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CHARACTERIZATION OF THE MAJOR METALLOPROTEASE ISOLATED FROM THE VENOM OF THE NORTHERN PACIFIC RATTLESNAKE, *CROTALUS VIRIDIS OREGANUS*

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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 or a homolog appears to be present in venoms from several rattlesnake species 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 toward fibrinogen and casein was assayed as described previously (Mackessy, 1993). Caseinolytic 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

Activity toward human and bovine fibrinogen was assayed using the method of Ouyang and Huang (1979). CVO protease V (10 μg) was added to 0.5 ml 2% fibrinogen and incubated at 37°C for 90 min. Aliquots were removed at 0, 3, 6, 20, 60 and 90 min, added to denaturing buffer and incubated overnight at 37°C. Samples were then electrophoresed on 14% acrylamide gels.

Determination of purity, molecular mass and pH optimum

Electrophoresis was performed in 14% total acrylamide gels (Novex) with or without reducing agent present. Buffers were essentially those of Laemmli (1970). Comparison with Novex Mark 12 mol. wt markers was used to estimate molecular mass. Optimum pH for digestion of casein yellow was determined using Good buffers (100 mM) at 0.5 pH unit increments (5.0–10.0). Since casein yellow is sparingly soluble at acidic pH, substrate was dissolved in 100 mM NaCl containing NaOH (65 μl 5 N NaOH/12.5 ml, pH ~ 8.0). Activity was then assayed 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 Bjarnason 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

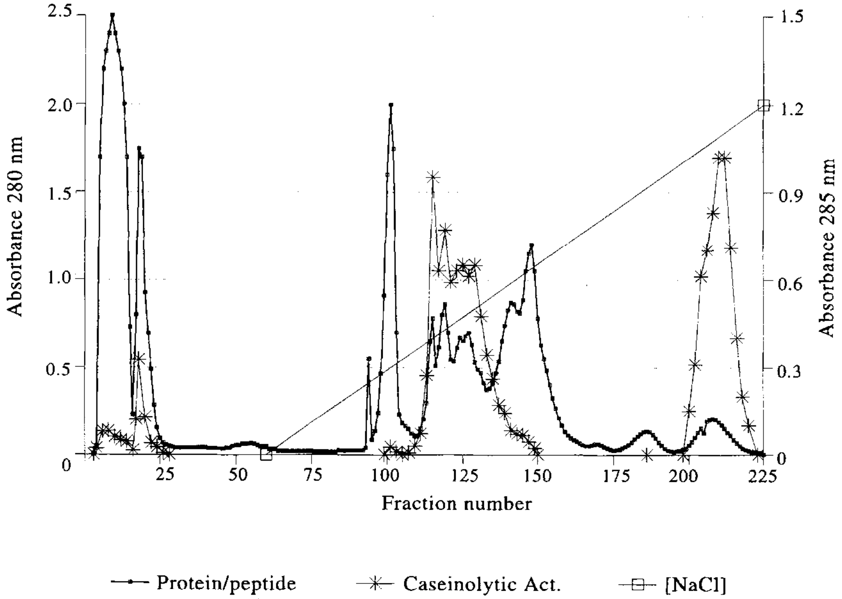

Fig. 1. DEAE-Sephadex ion-exchange fractionation of crude venom from adult *Crotalus viridis oreganus*.
Venom (235 mg) was applied to the column; the salt gradient (0–0.5 M) was initiated at fraction 60. CVO protease V eluted as the last peak.

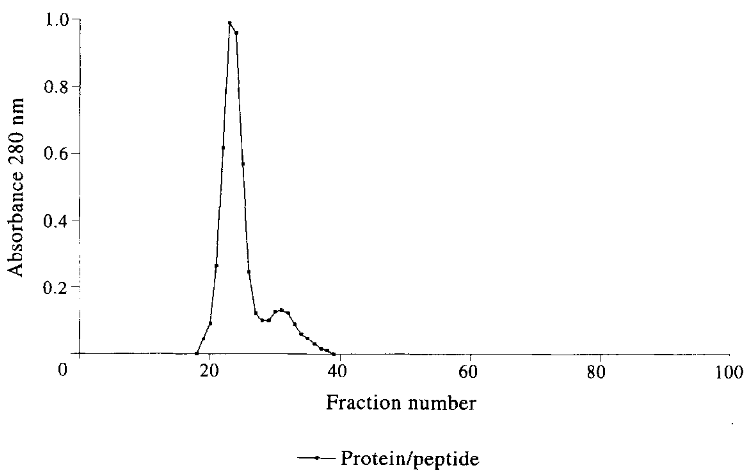

Fig. 2. Gel filtration of the combined material from the DEAE column.
CVO protease eluted as a symmetric peak; the shoulder represents autolytic degradation products.

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 ~ 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% of activity remained, and even at 100 mM citrate over 65% of enzyme activity is retained. Addition of $ZnCl_2$ (10–50 μM) to caseinolytic assay solutions resulted in slightly greater (< 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 transient clot (at ~ 15 min incubation) was formed during the assay on bovine fibrinogen. The Aα and Bβ chains were hydrolyzed preferentially, and the γ subunit was also slowly hydrolyzed (data not shown).

Metal analysis revealed that calcium was present at a ratio of 0.9 mole of calcium per mole protease. Neither zinc nor magnesium was detected in the sample (detection limit ~ 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 major characteristics of CVO protease V are summarized in Table 1.

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

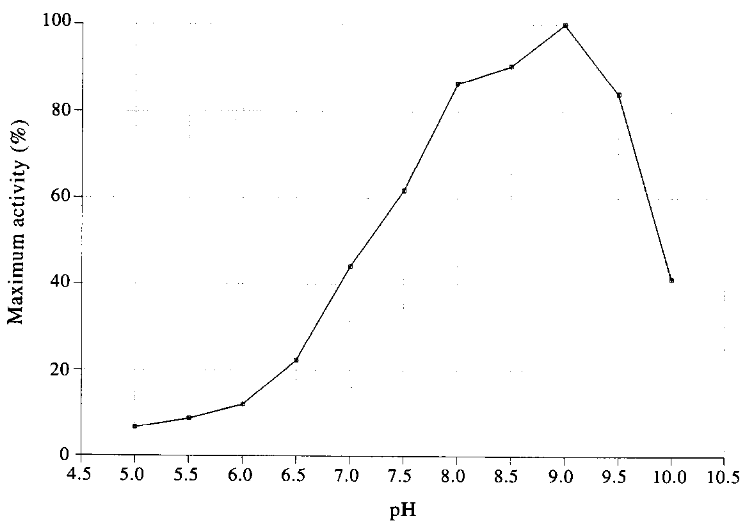

Fig. 3. Optimum pH of CVO protease V toward casein yellow.

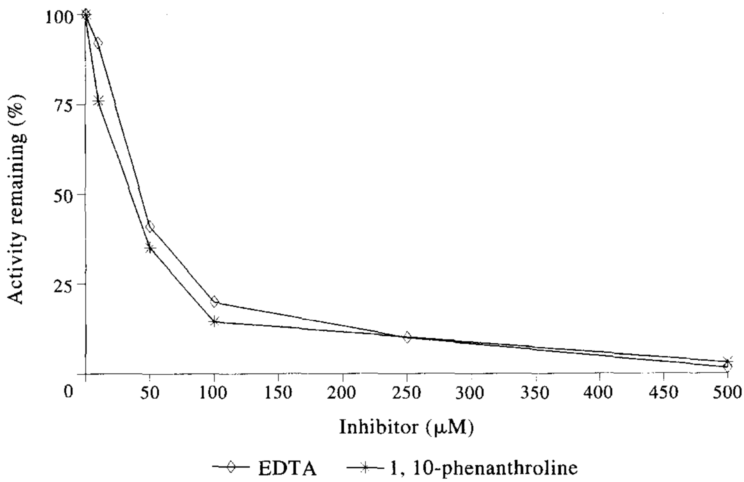

Fig. 4. Effect of EDTA and 1,10-phenanthroline on caseinolytic protease activity of CVO protease V.
Activity is abolished above 500 μM EDTA or 1,10-phenanthroline. Protease concentration, 10 μg/ml.

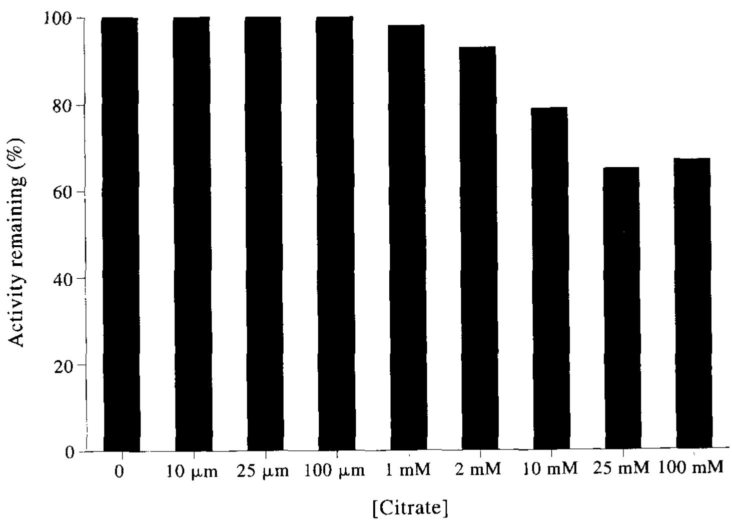

Fig. 5. Effect of citrate on caseinolytic activity of CVO protease V.
Note that more than 65% of activity is retained at citrate concentrations of up to 100 mM. Protease concentration, 10 μg/ml.

(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

Molecular mass	61 kDa
pH optimum (casein)	Protease V: 9.0 (8.0–9.5)
	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 adult venoms contain several proteases of lower mol. wt and there is evidence for secondary processing of proteases from other venoms (Hite *et al.*, 1992; Kini and Evans, 1992), the former seems most likely.

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 inactivation of CVO protease V during storage in the gland. However, significant protease 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 1,10-phenanthroline, it does not appear to contain significant amounts of zinc; instead, 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 mass metalloproteases such as HR1B (from *Trimeresurus flavoviridis* venom; Takeya *et al.*, 1990) and hemorrhagic toxin a (from *Crotalus atrox* venom; Bjarnason and Tu, 1978).

In conclusion, CVO protease V is the most abundant of at least five metalloproteases 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 this age-related change in venom activity. Bites from larger Pacific rattlesnakes result in much more extensive tissue damage than do bites by juvenile snakes (personal observations and Russell, 1980); when injected into prey, this tissue-damaging effect translates into a facilitation of digestion. Future work is aimed at deblocking the enzyme to obtain N-terminal sequence for comparison with known crotalid proteases. Such information may 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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REFERENCES

Bjarnason, J. B. and Fox, J. W. (1983) Proteolytic specificity and cobalt exchange of hemorrhagic toxin e, a zinc protease isolated from the venom of the western diamondback rattlesnake (*Crotalus atrox*). *Biochemistry* **22,** 3770–3778.

Bjarnason, J. B. and Fox, J. W. (1989) Hemorrhagic toxins from snake venoms. *J. Toxic. Toxin Rev.* **7,** 121–209.

Bjarnason, J. B. and Fox, J. W. (1995) Snake venom metalloendopeptidases: reprolysins. In: *Methods in Enzymology: Proteolytic Enzymes: Aspartic and Metallo Peptidases*, Vol. 248, pp. 345–368 (Barrett, A. J., Ed.). New York: Academic Press.

Bjarnason, J. B. and Tu, A. T. (1978) Hemorrhagic toxins from western diamondback rattlesnake (*Crotalus atrox*) venom: isolation and characterization of five toxins and the role of zinc in hemorrhagic toxin e. *Biochemistry* **17,** 3395–3404.

Fenton, A. W., West, P. R., Odell, G. V., Hudiburg, S. M., Ownby, C. M., Mills, J. N., Scroggins, B. T. and Shannon, S. B. (1995) Arthropod venom citrate inhibits phospholipase A_2. *Toxicon* **33,** 763–770.

Fox, J. W. and Bjarnason, J. B. (1995) Atrolysins: metalloproteinases from *Crotalus atrox* venom. In: *Methods in Enzymology: Proteolytic Enzymes: Aspartic and Metallo Peptidases*, Vol. 248, pp. 345–368 (Barrett, A. J., Ed.). New York: Academic Press.

Francis, B., Seebart, C. and Kaiser, I. I. (1992) Citrate is an endogenous inhibitor of snake venom enzymes by metal-ion chelation. *Toxicon* **30,** 1239–1246.

Hite, L. A., Shannon, J. D., Bjarnason, J. B. and Fox, J. W. (1992) Sequence of a cDNA clone encoding the zinc metalloproteinase hemorrhagic toxin e from *Crotalus atrox*: evidence for signal, zymogen and disintegrin-like structures. *Biochemistry* **31,** 6203–6211.

Jiang, W. and Bond, J. S. (1992) Families of metalloendopeptidases and their relationships. *FEBS Lett.* **312,** 110–114.

Kini, R. M. and Evans, H. J. (1992) Structural domains in venom proteins: evidence that metalloproteinases and nonenzymatic platelet aggregation inhibitors (disintegrins) from snake venoms are derived by proteolysis from a common precursor. *Toxicon* **30,** 265–293.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277,** 680–685.

Mackessy, S. P. (1988) Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *C. v. oreganus*. *Copeia* **1988,** 92–101.

Mackessy, S. P. (1993) Fibrinogenolytic proteases from the venoms of juvenile and adult northern Pacific rattlesnakes (*Crotalus viridis oreganus*). *Comp. Biochem. Physiol.* **106B,** 181–189.

Neurath, H. (1989) The diversity of proteolytic enzymes. In: *Proteolytic Enzymes: A Practical Approach*, p. 1 (Beynon, R. J. and Bond, J. S., Eds). Oxford: IRL Press.

Ouyang, C. and Huang, T.-F. (1979) α- and β-fibrinogenases from *Trimeresurus gramineus* snake venom. *Biochim. biophys. Acta* **571,** 270–283.

Russell, F.E. (1980) *Snake Venom Poisoning*. Philadelphia: J.B. Lippincott Co.

Shannon, J. D., Baramova, E. N., Bjarnason, J. B. and Fox, J. W. (1989) Amino acid sequence of a *Crotalus atrox* venom metalloproteinase which cleaves type IV collagen and gelatin. *J. biol. Chem.* **264,** 11575–11583.

Takeya, H., Oda, K., Miyata, T., Omori-Satoh, T. and Iwanaga, S. (1990) The complete amino acid sequence of the high molecular mass hemorrhagic protein HR1B isolated from the venom of *Trimeresurus flavoviridis*. *J. biol. Chem.* **265,** 16068–16073.

Utaisincharoen, P., Mackessy, S. P., Miller, R. A. and Tu, A. T. (1993) Complete primary structure and biochemical properties of gilatoxin, a serine protease with kallikrein-like and angiotensin-degrading activities. *J. biol. Chem.* **268,** 21975–21983.

Vassalli, J.-D. and Pepper, M. S. (1994) Membrane proteases in focus. *Nature* **370,** 14–15.