Biochemical Characterization of the Lizard Toxin Gilatoxin†

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ABSTRACT: The Gila monster (genus *Heloderma*) is the only known lizard to produce and inject a venomous secretion. Little is known about the venom from these lizards, and none of the toxins have been isolated until this time. This paper reports the isolation and characterization of a major lethal toxin (gilatoxin) from the venoms of *Heloderma suspectum* and *Heloderma horridum*. Gilatoxins from both species were similar in amino acid composition, electrophoretic mobility, p*I*, and immunological reactivity. They are acidic proteins possessing molecular weights of 35 000–37 500 and isoelectric points of 4.25 and consist of a single polypeptide chain. Neither is antigenically related to the venoms of snakes. The toxins are devoid of phospholipase A_2 activity and proteolytic, hemorrhagic, and hemolytic activities, with lethality being the only biological activity detectably expressed. The toxins appear to be unique and distinct from those of other venomous animals.

Only one genus of lizards (*Heloderma*) is proven to produce and inject a venomous secretion. The venom is a mixture of proteins containing several enzymatic activities (Tu & Murdock, 1967). The only component previously isolated in purity from Gila monster venom is bradykinin-releasing enzyme (Mebs, 1968). The crude venom is reported to be neurotoxic and hemorrhagic (Santesson, 1897; Loeb et al., 1913; Mebs, 1972). Although the majority of the enzymes have not been isolated in pure form, the crude venom shows a number of enzymatic activities with hyaluronidase and phosphodiesterase and arginine ester hydrolytic activities, peptidase and proteolytic activities being the most predominant (Mebs & Raudonat, 1966; Styblova & Kornalik, 1967; Tu & Murdock, 1967; Murdock, 1967; Mebs, 1968).

Heloderma venom has now been fractionated and the major toxin (gilatoxin) isolated. Gilatoxin was physically characterized and analyzed for associated enzymatic activity. Immunological relationships were examined by cross-reaction immunodiffusion experiments with antivenins from the families of poisonous snakes. The antigenicity of gilatoxin was investigated by antibody production in rabbits with subsequent cross-reaction to antigen from both *Heloderma suspectum* and *Heloderma horridum*. As judged by the immunological properties, amino acid composition, and molecular weight information, a new type of toxin has been isolated, and it is different from the well-characterized toxins of snakes, scorpions, spiders, and bees.

Materials and Methods

Crude *H. suspectum* and *H. horridum* venom was purchased from the Miami Serpentarium, Miami, FL. Spectropor dialysis tubing was from Fisher Scientific. Acrylamide and bis(acrylamide) (electrophoresis grade) were from Bio-Rad, Richmond, CA. Amino acid standards and amino acid analysis buffers (pH mixes and buffer concentrates) were from Pierce Chemical Co., Rockford, IL. Immunodiffusion disks and hyaluronidase were from Miles Laboratories. Trypsin and α-chymotrypsin were from Sigma, St. Louis, MO. Phospholipase A_2 was from Aldrich Chemical Co., Milwaukee, WI.

Isolation. The lethal fraction from *H. suspectum* and *H. horridum* was isolated by employing four steps, carried out at 4 °C. Crude venom (0.5–1.0 g) was dissolved in 5 mL of 0.05 M Tris-HCl, pH 8.5, containing 0.1 M NaCl and 0.002 M $CaCl_2$, applied to a Sephadex G-75 superfine column (5 × 90 cm), and separated by molecular sieve chromatography with a 55 mL/h flow rate, collecting 8-mL fractions (Figure 1A). The eluant flow was monitored by a Beckman DB-G spectrophotometer at 280 nm. The toxic fraction (Figure 1A, peak 2) was pooled, dialyzed, lyophilized, and rechromatographed according to the above procedure. The toxic fraction was pooled, dialyzed against water overnight, lyophilized, dissolved in 3 mL of 0.05 M Tris-HCl, pH 7.5, and applied to a DEAE-cellulose[1] ion-exchange column (2.5 × 10 cm) previously equilibrated with the same buffer. Separation was effected by using a 600-mL 0.5 M NaCl linear gradient with a flow rate of 25 mL/h and collecting 2.5-mL fractions (Figure 1B). The toxic fraction (Figure 1B, peak 6) was analyzed by analytical isotachophoresis and preparative isotachophoresis with the LKB Tachophor Model 2127 and LKB Uniphor Model 7900, respectively. The analytical system utilized an anionic electrolyte system consisting of 0.01 M HCl as the leading electrolyte and 0.01 M ε-aminocaproic acid as the trailing electrolyte with a current of 75 μA and a variable voltage of from 2 to 12 kV. Ampholine (pH 3.5–10) from Pharmacia was used as spacer ions. Preparative isotachophoresis used a Tris–H_2SO_4 buffer, pH 7.1, and a Tris–ε-aminocaproic acid buffer, pH 8.9, as the leading and trailing electrolytes, respectively. Current was maintained at 8 mA for 20–24 h with voltage ranging from 600 to 1500 V. Ampholine (2 mL of a 40% solution), pH range 3.5–10, was used as spacer ions (Figure 1C). The final step involved the LKB 7900 Uniphor for preparative electrophoresis by use of a 7.5% polyacrylamide gel with a 0.05 M Tris–glycine cathode buffer and a 0.05 M Tris-HCl anode buffer. The gel was run at 8 mA for 22 h, with 2-mL fractions collected and monitored at 280 nm with a Beckman DB-G spectrophotometer. Gilatoxin, showing one band by Tris–glycinate electrophoresis, was obtained by selective cutting of the shouldered peak (Figure 1D, peak 3; note insert showing gel).

Homogeneity. Tris–glycinate–polyacrylamide and β-alanine–polyacrylamide electrophoresis, utilized as criteria for

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; LD_{50}, lethal dose for 50% of the population; ip intraperitoneal; iv intravenous; DEAE, diethylaminoethyl; TAME, *p*-tosyl-L-arginine methyl ester; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; TRU/mg, turbidity reducing unit/milligram; Cl_3CCOOH, trichloroacetic acid.

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homogeneity, were according to Jovin et al. (1970). A 7% and 10% gel concentration was used for the Tris–glycinate system while a 7% gel concentration was used for the β-alanine system.

Molecular Weights. Molecular weights were determined by $NaDodSO_4$–polyacrylamide electrophoresis with the methods of Swank & Munkries (1971), Weber & Osborn (1969), Hedrick & Smith (1968), and Jovin et al. (1970). The Hedrick–Smith method used 7, 9, 12, and 15% polyacrylamide gel concentrations from which a Ferguson plot was constructed to determine the molecular weight. Gels were stained 1 h in 0.25% Coomassie Blue in 50% methanol and then diffusion destained in 1:3:10 acetic acid–isopropyl alcohol–water. Standards used were bovine serum albumin (68 000), ovalbumin (46 000), α-chymotrypsinogen (29 000), lysozyme (14 000), and cytochrome *c* (12 500). Isoelectric focusing with polyacrylamide gels was according to Horst et al. (1972) and by a modification of Jovin et al. (1970). The latter utilized the gel system of Jovin et al. (1970) but substituted glass-distilled water for the buffer system and incorporated 1 mL of 40% Ampholine (pH 3.5–10) for the pH gradient. Sulfuric acid (0.4% v/v) was used as the anode electrolyte, while 0.8% (v/v) monoethanolamine was used as the cathode electrolyte. The gels were maintained at 100 V for 1 h and then at 200 V for 2 h, or until the amperage had fallen to zero. Gels were soaked for 48 h in 20% Cl_3CCOOH with several changes, stained in 0.25% Coomassie Blue in 50% methanol for 1 h, and then diffusion destained. Concurrently, two blank gels were sliced in 2-mm sections and soaked in 1 mL of water for 24 h, and the pH was taken to establish the pH gradient throughout the gel.

Amino Acid Analysis. Amino acid analyses were carried out on a Model JLC-6AH amino acid analyzer. A minimum of three analyses from 24- and 72-h hydrolysates were used, and serine and threonine contents were obtained by the method of Moore & Stein (1963). Cysteine was measured by conversion to carboxymethylcysteine, and tryptophan was determined spectrophotometrically by the method of Edelhoch (1967). The analyzer was calibrated with a complete mixture of amino acids from Pierce Chemical Co. Due to the presence of carbohydrate, it was felt the data could be best presented by converting nanomoles to residues, with calculations based on the minimal number of residues. Phenylalanine was selected as 1.00, and the remaining residues were normalized to phenylalanine.

Reduction and Alkylation. Reduction and alkylation were carried out by dissolving 1–10 mg of protein in 1 mL of 8 M urea; 10 μL of 3 M Tris, pH 8.65, and 5 μL of β-mercaptoethanol were added, the tube was capped and wrapped in aluminum foil, and nitrogen was bubbled through for 10 min. The tubes were incubated at 37 °C for 6 h and cooled to 10–12 °C, and 30 mg of iodoacetamide, 1 mL of 3 M Tris, and 0.2 mL of 8 M urea were added. The tubes were kept in the dark and incubated for 30 min. The reaction was quenched by the addition of 15 μL of β-mercaptoethanol and dialyzed against glass-distilled H_2O.

Glycoprotein. Assays for glycoprotein were according to Segrest & Jackson (1972) to visualize glycoprotein on polyacrylamide gels by use of the periodic acid–Schiff reaction. Carbohydrate content was determined according to Hirs (1967).

Enzymes. Trypsin and chymotrypsin were assayed by the procedure of Walsh & Wilcox (1970) with *p*-tosyl-L-arginine methyl ester and *N*-benzoyl-L-tyrosine ethyl ester, respectively. Activity is expressed as units per milligram where 1 unit is equal to the hydrolysis of 1 μmol of substrate per min. Phospholipase A_2 activity was assayed by the method of Jeng et al. (1978). Activity is expressed as hydrolysis of moles per minute per milligram as determined by alkali consumption.

Hyaluronidase was assayed according to Tolksdorf et al. (1977) and Kass & Seastone (1977). Activity is expressed as turbidity reducing units/milligram = TRU/mg.

Immunology. Antibody against gilatoxin (antigilatoxin) was produced in 7–9-lb New Zealand white rabbits by intramuscular injection. The LD_{50} (intravenous injection) was calculated for animals of this weight in grams. One-tenth the LD_{50} was thoroughly emulsified with 5 mL of Freund's complete adjuvant. The emulsion was injected, containing 0.7 mg of gilatoxin (*Heloderma* major toxin), in six separate locations of the hind leg muscle. Three days later, another injection was made with the same amount of gilatoxin. Ten days elapsed to bleeding as this has been shown to be sufficient time for antibody production to reach high titer (Tu et al., 1980). At this time, blood was removed by ear vein puncture, red cells were removed by centrifugation, and the serum was tested by immunodiffusion against *Heloderma* crude venom and *Heloderma* major toxin. Gilatoxin, as necessary, was injected intraperitoneally (0.7 mg of gilatoxin in saline) to maintain high titer.

A solution of 10 mg/mL crude venom from *H. suspectum* and *H. horridum* was loaded in the center well. The outer wells contained solutions of antivenin to snakes from North, Central, and South America, Australia, Africa, and Southeast Asia and sea snakes. A control using Crotalidae venom in a well adjacent to Crotalidae antivenin was used to illustrate the effectiveness of the disk. The disks were incubated at 37 °C in high humidity for 24 h, deproteinized by washing with 0.9% saline in a motorized shaker for 24 h, desalinized by washing with water in the shaker for 24 h, and then stained for 15 min with 0.02% Amido Black. Destaining was accomplished by using acetic acid–isopropyl alcohol–water (1:3:10) with the motorized shaker. The disks were preserved by soaking in 2% glycerol.

Toxicity. Biological assays were carried out with white Swiss-Webster mice of 18–22-g weights. A constant 100-μL volume with varying protein concentrations was used for both intravenous and intraperitoneal injections. LD_{50} determinations were made with 5–10 mice per dose level with a 24-h time interval. Intraperitoneal injections were characterized by the method of Reed & Muench (1938), while later intraperitoneal and intravenous injections were characterized by the method of Litchfield & Wilcoxon (1949).

Hemorrhagic activity was assayed by the method of Bjarnason & Tu (1978) with 25–30-g Swiss-Webster mice. Protein was injected in 100 μL of 0.9% saline by a subcutaneous route. After 6 h, the mice were skinned, and hemorrhagic activity was noted.

Results

Purification. Isolation of the major lethal toxin (gilatoxin) from both species of *Heloderma* was achieved in similar fashion with the bulk of gilatoxin obtained in two steps. An additional two steps were incorporated to resolve a contaminant closely associated with the major toxin.

The first step involved fractionation of crude venom by using a Sephadex G-75 superfine column (Figure 1A). Biological assays show lethal activity occurring in a shouldered region seen on Figure 1A as peak 2. Tris–glycinate–polyacrylamide gels show major banding in the lower half of the gel, indicating the proteins possess a considerable negative charge, small size, or both. Peak 2 was rechromatographed, the resulting profile indicating that nothing was gained by this additional step. The

Table I: Yield and Activity of Purified Gilatoxins Isolated from the Venom of Two Species of *Heloderma*

venom	weight (g)	yield (%)[a]	LD_{50} (μg/g)	hemorrhage
H. suspectum (crude)			2.7	+
H. horridum (crude)			2.7	+
H. suspectum	0.5	4	2.9	−
H. suspectum	0.5	5	2.75	−
H. suspectum	1.0	5	2.75	−
H. horridum	0.5	3	2.6	−
H. horridum	0.5	3	2.6	−
H. horridum	0.5	4	2.6	−

[a] Percent of yield based on weight of proteins.

resulting peak was dialyzed, lyophilized, pooled, and applied on a DEAE-cellulose ion-exchange column (Figure 1B). Lethal activity was located in peak 6, a rather large peak, eluting from the column at 0.175 M NaCl concentration. Tris–glycinate–polyacrylamide gels show two major bands migrating very close to one another.

Preparative isotachophoresis was used to resolve these two bands, and the profile (Figure 1C) reveals a nonsymmetrical peak with inadequate resolution to separate the two proteins. Biological assays show lethal and hemorrhagic activity present under this peak.

The final purification step involved preparative electrophoresis (Figure 1D). Biological assays of selected tubes over the peak, corroborated by Tris–glycinate–polyacrylamide gels, indicate that hemorrhagic activity occurs in the ascending portion of Figure 1D, while lethal activity is found in the descending portion. With Tris–glycinate gels as a guide, the peak was selectively cut, separating the lethal protein from the hemorrhagic protein.

One of the primary concerns in the investigation of venom regards the yield of pure toxin. As Table I indicates, gilatoxin represents between 3 and 5% of the total protein content contained in crude venom.

Homogeneity. Capillary isotachophoresis was used to analyze the purification after DEAE-cellulose chromatography. Results indicate an almost symmetrical peak with a slight shoulder (Figure 2).

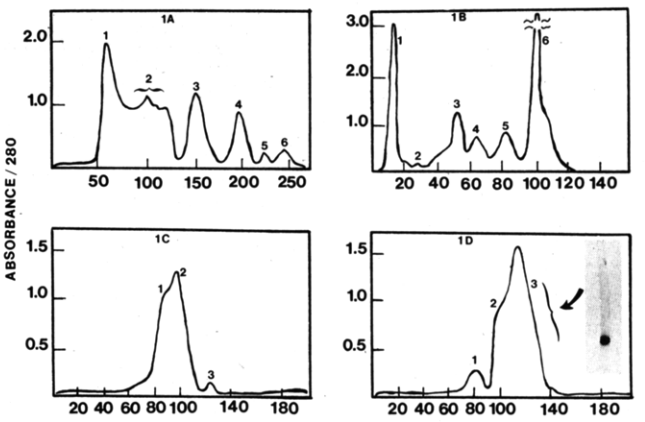

FIGURE 1: Fractionation profiles of *Heloderma* crude venom. (A) Crude *Heloderma* venom profile from Sephadex G-75 superfine column in 0.05 M Tris-HCl, pH 7.5, and 0.1 M NaCl. LD_{50} for peak 2 was found to be 2.7 μg/g in mice. (B) Peak 2 from (A) fractionated on DEAE-cellulose in 0.05 M Tris-HCl, pH 7.5, with a 600-mL 0.5 M linear gradient (NaCl). Peak 6 was lethal at 2.7 μg/g. (C) Peak 6 from (B) was fractionated by utilizing preparative isotachophoresis (see text for details). Toxicity of peaks 2 and 3 was 2.7 μg/g. (D) Peaks 2 and 3 from (C) were pooled and fractionated by preparative electrophoresis (see text for details). Gilatoxin was obtained by selective cutting over peak 3 from (D). Toxicity was assayed in mice, and the LD_{50} was found to be 2.7 μg/g.

Tris–glycinate–polyacrylamide and β-alanine–polyacrylamide gel electrophoresis shows *Heloderma* crude venom as a complex mixture of proteins. The Tris–glycinate system reveals 14–15 stainable bands, and the β-alanine system shows 4–6 stainable bands. Like snake venoms, the majority of the components are acidic and/or neutral proteins, with gilatoxin showing electrophoretic migration characteristics of an acidic protein. Upon completion of the fractionation procedure, one band, corresponding to gilatoxin, is present (insert, Figure 1D). This provides evidence that gilatoxin has been obtained in relatively pure form.

Glycoprotein. A positive periodic acid–Schiff reaction on polyacrylamide gels with 8, 10, and 12% gel concentrations confirmed the presence of a carbohydrate moiety. For determination of the extent of carbohydrate, the sulfuric acid–

Table II: Amino Acid Composition of Gilatoxin from *Heloderma suspectum*

amino[a] acid	min residues based on Phe = 1.0	residues/100 residues based on Phe = 1.0	wt contribution of min residues based on Phe = 1.0	residues/mol[d] for 32 200 M_r protein	nearest integer
Lys	1.81	4.38	232.02	12.99	13
His	1.45	3.51	198.88	10.41	10
Arg	1.00	2.42	156.20	7.18	7
Cm-Cys[b]	2.40	5.41	386.76	17.23	17
Asx	4.04	9.78	465.00	29.07	29
Thr	2.09	5.05	211.34	15.01	15
Ser	3.63	8.78	316.14	26.06	26
Glx	3.16	7.64	408.05	22.69	23
Pro	2.22	5.37	215.63	15.94	16
Gly	5.27	12.75	300.76	37.84	38
Ala	2.33	5.64	165.64	16.73	17
Val	3.11	7.52	308.36	22.33	22
Met	1.05	2.54	137.77	7.54	8
Ile	2.34	5.66	264.84	16.80	17
Leu	3.12	7.55	353.12	22.40	22
Tyr	1.32	3.19	215.41	9.48	9
Phe	1.01	2.44	148.66	7.25	7
Trp[c]				4.00	4
			4484.58[e]		311[e]

[a] Serine and threonine were obtained by the method of Moore & Stein (1963). [b] Carboxymethylcysteine was determined on separate samples of gilatoxin after reduction and alkylation. [c] Tryptophan was determined spectrophotometrically by the method of Edelhoch (1967). [d] Residues/mole was calculated by the following equation: (minimum residues)(observed molecular weight)/(minimal molecular weight) = residues/mole. [e] Total.

Table III: Amino Acid Composition of *Heloderma horridum*

amino[a] acid	min residues based on Phe = 1.0	residues/100 residues based on Phe = 1.0	wt contribution of min residues based on Phe = 1.0	residues/mol[d] for 32 200 M_r protein	nearest integer
Lys	2.09	4.68	267.92	13.99	14
His	2.07	4.63	283.92	13.85	14
Arg	1.07	2.39	167.13	7.16	7
Cm-Cys[b]	2.41	4.97	388.37	16.13	16
Asx	3.97	8.88	456.95	26.57	27
Thr	2.04	4.56	206.29	13.66	14
Ser	4.27	9.55	371.87	28.58	29
Glx	4.32	9.66	557.84	28.92	29
Pro	2.50	5.59	242.83	16.73	17
Gly	6.23	13.94	355.55	41.70	42
Ala	2.48	5.55	176.30	16.60	17
Val	3.02	6.76	299.43	20.22	20
Met	1.00	2.24	131.21	6.69	7
Ile	2.15	4.81	243.34	14.39	14
Leu	3.03	6.78	342.94	20.28	20
Tyr	1.05	2.35	171.35	7.03	7
Phe	1.00	2.24	147.19	6.69	7
Trp[c]					
			4810.43[e]		302[e]

[a] Serine and threonine were obtained by the method of Moore & Stein (1963). [b] Carboxymethylcysteine was determined on separate samples of gilatoxin after reduction and alkylation. [c] Tryptophan was determined spectrophotometrically by the method of Edelhoch (1967). [d] Residues/mole was calculated by the following equation: (minimum residues)(observed molecular weight)/(minimal molecular weight) = residues/mole. [e] Total.

Table IV: Enzymatic Activity of *Heloderma suspectum* and *Heloderma horridum* Crude Venom and Gilatoxin

enzyme and substrate	*H. suspectum* crude venom	*H. horridum* crude venom	*H. suspectum* gilatoxin	*H. horridum* gilatoxin
proteolytic[a]				
TAME	241	251	0.90	1.10
BTEE	1.0	2.5	0.03	0.05
hyaluronidase[b]				
hyaluronic acid	54	52	0.00	0.00
phospholipase A_2[c]				
egg yolk	2.0×10^{-6}	3.6×10^{-6}	0.00	0.00

[a] Assay expressed in units/mg, 1 TAME/BTEE unit being equal to the hydrolysis of 1 μmol of substrate/min. Trypsin expresses an activity of 250 TAME units/mg. α-Chymotrypsin expresses an activity of 60 units/mg. [b] Assay expressed as turbidity reducing units/mg (TRU/mg). Hyaluronidase expresses an activity of 50 TRU/mg. [c] Assay expressed as the hydrolysis of mol min^{-1} mg^{-1} as determined by alkali consumption. Phospholipase A_2 from *C. adamanteus* expresses an activity of 1.6×10^{-3} mol min^{-1} mg^{-1}.

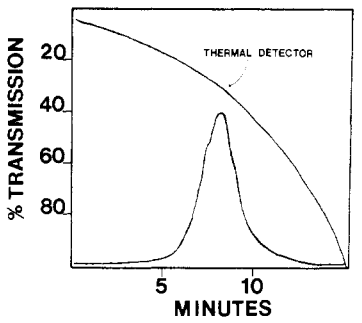

FIGURE 2: Capillary analytical isotachophoresis of 20 μg of purified gilatoxin.

phenol reaction of Hirs (1967) was used, with the results showing approximately 8% of the weight being accountable as carbohydrate.

Amino Acid Composition. The amino acid composition, given in Tables II and III, for both species of *Heloderma* was calculated on the basis of the smallest observed molecular weight from $NaDodSO_4$ gel electrophoresis. Since both toxins were shown to possess 8% carbohydrate, this value was subtracted from the observed molecular weight of 35 000 to give a value of 32 200 g/mol. The composition shows relatively high quantities of aspartic and glutamic acids, which would be expected with the acidic p*I* and electrophoretic mobility in Tris–glycinate gels. The most important aspect of these comparisons is the large amount of histidine present in both species. Carboxymethylation shows 16 carboxymethylcysteines present in *H. horridum* and 17 in *H. suspectum*. Amino acid composition adds more support to the similarity of the two gilatoxins.

Molecular Weights. The molecular weight of the lethal component from both *H. suspectum* and *H. horridum* was determined to be 35 000–37 000. All $NaDodSO_4$ electrophoretic systems used (with and without urea) corroborated this figure as did the Ferguson plot. Also, reduction/alkylation does not show differences in molecular weight, indicating that gilatoxin does not possess subunit structure.

The isoelectric point of the toxic fraction from both species of *Heloderma* was found to be pH 4.25–4.30 with an extinction coefficient of 2.64×10^7 M^{-1} cm^{-1}.

Enzymatic Activity. Gila monster crude venom possesses several enzymes. In order to clarify whether purified gilatoxin is associated with any of these enzymatic activities, proteolytic activities with TAME and BTEE as substrates and hyaluronidase were investigated (Table IV). None of these enzyme activities were found in the purified toxin from the venom of either species.

One of the most common enzymatic activities found associated with snake presynaptic neurotoxins is phospholipase A_2. Also, some snake neurotoxins, e.g., crotoxin and mojave toxin,

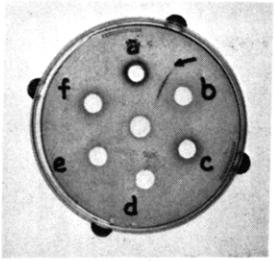

FIGURE 3: Ouchterlony immunodiffusion plates showing negative antigenic relationship between *Heloderma* crude venom and the antivenin from (b) Crotalidae (pit vipers), (c) Viperidae (vipers of South and Central Africa), (d) Elapidae (king cobra), (e) Elapidae (*Naja naja atra*), (f) Hydrophiidae (*Enhydrina shistosa*), and (a) control containing *Crotalus atrox* crude venom. Note the precipitin reaction to Crotalidae antibody.

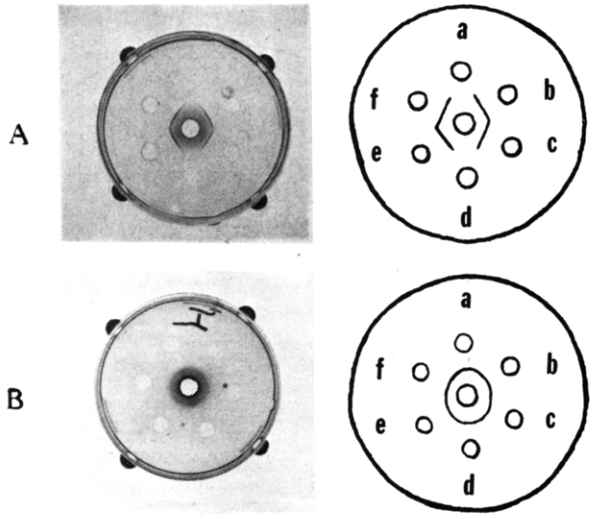

FIGURE 4: Ouchterlony immunodiffusion plates showing antigenic similarity of gilatoxin from *Heloderma suspectum* and *Heloderma horridum*. (A) Center well filled with antibody to gilatoxin from *H. horridum*; wells a and d filled with 0.9% saline. Wells b and c contain gilatoxin from *H. suspectum*, and wells e anf f contain gilatoxin from *H. horridum*. (B) Center well filled with antibody to gilatoxin from *H. horridum*; wells a, b, and c filled with gilatoxin from *H. suspectum*. Wells d, e, and f filled with gilatoxin from *H. horridum*. Note complete fusion of lines around the center well, indicating antigenic identity. Stylized drawings utilized to clearly indicate the results of these plates.

are known to have hemolytic activity. Both phospholipase A_2 activity and hemolytic activity (direct and indirect) were assayed. The results indicate that gilatoxin does not possess either of these activities.

Since these are the most common enzymes found associated with toxins and since gilatoxin from both species is lacking in all of them, it would appear that the toxic action is probably nonenzymatic.

Immunology. In order to ascertain that gilatoxin is not identical with snake venom components, immunodiffusion tests against different snake venoms were made. Immunodiffusion (Figure 3) shows that neither *Heloderma* crude venom nor *Heloderma* major toxin is immunologically related to the venom of Crotalidae, Viperidae, Elapidae, or Hydrophiidae, thus eliminating antigenic similarities with the poisonous snakes. Since no antivenin exists against the Gila monster, the antigenicity of gilatoxin was tested. Gilatoxin possesses the ability to elicit the immune response in rabbits with consequent production of antibodies as shown by Figure 4. Note that *H. suspectum* and *H. horridum* cross-react with one another with a fusion of lines. This evidence further corroborates the similarity of the major toxins from both species.

Table V: Synergistic Activity of *Heloderma* Venom As Determined by Recombination of DEAE-cellulose Fractions

combinations	LD_{50} (μg/g)	relative toxicity
crude venom	2.75	1.0
fraction: 1	>5	<0.5
2	>5	<0.5
3	>5	<0.5
4	>5	<0.5
5	>5	<0.5
6	2.75	1.0
fractions 1–5	>5	<0.5
fraction: 1 + 6	1.25	2.2
2 + 6	1.30	2.1
3 + 6	sublethal	<0.5
4 + 6	sublethal	<0.5
5 + 6	sublethal	<0.5

Lethality. Biological activity assayed by ip and iv routes of injection shows an LD_{50} for crude venom at 2.7 μg/g. Early in this investigation, DEAE-cellulose peak 6 was used, and, from gross observation, the mice which succumbed to the venom within 1–3 h exhibited the general signs of neuromuscular blockade, i.e., labored breathing and paralysis of locomotion, with the hind limbs being predominantly affected. Those which did not succumb demonstrated dramatic exopthalmia and were found to reverse this condition in 15–24 h. This condition raised questions about the contaminant as it was believed that exopthalmia was caused by the closely migrating protein. When the toxin was obtained in purest form, exopthalmia ceased, and hemorrhagic activity, associated with the major toxin, became negligible.

One interesting point is that the major toxin does not increase in potency on purification. The LD_{50} of the purified gilatoxin remains identical with that of crude venom, 2.7 μg/g. The reason for this remains uncertain; however, one possible explanation could be synergistic effects among the protein constituents of the crude venom. Gilatoxin, on a weight basis, actually loses toxicity on purification. Yet, by interaction with other venom proteins, toxicity is enhanced. The effects of recombination were investigated with the results shown in Table V. None of the peaks from DEAE-cellulose (except peak 6) singly, or in combination, were toxic. However, when peak 6 was added, toxicity was observed, and in combination with peaks 1 and 2 the LD_{50} was lowered from 2.7 to 1.25 μg/g. Neither peak 1 nor peak 2, in and of themselves, could elicit toxic action at 100-μg levels. There appears to be collective or synergistic activity among the proteins found in the DEAE-cellulose purification step.

Discussion

The majority of venom studies have concentrated on the venoms of poisonous snakes, especially those possessing powerful neurotoxins (Tu, 1973, 1977). Recently, toxins other than neurotoxins have been isolated, including cytotoxins, myotoxins, and hemorrhagic toxins. By comparison with the prolific amount of work on snake venoms, scant information is available concerning the Gila monster or beaded lizard of the southwestern United States and Mexico. One reason the lizards have not been studied is the difficulty of obtaining sufficient quantities of venom and their relatively innocuous nature.

Physically and chemically, gilatoxin most closely resembles hemorrhagic toxin *e* from *Crotalus atrox* (Bjarnason & Tu, 1978) and crotoxin from *Crotalus durissus terrificus* (Hendon & Fraenkel-Conrat, 1971). Common characteristics include molecular weights in excess of 20 000 and acidic isoelectric points. However, notable differences occur in that gilatoxin

is devoid of phospholipase A_2 (characteristic of presynaptic neurotoxins), hemorrhagic, and hemolytic activities. Also, the LD_{50} for gilatoxin is considerably high (2.5 μg/g vs. 0.06 μg/g for crotoxin) for a presynaptic neurotoxin, even though gross observation of envenomated animals shows neurotoxic symptoms. No evidence of subunit structure was found, and the most striking chemical difference occurs in the amino acid composition. Gilatoxin possesses a rather large amount of histidine, 5–7 times the quantity occurring in the venomous snakes.

Although LD_{50} values indicate a relatively weak toxin by comparison, this animal is considered highly dangerous because of the many fatal cases resulting from envenomation. This lends credence to the possibility of collective effects among the various venom components with consequent enhanced venom potency. This is further supported by recombination experiments which were found to decrease the LD_{50} by a factor of two.

One of the more dramatic effects of Gila monster envenomation is severe exopthalmia. The cause of this condition is unclear, although it associates with the major lethal toxin until removal of the hemorrhagic protein.

No immunological identity could be shown to venoms from sea snakes, cobras, vipers, or pit vipers. The antigenicity of gilatoxin is strong, with antibody production easily elicited in rabbits, probably due to the high molecular weight of this protein. Most important is the result that antigilatoxin can be produced to afford protection against lizard envenomation.

Although there is a close taxonomic relationship between snakes and lizards, it is not reflected in their main toxins. Gilatoxin, therefore, appears unique, not only from the toxins of poisonous snakes but also from those of scorpions, spiders, and bees.

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