

PURIFICATION AND CHARACTERIZATION OF PLATELET AGGREGATION
INHIBITORS FROM SNAKE VENOMS

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Abstract Proteins that inhibit glycoprotein (GP) IIb/IIIa mediated platelet aggregation have been purified from the venom of two snake species. A small platelet aggregation inhibitor (pl.AI), multisquamatin (Mr=5,700), was purified from *Echis multisquamatus* venom by hydrophobic interaction HPLC and two steps on C18 reverse phase HPLC. A larger pl.AI, contortrostatin (Mr=15,000), was purified by a similar HPLC procedure from the venom of *Agkistrodon contortrix contortrix*. Both pl.AIs inhibit ADP-induced human, canine and rabbit platelet aggregation using platelet rich plasma (PRP). Multisquamatin has an IC₅₀ of 97 nM, 281 nM and 333 nM for human, canine and rabbit PRP, respectively. Contortrostatin has an IC₅₀ of 49 nM, 120 nM and 1,150 nM for human, canine and rabbit PRP, respectively. In a competitive binding assay using ¹²⁵I-7E3 (a monoclonal antibody to GPIIb/IIIa that inhibits platelet aggregation) both contortrostatin and multisquamatin demonstrated GPIIb/IIIa specific binding to human and canine platelets. The IC₅₀ for contortrostatin displacement of 7E3 binding to human and canine GPIIb/IIIa is 27 nM and 16 nM, respectively and for multisquamatin it is 3 nM and 63 nM, respectively. Our results indicate that both pl.AIs inhibit platelet aggregation by binding with high affinity to GPIIb/IIIa.

Key words: platelet aggregation inhibitors, protein purification, snake venoms, GPIIb/IIIa, fibrinogen.

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Platelets serve an important role in mediating coronary artery thrombosis and rethrombosis in the genesis of acute myocardial infarction (1). Thus inhibition of platelet aggregation may provide an effective adjunctive approach for prevention of coronary artery reocclusion after successful thrombolytic therapy. Platelet aggregation involves the interaction of the platelet membrane glycoprotein (GP) IIb/IIIa receptor with plasma fibrinogen. GPIIb/IIIa belongs to the superfamily of integrin cell surface receptors (2). Integrins are heterodimers composed of α and β subunits that are noncovalently associated. They have been shown to be involved in cell-cell and cell-substratum interactions (3). It has been shown that both the α and the β subunits are required for fibrinogen binding (3). Integrins serve as receptors for extracellular matrix proteins such as fibronectin, fibrinogen, vitronectin, collagen and laminin (3). Some of these interactions have been shown to be mediated via an Arg-Gly-Asp (RGD) sequence present in the matrix proteins (3). For platelet aggregation an RGD sequence present in fibrinogen is essential for the interaction with GPIIb/IIIa (3).

A number of disintegrins or platelet aggregation inhibitors (pl.AIs) have been isolated from snake venoms (4-9). They are small proteins, rich in disulfide bonds and contain the RGD sequence. Recently pl.AIs have been characterized with respect to the amino acid sequence around the RGD site (10). The RGD sequence is present in all known pl.AIs except barbourin, the pl.AI purified from *Sistrurus m. barbouri*, which has a Lys-Arg-Asp (KGD) sequence instead of RGD and seems to be highly GPIIb/IIIa specific (8).

In this communication, we describe two pl.AIs, contortrostatin and multisquamatin, that have been purified from different snake venoms. Contortrostatin was purified from the venom of *Agkistrodon contortrix contortrix* (Southern copperhead) and multisquamatin was purified from the venom of *Echis multisquamatus*. We have studied the inhibitory activity of both proteins on platelets obtained from humans and two animal species. We have also studied the ability of these pl.AIs to compete for binding sites with a monoclonal antibody (7E3) directed to GPIIb/IIIa (11). In this report we present evidence indicating that contortrostatin and multisquamatin inhibit platelet aggregation by binding to the integrin receptor, GPIIb/IIIa.

MATERIALS AND METHODS

Materials:

Lyophilized venom from *Agkistrodon contortrix contortrix* was obtained from Biotoxins, Inc., St. Cloud, FL; venom from *Echis multisquamatus* was obtained from Latoxan, Rosans, France. All chemicals were of the highest grade available. Pierce protein assay kit using bicinchoninic acid was employed to determine protein concentrations (12).

High Performance Liquid Chromatography (HPLC):

For hydrophobic interaction (HIC)-HPLC a Perkin Elmer 410 LC pump was employed with a LC-95 UV/VIS detector and for reverse phase HPLC a Spectra Physics LC 8810 pump was employed with an SP 8450

UV/VIS detector. Absorbance for HIC-HPLC was monitored at 280 nm and for RP-HPLC at 215 nm. A polypropyl aspartamide (250 x 21 mm) column (Poly LC, Columbia, MD) was used for hydrophobic interaction HPLC. C18 (218TP54 and 218TP510) columns were used for reverse phase (RP) HPLC (Vydac, Hesperia, CA).

Purification of contortrostatin, the platelet aggregation inhibitor from *Agkistrodon contortrix contortrix* venom:

Contortrostatin was purified from *Agkistrodon contortrix contortrix* (Southern copperhead) venom using a three step HPLC procedure. For the first step of purification crude venom (1 g) was dissolved in 0.1 M phosphate buffer containing 1 M ammonium sulphate, pH 6.8 (buffer A) and applied to the polypropyl aspartamide HIC-HPLC column. Elution was achieved as follows: 50 minutes isocratically with 100% buffer A; a linear gradient for 90 minutes to 0.1 M phosphate, pH 6.8 (buffer B); 40 minutes isocratic at 100% buffer B. Fractions of 10 ml were collected in a Pharmacia Frac 100 fraction collector at 4°C using a flow rate of 5 ml/min. Fractions containing pl.AI activity were pooled and concentrated by ultra-filtration using an Amicon stir cell with a YM3 membrane. Further purification was achieved by C18 RP-HPLC. The C18 column (218TP510) was equilibrated with 95% of 0.1% TFA in water (solvent A) and 5% of 80% acetonitrile in 0.1% TFA in water (solvent B). Elution was achieved as follows: isocratic at 95% solvent A and 5% solvent B for 10 minutes; a linear gradient to 40% solvent B in 65 minutes; linear gradient to 100% solvent B in 20 minutes; isocratic at 100% solvent B for 25 minutes. Fractions were collected manually every minute at a flow rate of 7 ml/minute. Fractions containing pl.AI activity were pooled and rerun on the same C18 RP-HPLC column using a shallower gradient. Elution was achieved as follows: isocratic at 80% solvent A and 20% solvent B for 20 minutes; a linear gradient to 30% solvent B over 90 minutes; and a 25 minute linear gradient to 100% solvent B.

Purification of multisquamatin, the platelet aggregation inhibitor from *Echis multisquamatus* venom:

Multisquamatin was purified from 1 gram of *Echis multisquamatus* venom by a three step HPLC procedure. In the first step crude venom (1 g) was treated as *Agkistrodon contortrix contortrix* venom and applied to the HIC-HPLC column. Elution conditions were identical to the first step employed for the purification of contortrostatin. Pl.AI activity coeluted with platelet aggregatory activity in the HIC flow through. C18 RP-HPLC was employed to separate platelet inhibitory activity from platelet aggregatory activity. Fractions from the HIC flow through were pooled and concentrated using an Amicon stir cell with a YM3 membrane and subsequently applied to a C18 RP-HPLC column (218TP510). Elution was achieved as follows: 10 minute isocratic at 95% solvent A and 5% solvent B; a linear gradient to 30% solvent B over 110 minutes; and a linear gradient from 30% to 100% solvent B over 30 minutes; isocratic at 100% solvent B for 30 minutes. Fractions of 10.5 ml were collected at a flow rate of 7 ml/minute at 4°C. Active fractions were pooled, concentrated using a Savant Rotovac, and rerun on the same C18 RP-HPLC column. Elution was achieved as follows: 10 minute isocratic at 95% solvent A and 5% solvent B; a linear gradient to 30% solvent B over 80 minutes; a linear gradient

from 30% to 100% solvent B over 10 minutes; isocratic at 100% solvent B for 10 minutes. The peak containing pl.AI activity was collected manually.

Assay of platelet aggregation inhibitory activity:

Column fractions obtained during purification were assayed for pl.AI activity using fresh human platelet rich plasma (PRP) prepared from blood obtained from human volunteers who had no medication for at least two weeks. Blood (36 ml) was drawn into 4 ml of 0.1 M citrate and centrifuged at 150 x g for 20 minutes. The supernatant, PRP, was removed and the remaining blood was centrifuged at 10,000 RPM to obtain platelet poor plasma (PPP). Platelet counts were adjusted to 250,000 platelets/ μ l using a Coulter counter. A Bio-Data one channel aggregometer and a Helena four channel aggregometer were used to monitor platelet aggregation. Inhibition of ADP-induced platelet aggregation was monitored at 37°C by adding venom fractions one minute prior to the addition of ADP (10-20 μ M final concentration). Fractions exhibiting pl.AI activity were pooled and further purified. Rabbit and canine PRP was prepared by the same procedure and used in the studies described below.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE):

A Tris-Tricine 16.5% gel was used according to the protocol of Schagger and von Jagow under reducing and nonreducing conditions (13). The gel was run using a Biorad minigel system and stained with silver (14) or Coomassie blue R250.

Measurement of GPIIb/IIIa specific binding:

Measurement of contortrostatin and multisquamatin binding to platelet GPIIb/IIIa receptor was carried out using PRP prepared from blood obtained from human volunteers or male mongrel dogs. PRP was prepared as described above and the platelet count was determined with a H-10 cell counter (Texas International Laboratories, Inc., Houston, TX). PRP (180 μ l) was incubated with 20 μ l of varying concentrations of either contortrostatin or multisquamatin at room temperature. Radiolabelled antibody (125 I-7E3 IgG, 20 μ l, 18 mg/ml, 80,000 cpm), specific for GPIIb/IIIa, was then added and the mixture incubated for 30 minutes. To establish equilibrium binding, 50 μ l aliquots of the binding assay mixture were layered over 200 μ l of 30% sucrose in 0.4 ml microcentrifuge tubes and spun at 10,000 RPM for 4 minutes in a swinging bucket rotor to separate platelet-bound antibody from free antibody. The pellet and the supernatant were separated and counted in a Packard Minaxi 5000 series gamma counter. The number of molecules of 125 I-7E3 bound per platelet in the presence and absence of pl.AI was calculated by using the following formula:

$$\frac{(4) \times 0.9 \mu\text{g } 7\text{E}3 \times 3.76 \times 10^{12} \text{ molecules } 7\text{E}3/\mu\text{g}}{(5)}$$

where:

1 =	Pellet	2 =	Supernatant	3 =	Total CPM
	counts		counts		(1) + (2)

4 =	Fraction	5 =	Platelet
	bound		counts per
	(1)/(3)		$\mu\text{l} \times 45 \mu\text{l}$

RESULTS

Purification of contortrostatin:

Contortrostatin was purified from Southern copperhead venom using a three step chromatographic approach: HIC-HPLC, and two steps on C18 RP-HPLC. For the first step of purification crude venom was dissolved in a minimal volume of HIC buffer A and applied to the HIC-HPLC column. Proteins were eluted by a decreasing gradient of ammonium sulfate and were detected at 280 nm (Fig. 1 A). Pl.AI activity was observed in the flow through. Fractions containing pl.AI activity were pooled and concentrated for the second step of purification. In this step pooled fractions were applied to a C18 RP-HPLC column equilibrated with 0.1% TFA and 4% acetonitrile. Elution was achieved by an increasing gradient of acetonitrile (Fig. 2 A). Contortrostatin eluted at 28% acetonitrile (66 min). For the final step of purification, fractions containing pl.AI activity were applied to the C18 RP-HPLC column this time equilibrated with 0.1% TFA and 16% acetonitrile. Using a shallower gradient to 24% acetonitrile (Fig. 3 A), contortrostatin eluted as a sharp peak at 22% acetonitrile (82 min). The minor peak eluting just before contortrostatin also contained pl.AI activity and had a similar molecular weight to that of contortrostatin. Due to the low yield this peak was not further characterized. Yields of 1-2 mg of three step purified contortrostatin were obtained per gram of crude venom.

Purification of multisquamatin:

Multisquamatin was purified from *Echis multisquamatus* venom by a similar chromatographic approach to that used for the purification of contortrostatin. Crude venom was directly applied to the HIC-HPLC column under the same conditions used for Southern copperhead venom. Elution was achieved by a decreasing gradient of ammonium sulfate (Fig. 1 B). The major protein peak eluting in the flow through possessed platelet aggregating activity. This peak also contains pl.AI activity but it was difficult to detect due to the coeluting platelet aggregating activity. To confirm that the HIC flow through peak possessed pl.AI activity we applied aliquots to an analytical C18 RP-HPLC column (218TP54). One of the major peaks eluting from the C18 RP-HPLC column possessed pl.AI activity thereby indicating that the flow through peak from the HIC-HPLC column contains pl.AI activity. Active fractions from the HIC column were pooled, concentrated, and applied to the preparative C18 RP-HPLC column equilibrated with 0.1% TFA and 4% acetonitrile. Elution was obtained by an increasing gradient to 80% acetonitrile (Fig. 2 B). Pl.AI activity was obtained in the major peak eluting at 14% acetonitrile (61 mins). In order to remove minor contaminants multisquamatin was rerun on the C18 RP-HPLC column (Fig. 3 B). Multisquamatin eluted at 68 min (16% acetonitrile). Yields of 10-11 mg of three step purified multisquamatin were obtained per gram of crude venom.

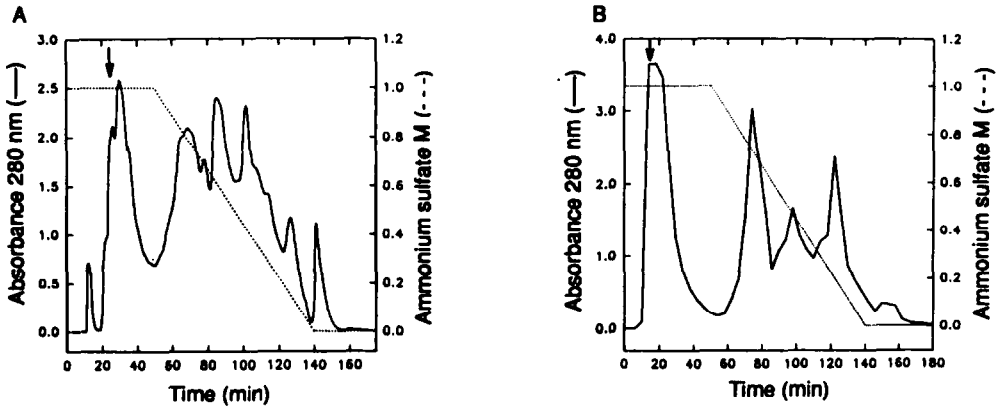


FIG. 1.

Separation of crude venoms by HIC-HPLC. (A) *Agkistrodon contortrix contortrix* was injected onto a HIC-HPLC column. (B) *Echis multisquamatus* venom was applied to the HIC-HPLC column under the same conditions as used for *Agkistrodon contortrix contortrix* venom. Refer to Materials and Methods for elution conditions. Arrows identify peaks containing pl.AI activity.

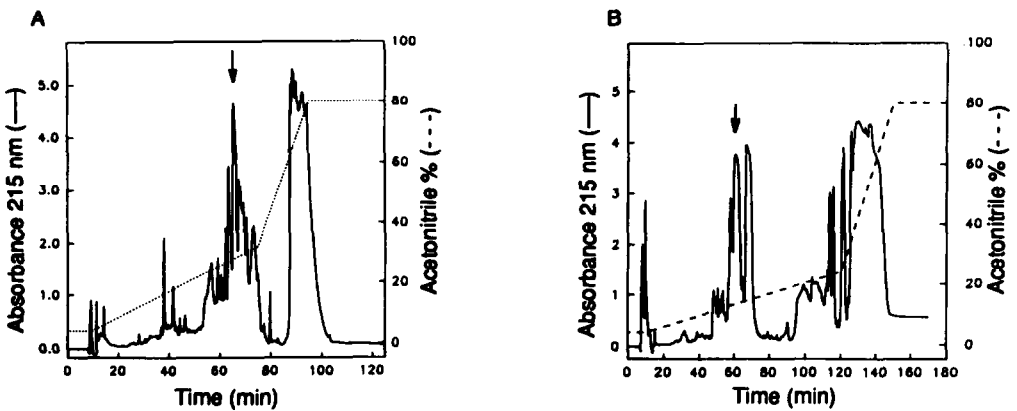


FIG. 2.

C18 RP-HPLC separation. (A) Pooled fractions from HIC-HPLC of *Agkistrodon contortrix contortrix* venom were applied to a C18 RP-HPLC column. (B) Pooled fractions from HIC-HPLC of *Echis multisquamatus* venom were applied to the C18 RP-HPLC column. Refer to Materials and Methods for elution conditions. Peaks containing pl.AI activity are indicated by the arrows.

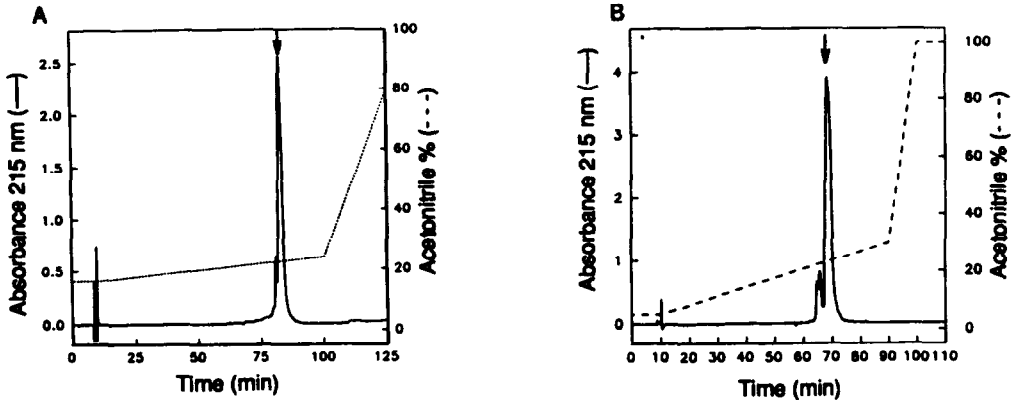


FIG. 3.

Final step of purification of contortrostatin and multisquamatin. (A) Contortrostatin was further purified by rerunning on the C18 RP-HPLC column but with a shallower gradient. It eluted at 82 min (22% acetonitrile) and was collected manually. Multisquamatin was further purified by rerunning on the C18 column using the same gradient as in the second step. It eluted at 68 min (16% acetonitrile) and was collected manually. Refer to Materials and Methods for elution conditions. Arrows identify peaks containing pl.AI activity.

SDS-PAGE analysis:

Reducing and nonreducing SDS-PAGE were used to assess homogeneity and to calculate molecular mass of purified pl.AIs (Fig. 4). SDS-PAGE analysis of contortrostatin revealed that it has a molecular mass of approximately 15,000 Da under nonreducing conditions and 5,000-7,000 Da under reducing conditions, thereby suggesting that it is composed of two or three subunits. Another possibility, although unlikely, is that the large difference in migration may be attributed to differential uptake of SDS under nonreducing and reducing conditions (5). Multisquamatin, on the other hand, has a molecular mass of 6,400 Da under nonreducing conditions and 5,700 Da under reducing conditions. Echistatin, the pl.AI purified from the venom of *Echis carinatus* ($M_r=5.4$ kDa) (5), exhibits a similar differential migration pattern to that of multisquamatin under reducing and nonreducing SDS-PAGE. This pattern in SDS-PAGE migration could be attributed to differential detergent binding based on structural differences under reducing and nonreducing conditions.

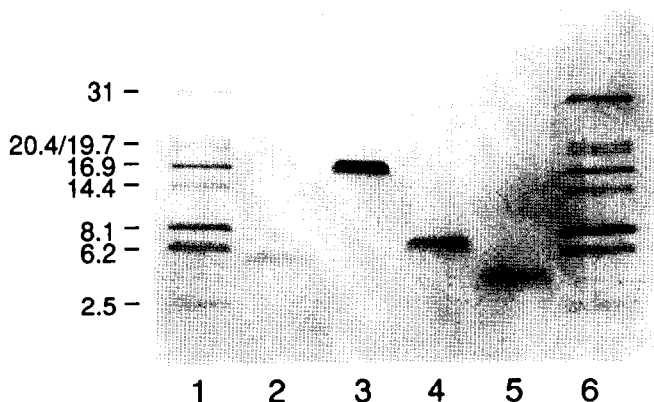


FIG. 4.

SDS-PAGE of contortrostatin and multisquamatin. Lanes 1 and 6 are low molecular weight markers: 31kDa, carbonic anhydrase; 20.4/19.7kDa, soybean trypsin inhibitor doublet; 16.9kDa, horse heart myoglobin; 14.4, lysozyme; 8.1 myoglobin CNBr fragment 1 (F1); 6.2kDa, myoglobin (F2); and 2.5kDa, myoglobin (F3). Lanes 2 and 3 are 1 μ g of reduced and nonreduced contortrostatin, respectively. Lanes 4 and 5 are 1 μ g of nonreduced and reduced multisquamatin, respectively. The gel was stained with Coomassie blue R250.

Inhibition of platelet aggregation:

Contortrostatin and multisquamatin inhibit ADP-induced platelet aggregation in human, canine, and rabbit PRPs (Fig. 5). Contortrostatin (0.73 μ g/ml) and multisquamatin (0.55 μ g/ml) inhibited 10 μ M ADP-induced human platelet aggregation by 50% (IC_{50}). The IC_{50} for 20 μ M ADP-induced canine platelet aggregation is 1.8 μ g/ml for contortrostatin and 1.6 μ g/ml for multisquamatin. Interestingly, the IC_{50} for contortrostatin mediated inhibition of rabbit platelet aggregation is considerably higher. The IC_{50} for 20 μ M ADP-induced rabbit platelet aggregation is 17.3 μ g/ml for contortrostatin, but only 1.9 μ g/ml for multisquamatin.

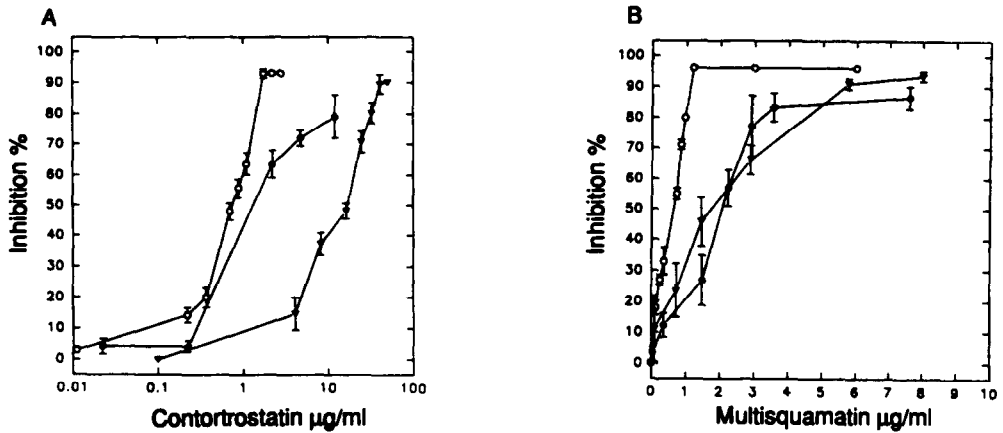


FIG. 5.

(A) Determination of IC_{50} for contortrostatin inhibition of human, canine and rabbit platelet aggregation. Empty circles represent human PRP, solid circles represent canine PRP, and empty triangles represent rabbit PRP. Varying concentrations of contortrostatin were preincubated with PRP prior to the addition of ADP. Contortrostatin has an IC_{50} of $0.73 \mu\text{g/ml}$, $1.8 \mu\text{g/ml}$ and $17.3 \mu\text{g/ml}$ for inhibition of human, canine and rabbit platelet aggregation, respectively. (B) Determination of IC_{50} for multisquamatin inhibition of human, canine and rabbit platelet aggregation. The symbols are the same as for contortrostatin. Varying concentrations of multisquamatin were preincubated with PRP as for contortrostatin. Multisquamatin has an IC_{50} of $0.55 \mu\text{g/ml}$, $1.6 \mu\text{g/ml}$ and $1.9 \mu\text{g/ml}$ for human, canine and rabbit platelet aggregation, respectively. Each point represents the average of three determinations.

Determination of GPIIb/IIIa specific binding of pl.AIs using the monoclonal antibody, 7E3:

The murine monoclonal antibody, 7E3, is a potent inhibitor of human and canine platelet aggregation (11). To determine if contortrostatin and multisquamatin inhibit platelet aggregation by binding to GPIIb/IIIa, we employed competitive binding studies using 7E3. These studies demonstrated specific platelet GPIIb/IIIa receptor binding for the two pl.AIs with both human and canine platelets (Fig. 6). The concentration of contortrostatin to inhibit 50% of 7E3 binding to human GPIIb/IIIa (IC_{50}) is $0.4 \mu\text{g/ml}$. Multisquamatin has an IC_{50} for human GPIIb/IIIa of $0.018 \mu\text{g/ml}$. The IC_{50} for contortrostatin and multisquamatin for canine GPIIb/IIIa is $0.24 \mu\text{g/ml}$ and $0.36 \mu\text{g/ml}$, respectively. These studies indicate that both contortrostatin and multisquamatin inhibit platelet aggregation by binding to GPIIb/IIIa.

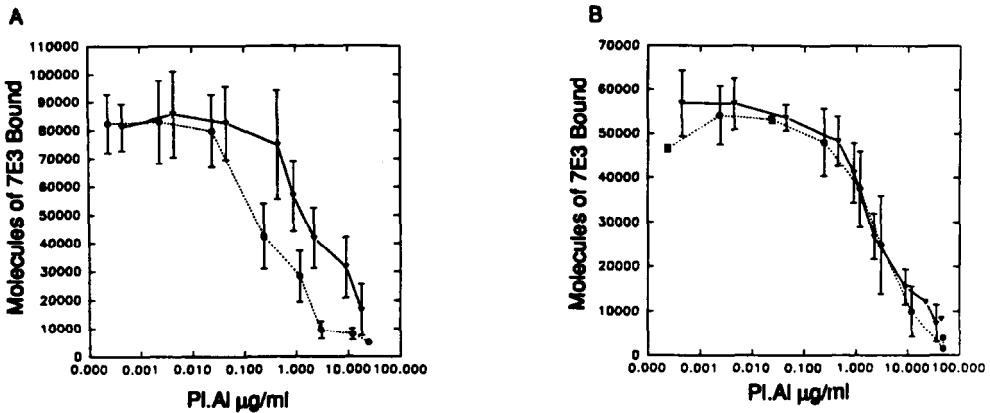


FIG. 6.

(A) Binding of contortrostatin and multisquamatin to human GPIIb/IIIa in the presence of a fixed saturating concentration of 7E3. Solid line with solid triangle represents contortrostatin and dotted line with solid circle represents multisquamatin. The IC_{50} for contortrostatin displacement of 7E3 is $0.4 \mu\text{g/ml}$ and for multisquamatin it is $0.018 \mu\text{g/ml}$. (B) Binding of contortrostatin and multisquamatin to canine platelet GPIIb/IIIa in the presence of a fixed saturating concentration of 7E3. The symbols are as in A. The IC_{50} for contortrostatin displacement of 7E3 is $0.25 \mu\text{g/ml}$ and for multisquamatin it is $0.36 \mu\text{g/ml}$. Refer to Materials and Methods for a detailed description. Each point represents the average of at least three determinations.

DISCUSSION

Recently platelet aggregation inhibitors have been purified from venoms of snakes of the *Crotalidae* and *Viperidae* families (4-10). The first pl.AI to be reported was trigramin from *Trimeresurus gramineus* venom (4). This pl.AI was shown to bind to the GPIIb/IIIa complex on the platelet surface (4). All pl.AIs purified thus far, with the exception of barbourin (8), possess the RGD sequence. This sequence motif has been implicated as being involved in the inhibition of integrin mediated interactions (1). Therefore, a number of investigators have examined the efficacy of linear and cyclic RGD peptides (15, 16) or cyclic KGD peptides (17) as antiplatelet agents. However, there appears to be increasing evidence that pl.AIs may have unique surface geometry which facilitates interactions with integrins by mechanisms other than those solely based on the RGD site. Recently it has been reported

that some snake venom metalloproteinases, which have no structural resemblance to mammalian matrix-degrading metalloproteinases, contain a domain which bears a striking resemblance to the pl.AI sequence (18-21). Although the pl.AI domain is devoid of the RGD sequence in at least one of the metalloproteinases (18), the proteinase is a potent inhibitor of ADP- and collagen-induced platelet aggregation. Furthermore, the finding that a mutated, chemically synthesized pl.AI derivative of echistatin (alanine substituted for arginine in the RGD sequence) still possessed some biological activity (22), suggests that other regions in the protein may be involved in binding and that there may be some flexibility in the RGD binding site. The synthetic RGD peptides, due to their small size, do not possess the molecular topography of the pl.AIs and, therefore, cannot interact with the multiplicity of mechanisms used by pl.AIs.

In view of the potential clinical use of pl.AIs as antithrombotic agents we have purified two pl.AIs, contortrostatin and multisquamatin, from *Agkistrodon contortrix contortrix* and *Echis multisquamatus* venoms, respectively. Both contortrostatin and multisquamatin are potent inhibitors of human, rabbit, and canine platelet aggregation. Interestingly, studies in collaboration with Dr. Joan Brugge and Edwin A. Clark, at Ariad Pharmaceuticals Inc., Cambridge, MA, have shown that contortrostatin does not inhibit platelet release reactions (unpublished results). The partial amino acid sequence of contortrostatin shows significant identity with applaggin (6). However, there appears to be an interesting difference in that the sequence for contortrostatin begins nine amino acid residues downstream from the applaggin start site (unpublished results). Presumably the amino terminal deletion has no effect on pl.AI activity since the IC_{50} values for contortrostatin and applaggin are very similar (6).

Multisquamatin appears to be similar to echistatin (5) based on the amino terminal sequence. However, the amino terminus of multisquamatin appears to start three amino acids upstream from the echistatin start site (unpublished results). The yield for multisquamatin (10-11 mg per gram of crude venom) is to our knowledge the highest yield of any pl.AI reported thus far. It appears that multisquamatin and contortrostatin are not very hydrophobic because they do not bind to the HIC-HPLC column. Furthermore, they elute early in the C18 RP-HPLC gradient which separates proteins based on their hydrophobicity (23). This is supported by their amino acid composition which is low in hydrophobic amino acids (unpublished results).

Several lines of evidence indicate that contortrostatin and multisquamatin inhibit platelet aggregation by binding specifically to the GPIIb/IIIa integrin receptor. Thus, in a fibrinogen-GPIIb/IIIa ELISA (24), in which the extent of purified GPIIb/IIIa bound to immobilized fibrinogen can be quantitated, both multisquamatin and contortrostatin effectively block GPIIb/IIIa binding (data not shown). Additionally, the partial amino acid sequences of both pl.AIs indicate considerable identity with other snake venom pl.AIs which are known to bind to GPIIb/IIIa. Finally, contortrostatin and multisquamatin block 7E3 binding to GPIIb/IIIa

(Fig. 6). 7E3 is a murine monoclonal antibody that specifically binds to GPIIb/IIIa, thereby inhibiting platelet aggregation (11). In the presence of low concentrations of either contortrostatin or multisquamatin 7E3 binding to platelets was significantly inhibited (Fig. 6).

An interesting observation is the relatively high IC_{50} for inhibition of rabbit platelet aggregation by contortrostatin. The IC_{50} of 17.3 $\mu\text{g/ml}$ is considerably higher than its IC_{50} of 0.73 $\mu\text{g/ml}$ and 1.8 $\mu\text{g/ml}$ for inhibition of human and canine platelet aggregation, respectively. However the IC_{50} values for multisquamatin inhibition of human, canine and rabbit platelet aggregation are very similar. This apparent difference raises two possible questions. First, does contortrostatin have a different mechanism of inhibition of platelet aggregation than multisquamatin? Second, is the rabbit GPIIb/IIIa different than GPIIb/IIIa present on human and canine platelets? Further study into the mechanisms involved in contortrostatin and multisquamatin mediated inhibition could provide more insight into the mechanistic differences between rabbit GPIIb/IIIa and its counterpart in human and canine platelets.

Two snake venom pl.AIs, kistrin (9) and bitistatin (25), have demonstrated a potential role as antithrombotic agents for use in thrombolytic therapy by enhancing and sustaining arterial thrombolysis in conjunction with recombinant tissue plasminogen activator. Based on the low IC_{50} values of contortrostatin we have tested its *in vivo* efficacy as an antithrombotic agent. Using a canine carotid reoccluding arterial thrombosis model, preliminary results with contortrostatin have been promising. In conjunction with APSAC contortrostatin efficiently sustains opening of the carotid artery, whereas APSAC alone is unable to prevent the rapid reocclusion of the carotid artery. Further *in vivo* characterization of both contortrostatin and multisquamatin is in progress.

Based on the experiments described above both contortrostatin and multisquamatin have been shown to be potent inhibitors of GPIIb/IIIa mediated platelet aggregation.

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