Collagenolytic Activity from Venom of the Rattlesnake *Crotalus atrox*[1] (35688)

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A specific collagenase from a vertebrate source was first demonstrated by Gross and Lapiere (1). Collagenase was subsequently found in the media from tissue cultures of a variety of normal animal tissues (2–7) as well as several human and animal tumors (8).

Delaunay and co-workers (9) reported that solutions of *Vipera aspis* venom contained weak collagenolytic activity and Hadidian reported that venom from *Agkistrodon piscivorus* is known to liquefy gelatin (10). We have demonstrated that venom from four species of snakes contains collagenolytic activity. Also, venom from *Crotalus atrox* degrades mesenteric native collagen fibers during tissue cultures (11). This report describes further evidence that the collagenolytic activity from *Crotalus atrox* venom is due to a specific collagenase.

Materials and Methods. Lyophilized venom from *Crotalus atrox* was purchased from Sigma Chemical Company, St. Louis, Missouri.

Rat-tail collagen solutions were prepared by extraction with acetic acid according to the procedure of Bornstein (12). Three times reconstituted rat-skin collagens were prepared by the method of Piez, *et al.* (13). The purity and native state of collagen was established by resistance to trypsin digestion.

Collagenase activity was determined by viscometry. Viscosity changes were measured in Ostwald viscosimeters with flow times for water ranging between 77 and 90 sec at 27°. The reaction mixture had a final volume of 7.0 ml and contained 3 mM Tris–HCl, pH 7.0, 3 mM $CaCl_2$, rat-tail collagen adjusted to a final specific viscosity of approximately 8.0 or rat-skin collagen with a final collagen concentration of 0.2%, and venom at various concentrations.

Samples to be electrophoresed were subjected to thermal denaturation at 45° for 10 min (15). Disc electrophoresis as modified by Reisfeld, *et al.* (16) for basic proteins and revised by Nagai (15) was used. However, the sample gel was not allowed to polymerize and 40% sucrose was substituted for water in the upper gel solution.

Electrophoresis was conducted at 2–3 mA/tube (0.5 cm diameter) for 135 min. The buffer boundary was monitored by methylene green mixed with the sample solution. Upon completion of the run, gels were transferred to staining tubes containing napthalene black stain (0.1% in acetic acid). Staining was allowed to progress at least 1 hr; then the gels were destained electrophoretically. Coomassie blue was utilized as a counter stain.

Venom solution exposure to low pH was accomplished by adjusting the pH to 2.5 with N HCl. After 30 min at the low pH the solutions were adjusted to pH 7.0 with N KOH and cleared by centrifugation.

Results. Various concentrations of *Crotalus atrox* venom reduced the specific viscosities of rat-tail tendon, acetic acid extracted rat-skin and sodium chloride extracted rat-skin collagen solutions (Table I). Reduction in specific viscosities of collagen solutions occurred at both 27° and 20°. Boiled venom solutions caused no reduction in the specific viscosities of collagen solutions. Venom solutions exposed to pH of 2.5 retained the capacity to reduce the specific viscosity of collagen solutions (Table II).

Collagen fragments resulting from venom collagenase activity, as well as full length α and β chains of collagen, were separated and

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[1] This study was supported by a grant from the Robert A. Welch Foundation, Houston, Texas and U.S. Public Health Service Grant No. DE 2743-03.

TABLE I. Effect of *Crotalus atrox* Venom on Collagen Solutions.[a]

Collagen source	Venom conc	(%) Initial η_{sp}
Acetic acid extracted rat-tail tendon	0.01	76
	0.11	57
	0.11	56
	0.57	47
	0.57[b]	100
Acetic acid extracted rat skin	0.29	64
	0.29[c]	86
	0.29[b]	100
Sodium chloride extracted rat skin	0.71[c]	90

[a] Venom concentrations are expressed as milligrams lyophilized venom/ml reaction mixture. One mg venom in solution contains 725 μg protein by the Lowry method (30). Percentage initial specific viscosity was calculated after 2 hr incubations.

[b] Boiled venom.

[c] Incubation at 20°.

disclosed on acrylamide gels (Fig. 1). Electrophoretic patterns on acrylamide gels demonstrated that controls exhibited typical α and β chains. Patterns from experimental reaction mixtures containing venom collagenase revealed fragments in addition to the α and β chains. When electrophoretic patterns from reaction mixtures containing venom exposed to low pH were compared with reaction mixtures containing venom which had not been exposed to low pH the patterns were quite different.

TABLE II. Effect on Rat-Tail Collagen Solutions by *Crotalus atrox* Venom after Exposure to pH 2.5.[a]

Venom conc	Hours of incubation	(%) Initial η_{sp}
0.71	24	68
0.71	42	57
0.71	92	57
0.71[b]	92	100

[a] Venom was exposed to pH 2.5 by methods described in the text. Venom concentrations expressed as mg venom/ml reaction mixture.

[b] Boiled venom.

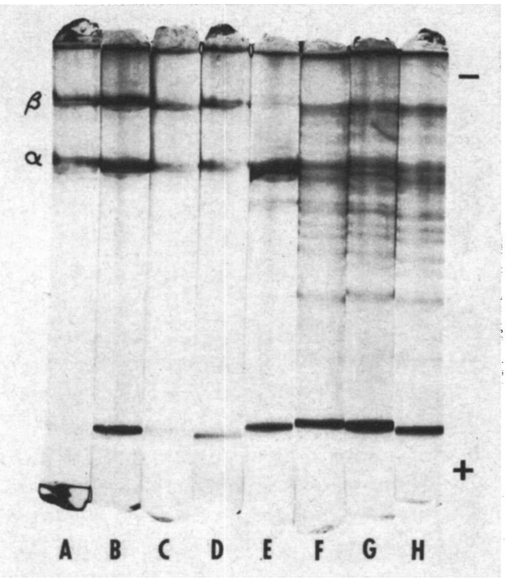

FIG. 1. Acrylamide gel patterns of thermally denatured venom-rat collagen reaction mixtures at 27°. A, Rat-skin collagen solutions incubated 24 hr without enzyme. B, Rat-skin collagen solutions, zero time control. C, Rat-tail collagen solutions incubated 24 hr without enzyme. D, Rat-tail collagen solutions incubated 96 hr with venom exposed to pH 2.5 and subsequently boiled. E, Rat-skin collagen solutions incubated 24 hr with crude venom. F, Rat-skin collagen solutions incubated 24 hr with venom exposed to pH 2.5. G, Rat-tail collagen solutions incubated 24 hr with venom exposed to pH 2.5. H, Rat-tail collagen solutions incubated 28 hr with venom exposed to pH 2.5.

Discussion. Crotalus atrox venom reduced the viscosity of three different preparations of collagens in solution. The acrylamide gel electrophoretic patterns derived from reaction mixtures containing collagen and venom preparations also showed collagen degradation. These observations, in conjunction with the fact that *Crotalus atrox* venom degrades mesenteric collagen fibers (11), indicate specific collagenase activity derived from *Crotalus atrox* venom.

The demonstration that *Crotalus atrox* venom collagenase activity is stable in an environment at pH 2.5 is consistent with the report that gelatin liquefying activity from *Agkistrodon piscivorus* venom is stable at low pH whereas some components of its proteolytic activities are not (10).

Collagenolytic activity of *Crotalus atrox* venom prior to low pH exposure resulted in the conversion of β chains to α chains and the formation of a fragment which traveled beyond the α chain on acrylamide gels during electrophoresis. The electrophoretic pattern obtained from reaction mixtures containing venom exposed to pH 2.5 showed a reduction of both α and β chains, with at least two fragments appearing between the α and β chains and several fragments below the α chains. The observed differences in electrophoretic patterns between the two venom preparations indicates that at least two enzymatic activities contribute to the reduction in viscosity of collagen solutions.

Collagenase obtained from tadpole and human tissue culture fluids acts on the collagen molecule to form fragments approximately 1/4 and 3/4 the original length of the molecule (4, 5, 17–25). The electrophoretic pattern of fragments after venom collagenolytic activity is apparently different from the patterns of human and tadpole collagenase activity, but is similar to the electrophoretic pattern of fragments obtained after the action of crude preparations of rat gingival collagenase (26). *Crotalus atrox* venom is known to contain a variety of proteolytic activities (27–29), and purified collagenase preparations from venom are required to determine with certainty the mechanism of attack on the collagen molecule.

We thank Mr. Mackwell Hickerson and Mr. Michael L. Reid for their technical assistance.

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Received Feb. 5, 1971 P.S.E.B.M., 1971, Vol. 137.