

# Purification, Characterization, and Amino Acid Sequence Determination of Acanthins, Potent Inhibitors of Platelet Aggregation from *Acanthophis antarcticus* (Common Death Adder) Venom

Geraldine Chow, Sivan Subburaju, and R. Manjunatha Kini<sup>1</sup>

Bioscience Centre, Faculty of Science, National University of Singapore, Singapore 119260

Received January 20, 1998, and in revised form March 23, 1998

**Venom of *Acanthophis antarcticus*, a common death adder, exhibits potent antiplatelet effects. By a combination of gel-filtration, cation-exchange, and reversed-phase chromatographic methods, two inhibitors of platelet aggregation, named acanthin I and II, were purified to homogeneity as assessed by capillary electrophoresis and electrospray mass spectrometry. These isoforms exhibit the most potent antiplatelet activity known thus far, with IC<sub>50</sub> values of 7 nM for acanthin I and 4 nM for acanthin II in human whole blood when collagen was used as an agonist, whereas with ADP the IC<sub>50</sub> values were 10 and 12 nM, respectively. Acanthin I and II are basic proteins with pI's of 10.2 ± 0.1 and 10.4 ± 0.1 and molecular weights of 12,844.58 ± 0.61 and 12,895.63 ± 0.48, respectively, as determined by electrospray mass spectrometry. They exhibit phospholipase enzyme activity, and acanthin I and II hydrolyzed 51.57 ± 1.30 and 46.85 ± 2.90 μmol of phosphatidylcholine/min/mg, respectively. The complete amino acid sequences of acanthin I and II showed that they have a high homology with each other and with other elapids' phospholipase A<sub>2</sub> neurotoxin, especially pseudexin A.** © 1998 Academic Press

**Key Words:** snake venom; venom phospholipase; platelet inhibitor; *Acanthophis antarcticus*.

Sequence data from this article have been deposited with the SWISS-PROT Databank under Accession number(s) P81236 for Acanthin I and P81237 for Acanthin II.

<sup>1</sup>To whom correspondence and reprint requests should be addressed at Bioscience Centre, Faculty of Science, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260. Fax: (65) 777 0052.

Platelet aggregation plays a vital role in hemostasis by maintaining the integrity of blood vessel walls (1–3), in facilitating the activation of coagulant factors (4–6), and in clot retraction (7, 8). Thus an aberration in platelet aggregation can cause havoc as seen in myocardial infarction and stroke wherein small clots formed in the blood vessels restrict the supply to the heart and brain. Hence, antiplatelet agents are sought after, as they would be useful in the prevention/treatment of atherosclerosis and cardiovascular and cerebrovascular diseases.

Several snake venom proteins interfere in thrombosis and hemostasis. Some of these proteins affect platelet aggregation and/or blood coagulation. A number of snake venom proteins that inhibit platelet aggregation have been purified and characterized (for reviews, see 9, 10). This heterogeneous group of proteins and polypeptides contains enzymes such as nucleotidases, L-amino acid oxidase, proteinases, and phospholipase A<sub>2</sub> as well as nonenzymatic proteins. They inhibit platelet aggregation by different mechanisms and hence provide useful and novel tools for the study of molecular mechanisms in platelet aggregation. In some instances they have also helped in developing new antiplatelet agents. For example, disintegrins, a group of potent nonenzymatic inhibitors from viperid and crotalid venoms (11, 12), have helped in the design of prototypes of new antiplatelet agents (13).

Australian snake venoms are among the most lethal in the world, containing interesting and distinctly different toxins, most probably because of different evolutionary pressures. Several venoms of Australian snakes are known to interfere with platelet aggregation as well as blood coagulation (14, 15). Recently, an inhibitor of platelet aggregation from *Austrelaps su-*

*perba* (16, 17) venom was purified and characterized. The inhibitor is a PLA<sub>2</sub> enzyme with molecular weight of 15,000. The inhibition by *A. superba* inhibitor is dependent on PLA<sub>2</sub> enzyme activity of the protein (16, 17). The mechanism of this inhibitor is not yet clear.

*Acanthophis antarcticus* contains numerous postsynaptic neurotoxins such as acanthophin a (18), and *A. antarcticus* toxins Aa b and Aa c (19, 20) and Aa e1 and e2 (21). This family of neurotoxins has been well studied and characterized. Recently, two isoforms of PLA<sub>2</sub> were also purified from this venom and were named acanthoxins (22). In this paper, we report the purification and partial characterization of two inhibitors of platelet aggregation from *A. antarcticus* venom. These inhibitors are PLA<sub>2</sub> enzymes and were named acanthin I and II. Acanthins are among the most potent antiplatelet inhibitors isolated thus far from snake venom.

## MATERIALS AND METHODS

**Materials.** Lyophilized *A. antarcticus* venom was obtained from Venom Supplies (Tanunda, SA, Australia). Bio-Gel P-2, Bio-Gel P-30, UNO-S1 (7 × 35 mm), and cartridge and reagents for pI determination were purchased from Bio-Rad (Hercules, CA). C<sub>5</sub> column (Jupiter 5, 4.6 × 250 mm) was from Phenomenex (Torrance, CA). Reagents for N-terminal sequencing and amino acid analysis were from Applied Biosystem (Foster City, CA) and Shimadzu (Kyoto, Japan), respectively. Collagen and ADP for platelet aggregation assay was purchased from Chrono-Log (Havertown, PA). Lecithin used as a substrate in phospholipase assay was obtained from ICN Biochemicals (Aurora, OH). The chromatographic reagents acetonitrile and TFA were from Fisher Scientific (Fair Lawn, NJ) and Fluka (Buchs, Switzerland), respectively. All other chemicals used were of analytical grade purchased from Sigma (St. Louis, MO).

**Isolation and purification of antiplatelet proteins.** Lyophilized crude venom (150 mg) was dissolved in 1.0 ml of distilled water and applied onto a Bio-Gel P-30 column (1.5 × 120 cm) equilibrated with 50 mM ammonium acetate, pH 5.0. Proteins were eluted with the same buffer at a flow rate of 18 ml/h and elution profile was monitored at 280 nm. Fractions that showed potent antiplatelet activity were pooled and purified further on UNO-S1, a cation-exchange column which was equilibrated with 50 mM ammonium acetate, pH 5.0. The bound proteins were eluted with a linear gradient of sodium chloride at a flow rate of 1.0 ml/min. Active fractions from UNO-S column were then subjected separately to further purification on Jupiter C<sub>5</sub> reversed-phase column. The column was equilibrated with 0.1% TFA and the bound proteins were eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min and the eluant was monitored at 280 nm. The purified proteins were named acanthin I and II.

**Electrospray mass spectrometry.** Samples were dissolved in 50% acetonitrile and 50% water and analyzed using Perkin-Elmer Sciex API 300 triple quadrupole instrument equipped with an ionspray interface. The ionspray voltage was set at 4600 V and the orifice voltage at 30 V. Nitrogen was used as a curtain gas with a flow rate of 0.6 L/min while compressed air was utilized as a nebulizer gas. The samples were infused into the mass spectrometer at a flow rate of 5 μl/min.

<sup>2</sup> Abbreviations used: PLA<sub>2</sub>, phospholipase A<sub>2</sub>; TFA, trifluoroacetic acid; PE, S-pyridylethylated; BNPS-skatole, 2-(2'-nitrophenylsulfonyl)-3-methyl-3-bromoindolenine.

**Capillary zone electrophoresis.** Capillary zone electrophoresis 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 + to - polarity mode in 0.1 M phosphate buffer (pH 2.5) at 15°C and at a constant potential of 12 kV. The proteins were detected by UV absorbance of 200 nm.

**Isoelectric point determination.** The isoelectric point (pI) for acanthin was performed in the same instrument used in capillary zone electrophoresis. The experiment was conducted under the following conditions: 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 + to - polarity mode with focusing and mobilizing voltages at 10 kV. The protein peaks were detected at 280 nm. To determine the pI of acanthin, BioMark synthetic pI markers (pI range: 10.4, 8.4, 7.4, 6.4, and 5.3) were also run under conditions identical to those of the samples. Both samples and standards were run in triplicate to determine the pI of acanthins.

**Amino acid composition determination.** Amino acid analysis was performed on a Shimadzu high-performance amino acid analyzer following hydrolysis in 6.0 M HCl for 24 h at 110°C. The amino acids were detected by a fluorescence detector after postcolumn derivatization with o-phthalaldehyde. The values for tryptophan were determined according to the method of Simpson *et al.* (23) using 4.0 M methanesulfonic acid containing 0.2% (w/v) tryptamine.

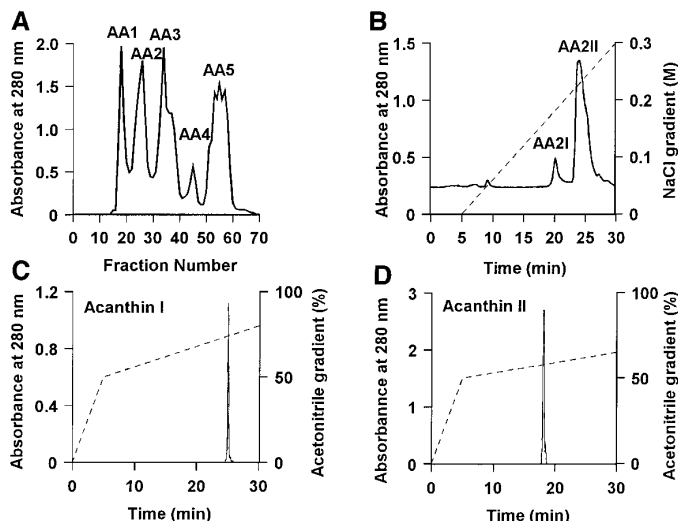
**Reduction and pyridylethylation.** PE acanthins were prepared by resuspending the samples in 100 μl of the denaturant buffer (6.0 M guanidinium hydrochloride, 0.13 M Tris, 1 mM EDTA, pH 8.0) containing 0.07 M β-mercaptoethanol. The solutions were heated at 37°C for 2 h. Subsequently, 1.5-fold molar excess (over sulfhydryl groups) of 4-vinylpyridine was added and incubated at room temperature. After 2 h, samples were desalted on Bio-Gel P-2 equilibrated with 9% formic acid.

**Protein digestion.** Digestion of PE acanthins with endoproteinase Lys-C was performed in 100 mM Tris-HCl, pH 8.5, in a molar ratio of 100:1 (substrate:protease) at 37°C for 18 h. PE acanthin II was also treated with 4 mg/ml BNPS-skatole in 70% acetic acid and the reaction carried out for 24 h at 37°C. The digests were fractionated on reversed-phase chromatography on Sephacil C<sub>8</sub> column.

**Amino-terminal sequencing.** Amino-terminal sequencing was carried out on PE acanthins and their peptides by an automated Edman degradation using an Applied Biosystems 494 pulsed-liquid-phase sequencer. Phenylthiohydantoin amino acids were identified using on-line reversed-phase high-performance liquid chromatography on a PTH-C18 column, in an Applied Biosystem 140C analyzer.

**Assay for phospholipase activity.** Phospholipase A<sub>2</sub> activity was determined by following the protocol of Kawauchi *et al.* (24) with slight modification. An aqueous emulsion of 20 mM phosphatidylcholine, in the presence of 10 mM Triton X-100 and 10 mM Ca<sup>2+</sup>, was used as a substrate. Released fatty acids were titrated at pH 8.0 with NaOH at room temperature with a 718 STAT Titrimo pH-stat (Metrohm, Switzerland). The phospholipase activity was expressed as micromoles of phosphatidylcholine hydrolyzed per minute per milligram of enzyme.

**Assay for antiplatelet effects.** Whole blood was taken from healthy volunteers who had not ingested any medication for 2 weeks. Freshly aspirated blood was collected in 3.8% trisodium citrate (9:1) and used within 3 h. Inhibition of platelet aggregation was determined by electrical impedance in a whole-blood aggregometer (Chrono-Log, Model 500-CA) according to the method of Cardinal and Flower (25). To an equal volume of phosphate-buffered saline and blood (1 ml total), acanthin was added and incubated at 37°C for 2 min with continual stirring at 1000 rpm. After 2 min platelet aggregation was induced by the addition of either 2 μl of collagen (2 μg/ml final concentration) or 5 μl of ADP (5 μM final concentration)



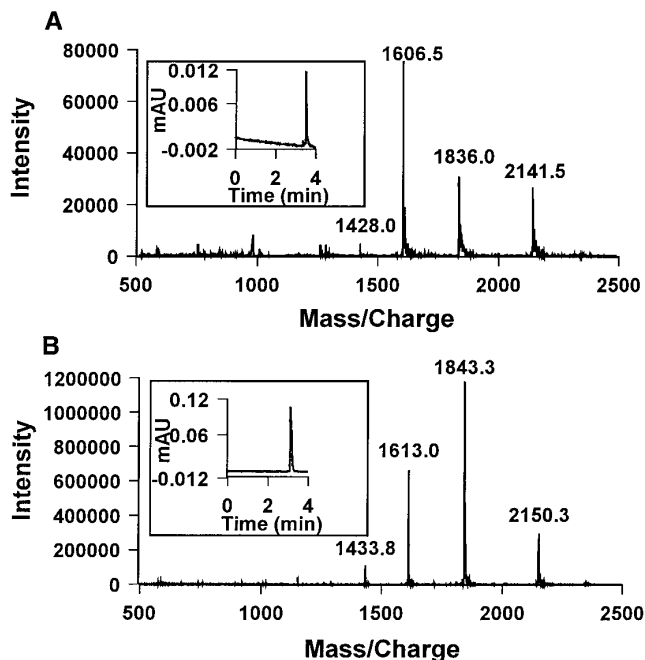
**FIG. 1.** Separation and purification of acanths. (A) Gel filtration of *A. antarcticus* venom on Bio-Gel P-30. 150 mg of crude venom was dissolved in 50 mM ammonium acetate buffer, pH 5.0, loaded onto Bio-Gel P-30 column (1.5 cm  $\times$  120 cm), and eluted with the same buffer at flow rate of 18 ml/h. Fractions of 4.0 ml were collected. (B) Cation-exchange chromatography of AA2 on UNO-S1 column. The fraction AA2 separated on Bio-Gel-P 30 was loaded onto UNO-S1 equilibrated with 50 mM ammonium acetate, pH 5.0, and eluted with a linear NaCl gradient (50 mM ammonium acetate, 1.0 M NaCl, pH 5.0) at a flow rate of 1.0 ml/min. (C and D) Reversed-phase chromatography of AA2I and AA2II on Jupiter C<sub>5</sub> column. The column was equilibrated with 0.1% TFA and eluted with acetonitrile gradient in 0.1% TFA at flow rate of 1.0 ml/min. The proteins contained in the peaks were named acanthin I and II.

and the aggregation was monitored for 5 min. Inhibition of platelet aggregation was calculated as the percentage difference in the rate of platelet aggregation in the presence or the absence of PLA<sub>2</sub>.

## RESULTS

### Isolation and Purification of Acanthins

Acanthins were purified from crude *A. antarcticus* venom by a three-step purification process. Initial separation of the venom on gel filtration on Bio-Gel P-30 yielded five major peaks of which only AA2 (Fig. 1A) contained antiplatelet proteins as determined by platelet aggregation assay. Subsequently, AA2 was subjected to a second purification step on UNO-S1, a cation exchanger, resulting in two fractions, namely, AA2I and AA2II (Fig. 1B). The fractions were then applied separately onto a reversed-phase column, Jupiter C<sub>5</sub>. AA2I eluted as a sharp peak separating away from minor contaminants at 62% acetonitrile while AA2II eluted at 48% acetonitrile (Figs. 1C and 1D) and these fractions were named acanthin I and II, respectively. Approximate yields of acanthin I and II from 100 mg of venom were 0.39 and 5.07 mg, respectively.



**FIG. 2.** Electrospray mass spectrometry of acanths. The spectra of (A) acanthin I and (B) acanthin II show a series of multiple-charged ions, related to molecules bearing six to nine protons. The respective insets depict the purity of acanthin I and acanthin II as determined by capillary zone electrophoresis.

### Characterization of Acanthins

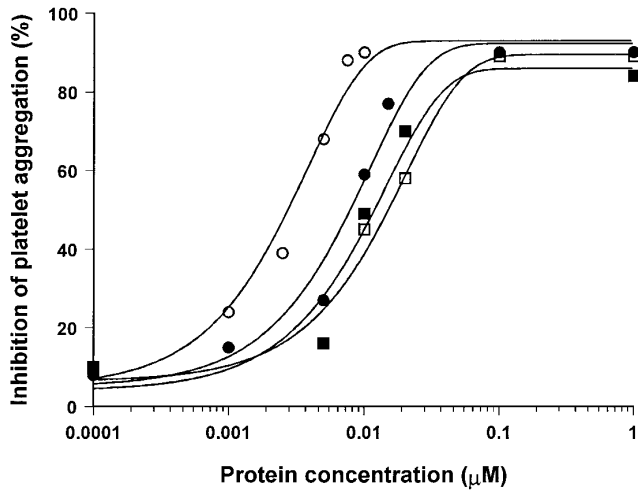
Both capillary electrophoresis and electrospray mass spectrometry (Figs. 2A and 2B) showed that acanthins purified in this manner were homogeneous. All subsequent experiments on biochemical and pharmacological characterization were performed using only purified acanthin I and II. Their physical characteristics are summarized in Table I.

### Inhibition of Platelet Aggregation by Acanthins

The antiplatelet effects of acanthins were studied by platelet aggregation assay using healthy human whole blood with either collagen or ADP as platelet agonists. The effects on platelet aggregation appear to be dose dependent with half-maximal (IC<sub>50</sub>) values of 7 nM for acanthin I and 4 nM for acanthin II when collagen was

**TABLE I**  
Properties of Acanthins

	Molecular mass (Da)	pI	PLA <sub>2</sub> activity ( $\mu$ mol phosphatidylcholine hydrolyzed/min/mg)
Acanthin I	12,844.58 $\pm$ 0.61	10.2 $\pm$ 0.1	51.57 $\pm$ 1.30
Acanthin II	12,895.63 $\pm$ 0.48	10.4 $\pm$ 0.1	46.85 $\pm$ 2.90



**FIG. 3.** Effects of acanthin I and II on platelet aggregation. Different concentrations of acanthin I (●,■) and acanthin II (○,□) were added to citrated blood and the effects on platelet aggregation were determined by electrical impedance in a whole-blood aggregometer using either collagen (●,○) or ADP (■,□) as platelet agonist.

used as an agonist. With ADP as the aggregation inducer, the  $IC_{50}$  values were 10 and 12 nM for acanthin I and II, respectively (Fig. 3).

#### *Amino Acid Composition and N-Terminal Sequence of Acanthins*

Acanthins were subjected to amino acid sequencing to confirm their identities. Prior to sequence analysis,

the proteins were reduced and pyridylethylated. By automated Edman degradation, the first 48 residues of acanthin II were identified unequivocally with a repetitive yield of 93%. The remaining sequence was determined by treatment with BNPS-skatole, which cleaves at the C-terminal side of tryptophan residues generating peptide fragments, W32-69 and W70-118. For overlapping peptides, acanthin II was digested with endoproteinase Lys-C with K64-81 providing the overlap between W32-69 and W70-118. The molecular weights of the peptides were identified by mass spectra analysis and their values matched the calculated masses deduced from the sequences of the respective peptides (Table II). The complete sequence of acanthin II is shown in Fig. 4A and its molecular weight corresponds with the value obtained from mass spectra analysis. Similarly with acanthin I, the first 58 residues were determined with a repetitive yield of 92% while the remaining sequence was determined by digesting acanthin I with endoproteinase Lys-C, generating peptide fragments as tabulated in Table III. By homology with acanthin II, the complete sequence of acanthin I was determined (Fig. 4B).

Acanthins I and II are 119- and 118-amino-acid proteins, respectively, with no posttranslational modification as the amino acid compositions (Table IV) correspond to the molecular weight of the proteins. Acanthins I composition reflects a 42% hydrophobic residue content while neutral and hydrophilic residues accounted for 31 and 27%, respectively. Acanthins II is a

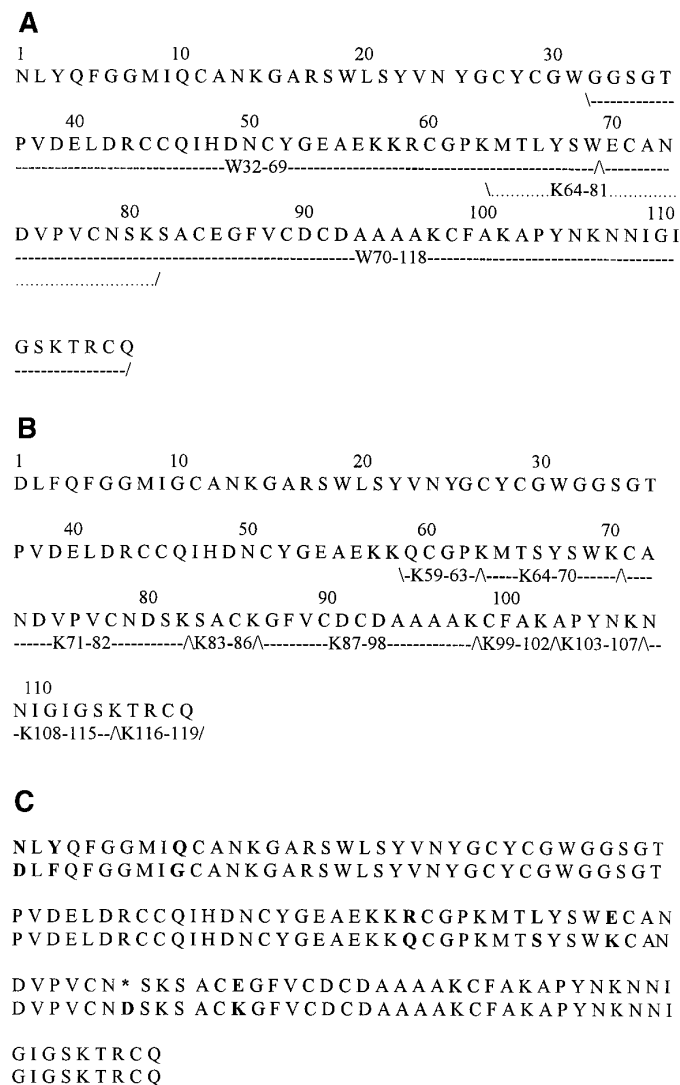
**TABLE II**  
Amino Acid Composition of Acanthins

Amino acids	Estimated values for acanthin I	Observed values for acanthin I	Estimated values for acanthin II	Observed values for acanthin II
Aspartic acid	15.50 ± 0.08 <sup>a</sup>	16 <sup>a</sup>	15.11 ± 0.11 <sup>a</sup>	15 <sup>a</sup>
Threonine	2.98 ± 0.03	3	1.97 ± 0.01	2
Serine	7.79 ± 0.01	8	7.11 ± 0.03	7
Glutamic acid	6.87 ± 0.02 <sup>b</sup>	7 <sup>b</sup>	8.70 ± 0.04 <sup>b</sup>	9 <sup>b</sup>
Proline	4.09 ± 0.04	4	3.89 ± 0.01	4
Glycine	14.28 ± 0.01	14	12.77 ± 0.01	13
Alanine	10.81 ± 0.05	11	11.13 ± 0.05	11
Valine	4.51 ± 0.01	5	4.63 ± 0.09	5
Methionine	1.84 ± 0.03	2	1.53 ± 0.04	2
Isoleucine	3.74 ± 0.01	4	3.74 ± 0.04	4
Leucine	3.42 ± 0.03	3	3.72 ± 0.03	4
Tyrosine	5.57 ± 0.02	6	7.04 ± 0.05	7
Phenylalanine	3.99 ± 0.01	4	2.91 ± 0.02	3
Histidine	0.07 ± 0.01	1	0.91 ± 0.07	1
Tryptophan	3.04 ± 0.02	3	2.77 ± 0.01	3
Lysine	10.75 ± 0.03	11	9.91 ± 0.06	10
Arginine	3.02 ± 0.01	3	3.85 ± 0.06	4
Cysteine	14.14 ± 0.01 <sup>c</sup>	14 <sup>c</sup>	14.18 ± 0.01 <sup>c</sup>	14 <sup>c</sup>

<sup>a</sup> Aspartic acid and asparagine residues in samples are represented as aspartic acid.

<sup>b</sup> Glutamic acid and glutamine residues in samples are represented as glutamic acid.

<sup>c</sup> Determined by mass difference between native and PE acanthins.



**FIG. 4.** Determination of the complete amino acid sequence of acanthins. (A) The sequence of acanthin II is shown along with the cleavage sites of endoproteinase Lys-C digestion and BNPS-skatole treatment and (B) acanthin I is depicted with the cleavage sites of endoproteinase Lys-C digestion. (C) Comparison of amino acid sequences of acanthins. Residues that are different are in boldface.

less hydrophobic protein with a hydrophobic content of 41% while neutral and hydrophilic residues accounted for 31 and 28%, respectively.

## DISCUSSION

Several platelet aggregation inhibitors have been purified and characterized from various snake venoms. They include enzymes, such as PLA<sub>2</sub> enzymes, nucleotidases, and fibrinogenases, and nonenzymatic polypeptides, such as disintegrins and mambin. Among these proteins disintegrins isolated from crotalid and viperid snake venoms are some of the most potent

**TABLE III**

Mass Spectrometry Analysis of Native, PE, Peptides of Endoproteinase Lys-C Digests, and BNPS-skatole Treatment of Acanthin II

Peptides	Calculated <sup>a</sup>	Observed <sup>b</sup>
Native acanthin II	12,896.63	12,895.63 ± 0.48
PE acanthin II	14,384.63	14,384.52 ± 0.78
W32-69	4,695.80	4,696.53 ± 0.87
W70-118	5,852.15	5,851.90 ± 0.69
K64-81	2,270.62	2,269.81 ± 0.40

<sup>a</sup> Molecular masses are calculated from the amino acid sequences of these fragments.

<sup>b</sup> Molecular masses are detected by electrospray mass spectrometry.

inhibitors of platelet aggregation with IC<sub>50</sub> values in the range between  $1.2 \times 10^{-8}$  and  $2.5 \times 10^{-8}$  M (12, 26). Disintegrins, as well as mambin, contain Arg-Gly-Asp tripeptide sequence in their structures and thus inhibit platelet aggregation by interfering in the interaction between fibrinogen and its receptor, glycoprotein IIb-IIIa complex (for references, see 27).

In our laboratories, several Australian snake venoms were initially screened for their antiplatelet effects. Among these the crude venom from *A. antarcticus* was found to be one of the most potent inhibitors of platelet aggregation. Thus, we initiated studies to identify the component(s) responsible for the antiplatelet effects of *A. antarcticus* venom. We have purified two proteins, acanthin I and II, using a three-step sequential purification process which included gel-filtration, cation-exchange, and reversed-phase chromatography. Both these components have similar molec-

**TABLE IV**

Mass Spectrometry Analysis of Native, PE, and Peptides of Endoproteinase Lys-C Digests of Acanthin I

Peptides	Calculated <sup>a</sup>	Observed <sup>b</sup>
Native acanthin I	12,842.53	12,844.58 ± 0.61
PE acanthin I	14,330.54	14,329.60 ± 0.77
K59-63	636.61	635.74 ± 1.47
K64-70	902.02	900.26 ± 1.76
K71-82	1,474.38	1,477.28 ± 3.16
K83-86	512.47	511.00 ± 1.42
K87-98	1,380.31	1,380.77 ± 1.03
K99-102	572.60	573.30 ± 0.10
K103-107	591.70	592.50 ± 0.20
K108-115	801.88	801.26 ± 0.37
K116-119	611.56	612.30 ± 0.20

<sup>a</sup> Molecular masses are calculated from the amino acid sequences of these fragments.

<sup>b</sup> Molecular masses are detected by electrospray mass spectrometry.

ular weights of 12,844.58 and 12,895.63, respectively. These molecular weights are similar to monomeric molecular weights of venom PLA<sub>2</sub> enzymes. As expected, acanthin I and II exhibited significant PLA<sub>2</sub> activity when assessed for phospholipase activity by titrimetric method using phosphatidylcholine as a substrate. Their specific activities, 51.57 and 46.85 μmol/min/mg, respectively, are similar to those of pseudexin A (43 μeq/min/mg), B (53 μeq/min/mg) and C (43 μeq/min/mg) (28). In addition, both are basic proteins with pI values of 10.2 and 10.4, respectively. However, these values differ from the pI value of acanthoxin, a PLA<sub>2</sub> purified from *A. antarcticus* venom (22). This could be due to differences in the methods used.

Both acanthins inhibited platelet aggregation in human whole blood. Dose-response studies indicate that acanthin I and II are among the most potent antiplatelet inhibitors known thus far, with IC<sub>50</sub> values of 7 and 4 nM, respectively, with collagen as an agonist. With ADP, a slighter higher concentration was required, giving IC<sub>50</sub> values of 10 nM for acanthin I and 12 nM for acanthin II. As mentioned earlier, several other PLA<sub>2</sub> enzymes also inhibit platelet aggregation and platelet release reactions. However, these inhibit platelet aggregation only at higher concentrations than acanthins. For instance, IC<sub>50</sub> values for inhibition of platelet aggregation by PLA<sub>2</sub> enzymes from *A. superba*, *Agkistrodon halys*, and *Ophiophagus hannah* venoms are 333, 785, and 340 nM, respectively (16, 29, 30). Thus acanthins appear to be comparatively more potent inhibitors of platelet aggregation than other PLA<sub>2</sub> enzymes.

As a first step toward understanding structure-function relationships of acanthins, we determined the complete amino acid sequence of these proteins. Amino acid and sequence analysis showed that acanthin I and II are 119- and 118-amino-acid proteins, respectively, with no posttranslational modification and are highly homologous (93%). In addition, acanthin I and II have the highest homology of 74 and 77%, respectively, with pseudexin A, a neurotoxic PLA<sub>2</sub> from red-bellied black snake venom (28). Amino acid sequences and the location of 14 half-cystine residues were typical of elapid and pancreatic phospholipases and hence acanthins can be classified as group I PLA<sub>2</sub> enzymes (31). In addition, amino acid residues that are implicated in enzymatic activity were highly conserved in both isoforms of acanthin. These include the Ca<sup>2+</sup> binding site (Y28, G30, G32, D49) (32-34) and the active site residues (H48, D49, D92, Y52, Y67) (35-38). It is interesting to note that both acanthins do not contain Arg-Gly-Asp tripeptide sequence. Therefore it is logical to conclude that acanthins may not interfere in the interaction between fibrinogen and its receptor, glycoprotein IIb-IIIa complex, unlike disintegrins and mambin. Disintegrins and mambin are the only other

venom polypeptides which inhibit platelet aggregation with inhibitory potencies similar to that of acanthins. Thus the mechanism of inhibition appears to be different from that of Arg-Gly-Asp-containing polypeptides.

Several PLA<sub>2</sub> enzymes interfere in platelet aggregation by specific mechanisms and they can be classified into three major classes (40). Class A contains PLA<sub>2</sub>s that induce platelet aggregation while in class B they cause inhibition in platelet aggregation and in class C PLA<sub>2</sub>s act as both inducer and inhibitor. Since both isoforms of acanthin inhibit platelet aggregation, they belong to class B. Class B can be further subdivided into two subgroups. In the first subgroup, the PLA<sub>2</sub> enzyme activity is involved in platelet inhibition as was observed with PLA<sub>2</sub> from *A. superba* (16, 17) and *Lachesis muta* (41) venoms. In these cases the antiplatelet effects were due to the hydrolysis of phospholipids from the plasma and/or from lipoproteins and the formation of lysophosphatidylcholine (17, 41). Lysophosphatidylcholine dose-dependently inhibits binding of fibrinogen to its glycoprotein IIb-IIIa complex and hence the inhibition of platelet aggregation (17). However, the second group inhibits platelet aggregation by mechanisms that are independent of its enzyme activity, such as PLA<sub>2</sub> from *A. halys* venom (29). These nonenzymatic mechanisms have not yet been deciphered. We are currently examining the role of PLA<sub>2</sub> activity of acanthins in the inhibition of platelet aggregation. Further studies are also under way to determine the structure-function relationships and mechanisms of these potent inhibitors of platelet aggregation.

#### ACKNOWLEDGMENTS

This work was supported by a financial grant from the Economic Development Board of Singapore as well as by Academic Research Grants RP 960304 and RP 940325.

#### REFERENCES

1. Packham, M. A., and Mustard, J. F. (1980) *Semin. Hematol.* **23**, 8-26.
2. Plow, E. F., Marguerie, G., and Ginsberg, M. H. (1987) *Biochem. Pharmacol.* **36**, 4035-4040.
3. Zucker, M. D. (1989) *Methods Enzymol.* **169**, 117-133.
4. Tracy, P. B., and Mann, K. G. (1986) in *Platelet Responses and Metabolism*, Vol. I, Responses (Holmsen, H., Ed.), pp. 297-324, CRC Press, Boca Raton, FL.
5. Tracy, P. B., and Mann, K. G. (1986) in *Biochemistry of Platelets* (Phillips, D. R., and Shuman, M. A., Eds.), pp. 295-318, Academic Press, Orlando, FL.
6. Ganz, P. R., Tackaberry, E. S., and Rock, G. (1988) in *Progress in Clinical and Biological Research*, Vol. 283, Platelet Membrane Receptors: Molecular Biology, Immunology, Biochemistry and Pathology (Jamieson, G. A., Ed.), pp. 255-261 A. R. Liss, New York.
7. 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.

8. Stormorken, H. (1986) in *Platelet Responses and Metabolism*, Vol. I, Responses (Holmsen, H., Ed.), pp. 3–32, CRC Press, Boca Raton, FL.
9. Brinkhouse, K. M., and Smith, S. V. (1988) in *Haemostasis and Animal Venoms*, Vol. 7, Hematology (Pirkle, H., and Markland, F. S., Jr., Eds.), pp. 363–376, Dekker, New York.
10. Kini, R. M., and Evans, H. J. (1990) *Toxicon* **28**, 1387–1422.
11. Huang, T. F., Holt, J. C., Kirby, E. P., and Niewiarowski, S. (1989) *Biochemistry* **28**, 661–666.
12. Dennis, M. S., Henzel, W. J., Pitti, R. M., Lipari, M. L., Napier, M. A., Deisher, T. A., Bunting, S., and Lazarus, R. A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2471–2475.
13. Scarborough, R. M., Naughton, M. A., Teng, W., Rose, J. W., Phillips, D. R., Nannizzi, L., Arfsten, A., Campbell, A. M., and Charo, I. F. (1993) *J. Biol. Chem.* **268**, 1066–1073.
14. Marshall, L. R., and Herrmann, R. P. (1983) *Thromb. Haemostasis* **50**, 707–711.
15. Marshall, L. R., and Herrmann, R. P. (1989) *Thromb. Res.* **54**, 269–273.
16. Yuan, Y., Jackson, S. P., Mitchell, C. A., and Salem, H. H. (1993) *Thromb. Res.* **70**, 471–481.
17. Yuan, Y., Jackson, S. P., Newnham, H. H., Mitchell, C. A., and Salem, H. H. (1995) *Blood* **86**, 4166–4174.
18. Sheumack, D. D., Spence, I., Tyler, M. I., and Howden, M. E. H. (1990) *Comp. Biochem. Physiol. B* **95**, 45–50.
19. Kim, H. S., and Tamiya, N. (1981) *Biochem. J.* **193**, 899–906.
20. Kim, H. S., and Tamiya, N. (1981) *Biochem. J.* **199**, 211–218.
21. Tyler, M. I., Retson-Yip, K. V., Gibson, M. K., Barnett, D., Howe, E., Stöcklin, R., Turnbull, R. K., Kuchel, T., and Mirtschin, P. (1997) *Toxicon* **35**, 555–562.
22. Van Der Weyden, L., Hains, P., Morris, M., and Broady, K. (1997) *Toxicon* **35**, 1315–1325.
23. Simpson, R. J., Neuberger, M. R., and Liu, T. Y. (1976) *J. Biol. Chem.* **251**, 1936–1940.
24. Kawachi, 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. Methods* **3**, 135–158.
26. Scarborough, R. M., Rose, J. W., Hsu, M. A., Phillips, D. R., Fried, V. A., Campbell, A. M., Nannizzi, L., and Charo, I. F. (1991) *J. Biol. Chem.* **266**, 9359–9362.
27. Kini, R. M., and Evans, H. J. (1992) *Toxicon* **30**, 265–293.
28. Schmidt, J. J., and Middlebrook, J. L. (1989) *Toxicon* **27**, 805–818.
29. Ouyang, C., Yeh, H. I., and Huang, T. F. (1983) *Toxicon* **21**, 797–804.
30. Huang, M. Z., Gopalakrishnakone, P., and Kini, R. M. (1997) *Life Sci.* **61**, 2211–2217.
31. Henrikson, R. L., Kreuger, E. T., and Keim, P. S. (1977) *J. Biol. Chem.* **252**, 4913–4921.
32. Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H., and Sigler, P. B. (1990) *Science* **250**, 1541–1546.
33. Van den Bergh, C. J., Slotboom, A. J., Verheij, H. M., and de Haas, G. H. (1989) *J. Cell. Biochem.* **39**, 379–390.
34. Dijkstra, B. W., Kalk, K. H., Hol, W. G. J., and Drenth, J. (1981) *J. Mol. Biol.* **147**, 97–123.
35. Drenth, J., Enzing, C. M., Kalk, K. H., and Vessies, J. C. A. (1976) *Nature* **264**, 373–377.
36. Dijkstra, B. W., Drenth, J., and Kalk, K. H. (1981) *Nature* **289**, 604–606.
37. Brunie, S., Bolin, J., Gewirth, D., and Sigler, P. B. (1985) *J. Biol. Chem.* **260**, 9742–9749.
38. Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J., and Sigler, P. B. (1985) *J. Biol. Chem.* **260**, 11627–11634.
39. Nathan, I., Dvilansky, A., Yirmiyahu, T., Aharon, M., and Livine, A. (1982) *Thromb. Haemostasis* **48**, 277–282.
40. Kini, R. M., and Evans, H. J. (1997) in *Venom Phospholipase A<sub>2</sub> Enzymes: Structure, Function and Mechanism* (Kini, R. M., Ed.), pp 373–391, Wiley, Chichester.
41. Fuly, A. L., Machado, O. L. T., Alves, E. W., and Carlini, C. R. (1997) *Thromb Haemostasis* **78**, 1372–1380.