Isolation and characterization of two disintegrins inhibiting ADP-induced human platelet aggregation from the venom of *Crotalus scutulatus scutulatus* (Mohave Rattlesnake)

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**Abstract**

Disintegrins and disintegrin-like proteins are molecules found in the venom of four snake families (Atractaspididae, Elapidae, Viperidae, and Colubridae). The disintegrins are nonenzymatic proteins that inhibit cell–cell interactions, cell–matrix interactions, and signal transduction, and may have potential in the treatment of strokes, heart attacks, cancers, and osteoporosis. Prior to 1983, the venom of *Crotalus scutulatus scutulatus* (Mohave Rattlesnake) was known to be only neurotoxic; however, now there is evidence that these snakes can contain venom with: (1) neurotoxins; (2) hemorrhagins; and (3) both neurotoxins and hemorrhagins. In this study, two disintegrins, mojastin 1 and mojastin 2, from the venom of a Mohave rattlesnake collected in central Arizona (Pinal County), were isolated and characterized. The disintegrins in these venoms were identified by mass-analyzed laser desorption ionization/time-of-flight/time-of-flight (MALDI/TOF/TOF) mass spectrometry as having masses of 7.436 and 7.636 kDa. Their amino acid sequences are similar to crotratroxin, a disintegrin isolated from the venom of the western diamondback rattlesnake (*C. atrox*). The amino acid sequence of mojastin 1 was identical to the amino acid sequence of a disintegrin isolated from the venom of the Timber rattlesnake (*C. horridus*). The disintegrins from the Mohave rattlesnake venom were able to inhibit ADP-induced platelet aggregation in whole human blood both having IC₅₀s of 13.8 nM, but were not effective in inhibiting the binding of human urinary bladder carcinoma cells (T24) to fibronectin.

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**Introduction**

The Mohave rattlesnake (*Crotalus scutulatus scutulatus*) is perhaps the most lethal rattlesnake in the United States, since its venom contains neurotoxins, and its LD₅₀ ranges between 0.12 to 0.5 mg/kg body weight (Glenn and Straight, 1978, 1982; Pattabhiran et al., 1978; Sánchez et al., 2003). It has been estimated that the amount of dried venom to kill an adult human is approximately 10–15 mg (Minton and Minton, 1969). The venom of *C. s. scutulatus* has been categorized into three types: (1) neurotoxic “Type A”; (2) hemorrhagic “Type B”; and (3) both neurotoxic and hemorrhagic “Type A + B” (Glenn and Straight, 1978, 1989; Glenn et al., 1983). The major toxins in venom of the Mohave rattlesnake consist of primarily pre-synaptic neurotoxins known as Mojave toxins (Bieber et al., 1975; Cate
and Bieber, 1978). It was later reported that a small group of snakes located in southern Arizona were hemorrhagic and lacked the Mojave toxin (Glenn et al., 1983). We have detected disintegrin activity from Mohave rattlesnake venom containing hemorrhagic activity and lacking the Mojave toxin (Sánchez et al., in press).

Disintegrins are obtained from proteolytic processing of the P-II or P-III class of proteins, they are either monomers or homo- or heterodimers, and they interact with integrin receptors (heterodimeric transmembrane proteins) of cells (Dennis et al., 1989; Gould et al., 1990; Kini and Evans, 1992; Niewiarowski et al., 1994; Yamada et al., 1999). Disintegrins were first thought to only inhibit platelet aggregation (Ouyang et al., 1983; Gould et al., 1990), and now there are over 90 disintegrins found in snake venom (McLane et al., 2004) which could have biomedical applications in strokes, heart attacks, diabetes, and osteoporosis (Niewiarowski et al., 1989; Huang et al., 1991; Sheu et al., 1992; Fisher et al., 1993; Nakamura et al., 1998, 1999; Brando et al., 2000; Marcinkiewicz et al., 2003). Inhibition of platelet aggregation occurs through the binding of disintegrins to the platelet fibrinogen receptors, α1β3 integrins. Many disintegrins contain an RGD (arginine–glycine–aspartic acid) sequence near the C-terminus that specifically binds to α1β3 integrins (Savage et al., 1990). These disintegrins prevent the binding of fibrinogen (a major component necessary for platelet aggregation also containing the RGD sequence; Plow et al., 1985) to the α1β3 integrin.

The purpose of this study was to isolate and characterize disintegrins in the venom of a Mohave rattlesnake void of Mojave toxin by a multidimensional liquid chromatography system (MDLC), followed by mass spectrometry (MS) characterization.

Materials and methods

Snake

The Mohave rattlesnake (C. s. scutulatus, Avid # 010-310-068) was obtained from Pinal Co., Arizona. It is currently housed at the Natural Toxins Research Center (NTRC), Texas A&M University-Kingsville, Kingsville, TX, U.S.A. The Avid # is a nine digit code given to individual specimens kept at the NTRC. With the Avid #, additional information can be obtained for a specimen via a database (http://ntrc.tamuk.edu).

Venom collection

Venom was extracted by allowing the snake to bite into a para-film stretched over a disposable plastic cup. The venom sample was centrifuged (500 × g for 10 min), and filtered through 0.45 μm filter under positive pressure. The venom was frozen in a −90 °C freezer and then lyophilized.

Hemorrhagic assay

Hemorrhagic activity for crude venom and its reverse phase fractions was determined by the procedure developed by Omori-Satoh et al. (1972). To test for activity, 0.1 mL of crude venom (0.0025 mg/mL) and its fractions (0.05 mg/mL) were injected intradermally into the backs of depilated New Zealand rabbits (Oryctolagus cuniculus). After 24 h, the rabbits were sacrificed and hemorrhagic spots measured (mm). The minimal hemorrhagic dose (MHD) was defined as the amount of protein (μg) that causes a 10 mm hemorrhagic spot.

Fibrinolytic assay

Fibrinolytic activity of crude venom and its reverse phase fractions was measured using a procedure modified from Bajwa et al. (1980). Fibrinogen solution from human plasma (9.4 mg/mL, 300 μL, SIGMA) and thrombin from bovine plasma solution (38.5 U/mL, 10 μL, SIGMA) were added to each well of a 24-well plate. The plate was shaken gently and the solution allowed to clot at room temperature. The plate was then incubated for 3 h, at 37 °C. Twenty microliters of crude venom (1 mg/mL) and its fractions (1 mg/mL) were added individually in the center of their corresponding wells, and incubated overnight, at 37 °C. Seven hundred microliters of 10% trichloroacetic acid (TCA) was placed in each well, and decanted after 10 min. If a zone of clearing was observed, the assay was considered positive. The assay was repeated three times for each sample.

Fibrinogenolytic assay

A modification of the method developed by Ouyang and Teng (1976) was used to test the fibrinogenolytic activity of crude venoms and their individual reverse phase fractions. Two hundred microliters of human fibrinogen solution (10 mg/mL) in 20 mM Tris–HCl buffer, pH 8.0, and 100 μL of C. s. scutulatus crude venom (0.03 mg/mL) was mixed and incubated at 37 °C. Fractionated venom samples were incubated for 4 h. Twenty microliter aliquots were taken and added to 20 μL of 20 mM Tris–HCl buffer, pH 8.0 containing 2 mM EDTA, 5% (w/v) SDS, 0.02% (w/v) bromophenol blue, and 10% (v/v) beta-mercaptoethanol. The samples were then boiled for 3 min, and analyzed by SDS using a homogeneous PhastGel 12.5, on a Pharmacia PhastSystem. The gels were stained with silver nitrate. The positive results were recorded by visible decrease of the α chain of fibrinogen on the gel.

Gelatinase assay

A modification of the method developed by Huang and Perez (1980) was used to test gelatinase activity. Twenty
microliters of crude venom (1 mg/mL) and its reverse phase fractions (1 mg/mL) were placed on a Kodak X-OMAT scientific imaging film with gelatin coating. Hydrolysis of gelatin on the X-ray film was determined by washing the film with tap water after 4 h incubation, at 37 °C in a moist incubator. A positive result was ascertained if there was clearing of the gelatin on the X-ray film.

**Protein purification**

**Reverse phase chromatography C18.** Five milligrams of venom sample was fractionated by reverse phase chromatography. Venom was separated using a Grace Vydac Reverse Phase C18 (250 × 4.6 mm) column. Fractions were eluted using a 0.1% TFA, and 80% acetonitrile in 0.1%TFA gradient over 60 min, with a flow rate of 1 mL/min. A Waters 484 tunable detector was used to monitor absorbances at 280 nm. Fractions were stored at −90 °C. Protein concentrations were determined by standard methods at 280 nm using an extinction coefficient of 1.

**Size exclusion chromatography.** The reverse phase C18 fraction (#8) with platelet aggregation activity was further fractionated by size exclusion. One hundred micrograms of protein was separated using a Waters ProteinPak 60 (7.8 × 300 mm) column, on a Waters High Performance Liquid Chromatography system. Fraction 8 was separated using a 0.02M Sodium phosphate buffer, pH 6.2. Proteins were detected at 280 nm. Fractions were stored at −90 °C. Protein concentrations were determined by standard methods at 280 nm using an extinction coefficient of 1.

**Anion exchange chromatography.** The size exclusion fraction (#2) with platelet aggregation activity was further fractionated by anion exchange chromatography. Two hundred and eight micrograms of protein were separated using a Waters DEAE 5PW column (7.5 × 75 mm) column, on a Waters High Performance Liquid Chromatography system. Fraction 2 was separated using 0.02M Tris–HCl, buffer, pH 8.0, at a flow rate of 1 mL/min. Proteins were detected at 280 nm. Fractions were tested for inhibition of platelet aggregation and retraction.

**Platelet aggregation assay**

A Chrono-Log Whole-Blood Aggregometer was used to monitor platelet aggregation, by impedance, of whole human blood when venom fractions were added. Four hundred and fifty micrograms of 10% citrated human blood was incubated to 37 °C, at least 5 min prior to use with equal amounts of 0.15 M Saline solution. Ten micrograms of venom fractions (1 mg/mL) were incubated with the blood sample for 2 min. An electrode was inserted in the blood sample, and 90 s later, 20 μL of a 1 mM ADP solution was added to the blood sample to promote platelet aggregation.

**Proteolytic activity on insulin B-chain by capillary electrophoresis (CE)**

A method described by Sánchez et al. (2001) was used to determine proteolytic activity on insulin chain B from bovine (SIGMA). A Beckman Capillary Electrophoresis P/ACE 5500 was used to observe the proteolytic activity of venom fractions. Ten microliters of 0.03 mg/mL venom or fractions were incubated with 10 μL of 0.5 mg/mL of insulin B chain, and 10 μL of 0.01M Borate buffer, pH 8.3. The mixture was immediately separated for 10 min at 20 kV, 19.5 μamps, using a 0.01 M Borate buffer, pH 8.3 through a 75 μm I.D. × 50 cm (100 × 800 aperture) free zone capillary. A P/ACE UV absorbance detector at 214 nm was used to detect the resulting peptides.

**Cell line and culture conditions**

The human urinary bladder carcinoma cell line (T24) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained as a monolayer culture with McCoy’s 5A minimum essential medium, supplemented with 10% fetal calf serum, sodium pyruvate, nonessential amino acids, L-glutamine, and vitamins incubated in a humidified 5% CO2–95% air incubator at 37 °C.

**Cellular adhesion inhibition assay**

Possible inhibition of T24 cell binding to fibronectin induced by venom components was evaluated as described by Wierzbicka-Patynowski et al. (1999). Cells were harvested, counted, and resuspended in medium containing 1% BSA at 5 × 10^5 cells/mL. Triplicate wells of a 96-well plate (Falcon Tissue Culture Plate) were coated with fibronectin at 10 μg/mL, in 0.01 M Phosphate buffer saline (PBS), pH 7.4, and incubated overnight at 4 °C. The plate was blocked by addition of 0.2 mL of PBS in 5% Bovine serum albumin (BSA) and incubated at 37 °C, for 1 h. Snake venom fractions were added to the cell suspension at concentrations of 60 μg/mL, and allowed to incubate at 37 °C for 1 h. The fraction that had disintegrin-like activity (inhibiting platelet aggregation) was tested at various concentrations (1210, 605, 302, 151, 75, and 38 nM). The blocking solution was aspirated, and the cell/venom fraction suspensions (0.2 mL) were added to the wells coated with fibronectin, and incubated at 37 °C for 1 h. Synthetic echistatin (SIGMA, Lot. 023K12301), a disinte-
Results

Proteolytic assays of crude C. s. scutulatus venom

The venom of C. s. scutulatus used in this study, contained hemorrhagic, fibrinolytic, gelatinase, fibrinogenolytic, and hydrolysis of insulin B-chain activities. The venom did not contain neurotoxic activity or the Mojave toxin (Sánchez et al., in press); and therefore, was considered a “Type B” venom.

Purification and sequence analysis of disintegrins

A total of 28 fractions were collected by reverse phase chromatography, and all fractions were tested for proteolytic activity and for inhibition of ADP-induced platelet aggregation on whole human blood. These fractions did not contain proteolytic activity, which is in part contributed by the denaturation of larger proteins by the solvents used for reverse phase chromatography. Fraction 8 (Fig. 1) inhibited platelet aggregation, but was unable to inhibit T24 cell adhesion to fibronectin.

Fraction 8 was further fractionated by size exclusion chromatography (Fig. 2). Ten fractions were collected, and disintegrin activity was present in fraction 2. Fraction 2 was further fractionated by anion exchange chromatography, and a total of 12 fractions were collected (Fig. 3). Fractions 3 and 6 inhibited platelet aggregation. Both fractions appeared highly purified by capillary electrophoresis (Fig. 4). Fraction 3 (mojastin 1) had a mass of 7.436 kDa and fraction 6 (mojastin 2) had a mass of 7.636 kDa as determined by MS (Fig. 5). The amino acid sequence was determined by tandem MS. Mojastin 1 contained 71 amino acids, and mojastin 2 had 73 amino acids (Fig. 6). Mojastin 1 and 2 had a 97% sequence identity with each other. Mojastin 1 had a 99% sequence identity with the disintegrins crotatroxin and durissin from venom of C. atrox and C. durissus, respectively, and a 93% identity with lutosin, the disintegrin from venom of C. lutosus. Mojastin 1 was identical to hordistatin 2, a disintegrin isolated from the venom of C. horridus, and was 97% identical to hordistatin 1.

Platelet aggregation and adhesion studies

Mojastin 1 and 2 inhibited ADP-induced platelet aggregation in whole blood and this effect was concentration dependent. The IC50s of mojastin 1 and 2 were 13.8 nM (Fig. 7).

Both disintegrins were not effective, at concentrations up to 1.210 μM, in inhibiting the binding of T24 cells to fibronectin (data not shown). T24 cells contain the integrins αvβ3 and αvβ5 suggesting the binding of these cells to fibronectin through αvβ3, since fibronectin does not mediate any type of cellular interaction with αvβ5 (Jin and Varner, 2004). Synthetic echistastin was able to significantly inhibit...
the binding of T24 cells to fibronectin with an IC\textsubscript{50} of 256 nM (data not shown).

Discussion

Disintegrins and disintegrin-like proteins are biologically important molecules found in snake venom or Duvernoy’s gland secretions (McLane et al., 2004). Disintegrins have the potential to be used in medical applications such as the treatment of strokes, heart attacks, diabetes, cancers, and osteoporosis (Sato et al., 1990; Fisher et al., 1993; Niewiarowski et al., 1994; Beviglia et al., 1995; Brando et al., 2000; Ritter and Markland, 2001). Over 90 disintegrins from snake venom have been isolated to date. In this study, two disintegrins (mojastin 1 and 2) of the Mohave rattlesnake venom, with masses of 7.436 and 7.636 kDa, respectively, were identified in venom of a snake collected in central Arizona (Pinal Co.). In a previous study (Sánchez et al., in press), a total of 14 venoms from Mohave rattlesnakes from various geographical locations in Arizona and Texas were analyzed for the presence of disintegrin and neurotoxic activities. Five of the venoms contained disintegrin activity, but these snakes lacked the Mojave toxin genes. In these five venoms, the two disintegrins identified by gene sequence (scutustatin and mojavestatin), are different than either disintegrin isolated from the venom analyzed in this study by biochemical methods. Scutustatin and mojavestatin were identified by PCR analysis of genomic DNA, and their deduced amino acid sequence (41 amino acids) did not extend past the RGD motif. In the 41 amino acid region, scutustatin and mojastin 1 and 2, shared 37

Fig. 1. Reverse phase C18 chromatography of \textit{C. s. scutulatus} (Avid # 011-064-358) venom. Five milligrams of venom was fractionated using a Grace Vydac C18 column (4.6 × 150 mm). The venom was separated using 0.1% TFA with the eluting solvent of 80% acetonitrile in 0.1% TFA on a Waters high performance liquid chromatography system. The fractions were tested for inhibition of platelet aggregation and retraction and T24 cell binding inhibition to fibronectin. Disintegrin activity was detected in fraction 8*. Snake was collected in Pinal Co., AZ.

Fig. 2. Size exclusion chromatography of the reverse phase, disintegrin-active, fraction 8. One hundred micrograms of fraction 8 was further separated using a Waters ProteinPak\textsuperscript{TM}60 column (7.8 × 300 mm). Fraction 8 was separated using 0.02 M Sodium Phosphate, pH 6.2, buffer on a Waters high performance liquid chromatography system. The fractions were tested for inhibition of platelet aggregation and retraction. Disintegrin activity was present in fraction 2*.
identical amino acids, with one conserved amino acid substitution. Mojavestatin and mojastin 1 and 2, only shared 28 out 41 amino acids in the same region.

Mojastin 1 and 2 have high amino acid sequence homology to crotatroxin and durissin disintegrins (Scarborough et al., 1993). Crotatroxin and durissin are monomeric disintegrins with a mass of 7.5 kDa isolated from the venom of *C. atrox* (Western diamondback rattlesnake) and *C. durissus durissus* (Central American rattlesnake), respectively. Mojastin 1 (Fig. 6) has a 99% amino acid identity with crotatroxin and durissin, lacking the amino acid alanine at the N-terminus. Mojastin 2 (Fig. 6) also has a 99% identity to crotatroxin and durissin having an additional amino acid (glutamic acid) at the N-terminus (Fig. 6). Mojastin 1 has a 100 and 97% amino acid identity with horrdistatin 2 and 1, respectively (Galán et al., in press). There is a 97% identity between mojastin 1 and 2 of the *C. s. scutulatus* venom. Mojastin 2 has two additional amino
acids than mojastin 1 at the N-terminal end (glutamic acid, and alanine). The difference in mass between the two proteins could be justified by the deletion of the first two amino acids on the N-terminus in mojastin 1 (E-A), which also accounts for the 0.2 kDa difference in mass difference between the two proteins (Fig. 5). It is highly possible that this difference could be the result of alternative processing of the pre-protein. Previous studies provide supporting evidence for the presence of common precursors for metalloproteases, disintegrins, and other related proteins (Kini and Evans, 1990; Hite et al., 1992, 1994; Paine et al., 1992, 1994; Takeya et al., 1992, 1993; Au et al., 1993).

Mojastin 1, 2, and crotatroxin contain the sequence RGDW, a motif with a high affinity to $\alpha_{IIb}\beta_3$ receptors, but weak affinity to $\alpha_v\beta_3$ receptors in solid-phase ligand binding assays (Scarborough et al., 1993). Crotratoxin

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**Fig. 5.** Intact mass of mojastin 1 (top panel (a)) and mojastin 2 (b) from the anion exchange chromatography purification step. Monoisotopic masses of 7.436 and 7.636 kDa were observed for mojastin 1 and 2, respectively. The samples were analyzed in reflectron mode using an Applied Biosystems 4700 Proteomics Analyzer and internal calibration.

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**Fig. 6.** Amino acid comparison of medium size disintegrins isolated from various snake venoms. The numbers in parenthesis are the NCBI accession numbers. Conserved amino acids are shaded in light gray. Conserved cysteines are shaded in dark gray. The RGD motif is highlighted in a black box. The KGD and MVD motifs are underlined. *Galán et al., 2005.
weakly inhibits cell adhesion to fibronectin as well as vitronectin on M21 melanoma cells (Scarborough et al., 1993). These cells have integrin receptors $\alpha_5\beta_1$ and $\alpha_v\beta_3$. Mojastin 1 and 2 should be expected to have comparable results to crotratoxin (since their amino acid sequences are almost identical), if the M21 cell line is used. Mojastin 1, 2, and the two disintegrins recently isolated from Timber rattlesnake (C. horridus) venom (Gala´n et al., in press), were unable to inhibit binding of T24 human urinary bladder carcinoma cells, containing integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Zhou et al., 2000), to fibronectin at concentrations up to 1.2 x 10^-5 M. The two C. horridus disintegrins also contain the RGDW sequence (Gala´n et al., in press). The effectiveness of these disintegrins to inhibit T24 cell binding was based on the ability of synthetic echistatin to inhibit binding with an IC50 of 256 nM (data not shown). According to the binding results obtained by Scarborough et al. (1993) with crotratoxin and the binding results obtain in our studies, we suggest that the RGDW group of disintegrins may inhibit cell adhesion and/or function mediated by the integrins $\alpha_5\beta_1$. Nonetheless, in all three studies, these disintegrins were more effective in inhibiting the binding of fibrinogen to the $\alpha_{IIb}\beta_3$ integrins found on human platelets with similar IC50s ranging between 7.3 and 17.2 nM (Scarborough et al., 1993; Galán et al., in press).

In conclusion, this study reports the isolation, determination of masses and amino acid sequences by MS, and the biological activities of mojastin 1 and 2, two effective inhibitors of ADP-induced platelet aggregation present in the venom of C. s. scutulatus lacking the Mojave toxin.

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