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A rapid and sensitive fluorometric method for the quantitative analysis of snake venom metalloproteases and their inhibitors

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Abstract

Metalloproteases are responsible for the hemorrhagic effects of many snake venoms and contribute to other pathways that lead to local tissue damage. Methods that quantify snake venom metalloproteases (SVMP) are therefore valuable tools in research on the clinical, physiological, and biochemical effects of envenomation. Comparative analysis of individual, population, and species differences requires screening of large numbers of samples and treatments, and therefore require a method of quantifying SVMP activity that is simple, rapid, and sensitive. This paper demonstrates the properties of a new fluorometric assay of SVMP activity that can provide a measure of metalloprotease activity in one hour. The assay is reliable, with variation among replicates sufficiently small to reliably detect differences in between species ($F_{19,60} = 2924$, $p < 0.001$), even for those venoms with low overall activity. It is also sensitive enough to detect differences among venoms using < 2 ng of whole venom protein. We provide an example use of this assay to detect the presence of natural SVMP inhibitors in minute samples of blood plasma from rock squirrels (*S. variegatus*), a natural prey species for North American rattlesnakes. We propose this assay is a useful addition to the set of tools used to characterize venoms, as well as high-throughput screening of natural or synthetic inhibitors, or other novel therapeutic agents against SVMP effects.

1. Introduction

Venoms from snakes within the family Viperidae are complex biochemical mixtures that function to immobilize prey and initiate digestion. Envenomation by viperid and crotalid snakes is typically characterized by hemorrhage and necrosis that can cause lasting tissue damage (Ownby, 1990) even if an individual survives the immediate context of predation. Hemorrhagic effects of snake venoms are commonly measured by some variation of the skin test of Kondo et al. (1960) using laboratory animals such as rabbits, rats, or mice (Theakston and Reid, 1983). In some cases measures of hemoglobin content in muscle or skin tissue exposed to venom have been used (Ownby et al. 1984; DeRoodt et al., 2000). Unfortunately, these methods require large numbers of animals, training and expertise in methodology, and may yield highly variable results.

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The recognition that snake venom metalloproteases (SVMP) are responsible for many of the toxic effects of viper venoms (Bjarnson and Fox, 1994) has guided the screening of candidate hemorrhagic toxins. SVMP are zinc-dependent reprolysins that can be divided into three groups based on analysis of venom transcriptomes (Fox and Serrano, 2008). These all possess a common metalloprotease domain, but may variously also contain disintegrin, high cysteine, or lectin-like domains (Hite et al., 1994; Fox and Serrano 2005, 2008). In addition to their role in producing hemorrhage (Baramova et al., 1989), SVMP also contribute to local myonecrosis, edema, ischemia, and skin lesioning (Gutiérrez and Rucavado, 2000; Matsui et al., 2000; see Gutiérrez et al., 2009 for a recent review). Therefore, researchers have used a variety of *in vitro* assays of metalloprotease activity as proxy measures of venom hemorrhagic activity, ranging from classic measures such as spot production on the gelatin emulsion on X-ray film to the gelatin-degradation ELISA method (Bee et al., 2001). These assays have advantages over *in vivo* methods because they don't require the use of large numbers of laboratory animals and yield measures with lower variability. However, these assays can consume significant amounts of sample, require multiple steps, and/or require long incubation times. Therefore, they place a limit on the rate and efficiency of screening of large numbers of venom samples, putative SVMP inhibitors, or other therapeutic agents.

Comparative studies of venom composition, and its function or evolution, commonly require large-scale screening. Although fundamental studies of function and evolution can be driven by a small number of well-chosen samples—Mackessy (2010) used single individuals of nine species to divide western rattlesnake venoms into two mutually exclusive categories of biochemical composition, with heterochrony as a proposed evolutionary mechanism—more complex questions of species, population, or individual variation necessitate larger data sets. For example, studies of the identity, distribution, and variation in the natural protective factors of mammalian prey against rattlesnake SVMP (Biardi, 2008) requires a combinatorial approach to experimental design. For example, investigating resistance of one groups of prey against venom from one sympatric and one allopatric rattlesnake species, using a minimum of ten prey individuals (to assess variation), would require 200 unique pairwise comparisions. Each comparison must in turn be replicated multiple times to provide appropriate controls and allow for statistical analysis of differences. In California ground squirrels, where there are clear differences in resistance among populations (Biardi et al., 2000; 2006) multiple groups of squirrels must be examined. Increasing individuals, populations, and/or species in this type of study increases the number of treatments and replicates in a non-linear way. For this research trajectory an ideal assay would have a minimum number of steps, yield rapid results, detect activity in small amounts of crude venom (and correspondingly small amounts of tissue from potentially resistant prey) and be inexpensive on a per-sample basis.

Fluorescent substrates have already been used successfully to quantify novel venom endopeptidase activities (Gasparello-Clemente and Silviera, 2002) and may provide a solution to the large scale analysis required by comparative studies of venoms and prey resistance. Here we validate a rapid and sensitive method detecting the hydrolysis of gelatin heavily conjugated with BODIPY-FL dye and evaluate its ability to quantify SVMP activity in sub-microgram amounts of whole venom protein. Since its development, this substrate has been used for *in situ* zymography and other assays of vertebrate metalloprotease activity (Oh et al., 1999; D'Angelo et al., 2001; Mook et al., 2003). Because of our interest in prey resistance to rattlesnake predation, we also demonstrate the utility of this assay in quantifying the effectiveness of natural SVMP inhibitors in whole blood plasma from a natural prey species, rock squirrels (*Spermophilus variegatus*).

2. Materials and methods

2.1. Venoms

Snake venoms were obtained in lyophilized form from the Natural Toxins Research Center (*Crotalus adamanteus, C. horridus, Crotalus m. molossus*) or Sigma Chemical Company, St. Louis, MO, USA (*Bitis gabonica, Bungaris multicinctus, C. atrox, C. o. oreganus, C v. viridis, Daboia russeli, Dentroaspis angusticeps, Echis coloratus, Lachesis muta, Naja melnaoleuca, N. n. kaouthia, N. nivea, Ophiphagous hannah, Pseudechis porphyiocus, Trimeresurus okinavensis*, and *Vipera berus*). Venoms were reconstituted at a concentration of 10 mg/ml in buffer containing 50 mM Tris HCl, 5 mM $CaCl_2$, and 0.05% Brij, pH 7.6, and stored at −20°C until use.

2.2. Fluorometric assay of metalloprotease activity

The assay was conducted at 22°C in white untreated flat bottom 96-well assay plates (Corning). Wells were filled to a volume of 200 μl with a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 0.2 mM sodium azide, pH 7.6. Self-quenched DQ gelatin (Molecular Probes, Eugene, OR) derived from pig skin was used as the substrate for protease activity. Quenching is removed as gelatin is hydrolyzed and releases peptides, exposing individual BODIPY-FL molecules to incident light.

Each reaction used 1 μg of DQ gelatin substrate per well, excepted as noted below (Section 2.5) during the estimation of kinetic parameters for select venoms.

Fluorescence was monitored using an excitation wavelength of 495 nm and emission wavelength of 515 nm. Unless otherwise indicated fluorescence was determined at 10-minute intervals for two hours. We used fluorimeters with both filter-based (Infinite F200, Tecan USA) and monochromator-based optics (LS50B Luminescence Spectrometer, Perkin Elmer). Metalloprotease activity is expressed as $RFU_{515nm} \times sec^{-1}$ after any background fluorescence of substrate-only blanks was subtracted.

2.3. Detection limits for metalloprotease activity

Two venoms with significant metalloprotease activity (*Crotalus atrox* and *Trimesurus okinavensis*) were diluted serially to yield a range of final protein concentrations from 1.25 ng/μl to 0.005 ng/μl. Individual dilutions were assayed for activity for 24 hours and the data then analyzed by least squares linear regression. The minimum limit of detection was estimated as the midpoint between the lowest dilution having and highest dilution not having activity significantly different from zero.

2.4. Effect of class-specific protease inhibitors

Two rattlesnake venoms (*Crotalus atrox* and *C. v. oreganus*) were treated with class-specific protease inhibitors using a modification of the method of Dunn (2001). Separate aliquots of each venom (0.005 μg/μl) were treated with 10mM EDTA, 0.1 mM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64), 0.1 mM phenlymethanesulfonyl fluoride (PMSF), or 0.1 mM (1,10)-phenanthroline monohydrate (1,10-PAM). Inhibitors were obtained from Sigma Chemical Co., St. Louis, MO, USA. Activity of treated and untreated venom samples was monitored at 10-minute intervals for two hours.

2.5. Estimation of kinetic parameters

We prepared a series of experiments to estimate the kinetic parameters of the metalloproteases present in a selection of crotalid venoms. The initial reaction velocity of crude venom (1 μg total protein) from seven crotalid species (*Crotalus atrox, C.*

adamanteus, C. horridus, C. m. molossus, C. v. viridis, C. v. oreganus, and *Trimesurus okinavensis*) was determined in the presence of serial dilutions of substrate from 5.00 to 0.02 μg/ml.

Nonlinear least-squares curve fitting was used to estimate V_{max} and K_M for whole venoms under a single-enzyme model assuming simple Michelis-Menten kinetics. We also used linear regression of $1/V_{obs}$ against 1/[S] as an alternative method of analysis. Individual slope and intercept parameters of regression models for each venom that differed significantly from zero at $\alpha = 0.05$ were used to derive estimates of V_{max} and K_M.

2.6. Screening for SVMP inhibitors from prey blood plasma

Rock squirrels (*Spermophilus variegatus*) from central New Mexico were screened for the ability to neutralize venom activity. Blood samples from squirrels were collected and processed according to the method in Biardi et al. (2006). Plasma from five adult *S. variegatus* was pooled and 1 μl aliquots were pre-incubated with 1 μg venom protein for 30 minutes at 22°C. We also prepared substrate- and venom-only controls. Samples were then assayed for activity at 10-minute intervals for two hours. Inhibition scores (%) were calculated as:

$$\frac{[(Venom{-}only\ RFU \cdot sec^{-1}) - (Venom{+}plasma\ RFU \cdot sec^{-1})] * 100}{(\text{Venom}-\text{only RFU} \cdot \text{sec}^{-1})}$$

We determined the capacity of squirrel plasma inhibitors to affect venoms from rattlesnakes sympatric with *S. variegatus* in this region (*C. atrox, C. v. viridis*, and *C. m. molossus*) as well as venoms from rattlesnakes outside this geographic region (*C. v. oreganus, C. adamanteus*, and *C. horridus*).

3. Results

3.1. Fluorometric assay of snake venoms

All 20 venoms assayed hydrolyzed the substrate. Because the activity of some venoms produced a rapid increase in fluorescence that exceeded the maximum sensitivity of the detector, values for venom activity were calculated using only the first 1 hour (six readings) for each venom (Table 1). Variation among replicates was sufficiently small to reliably detect differences in activity between species ($F_{19,60} = 2924$, $p < 0.001$), even for those venoms with low overall activity. Differences between taxonomic groups were also detected when venoms were analyzed at the taxonomic level of family and subfamily ($F_{2,17} = 9.65$, $p < 0.01$). Post-hoc analysis indicated that this was primarily due to Crotalinae venoms, which had significantly greater activity than elapid ($t_{0.05,7} = 3.72$, $p < 0.05$) and other viperid ($t_{0.05,3} = 3.43$, $p < 0.05$) venoms used in this assay (Figure 1).

3.2. Reaction mechanism of rattlesnake proteases

Pretreatment of venom with class-specific protease inhibitors clearly show that, at least for rattlesnake venoms, there is little or no contribution to increased fluorescence by cysteine or serine proteases (Figure 2). Venoms retained an average of 95.3% of activity when pre-treated with E64, and 96.6% of activity when pre-treated with PMSF. Since EDTA and 1,10 PAM—both potent metalloprotease inhibitors—strongly reduced venom activity, this assay appears to be a valid measure of SVMP.

3.3. Minimum detection limits for venoms with high SVMP activity

For the two venoms tested, measurable activity was detectable down to 15 ng after a 4-hour incubation. With a 24-hour incubation, detection of SVMP activity was possible for samples of less than two nanograms of whole venom protein (Table 2).

3.4. Kinetic parameters of Crotalinae venoms

A single enzyme Michaelis-Menten model shows a poor fit the seven venoms tested, suggesting the presence of multiple substances that affect whole venom activity. For example, analysis of *C. atrox* venom using least-squares nonlinear regreassion shows departures from model predictions at high and low concentrations of substrate (Figure 3). For six of seven venoms, however, this approximation explained more than 80% of variation in reaction rate. Slope and intercept parameters that were significantly different from 0 ($p < 0.05$ after Bonferonni correction) were used to estimate V_{max} and K_M values for crude venoms (Table 3).

3.5. Ground squirrel blood plasma inhibitors of SVMP

Rock squirrel blood plasma was able to inhibit the SVMP activity of all six crotalid venoms it was challenged with. The degree of inhibition varied from 87.7% against *C. atrox* venom, to only 18.3% against *C. m. molossus* venom (Figure 4). Differences among all venoms were statistically significant ($F_{5,18} = 1148$, $p < 0.001$). However, there was no significant difference between inhibition of venom from sympatric (60.8% average inhibition) and allopatric (62.1% average inhibition) rattlesnake species ($F_{1,2} = 3.40$, $p > 0.05$).

4. Discussion

This assay provides a rapid and reliable measure of proteolytic activity in a variety of snake venoms. Overall, activity of Viperinae and Crotalinae venoms are consistent with the known metalloprotease content, physiological effects, and biochemical properties of the venoms tested (Soto et al., 1988; Bjarnson and Fox, 1994), and correlate with the gelatin degradation activity reported by Bee et al. (2001) (Figure 5). The eight elapid venoms tested here showed low levels of metalloprotease activity, as expected (Table 1). Our results show a much smaller range of variation in the metalloprotease activity of elapid venoms. Further studies may clarify whether this is due to differential sensitivities of the two assays at low levels of metalloprotease activity, or whether the two assays may be detecting properties of venom unrelated to presence or activity of SVMPs.

It is clear that hydrolysis is due to metalloproteases in the three rattlesnake venoms tested. While viper venoms are a rich source of serine proteases that disrupt hemostasis (Braud et al., 2000) neither these enzymes, nor cysteine proteases, were not responsible for increased fluorescence (Figure 2). We did not assay specifically for the inhibition of aspartic proteases using, for example, pepstatin. Although venom gland transcriptome analysis of *Echis ocellatus* detected cDNA transcripts for renin-like proteases (Wagstaff and Harrison, 2006), there is little evidence high levels of aspartic protease activity in snake venoms (Matsui et al., 2000). We therefore assume that for the venoms tested this assay has high validity as a measure of SVMPs. Differences in the effectiveness of EDTA and 1,10-PAM may reflect an additional effect of EDTA by chelating calcium or other ions important for allosteric regulation of enzyme activity (Sela-Passwell et al., 2010). Our results, considered with other uses of DQ gelatin to investigate a variety of vertebrate matrix metalloproteases (Oh et al., 1999; D'Angelo et al., 2001; Zhang and Salamonsen, 2002; Hou et al., 2002; Mook et al., 2003) suggest this assay is likely to be a valid and reliable measure of the activity of metalloproteases in venoms from other taxonomic groups as well.

The sensitivity of the assay is comparable to the ELISA method of Bee et al. (2001). We were able to successfully detect SVMPs in two of the most active venoms when using less than 2 ng of whole venom protein (Table 2). The assay continued to reveal differences between *C. atrox* and *T. okinavensis* venoms even at this low concentration ($t_{0.05,3} = 5.48$, $p < 0.01$). It is likely that modifying assay parameters by increasing amount of substrate, incubation time, and/or temperature can lower the detection limit further if necessary.

An advantage of this assay is the ability to easily manipulate substrate concentration, facilitating studies of SVMP kinetics. Because snake venoms are complex mixtures of a variety of enzymes, polypeptides, and other factors, some of which still have unknown functions, the application of a single-enzyme Michalis-Menten model to the activity of whole venom samples is an oversimplification of the molecular interactions occurring upon envenomation. Our results confirmed that the Crotalinae venoms tested contain multiple factors that affect SVMP activity on DQ gelatin. Venoms that may have only a single enzyme with metalloprotease activity, such as has been suggested for venom from the elapid *Micropechis ikaheka* (Gao et al., 2002) may yield more interpretable kinetic parameters. The assay should also provide a rapid method for more detailed studies of the enzymatic properties of purified venom toxins, as well as the mechanism and effects of putative inhibitors on binding and reaction rate parameters.

Even for complex biochemical mixtures, the assay has utility as a rapid and sensitive screen for SVMP inhibitors. Previous research indicates that rock squirrels (*S. variegatus*) have a suite of behavioral adaptations to discourage rattlesnake predation (Owings et al., 2001), and there is evidence of serum protective factors that neutralize some rattlesnake venom toxins (Biardi, 2000; Biardi et al., 2010). We have used the current assay to demonstrate for the first time the presence of SVMP inhibitors in this prey species. The ability to detect inhibitors in small quantities of sample (~ 1 μl) shows this assay can be useful for the rapid screening of plasma, plasma fractions, or other tissue samples for the presence of naturally occurring SVMP inhibitors from prey, or venomous snakes themselves.

We believe this method is a useful addition to the set of assays commonly used to characterize hemorrhagic activity of snake venoms. This single-step assay is rapid (measures of SVMP activity in a time span of minutes to hours), facilitates analysis of the kinetic properties of venom SVMPs, and is readily adaptable to high-throughput screening of whole venoms, natural or synthetic inhibitors, or other novel therapeutic agents.

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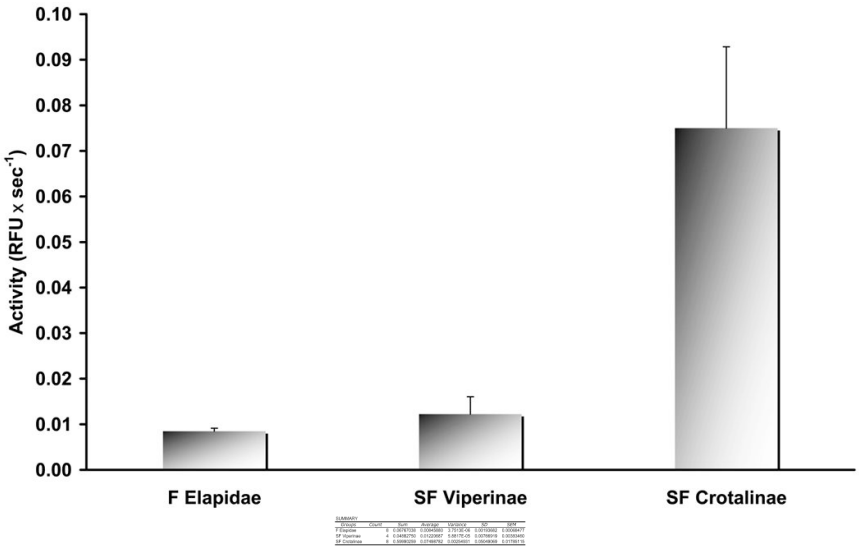


Figure 1.

Mean proteolytic activity (± SEM) of selected venoms in Family Elapidae (n = 8), Subfamily Viperinae (n = 4), and Subfamily Viperinae (n = 8).

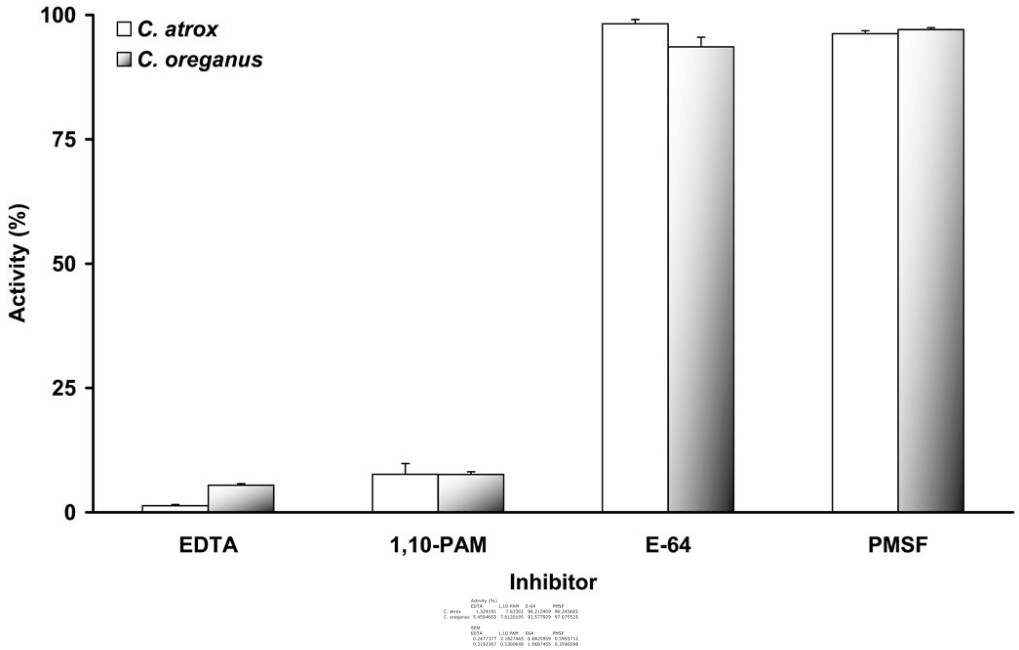


Figure 2.

Percent of protease activity remaining (± SEM) in two crotalid venoms after pretreatment with a metal ion chelator (EDTA), a metalloprotease-specific inhibitor (1,10-PAM), a serine protease-specific inhibitor (PMSF), and a cysteine protease-specific inhibitor (E-64). Low activity after treatment with EDTA and 1,10-PAM, and high activity after treatment with PMSF and E-64 supports the conclusion that the assay is a valid measure of SVMP activity.

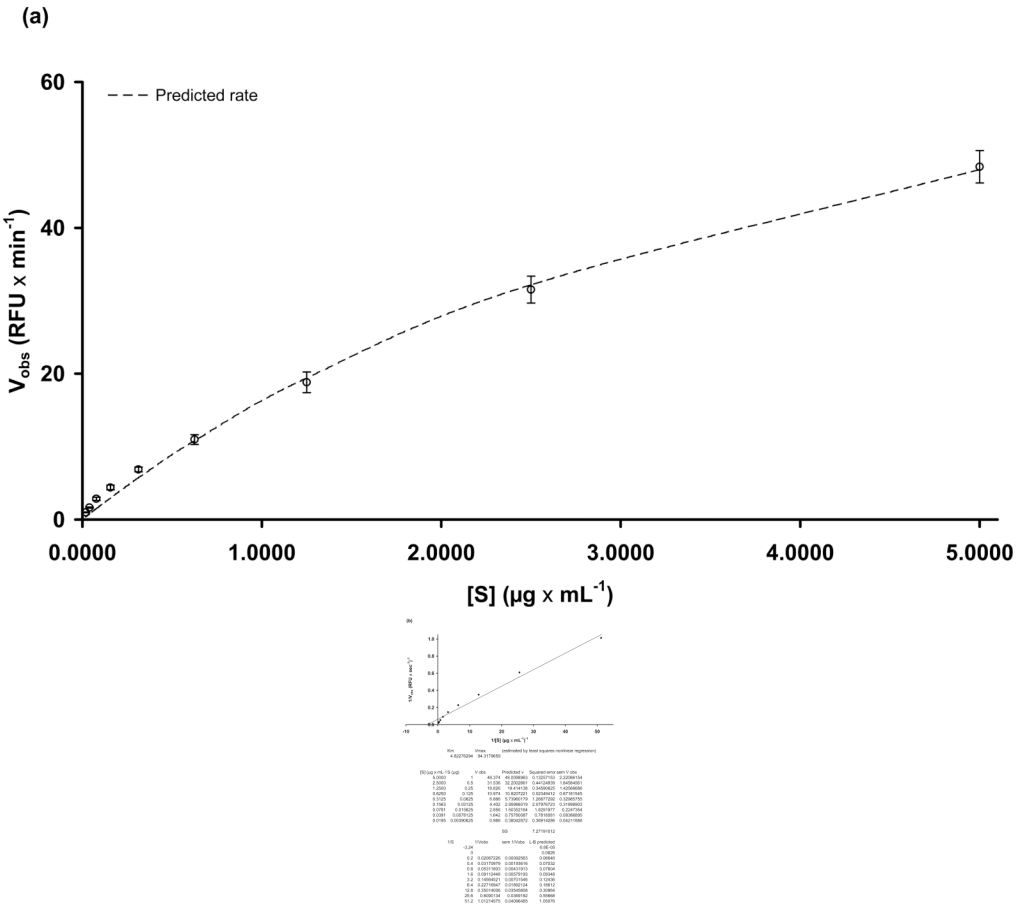


Figure 3.

(a) Reaction velocity was determined over a range of substrate concentrations for *C. atrox* venom. Values represent mean ± 1 standard deviation; the line represents the relationship predicted by least-squares nonlinear regression assuming a single enzyme obeying Michelis-Menten kinetics. (b) A single enzyme model also predicts a linear relationship with positive slope between the reciprocals of substrate concentration and reaction velocity. This can be visualized with a Lineweaver-Burke double reciprocal plot. As expected this model is a poor fit to *C. atrox* venom, which contains multiple SVMP, and shows significant nonlinearity at high substrate concentrations.

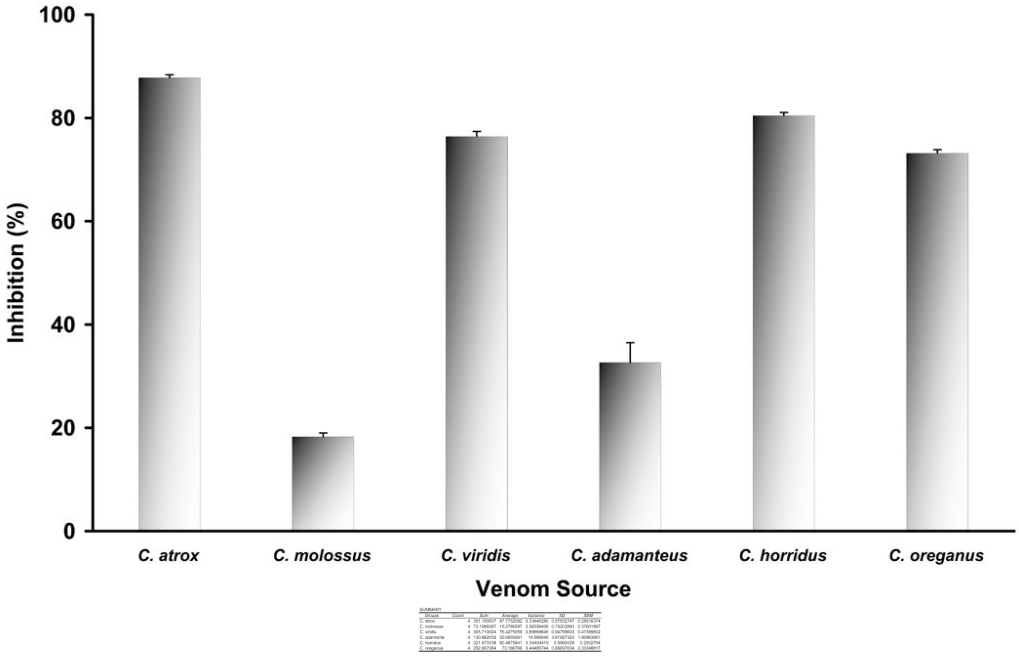
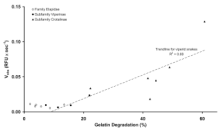


Figure 4.

Inhibition of rattlesnake SVMP activity by pooled blood plasma from rock squirrels (*S. variegatus*). These geographic range of these squirrels overlaps with that of *C. atrox*, *C. v. viridis*, and *C. m. molossus*, but not that of *C. adamanteus*, *C. v. oreganus*, and *C. horridus*. Values represent percent inhibition of venom activity (± SEM).



SUMMARY

Groups	RFU v sec-1	Bee et al.
Bungaris mu	0.0078266	3.7
Dendroaspis	0.0111714	2.0
Hemachatus	0.0076601	5.7
Naja melanc	0.0102463	13.5
Naja naja ka	0.0100751	4.0
Ophiphagou	0.0053971	8.7
Bitis gabonii	0.0096362	7.3
Daboia russ	0.0063984	11.4
Vipera berus	0.0092902	15.7
Echis colora	0.0235027	22.0
Crotalus ada	0.018338	42.4
Crotalus atro	0.1288354	60.8
Crotalus hor	0.0479597	41.7
Crotalus viri	0.0447369	44.4
Crotalus viri	0.0635788	49.0
Lachesis mu	0.0335559	22.3

Figure 5.

Correlation of activity in this SVMP fluorescence assay with measures of gelatinase activity (percent gelatin degradation) from Bee *et al.* (2001) for the 20 species analyzed by both groups.

Table 1

Activity of 20 snake venoms (± 1 s.e.m.) using DQ gelatin as a protease substrate. Venoms are arranged alphabetically within taxonomic unit.

Venom	Specific Activity[a] (RFU × sec^{-1} × mg^{-1})
Family Elapidae	
Bungaris multicinctus	78 ± 4.1
Dendroaspis angusticeps	111 ± 3.7
Hemachatus haemachatus	76 ± 3.2
Naja melanoleuca	102 ± 5.6
Naja naja kaouthia	101 ± 2.4
Naja nivea	68 ± 6.5
Ophiphagous hannah	54 ± 2.6
Pseudechis porphyiocus	85 ± 1.9
Family Viperidae	
Subfamily Viperinae	
Bitis gabonica	96 ± 7.8
Daboia russelli	64 ± 3.6
Echis coloratus	235 ± 5.6
Vipera berus	93 ± 4.2
Subfamily Crotalinae	
Crotalus adamanteus	183 ± 3.2
Crotalus atrox	1,288 ± 4.1
Crotalus horridus	480 ± 4.8
Crotalus molossus molossus	1,009 ± 3.4
Crotalus oreganus oreganus	447 ± 4.6
Crotalus viridis viridis	636 ± 7.6
Lachesis muta	336 ± 10.7
Trimeresurus okinavensis	1,620 ± 29.5

[a] Negative controls showed no activity and minimal background fluorescence.

Table 2

Effects of decreasing amounts of venom protein per well on the detection of SVMP activity. Reaction rate was determined using least squares linear regression.

Total Venom Protein in Assay (ng)	Reaction rate ($RFU_{515} \times sec^{-1}$)	
	Crotalus atrox	*Trimeresurus okinavensis*
250.0	0.01253	0.01948
125.0	0.00744	0.01275
62.5	0.00382	0.00669
31.2	0.00179	0.00346
15.0	0.00081	0.00177
7.8	0.00053	0.00085
3.9	0.00023	0.00042
1.9	0.00015	0.00026
0.97	*n.s.*[a]	*n.s.*[a]

[a] *n.s.* = not significantly different from 0

Table 3

Estimation of kinetic parameters by linear regression of the reciprocals of reaction rate and substrate concentration for seven Crotlinae venoms. A Michelis-Menten model of enzyme-substrate interactions was assumed for the purposes of calculating V_{max} and K_m values. (*n.d.* = not determined because slope and intercept estimates were not significantly different from 0 at $\alpha = 0.05$)

Venom	V_{max}	K_m	R^2	*F*
Trimeresurus okinavensis	0.0336	0.849	0.94	367.2
Crotalus atrox	0.0355	0.592	0.93	326.3
C. m. molossus	0.0388	0.778	0.94	292.8
C. horridus	0.0083	0.709	0.94	268.4
C. v. viridis	0.0092	0.357	0.86	119.7
C. o. oreganus	0.0066	0.365	0.84	101.0
C. adamanteus	*n.d.*[a]	*n.d.*[a]	–	–