

Comparative Studies on Angiotensins. IV.¹⁾ Structure of Snake (*Elaphe climocophora*) Angiotensin²⁾

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Three kinds of snake angiotensins have been isolated from the incubation product of snake (*Elaphe climocophora*) kidney extract with homologous plasma. The amino acid sequences were deduced by dansyl method as follows:

Asp-Arg-Val-Tyr-Val-His-Pro-Phe

X-Asx-Arg-Val-Tyr-Val-His-Pro-Phe-Tyr

X-Asx-Arg-Val-Tyr-Val-His-Pro-Phe-Tyr-Leu

Tyrosine in position 9 was different from histidine of mammalian angiotensins or serine of the fowl. Position 5 was valine as in so-called bovine angiotensin.

Keywords—snake angiotensin; *Elaphe climocophora*; dansyl method; tritium labelling; X-Asx¹-Val⁵-Tyr⁹-angiotensin I

Two angiotensins I from mammalian sources have been recognized. Native hog, human, or rat angiotensin I is Asp¹-Ile⁵-His⁹-decapeptide. The native angiotensin for a species has been defined as a naturally occurring form *in vivo*, or as a product resulting from the action of homologous renin on homologous angiotensinogen *in vitro*. The same angiotensin I is produced when hog renin is incubated with horse plasma (so-called equine angiotensin I). Asp¹-Val⁵-His⁹-angiotensin I is formed when rabbit renin acts on ox serum (so-called bovine angiotensin I).⁴⁾ We identified fowl angiotensin as Asp¹-Val⁵-Ser⁹-angiotensin I.¹⁾

Snake (*Elaphe climocophora*, Japanese name: Aodaisho) angiotensin was different from either Asp¹-Ile⁵-His⁹-angiotensin I, Asp¹-Ile⁵- or Asn¹-Val⁵-angiotensin II, or fowl angiotensin. The criteria used for differentiation were SE-Sephadex chromatographic behavior, ratio of oxytocic to pressor activity, and susceptibility to carboxypeptidase A.⁵⁾

We extended the study to elucidate chemical structure of snake angiotensin by separation, isolation and sequence analysis of the peptides in the active principle obtained by incubating the kidney extract with homologous plasma.

Experimental

Bioassay and Examination of Enzyme Susceptibility⁵⁾

Determination of the Pressor Activity—Activity of the materials in each step of separation was determined by their pressor action in the rat anesthetized with pentobarbital sodium (50 mg/kg, *ip*), and treated with pentolinium tartarate (5 mg/kg, *iv*), using synthetic Asn¹-Val⁵-angiotensin II as the standard.

Determination of the Oxytocic Activity—Rat uteri were suspended in an 8 ml-muscle chamber with de Jalon's solution and the assays were carried out by comparing the contraction height with synthetic Asn¹-Val⁵-angiotensin II.

- 1) Part III: T. Nakayama, T. Nakajima, and H. Sokabe, *Chem. Pharm. Bull.* (Tokyo), **21**, 2085 (1973).
- 2) This work was presented at the 94th annual meeting of the Pharmaceutical Society of Japan, Sendai, April 1974.
- 3) Location: a) 2-3, Kasumicho 1 chome, Hiroshima; b) Minamikawachimachi, Tochigi.
- 4) H. Sokabe, and M. Ogawa, *Int. Rev. Cytol.*, **37**, 271 (1974).
- 5) T. Nakajima, T. Nakayama, and H. Sokabe, *Gen. Comp. Endocrinol.*, **17**, 458 (1971).

Enzymatic Treatment for the Active Fraction—The enzyme solutions were prepared as follows: Both DFP-treated carboxypeptidases A and B (Worthington Biochemicals, 666 and 720 units/ml) were dissolved successively into 10% LiCl at concentrations of 50 and 12.5 μ l/ml. TPCK-trypsin, and γ -chymotrypsin (Worthington Biochemicals, 185 and 53 units/mg protein) were dissolved respectively to 100 μ g/ml with 0.1 N triethylamine-bicarbonate buffer (pH 8.0). These enzyme solutions were used without preincubation.

An aliquot of the active fraction equivalent to *ca.* 50 ng of angiotensin was dissolved in 100 μ l of 0.1 M phosphate buffer (pH 7.5) and 10 μ l of the enzyme solution was added. The mixture was incubated for 30 to 60 min at 37°. After the incubation, the mixture was heated at 100° for 10 min and lyophilized. The residue was redissolved in 1.5 ml of saline containing 0.001% Tween 80 for the bioassays.

Sequence Analysis of the Peptide

Dansylation of the Peptide⁶⁾—Peptide of 1 to 10 nmol was dissolved into 10 μ l of 0.1 N triethylamine and 10 μ l of 0.5% (w/v) dansyl chloride dioxane solution was added. The procedure was held in an ice bath. The mixture was kept for 16 hr in a refrigerator. After the reaction, the solvent was removed under nitrogen stream. Five μ l of HCOOH and 50 μ l of H₂O were added to the residue. The solution was kept 1 to 2 hr in a dark place and then dried.

Enzyme Degradation for Sequence Analysis of the Peptide—Trypsin or chymotrypsin solution was prepared as described above. The untreated or dansylated peptide of about 10 nmol was dissolved into 100 μ l of 0.1 N triethylamine-bicarbonate buffer (pH 8.0), to which 10 μ l of the enzyme solution was added. The mixture was incubated at 37° for 4 hrs or at 25° for overnight. After the incubation, the mixture was lyophilized. The residue was dissolved in 20 to 50 μ l of MeOH and an aliquot was served for further analysis.

Amino Acid Analysis—The peptide or the dansylated material was hydrolysed by 6 N HCl containing 0.005% phenol as a scavenger. The hydrolysis was performed at 110° for 24 hr or at 90° for 16 hr. Amino acid composition was determined by an amino acid analyser (JEOL 5-AH).

All procedures including extraction, bioassay, and purification were carried out with siliconized glass equipments.

Results and Discussion

Preparation of Crude Snake Angiotensin

A total of 128 snakes (*Elaphe climocophora*), weighing 46.7 kg, was used in four series of preparation. Frozen kidney tissue (total of 527 g) was homogenized with water (2 ml/g tissue), and centrifuged at 0°, 15000 \times g. The supernatant fluid was dialysed against 5.9 mM disodium ethylenediamine tetraacetic acid (EDTA) solution for 18 hrs, and acidified to pH 3.0 to inactivate angiotensinases. Plasma (total of 1073 ml) was also dialysed against 5.9 mM EDTA solution for 18 hrs. Dialysed plasma (total of 913 ml) was incubated with the kidney extract (1 ml/ml plasma proportionally) in the presence of Dowex 50W-X2(NH₄⁺) resin (200 μ l/ml plasma) at 20°, at pH 7.4, for 3 hrs. The reaction was stopped by freezing the incubation mixture to -20°. The reaction mixture was applied on the column of Dowex 50W-X2(NH₄⁺) resin (100 μ l/ml plasma); and the column was washed successively with 0.2 N ammonium acetate buffer (pH 6.0) (2 ml/ml plasma), 10% acetic acid (3 ml/ml plasma) and water (6 ml/ml plasma); and the active material was eluted with 0.1 N diethylamine (2 ml/ml plasma) and 0.5 N ammonia (2 ml/ml plasma) at room temperature. The eluate was evaporated by rotatory evaporator below 45°. Crude snake angiotensin of 745 μ g in activity (equivalent to Asn¹-Val⁵-angiotensin II) was obtained.

Separation of Snake Angiotensin

The brief detail of the separation of snake angiotensin (s) was summarized in Chart 1. Crude snake angiotensin was suspended to 5 ml of water and dissolved by adding a drop of 90% formic acid. The solution was chromatographed by SE-Sephadex column (12 \times 485 mm). The column was eluted with 100 ml of water and then eluted with a linear concentration gradient from water (200 ml) to 0.4 N ammonium formate (pH 7.0) (200 ml). The column was successively eluted with 300 ml of 0.5 N ammonium formate (pH 7.0). The pressor activities were eluted in a major and a minor peak as shown in Fig. 1. The major active peak, eluted at the concentration of 0.2 N ammonium formate, showed 90% of the total

6) Z. Tamura, T. Nakajima, T. Nakayama, J.J. Pisano, and S. Udenfriend, *Anal. Biochem.*, **52**, 595 (1973).

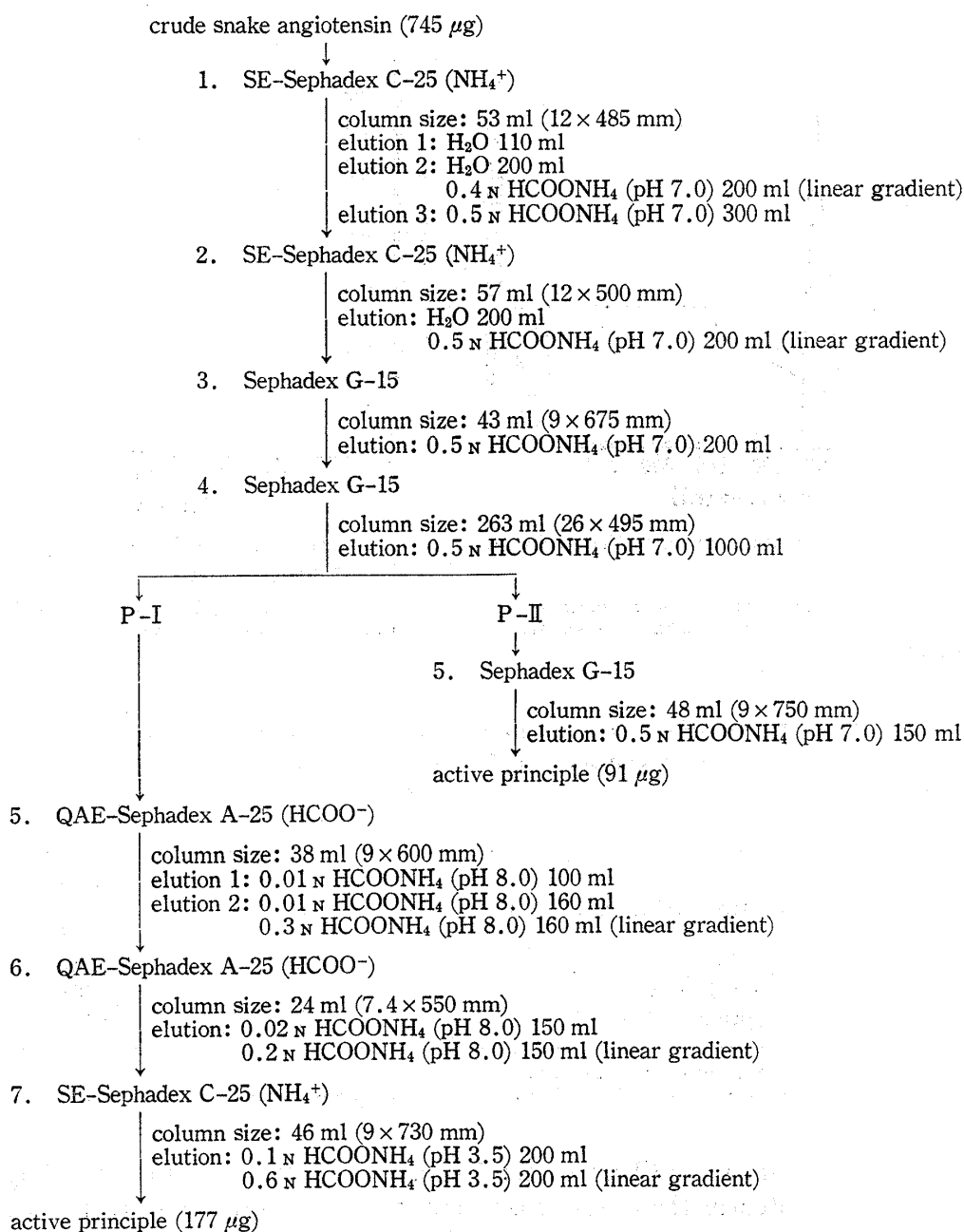


Chart 1. Purification of Snake Angiotensins

activity. This major active fraction was lyophilized and rechromatographed by the same system to the previous step. The active fraction of the second step of purification by SE-Sephadex chromatography was purified further by Sephadex G-15 gel permeation. In this step, the other minor activity was appeared as a shoulder behind the main peak. The gel permeation was repeated with the bigger size of Sephadex G-15 column. The active principles were separated into two peaks (P-I and P-II) as shown in Fig. 2. The oxytocic activity in the fraction P-I was almost equivalent to that of $\text{Asn}^1\text{-Val}^5$ -angiotensin II, while the fraction P-II was less active for the rat uterus contraction. The pressor activities in the both fractions were inactivated by trypsin or chymotrypsin digestion. By the carboxypeptidases A and B digestion, the pressor activity in the fraction P-I was lost, but that in the fraction P-II was increased twice after one hour (Table I). These characteristics in oxytocic and pressor activities or enzyme susceptibilities of the fraction P-I and P-II were similar to those of mammalian angiotensin II and I, respectively. These two active principles were

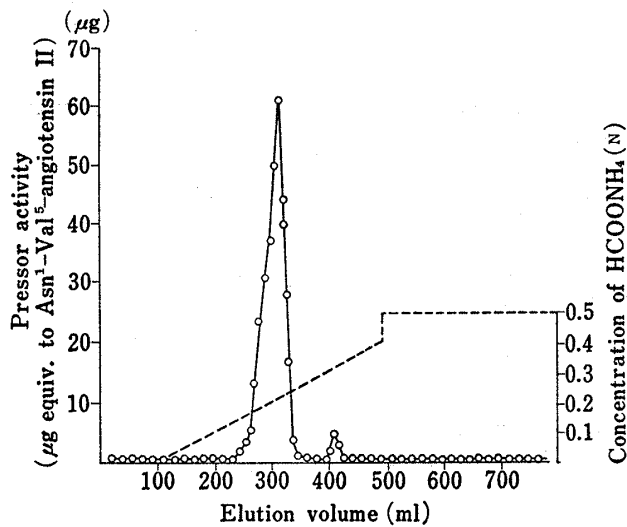


Fig. 1. SE-Sephadex Chromatogram of Crude Snake Angiotensin

The column was eluted at a flow rate of 20 ml/hr. Fractions of 5 ml each were collected. An aliquot of each fraction was assayed by rat pressor action.

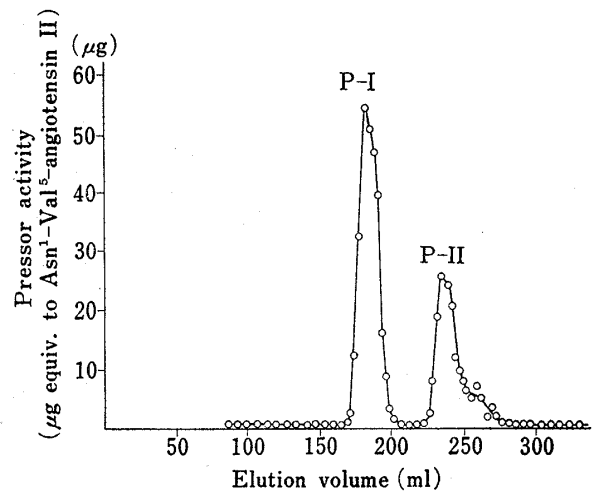


Fig. 2. Sephadex G-15 Chromatogram of Snake Angiotensin (Step 4)

The column was eluted at a flow rate of 6 ml/hr. Fractions of 3 ml each were collected.

TABLE I. Difference of Characteristics between Two Peaks appeared on Sephadex G-15 Chromatography

	P-I	P-II
O: P ratio ^{a)} (%)	93	5-6
Protease susceptibility ^{b)} (decrease %)		
Trypsin	88	85
Chymotrypsin	93	82
Carboxypeptidase A	14	-161
Carboxypeptidases A and B	32	-130

a) Oxytocic response/pressor response $\times 100$.

b) (initial response-final response)/(initial response) $\times 100$, activity was assayed by pressor response.

respectively purified as shown in Chart 1 (step 5-7). The active principle of 177 μg equivalent to $\text{Asn}^1\text{-Val}^5\text{-angiotensin II}$ in pressor activity from the fraction P-I, and 91 μg equivalent to $\text{Asn}^1\text{-Val}^5\text{-angiotensin II}$ in pressor activity from the fraction P-II were finally obtained.

Chemical Characterization of the Active Principle in the Fraction P-I

Amino acid composition of this fraction determined by an amino acid analyser was $\text{His}_{1.0}$, $\text{Arg}_{1.0}$, $\text{Asp}_{1.0}$, $\text{Pro}_{1.0}$, $\text{Val}_{2.0}$, $\text{Tyr}_{1.0}$, and $\text{Phe}_{1.0}$. N-terminal amino acid detected by dansyl method was aspartic acid. The dansylated peptide on the thin layer of Silica gel H was identical to $\text{DNS-Asp}^1\text{-O-DNS-Tyr}^4\text{-Val}^5\text{-angiotensin II}$ ⁷⁾ with the solvent system of *n*-butanol-acetic acid-water (4:1:5). Tryptic fragments of this dansyl peptide showed the same chromatographical behaviors as those of $\text{DNS-Asp}^1\text{-O-DNS-Tyr}^4\text{-Val}^5\text{-angiotensin II}$ by thin layer chromatography. These fragments were identical to DNS-Asp-Arg and $\text{Val-O-DNS-Tyr-Val-His-Pro-Phe}$ respectively. These results suggested that the active principle in the fraction P-I was identical to $\text{Asp}^1\text{-Arg}^2\text{-Val}^3\text{-Tyr}^4\text{-Val}^5\text{-His}^6\text{-Pro}^7\text{-Phe}^8$ (so-called bovine angiotensin II).

7) DNS: 1-dimethylaminonaphthalene-5-sulfonyl, dansyl.

Chemical Characterization of the Active Principle in the Fraction P-II

The following amino acids were determined from the acid hydrolysate of this fraction; His_{1.1}, Arg_{1.0}, Asp_{1.0}, Pro_{1.0}, Val_{1.9}, Leu_{0.4}, Tyr_{2.1}, and Phe_{1.0}. After dansylation of this active fraction, two adjacent yellow fluorescent bands (DNS-P-II-1; lower, DNS-P-II-2; upper) were observed on the thin layer chromatograms of Silica gel H with both solvent systems of *n*-butanol:acetic acid:water (4:1:5) and isopropanol:methyl acetate:28% ammonia(9:7:4). Each silica gel powder at the fluorescent bands after the chromatography of acidic solvent system, was scraped and applied to a small chromatographic tube. Both dansyl peptides were eluted from the column with the minimum volume of the mixture of acetone-water-acetic acid-pyridine (50:50:3:1). The purified DNS-P-II-1 and DNS-P-II-2 were hydrolysed with 6*N* hydrochloric acid at 110 ° for 24 hr and determined amino acid composition. The results were: His_{1.0}, Arg_{1.0}, Asp_{1.0}, Pro_{1.0}, Val_{2.0}, and Phe_{1.0} for DNS-P-II-1, and His_{1.0}, Arg_{1.0}, Asp_{1.0}, Pro_{1.0}, Val_{2.0}, Leu_{1.0}, and Phe_{1.0} for DNS-P-II-2.⁸⁾ N-terminal amino acid in both P-II-1 and -2 was blocked and was not detected by dansyl method.

C-terminal analysis by tritium labelling technique⁹⁾ was carried out for both peptides. Both DNS-P-II-1 (3 nmoles) and DNS-P-II-2 (2 nmoles) purified by thin layer chromatography, were dissolved respectively with 10 µl of pyridine in the small glass tubes, and 5 µl of tritium water (5Ci/ml) and 10 µl of acetic anhydride were successively added to the tubes at 0 °. The mixtures were stand for 5 min at 0 ° and for 15 min at room temperature, and after cooling again at 0 °, 20 µl of pyridine, 20 µl of acetic anhydride and 5 µl of tritium water were added to the reaction mixture at 0 ° and left again at room temperature for 1 hr. After the reaction, the solutions were evaporated under reduced pressure. The excess of tritium activity was removed by repeated lyophilization with adding 100 µl of 10% acetic acid (8 times) and finally 100 µl of water (3 times). Each residue was hydrolysed with 6*N* hydrochloric acid as an usual manner and the hydrolysates were chromatographed with cold amino acids on a thin layer of cellulose powder (Avicel SF) using the solvent system of *n*-butanol-acetic acid-water (4:1:5). Ninhydrin was sprayed on the thin layer and the plate was stand at room temperature until the pale pink spots appeared. The ninhydrin positive area were scraped into the counting vials. The results were shown in Table II.

TABLE II. C-Terminal Analysis of P-II-1 and -2 by Tritium Labelling Technique

Amino acid	P-II-1 (cpm)	P-II-2 (cpm)
His, Arg	803.7	932.8
Asp	502.4	350.7
Pro	354.3	311.3
Tyr	675.0	249.8
Phe	222.9	303.6
Val	316.7	298.0
Leu	452.3	3032.7
O-DNS-Tyr	2016.4	545.1

O-DNS-Tyrosine was the C-terminal for DNS-P-II-1 and leucine for DNS-P-II-2. These results showed that the fraction P-II was a mixture of closely similar peptides, and P-II-1 was a nonapeptide lacking 1 mol of C-terminal leucine of the amino acid composition of P-II-2.

8) O-DNS-Tyr was not determined by the usual system of amino acid analysis.

9) T. Matsuo, Y. Baba, R.M.G. Nair, A. Arimura, and A.V. Schally, *Biochem. Biophys. Res. Commun.*, **43**, 1334 (1971).

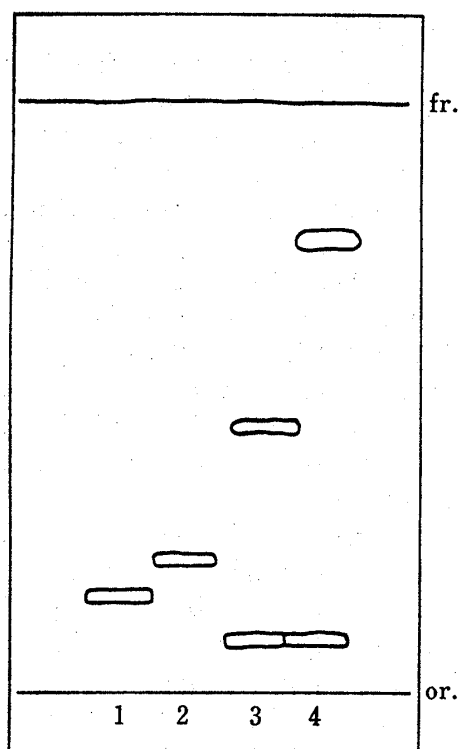


Fig. 3. Thin-Layer Chromatogram of DNS-P-II-1 and -2 and Their Chymotryptic Digests on Silica gel H

Solvent system: *n*-BuOH-AcOH-H₂O (4:1:5).
 1. DNS-P-II-1.
 2. DNS-P-II-2.
 3. chymotryptic digest of DNS-P-II-1.
 4. chymotryptic digest of DNS-P-II-2.

As shown in Fig. 3, chymotryptic digestion for DNS-P-II-1 produced two dansyl fragments which were observed by thin layer chromatography. Similarly DNS-P-II-2 was split by chymotrypsin to give two dansyl fragments and one of which was coincided to one of the chymotryptic fragments of DNS-P-II-1. The other dansyl fragment of DNS-P-II-1 and -2 were identical with O-DNS-Tyr and O-DNS-Tyr-Leu respectively by N-terminal analysis of each fragments and by co-chromatography with authentic peptides.

The chymotryptic product of the same *R_f* value obtained from DNS-P-II-1 and -2, was digested further with trypsin and the digest was chromatographed on a thin layer of Silica gel H. The Fluorescent digest was coincided with Val-O-DNS-Tyr-Val-His-Pro-Phe in both solvent systems of *n*-butanol-acetic acid-water (4:1:5) and isopropanol-methyl acetate-28% ammonia (9:7:4).

As a result of these experiments, it was suggested that the amino acid sequence of P-II-1 was X-Asx¹-Arg²-Val³-Tyr⁴-Val⁵-His⁶-Pro⁷-Phe⁸-Tyr⁹, and that of P-II-2 was X-Asx¹-Arg²-Val³-Tyr⁴-Val⁵-His⁶-Pro⁷-Phe⁸-Tyr⁹-Leu¹⁰.

Nature of Snake Angiotensin

We have identified three different angiotensin-like substances in the incubation product of snake kidney with homologous plasma. One was the same to so-called bovine angiotensin II and the other two were nona- and deca-peptide which had tyrosine at position 9 instead of histidine or serine. Both nona- and decapeptide were blocked at their N-terminals. We could not elucidate the N-terminal blocking group (s) because of minute amounts of materials available. Our results, however, suggest that at least two types of angiotensin are produced with snake kidney and plasma, one is a N-terminal free octapeptide (angiotensin II) and the other is N-terminal blocked decapeptide (angiotensin I). The peptide in the fraction P-II-1 might be an artifact produced by some proteases in the kidney or plasma during the preparation of crude angiotensin. The similar results have been observed in fowl angiotensin.¹⁾ The fowl kidney extract produced two kinds of angiotensins from fowl plasma, one was a decapeptide, and the other was a carboxypeptidase A-resistant material, probably a nona peptide.

The converting enzyme system probably presents in the snake, because we obtained both octa- and decapeptide in the crude angiotensin. Concentration of EDTA (5.9 mM) was not sufficient to inhibit this system in the snake. The reason of N-terminal block in nona- and decapeptide was unknown.

Amino acid in position 9 may be class specific: histidine for mammals, serine for birds, and tyrosine for reptiles, although more data are necessary to conclude this. Valine on position 5 seems to be more specific in non-mammalian classes.

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