HYPOTENSIN – A HYPOTENSIVE PEPTIDE ISOLATED FROM *CROTALUS ATROX* VENOM: PURIFICATION, AMINO ACID COMPOSITION AND TERMINAL AMINO ACID RESIDUES

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1. Introduction

Arterial hypotension is one of the most common clinical findings following severe bites by the North American rattlesnakes [1]. As has been indicated by Russell et al. [2] the immediate and profound decrease in systemic arterial pressure can not be attributed to direct cardiac effects since neither the heart rate nor the force of contraction are directly altered by intravenous administration of Crotalus venoms to a wide variety of experimental animals.

Indirect evidence for the potential role of bradykinin in snake venom-induced hypotension has been reported by Russell [3] who showed decreased bradykininogen levels in three patients following rattlesnake bites. Only one of the patients, however, was reported to be in shock when reporting to the hospital while the other two were normotensive and/or slightly hypotensive. It is obvious, then, that because of the great complexity of the venom secretion from the rattlesnakes little is known about the specific components which may induce the profound fall in blood pressure following envenomation.

In this paper the isolation of a potent hypotensive peptide* (heretofore called hypotensin) from the venom of the Western diamondback rattlesnake (*Crotalus atrox*), its amino composition, terminal residues and some of its biological properties are reported.

*Hypotensin: patent pending, Elars Bioresearch Laboratories, Inc., Fort Collins, Colorado 80522.

2. Materials and methods

2.1. *Materials*

Western diamondback rattlesnake (*C. atrox*) venom was collected from healthy specimens kept in our laboratory under controlled conditions of temperature, humidity and lighting. Immediately following collection the venom was cleared by centrifugation, lyophilized and stored at −40°C as described in earlier publications [4,5].

All chemical reagents were the best commercially available analytical grade; hydrochloric acid and other reagents for amino acid analysis were sequanal grade from Pierce Chemical Company; Sephadex gel filtration media from Pharmacia.

2.2. *Gel filtration on Sephadex G-100*

Venom (5–10 g) was dissolved in 50–100 ml of 0.12 M Tris-HCl–0.2 M NaCl, pH 8.6, centrifuged at 20 000 rev/min for 30 min in a refrigerated centrifuge (4°C), Sorval S-34 rotor. The supernatant fraction was then applied to a 5.0 × 100 cm column of Sephadex G-100 using upward flow adaptors (actual bed height: 90 cm) and elution carried out with the same buffer.

2.3. *Differential ultrafiltration*

Amicon PM-10 and UM-2 ultrafiltration membranes were used for further purification of the hypotensive peptide.

2.4. *Gel filtration on Sephadex G-25*

The retentate (10–15 ml) from the UM-2 ultrafiltration step was gel filtered on a 2.5 × 100.0 cm column of Sephadex G-25 (fine) using 0.005 M Tris-HCl, 0.1 M NaCl, pH 8.0.

2.5. *Amino acid analysis*

Amino acid analyses in duplicate were carried out on homogeneous samples of the hypotensive peptide according to the method originally described by Spackman, Stein and Moore [6] using a Durrum analyzer model D-500 with built in computer. Salt-free samples were prepared by exhaustive diafiltration (UM-2) against double-distilled deionized water.

Aliquots were hydrolyzed in duplicate for 24, 48 and 96 h at 110°C, and the results averaged. Tryptophane content was obtained from analysis of a 48 h alkaline hydrolyzate [7] and cysteine by performic acid oxidation followed by 24 h acid hydrolysis as described by Hirs [8].

2.6. *N- and C-terminal residue determinations*

Amino terminal sequence was determined using the manual method of Petersen et al. [9] and the automatic sequenator method as described by Hermodson et al. [10]. The carboxyl-terminal residue was determined following enzymatic digestion with carboxypeptidase-A using the Durrum analyzer.

2.7. *Polyacrylamide electrophoresis*

Electrophoresis in 14% polyacrylamide acidic gels containing 6 M urea was performed as described earlier [4,5,11] without modification.

2.8. *Bioassay*

The hypotensive activity of the venom peptide was studied in anesthetized beagle dogs (8–15 kg). A detailed investigation of the cardiovascular effects of varying doses of the peptide was carried out following a left and right heart catheterization as described in earlier communications [12,13] from this laboratory. The complete cardiovascular effects of the venom hypotensive peptide do not fall within the scope of the present paper and, therefore, will be presented elsewhere [14]. Its hypotensive action, however, is herein described.

3. Results

Gel filtration on Sephadex G-100 resolved the crude venom into five subfractions (fig.1) which have been partially identified [15] as follows: fraction I A, a large mol. wt. fraction excluded from the column contains most of the 5′-nucleotidase activity and other unidentified components; fraction I B, contains the L-amino acid oxidase, phosphodiesterase, some remnant 5′-nucleotidase activity and other components.

Subfractions II A and II B contain all of the phospholipase-A activity, the bulk of the proteolytically active proteins, the hemorrhagic components of the venom and the myocardial depressor protein [15]. Fraction III contains the small molecular weight components of the venom including the hypotensive peptide described in this work.

3.2. *Differential ultrafiltration*

Fraction III was pooled and subjected to ultrafiltration on a PM-10 membrane; the resulting ultrafiltrate mol. wt. below approx. 10 000) was dialyzed and ultrafiltered (diafiltration) using a UM-2 Amicon membrane. The retentate obtained after this procedure was then gel filtered on a Sephadex G-25 (fine) column.

3.3. *Sephadex G-25 gel filtration*

Fig.2 shows the elution profile obtained when the

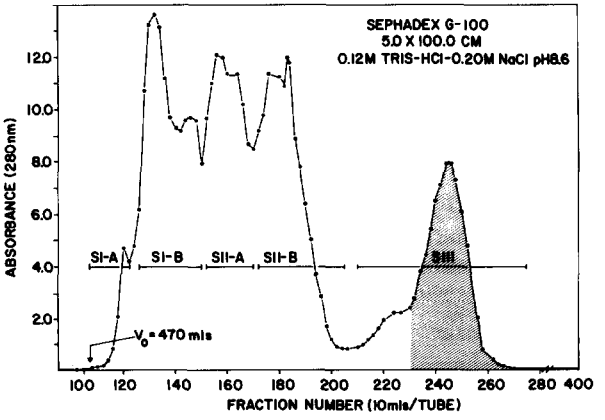

Fig.1. Elution profile of the gel filtration step (Sephadex G-100) used in the purification of hypotensin from the venom of *Crotalus atrox*. Load: 5 g venom in 80 ml Tris-HCl buffer pH 8.6. The fractions pooled for further purification are indicated by the cross hatched area. Further details in text.

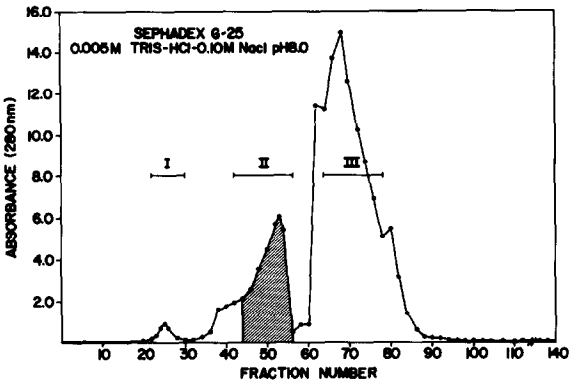

Fig.2. Chromatography of Sephadex G-100 purified hypotensin on Sephadex G-25. The fractions demarked by the cross-hatched area were pooled and concentrated by lyophilization.

retentate from the UM-2 diafiltration step was gel filtered on a 2.5 X 100 cm column of Sephadex G-25. The fractions constituting the second peak were pooled lyophilized and stored at −40°C until needed. Prior to amino acid analysis the highly purified hypotensive peptide was subjected to exhaustive diafiltration on a UM-2 Amicon membrane.

3.4. *Amino acid analysis*

The results of amino acid analysis of the highly purified peptide are shown in table 1. These values were derived from duplicate hydrolyzates at 24, 48 and 96 h. Values for serine and threonine were extrapolated to zero time hydrolysis and that reported for isoleucine was the maximum value obtained. Using the procedures described one tryptophan and one

Table 1
Amino acid analysis of hypotensin

Amino acid	μmoles/mg[a]	Residues/molecule Calculated[b]	Nearest integer
Alanine	0.080	0.86	1
Arginine	0.091	0.98	1
Aspartic acid	0.225	2.41	2
Cystine/2[c]	0.126	1.35	1
Glutamic acid	0.300	3.22	3
Glycine	0.144	1.55	1
Histidine	0.067	0.72	1
Isoleucine	0.088	0.94	1
Leucine	0.108	1.16	1
Lysine	0.111	1.19	1
Methionine	0.025	0.09	0
Phenylalanine	0.045	0.48	0
Proline	0.276	2.96	3
Serine[d]	0.107	1.15	1
Threonine[d]	0.084	0.90	1
Tryptophan[e]	0.117	1.26	1
Tyrosine	0.046	0.49	0
Valine	0.099	1.06	1
Total			Residues 20

[a]Average of: 2 values at 24 h, 1 value at 48 h, 1 value at 96 h.
[b]Assuming 0.093 μmoles/residue.
[c]Performic acid oxidized prior to acid hydrolysis.
[d]Extrapolated to zero time hydrolysis.
[e]48-h alkaline hydrolysis.

cysteic acid residues per mole of peptide could be identified.

3.5. *NH_2 and COOH terminal amino acid residues*

Identical results were obtained using either manual or automatic procedures to detect the N-terminal residue, which was identified as leucine; using the sequenator a second passage was used to determine the penultimate residue which was found to be glutamic acid. Thus, the amino terminal sequence of the hypotensive peptide from *C. atrox* venom is: H-Leu–Glu–

Among the amino acids released by enzymatic hydrolysis with carboxypeptidase-A, serine was the first one to appear followed by leucine and tyrosine. These results were repeated after incubation at 30 min, 2 h and 7 h and, therefore, it is concluded that the hypotensive peptide isolated in this study consists of approximately 20 residues (Ala, Arg, Asp_2, Cys, Glu_3, Gly, His, Ileu, Leu, Lys, Pro_3, Ser, Thr, Trp, Val), with leucine followed by glutamic acid at the amino terminal sequence. Serine is the C-terminal amino acid residue followed by leucine and tyrosine.

3.6. *Gel electrophoresis*

The electrophoretic pattern obtained with the purified hypotensive peptide is shown in fig.3 and indicates that, using the procedure herein described this peptide can be recovered in homogeneous or nearly homogeneous form.

3.7. *Bioassay of hypotensive peptide*

Table 2 shows the systemic arterial pressure response of anesthetized beagle dogs to 1.0 mg/kg intravenous injection of the purified hypotensive

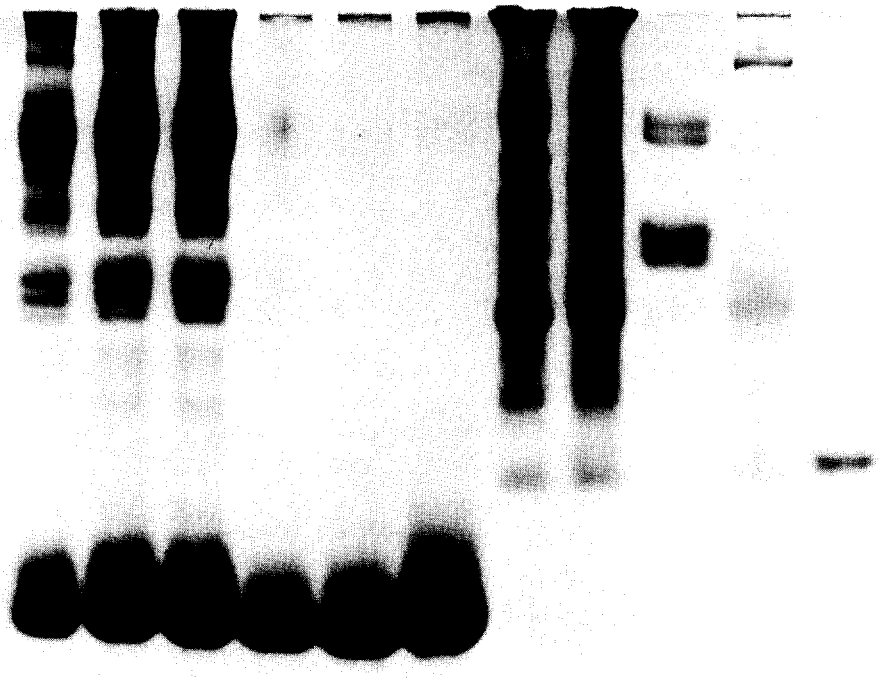

Fig.3. Polyacrylamide gel electrophoresis of (from left to right): pooled *Crotalus v. viridis* venom (10, 20 and 40 μl). Pure *Crotalus v. viridis* neurotoxin (10, 20 and 40 μl); pooled *Crotalus atrox* venom (10 and 20 μl); highly purified myocardial depressor protein from *C. atrox* venom; fraction I from the Sephadex G-25 gel filtration step; pure hypotensin from *C. atrox* venom. Conditions for electrophoresis: voltage, 250 V (constant); current, 100 mA; prerun, 4 h; electrolyte, 0.37 M glycine–0.1 M citric acid buffer, pH 2.9; length of separation, 4 h (3°C). Gel concentration, 14% Cyanogum-41. Migration is from top (+) to bottom (–)

Table 2
Aortic arterial pressure response to hypotensin administration[a]

	Time (min) Control	5	15	20	30	45	60	80	100
Mean[b]	142.7	60.5	71.7	80.0	92.5	95.5	114.0	114.5	118.0
Standard error (±)	4.5	9.4	11.1	10.8	9.6	8.3	7.7	5.2	4.8

[a]Hypotensin (1.0 mg/kg) was given as an I.V. bolus injection (2.0 ml saline), through a catheter in the femoral vein.

[b]Arterial pressure (mm Hg) was measured with an arterial catheter advanced via the femoral artery into the abdominal aorta. A statham P23Db strain gauge pressure transducer coupled to a 16 channel (Electronics for Medicine) photographic oscilloscopic system was used to continuously monitor the arterial blood pressure.

peptide. There was an immediate and profound systemic arterial hypotension evidenced by a fall from a control value of 142.7 ± 4.5 to 60.5 ± 9.4 mm Hg 5 min following I.V. administration of the venom peptide. A continuous gradual increase in the aortic pressure towards normal was then observed with values of 92.5 ± 9.6, 114.0 ± 7.7 and 118.0 ± 4.8 mm Hg at 30, 60 and 100 min respectively. Ten animals were used in the complete cardiovascular evaluation, results of which will be published elsewhere [14].

4. Discussion

The hypotensive peptide isolated in this study is of extreme importance for several reasons:

(1) It induces a rapid decrease in systemic arterial pressure which lasts for periods of up to two hours or longer.

(2) Its amino acid composition differs from that of bradykinin, bradykinin-like peptides and bradykinin-potentiating peptides isolated from natural sources.

(3) Hypotensin has leucine at the N-terminus while bradykinin-potentiating peptides have pyrrolidone carboxylic acid and bradykinin has anginine at the N-terminus.

Because of the above mentioned chemical and biological differences and to differentiate it from other well known vasoactive peptides we have named the hypotensive peptide from *Crotalus atrox* venom hypotensin.

Hypotensin does not appear to be destroyed by lung peptidases as judged by the duration of hypotensive response in intact dogs. It is, likewise, not destroyed by gastrointestinal peptidases since it induces a dose-dependant hypotension in normal and spontaneously hypertensive rats when administered by the oral route (Bonilla, unpublished).

The mechanism of action of hypotensin has not been elucidated; however, the fact that metiamide and chortrimeton do not block its hypotensive action indicate that histamine release is not responsible for the blood pressure decreased [14]. Other possible mechanisms such as central nervous system-induced hypotension or β-adrenergic blocking activity are now being investigated.

In conclusion, it should be noted that hypotensin represents a totally new type of naturally occurring vasoactive peptide which is very effective in lowering arterial blood pressure in experimental animals. The rapidity of onset of hypotensive response and its duration warrant further investigation of structure-activity relations, mechanism of action, and potential use as an antihypertensive drug.

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