Elastolytic Activity from Venom of the Rattlesnake *Crotalus atrox*[1] (37596)

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Tissue damage from the effects of severe envenomization by the western diamondback rattlesnake *Crotalus atrox* has been adequately described (1). Since destruction of connective tissues is one effect of venom on tissues, a series of studies were instituted to determine the mechanism by which venom degrades fibrous proteins of connective tissues. We have previously observed that rattlesnake venom degrades elastic as well as collagen fibers of mesentery (2). The present report gives evidence that rattlesnake venom contains elastolytic enzyme activity.

Materials and Methods. The aortas of 200 g rats were excised and incubated in Hanks' tissue culture medium with and without 500 mg % venom.[2] At various time intervals following incubation at 37° the aortas were fixed in 10% neutral formalin, embedded in paraffin, sectioned at 6 μm and stained with Verhoff's elastic tissue stain.

Elastinolytic activity of venom was also measured by the method of Shotton (3) using Congo red–elastin[2] as substrate. Elastin (2.5 mg) was suspended in 7.0 ml of 0.02 *M* sodium borate buffer (pH 8.8). Venom solutions (1 ml) were added and incubations were allowed to proceed at 37° with constant shaking. After centrifugation, solubilization of dye–protein complexes was monitored photometrically at 495 nm. Controls for spontaneous liberation of Congo red were included. An estimation of the amount of Congo red–elastin solubilized was made on the basis of complete solubilization with purified pancreatic elastase.[3]

t-BOC-L-alanine *p*-nitrophenyl esterase activity of venom was determined by a modification of the procedures described by Viser and Blout (4). The final reaction mixture to assay venom alanine *p*-nitrophenyl esterase activity contained 3.0×10^{-4} *M* *t*-BOC-L-alanine *p*-nitrophenol[4] and 10 m*M* Tris–HCl and 10 m*M* piperazine-*N*-*N*^{1}-bis-(2-ethane sulfonic acid) monosodium monohydrate (PIPES) at pH 8.0.[5] The release of *p*-nitrophenol was followed spectrophotometrically at 347.5 nm during incubations at 25°. The amount of *p*-nitrophenol liberated was calculated using an extinction coefficient of 5.5×10^3 liters/mole/cm (4). The effect of enzyme inhibitors was determined by assaying venom solutions preincubated 15 min with diethyldithiocarbamate, $HgCl_2$, KCN, EDTA and phenyl methyl sulfonyl fluoride at concentrations of 5 m*M*.

Results. Aorta incubated in tissue culture medium without venom for 4 hr (Fig. 1A) and 20 hr (Fig. 1B) showed the typical structure and stability of intact elastic fibers of the tunica media. After a 4 hr incubation with venom (Fig. 1C) there were definite breaks in the inner lamellae of elastic fibers, and after 20 hr incubation with venom (Fig. 1D) the elastic fibers had completely disappeared.

Rattlesnake venom catalyzed the solubilization of Congo red–elastin (Fig. 2). During a 3 hr incubation of Congo red–elastin with 99 μg protein, 31% of the elastin in the reaction mixture was solubilized. Elastin solu-

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[2] Sigma Chemical Co., St. Louis, MO.

[3] Worthington Biochemical Corp., Freehold, NJ.

[4] Cyclo Chemical Co., Los Angeles, CA.

[5] The K_m (apparent) for *t*-BOC-L-alanine *p*-nitrophenol was determined to be 1.6×10^{-4} *M*. Using 10 m*M* Tris and 10 m*M* PIPES, the optimal pH for the alanine *p*-nitrophenol esterase activity was found to be pH 8.0.

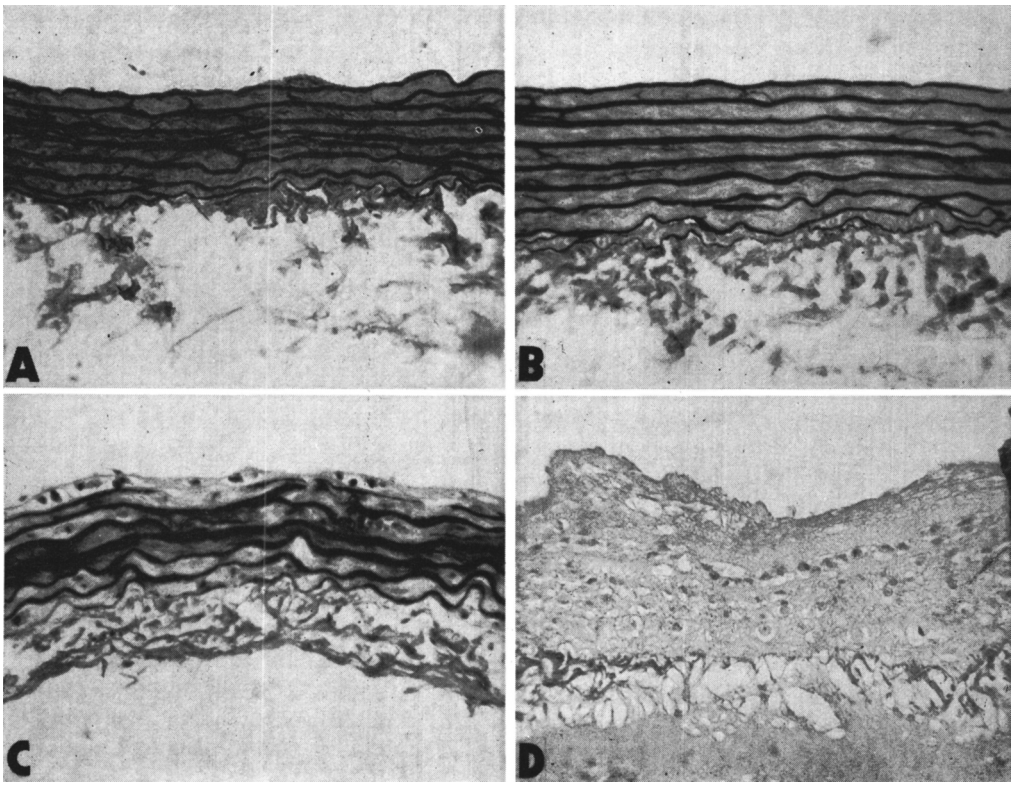

FIG. 1. Effect of *C. atrox* venom on native elastic fibers of rat aorta. Aorta segments were incubated with 0.5% venom at 37°: (A) 4 hr incubation without venom; (B) 20 hr incubation without venom; (C) 4 hr incubation with venom; (D) 20 hr incubation with venom.

bilization rate increased in a linear manner with increasing venom protein concentrations from 0.9 to 7.3 mg (Fig. 3). Sixty-seven percent of the elastin in the reaction mixture was solubilized during a 2 hr incubation with 7.3 mg venom protein.

In addition to elastinolytic activity, venom contained esterase activity against the elastase substrate *t*-BOC-L-alanine *p*-nitrophenol (Fig. 4). Venom solutions catalyzed the liberation of 0.0714 μmoles *p*-nitrophenol/mg protein/min with the assay employed. The reaction was linear over a venom protein concentration range from 0.1 to 0.5 mg. Alanine *p*-nitrophenyl esterase was inhibited 76% with diethyldithiocarbamate, 92% with $HgCl_2$, 65% with KCN, 85% with EDTA and 81% with phenyl methyl sulfonyl fluoride.

Discussion. Rattlesnake venom is known to contain a variety of proteolytic enzymes (5–10). The fact that *C. atrox* venom is able to degrade elastic fibers of rat aorta, solubilize Congo red–elastin, and exhibits esterase

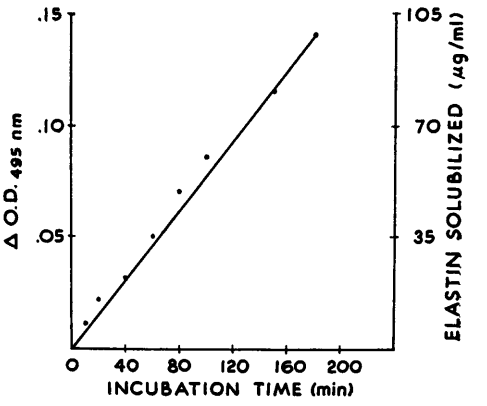

FIG. 2. Effect of incubation time on elastolysis with Congo red–elastin substrate. Values are corrected for spontaneous breakdown of substrate. Reaction mixtures contained 99 μg venom protein.

activity against the synthetic elastase substrate *t*-BOC-L-alanine *p*-nitrophenyl ester is the evidence that venom contains an elastase-like enzyme. Alanine esters have a high affinity for pancreatic elastase (4); however, they are not entirely specific for the elastases. Janoff (11) compared pancreatic and leukocyte elastase for their alanine *p*-nitrophenyl esterase activity with commercial preparations of nine hydrolytic enzymes. Chymotrypsin had the highest alanine *p*-nitrophenyl esterase activity among the enzymes tested; but, chymotrypsin had only one-tenth the specific activity of pancreatic elastase. Among purified enzymes from snake venoms a thrombin-like enzyme from *Crotalus adamanteus* (12) and Arvin from the Malayan pit viper *Ancistrodon rhodostoma* (13) contained significant alanine *p*-nitrophenyl esterase activity. Enzyme purification studies will be necessary to establish whether the elastolytic, elastinolytic, and alanine *p*-nitrophenyl esterase activity reside in the same enzyme.

Degradation of tissue elastic fibers by venom, in conjunction with the action of venom hyaluronidase (5, 14) and collagenolytic enzymes (2, 15) provides a plausible mechanism for the *in vivo* effects of rattlesnake venom on connective tissues and blood vessels.

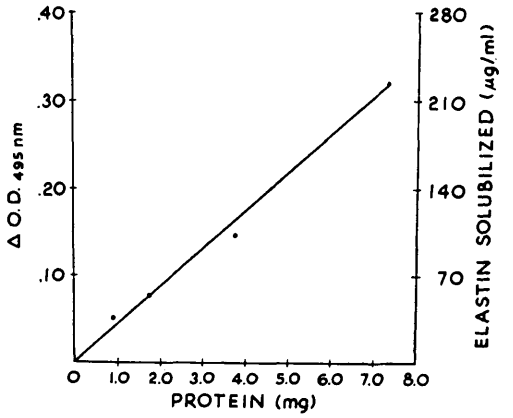

FIG. 3. Effect of venom protein concentration on elastolysis, with Congo red–elastin as substrate. Values are corrected for spontaneous breakdown of substrate. Incubations were carried out for 2 hr at 37°.

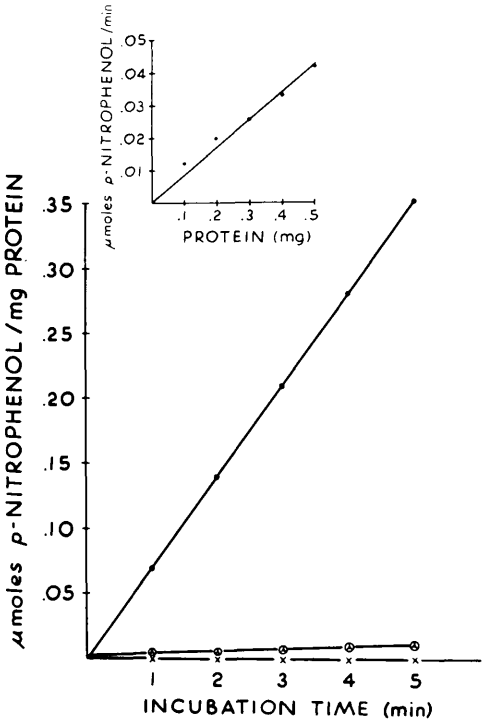

FIG. 4. *t*-BOC-L-alanine *p*-nitrophenyl esterase activity of *C. atrox* venom. The reaction mixture contained 3.0×10^{-4} *M* *t*-BOC-L-alanine *p*-nitrophenol, 10 m*M* Tris and 10 m*M* PIPES (pH 8.0), and 50 μg venom protein: (●) complete system; (○) less enzyme; (△) boiled enzyme; (×) less substrate.

Summary. In vitro incubation of rat aorta with rattlesnake venom led to the destruction of the elastic fibers of the aorta. Correlated with this action, venom contained enzyme activity which solubilized Congo red–elastin and catalyzed the release of *p*-nitrophenol from the synthetic elastase substrate *t*-BOC-L-alanine *p*-nitrophenol. Therefore, rattlesnake venom contains an elastase-like enzyme.

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