Novel Hypotensive Peptides from the Body of *Elaphe climacophora*[1)]

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Four hypotensive peptides called elapherine-A, -B, -C and -D were isolated from the body of *Elaphe climacophora* after removal of the internal organs. Elapherine-A, which had the lowest molecular weight, exhibited a prolonged fall for 5 min in the blood pressure of spontaneously hypertensive rats, whereas elapherin-B, -C and -D displayed a transient fall in the blood pressure. The molecular weights of elapherine-A—-D estimated from sodium dodecyl sulfate (SDS) gel disk electrophoresis were 6600, 6900, 7000 and 7200, respectively. The isoelectric points of these peptides were 10.4, 10.6, 10.7 and 10.5, respectively. They possessed a single polypeptide chain of 34—35 (elapherine-A), 56—58 (elapherine-B), 56—57 (elapherine-C) and 52—53 (elapherine-D) amino acid residues.

Keywords *Elaphe climacophora*; hypotensive peptide; elapherine; spontaneously hypertensive rat; snake; basic peptide; blood pressure; amino acid analysis; isoelectric point

Snakes belonging to the Elaphe family are widely distributed in Japan, with, *Elaphe climacophora* (Japanese name: aodaisho) being especially abundant. There have been many reports about the toxic components of snake venom.[2-5)] Interestingly, since ancient times the dried bodies of snakes have been used as an hypotensive agent in folk medicine. Recently, we isolated inosine as a hypotensive agent from the dried body of *Naja naja kaouthia* LESSON.[6)] Hypotensive peptides were isolated from *Elaphe climacophora* during the course of our study on the hypotensive principles present in the dried bodies of snakes. We also report here the isolation and physico-chemical properties of four basic peptides showing remarkable hypotensive activity from the body of *Elaphe climacophora*. Because of their origin, these peptides have been given the name elapherine.

Materials and Methods

Chemicals Hydrochloric acid was obtained from Pierce, IL, U.S.A. Phenol, methanesulfonic acid and Cosmosil ($_5C_{18}$-300) were obtained from Nacalai Tesque, Kyoto, Japan. Sephadex G-50 and CM-Sephadex C-25 were purchased from Pharmacia, Uppsala, Sweden, and ampholine (pH 3.5—10.0) was from LKB-Produkter AB, Bromma, Sweden. All other chemicals were of analytical grade purity.

Animals *Elaphe climacophora* was obtained from the Japan Snake Institute in Gunma Prefecture, Japan. Male spontaneously hypertensive rats (SH rats) weighing 260—300 g were obtained from the colony of the Department of Pharmacology, Jichi Medical School.

Hypotensive Activity in SH Rats Male SH rats weighing 250—300 g were anesthetized with sodium pentobarbital, 40 mg/kg, i.p. with supplementary doses as necessary. The trachea was isolated and cannulated with a short piece of polyethylene tubing. The systemic arterial blood pressure was measured *via* a carotid catheter connected to a pressure transducer (Nihon Kohden P23 ID, with WI-621 G chart recorder). Samples (1 mg) were dissolved in 1 ml of 0.9% saline and administered through cannula in the femoral vein. Injection volume was 1 ml/kg, and the pH of the sample solution was not changed (pH 6.3). As a control, only saline was administered.

Isolation of the Hypotensive Peptides from the Body of *Elaphe climacophora* The internal organs were removed from the body of *Elaphe climacophora* after decapitation and exsanguination. The body (300 g) was well washed with ice-cold saline and immediately lyophilized, and the dried body was then finely chopped. The powder (30 g) thus obtained, after being defatted with *n*-hexane (200 ml) and subsequently ether (200 ml), was extracted with 1 N AcOH containing 20 mM HCl (100 ml) on a boiling water-bath for 10 min according to the method of Matsuo *et al.*[7)] The same extraction was carried out three times. Each supernatant was combined and lyophilized to give an amorphous white powder (9.8 g). The powder (0.5 g) was extracted with 50 mM phosphate buffer (pH 7.0). The supernatant was concentrated by ultrafiltration using UHP-62 and UH-1 Toyo membrane filter and applied to a column (2.7 × 100 cm) of Sephadex G-50, using 0.1 N AcOH as an eluent at a flow rate of 0.6 ml/min. The eluate was separated into Fr-I (18—30), Fr-II (31—50) and Fr-III (51—60) based on the optical density (OD) at 230 nm. Among these, Fr-II (elution volume: 160 ml) showed the strongest hypotensive effect (−75 mmHg, 3 mg/kg, i.v.) on SH rats. The yield of Fr-II was 7.6 mg. The lyophilized powder of Fr-II was dissolved in 50 mM phosphate buffer (pH 8.0) and applied to a CM-Sephadex C-25 column (1.1 × 23 cm) equilibrated with the same buffer. Elution was carried out with a linear gradient of NaCl (0—1.0 M) in the same buffer at a flow rate of 0.3 ml/min. The eluate was separated into CM-1 (25—53), CM-2 (54—70), CM-3 (71—85), CM-4 (86—89), CM-5 (90—95) and CM-6 (96—120); CM-3 and CM-4 showed hypotensive activity (−90 mmHg, −100 mmHg, 3 mg/kg, i.v.). The active fractions were further purified by preparative high-performance liquid chromatography (HPLC). Apparatus, high-performance liquid chromatograph (JASCO, TRY ROTAR-VI); column, Cosmosil $_5C_{18}$-300 (10 × 250 mm); detector, UV 225 nm; flow rate, 3 ml/min; solvent system, linear gradient elution from (A) to (B), (A) 0.05% trifluoroacetic acid (TFA): CH_3CN (95 : 5, v/v), (B) 0.05% TFA: CH_3CN (75 : 25, v/v); temperature, ambient. Two main peaks were obtained from CM-3 and five peaks from CM-4. Elapherine-A (t_R: 37 min 50 s), elapherine-B (t_R: 48 min 30 s), elapherine-C (t_R: 49 min 0 s) and elapherine-D (t_R: 49 min 20 s) were isolated and lyophilized. Elapherine-A—-D showed potent hypotensive activity (-A, −105 mmHg; -B, −85 mmHg; -C, −70 mmHg; -D, −75 mmHg, 1 mg/kg, i.v.). The yields of elapherine-A—-D were 39.2, 19.2, 34.9 and 43.0 μg, respectively. The above-mentioned purification procedure was repeated more than 50 times to examine the hypotensive activity and amino acid analysis.

Disk Gel Electrophoresis Polyacrylamide disk gel electrophoresis was carried out in a 15% acrylamide gel at pH 4.5 by the method of Reisfeld *et al.*[8)]

Isoelectric Focusing Isoelectric focusing was performed using 2% carrier ampholyte by the method of Ritchie *et al.*[9)] The pI marker used was cytochrome C pI marker kit (Oriental Yeast Co.).

Molecular Weight Measurement The molecular weights of elapherine-A—-D were measured by sodium dodecyl sulfate (SDS) disk gel electrophoresis according to the method of Weber and Osborn.[10)] Molecular weight markers for SDS disk gel electrophoresis were chymotrypsinogen A (M.W. 25000, Sigma), myoglobin (M.W. 17800, Sigma), cytochrome c (M.W. 12300, Sigma) and trypsin inhibitor (M.W. 6500, Sigma). The molecular weight of elapherine-A was also measured by fast atom bombardment-mass spectrometry (FAB-MS, HX-110, JEOL).

Amino Acid Analysis Amino acid analysis of elapherine-A—-D was performed on an analyzer (CCP & 8000 series, Tosoh) by the method of Y. Ishida *et al.*[11)] after hydrolysis at 110 °C for 24 and 72 h in 6 N HCl containing 0.7% phenol.[12)] Determination of tryptophan was carried out after hydrolysis at 110 °C for 24 h in 4 M methanesulfonic acid.[13)]

Results and Discussion

The acidic extract of the dried body of *Elaphe clima-*

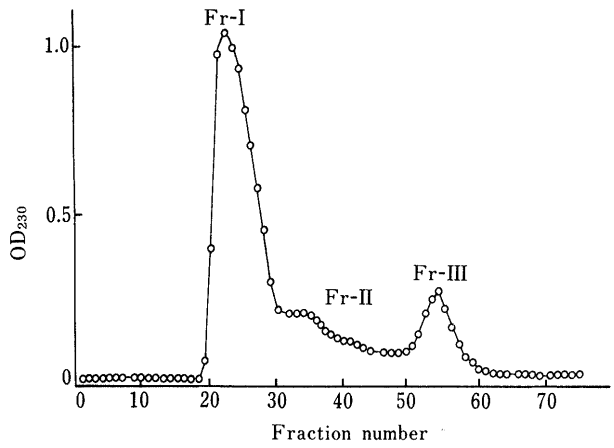

Fig. 1. Gel Filtration of Crude Extract on Sephadex G-50

Column size: 2.7 × 100 cm. Eluate: 0.1 N AcOH. Flow rate: 0.6 ml/min. Fraction size: 8 ml/tube.

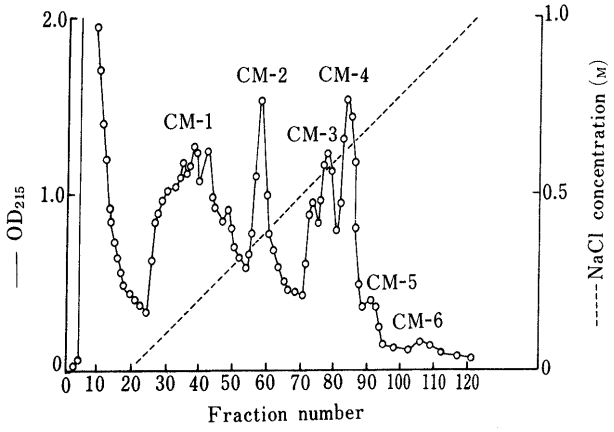

Fig. 2. Ion Exchange Chromatogram of Fr-II on CM-Sephadex C-25

Column size: 1.1 × 23 cm. Eluate: 50 mM NaH_2PO_4–Na_2HPO_4 (pH 8.0). Flow rate: 0.3 ml/min. Fraction size: 4 ml/tube.

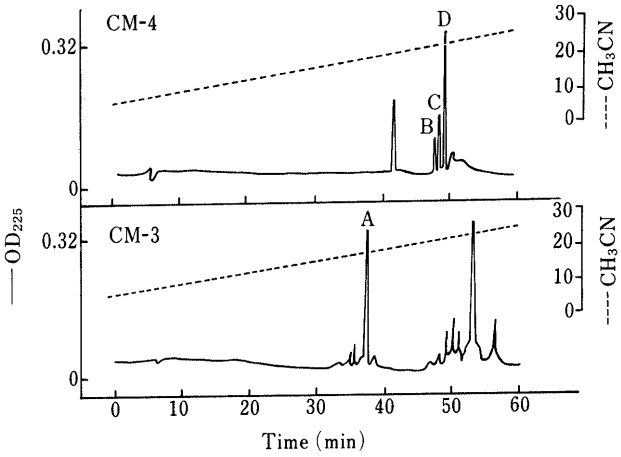

Fig. 3. Reverse-Phase High-Performance Liquid Chromatograms of CM-3 and CM-4

Column: Cosmosil ($_5C_{18}$-300), 10 × 250 mm. Solvent system: Linear gradient elution from (a) to (b). a) 0.05% TFA: CH_3CN = 95 : 5 (v/v). b) 0.05% TFA: CH_3CN = 75 : 25 (v/v). Flow rate: 3 ml/min. Temperature: Ambient.

cophora exhibited a hypotensive effect on SH rats. Examination of this acidic extract led to the isolation of four strongly basic peptides having remarkable hypotensive activity. The lyophilized powder, after extraction with 1 N AcOH containing 20 mM HCl, was extracted with 50 mM phosphate buffer (pH 7.0) and the supernatant was applied to a Sephadex G-50 column, yielding three peaks (Fig. 1). Fr-I—III were each lyophilized, and the hypotensive activity was detected only in Fr-II. The lyophilized material of Fr-II was chromatographed on CM-Sephadex C-25. The peptides were eluted with a linear gradient of NaCl (0—1.0 M) in 50 mM phosphate buffer (pH 8.0) (Fig. 2). The active fractions, CM-3 and CM-4 were pooled and lyophilized. Further purification of CM-3 and CM-4 was carried out by HPLC (Fig. 3). One major peak (elapherine-A) from CM-3 and three peaks (elapherine-B, -C and -D) from CM-4 were isolated and lyophilized. The hypotensive activity of the active fractions is summarized in Table I. Various biologically active peptides, such as calcitonin,[14] luteinizing hormone-releasing hormone (LH-RH)-like substance,[15] angiotensin II[16] and renin-like substance[17] have already been isolated from *Elaphe climacophora*; however, there is no report of the isolation of hypotensive peptides.

Elapherine-A—-D were homogenous in polyacrylamide gel electrophoresis (data not shown). The isoelectric points of each elapherine were pI 10.4 (-A), pI 10.6 (-B), pI 10.7 (-C) and pI 10.5 (-D) (data not shown). Molecular weights of purified elapherines were estimated by SDS polyacryl-

TABLE I. Effects of Active Fractions on the Blood Pressure in Anesthetized Spontaneously Hypertensive Rats

Sample	Dose (mg/kg, i.v.)	Mean arterial blood pressure (mmHg)
Acidic powder	5.0	−33
Fr-II	5.0	−75
CM-3	3.0	−90
CM-4	3.0	−100
Elapherine-A	1.0	−105
Elapherine-B	1.0	−85
Elapherine-C	1.0	−70
Elapherine-D	1.0	−75

Each value represents the mean of 3 rats. Route, intravenous injection; body weight, 260—280 g. Anesthetic: pentobarbital-Na (40 mg/kg).

TABLE II. Amino Acid Compositions of Elapherine-A, -B, -C and -D

Amino acid	Elapherine -A	-B	-C	-D
Asp	2	4	3	2
Thr[a]	2	0	0	0
Ser[a]	1	4	4	3
Glu	1	11—12	12	10—11
Pro	3	0	0	0
Gly	5	6—7	6	6
Ala	2	0	0	0
Cys[b]	2—3	3	3—4	3
Val	0	2	2	2
Ile	0	4	4	4
Leu	0	4	4	4
Tyr	2	5	6	6
Phe	3	0	0	0
His	4	0	0	0
Arg	6	13	12	12
Trp[c]	1	0	0	0
Total residues	34—35	56—58	56—57	52—53

a) Values extrapolated to zero hour. *b*) Values determined as cystine. *c*) Values obtained by hydrolysis for 24 h in 4 M methanesulfonic acid.

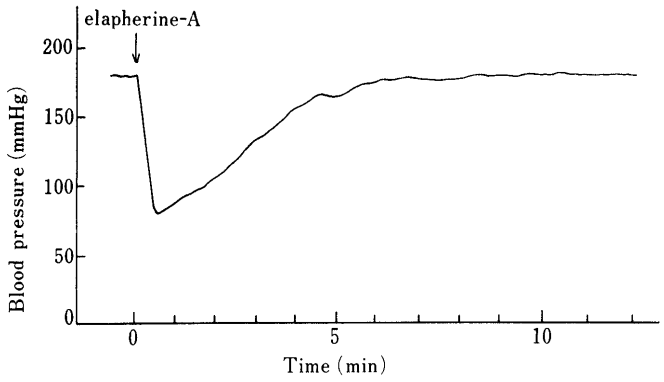

Fig. 4. Effect of Elapherine-A on the Blood Pressure in Anesthetized Spontaneously Hypertensive Rats

Body weight: 260 g. Arrow: Injection of 1 mg/kg of elapherine-A.

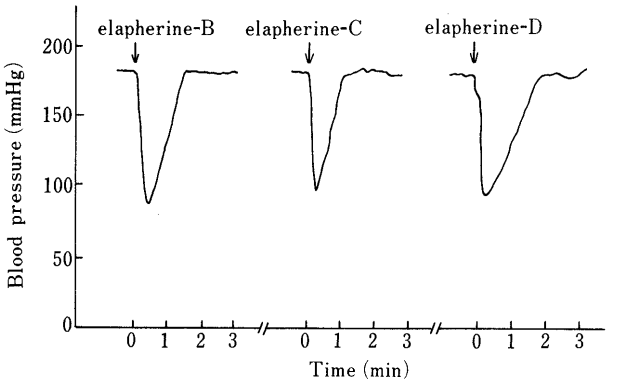

Fig. 5. Effects of Elapherine-B, -C and -D on Blood Pressure in Anesthetized Spontaneously Hypertensive Rats

Body weight: 260 g. Arrow: Injection of 1 mg/kg of each sample.

amide disk gel electrophoresis as follows: Elapherine-A (M.W. 6600), -B (M.W. 6900), -C (M.W. 7000) and -D (M.W. 7200) (data not shown). The molecular weight of elapherine-A was shown by FAB-MS to be 4418.8. The difference in the molecular weight of elapherine-A by the two methods was considered due to its physico-chemical properties, especially its strong basicity. The molecular weight of histone (basic protein) was estimated to be 1.5 times larger than the actual one by SDS polyacrylamide disk gel electrophoresis.[18] We considered that the present results were analogous to histone, and thus, the molecular weights of the elapherines were overestimated in SDS polyacrylamide disk gel electrophoresis. Elapherine-A, -B, -C and -D consisted of 34—35, 56—58, 56—57 and 52—53 amino acid residues containing cystine residues (Table II). Amino acid compositions of these peptides were characterized by the following points: 1) They lacked lysine and methionine. 2) Elapherine-A contained threonine, proline, alanine, phenylalanine and histidine residues, whereas elapherine-B—-D did not contain these amino acid residues. 3) Elapherine-B—-D contained hydrophobic amino acids such as valine, leucine and isoleucine, but elapherine-A did not contain these amino acids. 4) Elapherine-B—-D were characterized by the fact that glutamic acid and arginine were very rich. 5) Amino acid compositions of elapherine-B—-D were very similar to one another. Considering that elapherine-A—-D contained cystine residues, these peptides should have unique three-dimensional structures; the relationship between the hypotensive activity and structure is, therefore, of great interest. Many typical hypotensive peptides such as bradykinin,[19] bradykinin potentiator,[20] eledoisin,[21] kallidin,[22] kassinin,[23] neurotensin,[24] ranakinin[25] and substance P[26] contained no cystine residues, however, elapherine-A—-D did contain them. Elapherine-A showed a prolonged fall for 5 min, while elapherine-B—-D showed a transient fall in the blood pressure (Figs. 4 and 5). Elapherine-A—-D caused hypotensive activity not only in SH rats but also in normotensive rats, indicating that the effect is fundamental and not peculiar to SH rats (data not shown). These results show the peptides to be quite unique in both amino acid composition and hypotensive activity among the known hypotensive peptides.

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