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ABSTRACT

A mixture of venom and saliva from the lizard *Heloderma horridum* was analyzed for esterase, phosphomonoesterase, phosphodiesterase, 5'-nucleotidase, and protease activities. Hydrolysis of N-benzoyl-L-arginine ethyl ester occurred at a pH optimum between pH 8.6 and 9.1 with a maximum activity of 452 units per mg per min. Hydrolysis of p-toluenesulfonyl-L-arginine methyl ester occurred at a pH optimum between pH 8.1 and 8.5 with a maximum of only 36 units per mg per min. One mg of the venom mixture liberated 9.3 μM of p-nitrophenol from p-nitrophenyl phosphate per minute at an optimum pH between 8.2 and 8.3. Over a wide range of pH, only low phosphodiesterase and 5'-nucleotidase activities were observed. A trace of caesinolytic activity occurred at pH 9.0.

INTRODUCTION

Heloderma suspectum and *Heloderma horridum* of Mexico and Southwestern U.S.A. are the only known poisonous lizards (Styblova and Kornalik 1967). In both lizards, Mebs (1968) reported that the venom secretion is diluted with saliva. This venom-saliva mixture is very toxic and has phospholipase A, hyaluronidase, esterase, and kinin-releasing properties (Mebs and Raudonat 1967, Mebs 1968, 1972).

This report concerns the esterase, phosphomonoesterase, phosphodiesterase, 5'-nucleotidase, and protease activities in the venom-saliva mixture of *H. horridum*.

MATERIALS AND METHODS

The lyophilized *H. horridum* venom-saliva mixture was a gift from Dr. H.L. Stahnke, Director, Poisonous Animals Research Laboratory at Arizona State University. The BAEE (N-benzoyl-L-arginine ethyl ester), TAME (p-toluenesulfonyl-L-arginine methyl ester), 5'-adenylic acid, and bis-p-nitrophenyl phosphate sodium salt were purchased from Sigma Chemical Co.; disodium p-nitrophenyl phosphate from Nutritional Biochemicals Corp.; Tris-(hydroxymethyl)aminomethane, glycine, ammonium molybdate, hydroquinone, sodium sulfite, magnesium chloride, and trichloroacetic acid (TCA) from Fisher Scientific Co.; sodium hydrogen sulfite from J.T. Baker Chemical Co.; magnesium sulfate from Mallinckrodt Chemical Works; and the casein from ICN Pharmaceuticals, Inc.

All enzyme assays described herein were performed with 1.0 mg per ml stock solutions of the lyophilized venom-saliva mixture. At this concentration, linear rates of substrate hydrolysis were obtained by measuring absorbance changes with a Beckman Acta C III spectrophotometer. After determination of concentration of stock solution for use in the assays, buffer solutions over a wide pH range were prepared for determining the pH optimum of an enzyme activity. The factors used by Sulkowski et al. (1963) and Richards et al. (1965) for converting enzyme activities measured at 37C to values at 25C were followed.

Esterase activities were assayed at 25C by use of Tris-HCl buffers. Solutions of 0.87×10^{-3} M TAME (p-toluenesulfonyl-L-arginine methyl ester) and 0.25×10^{-3} M BAEE (N-benzoyl-L-arginine ethyl ester) served as substrates (Schwert and Takenaka 1955, Tu et al. 1965). The control cuvette contained 3.0 ml of substrate solution and the test cuvette contained 2.9 ml of substrate solution to which 0.1 ml of venom dissolved in H_2O was added and mixed. Then absorbance increases at 247 nm for TAME or at 253 nm for BAEE were recorded at 30-sec intervals for up to 10 min. Activity is expressed as:

$$\text{Units/mg} = \frac{\Delta \text{ Abs/min} \times 1000}{\text{mg of venom}}$$

For determination of phosphomonoesterase the procedure described by Richards et al. (1965) was used. The reaction mixture of 1.0 ml of 0.1 M glycine-NaOH buffer, 1.2 ml of 0.001 M disodium p-nitrophenyl phosphate, 0.3 ml of 0.01 M $MgCl_2$, and 0.5 ml of diluted venom was incubated at 37C for 30 min. Absorbance was measured at 400 nm. The blank contained all components except the venom. Specific activity was defined as:

$$\frac{\mu\text{M of substrate hydrolyzed/min}}{\text{mg of venom}} \quad \text{at 25C.}$$

A factor of 0.70 was used to convert results obtained at 37C to 25C.

Phosphodiesterase was determined by the procedure described by Richards et al. (1965). The reaction mixture of 1.0 ml of 0.1 M Tris-HCl buffers, 1.2 ml of 0.001 M Na-bis-p-nitrophenyl phosphate, and 0.8 ml of diluted venom was incubated at 37C for 30 min. Absorbance was measured at 400 nm against a blank that contained all components except the venom. Specific activity was calculated the same as for the phosphomonoesterase. The factor used for converting results obtained at 37C to 25C was 0.44.

5'-nucleotidase activity was determined by the procedure described by Lo et al. (1966). A mixture of 0.2 ml venom, 0.5 ml of 0.02 M 5'-adenylic acid, 0.1 ml of 0.1 M aq. $MgSO_4$, and 0.5 ml of 0.2 M glycine buffer was incubated at 37C for 15 min. Then 2.5 ml of 0.2 N H_2SO_4 was added. Liberated phosphate was assayed according to the method of Ging (1956). One ml of 5% aq. ammonium molybdate and 0.5 ml of 2% aq. hydroquinone were added to the solution containing the liberated phosphate, mixed, and incubated for 15 min. Then 0.5 ml of $Na_2SO_3 \cdot NaHSO_3$ solution (prepared by mixing 95 ml of 15% $NaHSO_3$ with 5 ml of 20% Na_2SO_3) was added and incubated for 15 min before absorbance was measured at 720 nm. In the controls, water was substituted for venom. Specific activity was calculated as:

$$\frac{\mu\text{M phosphate liberated/min}}{\text{mg of venom}} \quad \text{at 25C.}$$

A factor of 0.25 was used to convert results at 37C to 25C.

Protease activity was determined by using casein as substrate (Kunitz 1974, Rick 1965). The casein solutions were prepared by heating 1 gm of casein in 100 ml of buffer at 100C for 15 min. After cooling, H_2O was added to bring the solution back to 100 ml. One ml of venom solution was incubated with 1.0 ml of prewarmed substrate. After 20 min incubation at 35C, 3.0 ml of 5% TCA was added. After 30 min at room temperature, the solutions were centrifuged for

20 min at 3000 to 4000 g and the absorbance of the supernatants was determined at 280 nm. The blank was prepared by adding 3.0 ml of TCA to 1.0 ml of substrate solution followed by 1.0 ml of venom solution. A protease unit (PU^{cas}) is defined as the amount of venom which under the conditions (20 min, 35C, final incubation mixture of 2 ml, final volume after TCA of 5 ml) liberates TCA-soluble products, so that at 280 nm the absorbance increases by 1.00 in 1 min.

$$\text{Specific activity} = \frac{\text{Abs. in 20 min}}{\text{mg of venom/ml} \times 20}$$

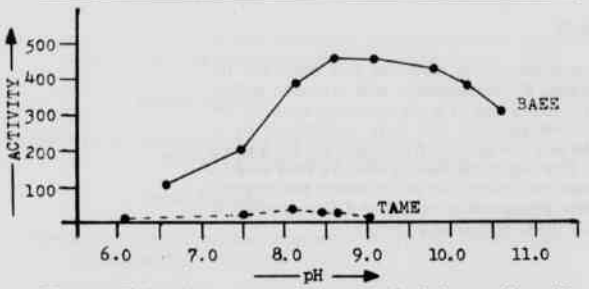

Figure 1. *H. horridum* venom arginine ester hydrolase activity with BAEE and TAME as substrates at various pH values of Tris • HCl (0.0667 M) buffer, 25C. Activities were calculated by methods described in text.

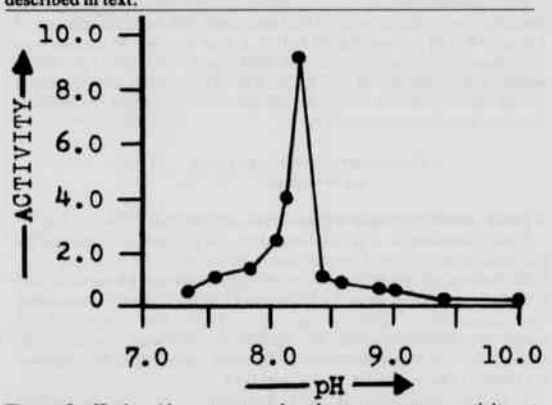

Figure 2. *H. horridum* venom phosphomonoesterase activity at various pH values. Activity is defined as the number of μM of p-nitrophenol liberated from p-nitrophenyl phosphate per min per mg of venom at 25C.

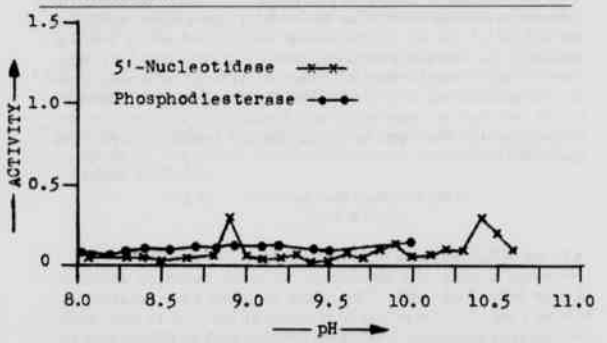

Figure 3. A comparison of *H. horridum* venom phosphodiesterase and 5'-nucleotidase activities at various pH values. Activity is defined as the number of μM of phosphate liberated from the substrate per min per mg of venom at 25C.

RESULTS AND DISCUSSION

Esterase, phosphomonoesterase, phosphodiesterase, 5'-nucleotidase, and protease activities were present in the venom-saliva mixture from the lizard *H. horridum*. Results plotted in Figure 1 show that hydrolysis of BAEE by *H. horridum* venom occurred at a pH optimum between 8.6 and 9.1 with a maximum activity of 452 units per mg per min. Hydrolysis of TAME occurred at a pH optimum between 8.1 and 8.5 with a maximum activity of only 36 units per mg per min (Fig. 1).

H. suspectum venom also hydrolyzes BAEE in preference to TAME (Tu et al. 1965, Tu and Murdock 1967, Mebs 1972). Mebs (1972) reported a pH optimum between 8.5 and 9.0 for BAEE and TAME hydrolysis by *H. suspectum* venom. Using snake venoms, Tu et al. (1965) reported that Crotalidae venoms have relatively high activities with BAEE and TAME substrates and that Viperidae venoms hydrolyze BAEE more rapidly than TAME. Neither BAEE nor TAME was hydrolyzed by Elapidae venoms.

One mg of *H. horridum* venom-saliva mixture liberated 9.3 μM of p-nitrophenol from p-nitrophenyl phosphate per minute at an optimum pH between 8.2 and 8.3 (Fig. 2). Although phosphomonoesterase has been observed in a variety of snake venoms (Gulland and Jackson 1938, Richards et al 1965) no report to the writers' knowledge has shown its presence in *Heloderma* venoms.

H. horridum phosphodiesterase and 5'-nucleotidase activities were low over a wide range of pH (Fig. 3). Phosphodiesterase activity is present also in *H. suspectum* venom (Styblova and Kornalik 1967) and in all snakes venoms tested (Russell 1972). 5'-nucleotidase is also common to most venoms (Russell 1972).

The *H. horridum* venom-saliva mixture had a trace of caseinolytic activity ($PU^{cas}_{mg} = 0.0015$/min) at pH 9.0. Higher proteinase activity was observed in *H. suspectum* venom (Styblova and Kornalik 1967, Mebs, 1972). Proteinases are also present in Crotalidae and Viperidae snake venoms although there is little or no proteinase activity in Elapidae and Hydrophiidae snake venoms (Russell 1972).

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