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Comparative Studies on Angiotensins. VI.[1] Structure of Angiotensin I produced by Renal Renin of the Dog, Guinea Pig and Rabbit, and Re-examination of the Peptides of the Pig, Horse and Ox using Homologous Renin Sources

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Native angiotensins of dog, guinea pig, rabbit, horse, pig and ox were isolated from plasma using homologous kidney extract as the renin source, and their sequences were analyzed by means of amino acid analysis and fluorescent peptide mapping.

Angiotensins of dog, guinea pig and rabbit were identical with Ile[5]-angiotensin I, and the sequences of native angiotensins of the other species were in accord with the results reported previously.

Keywords——sequence analysis; dansyl method; fluorescent peptide mapping; amino acid composition; dog angiotensin; guinea pig angiotensin; rabbit angiotensin; pig angiotensin; horse angiotensin; ox angiotensin

Two angiotensins, one containing 5-isoleucine and one containing 5-valine, have been found in mammals. Pharmacological studies showed that these two angiotensins have different specific activities for pressor action in the rat.[3] For physiological studies on the renin-angiotensin system, it is necessary to know which type of angiotensin is intrinsic to widely-used experimental animals.

$$Asp^1\text{–}Arg^2\text{–}Val^3\text{–}Tyr^4\text{–}\underset{Val^5}{\overset{Ile^5}{}}\text{–}His^6\text{–}Pro^7\text{–}Phe^8\text{–}His^9\text{–}Leu^{10}$$

Ile[5], and Val[5]-Angiotensin I

Ile[5]-angiotensin I was first produced *in vivo* by incubating horse plasma with pig kidney extract.[4] Ile[5]-angiotensin is common to humans,[5] rats[6] as the native form.[7] It has been suggested that native pig[8] and rabbit[9] angiotensins I may be identical to Ile[5]-angiotensin I. Val[5]-angiotensin I was first isolated by incubating ox serum with rabbit kidney extract.[10] Sheep angiotensin is considered to be the same as the ox form on the basis of its immunoreactivity.[11]

This report deals with the sequence analysis of angiotensins of such laboratory animals as the dog, guinea pig and rabbit, for which the angiotensin structure has not been chemically investigated. This report also includes a re-examination of a earlier work[4,8,10] on pig, horse and ox angiotensins, as they had been prepared with renin from heterogenous sources,[4,10] or amino acid analysis of the preparations had not been performed.[8]

Experimental

Preparation of Crude Angiotensin——Crude angiotensins were prepared by the method described previously.[1] Frozen kidney was homogenized with the same weight of 5.9 mM ethylenediaminetetraacetic acid (EDTA) solution and the homogenate was centrifuged at 0°C at 15000 *g*. The supernatant fluid was dialyzed 3 times against 10 volumes of the same solution and acidified to pH 3.0 with 1 N HCl. Homologous animal

plasma was also dialyzed 3 times against 10 volumes of 5.9 mM EDTA. The dialyzed plasma and kidney extract were mixed in a ratio of 10: 4 by volume and the mixture was incubated in the presence of Dowex 50-X2(NH_4^+) (1/5 volume of the plasma) at 37°C for 60 to 120 min, the optimum incubation period as determined in a preliminary experiment. The incubation mixture was poured with stirring into a column of Dowex 50-X2(NH_4^+) (1/10 volume of the plasma). The column was washed successively with 6 to 7 column volumes of 0.2 M CH_3COONH_4 (pH 6.0), 10 to 20 column volumes of 10% (v/v) AcOH, and 20 column volumes of H_2O. The pressor substance was eluted with 6 to 7 column volumes each of 0.1 M diethylamine and 0.5 M NH_4OH at 20 to 25°C. The eluates with diethylamine and NH_4OH were combined and evaporated to dryness under reduced pressure below 35°C, and the product was used as the starting material.

Determination of Pressor Activity[12]——Activity of the material at each step of separation was determined in terms of pressor action in the anesthetized rat, using synthetic Asp[1], Ile[5]-angiotensin II as the standard.

All glass equipment and droplet counter current tubes (Seikagaku Kogyo) were siliconized with dimethyl dichlorosilane and MeOH.

Chromatographic Procedures——The general procedure is described below in each case, unless otherwise noted.

Gel Permeation Chromatography——Gel permeation chromatography was carried out on a Sephadex G-15 column with 0.5 M $HCOONH_4$ (pH 7.0) as the elution solvent. Sephadex beads were pretreated with bovine serum albumin to prevent non-specific adsorption of angiotensins on the beads. The Sephadex beads were poured into 1% bovine serum albumin (Sigma A 4503) solution and stirred for 3 h, then collected on a Buchner's funnel and washed successively with 1 M $HCOONH_4$ and H_2O.

SP- or SE-Sephadex Chromatography——The sample was dissolved in 1/10 column volume of H_2O, adjusted to pH 3.0 with dilute HCOOH, and centrifuged at 3000 rpm for 10 min if necessary. The solution was charged on a column (NH_4^+) of SP- or SE-Sephadex. The chromatography was performed with a linear concentration gradient of $HCOONH_4$ from 0 to 0.5 M (pH 7.0) using 2 column volumes of each eluent.

QAE-Sephadex Chromatography——The sample was dissolved in 1/10 column volume of H_2O and the solution was adjusted to pH 8.0 with dilute NH_4OH then charged on a column ($HCOO^-$ form) of QAE-Sephadex. The column was eluted first with a linear concentration gradient from dilute NH_4OH (pH 8.0) to 0.03 M $HCOONH_4$ (adjusted to pH 8.0 with NH_4OH) and then with 0.05 M $HCOONH_4$ (adjusted to pH 5.3 with dilute HCOOH).

Droplet Counter Current Chromatography——The sample was dissolved in a minimum volume of the lower layer of 1-BuOH/AcOH/H_2O (4: 1: 5) and charged into the transfer tube filled with the upper layer of the solvent system. Counter current chromatography was performed using 50 transfer tubes with the lower layer as the moving phase.

Dansylation of Peptides and Fluorescent Peptide Mapping by Thin Layer Chromatography[1]——Angiotensins isolated from various sources were chromatographically identified by means of the peptide mapping technique in comparison with anthentic Val[5]- or Ile[5]-angiotensin and the respective enzymatic digests after being labelled with the dansyl (1-dimethylaminonaphthalene-5-sulfonyl, DNS) moiety.

A peptide (1 to 10 nmol) was dissolved in 10 μl of 0.1 M triethylamine and 10 μl of 0.5% (w/v) dansyl chloride dioxane solution was added. The mixture was kept for 16 h in the dark at 4°C. The solvent was removed under a nitrogen stream. Five μl of HCOOH (90%) and 50 μl of H_2O were added to the residue. The solution was kept for 1 to 2 h at room temperature in the dark and then dried. The dansylated peptide was purified by thin layer chromatography on Silica gel H (Merck) as described previously.[13]

TPCK-trypsin[3.4.21.4] and γ-chymotrypsin[3.4.21.1] (Worthington Biochem.; 185 and 53 units/mg protein, respectively) were each dissolved at 100 g/ml in 0.1 M triethylamine bicarbonate buffer (pH 8.0) and used for the fragmentation of angiotensins. The dansylated peptides (1 to 10 nmol) were dissolved in 10 μl of the same buffer and 10 μl of the enzyme solution was added. The mixture was incubated at 37°C for 4 h or at 25°C overnight. After removal of the solvent under nitrogen stream, the residue was dissolved in 10 to 20 μl of MeOH and 2 to 5 μl of the sample was applied to a thin layer of Silica gel H by the overlapping technique[13] together with authentic samples.

Two solvent systems, solvent A: upper layer of 1-BuOH/AcOH/H_2O (4: 1: 5) and solvent B: 2-PrOH/MeOAc/28% NH_4OH (9: 7: 4), were used for thin layer chromatography (TLC).

Authentic Peptides——Synthetic Ile[5]- and Val[5]-angiotensin I and II, their tryptic fragments (Asp–Arg, Asn–Arg, Val–Tyr–Val–His–Pro–Phe–His–Leu, Val–Tyr–Ile–His–Pro–Phe–His–Leu)[14,15] and chymotryptic fragment (His–Leu)[15] were provided by Dr. S. Sakakibara, Peptide Institute, Protein Research Foundation, Osaka.

Results and Discussion

Purification of Angiotensins

Table I summarizes the volumes of plasma used and the yields of crude and purified angiotensins of dog, guinea pig, rabbit, pig, horse and ox.

Table I. Yields of Crude and Purified Angiotensins from Plasma

Species	Plasma used	Crude angiotensin	Purified angiotensin
Dog	2460 (ml)	598 (μg)	294.0 (μg)
Guinea pig	970	131	65.2
Rabbit	1080	114	31.6
Pig	940	407	24.4
Horse	1000	402	37.0
Ox	960	198	60.8

Table II. Purification Steps of Angiotensins by Chromatography

	Dog	Guinea pig	Rabbit	Pig	Horse	Ox
Step 1	SE-Sephadex gradient	Droplet CC BAW[b] 4: 1: 5	Droplet CC BAW 4: 1: 5	SP-Sephadex gradient	Droplet CC BAW 4: 1: 5	Droplet CC BAW 4: 1: 5
Step 2	SE-Sephadex rechromatg. (step 1)	SP-Sephadex gradient	SP-Sephadex gradient	SP-Sephadex rechromatog (step 1)	SP-Sephadex gradient	SP-Sephadex gradient
Step 3	Droplet CC BAW 4: 1: 5	QAE-Sephadex 1. gradient 2. isocratic	QAE-Sephadex 1. gradient 2. isocratic	Droplet CC BAW 4: 1: 5	Sephadex G-15	Sephadex G-15
Step 4	Sephadex G-15	SP-Sephadex[a] stepwise		Sephadex G-15	QAE-Sephadex 1. gradient 2. isocratic	QAE-Sephadex 1. gradient 2. isocratic
Step 5	QAE-Sephadex 1. gradient 2. isocratic			QAE-Sephadex 1. gradient 2. isocratic		Sephadex G-15 rechromatog. (step 3)
Step 6				SP-Sephadex rechromatog. (step 1)		QAE-Sephadex rechromatog. (step 4)

a) Elution conditions: column size, 9 mmϕ × 310 mm; elution 1, 0.1 M $HCOONH_4$ (pH 7.0) 70 ml, elution 2, 0.3 M $HCOONH_4$ (pH 7.0) 40 ml.
b) BAW: 1-BuOH/AcOH/H_2O.

Table III. Amino Acid Compositions of Various Mammalian Angiotensins

	Dog	Guinea pig	Rabbit[a]	Pig	Horse[a]	Ox
Asp	1.1(1)	1.0(1)	0.3[b]	0.8(1)	0.4[b]	0.9(1)
Pro	1.1(1)	1.3(1)	1.0(1)	1.1(1)	1.0(1)	1.1(1)
Val	1.0(1)	1.2(1)	0.9(1)	0.9(1)	1.1(1)	1.9(2)
Ile	1.0(1)	1.0(1)	0.9(1)	0.8(1)	1.0(1)	0.0(0)
Leu	0.9(1)	1.1(1)	1.1(1)	0.9(1)	1.2(1)	1.1(1)
Tyr	1.0(1)	0.8(1)	0.0[b]	0.9(1)	0.0[b]	1.1(1)
Phe	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.2(1)
His	1.8(2)	2.2(2)	2.0(2)	1.7(2)	1.9(2)	1.7(2)
Arg	1.0(1)	1.2(1)	1.2(1)	1.0(1)	1.2(1)	1.0(1)

Numbers in parentheses indicate the integral ratios of the amino acids.
a) Dansyl derivatives were hydrolyzed.
b) Corresponding dansyl amino acids were detected on TLC.

Purification of angiotensins of these animals was performed as shown in Table I. The pressor activity from these mammals appeared as a single peak through all steps of the separation process, in contrast to the case of such nonmammalian verterbrates as fowl, snake or fishes.[9]

The purification was performed until the principle in the active fraction showed a single

fluorescent band after being dansylated, and its purity has been checked by thin layer chromatography with both solvent systems A and B.

An aliquot of each sample or its dansylated derivative was hydrolyzed with 6 N HCl at 110°C for 24 h. Amino acid compositions were determined with an amino acid analyzer (JEOL 5-AH).

As shown in Table III, the amino acid composition of dog, guinea pig, rabbit, pig and horse were the same as that of Ile^5-angiotensin I.

Identification of Angiotensins

Angiotensins were dansylated and chromatographed on a thin layer of silica gel. The fluorescent peptides from dog, guinea pig, rabbit, and horse overlapped with dansylated Ile^5-angiotensin I in both solvent systems (A, *Rf*: 0.18 and B, *Rf*: 0.45). Dansylated ox angiotensin coincided with dansylated Val^5-angiotensin I (A, *Rf*: 0.14 and B, *Rf*: 0.40).

The dansylated angiotensins were digested with trypsin and the digests were chromatographed similarly on a silica gel plate. Two fluorescent fragment were observed on the plate. One of them was common to both sources (ox and the others, such as dog, guinea pig, rabbit, *etc.*), and this fluorescent band showed the same *Rf* value (A, *Rf*: 0.51 and B, *Rf*: 0.14) as DNS–Asp–Arg.

Another fluorescent tryptic fragment of dansylated dog, guinea pig, rabbit, pig and horse angiotensins was identical with Val–(O-DNS)–Tyr–Ile–His–Pro–Phe–His–Leu (A, *Rf*: 0.48 and B, *Rf*: 0.64) and that from ox was identical with Val–(O-DNS)–Tyr–Val–His–Pro–Phe–His–Leu (A, *Rf*: 0.45 and B, *Rf*: 0.60) respectively.

Chymotrypsin split dansylated angiotensin into two kinds of peptide fragments. One was fluorescent and the other was non-fluorescent. The fluorescent chymotryptic fragments derived from dog, guinea pig, rabbit, pig and horse showed the same chromatographic behavior as the dansyl derivative of Ile^5-angiotensin II, and that from ox showed the same behavior as that of Val^5-angiotensin II. The non-fluorescent chymotryptic fragments from these animals including dansylated ox angiotensin were identified as DNS–His–Leu after the digestion mixture had been re-dansylated and chromatographed on a silica gel plate.

The results obtained from amino acid analysis and chromatographic investigation of these angiotensins indicated that angiotensins of dog, guinea pig, rabbit are Ile^5-angiotensin I and the results for native angiotensins of pig, horse and ox were in accord with those reported previously, *i.e.* Ile^5-angiotensin I for horse and pig, and Val^5-angiotensin I for ox.

Many angiotensins in mammals, as shown in Table IV, are Ile^5-angiotensin I. Two mammals, ox and probably sheep, produce Val^5-angiotensin. It is of interest that the Ile^5-peptide appears to be predominant in mammals and histidine at position 9 is common in these angiotensins, while the Val^5-peptides occur widely in non-mammals with additional replacement of the amino acid at position 9 by serine (fowl),[18] tyrosine (snake)[19] or asparagine.[20]

Table IV. Mammalian Native Angiotensin I

Angiotensin	Species
Ile^5–Angiotensin I	Human, horse, pig, dog, rabbit, guinea pig, rat
Val^5–Angiotensin I	Ox, sheep[a]

a) Only indirect evidence is available. No amino acid analysis was carried out.[11]
The table summarizes mammalian native angiotensins produced by renin in the kidney. Rabbit angiotensin produced by renin in the uterus is considered to be identical to renal angiotensin on the basis of its behavior in countercurrent distribution.[16] Kangaroo renal angiotensin is suggested to have a different amino acid composition from those of known angiotensins.[17]

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