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CHARACTERIZATION OF THE MAJOR METALLOPROTEASE ISOLATED FROM THE VENOM OF THE NORTHERN PACIFIC RATTLESNAKE

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S. P. Mackessy. Characterization of the major metalloprotease isolated from the venom of the northern Pacific rattlesnake, *Crotalus viridis oreganus*. *Toxicon* **34,** 1277–1285, 1996.—Rattlesnake venoms typically contain several different metalloproteases, some of which are hemorrhagic toxins. Metalloproteases contribute significantly to the often severe necrotic changes in tissues following envenomation, and these prominent components are important to the predigestive role of venoms. Venom of the northern Pacific rattlesnake (*Crotalus viridis oreganus*) contains at least five distinct metalloproteases, and the dominant protease (trivial name, CVO protease V) has been isolated and characterized as being a single polypeptide chain acidic protein with a molecular mass of 61 kDa and a pH optimum of ~ 9.0. It catalyzes the hydrolysis of several protein substrates, including casein, and is inhibited by metal chelators such as EDTA, EGTA and 1,10-phenanthroline but not by serine protease inhibitors such as PMSF. Calcium is present at a molar ratio of approximately 1:1, but, unlike other described venom metalloproteases, this protease does not appear to contain zinc. Caseinolytic activity is not significantly inhibited by citrate (at pH 9.0) at levels up to 2.0 mM; at 100 mM citrate (at pH 9.0) more than 65% of activity is retained. It is partially inhibited by nanomolar concentrations of ATP, but higher amounts (micromolar) do not result in further inhibition of activity. The protease shows fibrinolytic and fibrinogenolytic activity, but is only weakly hemorrhagic in rats. When stored in solution for long periods it undergoes autolytic degradation. This protease [redacted] but is not present in venoms from juvenile *C. v. oreganus*. The presence of this component in venoms from adult Pacific rattlesnakes is responsible for the age-related increase in metalloprotease activity of the crude venom.

INTRODUCTION

Metalloproteases are one of several classes of proteolytic enzymes (see, e.g., Neurath, 1989) which are produced by a variety of bacteria (Jiang and Bond, 1992) and venomous

snakes (Bjarnason and Fox, 1995; Kini and Evans, 1992; Bjarnason and Tu, 1978). In addition, metalloproteases are endogenous components of many tissues and are intimately involved in metastasis (see Vassalli and Pepper, 1994). Venoms from crotalid snakes are generally characterized by a high level of metalloprotease activity toward synthetic substrates and native substrates such as basement membrane proteins (Shannon *et al.*, 1989), and venom from a single species may contain several enzymes of distinctly different molecular masses with seemingly similar activities for example (see, e.g., Bjarnason and Tu, 1978; Mackessy, 1993; Fox and Bjarnason, 1995). Several of these appear to be absent from venoms of juvenile snakes, and overall caseinolytic protease activity of venoms from juveniles is approximately 5-fold lower than venoms from adult Pacific rattlesnakes (Mackessy, 1988).

In the present study, the isolation and characterization of the major protease from Pacific rattlesnake venom is discussed. Metalloproteases are abundant in crotalid venoms, and they are important to the biological roles of venoms as they apply to the snakes. In addition, they may represent a model class of metalloproteases well-suited for the study of tissue invasion, hemorrhage, necrosis and perhaps apoptosis. Many venom proteases have been shown to be zinc-dependent metalloproteases (Bjarnason and Fox, 1989), most are also hemorrhagic toxins and several have been sequenced (e.g. Hite *et al.*, 1992; Takeya *et al.*, 1990). Crotalid venoms thus represent a rich source of these enzymes, and their prevalence has made venoms an important resource for the study of metalloprotease structure and function.

MATERIALS AND METHODS

Reagents and venoms

Ion-exchange media were obtained from Pharmacia. Casein yellow was purchased from CalBioChem. Acrylamide gels (14%) were obtained from Novel Experimental. All other biochemicals were obtained from Sigma Chemical Corp. (St Louis, MO, U.S.A.). Using standard methods, venoms were extracted from adult snakes (> 700 mm total length) collected in San Luis Obispo (CA, U.S.A.). Venoms were then lyophilized and stored frozen with desiccant until used.

Isolation of the major metalloprotease

Crude venom (235 mg, from three snakes) was dissolved in 3.0 ml 10 mM Tris–HCl pH 8.2, centrifuged for 5 min to pellet solids and applied to a 1.5 × 28 cm column of DEAE–Sephadex A-50 equilibrated with the same buffer. Adsorbed proteins were eluted with an increasing linear NaCl gradient (0–0.5 M NaCl, 400 ml of each buffer). The major metalloprotease eluted in the last peak and was > 90% homogeneous. Fractions were combined, dialyzed and lyophilized, and this material was redissolved in 10 mM HEPES (pH 6.8, containing 60 mM NaCl) and chromatographed on a 1.5 × 75 cm column of BioGel P-100. Fractions from the single major peak were combined, dialyzed and lyophilized. The material obtained from this step (CVO protease V) was homogeneous, as judged by SDS–PAGE.

Protease assays

activity in the presence of $ZnCl_2$ (10–500 μM) was also evaluated.

Inhibitor assays

Inhibition of CVO protease V by EDTA, EGTA and 1,10-phenanthroline was assayed as described previously (Mackessy, 1993) with a slight modification. In preliminary studies, DMSO was found to inhibit protease activity in concentrations of greater than 5 μl/ml. Therefore, a 1.0 M solution of 1,10-phenanthroline in DMSO was diluted to 10 mM in buffer (100 mM HEPES, pH 8.0, with 100 mM NaCl) and used at the appropriate concentration for assays. The effects of citrate, ATP and ADP on caseinolytic activity were assayed by placing

the appropriate concentration of potential inhibitor in a test tube containing 10 μg CVO protease V in 0.5 ml 100 mM CHES buffer, pH 9.0, containing 100 mM NaCl, vortexing and incubating at RT (25°C) for 15 min. Casein yellow substrate (0.5 ml, 12 mg/ml in same buffer) was added and activity was assayed as above. Residual activity (compared with controls) was expressed as per cent activity remaining.

Determination of fibrinogenase activity

Determination of purity, molecular mass and pH optimum

as above.

Metal analysis

CVO protease V was analysed for zinc, magnesium and calcium by atomic absorption spectrophotometry. Samples were dissolved in Millipore-filtered water, and parallel water controls were also analysed for metal content.

N-*terminal sequence*

N-terminal sequence was attempted on intact CVO protease V after N-ethylpyridylation (Utaisincharoen *et al.*, 1993). Automated Edman degradation was performed using an ABI 473A Sequencer (Macromolecular Resources, Colorado State University).

Hemorrhagic activity

Hemorrhagic activity toward Sprague–Dawley (SD) rats was evaluated following injection of 10 μg/100 μl saline of CVO protease V (essentially the method of Biarnason and Fox, 1983). After 24 hr, animals were killed by CO_2 asphyxiation and skinned, and the visceral side of the skin was inspected for hemorrhage.

RESULTS

Ion-exchange chromatography of crude venom produced 11 discrete protein/peptide peaks, five of which showed activity toward casein yellow (Fig. 1). The highest specific activity resided in the last peak, and this material was then subjected to gel filtration (Fig. 2). Two peaks resulted, and CVO protease V was contained in the first and major peak. This material appeared to be homogeneous as demonstrated by SDS–PAGE and was used in all subsequent analyses. The protein migrated as a single band in the presence or absence of 2-mercaptoethanol, indicating that it consisted of a single polypeptide chain. The relative molecular mass of CVO protease V was 61 kDa. From its binding to the DEAE–Sephadex column it was apparent that the protein was acidic (p*I* not determined).

CVO protease V had a specific activity toward casein yellow of 0.68 $AU_{285\ nm}$/min/mg protein. The pH optimum for the purified enzyme was between 8.0 and 9.5 (Fig. 3); for

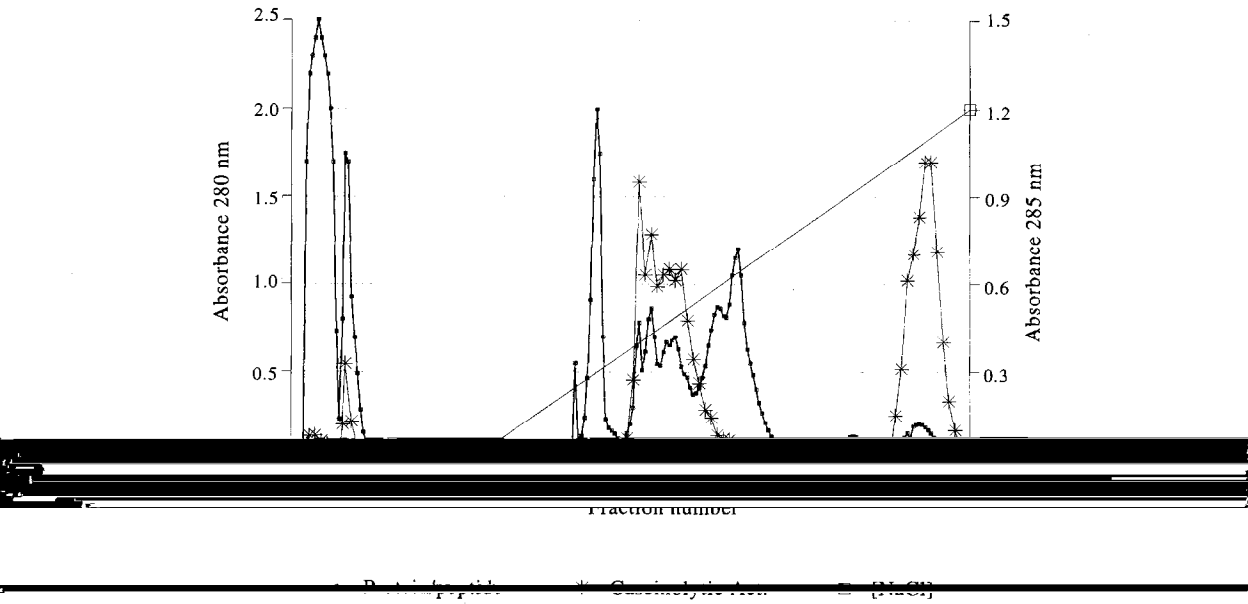

Fig. 1. DEAE-Sephadex ion-exchange fractionation of crude venom from adult *Crotalus viridis oreganus*.
Venom (235 mg) was applied to the column; the salt gradient (0–0.5 M) was initiated at fraction 60. CVO protease V eluted as the last peak.

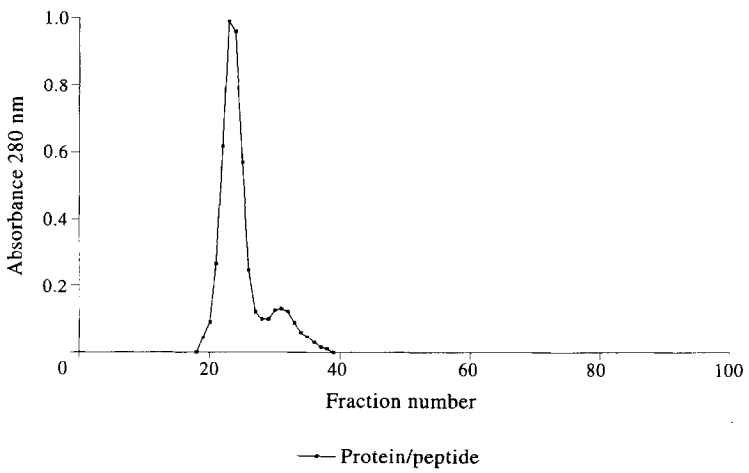

Fig. 2. Gel filtration of the combined material from the DEAE column.
CVO protease eluted as a symmetric peak; the shoulder represents autolytic degradation products.

the crude venom, the pH optimum toward casein yellow was approximately 8.0. Hide powder azure is also readily hydrolyzed by this protease. No activity was shown toward serine protease substrates, including BzPheValArg-*p*-nitroaniline or BzProPheArg-*p*-nitroaniline. Caseinolytic activity was strongly inhibited by metal ion chelators such as

1,10-phenanthroline, EDTA (Fig. 4) and EGTA; the IC_{50} for EDTA was 43 μM, and for 1,10-phenanthroline it was 35 μM (CVO protease V, 10 μg/ml). CVO protease V is therefore a metalloprotease. A slight effect of ATP and ADP on caseinolytic activity was observed; at micromolar concentrations, ATP induced an apparent decrease in activity of $\sim$ 20%, but above 100 μM no further decrease in activity was noted. Citrate also has little or no effect on caseinolytic activity at pH 9.0 (Fig. 5); at 2.0 mM citrate, more than 90%

(< 10% higher) activity, and at higher concentrations (100–500 μM) zinc was slightly inhibitory.

Bovine and human fibrinogen was readily hydrolyzed by the purified enzyme, and a

hydrolyzed (data not shown).

Metal analysis revealed that calcium was present at a ratio of 0.9 mole of calcium per

$\sim$ 0.02 mole/mole protease). Water controls were negative (below detection limit) for all metals tested.

N-terminal sequencing attempts were unsuccessful, suggesting that the enzyme was N-terminally blocked. CVO protease V showed little hemorrhagic activity in rats, and at 10 μg protease, a weak, diffuse subdermal spot, < 5 mm in diameter was produced. The

DISCUSSION

The major metalloprotease found in the venom of adult Northern Pacific rattlesnakes (designated CVO protease V) has been isolated. Previously, it has been shown that this protease is apparently lacking in the venoms of neonate and juvenile Pacific rattlesnakes

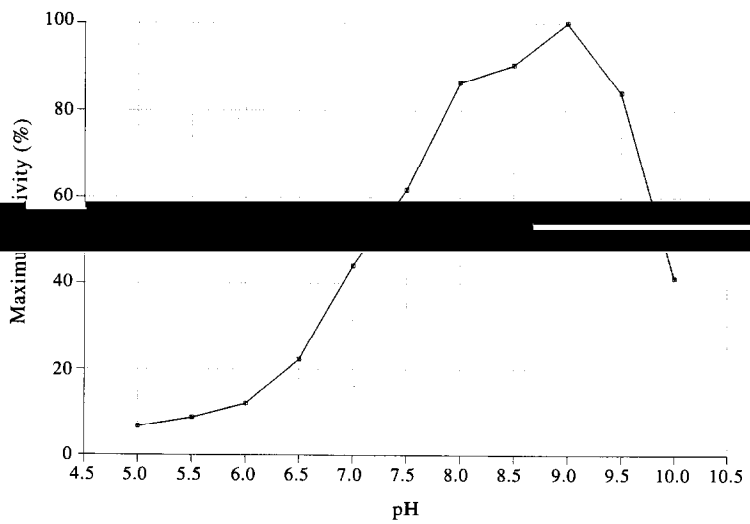

Fig. 3. Optimum pH of CVO protease V toward casein yellow.

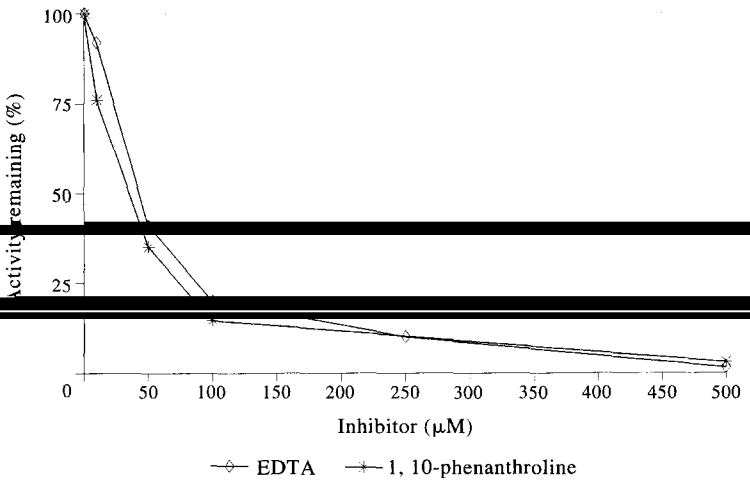

Fig. 4. Effect of EDTA and 1,10-phenanthroline on caseinolytic protease activity of CVO protease V.
Activity is abolished above 500 μM EDTA or 1,10-phenanthroline. Protease concentration, 10 μg/ml.

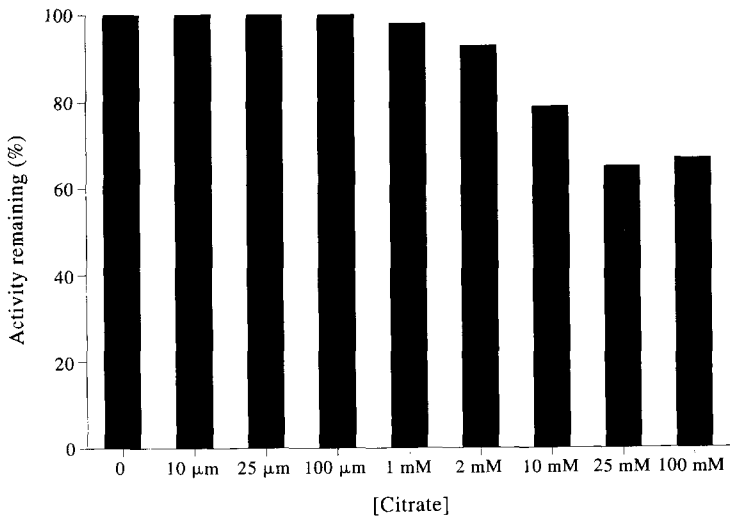

Fig. 5. Effect of citrate on caseinolytic activity of CVO protease V.
Note that more than 65% of activity is retained at citrate concentrations of up to 100 mM. Protease concentration, 10 μg/ml.

(Mackessy, 1993), and this protease was further characterized in an attempt to clarify the biological role of this venom component. It is an acidic single polypeptide with a molecular mass of approximately 61 kDa, and the presence of this protease in the venom of adult snakes greatly contributes to the 5-fold higher (caseinolytic) protease activity of adult

Table 1. Summary of characteristics of CVO protease V

[redacted]	[redacted]
	Crude venom: 7.8 (~ 8.0)
Metal content	0.9 mole calcium/mole protease
Subunits	Single polypeptide chain, acidic
Inhibitors	EDTA (IC_{50} = 35 μM)
	1,10-phenanthroline (IC_{50} = 35 μM)
	Also: EGTA, ATP (weak), ADP (weak), citrate (weak)
Other characteristics	Substrates include fibrinogen, fibrin, casein, hide powder
	N-terminally blocked (pGlu?)
	Weakly hemorrhagic in SD rats

venoms. Automated Edman degradation sequencing of the ethylpyridylated enzyme was blocked, indicating an N-terminal modification; based on the sequences of other crotalid metalloproteases (see, for example, Takeya *et al.*, 1990), this residue is likely to be pyroglutamate. At present it is not known whether this protease represents a secondarily processed venom component (venom from juvenile *C. v. oreganus* contains a 100 kDa metalloprotease) or if it is a novel enzyme whose expression is age dependent. Since the [redacted]

A sensitivity to metal chelators (EDTA, EGTA and 1,10-phenanthroline) confirmed the identification of this enzyme as a metalloprotease. The nucleotides ATP and ADP, weak inhibitors of activity, may act via partial competition for metal ion (I. Kaiser, personal communication). Citrate, an endogenous component of venoms from several sources and inhibitor of other venom enzyme components (Fenton *et al.*, 1995; Francis *et al.*, 1992), does not result in significant inhibition of activity at pH 9.0 until concentrations reach the high millimolar range. It is therefore unlikely that citrate has an important role in the [redacted] inhibition may occur at lower pH (not evaluated) and contribute to inactivation of the enzyme until it is introduced into prey tissues.

CVO protease V appears to differ from most crotalid metalloproteases in several respects. Although it is inhibited by metal chelators such as EDTA, EGTA and [redacted] calcium seems to be the only metal ion present. It is extremely unlikely that the lack of detectable zinc has resulted from dietary deficiencies, since these venoms were extracted from healthy snakes caught in the wild. Also, this protease is only weakly hemorrhagic, whereas most venom-derived zinc metalloproteases are strongly hemorrhagic. Judging from its molecular mass (~ 61 kDa), it may share similarities with other high molecular [redacted] 1990) and hemorrhagic toxin a (from *Crotalus atrox* venom; Bjarnason and Tu, 1978). [redacted] found in the venom of adult northern Pacific rattlesnakes (*Crotalus viridis oreganus*). Along with these other proteases, protease V is responsible for the tissue degradation and necrosis that occurs upon envenomation of prey, and it likely contributes to the localized tissue damage frequently seen in human envenomations. Ontogenetic changes in venom composition are pronounced in this species, and CVO protease V plays a major role in

[redacted]

[redacted]

[redacted]

shed light on the mechanism of production of increased protease activity of adult venoms and on the relation of the larger metalloproteases to smaller enzymes present in the same venom.

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