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Research paper

Alsophinase, a new P-III metalloproteinase with α-fibrinogenolytic and hemorrhagic activity from the venom of the rear-fanged Puerto Rican Racer *Alsophis portoricensis* (Serpentes: Dipsadidae)

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ABSTRACT

Metalloproteinases from snake venoms are often multi-domain enzymes involved in degradation of a variety of structural proteins. Hemorrhage and tissue necrosis are common manifestations of viperid envenomations in humans, largely due to the actions of prominent metalloproteinases, and envenomation by rear-fanged snakes may also cause hemorrhage. We purified the major metalloproteinase in *Alsophis portoricensis* (Puerto Rican Racer) venom through HPLC size exclusion and ion exchange chromatography. Named alsophinase, it is the first protein purified and characterized from the venom of *Alsophis*. Alsophinase is a single polypeptide chain protein, and based on mass, activity and complete inhibition by 1,10-phenanthroline, it is a class P-III snake venom member of the M12 ADAM family of metalloproteinases. Alsophinase has a molecular mass of 56.003 kDa and an N-terminal sequence of QDTYLNAKKYIEFYLVVDNGMFxKYSxxFTV, with 67% sequence identity to a metalloproteinase isolated from venom of *Philodryas olfersii* (another rear-fanged species). Alsophinase rapidly catalyzed cleavage of only the Ala14–Leu15 bond of oxidized insulin B chain, had potent hemorrhagic activity in mice, and degraded only the α-subunit of human fibrinogen *in vitro*. Alsophinase is responsible for hemorrhagic and fibrinogenolytic activity of crude venom, and it may contribute to localized edema and ecchymosis associated with human envenomations by *A. portoricensis*. It may be more specific in peptide bond recognition than many well-characterized viperid P-III metalloproteinases, and it could have utility as a new protein fragmentation enzyme for mass spectrometry studies.

1. Introduction

Snake venom metalloproteinases (SVMPs) belong to the M12 subfamily of metalloproteinases (E.C. 3.4.24) and are thought to have evolved among snakes from the ADAM (A Disintegrin and Metalloproteinase) family of proteins [1]. These metalloproteinases are commonly inhibited by metal ion chelating agents, and they are classified based on the presence of domain structures into classes P-I–P-III, all of which contain pre-, pro-, and proteinase domains with several stabilizing disulfide bonds [2,3]. The classes differ in molecular weight and domain composition. Class P-I has only the metalloproteinase domain, while P-II has an additional disintegrin-like domain; class P-IIIa–c have the metalloproteinase, disintegrin-like and cysteine-rich domains, and class P-IIId also has a lectin domain [3]. The class P-III SVMPs possess the most varied activities and are typified by the hemorrhagic metalloproteinases, common in viper venoms, which also induce muscle cell degeneration and necrosis [4–6]. The mechanism of hemorrhagic activity is thought to involve degradation of components of the extracellular matrix (ECM), damaging the integrity of the interaction between the ECM and endothelial cells and leading to hemorrhage [7–9]. Muscle necrosis develops secondarily to the ischemia provoked in skeletal muscle as a consequence of microvasculature injury [10]. Hemorrhagic metalloproteinases are characteristic of many viperid venoms (e.g., rattlesnakes [11]) but are typically found in low abundance in elapid venoms; their distribution in rear-fanged snake venoms is poorly known, but they have been documented in venoms of several species [12–17].

Metalloproteinases are often the most abundant transcripts/proteins present in transcriptomic and proteomic studies of viperid snake venom glands [18–21]. These prominent venom components can cause severe tissue necrosis and morbidity [22,23], in part because of the capacity to localize to the basement membrane and promote degradation of collagen and other ECM proteins [24,25],

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resulting in often grave hemorrhage and tissue damage [9,10]. Accordingly, metalloproteinases from viperids venoms have been extensively studied, and their activities and catalytic mechanism, gene and protein structures, and evolution and relation to mammalian ADAMs have been recently reviewed [26,27]. In addition, because of their multi-domain structure and the exploration of disintegrins and C-type lectins as anticancer drug leads [28,29], and the similarities in activity with several proteinases involved in metastasis [26,30–31], venom metalloproteinases have particular pharmacological significance.

Alsophis portoricensis is a common rear-fanged snake in much of the Caribbean [32], and human envenomations, though uncommon, may result in localized but moderately severe hemorrhage [33]. Although hemorrhagic effects of envenomation have not been studied, previous investigations revealed that when envenomated by *A. portoricensis*, *Anolis* lizards appear to suffer respiratory distress due to hemorrhage in the lungs [33–35]. Hemolytic and proteolytic activities were reported in *A. portoricensis* venom [34], and in a recent characterization of *A. portoricensis* venom, we detected substantial metalloproteinase activity [33]. The presence of potent metalloproteinase(s) could explain documented hemorrhagic effects and suggested that *A. portoricensis* may use venom in much the same way as other venomous snakes such as vipers (facilitation of tissue breakdown of prey). Rear-fanged snake venoms are difficult to obtain and are poorly known, and because these snakes are evolutionarily distinct from and utilize different prey than most vipers, we hypothesized that this venom metalloproteinase could possess novel characteristics. The present study details the isolation and characterization of the major metalloproteinase in the venom using chromatography, mass spectrometry, substrate specificity assays, N-terminal sequencing and structural comparison with other metalloproteinases. This hemorrhagic metalloproteinase, named alsophinase, is the first protein characterized from the venom of *Alsophis*.

2. Materials and methods

2.1. Materials

Sephacel diethylaminoethyl (DEAE) ion exchange medium was obtained from GE Biosciences (Piscataway, NJ, USA). Protein concentration standard reagents were purchased from BioRad Inc. (San Diego, CA, USA). Mark 12 unstained molecular mass standards and pre-cast electrophoretic gels (NuPAGE Novex Bis-Tris and Novex Zymogram) were purchased from Invitrogen, Inc. (San Diego, CA, USA). All other buffers and reagents were of analytical grade and were obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Venom was extracted from *A. portoricensis* and protein content was determined as described previously [33].

2.2. Purification of alsophinase

Purification was achieved using size exclusion high performance liquid chromatography (SE-HPLC) followed by separation on a weak anion exchange (DEAE) column. Crude venom (5 mg per run) was dissolved in 200 μL of 25 mM HEPES buffer, pH 6.8, with 100 mM NaCl and 5.0 mM $CaCl_2$, injected onto a TosoHaas TSK G2000 SWxl size exclusion column (7.8 mm ID × 300 mm, 5.0 μm) and fractionated at a flow rate of 0.3 mL/min using a Waters HPLC system and the same buffer [36]. Fractions were collected at 1 min intervals and absorbance was recorded at 280 and 220 nm using Empower software. Fractions were assayed for proteinase activity using azocasein as a substrate [37]. Fractions from peak 1 contained all proteinase activity and showed multiple bands following SDS-PAGE (data not shown). These fractions (from four separate SE-HPLC runs; total 20 mg crude venom) were combined and dialyzed against 2 × 2 L ddH_2O using 8K MWCO dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA, USA).

The dialyzed sample was then lyophilized and 12.0 mg was dissolved in 2.0 mL of buffer A (10 mM Tris–HCl, pH 8.2) and applied to a 1.5 × 28 cm column of DEAE Sephacel equilibrated with buffer A. Unbound proteins were eluted with buffer A at a flow rate of 0.1 mL/min for 12 h. Bound proteins were eluted with a linear gradient of 0–0.5 M NaCl (buffer B: 10 mM Tris–HCl, pH 8.2 with 0.5 M NaCl) at a flow rate of 0.18 mL/min and 30 min fractions [38]. 20 μL of each of the fractions was assayed for proteinase activity as above. SDS-PAGE was then performed on all fractions to verify purity (alsophinase was located in the first peak). The resulting fractions were desalted using 10K MWCO spin concentrators (Nanosep, Pall Life Sciences, East Hills, NY, USA). Final purification before mass spectrometry and sequencing was achieved using a Phenomenex Jupiter C4 (5 μm, 150 × 4.6 mm) reversed phased HPLC column on a Waters HPLC system using Empower software.

2.3. Reduction/alkylation followed by RP-HPLC

Alsophinase was reduced and alkylated prior to mass spectrometry and N-terminal sequencing. 30 μg of purified alsophinase was dissolved in 250 μL of buffer (0.5 M ammonium bicarbonate with 6.0 M urea) and 200 μL of the same buffer was added in a 1.5 mL microcentrifuge tube. 50 μL of 100 mM dithiothreitol (DTT) was then added to the tube (final concentration 10 mM, 15 mg/mL), overlain with nitrogen, and incubated for 1 h at 50 °C. Next, 50 μL of 500 mM iodoacetamide (IAA) was added to the tube (final concentration of ~50 mM, 90 mg/mL), overlain again with nitrogen, and incubated for 1 h at room temperature. The resulting solution was loaded onto a Phenomenex Jupiter C4 (5 μm, 150 × 4.6 mm) HPLC column equilibrated with buffer A (0.1% TFA in water). A linear gradient was applied from 0 to 100% buffer B (95% acetonitrile, 0.1% TFA) over 30 min on a Waters HPLC system [17]. The absorbance was monitored at 280 and 220 nm, and 1 min fractions were collected. Fractions of interest were pooled, dried on a SpeedVac and used for mass analysis or N-terminal sequencing.

2.4. MALDI-TOF MS

1 μg of reduced and alkylated alsophinase was dissolved in 1.0 μL 50% acetonitrile (50% ACN in 0.1% TFA), mixed with an equal volume of sinapinic acid matrix (10 mg/mL 50% ACN in 0.1% TFA) and analyzed using matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a Bruker Ultraflex spectrometer (Metabolomics and Proteomics Facility, Colorado State University, Fort Collins, CO, USA).

2.5. N-terminal sequencing

Snake venom metalloproteinases are commonly N-terminally blocked with pyroglutamate (e.g., [3,17]), necessitating removal before sequencing. Approximately 10 μg of alsophinase was digested with pyroglutamate aminopeptidase prior to sequencing. Deblocked sample was then subjected to N-terminal sequencing on an ABI Procise protein sequencer (Protein Structure Core Facility, University of Nebraska Medical Center, Omaha, NE, USA). Sequence data generated for alsophinase were compared against the BLASTP database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, 5 December 2011).

2.6. Fibrinogen digest

Fibrinogenase activity was determined using 1.5 μg of purified alsophinase incubated with human fibrinogen (final fibrinogen concentration of 1.0 mg/mL) in a total volume of 200 μL. 20 μL aliquots were removed at 0, 1, 5, 10, 30, and 60 min, combined with 20 μL 5% 2-mercaptoethanol and 4% SDS, and heated at 70 °C for 10 min [39]. 10 μL of the resulting solutions were electrophoresed on a 12% NuPAGE Bis-Tris gel, stained using Coomassie Brilliant Blue R250, and imaged using an Alpha Imager system.

2.7. Gelatinase assay

Endoproteinase activity was assayed using varying quantities of alsophinase (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, and 5.0 μg/lane) on a 10% acrylamide–gelatin Novex zymogram gel [40]. The gel was incubated in renaturing buffer (2.5% Triton X-100 in ddH_2O) at RT for 1 h, transferred to developing buffer and incubated at 37 °C overnight with gentle agitation. The gel was stained with Coomassie, destained and imaged. Areas of gelatinase activity appeared clear on a blue field.

2.8. Oxidized insulin B-chain digest

Activity toward oxidized B-chain of insulin was used to screen for specificity of peptide bond hydrolysis by alsophinase [41]. 5 μg of purified alsophinase was added to bovine insulin B-chain (0.75 mg/mL) in 300 μL buffer (0.1 M HEPES with 0.1 M NaCl, pH 8.0). A control was also run with the same concentration of insulin B-chain and no alsophinase. Tubes were incubated at 37 °C, and 75 μL aliquots of the assay solution were removed after 1, 4, and 24 h to fresh tubes containing 125 μL of 0.1% TFA in water (HPLC buffer A). The resulting solutions (200 μL) were loaded onto a Phenomenex Jupiter C4 (5 μm, 150 × 4.6 mm) reverse-phase column equilibrated with buffer A, and peptides were eluted using a linear gradient of 0–60% buffer B (95% acetonitrile, 0.1% TFA) over 60 min. Peptides were SpeedVac dried and subjected to MALDI-TOF MS. Masses of resulting peptides were used to deduce cleavage sites; cysteine residues of the intact peptide substrate were sulfated, resulting in altered masses of the fragments containing cys residues, and expected masses were corrected for sulfation.

2.9. Proteinase inhibition assays

The effects of a metalloproteinase inhibitor (1,10-phenanthroline) [38] and a serine proteinase inhibitor (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; AEBSF) [42] on alsophinase were evaluated. Assays were conducted in triplicate, with three sets of controls: with and without inhibitor (no alsophinase), and alsophinase alone (no inhibition). A 100 mM stock solution of 1,10-phenanthroline in DMSO was diluted to assay concentrations of 0.001–0.5 mM with buffer (100 mM HEPES with 100 mM NaCl, pH 8.0) in a final volume of 500 μL for each assay tube. 100 mM AEBSF in 70% ethanol was diluted to assay concentrations of 0.1, 0.5, 1.0 and 5.0 mM with the same buffer. 1 μg of alsophinase was added to all assays tubes, incubated for 30 min at RT and then assayed for proteinase activity using azocasein substrate [37]. Percent proteinase activity remaining after incubation with inhibitor was plotted against the concentration of inhibitor used.

2.10. Hemorrhagic activity

Hemorrhagic activity was evaluated using lyophilized crude venom or purified alsophinase dissolved in 25 μL of 0.9% sterile saline and injected intradermally into four week old female non-Swiss albino (NSA) mice [43]. After a 2 h incubation period, the mice were euthanized using CO_2. Skins were then removed and internal surfaces were photographed. Doses were given in duplicate at 0 (control), 0.1, 0.5, 1.0, 5.0, and 10.0 μg alsophinase. Mice used in this assay were bred in the UNC Animal Facility, and all procedures were reviewed and approved by the UNC IACUC (protocol No. 9401).

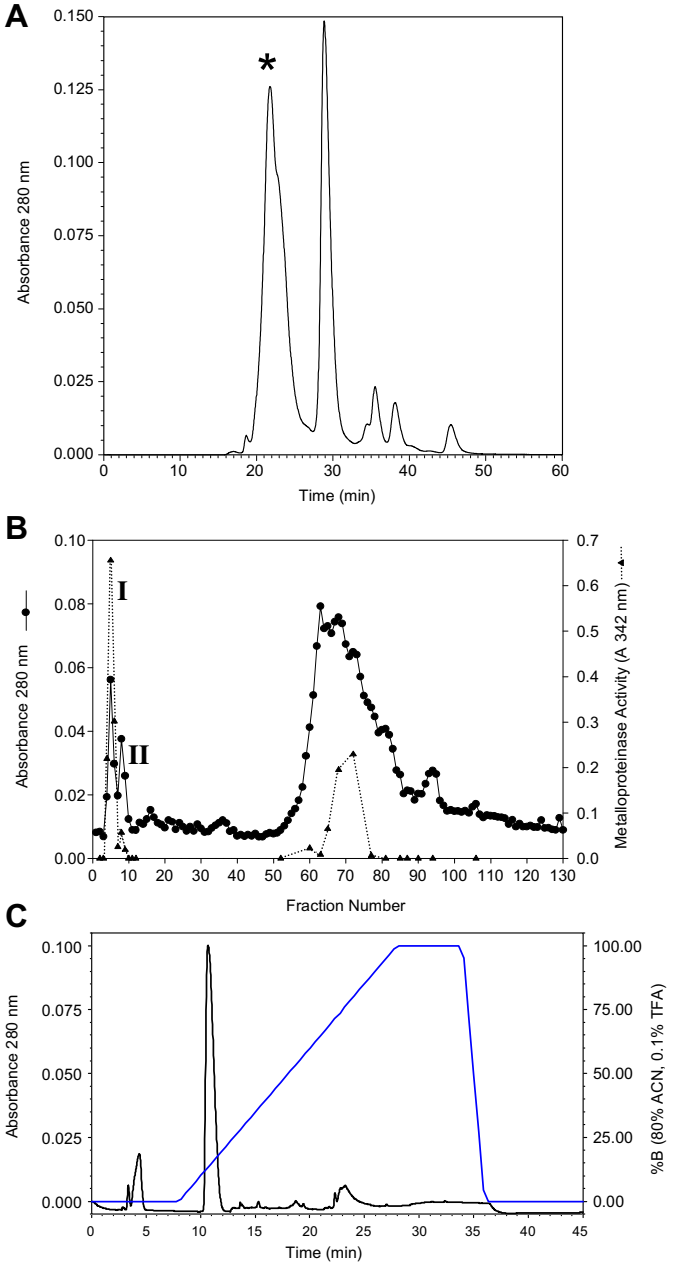

Fig. 1. Purification of alsophinase. A. Size exclusion HPLC of 500 μg *A. portoricensis* venom; flow rate of 0.30 mL/min. The first peak (*) in the chromatogram contains metalloprotease and phosphodiesterase activities, the second large peak is a cysteine-rich secretory protein (CRiSP), and the small peaks further downstream are lower molecular mass toxins. B. Anion exchange FPLC (DEAE Sephacel) fractionation of SE-HPLC peak I (four separate runs combined: 20 mg crude venom total); metalloproteinase activity was located in two peaks, and peak I contained alsophinase. C. RP-HPLC of alsophinase (DEAE peak I).

2.11. Local and systemic damage

To evaluate the local and systemic effects of crude *A. portoricensis* venom and alsophinase, two NSA mice were injected in the right gastrocnemius muscle with purified alsophinase or crude *A. portoricensis* venom (UNC IACUC protocol No. 9401). Doses were 1.0 and 4.0 μg alsophinase and 5.0 and 10.0 μg crude venom, as well as a saline control, all delivered in a 25 μL bolus. After 12 h, mice were euthanized with CO_2, and the lungs and gastrocnemius muscle were removed and preserved in buffered formalin. Tissue samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin/eosin (Colorado HistoPrep, Fort Collins, CO, USA). Light micrographs were then recorded digitally using an Olympus CX41 microscope with a CCD camera, and images were captured using SPOT Digital software (Diagnostics Instruments, Inc.) version 4.0.5. Images were optimized using Adobe Photoshop Creative Suites 2.0 and examined for pathological changes [17].

3. Results

3.1. Purification of alsophinase

Size exclusion HPLC of crude *A. portoricensis* venom yielded five peaks, with proteinase activity confined to peak I (Fig. 1A). The fractions comprising peak I (from four separate SE runs) were combined, dialyzed and lyophilized, dissolved in anion exchange buffer A and loaded onto a DEAE column. When assayed for proteinase activity using azocasein, the first unbound peak showed the highest activity, but the broad bound peak also showed some proteinase activity (Fig. 1B). Peak I fractions (3–6) were pooled and subjected to final purification on a Phenomenex C4 column (Fig. 1C). When electrophoresed on a 12% polyacrylamide gel, the unbound DEAE peak I revealed a single large band at ~57 kDa with a faint band (autolytic degradation product) at approx. 37 kDa (Fig. 2A), whereas the bound peak pool remained quite complex, with several protein bands present. A 30 μg sample of the unbound DEAE peak I was reduced, alkylated, and purified from reduction chemistry via reverse-phase HPLC using a Phenomenex C4 column. Analysis using MALDI-TOF MS revealed that alsophinase has a mass of 56,003.7 kDa (Fig. 2B).

3.2. Protein sequencing

Edman degradation N-terminal sequencing of the reduced and alkylated 56 kDa protein (following deblocking and RP-HPLC) yielded the metalloproteinase domain sequence QDTYLNAKKYIEFYLVVDNGMFxKYSxxFTV. A search of the BLASTP database revealed that this N-terminal region of alsophinase shared 67% identity with a SVMP from another dipsadid rear-fanged snake, *Philodryas olfersii*, as well as lower identity with several other SVMPs (Fig. 3). Comparison with these homologous enzymes showed that the N-terminus of elapid, atractaspidid and "colubrid" metalloproteinases all possess an N-terminal block of pyroglutamate, as is typical of many viperid metalloproteinases.

3.3. Fibrinogen and gelatin digests

Alsophinase (1.5 μg) hydrolyzed the α-subunit of fibrinogen almost instantly, and degradation products were observed at approx. 25, 11, and 10 kDa; the latter two appear to result from secondary cleavage of the 25 kDa band. Prolonged exposure to alsophinase resulted in very slight β-subunit degradation, as evidenced by a decrease in band intensity (Fig. 4). This was also observed in the crude venom assay [33]. There was no apparent gelatinase activity on zymogram gels for alsophinase, as had been observed for the crude venom (data not shown), perhaps due to inhibition of the purified proteinase by SDS.

3.4. Peptide bond specificity of alsophinase

There was nearly complete degradation by alsophinase of the oxidized insulin B-chain by 1 h, whereas no degradation was observed in controls (Fig. 5), even after 24 h at 37 °C. Four peaks, with elution times of 33, 37, 40 and 41 min, were observed after 1 h incubation chromatogram, and this same elution pattern, with no

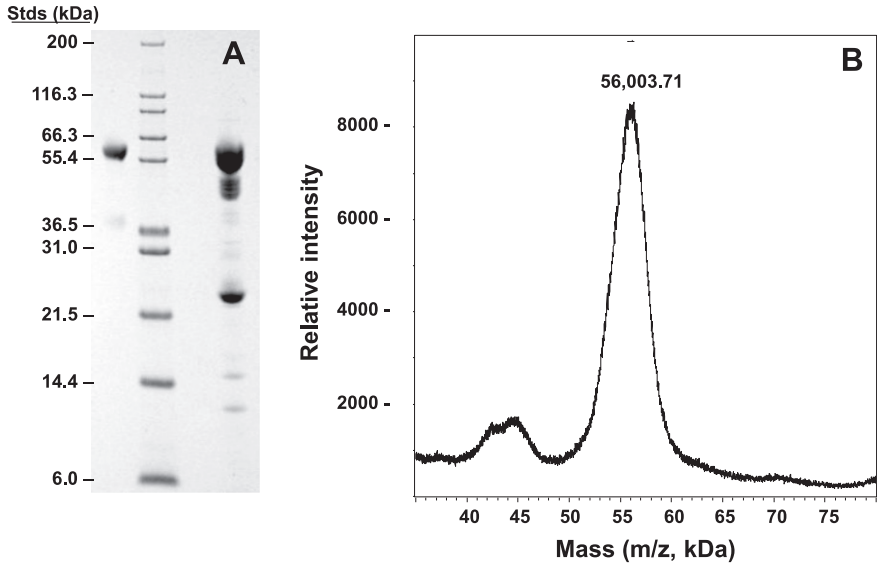

Fig. 2. Purity and mass determination of alsophinase. A. 1D SDS-PAGE of purified alsophinase (left lane) and crude *A. portoricensis* venom (right); molecular mass standards are in center lane. Alsophinase appeared as a single band at ~56 kDa with a faint degradation product band visible at ~37 kDa. B. MALDI-TOF mass spectrogram of alsophinase, 35–80 kDa mass window. Alsophinase appears as a broad peak at 56.003 kDa, suggesting probable glycosylation.

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                                    10          20          30
Alsophinase              1   QDTYLNAKKY IEFYLVVDNG MFxKYSxxFT V     31
Patagonfibrase(fragment)             F-IAVDNG MF
ACS74988.1_PHIOL 194   QNAYLDAPKY IEFFIVVDNG MFRKYSSDLT A    224
ABU68504.1_PHIOL 194   QNAYLDAPKY IEFFIVVDNG MFxKYSSDLT A    224
Q10749.3_NAJMO   196   QDRYLQAKKY IEFYVVVDNV MYRKYTGKLH V    226
ABQ01135.1_HOPST 196   QDSYLQVKKY IEFYVVVDNR MYKKYNSSRD A    226
AAF01041.1_ATRMI 190   QDRYLHSKKY IEFVVVVDNR MFRKYSNNSS T    220
AAF01039.1_ATRMI  15   QDSYLKAPKY IEFVVVVDNR MFRKYSNDSN A     45
ABG26978.1_SIST  191   QQAYLDAKKY VEFVVVLDHG MYTKYKDDLD K    221
                       *  **   **  **    *   *  **
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Fig. 3. N-terminal sequence alignment (CLUSTALW 2.0.8) of alsophinase with selected venom metalloproteinases. Pfb is an N-terminally-located fragment of patagonfibrase from *Philodryas patagoniensis* venom; ACS74988.1 and ABU68504.1 (GenBank accession numbers) are fragments Phi2 and Phi1, respectively, of a SVMP from *P. olfersii* (Green Snake); Q10749.3 is the zinc metalloproteinase precursor (mocarhagin) from *Naja mossambica* (Mozambique Spitting Cobra); ABQ01135.1 is Stephensase-1, a SVMP from *Hoplocephalus stephensii* (Stephen's Banded Snake); AAF01041.1 and AAF01039.1 are metalloproteinase precursors from *Atractaspis microlepidota andersoni* (Small-scaled Burrowing Asp); ABG26978.1 is metalloproteinase isoform 1 from *Sistrurus catenatus edwardsii* (Desert Massasauga). Residue number (alsophinase) is given above aligned sequences, numbers preceding and following sequences indicate positions in published sequences, and invariant residues are indicated by an asterisk.

additional peaks, was also observed after 4 or 24 h incubation (data not shown). The fractions comprising these peaks (1 h incubation) were SpeedVac dried and analyzed via MALDI-TOF MS. The observed molecular masses, the corrected expected masses, and the corresponding sequences of the fragments (as well as the intact peptide) are given in Table 1. The most labile bond was Ala14–Leu15, while the Tyr16–Leu17 bond was hydrolyzed at a much lower frequency under these assay conditions.

3.5. Inhibition assays

When alsophinase was incubated with the metalloproteinase inhibitor 1,10-phenanthroline, complete loss of proteinase activity was observed at concentrations as low as 50 μM 1,10-phenanthroline (Fig. 6A); the concentration at which 50% metalloproteinase activity was inhibited (IC_{50}) was approximately 15.2 μM. There appeared to be only minor inhibitory effects (at 1.0 and 5.0 mM) of the serine protease inhibitor AEBSF (Fig. 6B), as ~62% activity remained at both of these concentrations.

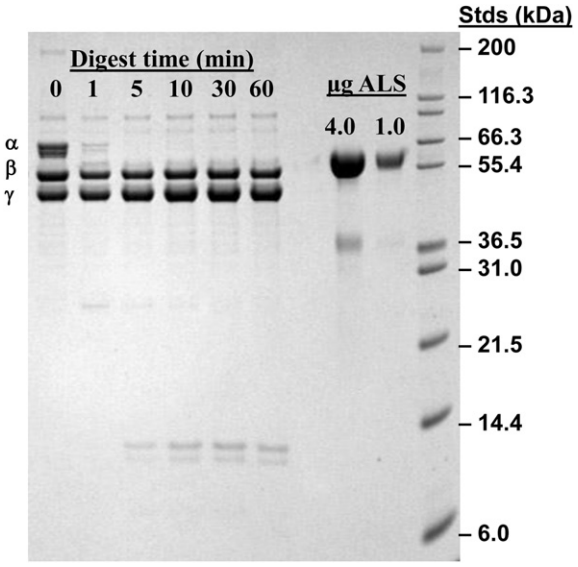

Fig. 4. Degradation of fibrinogen by alsophinase. 1.5 μg alsophinase was incubated for 0, 1, 5, 10, 30, and 60 min (lanes 1–6, left to right) with human fibrinogen. Note the rapid loss of the α-subunit, and the appearance of degradation products at 25, 11, and 10 kDa. 4.0 and 1.0 μg of purified alsophinase (ALS) are included for comparison (lanes 8 and 9), and molecular mass standards are in far right lane.

3.6. Hemorrhagic activity

Hemorrhagic activity of alsophinase was evaluated using the mouse dermal assay (Fig. 7), and purified toxin effects were compared with those of saline control (Fig. 7A) and 5.0 μg crude venom (Fig. 7B). A small hemorrhagic spot was visible at the 0.1 μg dose (Fig. 7C), and more profound hemorrhages were present at the 5 and 10 μg doses (Fig. 7D) of alsophinase. The 10 μg alsophinase

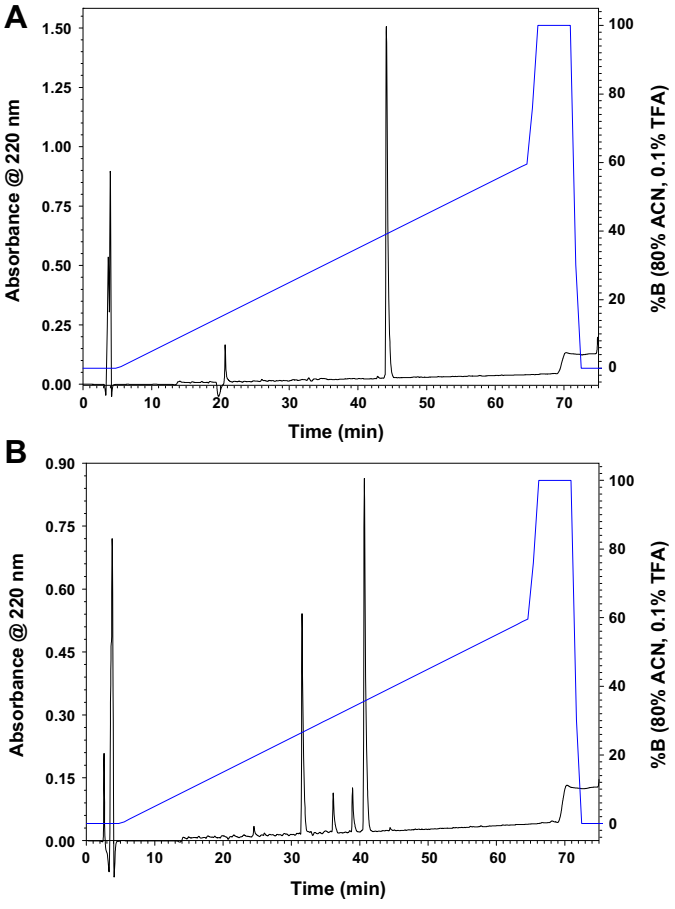

Fig. 5. Oxidized insulin B-chain digest by alsophinase following 1 h incubation at 37 °C. Control (A) and with alsophinase (B); 4 and 24 h digests showed an identical pattern. Masses of digest peaks in B were determined by MALDI-TOF-MS. Solvent A: 0.1% TFA in water; flow rate 1 mL/min.

Table 1
Masses and sequences of oxidized bovine insulin B-chain fragments following 1 h digestion with alsophinase.

HPLC fraction number	Observed value	Expected value		
	Experimental mass (Da)	Sulfated peptide mass (Da) = M + 48 (per C)	Unmodified peptide mass (Da)	Sequence
33	1601.80	1601.76	1553.76	FVNQHLCGSHLVEA
37	1635.73	1635.86	1587.86	LVCGERGFFYTPKA
40	1877.94	1878.09	1830.09	FVNQHLCGSHLVEALY
41	1912.07	1912.19	1864.19	LYLVCGERGFFYTPKA
45	3494.86	3495.91	3399.93	FVNQHLCGSHLVEALYLVCGERGFFYTPKA (Intact peptide)

injection also induced intramuscular hemorrhage, as evidenced by deep subcutaneous discoloration on the mouse intercostal muscles (not shown), and this extradermal effect may have contributed to the lower apparent dermal effects of the purified toxin.

3.7. Local and systemic damage

When crude *A. portoricensis* venom or purified alsophinase was injected intramuscularly into the gastrocnemius muscle of mice, hemorrhage and necrosis at the injection site were observed after 12 h for all dosages. Systemic damage was incurred in both the crude venom and alsophinase experimental groups, as evidenced by erythrocytic extravasation in the alveolar spaces and congestion of blood vessels in the lung tissue (Fig. 8A–C). Hematoxylin and eosin-stained muscle tissue samples reflected this observation; extravasation of erythrocytes and the presence of polymorphonuclear leukocyte infiltrate, which characterizes an inflammatory reaction, were seen in sectioned muscle (Fig. 8D–F).

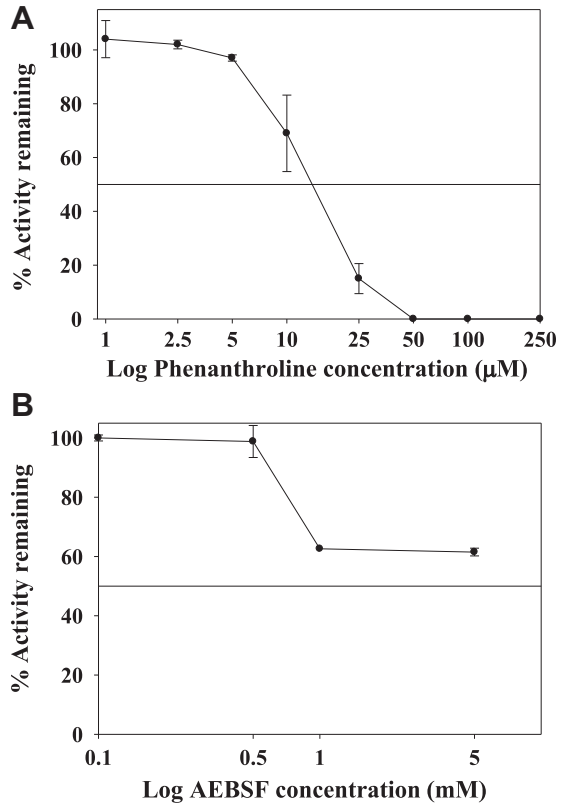

Fig. 6. Effects of proteinase inhibitors on alsophinase caseinolytic activity. A. Effect of 1,10-phenanthroline, a metalloproteinase inhibitor. Metalloproteinase activity was completely inhibited at 50 μM 1,10-phenanthroline and the IC_{50} was approximately 15.2 μM. B. Effect of AEBSF, a serine proteinase inhibitor. Partial inhibition was observed at concentrations 100-fold higher than for 1,10-phenanthroline, and inhibitory effect appears to plateau at concentrations of 1.0 mM or higher. Mean ± 1 SD; horizontal line indicates 50% activity.

4. Discussion

In this study, we purified and characterized the major metalloproteinase in *A. portoricensis* venom through size exclusion and ion exchange chromatography. Named alsophinase for *A. portoricensis* metalloproteinase, it is the first protein purified and characterized from the venom of a snake in the genus *Alsophis* and the first report of N-terminal sequence data for a metalloproteinase purified from a dipsadid snake venom.

A single band was present at ~57 kDa under non-reducing and reducing SDS-PAGE, demonstrating that alsophinase is a single polypeptide chain protein. This approximate molecular mass was confirmed to be 56.003 kDa upon MALDI-TOF MS analysis, similar to a 58 kDa metalloproteinase PofibH from the dipsadid snake *P. olfersii* [12] and to the 53 kDa metalloproteinase, patagonfibrase, from the dipsadid snake *Philodryas patagoniensis* [17]. Both PofibH and patagonfibrase have acidic isoelectric points (4.6 and 5.8, respectively), and other proteins in the 50–60 kDa range of *Alsophis* venom (some likely metalloproteinase isoforms) were also acidic; based on ion exchange chromatographic behavior, however, alsophinase is somewhat basic. The N-terminal sequence of alsophinase metalloproteinase domain (QDTYLNAKKYIEFYLVVDNGMFxKYSxxFTV) shares 67% identity with the deduced sequence of an SVMP from *P. olfersii*. This relatively low sequence identity with a closely related proteinase is likely due to the generally higher variability in the N-terminal domains of SVMPs and may not reflect total sequence homology. SVMP sequences are generally highly conserved [3,44], and with complete sequencing, it is probable that sequence identity with other known SVMPs will increase.

Alsophinase showed high activity toward azocasein and was strongly inhibited by micromolar concentrations of the metal chelator 1,10-phenanthroline, demonstrating that it is a metalloproteinase. Serine proteinases, derived from regulatory enzymes involved in maintaining vertebrate hemostasis, are commonly found in viperid venoms [45–47], and some have also been identified in dipsadid venoms (PofibS, from *P. olfersii* venom [12]). The partial inhibitory effect of AEBSF suggests that a serine residue may be important for activity, but it is unlikely involved in the catalytic site, as high concentrations (millimolar) of AEBSF failed to abolish proteinase activity. The complete degradation of the α-subunit of human fibrinogen is also consistent with the observation that alsophinase is an α-fibrinogenase SVMP [48]. The exceptionally rapid hydrolysis of the Aα-chain is an important defining character, as other α-fibrinogenases, such as jararhagin (from the viper *Bothrops jararaca* venom), PofibH and patagonfibrase also exhibit this behavior [12,17,49]. A report by Mazzi et al. [50] described the eventual degradation of the Bβ-chain of fibrinogen by BjussuMP-I from *Bothrops jararacussu* venom after an incubation period of

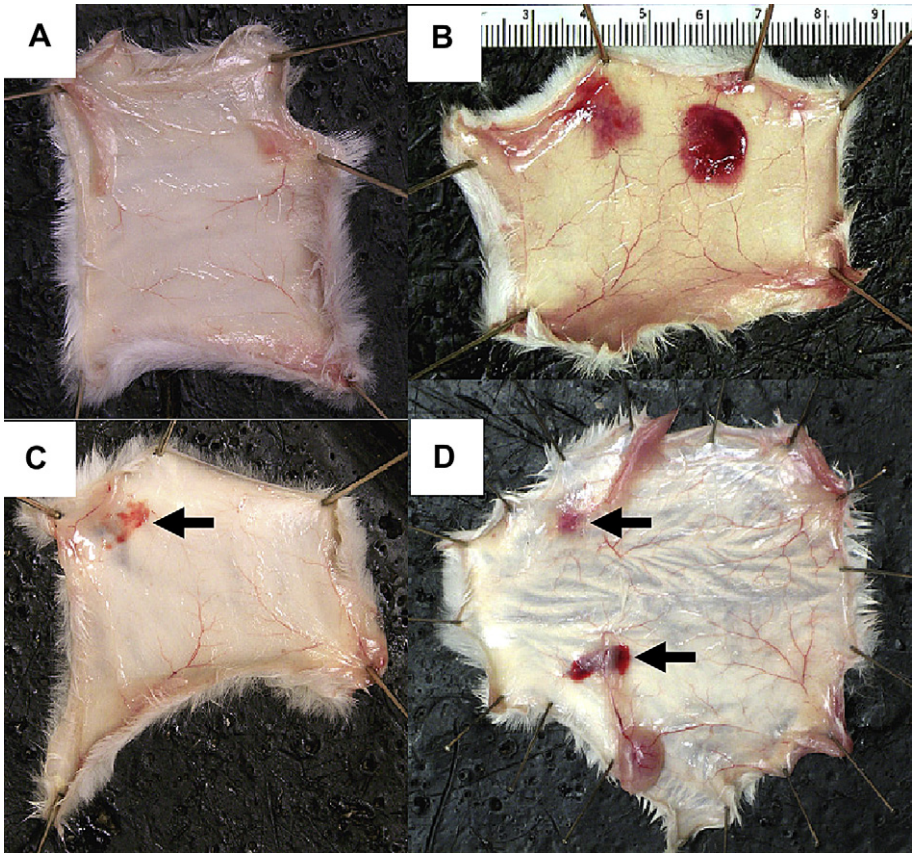

Fig. 7. Hemorrhagic assay – inner surfaces of mouse skins are shown. A. 0.9% saline control; B. 5.0 μg crude venom; C. 0.1 μg alsophinase (arrow); D. 5.0 μg (top arrow) and 10 μg (bottom arrow) alsophinase. Metric rule (mm) is at top right.

6 h, but very minimal loss of the Bβ-chain was observed following incubation with alsophinase. Although the crude venom showed substantial gelatinase activity on zymogram gels, alsophinase did not. However, it is possible that in the presence of SDS, purified alsophinase was irreversibly inactivated, as has been observed with some colubrid metalloproteinases [36].

Snake venom metalloproteinases typically display hydrolytic activity toward the B-chain of insulin, and upon incubation, they commonly cleave the 30 residue peptide at Ala14–Leu15, Tyr16–Leu17, His10–Leu11, Leu15–Tyr16, and Phe24–Phe25 [51,52]. The oxidized B-chain of insulin was cleaved by alsophinase predominantly between Ala14 and Leu15, resulting in two fragments with masses of 1912.1 Da and 1601.8 Da; this cleavage site is common to many SVMPs [51,52]. There was also minor cleavage at Tyr16–Leu17, which yielded two additional fragments of 1877.9 Da and 1635.7 Da. Although this second cleavage site was observed, it appeared to be considerably less prominent, as evidenced by the much smaller degradation peaks (approximately 5:1 ratio). This cleavage pattern was seen for PoFibH, which also showed three additional cleavage sites [12]. However, unlike many other SVMPs, alsophinase cleaves the oxidized B-chain of insulin primarily at the Ala14–Leu15 bond, minimally at the Tyr16–Leu17 bond, and no other cleavage products were detected. Alsophinase catalytic activity toward the oxidized B-chain of insulin therefore appears to be more specific than most other SVMPs characterized to date.

Upon envenomation of prey by viperid snakes, hemorrhage and necrosis are evident at the site of the bite (localized), and systemic damage is often sustained to vital organs as well. This has also been noted in envenomations by several colubrid snake species, including *A. portoricensis* [33,35]. Patagonfibrase, a metalloproteinase purified from *P. patagoniensis* venom, was also shown to have hemorrhagic and necrotic effects [17]. Both crude *A. portoricensis* venom and alsophinase caused substantial morphological changes in mouse skin, gastrocnemius muscle and lung tissue. Effects are comparable to those shown in studies of *P. patagoniensis* crude venom and patagonfibrase, where interfibrillar hemorrhage and edema, polymorphonuclear neutrophil inflammatory infiltrate and necrosis were observed [17,53]. *P. olfersii* venom induced similar necrotic effects in mouse skeletal muscle due to local ischemia resulting from the action of hemorrhagic and edematogenic toxins and of proteolytic enzymes [54]. As in our study, these changes were detected in histological sections through the accumulation of fluid, erythrocytes and leukocytes in the damaged tissue. The SVMP with the greatest sequence identity to alsophinase, PoFibH, was also shown to have high hemorrhagic activity [12]. The pathology of systemic hemorrhaging in the lungs of experimental animals was of particular interest, as this was a sign that had been noted in previous studies [34] as well as in our study with crude *A. portoricensis* venom [33]. These signs are not limited to colubrid venoms, as jararhagin was also found to induce systemic bleeding in the lungs [55].

Based on the molecular mass, hemorrhagic activity, and sequence similarity to other class P-III SVMPs, we hypothesize that alsophinase belongs to class P-III SVMPs, which contain proteinase, disintegrin-like and cysteine-rich domains [8]. The venom gland transcriptome of *P. olfersii* revealed that class P-III SVMPs are major components of this species' venom, and they were hypothesized to be the main cause of observed toxic effects [56]. *P. olfersii* has a venom similar in composition to that of *Alsophis*, so it is likely that

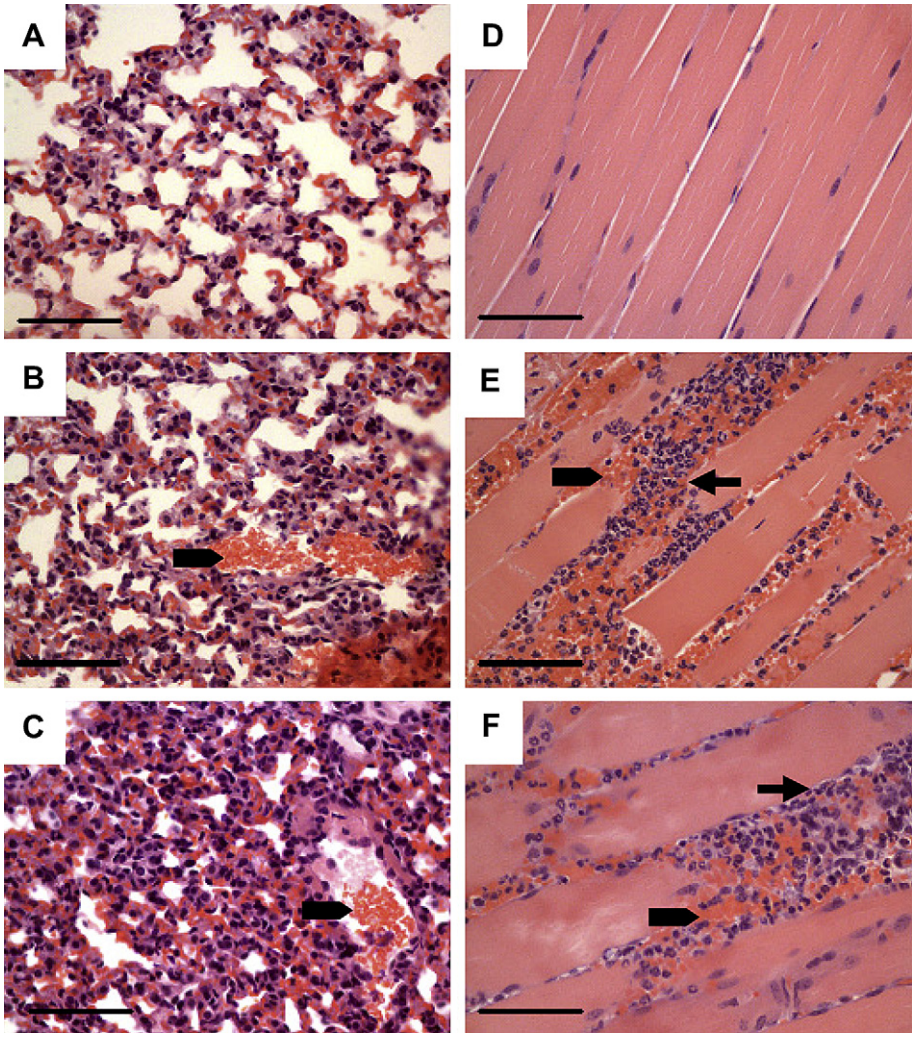

Fig. 8. Histopathology of mouse lung and muscle tissue following treatment with *A. portoricensis* crude venom or purified alsophinase. (A) and (D) show control lung and gastrocnemius muscle, respectively. (B) lung tissue, 10 μg venom, (E) muscle tissue, 5 μg venom, (C) and (F) are lung and muscle tissue, respectively, from mouse treated with 4 μg alsophinase. Erythrocytic extravasation is apparent in both tissues treated with either venom or alsophinase (arrowheads), and necrosis with infiltration of polymorphonucleocytes and monocytes is seen in treated muscle tissue (arrows). Scale bars are 50 μm.

alsophinase is a major contributor to the hemorrhage observed in human bites and prey envenomations by *A. portoricensis*.

The reactivity of class P-III SVMPs can cause them to undergo autolysis under non-physiological conditions [3,57]. When purified alsophinase was frozen and thawed repeatedly, a lower molecular weight band of ~37 kDa was present on SDS-PAGE gels (see Fig. 2). In patagonfibrase, this autolytic cleavage occurs precisely at the start of the disintegrin domain, producing a fragment of approximately 32.6 kDa [57]. We did not sequence this fragment in alsophinase, but because it is similar in sequence and mass to patagonfibrase and because alsophinase appears to show preference for a leucine residue in the P1 substrate site (also found at the start of the disintegrin domain), we speculate that autolytic degradation of dipsadid metalloproteinases shows a similar pattern.

Alsophis venom has potent hemorrhagic activity and degraded fibrinogen, which *in vivo* should promote bleeding in prey animals. Alsophinase alone produces hemorrhage and necrosis in skin, muscle and lung tissue, and we believe that it is a major contributor to the damaging effects of the whole venom. However, there are additional metalloproteinases and likely a number of other enzymes and toxins present in the venom that could damage structural proteins in prey tissue, leading to "predigestion" of prey, and alsophinase is only one of these. Similar mechanisms of tissue degradation and necrosis can be seen in all families of venomous snakes, and the biological roles of venom in prey handling and digestion appear to be universal [36,58]. A transcriptomic study of the *A. portoricensis* venom gland will complement the functional and proteomic data presented here and would lead to a more complete understanding of how *A. portoricensis* venom components vary phylogenetically. Finally, alsophinase can serve as an important model in future structure/function studies of rear-fanged snake venom proteins, and its apparent specificity of peptide cleavage may make it useful as a biochemical tool.

5. Conclusions

We report the purification and characterization of a new metalloproteinase, alsophinase, from the venom of a rear-fanged snake (*A. portoricensis*). It is a P-III metalloproteinase possessing N-terminal sequence homology with multi-domain metalloproteinases from other snake venoms, and it hydrolyzed the oxidized B chain of insulin (a model substrate) at Ala14–Leu15 and the Aα subunit of human fibrinogen. *In vivo*, it is responsible for

hemorrhage and tissue necrosis which develops following envenomations. Apparent peptide bond specificity suggests that it may have applications for limited cleavage of proteins, such as generation of peptide fragments for mass spectrometric characterization. Results demonstrate that rear-fanged snakes (such as *Alsophis*) represent an under-utilized source of new enzyme activities with conserved structural motifs.

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