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ENZIMAS HIDROLITICAS DEL VENENO DE *Heloderma horridum horridum*: FOSFOLIPASA A2 Y CALICREINA

T E S I S

QUE PARA OBTENER EL TITULO DE:

Doctor en Investigación Biomédica Básica

P R E S E N T A :

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PRESENTACION

Esta tesis se basa en parte de las investigaciónes que hemos realizado sobre la bioquímica del veneno del Monstruo de Cuentas (Heloderma horridum) y la componen los siguientes artículos:

1. Alagón, A. C., Maldonado, M. E. A., Julià, J. Z., Sánchez, C. R. y Possani, L. D. (1982) Venom from two sub-species of Heloderma horridum (Mexican Beaded Lizard): General characterization and purification of N-benzoyl-L-arginine ethyl ester hydrolase. Toxicon 20: 463-475.

2. Sosa, B. P., Alagón, A. C., Martin, B. M. y Possani, L. D. (1986) Biochemical characterization of the phospholipase A2 purified from the venom of the Mexican Beaded Lizard (Heloderma horridum horridum Wiegmann) Biochemistry (USA) 25: 2927-2933.

3. Alagón, A. C., Possani, L. D., Smart, J. y Schleuning, W. D. (1986) Helodermatine, a kallikrein-like, hypotensive enzyme from the venom of Heloderma horridum horridum (Mexican Beaded Lizard). J. Exp. Med. 164: 1835-1845.

Los estudios que aquí se presentan los iniciamos hace 10 años en el laboratorio del Dr. Lourival D. Possani en el entonces Departamento de Biología Experimental del Instituto de Biología. Comenzamos desde la captura de los animales y aprendiendo su manipulación y manutención en cautiverio, así como la técnica para extraeles el veneno. Esta labor inicial hubiera sido muy difícil si no hubiésemos contado con la ayuda del finado Profesor Jordi Julià Zertuche y del Biólogo Cornelio Sánchez Reyna.

Por aquellas fechas, el veneno de los Heloderma era poco conocido, por lo menos, desde el punto de vista molecular. Desde entonces, varios grupos de investigación han caracterizado varios de sus componentes: dos toxinas (Hendón & Tu, 1981; Mochca, 1988), una hialuronidasa (Tu & Hendon, 1983), varios péptidos relacionados con la familia de la secretina y del péptido

intestinal vasoactivo (Parker et al.,1984; Robberecht et al., 1984), una fosfolipasa A2 (Sosa, 1984; Sosa et al., 1986) y dos hidrolasas de ésteres de arginina (Alagón et al., 1982) identificadas posteriormente una como calicreína (Alagón et al., 1986) y la otra como una proteasa capaz de activar plasminógeno (González, 1983; Sosa, 1988).

Nuestras investigaciones en este campo se han desarrollado en forma intermitente y en paralelo con otros proyectos; sin embargo, han tenido una influencia definitiva en los proyectos que actualmente realizamos. El conocimiento y experiencia logrados con los tripéptidos sintéticos empleados en la caracterización de la calicreína nos han permitido plantear alternativas experimentales para la generación de señales fluorescentes en ensayos de hibridación por ácidos nucleicos. La caracterización de la proteasa que activa plasminógeno nos ha facilitado el estudio de los principios fibrinolíticos presentes en los trofozoitos de Entamoeba histolytica (Alagón et al, 1988; sometido para su publicación en Molecular Microbiology) y la saliva del vampiro Desmodus rotundus. El manejo de la fosfolipasa A2 fue muy importante como modelo para desarrollar varias técnicas novedosas de purificación (patente en proceso) y análisis de este tipo de enzimas que estamos usando con éxito en la caracterización de enzimas membranales similares de la amiba.

Los estudios realizados con el veneno de este saurio han servido para la graduación de dos estudiantes de licenciatura (González, 1983; Sosa, 1984) y uno de maestría (Sosa, 1988). Debemos mencionar que la tesis de Sosa (1984) comprende parte de los resultados formalizados en el artículo de Sosa et al (1986) que forma parte de la la presente tesis. Las dos restantes cubren materia que no estamos presentando en ésta.

Un producto colateral de nuestros trabajos con este veneno fue la elaboración de un suero antiheloderma en borregos, el cual fue procesado en colaboración con el Instituto Nacional de Higiene de la SSA para su empleo en humanos. De hecho, ya se usó

exitosamente para tratar a un médico veterinario accidentado en el Zoológico de Chapultepec de la Ciudad de México. Sobra decir que este suero es de mayor importancia para las personas que manejamos helodermas.

ASPECTOS DESTACABLES DE LOS ARTICULOS

Sobre el primer artículo (Alagón et al., 1982):

El trabajo compara el veneno de dos subespecies de Heloderma horridum: la típica horridum, de distribución muy amplia, y la alvarezi, localizada en la región central del Estado de Chiapas. Los venenos de las dos subespecies resultaron muy similares respecto a su toxicidad en ratones, a su contenido de varias actividades enzimáticas (fosfolipasa, hialuronidasa, e hidrolasas de ésteres de tirosina y arginina), y a sus patrones electroforéticos y de inmunodifusión.

La hidrolasa de ésteres de arginina con mayor peso molecular fue, también, purificada por medio de un esquema más bien ineficiente. No obstante, fue el primer componente de los venenos de Heloderma que haya sido purificado a homogeneidad.

Sobre el segundo artículo (Sosa et al., 1986):

En este trabajo purificamos y caracterizamos la fosfolipasa del veneno del saurio objeto de nuestros estudios. Los intentos para aislarla, usando un medio de afinidad descrito en la literatura, fueron infructuosos, por lo que recurrimos a cromatografía por interacción hidrofóbica en fenil-Sefarosa y otros tipos de cromatografía convencionales.

Pudimos definir su especificidad, del tipo A2, a través del análisis de los productos de hidrólisis de substratos sintéticos. Su actividad mostró dependencia a Ca^{++}. Se trata de una glicoproteína acídica de 19 Kda. Determinamos la secuencia de los primeros 39 aminoácidos a partir de su extremo amino. La

secuencia de esta región es distinta a la reportada para otras fosfolipasas A2 purificadas de páncreas de mamíferos y de venenos de crotálidos y elápidos, y, sin embargo, es homóloga (56%) a la fosfolipasa A2 del veneno de la abeja común. Al alinearla, usando los residuos de cisteína como puntos de referencia, nos fue posible identificar varios aminoácidos que han sido postulados, en las otras fosfolipasas A2, como importantes en el pegado del Ca^{++}.

Sin ser materia de ésta tesis, quisiéramos comentar que hemos continuado su secuenciación; en este momento nos falta por definir menos del 10% del total de la molécula. Nos resulta muy interesante el que, aun sin que hayamos realizado un estudio por computadora, la secuencia nueva parece ser más similar a las de páncreas y de venenos de serpientes; como si se tratara de una proteína híbrida, intermedia, entre las fosfolipasas A2 conocidas.

Este trabajo despertó interés en los dos grupos que más han estudiado, estructuralmente, a las fosfolipasas A2, por lo que nos hemos permitido anexar sus comentarios.

Sobre el tercer artículo (Alagón et al., 1986):

En este artículo reportamos la purificación y caracterización de una endoproteinasa de 63 Kda, la Helodermatina, presente en el veneno del Heloderma horridum horridum. La purificación de la Helodermatina fue lograda en dos pasos: cromatografía de afinidad a través de benzamidina-Sefarosa y de intercambio aniónico en DEAE-celulosa. La proteína resultante fue homogénea en electroforesis en geles de poliacrilamida; el rendimiento del derivado del primer aminoácido, cuando sujeta a degradación de Edman, fue mayor de 90% A través de secuenciación automática se identificaron 19 de los primeros 21 residuos y encontramos que esta región es significativamente homóloga a la calicreína pancreática porcina y a las enzimas semejantes a calicreína de los veneno de Crotalus

atrox y C. adamanteus.

También obtuvimos varias constantes cinéticas con tripéptidos sintéticos (S-2302, S-2266, S-2160 y S-2227) como substratos específicos. Estos datos clasifican a la Helodermatina como una enzima semejante a calicreína, concepto que se ve apoyado por la fuerte inhibición de la enzima por los inhibidores de tripsina pancreático (del tipo Kunitz, aprotinina, Trasylol) y de soya.

Por último, también mostramos que la Helodermatina es una quininogenasa muy potente, es decir, es capaz de liberar quininas vasoactivas a partir de quininógeno y producir hipotensión sanguínea. Así, por criterios estructurales, enzimáticos y fisiológicos pudimos concluír que la Helodermatina pertenece a la familia de las calicreínas, siendo la primera enzima descrita en saurios con tales propiedades.

PERSPECTIVAS

Nuestras áreas de interés actuales están centradas en la parasitología molecular, en particular con la caracterización, por métodos bioquímicos y de biología molecular, de proteínas que pudieran estar interviniendo en los mecanismos patogénicos de Entamoeba histolytica, y, asimismo, con el desarrollo de alternativas para generar señales no-radioactivas aplicables a sistemas de detección de parásitos que emplean hibridación de ácidos nucléicos. Sin embargo, queremos finalizar dos proyectos con el veneno de Heloderma:

1. Estamos complentando, en colaboración con los Dres. L. D. Possani y B. Martin, la estructura primaria de la fosfolipasa A2 y tenemos planeado un estudio comparativo por computadora que sirva para definir su homología con otras enzimas análogas. Dado el interés por el Dr. B. Dijstra para analizar a la fosfolipasa por difracción de rayos X, estamos intentado la obtención de buenos cristales para su envío a Holanda.

2. Nuestras investigaciones con la proteasa que es capaz de activar plasminógeno (Helodermatidina) han demostrado que en el sistema del mamífero lo hace en forma similar a urocinasa pero con una actividad específica muy baja, por lo que podemos descartarlo como un activador de plasminógeno verdadero (Sosa, 1988). Entonces, ¿cuál es su función biológica?. Queremos contestar esta pregunta. En este sentido tenemos indicaciones preliminares que apuntan a que su eficiencia es muy alta empleando plasminógeno de pollo como substrato, por lo que podemos postular se trata de un componente digestivo más bien que tóxico. Esta hipótesis resulta más evidente a la luz de que los Helodermas tienen como alimento principal huevos de aves (Bogert y Martín del Campo, 1956) y que las yemas de los mismos son muy ricas en plasminógeno (Valinsky y Reich, 1981).

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VENOM FROM TWO SUB-SPECIES OF *HELODERMA HORRIDUM* (MEXICAN BEADED LIZARD): GENERAL CHARACTERIZATION AND PURIFICATION OF *N*-BENZOYL-L-ARGININE ETHYL ESTER HYDROLASE

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A. C. ALAGÓN, M. E. A. MALDONADO, J. Z. JULIÀ, C. R. SÁNCHEZ and L. D. POSSANI. Venom from two sub-species of *Heloderma horridum* (Mexican beaded lizard): General characterization and purification of *N*-benzoyl-L-arginine ethyl ester hydrolase. *Toxicon*, 20, 463–475, 1982.—The venom from the lizards *Heloderma horridum horridum* and *Heloderma horridum alvarezi* was obtained at a protein concentration of 80 mg/ml with a pH value of 6.9–7.0. The volume of venom obtained is approximately 0.5 ml per extraction. The i.p. LD_{50} value in mice for both sub-species is 2 mg/kg body weight. The electrophoretic pattern of the venom applied to polyacrylamide gels shows at least 18 protein bands and this pattern is constant for the same animal during all 12 months of the year, although different animals from the same population may present a slightly different pattern. The venom has the following enzymatic activities: phospholipase A, hyaluronidase, and Bz-Arg-OEt and Bz-Tyr-OEt hydrolase. Some of the venom components can be selectively and reversibly precipitated at acidic pH (4.7). The venom is very immunogenic and the sheep anti-sera against both sub-species cross-react quite extensively. A Bz-Arg-OEt hydrolase was purified from the venom of *H. h. horridum* by column chromatography in Sephadex G-75 followed by two steps on DEAE-cellulose columns at two different pH values (7.55 and 8.6). The last step was chromatography in a phenyl-sepharose column. The molecular weight of this enzyme, as obtained by SDS-gel electrophoresis, is approx. 65,000.

INTRODUCTION

THERE are only two species of venomous lizards in the world: *Heloderma suspectum*, with two sub-species, *H. suspectum suspectum* and *H. suspectum cinctum*, distributed in Arizona (USA) and the desert of Sonora in Mexico, and *H. horridum*, with three sub-species, *H. horridum exasperatum*, *H. horridum horridum* and *H. horridum alvarezi*, mainly distributed in the Pacific region of Mexico. As early as 1864, Sumichrast [mentioned by LOEB (1913)] reported some of the physiological effects of the venom from these lizards. Leo Loeb, in 1913, published a book dealing with the venom of *Heloderma*. Several publications can be found in

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Abbreviations used: Bz-Arg-OEt, *N*-benzoyl-L-arginine ethyl ester; Bz-Tyr-OEt, *N*-benzoyl-L-tyrosine ethyl ester; SDS, sodium dodecyl sulphate.

the literature on the general effects associated with gila monsters (TINKHAM, 1956; BOGERT and MARTIN DEL CAMPO 1956; TU, 1977). MEBS and RAUDONAT (1966), TU and MURDOCK (1967) and STÝBLOVÁ and KORNALIK (1967) have published a general biochemical characterization of the *H. suspectum* venom. MEBS (1972) reported the isolation of a kinin-releasing enzyme as well as *N*-benzyoyl-L-arginine-ethyl ester hydrolase from the *H. suspectum* venom. Despite this literature, there are no chemical data on any of the components isolated or characterized from the Mexican sub-species of *Heloderma* (*exasperatum, horridum* or *alvarezi*). In this communication we report several observations and properties related to the venom of *H. horridum horridum* and *H. horridum alvarezi*, as well as the purification of an enzyme from *H. horridum horridum*. Part of this work was presented as an abstract during the meeting of the Mexican Society for Biochemistry (October 1978, San Luis Potosi, México).

MATERIAL AND METHODS

Venum

The venom was pipetted directly from the mouth of the lizards by the technique described by LEOB (1913). The *H. horridum horridum* were collected by us in the field (Estación Experimental de Chamela, State of Jalisco, México) and the *H. horridum alvarezi* were a gift from Professor Miguel Alvarez del Toro (Zoological Museum of Tuxtla Gutierrez, Chiapas, México). The freshly collected venom (in ice) was centrifuged at 18,000 *g* for 10 min in a Sorvall Centrifuge (rotor SS-34, r_{max} = 10.8 cm) and the supernatant (soluble venom) was directly used or lyophilized and stored at − 20°C until needed.

Chemicals and resins

Only analytical grade reagents and solvents were used. Sephadex G-75 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Diethyl-aminoethyl-cellulose (DEAE-Cellulose), type DE-32 was from Whatman Inc., (Clifton, NJ, U.S.A.). Hyaluronic acid was from Nutritional Biochemical Co. Bz-Arg-OEt and Bz-Tyr-OEt were from Sigma Chemical Co.

Lethality and enzymatic assays

Lethality tests were conducted by i.p. injection of 0.1–0.3 ml solution into albino mice (Mexican strain). The LD_{50} of the soluble venom or its fractions were obtained graphically by plotting the % of deaths at 20 hr vs the logarithm of the dose (ten mice at each dose and nine to eleven different doses of increasing concentration). To define the toxicity of the various chromatographic components the following definitions were used. "Lethal" means that the component at the dose injected was enough to kill the mouse within 20 hr after injection. "Toxic" means that the mouse showed any of the following symptoms: excitability, sporadic convulsions, hemorrhage in the eye or dyspnea, but the mouse did not die within the first 20 hr after injection. "Non-toxic" means normal behaviour, similar to injection of 0.9% NaCl.

Phospholipase A activity was determined by a titrimetric procedure with egg yolk as substrate (SHILOAH *et al.*, 1973). One unit of phospholipase activity is defined as the amount of enzyme releasing 1 μmole of acid per min at pH 8 and 25°C in 3.0 ml of a 10% (w/v) solution of egg yolk in 0.1 M NaCl.

Hyaluronidase activity was measured by a turbidimetric method (TOLKSDORF *et al.*, 1949) at 25°C. One unit of activity is defined as the amount of enzyme required to hydrolyse 1 μg of hyaluronic acid per min at 25°C in 200 μl of a 200 μg/ml solution of hyaluronic acid in pH 5.3 buffer. Esterase activity was measured spectrophotometrically with benzoyl esters (Bz-Arg-OEt and Bz-Tyr-OEt) as substrates. These assays were carried out at 25°C by adding the enzyme in solution to a cuvette containing 50 mM $CaCl_2$, 1 mM Bz-Arg-OEt or 0.5 mM Tris–HCl buffer (pH 7.8) and measuring the absorbance at 255 nm at time intervals of 30 sec for 3–5 min periods in 1.5 ml total volume. One unit of enzymic activity is defined as the amount of protein needed to hydrolyse 1 μmole of substrate in 1 min in the above conditions. Dialysis to remove excess salts after DEAE-cellulose chromatography was performed against the proper buffer solution in Spectrapor type 3 dialysis tubing (molecular-weight 'cut-off' approx. 3500; Spectrum Medical Industries). Protein concentrations, unless otherwise specified, were measured by spectrophotometry, assuming that $1\ A_{280}^{1cm}$ unit = 1 mg/ml. Columns were run at room temperature (25°C).

Electrophoresis

Discontinuous electrophoresis in polyacrylamide gel (Bio-Rad Laboratories) was performed by the method of JOVIN and his co-workers (1964), and gels were stained with Coomassie Brilliant Blue G-250 (Serva Laboratories) (RESNER *et al.*, 1975). SDS polyacrylamide gels were run in cylindrical tubes containing 12% acrylamide, 0.6% methylene bisacrylamide and 0.2% SDS (SWANK and MUNKRES, 1971). Slab gel electrophoresis in

polyacrylamide was run according to the technique described by SHUTER (1971) in a Canalco apparatus (Prep. Disc Instruction Corporation, Rockville, Maryland, 1968).

Immunological studies

Specific sheep anti-venom sera were prepared using three sheep (*Ovis aries*), commonly named "peligüey" in México. Each sheep received ten injections of increasing concentrations of venom: 0.1, 0.5, 1.0, 5.0, 10.0 and repeated doses of 25.0 mg respectively, injected every week. The total volume of venom injected each time was adjusted to 1 ml with 0.1 M Tris–HCl buffer, pH 7.95, and emulsified with an equal volume of complete Freund's adjuvant. From two to three sites were chosen for s.c. injection behind the neck of the animals. Bleedings were made on weeks four, five, six, eight and ten, and several subsequent weeks. Sera of the 6th-week bleeding were already useful for immunodiffusion studies. Immunodiffusion was carried out in 0.8% purified agar containing 0.1 M Tris–HCl buffer, pH 7.95; wells of 4 mm diameter were spaced 10 mm apart from center to center. The gels were run at room temperature for 48 hr and when needed washed, dried and stained with amido black (1% in 10% acetic acid).

Acid precipitation treatment

The venom was adjusted to a final concentration of 2–80 mg/ml in a final buffer concentration of 20 mM ammonium acetate, pH 4.7. The mixture of venom and buffer was allowed to stand for 10 min at 25°C and then centrifuged for 20 min. The rotor (Sorvall no. SS-34) was operated at 4°C and 25,000 *g* (r_{max} = 10.8 cm). The precipitate was resuspended in 25 mM potassium phosphate buffer, pH 7.6, and both fractions (supernatant and resuspended precipitate) were read at 280 nm for calculating the percentage of reversible precipitation. For the purification of the enzyme we have chosen a venom concentration of 10 mg/ml for the acidic precipitation.

RESULTS

General characterization of the venom from H. horridum horridum and H. horridum alvarezi

Mice injected with the venom from both sub-species of lizards present the same symptoms of intoxication: dyspnea, sporadic convulsions, hemorrhage in the eye and in the gastrointestinal tract, congestion and edema in the lungs and finally respiratory failure. The same symptoms were described by LOEB (1913) for *H. suspectum* envenomation. The LD_{50} values for *H. h. horridum* and *H. h. alvarezi* are 2 mg/kg mouse weight (Table 1). The amount of venom obtained from *H. h. horridum* is approximately 0.5 ml per animal (27 extractions from eight different specimens). This venom was collected in captivity (Mexico City). For *H. h. alvarezi* the volume of venom is approx. 0.92 ml per animal (eleven extractions from eleven animals) when collected in their natural habitat (Chiapas, México) and the volume is 0.55 ml per animal (six extractions from two animals) when extracted in captivity (México City). For

TABLE 1. LETHALITY AND ENZYMATIC ACTIVITIES IN THE VENOM OF TWO SUB-SPECIES OF *Heloderma horridum*

Fraction (Figs. 1, 3)	*H. h. horridum* WV	S	P	*H. h. alvarezi* WV	S	P
LD_{50} (μg/g mouse)	2	—	—	2	—	—
Phospholipase*	68.0 ± 0.5	52.3 ± 0.3	50.6 ± 0.5	91.4 ± 1.1	75.9 ± 0.7	77.7 ± 0.3
Hyaluronidase*	6.6 ± 0.2	6.7 ± 0.4	none†	9.2 ± 0.06	8.6 ± 0.3	none†
Bz-Arg-OEt hydrolase*	2.7 ± 0.3	3.4 ± 0.1	none†	4.2 ± 0.5	4.3 ± 0.8	none†
Bz Tyr-OEt hydrolase‡	0.08	0.13	none†	0.05	0.46	none†

*Enzymatic activity was averaged after three independent determinations (mean ± S.D.) and are expressed as units/mg (see Material and Methods for definition of units).

†Negative with enzyme samples up to 500 μg protein.

‡The *N*-benzoyl-L-tyrosine-ethyl ester hydrolase activity is based upon a single measurement and is also expressed as units/mg (see Material and Methods for definition of units).

WV = whole venom; S = soluble venom after acid treatment (10 min in 20 mM ammonium acetate buffer, pH 4.7). P = precipitated venom resuspended in 25 mM phosphate buffer, pH 7.6 (see Material and Methods for details).

both sub-species the protein concentration of the freshly collected venom is approx. 80 mg/ml. The electrophoretic pattern of the venom (Fig. 1) indicates at least 18 different protein components using the Tris-buffer system described by JOVIN *et al.* (1964). There is no difference in the electrophoretic pattern of the two venoms (data not shown). There is also no apparent difference in the electrophoretic pattern of the venom extracted once per month from the same animal over a period of one year (results not shown), although a small difference is apparent among the venom extracted from different individuals at the same time (Fig. 2). The venom has the following enzymatic activities: phospholipase, hyaluronidase, and Bz-Arg-OEt and Bz-Tyr-OEt hydrolases (Table 1). The venoms, freshly collected from both sub-species of lizards, have a neutral pH (6.9–7.0). The venom, diluted in 15 mM potassium phosphate buffer (pH 7.6 or 8.6), is very stable, even at a concentration of 2 mg/ml, but when diluted in ammonium acetate buffer (pH 4.7) a precipitate is formed. We have found that this precipitation is selective and reversible and inversely proportional to the logarithm

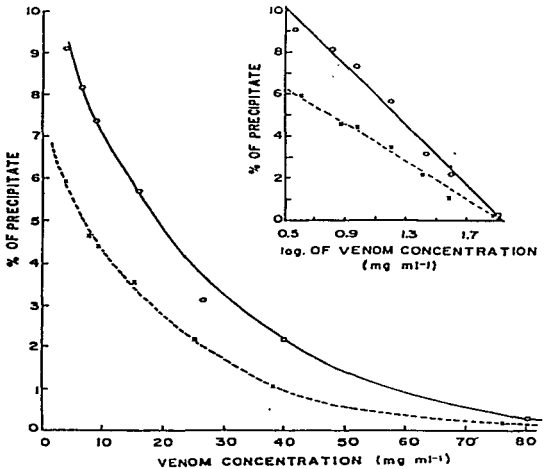

FIG. 3. ACID PRECIPITATION OF THE VENOM.
The soluble venom from *H. h. horridum* was diluted in 20 mM ammonium acetate buffer, pH 4.7, incubated at 25°C for 10 min and centrifuged at 25,000 *g* for 20 min. The precipitate was recovered in 25 mM potassium phosphate buffer, pH 7.6, and the protein content was determined by absorbancy at 280 nm, as described in Material and Methods. The inset represents the percentage of venom which is precipitated with acid vs the log of the venom concentration used for every point of the graph. *H. h. horridum* (o), *H. h. alvarezi* (x).

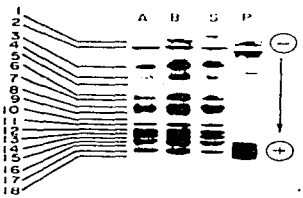

FIG. 1. ELECTROPHORESIS OF SOLUBLE VENOM AND ACID PRECIPITATED VENOM FROM *H. h. horridum*. Polyacrylamide cylindrical gels (5.5 cm, 2 mA) in Tris–glycine buffer, pH 8.3, were prepared according to the method of JOVIN *et al.* (1964). The samples, A and B (100 and 200 μg of the soluble venom respectively), and S and P (100 μg each of the soluble fraction after acid treatment and the precipitated venom resuspended in phosphate buffer respectively; see Fig. 3), were run and stained with Coomassie Brilliant Blue G-250. The proteins run toward the anode. Initially, 18 discrete bands were visible; eight strong (3, 7, 8, 11, 12, 13, 14, 17), six moderate (1, 2, 4, 6, 9, 16) and four weak (5, 10, 15, 18).

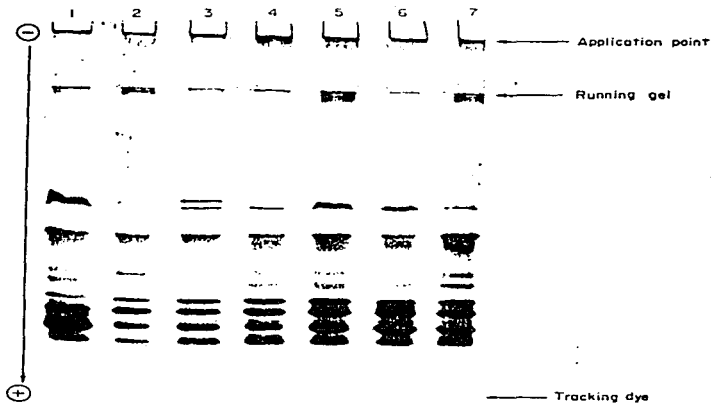

FIG. 2. ELECTROPHORESIS OF SOLUBLE VENOM FROM *H. h. horridum* OBTAINED FROM SEVEN DIFFERENT ANIMALS.
Venom from seven (1–7) different animals (100 μg per lane) was run on polyacrylamide plate gel [(10 cm, 15 mA in Tris–glycine buffer, pH 8.3 (SHUTER, 1971)] and stained with Coomassie Brilliant [Blue R-250. The proteins run toward the anode.]

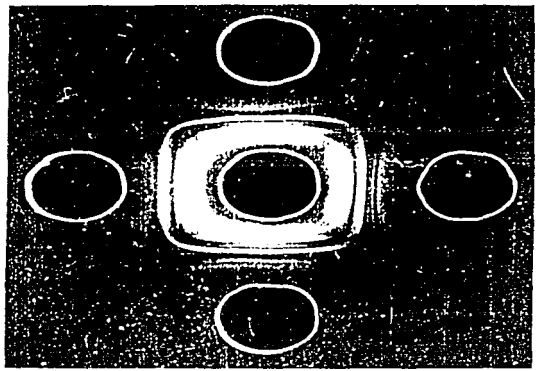

FIG. 4. IMMUNODIFFUSION TEST.

An immunodiffusion test from the sheep anti- *H. h. horridum* venom was carried out in 0.8% agarose containing 0.1 M Tris–HCl buffer at pH 7.95; wells of 4 mm diameter were spaced 10 mm apart from center to center. The gels were run at 4°C and a direct picture was taken after 48 hr incubation. The central well contains sheep anti-venom serum (20 μl). The upper and lower wells contain 20 μl (200 μg) of *H. h. horridum* venom. The left and right wells contain 20 μl (200 μg) *H. h. alvarezi* venom.

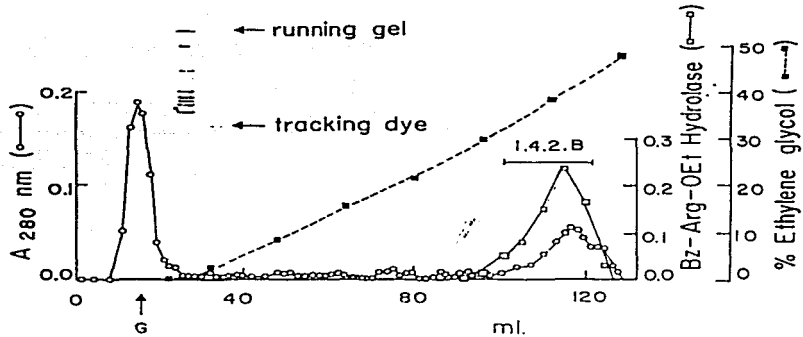

FIG. 7. HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF THE FRACTION I.4.2 FROM FIG. 6B.
The fraction I.4.2 (1.6 mg) in 0.01 M sodium phosphate buffer (pH 6.8) 25% saturated with ammonium sulfate, was applied to a phenyl-sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) column (0.9 × 10 cm). The column was eluted with a linear ethylene glycol gradient in the above-noted buffer (120 ml total volume). The flow rate was 20 ml/hr. Tubes of 1.6 ml volume were collected and pooled to give the pure enzyme, fraction I.4.2.B (horizontal bar). G means the same as Fig. 6. Inset: gel electrophoresis of the soluble venom (left lane, 100 μg) and the purified enzyme, fraction I.4.2.B (right lane, 40 μg). Cylindrical polyacrylamide gels were run in the same conditions as in Fig. 1.

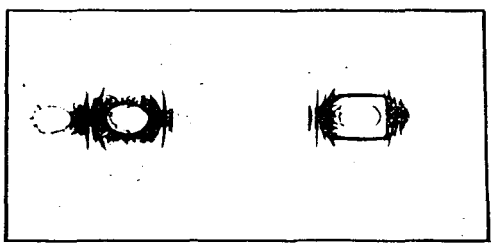

FIG. 8. IMMUNODIFFUSION TEST OF THE PURIFIED Bz-Arg-OEt HYDROLASE.
The agarose gel was prepared as described in Fig. 4. After 48 hr of diffusion the plate was washed, dried and stained with amido black (1% in 10% of acetic acid). The left part of the picture (set of five wells) was obtained with sheep anti-*H. h. horridum* venom (20 μl of serum in the central well); upper and lower wells contain 10 μl (30 μg) of purified Bz-Arg-OEt hydrolase; left and right wells contain 10 μl (100 μg) of whole venom from *H. h. horridum*. The right part of the picture (set of five wells) was obtained in the same conditions as the left portion of the picture, but the upper and lower wells contain only buffer (the enzyme was ommitted).

precipitation phenomenon. Phospholipase seems to be one of the enzymes which preferentially is precipitated in this condition. When resuspended in 15 mM phosphate buffer pH 7.6 the precipitate dissolves and can easily be quantified. The venoms are good antigens as can be observed in Fig. 4. The venoms from both sub-species (*horridum* and *alvarezi*) cross-react extensively with the sera against the *H. h. horridum* venom (Fig. 4).

Isolation of a Bz-Arg-OEt hydrolase from the venom of H.h. horridum

Since no important differences were found between the general biochemical characterization of the venom from *H. h. horridum* and *H. h. alvarezi* we have decided to use only the venom from the typical sub-species *H. h. horridum* for the isolation of the enzyme. The first step in the purification of the Bz-Arg-OEt hydrolase was acid precipitation, as described in Material and Methods. The supernatant was applied to a Sephadex G-75 column resolving six fractions (Fig. 5). Fraction I contains most of the hyaluronidase and phospholipase activities and also shows Bz-Arg-OEt hydrolase activity. Fraction II has the highest proportion of the Bz-Arg-OEt hydrolase and little of the other enzyme activities. All other fractions, III–VI, contain almost none of the enzymatic activities tested. Figure 5 (legend) also summarizes the enzymatic activities obtained during gel filtration in Sephadex G-75.

The lethality to mice of the various fractions from Sephadex G-75 was also investigated. Fraction I is lethal at doses of 20 mg/kg mouse weight. Fraction II is possibly the fraction that contains the most toxic component. Its LD_{50}, determined at eleven different doses using ten mice for each dose, was 0.46 mg/kg mouse weight. The toxic potency of this fraction was increased more than four-fold compared with the whole venom (2 mg/kg weight). Fraction III was lethal at a dose of 5 mg/kg mouse weight and all remaining fractions (III-IV) were not lethal at doses up to 5 mg/kg mouse weight, the mice injected with the latter fractions presenting no symptoms of intoxication. Fractions I and II correspond to 36 and 25% of the

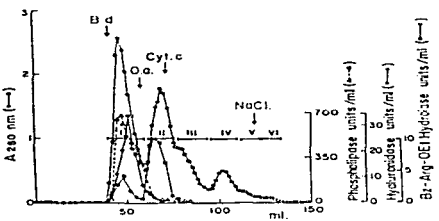

FIG. 5. SEPARATION OF *H. h. horridum* VENOM BY SEPHADEX G-75 COLUMN CHROMATOGRAPHY.
The supernatant (S) fraction of the soluble venom (65 mg) after acid precipitation (see Figs 1 and 3) was applied to a Sephadex G-75 column (0.9 × 200 cm) and run in 25 mM potassium phosphate buffer, pH 7.6, at a flow rate of 15 ml/hr. Tubes containing 1.9 ml volume were collected and pooled as fractions I–VI (horizontal bars) based on the absorbancy at 280 nm and on the enzymatic activities (right portion of the graph). Fractions I, II and III contained, respectively, 92.1, 7.5 and 0.4% of the total phospholipase acitivity. Fractions I and II contained, respectively, 95.2 and 4.8% of the total hyaluronidase activity. Fractions I, II and III contained, respectively, 27.2, 71.0 and 1.8% of the total Bz-Arg-OEt hydrolase activity. Vertical arrows indicate the elution volume of B.d (blue dextran, molecular weight over two million), O.a. (ovoalbumin, molecular weight 45,000), Cyt. c (cytochrome c, molecular weight 12,400) and NaCl (sodium chloride) used as molecular weight markers of the column.

total protein recovered respectively. For further purification we have started with fraction I from Fig. 5. A sample of this fraction was applied to a DEAE-cellulose column in 15 mM potassium phosphate buffer, pH 7.55 (Fig. 6A). The chromatogram of Fig. 6A shows the separation of fraction I from Fig. 5 into eight sub-fractions. Fractions I.5, I.6 and I.8 contain some phospholipase activity. This result indicates that the venom has different forms of phospholipases. Fraction I.4, containing the Bz-Arg-OEt hydrolase activity, was re-chromatographed in the same DEAE-cellulose column, but with 30 mM potassium phosphate buffer at pH 8.6 (see Fig. 6B). In this figure fraction I.4 is shown to be composed of two subfractions; only fraction I.4.2 has the Bz-Arg-OEt hydrolase activity. Gel electrophoresis of fraction I.4.2 has shown that the enzyme still had protein contaminants. Only after chromatography of fraction I.4.2 on a phenyl-sepharose column was this enzyme

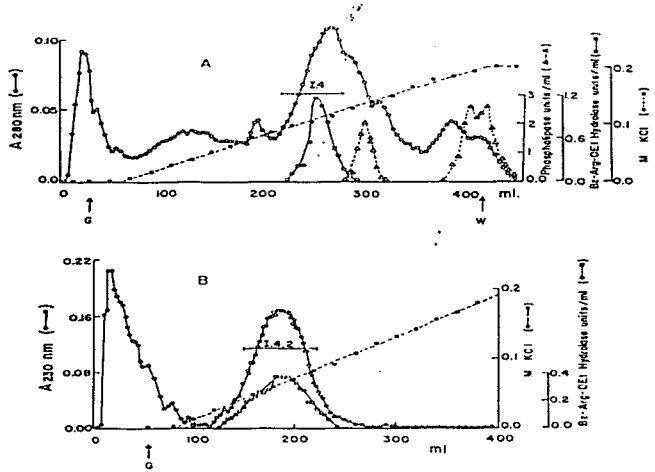

FIG. 6. ION EXCHANGE CHROMATOGRAPHY OF THE FRACTION I FROM SEPHADEX G-75 (FIG. 5). (A). The fraction I from Fig. 5 (19 ml) containing 22.2 mg of protein was adjusted to 15 mM potassium phosphate buffer pH 7.55, and applied to a DEAE-Cellulose Column (0.9 × 22.5 cm). The enzyme was eluted with a linear gradient (200 ml each) of 0–0.2 M KCl in the same 15 mM potassium phosphate buffer at pH 7.55, with a flow rate of 40 ml/hr. Tubes containing 2 ml volume were pooled to give fraction I.4 (horizontal bar) based on absorbancy at 280 nm and on the Bz-Arg-OEt hydrolase activity. The phospholipase activity is also indicated. The vertical arrow label with G indicates the point where the gradient started, and W indicates the stepwise wash with 0.2 M KCl. (B). The fraction I.4 (5.2 mg) from Fig. 6A was dialyzed against 30 mM potassium phosphate buffer, pH 8.6, and applied to a DEAE-Cellulose (similar to Fig. 6A) column (0.9 × 14 cm). The enzyme was eluted with the same 30 mM K phosphate buffer, pH 8.6, at a flow rate of 40 ml/hr. A linear gradient, containing 200 ml buffer (each), was applied from 0 to 0.2 M KCl to give fraction I.4.2 (horizontal bar). Tubes containing 2 ml volume were read at 280 nm and the Bz-Arg-OEt hydrolase activity determined before pooling. G means the same as in Fig. 6A.

obtained in pure form (Fig. 7). The inset of Fig. 7 represents the pattern of the Bz-Arg-OEt hydrolase in gel electrophoresis compared to the native venom. In a separate experiment the cylindrical gel containing the purified enzyme was cut into 1.5-mm slices and the Bz-Arg-OEt hydrolase activity was measured directly in the gel slices. The enzyme activity corresponded to the protein band in the gel (data not shown). Immunodiffusion gels have shown that the purified enzyme presents only one band (see Fig. 8). Sodium dodecyl sulphate gel electrophoresis of this enzyme has shown only one protein band (data not shown) with a molecular weight of 65,000, similar to bovine serum albumin (Fig. 9). Table 2 summarizes all steps of purification of the Bz-Arg-OEt hydrolase, giving the values of the final recoveries for each step. In reference to the other enzymes found in fraction I (Fig. 5), it is interesting to observe that the phospholipase activity applied to the DEAE-Cellulose column (Fig. 6A) was only partially recovered (30%), whereas the hyaluronidase activity was completely lost in this step.

DISCUSSION

In the literature there is no report on the electrophoretic pattern of the venom from *H. h. horridum* or *H. h. alvarezi* in polyacrylamide gels. The only reference (Mebs and Raudonat, 1966) reporting electrophoresis of *Heloderma* venom was obtained on a paper support; no mention is made as to which species it corresponds (probably to *H. suspectum suspectum*) and it is not comparable to our results. The constant relative composition of the venom during the year is also peculiar to *Heloderma* venom. It is known from other species, mainly wasps and snakes (reviewed by Tu, 1977) that there exists a certain variation in the proportion of some components of the venom according to the season of the year. The small difference found among individuals of the same population is not necessarily consistent; it could be a variation on the degree of contamination of the venom with saliva, although this did not happen in the

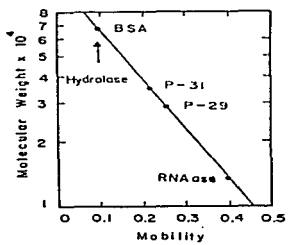

Fig. 9. Molecular weight determination of the Bz-Arg-OEt hydrolase by SDS-polyacrylamide gels. The purified fraction I.4.2.B (Fig. 7) was run in parallel with standards of known molecular weight (BSA, bovine serum albumin, molecular weight 65,000; P-31 peptide from BSA, molecular weight 31,000; P-29 peptide from BSA, molecular weight 29,000; RNAse, ribonuclease, molecular weight 12,600) in cylindrical polyacrylamide gels containing 12% SDS (Swank and Munkres, 1971). The molecular weight of the enzyme (Hydrolase) corresponds exactly to the point (vertical arrow) of bovine serum albumin, with an approx. molecular weight of 65,000.

case of the *Heloderma*, from which we have analyzed the venom extracted throughout the year (twelve extractions from the same animal). In other words, if the difference was due to contamination with saliva it would probably have appeared in the gel electrophoresis pattern from the venom extracted in different seasons from the same individual. The potency of the venom is high compared to other venomous animals (see TU, 1977) and the amount of venom obtained by a single extraction (approx. 40 mg of venom) is enough to kill hundreds of mice of 20 g each (LD_{50} 40 μg/20 g mouse weight). Another interesting property of the venom is its heat-stability, as already reported by LOEB (1913) and MEBS (1972). The venom is still active, even after autoclaving at 120°C for several min!

The enzymes that we have found in the venom of *H. h. horridum* confirm the findings by MEBS (1972), TU and MURDOCK (1967) and STÝBLOVÁ and KORNALIK (1967) for the venom of *H. suspectum suspectum*. Both species of lizard have the same kind of enzymatic activities. The results of gel filtration of the venom from *H. h. horridum* seem to be very similar to those obtained by MEBS (1972) for the venom of *H. suspectum*. In the venom of *H. h. horridum* there is, apparently, a toxic protein which is excluded in fraction II, as already discussed in the section of Results. The final purification of the Bz-Arg-OEt hydrolase enzyme was not a straightforward procedure. The enzyme is eluted with other components of the venom and a column of hydrophobic interaction-type (phenyl-sepharose) had to be used as the last step in the purification procedure (Fig. 7). This problem explains the low yield finally obtained during the purification of the Bz-Arg-OEt hydrolase, which is in the order of 1.5%, as shown in Table 2. An additional problem is related to the specific activity of the enzyme. Due to the fact that the starting material (soluble venom) contains several different enzymes that split the substrate (*N*-benzoyl-L-arginine ethyl ester), it is difficult to report the exact value for the purification of the enzyme. We do not know the individual contribution of each enzyme in the initial sample.

Comparison of the above results with MEBS's (1972) report suggests that the Bz-Arg-OEt hydrolase here described corresponds to the kinin-releasing enzyme found by him in the venom of *H. s. suspectum*.

TABLE 2. SUMMARY OF THE PURIFICATION PROCEDURE OF THE *N*-BENZOYL-L-ARGININE-ETHYL ESTER HYDROLASE FROM THE VENOM OF *Heloderma horridum horridum*

Fraction	Source of fraction	Protein (mg)*	Total activity (units)†	Specific activity (units/mg)	Recovery	Ratio to the Specific activity of the whole venom
Whole venom	Lyophilized venom	100.0	267.0	2.67	100.0	1.00
S	Supernatant acid precipitated	92.0	310.0	3.37	116.1	1.26
I	Sephadex G-75	34.5	90.1	2.61	33.7	0.98
I.4	DEAE-Cellulose (pH 7.55)	8.06	45.5	5.64	17.0	2.11
I.4.2	DEAE-Cellulose (pH 8.6)	3.93	30.9	7.86	11.6	2.94
I.4.2.B	Phenyl-Sepharose	0.55	3.7	6.74	1.4	2.52

*Assuming 1 absorbancy unit at 280 nm equal to 1 mg/ml.

†One unit is the amount of enzyme that hydrolyzes 1 μmol of substrate per min in the conditions described in Material and Methods.

S, I, I.4, I.4.2 and I.4.2.B are fractions obtained during the purification procedure (chromatrographic steps) indicated in the second column of the Table.

Acknowledgements—The authors are indebted to Professor MIGUEL ALVAREZ DEL TORO for allowing us to obtain the venom from the *H. h. alvarezi* from the Zoologic Museum of Chiapas, México. The technical assistance of FREDY V. CORONAS is aknowledged.

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Biochemical Characterization of the Phospholipase A_2 Purified from the Venom of the Mexican Beaded Lizard (*Heloderma horridum horridum* Wiegmann)†

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ABSTRACT: A phospholipase A_2 was isolated from the venom of the mexican beaded lizard (*Heloderma horridum horridum*) by phenyl-Sepharose chromatography followed by Sephadex G-75 gel filtration and two additional steps on ion exchange resins (DE-32 cellulose). The affinity chromatographic method (PC–Sepharose 4B) reported for the isolation of other phospholipases [Rock, Ch. O., & Snyder, F. (1975) *J. Biol. Chem. 250*, 2564–2566; King, T. P., Alagon, A. C., Kwan, J., Sobotka, A. K., & Lichteinstein, L. M. (1983) *Mol. Immunol. 20*, 297–308; King, T. P., Kochoumian, L., & Joslyn, A. (1984) *Arch. Biochem. Biophys. 230*, 1–12] was ineffective for the separation of this enzyme. The monomeric form of the *Heloderma* phospholipase has an apparent M_r of 18 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and 19 060 as calculated from amino acid analysis. It also contains on the order of 7% carbohydrates per mole of enzyme. The N-terminal amino acid sequence was shown to be very different from that of phospholipases isolated from mammalian pancreas and crotalids and elapids snake venoms. The first 39 amino acid residues at the N-terminal region have 56% homology with bee venom phospholipase but differ from the bee phospholipase in that its isoelectric point is acidic ($pI = 4.5$), instead of basic, and it has approximately 50 amino acid residues more in the molecule. The specificity of the enzyme is mainly A_2 type with possible residual B-type activity. The enzymatic activity is Ca^{2+}-dependent. Half-cystine alignment of the *Heloderma* phospholipase sequence with those of other known phospholipases shows the lack of an octadecapeptide at the N-terminal region, the existence of an extra hexapeptide at positions 42–47, and an exact correspondence of *Heloderma* Gly-12, Gly-14, His-36, and Asp-37 with Gly-30, Gly-32, His-48, and Asp-49 from other phospholipases shown to be important for Ca^{2+} binding [Dijkstra, B. W., Drenth, J., Kalk, K. H., & Vandermaalen, P. J. (1978) *J. Mol. Biol. 124*, 53–60]. The *Heloderma* phospholipase like the bee phospholipase has a Trp at position 10 corresponding to Tyr-28 of other phospholipases, also claimed to be important for calcium binding. Although the present enzyme is structurally very different, it could be sharing similar peptide sequences around the Ca^{2+} binding site of other phospholipases A_2 reported, thus far.

The enzymes with phospholipase A_2 (EC 3.1.1.4) activity are calcium-dependent esterases. They hydrolyze the 2-acyl bond of 3-*n*-phosphoglycerides. Most of the phospholipases A_2 isolated so far, from pancreas; snake venoms, and bee venoms, are relatively small and rigid molecules, having 6–7 disulfide bridges in a protein approximately 125 residues in length (Verheij et al., 1981; Heinrikson, 1982).

Comparison of the primary structure of more than 30 phospholipases has revealed a high degree of homology in the amino acid sequence of these enzymes. Two classes of phospholipases have been proposed: group I comprises phospholipases from pancreatic juice, elapids (cobras), and hydrophids (sea snakes) and group II is composed by phospholipases from many crotalids (rattlesnakes) (Heinrikson et al., 1977). The two different structural groups were separated on the basis of specific disulfide bridges of the molecules.

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Crystallographic studies of the bovine pancreatic enzyme, group I (Dijkstra et al., 1978), and a phospholipase from the western diamondback rattlesnake (*Crotalus atrox*) (Keith et al., 1981) have shown great similarities in their three-dimensional structures. The phospholipase A_2 enzymes are Ca^{2+}-dependent enzymes. Among the amino acid residues shown to be important for the Ca^{2+} binding site are Tyr-28, Gly-30, Gly-32, His-38, and Asp-49, as shown by a X-ray crystallographic analysis of a Ca^{2+}–enzyme complex in bovine phospholipase (Dijkstra et al., 1978).

Despite the structural similarities and the knowledge of the Ca^{2+} binding site of phospholipases, the actual catalytic mechanism of these enzymes is yet to be established (Maraganore et al., 1984). The discovery of new structural types of phospholipases A_2 will certainly help in understanding the important characteristics of the structure of the enzyme that could be related to the catalytic activity.

The purpose of this paper is to report the purification and an extended characterization of a phospholipase enzyme present in the venom of the mexican beaded lizard. The N-terminal amino acid sequence of the *Heloderma horridum horridum* phospholipase A_2 reveals the uniqueness of the venom of these animals, considered to be "fossil animals" in danger of extinction (Tu, 1977), and the key role they might play in understanding comparative biochemical and structural features of certain types of proteins during evolution of the animal kingdom.

Experimental Procedures

Materials. Lyophilized whole venom was obtained as previously described (Alagón et al., 1982). Sephadex G-75, phenyl-Sepharose 4B, and AH-Sepharose 4B were from Pharmacia Fine Chemicals. DE-32 cellulose was from Whatman, Inc. Synthetic phospholipids were from Sigma Chemical Co. Lecithin analogue PC[1] was obtained from Berchtold Chemical Laboratory, Bern, Switzerland. Bee venom phospholipase was purified according to King et al. (1976). Specific anti-venom sera were prepared in three sheep as previously described (Alagón et al., 1982).

Methods. The quantitation of the phospholipase activity was determined by the titrimetric procedure of Shiloah et al. (1973). One unit of enzymic activity was defined as the amount of enzyme releasing 1 μmol of acid per minute at pH 8.0 and 25 °C in 3.0 mL of a 10% (w/v) solution of egg yolk in 0.1 M NaCl. When required, the phospholipase activity in slab gels was detected by clearing of egg yolk suspension with erythrocytes in 1.2% agarose gel (Haberman & Hardt, 1972).

For the purpose of studying the products formed on phospholipase digestion, we followed the standard procedure described by King et al. (1984), with some minor modifications. Synthetic phospholipids 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphocholine and 1-oleoyl-2-stearoyl-3-*sn*-glycerophosphocholine were used as substrates. Fine suspensions of phospholipids (4 mg/ mL) were prepared by sonication in 10 mM Tris-HCl buffer (pH 8) containing 10 mM $CaCl_2$ and 0.5% Triton X-100, in an ultrasonic cleaner bath. The digests were examined by thin-layer chromatography on silica gel plates (DC Alufolien Kieselgel 60, Merck); 10 μL of digest was applied to the plate and developed in a system of chloroform–methanol–0.1 N HCl (70:30:5). Spots were visualized after exposure to iodine vapor. They were also examined directly for saturated and unsaturated fatty acids by chromatography on silica gel plates previously soaked by capillarity in 10% $AgNO_3$ dissolved in 82% methanol and dried at 100 °C for 30 min. After the plates were developed in a system of hexane–diethyl ether–acetic acid (80:20:1), the spots were visualized under UV light after being sprayed with 0.2% dichlorofluorescein in ethanol. R_f values for saturated and unsaturated fatty acids were 0.6 and 0.47, respectively.

The absorbent for affinity chromatography of phospholipase (Rock & Snyder, 1975) was prepared by a carbodiimide-promoted coupling of 240 mg of PC to 6 g of AH-Sepharose 4B. Protein solutions were concentrated by ultrafiltration with an Amicon PM-10 membrane. Dialysis was carried out in a Visking 8/32 membrane. Protein concentration was measured spectrophotometrically by assuming that 1 A_{280nm}^{1cm} unit = 1 mg/mL.

Electrophoresis was carried out in 7.5% polyacrylamide slab gels (0.1 × 10 × 12 cm) containing Tris buffer (Jovin et al., 1964) or in 15% gels containing Tris–SDS buffer (Laemmli, 1970). Proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Swank & Munkres, 1971). Isoelectrofocusing was performed in 5.6% polyacrylamide gels with 2% ampholytes (pH 3–10) and 6 M urea (Awdeh et al., 1968).

Indirect hemolytic activity was measured as previously described (Sosa et al., 1979). The 50% hemolytic unit (HU_{50}) was graphically determined following Mayer's immune hemolytic technique as modified by De Hurtado and Layrisse (1964).

Immunodiffusion and immunoelectrophoresis were carried out in 1% agarose gels (King et al., 1978), and the precipitates were visualized after dye staining of dried and washed gels (Axelsen et al., 1973).

Amino acid composition was obtained by the time course hydrolysis method described by Moore (1963). Duplicate samples of protein were hydrolyzed under vacuum in individually sealed tubes with 6 M HCl containing 0.5% phenol, at 110 °C for 20, 48, and 72 h. Half-cystine content was determined as cysteic acid after performic acid oxidation. Approximately 1 nmol of protein was treated with 50 μL of 3% H_2O_2 in 90% formic acid for 3 h at 25 °C. The reagent was blown off under nitrogen at 50 °C. After being dried in vacuo, the sample was hydrolyzed as usual. The analysis of the hydrolysates was carried out on a Durrum D-500 amino acid analyzer.

The glucosamine and galactosamine contents were determined from the amino acid analysis after calibration with the proper standards. Carbohydrate content, excluding amino sugars, was measured by the orcinol–sulfuric acid method (Tsugita & Akabori, 1959).

For N-terminal sequence determination, phospholipase (10 nmol) was reduced, pyridylethylated (Friedman et al., 1970), exhaustively dialyzed against double-distilled water, and lyophilized. Two nanomoles in 0.2 mL of 20% acetic acid was evaporated to dryness in the spinning cup (previously treated with 3.0 mg of polybrene) of a Beckman Model 890 M sequencer, using program 3 (Possani et al., 1985). PTH-amino acid derivatives were identified and quantitated by high-performance liquid chromatography on a Hewlett-Packard 1084 B equipped with a variable-wavelength detector set at 269 nm.

Results

Purification of Phospholipase. Lyophilized whole venom was dissolved in 10 mM phosphate buffer (pH 6.8) and applied

[1] Abbreviations: AH, aminohexyl; DE, diethylaminoethyl; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PC, 1-(11-carboxyundecyl)-2-hexadecyl-*rac*-glycero-3-phosphocholine; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

Table I: Summary of the Purification Procedure of the Phospholipase A_2 from *H. horridum horridum* Venom

fraction	source of fraction	protein (mg)[a]	total act. (units)[b]	sp act. (units/mg)	recovery (%)	ratio to sp act. of whole venom
whole venom	lyophilized venom	100.0	12 500	125	100.0	1.0
V	phenyl-Sepharose	8.0	5 160	645	41.3	5.2
V.2	Sephadex G-75	4.8	3 070	640	24.6	5.1
V.2.2	DE-32 cellulose	0.6	1 225	2040	9.8	16.3
V.2.2.1	DE-32 cellulose	0.5	1 295	2590	10.4	20.7

[a] Assuming 1 absorbancy unit at 280 nm equal to 1 mg/mL. [b] One unit is the amount of enzyme that releases 1 μmol of acid per minute in the conditions described under Experimental Procedures.

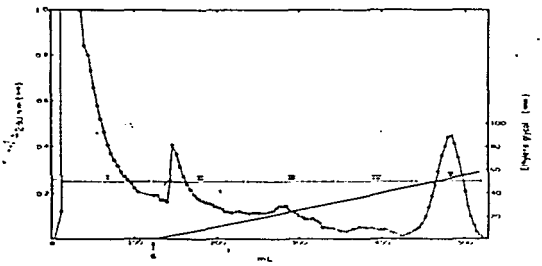

FIGURE 1: Fractionation of soluble venom. Soluble venom (8.0 mL; 158.8 A_{280}^{1cm} units) in 0.01 M sodium phosphate buffer (pH 6.8) was applied to a phenyl-Sepharose column (41.5 × 7.0 cm). The column was eluted with a linear gradient (200 mL each) from 0 to 58% ethylene glycol in the same buffer, at 60 mL/h. Fractions (4 mL/tube) were collected and pooled as indicated (horizontal bars; I–V). G indicates the starting of the gradient. Fraction V (with the phospholipase activity) was dialyzed against the buffer of Figure 2 and concentrated by ultrafiltration.

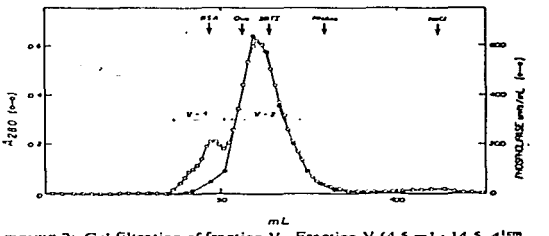

FIGURE 2: Gel filtration of fraction V. Fraction V (4.5 mL; 14.5 A_{280}^{1cm} units) in 0.05 M sodium phosphate buffer (pH 8) was applied to a Sephadex G-75 column (0.9 × 200 cm) and eluted with this same buffer, at 15 mL/h. Fractions (2 mL/tube) were collected, pooled as indicated (horizontal bars), and concentrated. Vertical arrows indicate the elution volume of BSA (bovine serum albumin, M_r 65 000), Ova (ovalbumin, M_r 45 000), SBTI (soybean trypsin inhibitor, M_r 21 500), RNase (ribonuclease, M_r 13 500), and NaCl (sodium chloride), used as molecular weight markers.

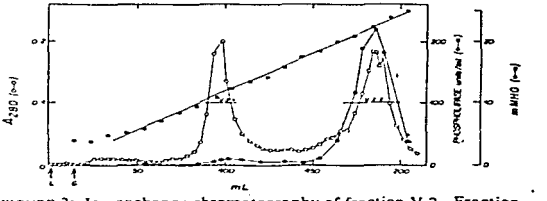

FIGURE 3: Ion-exchange chromatography of fraction V.2. Fraction V.2 (5.0 mL; 7.05 A_{280}^{1cm} units) in 0.05 M sodium phosphate buffer (pH 8) was applied to a DE-32 cellulose column (0.9 × 30 cm) and eluted with a linear NaCl gradient (200 mL each) from 0 to 0.5 M in the same buffer, at 60 mL/h. Fractions (2 mL/tube) were collected as indicated by the horizontal bars. Fractions V.2.2. was dialyzed against the starting buffer, concentrated, and rechromatographed in this column under the same conditions. L and G indicate loading of the sample and the starting of the gradient, respectively.

to a phenyl-Sepharose CL-4B column (Figure 1). Proteins were eluted with a linear ethylene glycol gradient to yield five fractions. Fraction V, which contained the phospholipase activity, was concentrated and used for gel filtration on Sephadex G-75 (Figure 2). Tubes containing phospholipase were applied to a DE-32 cellulose column eluted with a linear salt gradient; two fractions were collected (Figure 3). Fraction V.2.2, which accounted for most of the phospholipase activity, was dialyzed and rechromatographed in the same DE-32 cellulose column under identical conditions (results not shown). Tubes containing phospholipase activity (fraction V.2.2.1) were pooled, dialyzed against 5 mM ammonium acetate buffer (pH 8.3), concentrated, and stored at −22 °C until used. Table I summarizes the isolation procedures and recoveries of protein and phospholipase activity.

Characterization of Purified Phospholipase. The gel electrophoretic pattern in the presence of SDS of the purified enzyme (fraction V.2.2.1) showed a major protein band with an apparent M_r of 18 000 and a minor contaminant of 16 000; identical results were obtained after reduction with 1% 2-mercaptoethanol (data not shown). Under nondenaturating conditions, the above sample showed three protein bands. In order to identify the protein containing phospholipase activity, unstained gels without SDS were placed on the surface of agarose gels containing egg yolk suspension with erythrocytes. Two bands with clearing activity were located, either with crude venom or with purified phospholipase. Most of the activity matched the major band detected with Coomassie Blue, while the rest of the activity corresponded to a very faint band of slower mobility. This band is apparently a dimer of phospholipase, noncovalently bound, since it did not appear in SDS gels electrophoresis, even when higher concentrations of the sample were used.

The isoelectric point determined for the purified phospholipase was 4.5, and that of the small contaminant was 4.6 (data not shown). Immunodiffusion of the enzyme showed a single, rather broad, precipitin line (Figure 4A), while immunoelectrophoresis of the same sample gave one main arc and a smaller one, farther, in the direction of the anode (Figure 4B).

The enzymatic activity of the purified protein is Ca^{2+}-dependent. Preincubation of the enzyme, for 10 min, with 10 mM EDTA completely abolished its activity, when measured

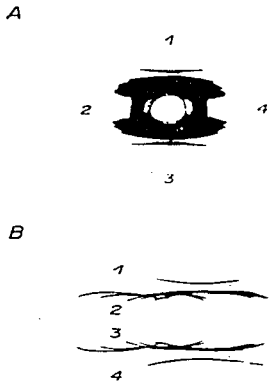

FIGURE 4: Immunodiffusion and immunoelectrophoresis tests. (A) Immunodiffusion test was carried out in 1% agarose containing 0.1 M Tris-HCl buffer, pH 7.2. The central well contained sheep anti-venom serum (20 μL); wells 2 and 4 contained 20 μg of purified phospholipase; wells 1 and 3 contained 200 μg of *H. horridum horridum* venom. (B) Immunoelectrophoresis in 1% agarose containing 0.025 M Tris-Hcl, pH 8.65, with sheep anti-venom serum (50 μL). (1 and 4) Purified phospholipase (20 μg); (2 and 3) *H. horridum horridum* venom (200 μg). The anode was at the right side.

titrimetrically with a suspension of egg yolk containing 10 mM EDTA. The activity is restored upon addition of calcium chloride to a final concentration of 25 mM.

The specificity of *H. horridum horridum* phospholipase was examined by thin-layer chromatography. Two synthetic phospholipids (4 mg/mL) were digested by phospholipase at 1.4 μg/mL final concentration, at pH 8 and 25 °C. Under these conditions, both 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphocholine and 1-oleoyl-2-stearoyl-3-*sn*-glycerophosphocholine were completely converted to their respective monoacyl phospholipid in 2–5 min. The second substrate was also converted to glycerophosphocholine in about 6 h. Qualitative estimation of $AgNO_3$-treated chromatograms showed that after 10-min digestion only fatty acids at position 2 were released from the two substrates. After 6-h digestion, oleic acid was also released from 1-oleoyl-2-stearoyl-3-*sn*-glycerophosphocholine. Since the specificity of bee venom phospholipase A_2 is well-known (Shipolini, 1971), digests of this phospholipase at a final concentration 10 μg/mL were also examined in control experiments; only products corresponding to the hydrolysis of the acyl group at the 2-position were obtained, even after 6-h digestion.

The graphical determination of the 50% hemolytic unit for crude venom and for purified phospholipase A_2 gave 210 and 760 HU_{50}/mg, respectively. The hemolytic potency was only 3.6 times higher for the purified enzyme than for the unfractionated venom, which is less than the calculated ratio between the specific activity of the two samples (20.7 times; Table I). The above results suggest the presence of some other factors in the crude venom, which synergistically potentiate the hemolytic effect of phospholipase A_2, as previously discussed (Sosa et al., 1979).

Table II: Amino Acid Composition of Phospholipase A_2 from *H. horridum horridum* Venom[a]

amino acid	found[b]	nearest integer
aspartic acid	16.1 ± 0.4	16
threonine	9.0 ± 0.2	9
serine	11.9 ± 0.2	12
glutamic acid	21.2 ± 0.0	21
proline	5.4 ± 0.0	5
glycine	16.5 ± 0.6	16
alanine	12.8 ± 0.1	13
half-cystine	10.5 ± 0.6	10
valine	5.8 ± 0.4	6
methionine	5.2 ± 0.2	5
isoleucine	6.0 ± 0.4	6
leucine	11.7 ± 0.5	12
tyrosine	8.9 ± 0.4	9
phenylalanine	6.0 ± 0.5	6
histidine	5.7 ± 0.3	6
lysine	9.3 ± 0.4	9
arginine	3.8 ± 0.3	4
tryptophan	5.1	5
total		170
calculated M_r		19060

[a] Calculated on the assumption that the number of aspartic acid, glutamic acid, alanine, and leucine are 16, 21, 13, and 12 per mole of phospholipase A_2, respectively. [b] Average values ± SEM of duplicate hydrolysates at 20, 48, and 72 h. The serine and threonine contents were estimated by extrapolation to zero time. The value for half-cysteine was determined as cyteic acid after performic acid oxidation. Tryptophan was estimated from its molar extinction coefficient, according to King and Spencer (1970).

The amino acid composition of phospholipase A_2 from *H. horridum horridum* shown in Table II suggests that the enzyme is composed of 170 residue with a calculated M_r of 19060 in agreement with the apparent M_r of 18000 calculated by SDS gel electrophoresis. Phospholipase A_2 contained three residues of galactosamine (possibly acetylated) per mole of protein; glucosamine was not detected. The carbohydrate content of the enzyme by the method used (Tsugita & Akabori, 1959), which does not detected amino sugars, gave a value of 3.77 ± 0.32%. Thus, the peptide molecular weight (19060) is increased to 20420 if the total carbohydrate content is taken into account.

The N-terminal amino acid sequence of a reduced and pyridylethylated sample of the enzyme showed the following sequence: H_2N-Gly-Ala-Phe-Ile-Met-Pro-Gly-Thr-Leu-Trp-Cys-Gly-Ala-Gly-Asn-Ala-Ala-Ser-Asp-Tyr-Ser-Gln-Leu-Gly-Thr-Glu-Lys-Asp-Thr-Asp-Met-Cys-Cys-Arg-Asp-His-Asp-His-Cys. Only one amino acid was identified per cycle, and the repetitive yield was 95%. From this, we conclude that either the minor contaminant is blocked at the N-terminal position or more likely its concentration is too low to be detected.

Discussion

Many phospholipase enzymes have been purified from various sources: pancreatic tissue (Drenth et al., 1976; Evenberg et al., 1977; Fleer et al., 1978), honey bee venom (Shipolini, 1971), and numerous snake venoms (Randolph & Heirikson, 1982). The procedure described above for purification of the phospholipase from the *H. horridum horridum* venom was not a straightforward procedure. Initially, we have used the affinity column method described by Rock and Synder (1975) and King et al. (1983, 1984) in order to purify this enzyme. For comparative purposes and with the aim to stress the point, we are including in Figure 5 the chromatographic behavior of *Heloderma* venom in a PC–Sepharose 4B column prepared as described by the above-refered authors.

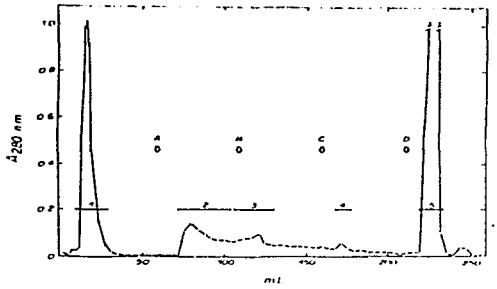

FIGURE 5: Affinity chromatography of crude venom on a column of Sepharose 4B with a bound substrate analogue of phosphatidylcholine. A total of 48 A_{280}^{1cm} units of soluble venom in 4 mL of 25 mM Tris-HCl buffer (pH 7.95) containing 25 mM calcium chloride was applied into a column of PC–Sepharose 4B (0.9 × 7.5 cm) and washed with 50 mL of the same buffer. Proteins fractions were eluted successively with 50 mL of each of the following solutions: (A) 0.05 M Tris-HCl buffer (pH 7.95) containing 0.5 M NaCl, (B) 0.05 M Tris-HCl buffer (pH 7.45) containing 50 mM EDTA, (C) 0.1 M acetic acid containing 0.5 M NaCl, and (D) 25% 2-propanol in 0.05 M Tris-HCl buffer (pH 7.95). Tubes of fraction 4 contained 0.3 mL of 1 M Tris-HCl buffer (pH 7.95). The column was eluted at 60 mL/h, and fractions of 2 mL were collected.

A control for the efficacy and goodness of the PC–Sepharose 4B resin that we have synthesized was established by isolating a wasp phospholipase from the venom of *Polistes dorsalis* (Amezcua & Alagon, 1984). As shown in Figure 5, several conditions were used to elute the enzyme from the column. However, no phospholipase activity could be detected in fractions 1–4. Fraction 5 was obtained with a more severe step and represented 20.3% of the protein applied to the absorbent. Fractions 5 from repetitive experiments contained from 2 to 60% of the initial phospholipase activity, when measured after dialysis against 25 mM Tris-HCl buffer (pH 7.95) containing 25 mM calcium chloride. The highest value was obtained when the protein was dialyzed immediately after its elution from the column. Electrophoretic analysis showed that fraction 5 was still a rather complex mixture of proteins with six major and five minor bands (data not shown). The average protein recovery was 75%. Attempts to purify the enzyme by means of increasing linear gradients of 2-propanol also failed to separate it from other proteins.

Since the PC–Sepharose 4B resin has large alkyl groups, it is conceivable that strong hydrophobic interactions between *Heloderma* proteins and the matrix of the affinity column prevent successful isolation of the phospholipase. For this reason, instead of the affinity PC–Sepharose 4B resin we have used a hydrophobic support (phenyl-Sepharose, see Figure 1) as the first step in the purification procedure. Three additional purification steps were included that used molecular weight sieving properties of the Sephadex gel (Figure 2) and ion exchanger resin (Figure 3) in order to obtain this enzyme at a degree of homogeneity that would allow further appropriate characterization. It was necessary to include the Sephadex G-75 step because the phospholipase activity was lost when the phenyl-Sepharose-eluted fractions were directly applied to a DE-32 cellulose column, at pH 8.0. This was interpreted as due to proteolytic cleavage from contaminants present in the venom at this stage of the purification procedure. After the last chromatographic step a highly purified fraction was obtained as mentioned under Results, containing a monomeric and a dimeric form of the enzyme as shown by the combined results of slab gel in nondenaturing and denaturing conditions. Because of similar molecular weights and isoelectric points of the phospholipase and the minor contaminant, it was impossible to separate them by these methods. In a separate experiment (results not shown), a slab gel run on this purified material was cut in the position of the main band corresponding to the phospholipase activity, and after this was eluted from the gel, it was used for amino acid analysis determination. In the sample obtained after the column shown in Figure 3, no proteinase activity was detected by the method described by Alagon et al. (1982), and since the main component (over 95%) was shown to have the enzyme activity, we decided to proceed with the chemical and enzymatic characterization of the phospholipase A_2.

The amino acid analysis of the phospholipase was obtained with samples purified independently from different batches of venom and gave a minimum M_r of 19 060. The molecule contained 170 amino acid residues. Because it has five methionines, a sample of the enzyme was reduced, alkylated, and cleaved with cyanogen bromide. The six resulting peptides purified by HPLC (results not shown) are consistent with the calculated M_r of 19 060. The high content of hydrophobic amino acids (13 Ala, 6 Val, 12 Leu, 6 Ile, 6 Phe, 9 Tyr, 6 Trp) is also consistent with the chromatographic behavior of the enzyme on solid supports containing alkyl or aryl groups (PC–Sepharose, phenyl-Sepharose). The immunodifusion and immunoelectrophoretic results obtained with both crude venom and purified enzyme (Figure 4) confirmed the results obtained by gel electrophoresis.

The specificity of the phospholipase activity assayed with two synthetic substrates indicates that this enzyme predominantly hydrolyzes the fatty acids at position 2 in the phospholipid molecule (type A_2 activity); also, when fatty acid at the 1-position in the monoacylglycerophosphocholine molecule is unsaturated, it can be hydrolyzed although at a slower rate (more than 6 h; type B activity). This does not occur with some other phospholipases A_2, as is the case for bee venom enzyme, which was used in these experiments as a control for type A_2 activity. The selective preference of purified phospholipase to hydrolyze unsaturated fatty acids has been reported previously for wasp venom phospholipase A_1 by King et al. (1984). Since the purified phospholipase still had a minor contaminant of lower molecular weight, we cannot eliminate the possibility that A_2 and B types of activities are in different protein molecules.

The activity of the phospholipase from *H. horridum horridum* is possibly related to a similar protein in the venom of the Gila monster (*Heloderma suspectum suspectum*) reported by Dehaye et al. (1984). These authors have described a pancreatic secretory protein (M_r 17 500), which also displays phospholipase activity. Thus far, no chemical data are available on the Gila monster secretory protein molecule.

Sequence comparison of the N-terminal regions from bee venom, bovine pancreatic, and other snake venom phospholipases shows *H. horridum horridum* to be more homologous to the bee venom phospholipase A_2 than to the others (Figure 6). The amino-terminal amino acid sequence of phospholipase A_2 from hornet venom is also very different from the *Heloderma* phospholipase (Tuichibaev et al., 1985). Direct sequence comparison of the first 39 amino acid residues at the N-terminal region of *Heloderma* and bee venom phospholipase (Figure 7) shows a 56% homology. This somehow unexpected result makes the venom of *Heloderma* a more interesting one,

	1									10										20									
B. pancreas	A	L	W	Q	F	N	G	M	I	K	C	K	I	P	S	S	E	P	L	L	D	F	N	N	Y	G	C	Y	C
H. h. horridum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	A	F	I	M	P	G	T	L	W	C
A. mellifera	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	I	Y	P	G	T	L	W	C
N. melanoleuca (group I)	N	L	Y	E	F	K	N	M	I	Q	C	T	V	P	N	R	S	W	W	H	-	F	A	N	Y	G	C	Y	C
C. adamanteus (group II)	S	L	V	Q	F	E	T	L	I	M	K	V	A	K	R	S	G	L	L	W	-	Y	S	A	Y	G	C	Y	C

	30									40										50										
B. pancreas	G	L	G	G	S	G	T	P	V	D	D	L	-	-	-	-	-	-	D	R	C	C	Q	T	H	D	N	C	...
H. h. horridum	G	A	G	N	A	A	S	D	Y	S	Q	L	G	T	E	K	D	T	D	M	C	C	R	D	H	D	H	C	...
A. mellifera	G	H	G	N	K	S	S	G	P	N	E	L	G	R	F	K	H	T	D	A	C	C	R	T	H	D	M	C	...
N. melanoleuca	G	R	G	G	S	G	T	P	V	D	D	L	-	-	-	-	-	-	D	R	C	C	Q	I	H	D	N	C	...
C. adamanteus	G	W	G	G	H	G	R	P	Q	D	A	T	-	-	-	-	-	-	D	R	C	C	F	V	H	D	C	C	...

FIGURE 6: Comparison of amino-terminal sequences for phospholipases A_2 from bovine pancreas with others from the venom of *H. horridum horridum*, *A. mellifera*, *Naja melanoleuca*, and *Crotalus ademanteus* [these two last are representative of group I and II phospholipases A_2, respectively, according to Heinrikson et al. (1977)]. Gaps (-) are introduced to provide proper alignment of half-cystine residues and the greatest homology. Invariant residues are enclosed in boxes.

	1									10										20		
Heloderma	G	A	F	I	M	P	G	T	L	W	C	G	A	G	N	A	A	S	D	Y		11/20
Bee			I	I	Y	P	G	T	L	W	C	G	H	G	N	K	S	S	G	P		0.55
	*	*	*		*								*			*	*		*	*		

	31									30									40		
Heloderma	S	Q	L	G	T	E	K	D	T	D	M	C	C	R	D	H	D	H	C...		11/19
Bee	N	E	L	G	R	F	K	H	T	D	A	C	C	R	T	H	D	M	C...		0.58
	*	*			*	*		*			*				*			*			

Total	22/39	
	0.56	

FIGURE 7: Comparison of the N-terminal amino acid sequences of the phospholipases A_2 from bee and *Heloderma* venoms. A Beckman program (Genes) was used to compare both sequences. Asterisks (*) mean different amino acids. The values on the right hand of the figure mean identical amino acids/total amino acids compared in that line, and the fractional number means the proportion of homology.

as mentioned in the introduction. The N-terminal sequence of the phospholipase from the venom of *Heloderma* is similar to that of the enzyme from been venom. It would be more logical to find greater homologies in the sequences of proteins coming from animals that belong to the same taxonomic group, like *Heloderma* and snakes (both from the class Reptilia, order Squamata), than to the bees (from the class Insecta, order Hemiptera). However, if we compare some properties of bee and *Heloderma* venom phospholipases, the bee phospholipase A_2 has a basic isoelectric point ($pI = 10$), and the *Heloderma* has an acidic phospholipase ($pI = 4.5$). The amino acid composition of *Heloderma* phospholipase (Table II) shows that it is rich in Asx (16 residues) and Glx (26 residues). Most of the Asx and Glx residues in bee venom phospholipase as reported by Shipolini (1971) are in the amidated form. It could well be that in the *Heloderma* the Asx and Glx residues are in the acidic form. Bee venom phospholipase A_2 contains four residues of glucosamine (presumed to be N-acetylated) per mole of protein (Shipolini et al., 1974), while *Heloderma* venom phospholipase A_2 has three residues of galactosamine per molecule. The total carbohydrate content is 7.88 and 6.66% for the bee and *Heloderma* venom phospholipases, respectively. These differences and possible differences at the C-terminal sequence (unknown so far) of the *Heloderma* phospholipase will certainly shed light on this interesting structural feature of phospholipases from different origins. It will also probably aid in the classification of phospholipase groups proposed by Heinrickson et al. (1977) and Maraganore et al. (1984).

Another interesting aspect of the results shown in Figure 6 is the positions of invariant amino acids. In this figure we have compared the two well-described groups of phospholipases with that of *Heloderma* and *Apis mellifera* (bee). The rectangular boxes in the figure show that Gly-30, Gly-32, His-48, and Asp-49 are found in all sequences. Since the catalytic activity of *Heloderma* phospholipase, as well the others, is Ca^{2+}-dependent and the above-mentioned amino acids were shown to be involved at the Ca^{2+} binding sites of bovine

pancreatic phospholipase (Dijkstra et al., 1978), it is conceivable that both the *Heloderma* and the bee venom phospholipases also share the same type of amino acid at a similar Ca^{2+} binding site or catalytic active site (Dijkstra et al., 1981) of group I and II phospholipases from pancreatic juice and snake venoms. We are presently completing the amino acid sequence of the *Heloderma* phospholipase A_2 and expect this to contribute to a better understanding of this interesting enzyme.

ACKNOWLEDGMENTS

We thank Fernando Zamudio and Denise Merkle-Lehman for technical assistance during the amino acid analysis and automatic sequence of the *Heloderma* phospholipase.

Registry No. Phospholipase A_2, 9001-84-7.

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Dr. Alejandro C. Alagón
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UNAM, Mexico D.F., 04510, MEXICO

Dear Dr. Alagón:

I would like to commend you on your exciting discovery that the phospholipase A_2 from *H.h.horridum* is structurally related to the bee venom enzyme (Biochemistry 25, 2927). Our laboratory has been quite interested in assessing the relationship between PLA2 from venom and pancreas and from bee venom. I believe that some of our results may prove interesting and, in fact, applicable to your studies. Enclosed is a copy of a manuscript currently in press in the J. Protein Chem.. The manuscript details a reassessment of the bee venom enzyme structure relative to its homology to more conventional phospholipases in amino acid sequence and conformation. In light of your recent findings, I think you'll find this paper enjoyable.

Sincerely,

John Mallary

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Onze ref.: BWD/RAH
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GRONINGEN, October 6, 1986

Onderwerp:

Dear Dr. Alagón,

With interest I read your paper in Biochemistry on the PLA_2 from the Mexican beaded lizard. Especially the N-terminal sequence homology with the bee venom enzyme was very unexpected to me. I think that for our understanding of the phospholipase A_2 catalytic mechanism, and also for our understanding of the evolution of the phospholipase 3D structure, knowledge of the 3D structure of this or a closely related enzyme might give new insights. Would it be possible for you to try to crystallize the protein? For an X-ray structure determination crystals of minimum dimensions of about 0.2 x 0.2 x 0.2 mm are needed. If you could grow these crystals we might try to solve the X-ray structure in a collaborative project.

For your information I enclose a reprint of an article on the 3D structure of porcine phospholipase A_2. It is a correction of the paper from 1976 (Nature 264, 373-377) on the porcine structure.

I wish you the best of luck with the completion of the aminoacid sequence determination of the Heloderma horridum horridum phospholipase A_2.

Yours sincerely,

Bauke W. Dijkstra

HELODERMATINE, A KALLIKREIN-LIKE, HYPOTENSIVE ENZYME FROM THE VENOM OF *HELODERMA HORRIDUM HORRIDUM* (MEXICAN BEADED LIZARD)

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The Mexican beaded lizard (*Heloderma horridum horridum*) is one of the two members of venomous lizards of the family *Helodermatidae* (suborder, *sauria*) that inhabit the arid regions of the southwestern United States and the Pacific coast of Mexico. The *Heloderma* venom is less well characterized than many snake venoms. A neurotoxin (1), a hyaluronidase (2), peptides related to the vasoactive intestinal peptide-secretin family (3, 4), and two arginine esterases (*M*, 22,000 and 63,000) have been described. Mebs (5) has seen a kinin-generating (or kallikrein-like) activity in the venom of the family member Gila monster (*Heloderma suspectum*), but no purification to homogeneity or detailed characterization of this activity was achieved. In the following report we describe a protein purification scheme that yields in two steps the electrophoretically homogeneous arginine esterase (*M*, 63,000) from lyophilized *H. h. horridum* venom. A detailed examination of its catalytic activity revealed properties closely related to kallikrein.

Materials and Methods

Microgranular DEAE-cellulose (DE-52) was from Whatman Inc., Clifton, NJ, and ε-aminocaproyl-*p*-aminobenzamidine agarose (benzamidine Sepharose) was from Pierce Chemical Co., Rockford, IL. Diisopropylphosphofluoridate (DFP), Omnifluor, and 1,4-bis 2-(5-phenyloxazolyl) benzene (POPOP)[1] were purchased from New England Nuclear, Boston, MA. All reagents for PAGE were obtained from Eastman Kodak Co., Rochester, NY or Bio-Rad Laboratories, Richmond, CA. *N*-benzoyl-L-arginine ethyl ester (BAEE), benzoyl-arginine-*p*-nitroanilide (BAPNA), 4-methylumbelliferyl-*p*-guanidinobenzoate hydrochloride (MUGB) and 4-methyl-umbelliferone were purchased from Sigma Chemical Co., St. Louis, MO. D-Pro-Phe-Arg *p*-nitroanilide (S-2302), D-Val-Leu-Arg-*p*-nitroanilide (S-2266), D-Phe-Val-Arg *p*-nitroanilide (S-2160), and D-Glu-Gly-Arg *p*-nitroanilide (S-

Preliminary results of this work were presented at the Symposium on Animal Venoms and Hemostasis, July 20–21, 1985, San Diego, CA.; and they were published in abstract form (26).

[1] *Abbreviations used in this paper:* BAEE, *N*-benzoyl-L-arginine ethyl ether; BAPNA, benzoyl-arginine-*p*-nitroanilide; HMW, high molecular weight; MUGB, 4-methylumbelliferyl-*p*-guanidinobenzoate-hydrochloride; POPOP, 1,4-bis 2-(5-phenyloxazolyl) benzene; tPA, tissue-type plasminogen activator.

2227) were generous gifts of Dr. Petter Friberger, Kabi Vitrum, Stockholm, Sweden. Soybean trypsin inhibitor and limabean trypsin inhibitor were from Sigma Chemical Co. Aprotinin, the bovine pancreatic trypsin inhibitor of the Kunitz type (Trasylol), was kindly provided by Dr. H. Trusheit, Bayer Pharmaceuticals, Wuppertal, Federal Republic of Germany. Mainly single-chain, tissue-type plasminogen activator (tPA) was purified from melanoma cell–conditioned medium, as described (6). Human high-molecular-weight (HMW) kininogen was generously provided by Drs. Brigitte Dittmann and Hans Fritz, University of Munich, Federal Republic of Germany.

Collection of Venom. *Heloderma* venom was obtained by the technique initially described by Loeb (7). Collected venom was separated into aliquots, lyophilized, and kept at −20°C until further use (8).

Measurement of Enzymatic Activities. BAEE-hydrolyzing activity was measured as described (8). Amidolytic activity vs. the substrates S-2302, S-2160, S-2227, S-2266, and BAPNA was quantified by monitoring the increase of absorbance at 405 nm in a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) after the conditions described by Latallo et al. (9). K_m values were determined according to the double reciprocal method of Lineweaver and Burk (9a), plotting data that were obtained after least square analysis of the measured values. K_i values were determined by measuring substrate inhibitor competition according to Dixon (10). Active site titration was performed with MUGB as described by Jameson et al. (11). Plasminogen activator activity was determined by the ^{125}I-fibrin lysis method of Unkeless et al. (12), and we determined thrombin-like activity by standard methods in a clinical coagulometer. Kinin-liberating activity was determined by incubating the enzyme with highly purified human HMW-kininogen. The kinins formed were measured by monitoring the contractions of an isolated rat uterus preparation using a polygraph recorder by a variation of the method of Trautschold (13): 1 μg of helodermatine and 150 μg of human HMW-kininogen were incubated in 1 ml of 0.1 M Tris/HCl (pH 8.5), 10^{-5} M phenanthroline for 30 min at 25°C. The reaction was stopped by heating the sample for 10 min at 95°C. The kinins generated were quantified by comparing the activity with a standard curve, derived from values obtained through the effects of 10–200 pg of synthetic bradykinin triacetate.

Column Chromatography. All procedures were performed in standard laboratory glassware and polyethylene tubing connections at ambient temperature. Elution rates were maintained constant by using peristaltic pumps. Fractions were collected in 10-ml glass tubes.

Measurement of Protein Concentration. Protein concentrations were determined by the method of Lowry et al. (14) or by absorbance measurements at 280 nm, assuming an extinction coefficient of $E^{1\%}_{280}$ of 10.0.

Electrophoretic procedures. We performed SDS-PAGE using the conditions described by Laemmli (15). 10% polyacrylamide slab gels (150 × 120 × 1.5 mm) were used. Electrophoresis was performed at 25 mA constant current. Gels were fixed and stained in a solution of 0.25% Coomassie Brilliant Blue R in 20% methanol (vol/vol), 10% acetic acid (vol/vol) in double-distilled H_2O. Destaining was achieved in a solution of 50% (vol/vol) methanol, 10% (vol/vol) acetic acid in double-distilled H_2O.

Iodination Procedure. 2 μg of helodermatine were iodinated to a specific activity of ~2 μCi/g using New England Nuclear (Boston, MA) Bolton and Hunter reagent according to the instructions provided by the manufacturers.

Labeling with DIFP. 20 ng helodermatine were labeled at the active site with [^{3}H]iPr$_2$-P-F, essentially as described (16).

Amino Acid Analysis. We performed amino acid analysis after acid hydrolysis of sample duplicates according to Moore and Stein (17), as modified by Liao et al. (18) to avoid oxidation of cysteine, methionine, and tyrosine. Serine and threonine values were obtained by extrapolation to time 0 from the 24-, 48-, and 72-h hydrolysis time. Values for valine, leucine, and isoleucine were obtained from the 72-h hydrolysis.

Amino Acid Sequence Determination. The amino terminal covalent structure of helodermatine was determined by sequential chemical degradation as described by Edman (19), applying the technical specifications of Hewick et al. (20).

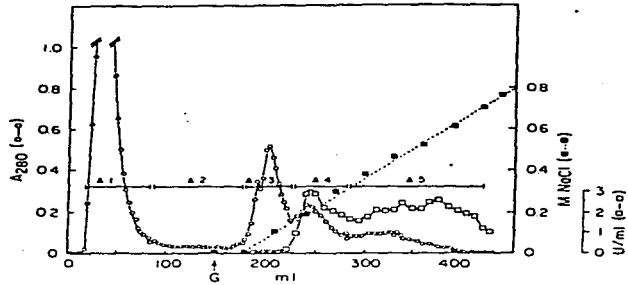

FIGURE 1. Benzamidine-Sepharose affinity chromatography of *H. h. horridum* whole venom. Whole soluble venom (100 mg) was applied to the column. Elution rate was maintained 40 ml/h. 3-ml fractions were collected. The start of the gradient is indicated by an arrow (*G*). Fractions were pooled as indicated by horizontal bars.

Blood Pressure Recording from the Carotid Artery of an Anesthetized Rabbit. A New Zealand white rabbit (2.5 kg) was anesthetized with phenobarbital and the carotid artery was cannulated with a polyethylene catheter connected to a transducer and a polygraph recorder. Injections were made into the femoral artery.

Results

Purification of Helodermatine: Benzamidine Sepharose Affinity Chromatography. Lyophilized venom (100 mg) was dissolved in 0.1 M Tris/HCl, pH 7.95 (starting buffer), and insoluble material was removed by centrifugation. The clear supernatant was applied to a 10 ml (0.9 × 15 cm) column of benzamidine-Sepharose. The column was washed with 15 volumes of the same buffer and was eluted by using a linear salt gradient from 0 to 0.8 M NaCl in a total volume of 300 ml. The results are presented in Fig. 1.

Purification of Helodermatine: DEAE-Cellulose Anionic Exchange Chromatography. Pooled active fractions from five independent benzamidine-Sepharose chromatographies (A4 in Fig. 1) were combined, dialyzed against starting buffer (15 mM potassium phosphate, pH 7.55), and applied to a 0.9 × 30 cm column of DEAE-cellulose (DE 52; Whatman Inc.) equilibrated in the same buffer. The column was washed with two volumes of starting buffer and developed using a linear gradient from 0 to 0.3 M KCl in a total volume of 500 ml. The active fractions were pooled, dialyzed vs. 50 mM $(NH_4)HCO_3$, and were lyophilized. The results of the chromatography are presented in Fig. 2 and a summary of the purification procedure is given in Table I.

Criteria of Purity. The purity of the helodermatine preparation was established by four different independent methods: (*a*) SDS-PAGE of the reduced protein followed by staining with Coomassie Brilliant Blue (Fig. 3); (*b*) SDS-PAGE of the iodinated nonreduced and reduced protein followed by radioau-

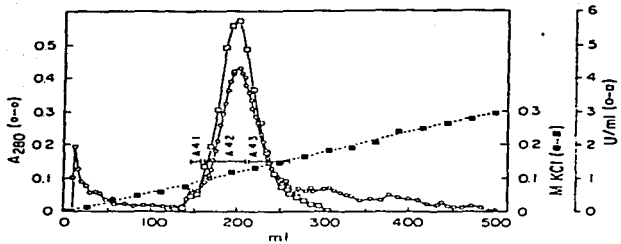

FIGURE 2. DEAE-cellulose anionic exchange chromatography of fraction A.4 from Fig. 1. 4.5 ml of fraction A.4 from Fig. 1 were applied to the column. The flow was maintained at 30 ml/h. 2.5-ml fractions were collected. Fractions were pooled as indicated by horizontal bars. Fraction A.4.2 was used for further characterization. Fractions A.4.1 and A.4.3, which contained minor contamination, were recycled in the next batch of purification.

TABLE I

Purification of Helodermatine from Lyophilized H. h. horridum Venom

Fraction	Source of fraction	Protein	Specific activity (BAEE)*	Recovery of activity
		mg	*U/mg*	%
Whole venom	Lyophilized venom	100	2.67	100
A.4	Benzamidine-Sepharose	9.1	20.02	81
A.4.2	DEAE-32 cellulose	6.4	22.64 (8.77‡)	55

* Assuming $E^{1\%}_{278} = 10$

‡ Assuming $E^{1\%}_{280} = 25.8$, as derived from the amino acid analysis of the pure protein.

tography (Fig. 4); (*c*) SDS-PAGE of the nonreduced and reduced protein after active site affinity labeling with DIFP (Fig. 5); and (*d*) identification of only one amino acid in each step of the *N*-terminal amino acid sequence (Fig. 6).

Amino Acid Analysis. The results of the amino acid analysis are presented in Table II. There is no unusual or conspicuous feature in the amino acid composition. The A_{280} of a solution of 1 mg/ml helodermatine as determined by amino acid analysis was found to be 2.58.

NH_2-Terminal Protein Sequence. The sequence of the 20 NH_2-terminal amino acids is presented in Fig. 6 and compared with the sequence of porcine pancreatic kallikrein (21), crotalase, a kallikrein-like enzyme from the venom of the diamond back rattle snake *Crotalus adamanteus* (22) and kallikrein-like enzymes from *Crotalus atrox* (23). There is a significant homology to all four enzymes, but the closest (8 of 19 residues) is to porcine pancreatic kallikrein.

Enzymatic Properties. Both D-Pro-Phe-Arg pNa (S-2302) and D-Val-Leu-Arg pNa (S-2266), originally developed, respectively, for human serum kallikrein and glandular kallikrein proved to be excellent substrates for helodermatine. Kunitz-type bovine pancreatic trypsin inhibitor (aprotinin, Trasylol) and soybean trypsin inhibitor are strong competitive inhibitors of helodermatine. DIFP inactivated helodermatine irreversibly in a time-dependent fashion consistent with a second-

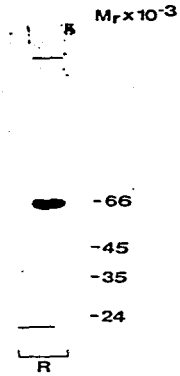

FIGURE 3. SDS-PAGE of 5 μg of purified helodermatine under conditions described in the text. The sample was reduced by boiling for 10 min in sample buffer containing 1% 2-ME. Acrylamide concentration was 7.5%.

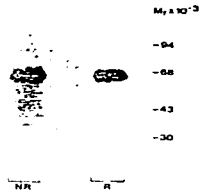

FIGURE 4. SDS-PAGE of ^{125}I-labeled helodermatine. 0.01 μCi of sample were loaded per well. *NR*, native sample denatured at room temperature in sample buffer; *R*, sample denatured and reduced by boiling for 5 min in presence of 10 mM DTT. The gel was dried and exposed over night on XAR film, Eastman Kodak Co., Rochester, NY.

order rate constant ~1/M·s. Active site titration with MUGB revealed a good correlation between molarity calculated on the basis of available active sites or on the basis of M_r and protein content. These experiments are summarized in Table III. Helodermatine released 75% of the amount of kinin from human HMW-kininogen, as human plasma kallikrein did in equivalent amounts and otherwise identical conditions. Incubation of 40 μg single chain tPA with 2 μg helodermatine (100 μl reaction volume, 60 min at 25°C) resulted in a 50% conversion of single-chain tPA into double-chain tPA. Without addition of helodermatine, the single-chain tPA preparation was perfectly stable (Fig. 7*A*).

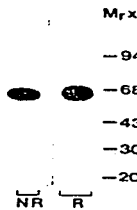

FIGURE 5. SDS-PAGE of DIFP-labeled helodermatine. 1 μg of sample was loaded per well. *NR*, native sample denatured at room temperature in sample buffer; *R*, sample denatured and reduced by boiling for 5 min in presence of 10 mM DTT. The gel was processed for fluorography, dried, and exposed to film as described (16).

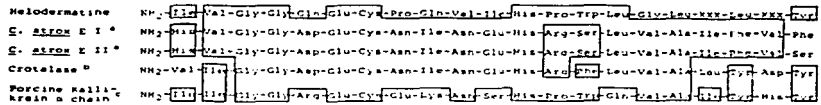

FIGURE 6. NH_2-terminal sequence of helodermatine. Comparison with similar proteinases. Regions of sequence identity are *boxed* (21–23).

The enzyme neither coagulated fibrinogen, nor did it activate plasminogen (data not shown).

Effect on Rabbit Blood Pressure. The effect of helodermatine on the blood pressure of an anesthetized rabbit is shown in Fig. 8. If 40 μg of helodermatine were incubated with 200 μg of aprotinin for 5 min at ambient temperature before injection, the hypotensive effect was completely abolished (data not shown).

Discussion

We describe a simple procedure for the purification of the major *N*-benzoyl-L-arginine esterase from crude venom of *H. h. horridum*. The procedure compares favorably, in terms of yield, speed, and final purity, to previously published methods (5, 8). The principal step consists of affinity chromatography on commercially available benzamidine-Sepharose. The purity of the preparation appears well established by several criteria, notably the migration as a single band in SDS-PAGE of the iodinated protein and the initial yield of one terminal amino acid residue obtained through the Edman degradation procedure. Affinity labeling with DIFP established that the enzyme belongs to the serine proteinase family. Several lines of evidence indicate a close relationship of this enzyme to the kallikreins. Among them stands foremost the high efficiency in liberating the vasoactive kinin from HMW-kininogen, the high catalytic efficiency by which the substrate S-2302 and S-2266 are hydrolysed and the low K_i values are derived

TABLE II
Amino Acid Composition of Purified Helodermatine

Amino acid	20 h*	48 h*	72 h*	Nearest integer
Lysine	12.35	11.89	12.11	12
Histidine	12.57	12.69	12.64	13
Arginine	25.59	26.63	25.43	26
Tryptophan	ND	ND	ND	ND
Aspartic acid	57.05	57.33	55.56	57
Threonine	39.25	36.47	33.23	42‡
Serine	45.76	38.14	32.31	51‡
Glutamic acid	61.38	60.84	61.47	61
Proline	34.62	32.69	33.68	34
Glycine	69.60	69.61	69.60	70
Alanine	19.61	19.19	20.40	20
Half-cystine	24.34	22.95	21.56	23
Valine	42.16	44.40	45.08	45
Methionine	6.01	6.00	6.67	6
Isoleucine	35.36	38.83	39.00	39
Leucine	40.95	40.93	41.22	41
Tyrosine	22.72	21.97	21.62	22
Phenylalanine	11.08	11.83	11.64	12
Glucosamine	8.51	3.36	0.94	11‡

Amino acid compositions were calculated by taking the number of residues per mole of aspartic acid, glutamic acid, alanine, and leucine to be, respectively, 57, 61, 20, and 41.
* Averages of duplicate analyses.
‡ Calculated by extrapolating to time 0 of hydrolysis.

TABLE III
Enzymatic Properties of Helodermatine

Substrate	K_m	K_{cat} (1/s)	K_{cat}/K_m (1/M·s)
	μM		
S-2302	16	1.31	81,900
S-2160	—	0.05	—
S-2227	—	0.37	—
S-2266	75	1.46	17,600
BAEE	83	9.19	110,500

Inhibitor	K_i (M)*
Aprotinin (Trasylol)	2.8×10^{-8}
Soybean trypsin inhibitor	3.0×10^{-8}

* Derived from Dixon plots, using S-2302 as competing substrate.

from the interaction with aprotinin and soybean trypsin inhibitor. Finally, N-terminal sequence analysis revealed a significant homology to kallikrein from porcine pancreas and kallikrein-like enzymes from the venoms of *C. atrox* and *C. adamanteus*. The hypotensive shock almost immediately after bites of crotalid snakes is most likely due to the kallikrein-like enzymes in the respective venoms (23). In analogy, the hypotensive effect of heloderma venom after injection into rabbits, already noted by Fleisher (24), is due to helodermatine, either alone or in combination with other still unknown reagents, because we have clearly shown

FIGURE 7. SDS-PAGE of (*A*) single-chain tPA incubated for 60 min at 25°C in 50 mM Tris/HCl, pH 7.4; (*B*) single-chain tPA incubated in presence of 5% (wt/wt) helodermatine under otherwise identical conditions. 15 μg of sample were loaded per well. Polyacrylamide concentration was 10%. H and L chain of tPA are indicated by *arrows*. Helodermatine is not visible under the staining conditions used. *NR* and *R*, see footnote to Fig. 5.

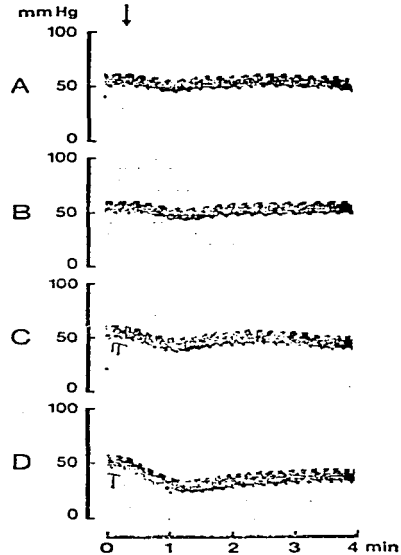

FIGURE 8. Effect of helodermatine on the blood pressure of an anesthetized rabbit. Panels *A*–*D* show the effect of the injection of 2, 4, 8, and 16 μg/kg.

its hypotensive effect. In contrast to the snake enzymes, however, no thrombin-like activity appears to be associated with this enzyme. The conversion of single-chain into double-chain tPA by helodermatine may contribute to the maintenance of a hyperfibrinolytic state at the site of a bite.

Recently, peptides related to glucagon and vasoactive intestinal peptide (VIP) have been described (3) in the venom of *H. suspectum*. Glandular kallikreins have been associated with the processing of prohormone precursors (25). Thus, another possible function of helodermatine might be the generation of pharmacologically active peptides from protein precursors in the venom.

We present for the first time a chemical and enzymatic characterization of a kallikrein-like enzyme in the *Sauria* family. Further research will have to address the question whether this enzyme is more related to the glandular or the serum kallikreins. The M_r of helodermatine is significantly higher than that of glandular kallikreins (21) but lower than the M_r of serum kallikrein. Like the glandular kallikreins, helodermatine is strongly inhibited by aprotinin. It cleaves synthetic peptide substrates, however, with similar kinetics as serum kallikrein. Our findings are of possible relevance for the evolutionary history of the kallikrein/kinin system or proteolytic regulatory systems in general.

Summary

We have purified and characterized the major *N*-benzoyl-L-arginine ethyl ester hydrolase from the venom of *Heloderma horridum horridum*. The enzyme belongs to the serine proteinase family, and its activity vs. peptide amide substrates and human high-molecular-weight kininogen suggests a similarity to the family of kallikreins. This interpretation is corroborated by its reactivity with the natural inhibitors soybean trypsin inhibitor and Kunitz-type bovine pancreatic trypsin inhibitor (aprotinin). Injection of the enzyme (2–16 μg/kg) into anesthetized rabbits leads to a rapid dose-dependent transient decrease of the arterial blood pressure. Like glandular kallikrein it specifically converts single-chain tissue type plasminogen activator into its double chain form. In contrast to other kallikrein-like enzymes from snake venoms it shows no thrombin-like or plasminogen activator activity. The enzyme is a single-chain glycoprotein (M_r 63,000). The *N*-terminal sequence revealed significant homology to pig pancreatic kallikrein and to kallikrein like enzymes from *Crotalus atrox* and *Crotalus adamanteus* venom. This enzyme, which we name Helodermatine, is the first purified from *Sauria* with kallikrein-like properties.

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