Primary structure of helodermin, a VIP-secretin-like peptide isolated from Gila monster venom

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The complete amino acid sequence of helodermin isolated from the venom of Gila monster was elucidated. The peptide was shown to be a basic pentatriacontapeptide amide: His-Ser-Asp-Ala-Ile-Phe-Thr-Gln-Gln-Tyr-Ser-Lys-Leu-Leu-Ala-Lys-Leu-Ala-Leu-Gln-Lys-Tyr-Leu-Ala-Ser-Ile-Leu-Gly-Ser-Arg-Thr-Ser-Pro-Pro-Pro-NH_2. A high degree of sequence similarities to secretin/VIP/PHI/(PHM)/GRF from mammal and bird was observed over the entire N-terminal 1–27 sequence. In particular, the amino acid residues in positions 3, 6 and 7 were found to be common to 9 peptides of the family. Another interesting feature of the structure of helodermin was its C-terminal -Pro-Pro-Pro-NH_2 sequence. Isolation of helodermin was the first demonstration of the existence of a secretin/VIP-related peptide in an animal that is neither mammal nor bird.

Gila monster venom *Helodermin* *Amino acid sequence* *Sequence analysis* *Solid-phase peptide synthesis* *Secretin/VIP (vasoactive intestinal peptide) peptide family*

1. INTRODUCTION

Helodermin has been purified [1] from the venom of Gila monster (*Heloderma suspectum*). The biological and pharmacological activities so far demonstrated [1,2] have suggested that the newly discovered peptide could be classified in the secretin/VIP family of peptides. This paper describes the structure elucidation of helodermin.

2. MATERIALS AND METHODS

HPLC for purification and identification of natural and synthetic peptides was performed on a Toyo-Soda high speed liquid chromatograph model SP 8700 and the eluates were monitored with a Toyo-Soda Spectrophotometer model UV-8-II at 210 nm wavelength. Amino acid analysis was carried out with a Hitachi amino acid analyzer model 835 using *o*-phthalaldehyde. Pro and Cys were determined after treatment of the column eluates with hypochlorous acid. Peptide sequence analysis was performed on an Applied Biosystems gas phase protein sequencer model 470 A. Phenylthiohydantoin (PTH)-amino acids liberated in sequence analysis were identified by HPLC on a Beckman HPLC instrument consisting of a pump system model 112, a controller model 421 and a detector model 165 with a Waters automatic sampler model 710 B and a SIC data processor model 7000 A. Peptide synthesis was carried out by a solid-phase technique [3] with a Beckman peptide synthesizer model 990 B. The

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Abbreviations: VIP, vasoactive intestinal peptide; PHI, peptide histidine isoleucine; PHM, peptide histidine methionine; GRF, growth hormone-releasing factor; HPLC, high-performance liquid chromatography

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reagents used included acetonitrile and water for HPLC (E. Merck, Darmstadt), bovine trypsin (EC 3.4.21.4, TPCK-treated) and bovine α-chymotrypsin (EC 3.4.21.1, TLCK-treated) (Sigma, St Louis, MO), and all the reagents needed for sequence analysis (Applied Biosystems, Foster City, CA) and for peptide synthesis (Protein Research Foundation, Osaka).

2.1. *Isolation of helodermin*

Helodermin was isolated from Gila monster venom as in [1]. The final purification was performed by HPLC using a μBondapak C-18 column (Waters Assoc., Milford, MA) (3.8 × 300 mm) in a 0.01 N HCl/acetonitrile gradient system (0/100–20/80, v/v) over 80 min at a flow rate of 1.0 ml/min.

2.2. *Enzymatic fragmentation*

Purified helodermin (0.4 nmol) was dissolved in 10% acetic acid (5 μl), to which 1% aqueous ammonium bicarbonate (500 μl) and a solution of trypsin in 1% aqueous ammonium bicarbonate (0.1 μg in 10 μl) were added. The reaction mixture was kept at 25°C for 4 h, then heated in a boiling water bath for 5 min and lyophilized. The lyophilizate was dissolved in 0.01 N HCl (100 μl) and the solution was applied to a μBondapak C-18 column (3.8 × 300 cm) for HPLC. The column was eluted with a linear gradient system of 0.01 N HCl/acetonitrile (0/100–40–60, v/v) over 40 min at a flow rate of 1.0 ml/min. The fractions containing peptides were collected and lyophilized, then submitted to amino acid analysis and sequence determination. Chymotryptic digestion was similarly performed on 1.2 nmol helodermin with 0.4 μg α-chymotrypsin.

2.3. *Amino acid analysis*

Each peptide (≃50 pmol) was hydrolyzed in 6 N HCl (20–50 μl) containing 4% thioglycollic acid at 110°C for 24 h in an evacuated tube. The solution was evaporated to dryness and the residue was submitted to amino acid analysis.

2.4. *Sequence analysis*

The amino acid sequences of a purified preparation of helodermin (≃1.0 nmol) and its tryptic and chymotryptic fragments (≃0.2 nmol each) were analyzed according to operation programs 01PREP and RUNTFA provided for the sequencer. In each step of degradation, the reaction mixture was applied to an Ultrasphere column (Beckman Instruments, Palo Alto, CA) (2 × 250 mm) heated at 50°C and the column was eluted with a gradient solvent system of acetonitrile/20 mM sodium acetate (pH 4.5) (10/90, v/v, for 0–1 min, 10/90–35/65 for 1–3 min, 35/65–40/60 for 3–18 min, and 40/60 for 18–35 min). The eluates were monitored at wavelength 269 and 322 nm to identify the PTH-amino acids liberated.

2.5. *Peptide synthesis*

Helodermin-related peptides synthesized here were H-Leu-Ala-Ser-Ile-Leu-Gly-Ser-Arg-Thr-Ser-Pro-Pro-Pro-$\underline{NH_2}$ and H-Leu-Ala-Ser-Ile-Leu-Gly-Ser-Arg-Thr-Ser-Pro-Pro-Pro-$\underline{OH}$ (positions 23–35). The peptide chain was elongated on a chloromethylated polystyrene resin for the peptide having a C-terminal free α-carboxylic acid and on a benzhydrylamine polystyrene resin for the peptide having a C-terminal amide, according to a program similar to those employed previously [4,5]. Protected amino acids used were $\underline{N}^{\alpha}$-Boc derivatives of Pro, Gly, Leu, Ile, Ala, Ser(Bzl), Thr(Bzl) and Arg(Tos). When the synthesis of the desired peptide was completed, the protected peptide-resin was treated with HF in the presence of anisole. The resulting peptide was purified by gel filtration and reverse-phase HPLC. The purity of the synthetic peptides was confirmed by HPLC and amino acid analysis of their acid hydrolysates and aminopeptidase M digests. Details of the synthesis and physicochemical characterization of the peptides will be presented elsewhere. The two synthetic tridecapeptides corresponding to helodermin (23–35) were used for identification of chymotryptic fragment C-4 by HPLC on a TSK-GEL-ODS-120T column (Toyo Soda, Tokyo) (4.6 × 250 mm) in a gradient solvent system of acetonitrile/30 mM phosphate buffer (pH 7.5) (20/80–40/60, v/v) over 20 min at a flow rate of 1 ml/min.

3. RESULTS AND DISCUSSION

Fig.1 shows the final purification profile of helodermin on HPLC. The main peak fractions of biological activities [1,2] (retention time 41.2 min)

were collected and used for structure determination. An acid hydrolysate of helodermin contained 14 different amino acid species in the ratios shown in table 1, indicating a pentatriacontapeptide structure for the peptide. Sequence analysis with the whole molecule of helodermin revealed its N-terminal partial structure as being:

$$\overset{1}{\text{His}}\text{-Ser-Asp-Ala-}\overset{5}{\text{Ile}}\text{-Phe-Thr-(Gln)-(Gln)-}$$
$$\overset{10}{\text{Tyr}}\text{-(Ser)-Lys-Leu-Leu-}\overset{15}{\text{Ala}}\text{-(Lys)-(Leu)-}$$
$$\text{(Ala)-(Leu)-}\overset{20}{\text{(Gln)}}\text{-(Lys)-}\overset{22}{\text{Tyr}}\text{-(Leu)-----.}$$

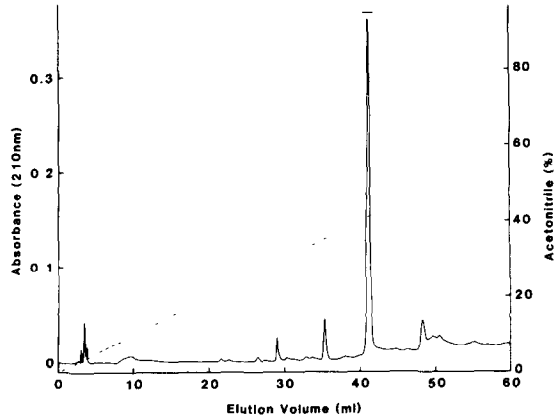

Fig.1. Final purification of helodermin preparation on HPLC (helodermin preparation: 9.6 μg).

The ambiguous amino acid residues and the C-terminal part of the structure were confirmed using tryptic and chymotryptic fragments of helodermin. HPLC of the tryptic digest yielded 4 major components, T-1 to T-4 (fig.2), and the chymotryptic digest contained 5 major components, C-1 to C-5 (fig.3). Amino acid compositions of acid hydrolysates of the 4 tryptic fragments (table 1) accounted for 30 of the 35 residues constituting the helodermin molecule. Sequence analysis of the tryptic fragments confirmed the 1-21 sequence of helodermin which corresponded to T-1, T-2 and T-3. Since the amino acid residues in positions 22 and 23, i.e., Tyr and Leu, were found to correspond to the N-terminal two residues of T-4, fragment T-4 was supposed to be placed next to T-3 and followed by the 5 amino acid residues, Pro(3), Ser(1) and Thr(1), missed in HPLC of the tryptic

Table 1

Amino acid compositions of helodermin and its tryptic fragments

Amino acid	Helodermin	T-1	T-2	T-3	T-4	T-5[a]
Ala	3.98(4)	1.17(1)	1.01(1)	0.80(1)	1.22(1)	
Arg	1.02(1)				1.00(1)	
Asx	1.01(1)	1.11(1)				
Glx	2.87(3)	2.12(2)		1.04(1)		
Gly	1.08(1)				1.78(1)	
His	0.90(1)	0.98(1)				
Ile	2.16(2)	0.97(1)			0.88(1)	
Leu	5.92(6)		1.73(2)	1.38(2)	1.79(2)	
Lys	2.95(3)	1.14(1)	0.99(1)	0.82(1)		
Phe	0.97(1)	1.01(1)				
Pro	3.19(3)[b]					(3)
Ser	5.05(5)	1.87(2)			2.07(2)	(1)
Thr	1.75(2)	1.04(1)				(1)
Tyr	2.28(2)	1.00(1)			0.91(1)	
Position	1–35	1–12	13–16	17–21	22–30	31–35

[a] Not isolated

[b] Determined by using hypochlorous acid

The values in parentheses represent the theoretical number of residues

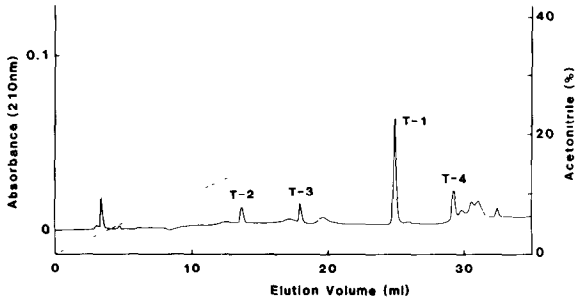

Fig.2. Elution profile of tryptic digest of helodermin on HPLC.

digest. These results did not conflict with amino acid analysis (table 2) and with the sequencing of the 5 chymotryptic fragments C-1 to C-5. Among these fragments, C-4 was assigned to the C-terminal fragment of helodermin and shown to contain the Thr-Ser-Pro-Pro-Pro sequence at the end of its C-terminus. Since the amino acid residue in position 30 was found to be Arg, the 5 residues which had been missed in HPLC of the tryptic digest were inferred to constitute the C-terminal sequence, Thr-Ser-Pro-Pro-Pro, corresponding to positions 31–35, which was tentatively designated T-5. Although the reason why fragment T-5 was not detected by HPLC has not yet been clarified, the high hydrophobicity of this fragment might be considered as at least partly responsible.

The question remaining with respect to the chemical structure of the C-terminal end group of helodermin was answered by a synthetic approach. Namely, two tridecapeptides, corresponding to fragment C-4 (positions 23–35), were synthesized with, respectively, a C-terminal free carboxylic acid and the corresponding amide. When compared with fragment C-4 in terms of retention time in HPLC (fig.4), the synthetic tridecapeptide amide coeluted with fragment C-4 at a retention time of 13.9 min in the system employed and the other synthetic peptide showed a different retention time (12.0 min). This confirmed the C-terminal amide structure of the helodermin molecule. Our results, summarized in fig.5, show that helodermin is a basic pentatriacontapeptide amide.

Christophe and co-workers have previously demonstrated that helodermin interacts with secretin or VIP receptors, depending on the system tested. The peptide binds to secretin-preferring receptors in acini and membranes from rat pancreas [2,6]. It activates adenylate cyclase in rat heart through the occupancy of receptors that are

Table 2

Amino acid compositions of the chymotryptic fragments of helodermin

Amino acid	C-1	C-2	C-3	C-4	C-5
Ala	1.07(1)	1.04(1)	1.05(1)	1.07(1)	1.68(1)
Arg				0.91(1)	
Asx	1.06(1)				1.01(1)
Glx	1.86(2)		0.96(1)		1.82(2)
Gly				1.06(1)	
His	0.87(1)				
Ile	0.98(1)			0.97(1)	1.56(1)
Leu	1.08(1)	2.07(2)	1.02(1)	1.95(2)	1.44(1)
Lys	0.94(1)	0.96(1)	0.94(1)		0.99(1)
Phe	0.98(1)				0.95(1)
Pro				(3)	
Ser	1.62(2)			3.14(3)	2.49(2)
Thr	0.96(1)			0.96(1)	1.34(1)
Tyr	1.01(1)		1.01(1)		1.02(1)
Position	1–13	14–17	18–22	23–35	2–13

The values in parentheses represent the theoretical number of residues

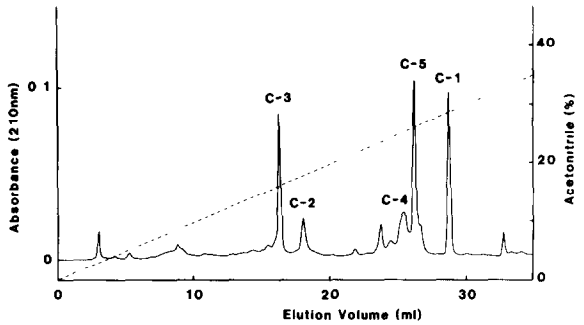

Fig.3. Elution profile of chymotryptic digest of helodermin on HPLC.

common for secretin and VIP; it recognizes specific VIP receptors in rat brain and human heart [2]. In addition, ^{125}I-labelled helodermin binds to a class of high-affinity VIP receptors in rat liver membranes [7].

Now, the newly elucidated sequence of helodermin substantiates chemically the close relationship of helodermin with secretin/VIP/PHI/GRF (fig.6). Located in identical positions to those of helodermin are as many as 14–15 amino acid residues in porcine, human, and chicken VIP; 12–13 residues in porcine PHI and human PHI (PHM); 10 residues in porcine and chicken secretin, and 12 residues in human and rat GRF. In particular, the residues in positions 3, 6 and 7 are common to all 9 peptides.

In several precursors of bioactive peptides, the formation of a carboxyl terminal α-amide residue depends on the sequence X-Gly-basic-basic [8]. Helodermin has a C-terminal prolinamide residue (like calcitonin and thyroliberin). This residue, representing X in the putative precursor, is in the last 35 position of an interesting -Pro-Pro-Pro-NH_2 sequence. Helodermin is longer than secretin, VIP, and PHI/PHM, perhaps due to the fact that its glycine in position 28 cannot serve as an amino donor to leucine in position 27, because a transition mutation has provoked the replacement of an AGG codon (for the first of a pair of Arg residues) by AGC (for serine 29), in the mRNA encoding the peptide.

Helodermin, a secretory peptide from the sublingual venomous gland of a lizard, is the first demonstration of the production of a secretin/VIP/PHI/GRF related peptide in an animal other than mammal and bird. Despite

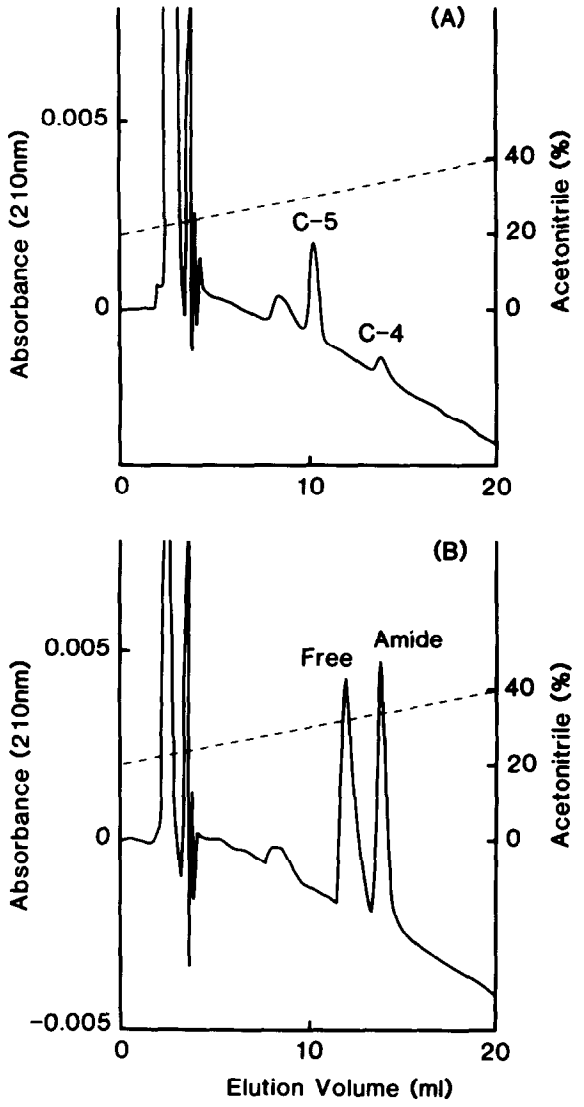

Fig.4. Identification on HPLC of chymotryptic fragment C-4 with synthetic peptides. (A). Elution profile of chymotryptic fragments C-4 and C-5. (B) Elution profile of synthetic tridecapeptides corresponding to helodermin (23–35) which have α-carboxylic acid (Free) and α-carboxyamide (Amide) groups, respectively.

strong homology, helodermin, due to its original amino acid sequence (fig.6), displays an original pattern of biological activities and might well be the prototype of a new class of regulatory peptides and not the mere ontogenic variant of a well known model. The structure of each peptide in the

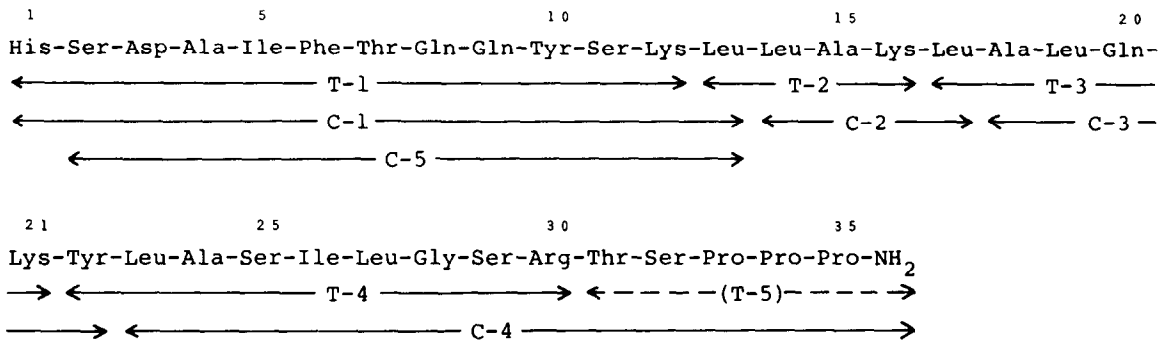

Fig.5. The complete amino acid sequence of helodermin. T-1 to T-5 represent the tryptic fragments and C-1 to C-4 the chymotryptic fragments.

secretin/VIP/PHI/GRF family is, indeed, quite conservative throughout evolution. This hypothesis is now being actively tested in our laboratories.

ACKNOWLEDGEMENTS

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THE VIP-SECRETIN-PHI FAMILY OF PEPTIDES.

SPECIES	PEPTIDE	1 5 10 15 20 25 30 35 40 45
g	**HELODERMIN** :	H-S-D-A-I-F-I-Q-Q-Y-S-K-L-L-A-K-L-A-L-Q-K-Y-L-A-S-I-L-G-S-R-T-S-P-P-P-*
p/h	**VIP** :	H-S-D-A-V-F-I-D-N-Y-T-R-L-R-K-Q-M-A-V-K-K-Y-L-N-S-I-L-N-*
c	**VIP** :	H-S-D-A-V-F-I-D-N-Y-S-R-F-R-K-Q-M-A-V-K-K-Y-L-N-S-V-L-T-*
p	**PHI** :	H-A-D-G-V-F-I-S-D-F-S-R-L-L-G-Q-L-S-A-K-K-Y-L-E-S-L-I
h	**PHM** :	H-A-D-G-V-F-I-S-D-F-S-K-L-L-G-Q-L-S-A-K-K-Y-L-E-S-L-M
p	**SECRETIN** :	H-S-D-G-T-F-I-S-E-L-S-R-L-R-D-S-A-R-L-Q-R-L-L-Q-G-L-V-*
c	**SECRETIN** :	H-S-D-G-L-F-I-S-E-Y-S-K-M-R-G-N-A-Q-V-Q-K-F-I-Q-N-L-M-*
h	**GRF** (1-44) :	Y-A-D-A-I-F-I-N-S-Y-R-K-V-L-G-Q-L-S-A-R-K-L-L-Q-D-I-M-S-R-Q-Q-G-E-S-N-Q-E-R-G-A-R-A-R-L-*
r	**GRF** (1-43) :	H-A-D-A-I-F-I-S-S-Y-R-R-I-L-G-Q-L-Y-A-R-K-L-L-H-E-I-M-N-R-Q-Q-G-E-R-N-Q-E-Q-R-S-R-F-N
		1 5 10 15 20 25 30 35 40 45

* : NH_2

SPECIES : b: bovine; c : chicken; g : Gila monster; h : human; p : porcine; r : rat;

Fig. 6. Comparison of the amino acid sequence of helodermin with those of VIP, PHI, PHM, secretin and GRF. Identities are represented by bold letters and bars. * = α-carboxyamide group.

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