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SHORT COMMUNICATIONS

FRACTIONATION OF RED DIAMOND RATTLESNAKE (*CROTALUS RUBER RUBER*) VENOM: PROTEASE, PHOSPHODIESTERASE, L-AMINO ACID OXIDASE ACTIVITIES AND EFFECTS OF METAL IONS AND INHIBITORS ON PROTEASE ACTIVITY

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S. P. MACKESSY. Fractionation of red diamond rattlesnake (*Crotalus ruber ruber*) venom: protease, phosphodiesterase, L-amino acid oxidase activities and effects of metal ions and inhibitors on protease activity. *Toxicon* **23**, 337–340, 1985. — *Crotalus ruber ruber* venom contains several different proteases, and the proteolytic activity of the crude venom is 6–15 times greater in adult than in juvenile venom. Venom samples were assayed for proteolytic, phosphodiesterase, L-amino acid oxidase and elastinase-like activities and were subjected to gel filtration on BioGel P-100. Two major size classes of proteases were resolved (mol. wt 67,000 and 20,500). EDTA, *N*-ethylmaleimide (*N*-EM) and 1,10-phenanthroline inhibited proteolytic activity of crude venom, and EDTA, Zn^{2+} and Cu^{2+} inhibited proteolytic activity of the fractionated venom.

THE RED DIAMOND RATTLESNAKE (*Crotalus ruber*) is a large species whose chaparral habitats are increasingly encroached upon by human activity in southwestern California. Though behaviorally inoffensive, its large size (to 1.5 m; KLAUBER, 1972), high venom yield (GLENN and STRAIGHT, 1982) and evidence of severe tissue damage on envenomation (RUSSELL, 1969; LYONS, 1971) combine to make this snake of clinical concern within its restricted range. However, with few exceptions, very little is known about the chemical nature of this venom (e.g. TU *et al.*, 1966; DURKIN *et al.*, 1981).

Initial investigations in this laboratory of crotalid proteolytic enzymes indicated high activity levels in *C. ruber* venom. Proteolytic enzymes are most prevalent among crotalids and viperids (TU *et al.*, 1966) and the severe tissue damage associated with rattlesnake envenomation results chiefly from the action of proteases and related enzymes (OWNBY, 1982). The partial purification of two size classes of proteases and their response to inhibitors and metal ions is presented here as a preliminary characterization of *C. ruber* venom.

Venom from juvenile and adult snakes collected in the vicinity of Hemet, Riverside Co., California was extracted manually, quick-frozen and lyophilized. Hide powder azure (lot No. 810279) and casein yellow (lot No. 610029) were obtained from CalBioChem. BioGel P-100 (100–200 mesh) was purchased from Bio-Rad Laboratories. Molecular weight protein standards and other biochemicals (analytical grade) were obtained from Sigma Chemical Co.

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Crude venom was assayed for proteolytic activity (STEYN and DELPIERRE, 1973), L-amino acid oxidase activity (essentially the method of WEISSBACH *et al.*, 1961; reaction terminated by the addition of 10% (w/v) trichloracetic acid), elastinase-like activity (SIMPSON and TAYLOR, 1973) and phosphodiesterase activity (BJÖRK, 1963). The effects of phenylmethylsulfonyl fluoride (PMSF), 1,10-phenanthroline, *N*-ethylmaleimide (*N*-EM) and EDTA at three concentrations (1, 10 and 100 μg/ml) were evaluated using hide powder azure as substrate. Venom (30 μg), inhibitor and 0.05 M *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.8, were allowed to stand at room temperature (21 – 23°C) for 30 min. Proteolytic activity was then assayed as above. All assays were run in duplicate and compared to control hydrolysis.

Adult *C. ruber* venom (150 mg) was fractionated on a 2.8 × 96 cm column of BioGel P-100 using 0.05 M ammonium acetate buffer, pH 7.0, with a flow rate of 4.8 ml/hr. Prior to fractionation, the column was calibrated with albumin (mol. wt 67,000), ovalbumin (mol. wt 43,000), chymotrypsinogen A (mol. wt 25,000) and ribonuclease A (mol. wt 13,700). Column effluent was assayed for proteolytic, L-amino acid oxidase and phosphodiesterase activities. The effects of a single concentration (100 μg/ml) of metal ions (Ca^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+}) and inhibitors (EDTA and *N*-EM) on proteolytic activity of peaks Ib and III were assayed using casein yellow. Column effluent (peak Ib: 20 μl; peak III: 3μl) and inhibitor or metal ion were added to 0.1 M *N*-2-hydroxyethylpiperazine propanesulfonic acid (EPPS) buffer, pH 8.0, (total 1.0 ml) and allowed to stand for 30 min. One milliliter of substrate (12 mg/ml EPPS) was then added and tubes were incubated at 37°C with constant stirring for 30 min. At this time 1.0 ml of 0.5 N $HClO_4$ was added, unreacted casein yellow removed by filtration and absorbance read at 285 nm.

Venom yields from adult (1.1 m total length) *C. ruber* ranged from 300 – 350 mg lyophilized venom/snake, while yields from juvenile snakes (530 – 580 mm) were 28 – 35 mg. Adult venom (N = 3) hydrolyzed 14.1% of hide powder azure substrate/10 μg venom/30 min, while juvenile venom (N = 3) hydrolyzed 1.3% of substrate/10 μg venom/30 min. Adult venom (100 μg) solubilized 11% of the substrate Congo red – elastin, corresponding to approximately 40 μg elastin per ml solubilized. Incubation of 50 μg of adult venom with 0.5 μmole L-kynurenine resulted in the formation of 0.12 μmole kynurenic acid, the deamination product of L-amino acid oxidase. Using the units of BJÖRK (1963), 75 μg adult venom liberated 1.0 μmole *p*-nitrophenol/min. Assays of adult venom for NAD nucleosidase (TATSUKI *et al.*, 1975) and acetylcholinesterase (ELLMAN *et al.*, 1961) were negative, consistent with findings for the related crotalids *Crotalus adamanteus, C. atrox* and *C. viridis viridis* (TATSUKI *et al.*, 1975; ZELLER, 1948).

Gel filtration of adult *C. ruber* venom resolved 6 peaks, and the distribution of proteolytic, L-amino acid oxidase and phosphodiesterase activities is shown in Fig. 1. Two distinct size classes with proteolytic activity were resolved: peak Ib, approximate mol. wt 64,500 – 67,000 and peak III, approximate mol. wt 19,000 – 20,500. Peak III exhibited much higher specific activity toward casein yellow than the higher mol. wt fractions and also showed considerable activity toward Congo red – elastin. Phosphodiesterase and L-amino acid oxidase eluted very near the void volume and thus approximate mol. wts were not estimated. The results of inhibitor and metal ion effects on proteolytic activity of crude and fractionated venom are shown in Table 1. Metal chelators inhibited proteolytic activities, as did copper and zinc ions, while calcium (and magnesium: peak III) enhanced activity. Conflicting results were obtained using *N*-EM; these differences are being investigated.

Crude venom assays and fractionation demonstrated that venom from adult *C. ruber* contains several potent proteases and, as is seen among other species of large rattlesnakes

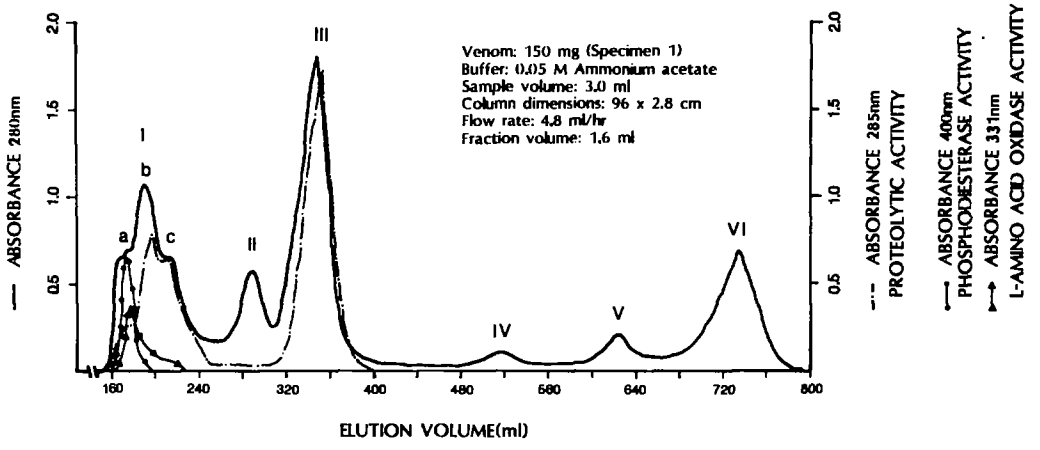

FIG. 1. ELUTION PROFILE OF CRUDE *C. ruber* VENOM ON BIOGEL P-100: PROTEOLYTIC, PHOSPHODIESTERASE AND L-AMINO ACID OXIDASE ACTIVITIES.

(e.g. BONILLA *et al.*, 1973), proteolytic activity is much higher in adult than in juvenile venoms. The apparent ontogenetic difference in activity underscores the importance of obtaining size information in snakebite cases, since size may directly affect symptoms of envenomation (REID and THEAKSTON, 1978). *C. ruber* venom proteases apparently require divalent metal ion cofactors, as demonstrated by inhibition by EDTA and 1,10-phenanthroline. Similar sensitivity to metal chelators has been observed with *C. atrox* hemorrhagins (BJARNASON and TU, 1978).

Crotalus ruber and *C. atrox* are considered to be closely related (BRATTSTROM, 1964; KLAUBER, 1972) and it may be expected that venom chemistry is similar as well. Results of

TABLE 1. EFFECT OF METAL IONS AND POTENTIAL INHIBITORS ON PROTEOLYTIC ACTIVITY OF CRUDE AND FRACTIONATED *Crotalus ruber* VENOM

Inhibitor/ion	% Activity after treatment				
	Crude venom*			Fractionated venom†	
	1.0 μg/ml	10 μg/ml	100 μg/ml	Peak Ib	Peak III
PMSF	100	85.5	94		
1,10-phenanthroline	100	76.5	50		
N-ethylmaleimide	91	54	62	113	118.6
EDTA	100	87.5	2	6.3	1.0
Ca^{2+}				114	110.5
Cu^{2+}				7.4	18.7
Mg^{2+}				98.4	109
Zn^{2+}				31.8	67.8

All values are the average of 2 replicates. Control absorbances (100% activity) were: crude venom, 595 nm, pH 7.8, 1.04; peak Ib, 285 nm, pH 8.0, 0.90; peak III, 285 nm, pH 8.0, 1.08.

*Three concentrations of inhibitors (pH 7.8) were used as described in text.

†A single concentration of inhibitor or ion (100 μg/ml) was used at pH 8.0.

this study show similarities to the elution profiles and mol. wt estimates for fibrinolytic (and caseinolytic) enzymes of *C. atrox* venom (BAJWA *et al.*, 1981) and to the sensitivity to chelators found in four proteolytic and hemorrhagic toxins isolated from *C. atrox* venom (BJARNASON and TU, 1978). Morphological similarities may thus correlate with venom chemistry characteristics; however, further purification of *C. ruber* venom fractions is necessary to address this question adequately.

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