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_r 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_r 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 (DIFP), 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-methyllumbelliferyl-*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-

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[1] *Abbreviations used in this paper:* BAEE, *N*-benzoyl-L-arginine ethyl esther; 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 kindy provided by Dr. H. Truscheit, 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^{-3} 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 30% 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).

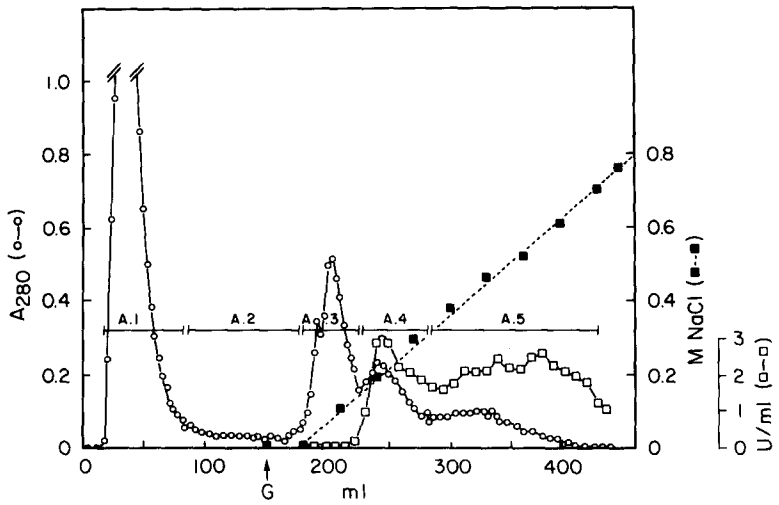

FIGURE 1. Benzamidine-Sepharose affinity chromatography of *H. h. horridum* whole venom. Whole soluble venom (100 mg) was applied to the column. Elution rate was maintained 40 ml/h. 3-ml fractions were collected. The start of the gradient is indicated by an arrow (*G*). Fractions were pooled as indicated 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 (*A.4* 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-

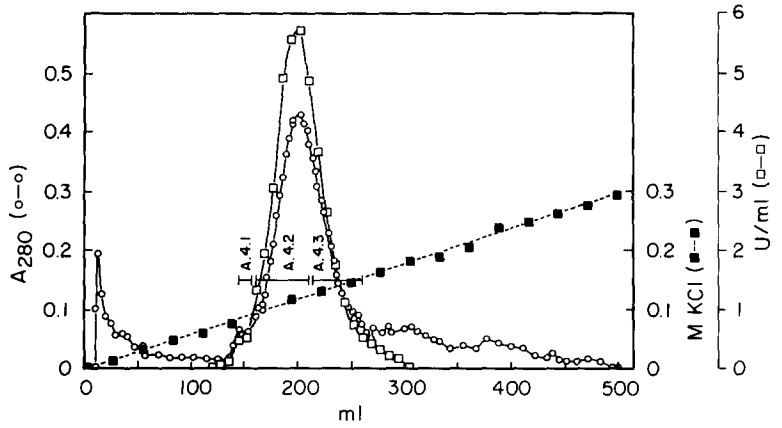

FIGURE 2. DEAE-cellulose anionic exchange chromatography of fraction A.4 from Fig. 1. 4.5 ml of fraction A.4 from Fig. 1 were applied to the column. The flow was maintained at 30 ml/h. 2.5-ml fractions were collected. Fractions were pooled as indicated by horizontal bars. Fraction A.4.2 was used for 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 $E^{1\%}_{280} = 10$

‡ 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-

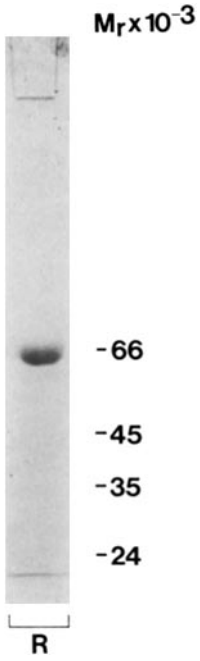

FIGURE 3. SDS-PAGE of 5 μg of purified helodermatine under conditions described in the text. The sample was reduced by boiling for 10 min in sample buffer containing 1% 2-ME. Acrylamide concentration was 7.5%.

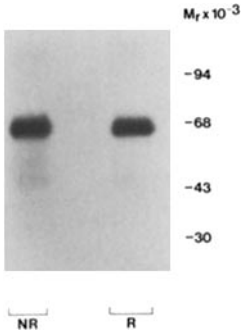

FIGURE 4. SDS-PAGE of ^{125}I-labeled helodermatine. 0.01 μCi of sample were loaded per well. *NR*, native sample denatured at room temperature in sample buffer; *R*, sample denatured and reduced by boiling for 5 min in presence of 10 mM DTT. The gel was dried and exposed over night on XAR film, Eastman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

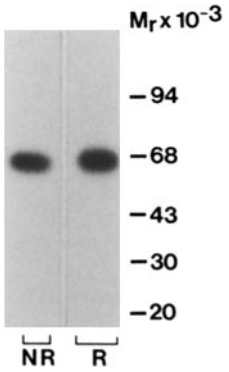

FIGURE 5. SDS-PAGE of DIFP–labeled helodermatine. 1 μg of sample was loaded per well. *NR*, native sample denatured at room temperature in sample buffer; *R*, sample denatured and reduced by boiling for 5 min in presence of 10 mM DTT. The gel was processed for fluorography, dried, and exposed to film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

Helodermatine	NH_2-Ile-Val-Gly-Gly-Gln-Glu-Cys-Pro-Gln-Val-Ile-His-Pro-Trp-Leu-Gly-Leu-XXX-Leu-XXX-Tyr
C. atrox E I [a]	NH_2-His-Val-Gly-Gly-Asp-Glu-Cys-Asn-Ile-Asn-Glu-His-Arg-Ser-Leu-Val-Ala-Ile-Phe-Val-Phe
C. atrox E II [a]	NH_2-His-Val-Gly-Gly-Asp-Glu-Cys-Asn-Ile-Asn-Glu-His-Arg-Ser-Leu-Val-Ala-Ile-Phe-Val-Ser
Crotalase [b]	NH_2-Val-Ile-Gly-Gly-Asp-Glu-Cys-Asn-Ile-Asn-Glu-His-Arg-Phe-Leu-Val-Ala-Leu-Tyr-Asp-Tyr
Porcine Kallikrein a chain [c]	NH_2-Ile-Ile-Gly-Gly-Arg-Glu-Cys-Glu-Lys-Asn-Ser-His-Pro-Trp-Gln-Val-Ala-Ile-Tyr-His-Tyr

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

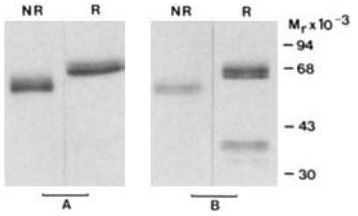

FIGURE 7. SDS-PAGE of (*A*) single-chain tPA incubated for 60 min at 25°C in 50 mM Tris/HCl, pH 7.4; (*B*) single-chain tPA incubated in presence of 5% (wt/wt) helodermatine under otherwise identical conditions. 15 μg of sample were loaded per well. Polyacrylamide concentration was 10%. H and L chain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

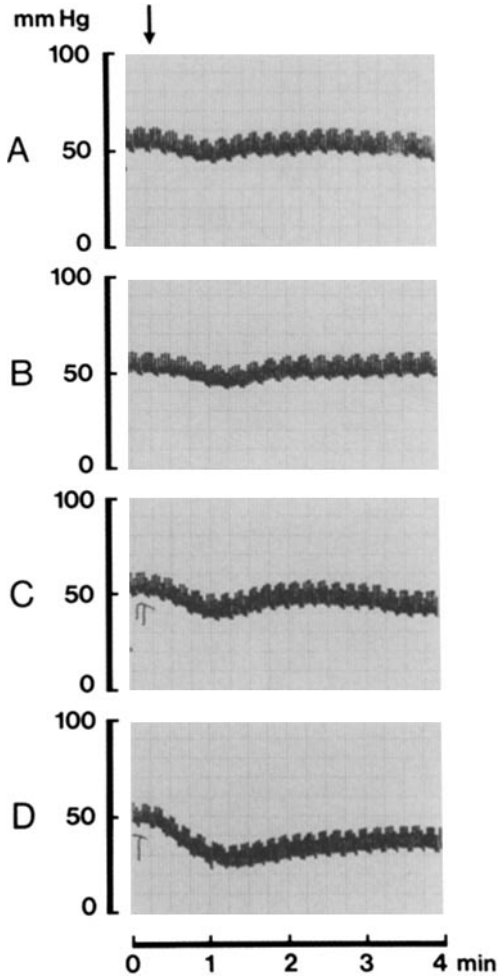

FIGURE 8. Effect of helodermatine on the blood pressure of an anesthetized rabbit. Panels *A–D* show the effect of the injection of 2, 4, 8, and 16 μg/kg.

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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References

1. Hendon, R. A., and A. T. Tu. 1981. Biochemical characterization of the lizard toxin gilatoxin. *Biochemistry.* 20:3517.

2. Tu, A. T., and R. R. Hendon. 1983. Characterization of lizard venom hyaluronidase and evidence for its action as a spreading factor. *Comp. Biochem. Physiol.* 76:377.
3. Parker, D.-S., J. P. Raufman, T. L. O'Donohue, M. Bledsoe, H. Yosida, and J. J. Pisano. 1984. Amino acid sequences of helospectins, new members of the glucagon superfamily, found in Gila monster venom. *J. Biol. Chem.* 259:11751.
4. Robberecht, P., M. Waelbroeck, J. P. Dehaye, J. Winand, A. Vandermeers, M. C. Vandermeers-Piret, and J. Christophe. 1984. Evidence that helodermin, a newly extracted peptide from Gila monster venom, is a member of the secretin/VIP/PHI family of peptides with an original pattern of biological properties. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 166:277.
5. Mebs, D. 1969. Isolierung und Eigenschaften eines Kallikreins aus dem Gift der Krustenechse Heloderma suspectum. *Hoppe Seyler's Z. Physiol. Chem.* 350:821.
6. Kruithof, E. K. O., W. D. Schleuning, and F. Bachmann. 1985. Human tissue-type plasminogen activator. Production in continuous serum-free cell culture and rapid purification. *Biochem. J.* 226:631.
7. Cooke, E., and L. Loeb. 1913. General properties and actions of the venom of Heloderma, and experiments in immunization. *In* The Venom of Heloderma. L. Loeb, editor. Carnegie Institution, Washington, D.C. 49–95.
8. Alagon, A. C., M. E. Maldonado, J. Z. Julia, C. R. Sanchez, and L. D. Possani. 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.
9. Latallo, Z. S., E. Teisseyre, and E. Raczka. 1979. Activators of plasminogen measurement using chromogenic substrates. *In* Chromogenic Peptide Substrates. M. F. Scully and V. V. Kakkar, editors. Churchill-Livingstone, Edinburgh, United Kingdom. 154–164.
9a. Lineweaver, H., and D. Burk. 1934. The determinatioin of enzyme dissociation constants. *J. Amer. Chem. Soc.* 56:658.
10. Dixon, M. 1953. The determination of enzyme inhibitor constants. *Biochem. J.* 55:170.
11. Jameson, G. W., D. V. Roberts, R. W. Adams, W. S. A. Kyle, and D. T. Elmore. 1973. Determination of the operational molarity of solutions of bovine alpha-chymotrypsin, trypsin, thrombin and factor Xa by spectrofluorimetric titration. *Biochem. J.* 131:107.
12. Unkeless, J. C., A. Tobia, L. Ossowski, J. P. Quigley, D. B. Rifkin, and E. Reich. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. I. Chick embryo fibroblast cultures transformed by avian RNA tumor viruses. *J. Exp. Med.* 137:85.
13. Trautschold, I. 1970. Assay methods in the kinin system. *In* Handbook of Experimental Pharmacology. Vol. XXV. E. G. Erdös, editor. Springer-Verlag, Heidelberg, Federal Republic of Germany. 52–81.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
16. Schleuning, W. D., M. Sudol, and E. Reich. 1983. A proenzyme from chicken plasma similar to human plasma prekallikrein. *J. Biol. Chem.* 258:14106.
17. Moore, S., and W. H. Stein. 1963. Chromatographic determination of amino acids by the use of automatic recording equipment. *Methods Enzymol.* 6:819.
18. Liao, T. H., G. W. Robinson, and J. Salnikow. 1973. Use of narrow-bore columns in amino acid analysis. *Anal. Chem.* 45:2286.

19. Edman, P. 1956. On the mechanism of the phenol isothiocyanate degradation of peptides. *Acta Chem. Scand.* 10:761.
20. Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer. 1981. A gas-liquid solid phase peptide and protein sequenator. *J. Biol. Chem.* 256:7990.
21. Tschesche, H., G. Mair, and G. Godec. 1979. The primary structure of porcine glandular kallikreins. *Adv. Exp. Med. Biol.* 120A:245.
22. Pirkle, H., F. S. Markland, I. Theodor, R. Baumgartner, S. S. Bajwa, and H. Kirakossian. 1981. The primary structure of crotolase, a thrombin-like venom enzyme, exhibits closer homology to kallikrein than to other serine proteases. *Biochem. Biophys. Res. Commun.* 99:715.
23. Bjarnason, J. B., A. Barish, G. S. Direnzo, R. Campbell, and J. W. Fox. 1983. Kallikrein-like enzymes from Crotalus atrox venom. *J. Biol. Chem.* 258:12566.
24. Fleisher, M. S. 1913. Influence of Heloderma venom upon blood-pressure, diuresis, peritoneal transudate and intestinal fluid. *In* The Venom of Heloderma. L. Loeb, editor. Carnegie Institution, Washington, D.C. 101–117.
25. Mason, A. J., B. A. Evans, D. R. Cox, J. Shine, and R. I. Richards. 1983. Structure of mouse kallikrein gene family suggests a role in specific processing of biologically active peptides. *Nature (Lond.).* 303:300.
26. Alagon, A., L. D. Possani, J. Smart, W.-D. Schleuning. 1985. Helodermatine, a kallikrein-like enzyme from the venom of Heloderma horridum horridum. *Thromb. Haemostasis.* 54:312 (Abstr.)