features of Group 1 PLA_2 enzymes. There are seven pairs of cysteine residues in all but one clone. The degree of homology with other venom PLA_2 enzymes was found to be more than 85%.

The primary structure also revealed that all the residues necessary for enzymatic action, catalytic network, and formation of a hydrophobic wall around the enzymatic site and responsible for Ca^{2+} binding (35) are conserved fully in 88% of the isoforms. Only the two distinct clones possessing the pancreatic loop did not adhere fully to this proposed network. These clones, with extra amino acid residues (62–66), were found to belong to Group IB PLA_2 enzymes (36, 57). These isoforms are commonly found in pancreatic PLA_2 enzymes and are almost absent among snake venom PLA_2 enzymes. Among snake venom PLA_2 enzymes, only a few enzymes from Australian "elapids" have been reported to contain such pancreatic loops. They are the three nontoxic PLA_2 enzymes: the γ subunit of taipoxin, the D subunit of textilotoxin and OS_1, and a toxic PLA_2, HT_e (58–62). These sequences are known to have an octapeptide of the signal peptide attached to the mature protein, which may even be true for these clones.

Variations in snake venom composition have been associated with factors such as age, sex, geographic origin, season of the year, and diet (63). Sasa (64) has hypothesized three reasons for these variations: venom variation could be a function of geographic distance or it may result from the phylogenetic relationships among populations or it could even be associated with diet variation among populations.

Studies on *Naja haje* venoms have revealed a great heterogeneity even in litters born in the same snake farm (65). Also, individual specimens of *Bothrops jara-*

raca (66) have been shown to exhibit different PLA_2 activities among individual snakes, maintained under seasonally invariant conditions. Individual and age-dependent variations have been shown in *Bothrops atrox* venoms (67) whereas geographic and ontogenic variations have been shown in *Crotalus atrox* snake venom (68).

Another reason for differences in cDNA isoforms and superbins can be seen at the genetic level. Vandenplas *et al.* (69) reported that there was an increase in the transcription level of mRNA in the snake venom gland following milking of the venom. The increase was demonstrated to be at the maximum level between 3 and 5 days after milking the venom (70), presumably because once the protein is depleted the toxin genes become activated with the production of corresponding mRNAs. However, in the present study, the *A. superbus* venom gland was isolated immediately after milking since the sample was procured commercially. Considering all these factors, the probability of finding 100% homology seems quite low. Only one of the clones, S11-61, showed more than 85% similarity, besides being basic, with Superbin a. The mRNAs cloned may possibly represent the low-abundance isoforms of superbins in the venoms of *A. superbus.* The present study, however, has revealed the presence of at least 21 isoforms of PLA_2 enzymes in *A. superbus* venom. More cloning and protein purification studies have to be carried out to confirm these findings. The characterization of these isoforms by expressing their cDNAs in hosts such as *E. coli* may also help to identify the functional and pharmacological properties of these proteins.

ACKNOWLEDGMENTS

This work was supported by academic research grants, RP960324 and RP950377, to K.J. and R.M.K., respectively, from the National University of Singapore. S.B. received a Research Scholarship from the University.

REFERENCES

1. Shuman, M. A., and Greenberg, C. S. (1986) *in* Biochemistry of Platelets (Phillips, D. R., and Shuman, M. A., Eds.), pp. 319–346, Academic Press, Orlando, FL.
2. Stormorken, H. (1986) *in* Platelet Responses and Metabolism (Holmsen, H., Ed.), Vol. I, pp. 3–32, CRC Press, Boca Raton, FL.
3. Rickles, F. R., and Edwards, R. L. (1983) *Blood* **62,** 14–31.
4. Gastpar, H. (1982) *Ann. Chir. Gynaecol.* **71,** 142–150.
5. Verheij, H. M., Boffa, M. C., Rothen, C., Bryckert, M. C., Verger, R., and de Haas, G. H. (1980) *Eur. J. Biochem.* **112,** 25–32.
6. Kini, R. M., and Evans, H. J. (1989) *Toxicon* **27,** 613–635.
7. Rosenberg, P. (1986) *in* The Relationship between Enzymatic Activity and Pharmacological Properties of Phospholipases in Natural Poisons (Harris, J. B., Ed.), pp. 129–174, Clarendon Press, Oxford.
8. Ouyang, C., Teng, C. M., Chen, Y. C., and Lin, S. C. (1978) *Biochim. Biophys. Acta* **541,** 394–407.
9. Ouyang, C., and Huang, T. F. (1984) *Toxicon* **22,** 705–718.
10. Teng, C. M., Chen, Y. H., and Ouyang, C. (1984) *Biochim. Biophys. Acta* **772,** 393–402.
11. Teng, C. M., Kuo, Y. P., Lee, L. G., and Ouyang, C. (1986) *Thromb. Res.* **44,** 875–886.
12. Chen, R. H., and Chen, Y. C. (1989) *Toxicon* **27,** 675–682.
13. Laing, G. D., Kamiguti, A. S., Wilkinson, M. C., Lowe, G. M., and Theakston, R. D. G. (1995) *Biochim. Biophys. Acta* **1250,** 137–143.
14. Sim, K. L. (1997) *Biochim. Biophys. Acta* **1379,** 198–206.
15. Chow, G., Subburaju, S., and Kini, R. M. (1998) *Arch. Biochem. Biophys.* **354,** 232–238.
16. Yuan, Y., Jackson, S. P., Mitchell, C. A., and Salem, H. H. (1993) *Thromb. Res.* **70,** 471–481.
17. Subburaju, S., and Kini, R. M. (1997) *Toxicon* **35,** 1239–1250.
18. Guignery-Frelat, G., Ducancel, F., Menez, A., and Boulain, J. C. (1987) *Nucl. Acid Res.* **15,** 5892.
19. Pan, F. M., Chang, W. C., and Chiou, S. H. (1994) *Biochem. Mol. Biol. Int.* **33,** 187–194.
20. Pan, F. M., Yeh, M. S., Chang, W. C., Hung, C. C., and Chiou, S. H. (1994) *Biochem. Biophys. Res. Commun.* **199,** 969–976.
21. Armugam, A., Earnest, L., Chung, M. C., Gopalakrishnakone, P., Tan, C. H., Tan, N. H., and Jeyaseelan, K. (1997) *Toxicon* **35,** 27–37.
22. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Gene* **33,** 103–119.
23. Roepstorff, P. (1993) *Trends Analy. Chem.* **12,** 413–421.
24. Kawauchi, S., Iwanaga, S., Samejima, Y., and Suzuki, T. (1970) *Biochim. Biophys. Acta* **236,** 142–160.
25. Cardinal, D. C., and Flower, R. J. (1980) *J. Pharmacol.* **3,** 135–158.
26. Chomzynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
27. Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. (1977) *Biochemistry* **16,** 4743–4751.
28. Goldberg, D. A. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5794.
29. Ghadessy, F. J., Chen, D., Kini, R. M., Chung, M. C. M., Jeyaseelan, K., Khoo, H. E., and Yuen, R. (1996) *J. Biol Chem.* **271,** 25575–25581.
30. Jeyaseelan, K., Armugam, A., Lachumanan, R., Tan, C. H., and Tan, N. H. (1998) *Biochim. Biophys. Acta* **1380,** 209–222.
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning—A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32. Chen, E. Y., and Seeberg, P. H. (1985) *DNA* **4,** 165–170.
33. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410.
34. Kini, R. M. (1998) *Toxicon* **36,** 1659–1670.
35. Bouchier, C., Boulain, J. C., Bon, C., and Menez, A. (1991) *Biochim. Biophys. Acta* **1088,** 401–408.
36. Davidson, F. F., and Dennis, E. A. (1990) *J. Mol. Evol.* **31,** 228–238.
37. Takagi, J., Sekiya, F., Kasahara, K., Inada, Y., and Saito, Y. (1988) *Toxicon* **26,** 199–206.
38. Li, Y. S., Liu, K. F., and Wang, Q. C. (1986) *Toxicon* **24,** 875–883.
39. Fry, B. G. (1999) *Toxicon* **37,** 11–32.
40. Kini, R. M., and Evans, H. J. (1997) *in* Venom Phospholipase A_2 Enzymes: Structure, Function and Mechanism (Kini, R. M., Ed.), pp. 373–391, Wiley, Chichester.
41. Yuan, Y., Jackson, S. P., Newnham, H. H., Mitchell, C. A., and Salem, H. H. (1995) *Blood* **86,** 4166–4174.

42. Fuly, A. L., Machado, O. L., Alves, E. W., and Carlini, C. R. (1997) *Thromb. Haemost.* **78,** 1372–1380.
43. Ouyang, C., Yeh, H. I., and Huang, T. F. (1983) *Toxicon* **21,** 797–804.
44. Carver, J. P., and Blout, E. R. (1967) *in* Treatise on Collagen (G.N., Ed.), p. 441, Academic Press, New York.
45. Kini, R. M. (1994) *Curr. Top. Peptide Protein Res.* **1,** 297–311.
46. Wang, Y. M., Lu, P. J., Ho, C. L., and Tsai, I. H. (1992) *Eur. J. Biochem.* **209,** 635–641.
47. Smith, L. A., and Schmidt, J. J. (1990) *Toxicon* **28,** 575–585.
48. Bouchier, C., Ducancel, F., Guignery-Frelat, G., Bon, C., Boulain, J. C., and Menez, A. (1988) *Nucleic Acids Res.* **16,** 9050.
49. Danse, J. M., Toussaint, J. L., and Kempf, J. (1990) *Nucleic Acids Res.* **18,** 4609.
50. Ogawa, T., Oda, N., Nakashima, K., Sasaki, H., Hattori, M., Seaquake, Y., Kihara, H., and Ohno, M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8557–8561.
51. Nakashima, K. I., Ogawa, T., Oda, N., Hattori, M., Sakaki, Y., Kihara, H., and Ohno, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5964–5968.
52. Krizaj, I., Liang, N. S., Pungercar, J., Strukelj, B., Ritonja, A., and Gubensek, F. (1992) *Eur. J. Biochem.* **204,** 1057–1062.
53. Watson, M. E. (1984) *Nucleic Acids Res.* **12,** 5145–5164.
54. Ducancel, F., Guignery-Frelat, G., Bouchier, C., Menez, A., and Boulain, J. C. (1988) *Nucleic Acids Res.* **16,** 9048.
55. Ducancel, F., Boulain, J. C., Tremeau, O., and Menez, A. (1989) *Protein Eng.* **3,** 139–143.
56. Takasaki, C., Suzuki, J., and Tamiya, N. (1990) *Toxicon* **28,** 319–327.
57. Heinrikson, R. L., Krueger, E. T., and Keim, P. S. (1977) *J. Biol. Chem.* **252,** 4913–4921.
58. Fohlman, J., Lind, P., and Eaker, D. (1977) *FEBS Lett.* **84,** 367–371.
59. Pearson, J. A., Tyler, M. I., Retson, K. V., and Howden, M. E. (1991) *Biochim. Biophys. Acta* **1077,** 147–150.
60. Lambeau, G., Ancian, P., Nicolas, J. P., Beiboer, S. H., Moinier, D., Verheij, H., and Lazdunski, M. (1995) *J. Biol. Chem.* **270,** 5534–5540.
61. Francis, B., Coffield, J. A., Simpson, L. L., and Kaiser, I. I. (1995) *Arch. Biochem. Biophys.* **318,** 481–488.
62. Huang, M. Z., Gopalakrishnakone, P., Chung, M. C. M., and Kini, R. M. (1997) *Arch. Biochem. Biophys.* **338,** 150–156.
63. Monteiro, R. Q., Dutra, D. L., Machado, O. L., Carlini, C. R., Guimaraes, J. A., Bon, C., and Zingali, R. B. (1998) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **120,** 791–798.
64. Sasa, M. (1999) *Toxicon* **37,** 249–260.
65. Nkinin, S. W., Chippaux, J. P., Pietin, D., Doljansky, Y., Tremeau, O., and Menez, A. (1997) *Bull. Soc. Pathol. Exot.* **90,** 277–281.
66. Monteiro, R. Q., Yamanouye, N., Carlini, C. R., Guimaraes, J. A., Bon, C., and Zingali, R. B. (1998) *Toxicon* **36,** 153–163.
67. Meier, J. (1986) *Toxicon* **24,** 41–46.
68. Minton, S. A., and Weinstein, S. A. (1986) *Toxicon* **24,** 71–80.
69. Vandenplas, M. L., Vandenplas, S., Brebner, K., Bester, A. J., and Boyd, C. D. (1985) *Toxicon* **23,** 289–305.
70. Paine, M. J., Desmond, H. P., Theakston, R. D., and Crampton, J. M. (1992) *Toxicon* **30,** 379–386.