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Comparative Studies on Angiotensins. V.[1] Structure of Angiotensin formed by the Kidney of Japanese Goosefish and Its Identification by Dansyl Method

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Goosefish angiotensin produced by incubation of the kidney extract with homologous plasma, was purified. The structure was identified as Asn[1]–Val[5]–His[9]–angiotensin (I) decapeptide by amino acid composition and fluorescent peptide mapping technique.

Keywords——teleostian angiotensin; Japanese goosefish; *Lophius litulon*; aglomerular kidney; corpuscles of Stannius; dansyl method; peptide map

Angiotensin-like substances in nonmammalian species produced by incubation of homologous plasma with renin[3] in the kidney or the corpuscles of Stannius, have been demonstrated.[4] The kidney of Japanese goosefish, *Lophius litulon* (Japanese name: Kiankou), an aglomerlar teleost, produced angiotensins,[3] of which a fraction was the most basic, and eluted at the highest concentration of ammonium formate buffer from the SE-Sephadex column.

This paper described the purification and the chemical characterization of goosefish angiotensin.

Experimental

Methods used in this study were the same as reported previously,[1] unless otherwise noticed. Enzymatic degradation of the peptides or dansyl-peptides was carried out by the procedure described elsewhere.[5] All procedure including extraction, bioassay and purification were carried out with siliconized glass equipments.

Determination of the Pressor Activity[6]——Activity of the materials in each step of separation was determined by their pressor action in the anesthetized rat, using synthetic Asn[1]–Val[5]-angiotensin II as the standard.

Dansylation of the Peptides[7]——The peptide of 1 to 10 nmol was dissolved in 10 μl of 0.1 N triethylamine and 10 μl 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 at 4°. After the reaction the solvent was removed to dryness under nitrogen stream. Five μl of HCOOH and 50 μl of H_2O was added to the residue. The solution was kept to 1 to 2 hr in a dark place and then dried.

Thin-Layer Chromatography——Thin-layer of Silica gel H was used for the fluorescent peptide mapping analysis of angiotensins with following solvent systems: *n*-BuOH: AcOH: H_2O (4: 1: 5), and iso-PrOH: MeOAc: 28% NH_4OH (9: 7: 4). Authentic angiotensins and the related peptide fragments in this analysis were gifts from Dr. S. Sakakibara, Peptide Institute, Protein Research Foundation.

1) Paper IV in this series, T. Nakayama, T. Nakajima, and H. Sokabe, *Chem. Pharm. Bull.*, (Tokyo), **25**, 3255 (1977)
2) Location: *a) 2-3 Kasumicho 1-chome, Hiroshima*; *b) Minamikawachimachi, Tochigi-ken.*
3) We used renin and angiotensin in the broadest sense to refer to renin-like activity and angiotensin-like substance.
4) T. Nakajima, T. Nakayama, and H. Sokabe, *Gen. Comp. Endocrinol.*, **17**, 458 (1971).
5) M. Watanabe, T. Yasuhara, and T. Nakajima, *Animal, Plant, and Microbial Toxins*, II, 105 (1976).
6) H. Nishimura, and H. Sokabe, *Japan. Heart J.*, **5**, 494 (1968).
7) Z. Tamura, T. Nakajima, T. Nakayama, J.J. Pisano, and S. Udenfriend, *Anal. Biochem.*, **52**, 595 (1973).

Results

Preparation of Crude Goosefish Angiotensin

Goosefish angiotensin was prepared by incubating the kidney extract with homologous plasma in a series of experiment. A typical result is described as follows: Frozen kidney tissue weighing 200 g was homogenized with 200 ml of 5.9 mM disodium ethylenediamine tetraacetate (EDTA) and the homogenate was centrifuged at 0° at 15000 $\times g$. The supernatant fluid was dialyzed against the same solution for overnight, and then acidified to pH 3.0 with 1 N hydrochloric acid to inactivate angiotensinases. Goosefish plasma of 400 ml was also dialyzed against 5.9 mM EDTA solution. The dialyzed plasma of 325 ml was incubated with 160 ml of kidney extract in the presence of 60 ml of Dowex 50W-X2 (NH_4^+) (pH 7.4) at 20°, pH 7.4, for 80 min. The incubation mixture was applied to the column of 30 ml of Dowex 50W—X2(NH_4^+) (pH 6.0), and the column was washed successively with 600 ml of 0.2 N ammonium acetate buffer (pH 6.0), 900 ml of 10% (v/v) acetic acid and 1800 ml of water, and the active material was eluted with each 600 ml of 0.1 N diethylamine and 0.5 N ammonia at 20 to 25°. The eluate was evaporated by rotatory evaporator below 45°. Crude goosefish angiotensin of 75 μg equivalent to Asn^1–Val^5–angiotensin II was obtained.

A total of 220 μg of the crude material was finally obtained from 1440 ml of plasma.

Purification of Goosefish Angiotensin

Brief details of the purification was summarized in Chart 1.

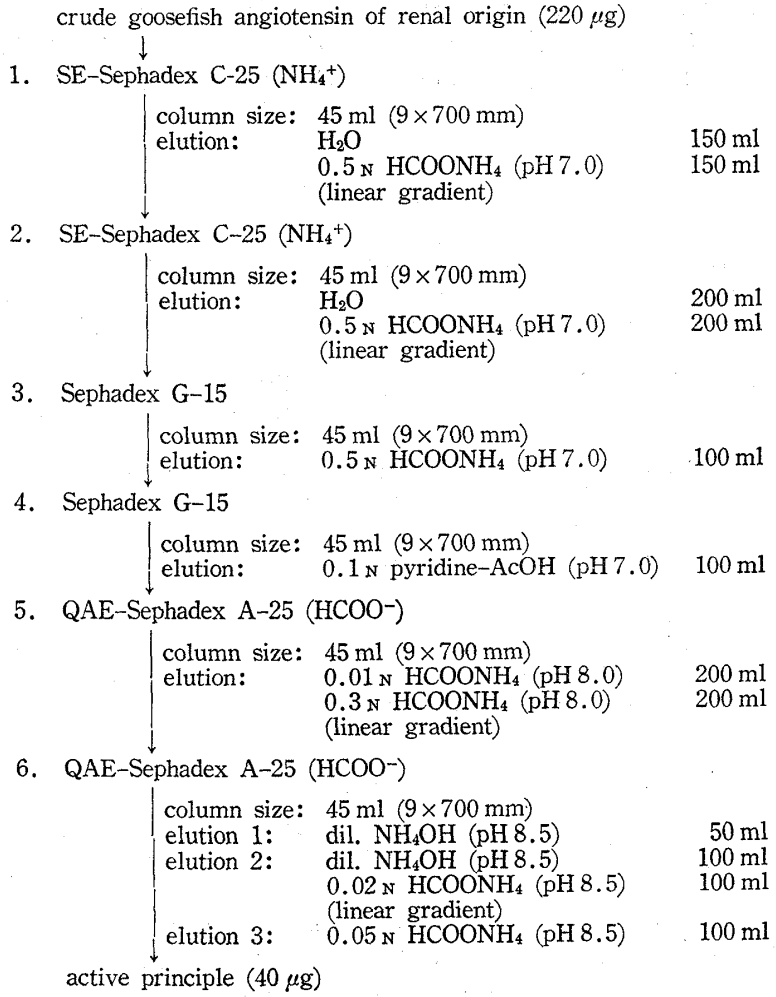

Chart 1. Purification of Goosefish Angiotensin

Crude goosefish angiotensin (220 μg) was suspended in 7 ml of dilute formic acid (pH 3.0) and centrifuged at 3000 rpm for 10 min. The supernatant fluid was applied to a SE-Sephadex column (9×700 mm) by sandwich system with each 3 ml of formic acid (pH 3.0). The column was equilibrated with 0.5 N ammonium formate-formic acid (pH 3.5) and washed with water prior to chromatography. The column was eluted initially with a linear concentration gradient from 150 ml of water to 150 ml of 0.5 N ammonium formate (pH 7.0). The pressor activity was separated into two peaks, major and minor one, as shown in Fig. 1. The major active peak which contained 85% of its total activity, was more basic than the minor peak and was eluted at the concentration of 0.35 N of ammonium formate buffer. The result accords with the previous one.[4] The major active principle was rechromatographed with the similar system as shown in Chart 1. The active fraction was lyophilized and purified further by Sephadex G-15 gel permeation chromatography (column size: 9×700 mm) with 0.5 N ammonium formate (pH 7.0) as elution buffer. The pressor activity was appeared as a single peak at three void volumes of the column. While, in the next step of rechromatography using the same column with different elution buffer, 0.1 N pyridine–acetic acid (pH 7.0), the pressor activity was appeared at two void volumes of the column. This result suggests that the active principle is adsorbed in some extent to Sephadex G-15 when the column is eluted with ammonium formate buffer. Some yellow fluorescent bands were still observed by Silica gel thin-layer chromatography after dansylation of the active fraction. Purification by repeated QAE-Sephadex chromatography was performed for the active principle. In the last step, the active principle was dissolved in 1 ml of dilute ammonia (pH 10.0) and applied to the column by sandwich system with each 1 ml of dilute ammonia (pH 10.0). The column was successively eluted with 50 ml of dilute ammonia (pH 8.5), with a linear concentration gradient from 0.0001% ammonia (pH 8.5) to 0.02 N ammonium formate, of which pH was adjusted to 8.5 with ammonia, and finally, with 0.05 N ammonium formate (pH 8.5).

The active principle of 40 μg equivalent to Asn^1–Val^5–angiotensin II was eluted as a single peak as shown in Fig. 2.

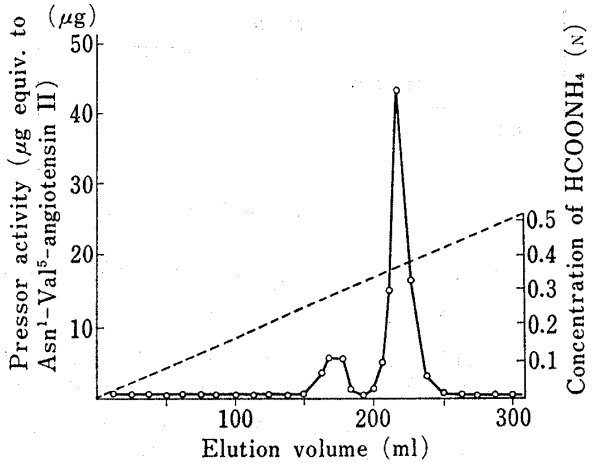

Fig. 1. SE-Sephadex Chromatogram of Crude Goosefish Angiotensin of Renal Origin

The column was eluted at a flow rate of 12 ml/hr. Fractions of 5 ml each were collected. An aliquot of each fraction was assayed by pressor activity in the rat.

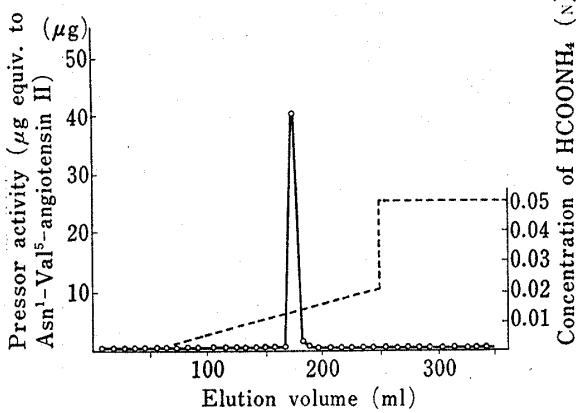

Fig. 2. QAE-Sephadex Chromatogram of Goosefish Angiotensin (step 6)

The column was eluted at a flow rate of 45 ml/hr. Fractions of 5 ml each were collected.

The dansylated principle showed a single fluorescent band on a thin layer chromatogram of Silica gel H. The amino acid composition obtained by an amino acid analyser (JEOL 5-AH) after acid hydrolysis was His_2, Arg_1, Asp_1, Pro_1, Val_2, Leu_1, Tyr_1, and Phe_1. This composition is the same to that of so-called bovine angiotensin I.[7] Dansyl aspartic acid was also identified from the acid hydrolysate of the dansylated peptide. But dansylated

goosefish angiotensin did not coincide with dansylated bovine angiotensin I by thin-layer chromatography.

Fluorescent Peptide mapping of Dansyl Angiotensins and Their Fragments

So-called bovine angiotensin I is a decapeptide of following sequence[8];

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

When the peptide is digested with trypsin, the produced fragments are Asp–Arg and Val–Tyr–Val–His–Pro–Phe–His–Leu. By the chymotrypsin digestion, the fragments are Asp–Arg–Val–Tyr, Val–His–Pro–Phe, and His–Leu. When the same enzymatic digestions are performed for the dansylated peptide, the fragments are DNS–Asp–Arg and Val–O–DNS–Tyr–Val–His–Pro–Phe–His–Leu for the tryptic digest, and DNS–Asp–Arg–Val–O–DNS–Tyr–Val–His–Pro–Phe (dansyl angiotensin II) and His–Leu for the chymotryptic digest.[9] Using these peptides and dansylated peptides, the fluorescent peptide maps were established. The chromatograms with two solvent systems are shown in Fig. 3. Goosefish angiotensin was compared with these fluorescent peptide maps after treatment of trypsin or chymotrypsin digestion. Dansylated goosefish angiotensin was cleft by chymotrypsin digestion to produce a fluorescent spot coincident with DNS–Asn–Arg–Val–O–DNS–Tyr–Val–His–Pro–Phe. After redansylation of the digest, DNS–His–Leu was identified. The cleft peptides by trypsin digestion were redansylated and DNS–Asn–Arg and DNS–Val–O–DNS–Tyr–Val–His–Pro–Phe–His–Leu were identified (Fig. 4).

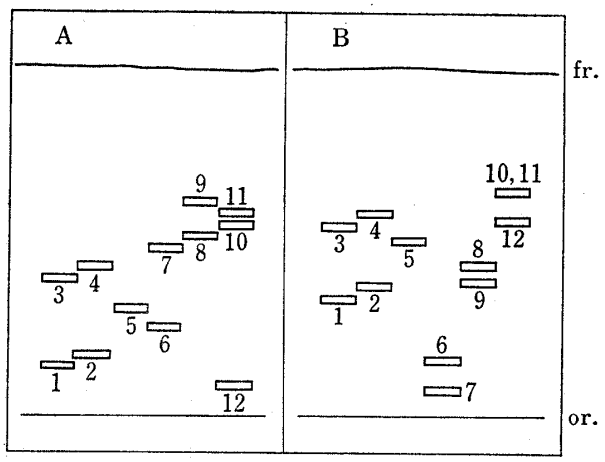

Fig. 3. Thin-Layer Chromatogram of Dansylated Angiotensins and Their Fragments on Silica Gel H

A, *n*-BuOH: AcOH: H_2O (4: 1: 5), B, iso-PrOH: MeOAc: 28% NH_4OH (9: 7: 4).

1. DNS-Asp-O-DNS-Tyr-Val[5]-angiotensin I.
2. DNS-Asp-O-DNS-Tyr-Ile[5]-angiotensin I.
3. DNS-Asp-O-DNS-Tyr-Val[5]-angiotensin II.
4. DNS-Asp-O-DNS-Tyr-Ile[5]-angiotensin II.
5. DNS-Asn-O-DNS-Tyr-Val[5]-angiotensin II.
6. DNS-Asn-Arg.
7. DNS-Asp-Arg.
8. DNS-Asn-Arg-Val-O-DNS-Tyr.
9. DNS-Asp-Arg-Val-O-DNS-Tyr.
10. DNS-Val-His-Pro-Phe.
11. DNS-Ile-His-Pro-Phe.
12. DNS-His-Leu.

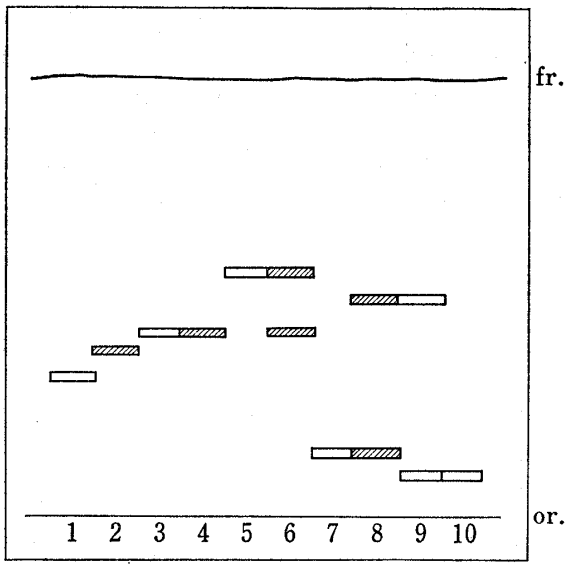

Fig. 4. Thin-Layer Chromatogram of Dansylated Goosefish Angiotensin and Its Enzymatic Digests

Solvent system: iso-PrOH: MeOAc: 28% NH_4OH (9: 7: 4).

1. DNS-Asp-O-DNS-Tyr-Val[5]-angiotensin I.
2. DNS-goosefish angiotensin.
3. DNS-Asn-O-DNS-Tyr-Val[5]-angiotensin II.
4. Chymotryptic digest of 2.
5. DNS-His-Leu.
6. Redansylated products of 4.
7. DNS-Asn-Arg.
8. Redansylated products of tryptic digest of DNS-goosefish angiotensin.
9. Redansylated products of tryptic digest of DNS-Asp-O-DNS-Tyr-Val[5]-angiotensin I.
10. DNS-Asp-Arg.

8) H. Sokabe and M. Ogawa, *Int. Rev. Cytol.*, 37, 271 (1974).
9) DNS: 1-dimethylaminonaphthalene-5-sulfonyl, dansyl.

The results from the amino acid analysis and thin-layer chromatography indicated that the amino acid sequence of goosefish angiotensin was deduced to be;

Asn-Arg-Val-Tyr-Val-His-Pro-Phe-His-Leu.

Discussion

Goosefish angiotensin of 40 μg was purified from the incubation product of 1440 ml of plasma with the kidney extract. Amino acid sequence was suggested as Asn^1–Val^5–His^9–angiotensin (I)-decapeptide by the dansyl method. The dansyl procedure used in this experiment differed from the method of Gray and Hartley,[10] specifically in the use of higher pH for dansylation to react dansyl chloride completely with the hydroxyl group of tyrosine, and in the treatment with formic acid for elimination of dansyl group from imidazole ring of histidine. Tyrosin and histidine containing peptides formed a single fluorescent spot by this procedure. It can be applied to less than 10 μg of peptides for the fluorescent mapping of angiotensin and the replacement of the amino acid residues in the sequence.

Goosefish angiotensin from the kidney was the most basic angiotensin of a new type of structure ever recognized in the vertebrates. It was the first example of asparagine at N-terminal obtained from natural sources. Valine at position 5 was the same to so-called bovine,[8] fowl,[11] and snake,[1] and may be dominant in nonmammalian species over isoleucine in mammals: the hog, human, rat, and dog.[12] Histidine at position 9 in goosefish angiotensin was the same to the above mammalian species and different from fowl, snake or eastern gray kangaroo.[13]

In the teleostian angiotensins, two peaks were always seen in the SE-Sephadex chromatograms. In goosefish angiotensin of present study, major and minor peaks were also observed, although the minor one was not investigated further. The reason why two peaks were formed is unknown. Separation of the crude active materials into some peaks after further chromatographies was commonly seen in the fowl,[11] and snake.[1]

The corpuscles of Stannius in Japanese goosefish contained renin, which produced angiotensin when incubated with the homologous plasma.[14] SE-Sephadex chromatogram of this angiotensin was different from that of renal origin.[15] Two peaks appeared earlier than those of renal origin, indicating greater acidity of the substances.

Acknowledgement We thank Dr. S. Sakakibara, Peptide Institute, Protein Research Foundation for supplying the authentic peptides. Thanks are also due to Mrs. M. Sokabe for her technical assistance.

10) W.R. Gray and B.S. Hartley, *Biochem. J.*, **89**, 379 (1963).
11) T. Nakayama, T. Nakajima, and H. Sokabe, *Chem. Pharm. Bull.* (Tokyo), **21**, 2085 (1973).
12) T. Hayashi, T. Nakayama, T. Nakajima, and H. Sokabe, Abstracts of papers, C.S. Region Meeting Pharm. Soc. Jap., Hiroshima, October, (1975), p. 13.
13) J.B. Best, J.R. Blair-West, J.P. Coghlan, E.J. Cran, R.T. Fernley, and P.A. Simpson, *Clin. Exptl. Pharmacol. Physiol.*, **1**, 171 (1974).
14) H. Sokabe, H. Nishimura, M. Ogawa, and M. Oguri, *Gen. Comp. Endocrinol.*, **14**, 510 (1970).
15) T. Nakayama, T. Hayashi, T. Nakajima, and H. Sokabe, to be published.