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The Studies on the Active Peptide on Smooth Muscle in the Skin of *Rana rugosa*, Thr^6-Bradykinin and Its Analogous Peptide, Ranakinin-R[1]

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Thr^6-bradykinin and three kinds of its analogous peptides have been isolated from the methanol extract of the fresh skin of *Rana rugosa*. The longest bradykinin analogue, Thr^6-bradykinyl-Ile-Ala-Pro-Glu-Ile-Val, was named ranakinin-R. Serotonin was also identified in the extract. The new peptide which contracts the rat uterus, guinea pig gall bladder and is hypertensive, was isolated and suggested to be the ranatensin or bombesin analogue by these pharmacological properties and by partial sequence analysis of the peptide.

Keywords——vasoactive polypeptide; bradykinin analogue; Thr^6-bradykinin; ranakinin-R; amphibian; skin peptide; *Rana rugosa*; sequence analysis

The methanol extract of amphibian skin contains various types of biological active peptides such as bradykinins, physalaemins, caeruleins *etc.*[3] Erspamer *et al.*[4] have been demonstrated the presence of new biological active peptides in the dry skin of *Rana rugosa* (Japanese name: Tsuchigaeru) by the pharmacological screening. We have isolated these peptides and characterized chemically as the new peptides analogous to bradykinin and a new ranatensin or bombesin analogous peptide.

Experimental

Assay Methods——Assay methods employed in this experiment were oxytocic assay, pressor assay and cholecystokinin-pancreozymin assay. The rat uteri were used in oxytocic assay according to the method described previously.[5] The pressor activity was assayed by measuring the rat arterial blood pressure.[6] The gall bladder strip of quinea pig was used for cholecystokinin assay by the method of Ljungberg.[7] The rat pancreatic secretion was measured for pancreozymin assay by the method of Tachibana.[8]

Reagents and General Procedures

The Enzyme Solutions——TPCK-trypsin and γ-chymotrypsin (Worthington Biochemicals, 185 and 53 units/mg protein) were dissolved respectively to 100 μg/ml with 0.1N triethylamine bicarbonate buffer (pH 8.0). Carboxypeptidase A (DFP-treated, Worthington Biochemicals, 666 units/ml) was dissolved into 10% LiCl at concentration of 400 μg/ml.

1) A part of this work was presented at the 12th Symposium on Peptide chemistry, Kyoto, November 1975.
2) Location: *a) Kasumi Hiroshima, 734, Japan*; *b) Koishikawa-4 Bunkyo-ku Tokyo, 112, Japan.*
3) *a)* V. Erspamer, G.F. Erspamer, and L. Negri, "Chemistry and Biology of the Kallirein-Kinin System in Health and Disease," ed. by J.J. Pisano and K.F. Austen, DHEW Publication No. (NIH) 1976, pp. 76—791, Chapter 21, "Naturally Occurring Kinins"; *b)* T. Nakajima, *ibid.*, Chapter 22, "New Vasoactive Peptides in Nonmammalian Origin."
4) V. Erspamer, G. Bertaccini, and N. Urakawa, *Jpn. J. Pharmacol.*, **14**, 468 (1964).
5) I. Trautschold, "Hand Book of Experimental Pharmacology," Vol. XXV, ed. by E.G. Erdös, Springer-Verlag, New York, 1970, p. 57.
6) T. Nakajima, T. Nakayama, and H. Sokabe, *Gen. Comp. Endocrinol.*, **17**, 458 (1971).
7) S. Ljungberg, *Svensk. Farm. Tidskr.*, **68**, 351 (1964).
8) S. Tachibana, *Jpn. J. Pharmacol.*, **21**, 325 (1971).

Enzymatic Digestion of the Peptide——The peptide of 1 to 10 nmol was dissolved into 10 μl of 0.1 N triethylamine bicarbonate buffer and 10 μl of the enzyme solution was added and incubated at 37° for 4 hr or at 25° for overnight.

Dansyl Procedures——Dansylation of the peptide and Dansyl-Edman procedure were performed according to the method of Tamura *et al.*[9] and Gray[10] respectively.

High Performance Liquid Chromatography——LC4-4 (Toyo Soda Co.) was packed in a thick glass tube of 5×500 mm size and the column was maintained at 55° in an oven. The mixture of MeOH: H_2O: AcOH: triethylamine (45: 55: 1: 1) was eluted at the flow rate of 1.2 ml/min with double plunger pump. The eluate was detected by ultraviolet (UV) absorbance at 254 or 280 nm.

Thin-Layer Chromatography——Thin-layer of Silica gel H and polyamide layer were used to identify the dansylated peptide with the solvent system of *n*-BuOH: AcOH: H_2O (4: 1: 5), iso-PrOH: MeOAc: 28% NH_4OH (9: 7: 4) for Silica gel H, and benzene: AcOH (9: 1), 1.5% HCOOH for polyamide layer respectively. The combination of Silica gel H and the mixture of benzene: dioxane: AcOH (90: 25: 4) was used for the identification of dansylated active amines. All of the reagents employed for the Edman degradation procedure were redistilled, sealed under N_2 gas and stored in a refrigerator. The other reagents used in this experiment were guaranteed grade. Glass equipments including glass chromatotubes were siliconized by dimethyl dichloro silane and MeOH.[11]

Results and Discussion

Preparation of the Starting Material

The fresh skin obtained from 170 adult frogs (*Rana rugosa*) collected at the suburbs of Hiroshima city, was dipped immediately in 50 ml of 6% trichloroacetic acid solution and then 300 ml of methanol was poured into the mixture. The mixture was kept for 2 days in a cold room (4°). The skin was taken out and extracted further by adding 300 ml of fresh methanol in a cold room for 1 day. The re-extraction was repeated twice in the same method. All the extracts were combined, evaporated in a reduced pressure below 0° and finally lyophilized. Fifty ml of water was added to the residue and the suspension was extracted 3 times with 100 ml of *n*-hexane. The aqueous layer was centrifuged at 3000 rpm for 10 min and the supernatant was served for the starting material.

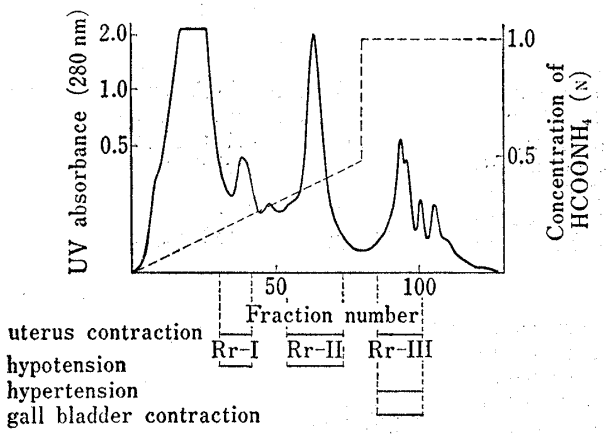

Fig. 1. SE-Sephadex Chromatogram of the Crude Skin Extract

The column (25 mm×450 mm) was eluted with a linear gradient elution from H_2O to 0.5 N $HCOONH_4$ (pH 6.5) (540—540 ml) and successively eluted with 1.0 N $HCOONH_4$ (pH 9.5) (750 ml). 15 ml fraction were collected.

The biological activities were shown by solid lines below the chromatogram.

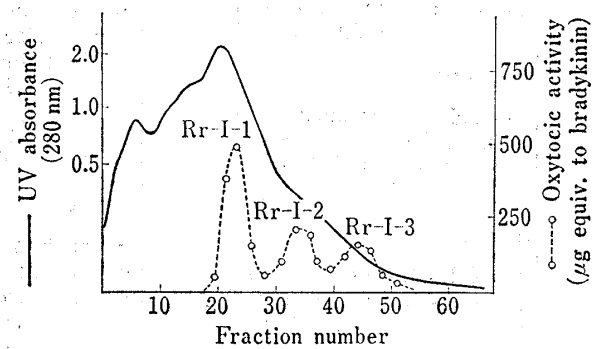

Fig. 2. Droplet Counter Current Chromatogram of Rr-I-1, 2 and 3

Transfer tubes of 75 were used for the separation. The lower layer of *n*-BuOH: AcOH: H_2O (4: 1: 5) was used as the moving phase. The eluate of 3 g was collected in each fraction tube.

9) Z. Tamura, T. Nakajima, T. Nakayama, J.J. Pisano, and S. Udenfriend, *Anal. Biochem.*, **52**, 595 (1973).
10) W.R. Gray, "Method in Enzymology," Vol. XI, ed. by C.H.W. Hirs, Academic Press, New York, London, 1967, p. 469.
11) H. Yoshida, K. Matsumoto, T. Nakajima, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **19**, 1691 (1971).

Separation of the Active Principles

The starting material of 50 ml being adjusted pH to 3.0 by adding formic acid, was applied to an SE-Sephadex column and chromatographed by a linear gradient elution with water to 0.5 N ammonium formate (pH 6.5) and then flat elution with 1.0 N ammonium formate (pH 9.5). The chromatogram was shown in Fig. 1. Oxytocic active principle was separated into 3 peaks (Rr–I, Rr–II and Rr–III) and their activities were 1.8 mg for Rr–I, 16.2 mg for Rr–II and 2.2 mg for Rr–III respectively equivalent to synthetic bradykinin. Preliminary experiment showed that the fraction Rr–I increased its oxytocic activity with treatment with trypsin, while activities in Rr–II and –III did not varied with this procedure, and by chymotryptic treatment, the activities in Rr–I and –III were lost but that in Rr–II was not reduced completely. Both Rr–I and –II were hypotensive but Rr–III was hypertensive for the rat arterial blood pressure. In addition, the fraction in Rr–III showed the gall bladder contracting activity.

Purification of the Active Principles in Rr-I

The lyophilized material obtained from the fraction Rr–I was dissolved in 5 ml of the lower layer of *n*-butanol: acetic acid: water (4: 1: 5) and separated by droplet counter current chromatography using the lower layer of the same solvent system as the moving phase. As shown in the chromatogram in Fig. 2, the active principle was separated further into 3 peaks

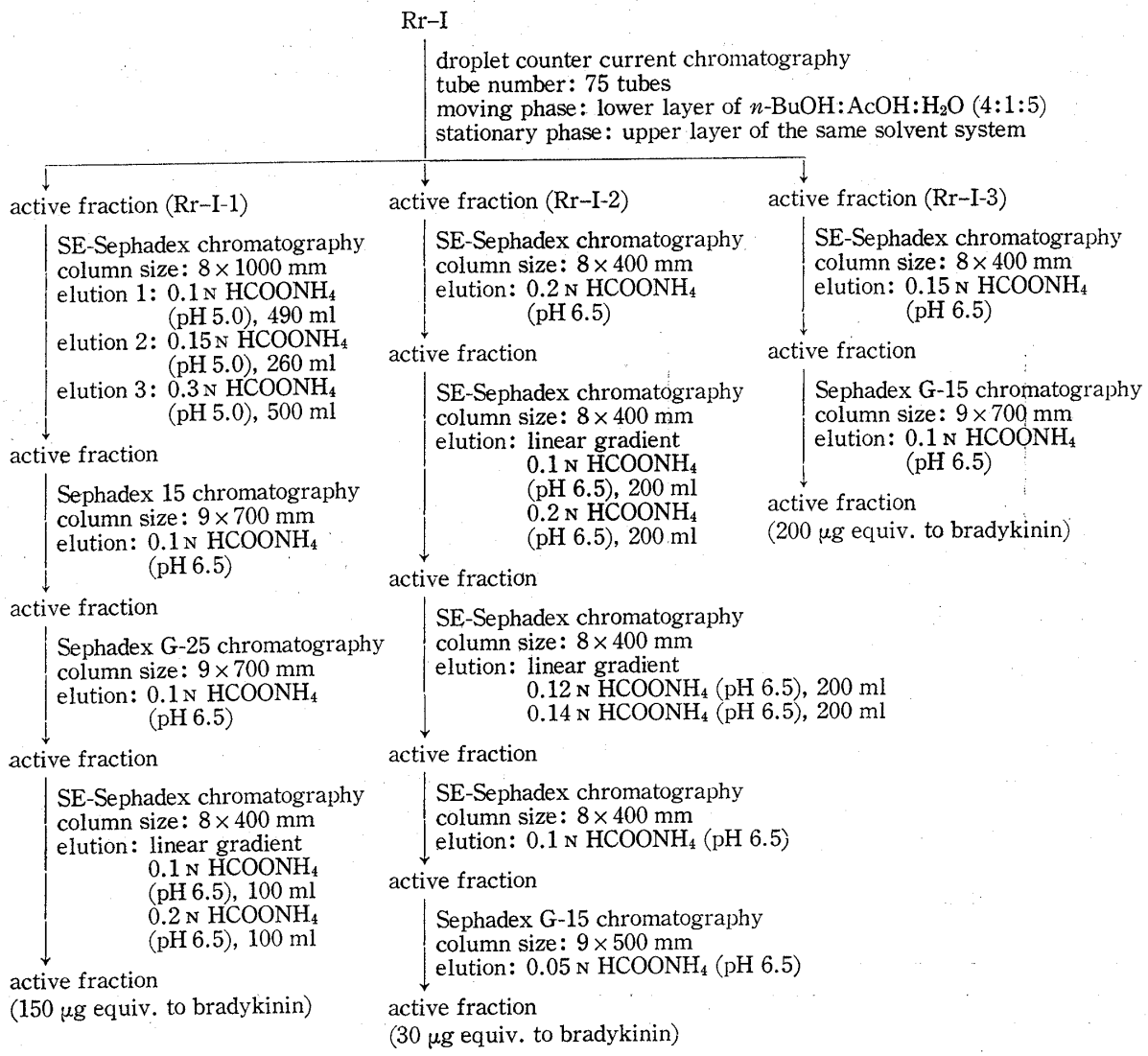

Chart 1. Purification of the Active Principles in the Fraction Rr–I

(Rr–I–1, Rr–I–2, and Rr–I–3). These active fractions were respectively purified as shown in Chart 1, until the single fluorescent band on a thin-layer of silica gel was observed when the aliquot of the fraction was dansylated and chromatographed. The oxytocic bioassay was employed for the purification of these materials.

Sequence Analysis of the Active Principles in Rr-I-1, -2 and -3

Amino acid compositions of these peptides after usual amino acid hydrolysis of 6 N hydrochloric acid for 24 hr at 110° were as follows:

Rr–I–1; $Arg_{1.40}$, $Thr_{0.80}$, $Glu_{1.22}$, $Pro_{3.90}$, $Gly_{1.10}$, $Ala_{0.90}$, $Ile_{1.30}$, $Phe_{2.00}$

Rr–I–2; $Arg_{2.00}$, $Thr_{1.00}$, $Glu_{1.20}$, $Pro_{3.50}$, $Gly_{1.30}$, $Ala_{1.70}$, $Ile_{1.90}$, $Phe_{2.00}$

Rr–I–3; $Arg_{1.30}$, $Thr_{0.90}$, $Glu_{0.90}$, $Pro_{3.80}$, $Gly_{1.20}$, $Ala_{1.00}$, $Val_{1.30}$, $Ile_{1.80}$, $Phe_{2.00}$

The compositions were similar to each other. Rr–I–1 was lacking 1 mol of isoleucine comparing to Rr–I–2, and Rr–I–3 contained an additional amino acid valine to Rr–I–2. The N-terminal amino acids of these peptides were the same and it was identified to arginine by dansyl method. The dansylated peptides of the three were split by trypsin digestion to produce the same dansylated core peptide which was identified as Thr^6-bradykinin by its amino acid composition, and by co-chromatography with the authentic sample using two different solvent systems on Silica gel H. This indicated that Thr^6-bradykinin sequence was located at the N-terminal part of these peptides. After the digestion of the dansylated Rr–I–3 with trypsin, C-terminal portion of the peptide which appeared non-protected amino terminal was successively analysed by dansyl Edman procedure and the sequence of $\underrightarrow{\text{Ile–Ala–Pro–Glx–}}$ was decided. Rr–I–3 of 10 nmol was dissolved in 1 ml of triethylamine bicarbonate buffer (pH 7.9), and 5 μl of the carboxypeptidase A solution was added. The mixture was incubated at 35° and an aliquot of the incubation product was taken out at 5, 10, 30, 60 and 360 min later and the liberated amino acids were determined by an amino acid analyser. The result was shown in Table I. This indicated that the C-terminal sequence of Rr–I–3

Table I. The Liberation of Amino Acids from Rı–I–3 by Carboxypeptidase Digestion

Incubation time (min)		5	10	30	60	360
Liberated amino acids[a] ($\times 10^{-9}$ mol)	Val	—	0.38	0.63	0.84	1.49
	Ile	—	—	—	0.10	0.59
	Glu	—	—	—	—	0.32

a) Amino acids were determined directly by an amino acid analyser (JEOL 5AH).

was considered to be $\overleftarrow{\text{–Glu–Ile–Val.}}$ At the same time, the another aliquot of the incubation product was dansylated and chromatographed on a thin layer of Silica gel H with the solvent system of isopropanol: methyl acetate: 28% ammonia (9: 7: 4). The dansyl peptide which was liberated valine from the C-terminal of Rr–I–3 was coincided to dansylated Rr–I–2. When the second liberation had occurred, the dansylated peptide was coincided to dansylated Rr–I–1.

As the results of these facts, the full sequence of Rr–I–3 was deduced to be Thr^6-bradykinyl–Ile–Ala–Pro–Glu–Ile–Val. We have called this peptide ranakinin-R. Rr–I–1 and Rr–I–2 were so far deduced to be Thr^6-bradykinyl–Ile–Ala–Pro–Glu and Thr^6-bradykinyl–Ile–Ala–Pro–Glu–Ile respectively.

The oxytocic activity of ranakinin-R was approximately 1/5 of that of bradykinin and hypotensive activity was approximately 1/4 comparing to that of bradykinin.

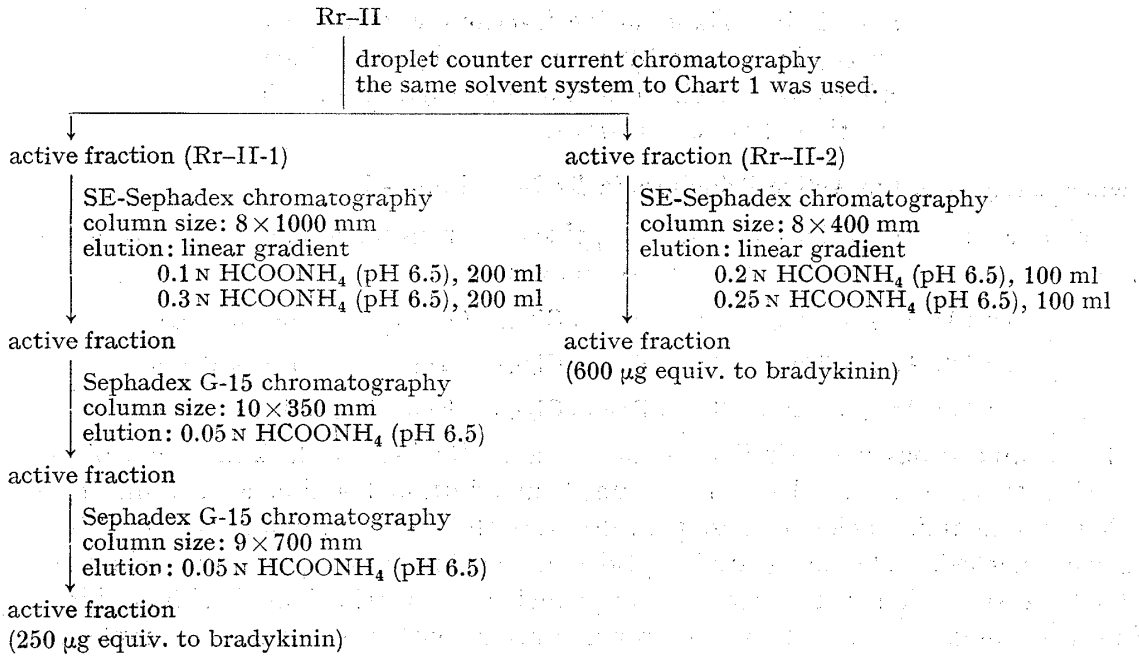

Chart 2. Purification of the Active Principles in the Fraction Rr–II

Purification and Chemical Characterization of the Active Principles in Rr-II

Purification of this fraction was summarized in Chart 2. One forth of the active principle was dissolved in 2.5 ml of the lower layer of *n*-butanol: acetic acid: water (4: 1: 5) and purified by droplet counter current chromatography using the same lower layer as the moving phase. The active principle was separated into 2 peaks (Rr–II–1 and Rr–II–2). Active principle in the fraction Rr–II–1 was inactivated by chymotrypsin digestion but not by trypsin and was considered to be the bradykinin-like peptide. Rr–II–1 was further purified with SE-Sephadex and Sephadex G-15 column chromatography. The purified Rr–II–1 showed the amino acid composition of Arg_2, Thr_1, Pro_3, Gly_1 and Phe_2. The peptide was identified with Thr^6-bradykinin by dansyl-Edman degradation. The dansylated peptide and its tryptic or chymotryptic fragments were also coincided to their corresponding peptide or peptide fragments of similarly treated authentic Thr^6-bradykinin by the method described in elsewhere.[12]

The activity of Rr–II–2 was not inactivated by trypsin or chymotrypsin treatment. The principle showed a maximum absorption at 275 nm and a shoulder at 295 nm, a typical absorption spectrum of serotonin. In the thin-layer chromatography of Silica gel H, the dansyl derivative of this principle showed overlapping to dansylated serotonin with a solvent system of benzene: dioxane: acetic acid (90: 25: 4). The principle was eluted also the same retention volume to serotonin by the high performance liquid chromatography.

Characterization of the Active Principle in Rr-III

The active principle in Rr–III was pharmacologically different from Rr–I or Rr–II. The principle in this fraction contracted the rat uterus preparation similar to bradykinin, but the contraction was longlasting. The bradykinin and its analogous peptides are the hypotensive, while this principle was hypertensive for the rat arterial blood pressure. These pharmacological properties involving the guinea pig gall bladder contraction, were similar to ranatensin or bombesin analogous peptide.[13]

12) M. Watanabe, T. Yasuhara, and T. Nakajima, "Animal, Plant, and Microbial Toxins," Vol. II, ed. by Y. Sawai, Plenum Publishing Co., New York, London, 1976, p. 105.
13) *a*) T. Nakajima, T. Tanimura, and J.J. Pisano, *Fed. Proc.*, **29**, 282 (1970); *b*) V. Erspamer, G.F. Erspamer, and M. Inselvini, *J. Pharm. Pharmacol.*, **22**, 876 (1970).

Rr-III was purified by the procedure as shown in Chart 3. The purified principle showed a UV spectrum of a λ_{max} at 280 nm and a shoulder at 295 nm due to the tryptophyl residue. The amino acid composition determined after the usual acid hydrolysis was; $His_{0.95}$, $Arg_{2.00}$, $Asp_{2.00}$, $Thr_{1.90}$, $Ser_{1.00}$, $Glu_{1.00}$, $Gly_{1.10}$, $Ala_{2.00}$, $Met_{0.95}$, $Leu_{1.00}$, $Tyr_{0.95}$, $Phe_{1.00}$, and $Trp_{1.10}$ (UV absorption). The activity of this principle was not inactivated by trypsin or carboxypeptidase A but chymotrypsin. The dansyl Edman procedure resulted the following sequence at the N-terminal portion of the peptide; Ser–Asx–Ala–Thr–Leu–Arg–Arg–, and this sequence suggested that this peptide was a new one ever isolated from the amphibian skin. The full sequence, however, could not be determined because of its minute amount. Further investigation should be succeeded to identify its chemical characters.

Rr-III
↓ Sephadex G-25 chromatography
column size: 12×450 mm
elution: 0.1 M pyridine acetate (pH 7.0)
active fraction
↓ SE-Sephadex chromatography
column size: 7×500 mm
elution: 0.3 N $HCOONH_4$ (pH 6.5)
active fraction
↓ XAD-II chromatography
column size: 10×250 mm
adsorption: 0.1% AcOH, 12 ml
elution: linear gradient
0.1% AcOH, 45 ml
MeOH, 45 ml
active fraction
↓ SE-Sephadex chromatography
column size: 7×500 mm
elution: 0.25 N $HCOONH_4$ (pH 6.5)
active fraction
↓ SE-Sephadex chromatography
column size: 7×250 mm
elution: 0.1 N $HCOONH_4$ (pH 6.5)
active fraction
(300 μg equivalent to bradykinin)

Chart 3. Purification of the Active Principle in the Fraction Rr–III

The skin of *Rana rugosa* contained six kinds of vasoactive principles, these were four kinds of bradykinins, serotonin and the new peptide. All of the bradykinins were replaced with threonine at the sixth position of the amino acid sequence of bradykinin. Since the fresh skin was immediately dipped in 6% trichloroacetic acid to inactivate some proteases which might exist in the skin, and was diluted with methanol as described previously, it has possibly considered that Thr^6-bradykinin, Rr–I–1 or Rr–I–2 were not the artifacts being produced during the extraction or separation process and the skin of *Rana rugosa* had contained in various forms of the bradykinin analogous peptides in a living state. The contents of these peptides in the first step of separation, were approximately 870 μg for Rr–I–1, 540 μg for Rr–I–2, 400 μg for Rr–I–3 and 3000 μg for Thr^6-bradykinin, although these values did not represent exactly the contents of these peptides in the fresh skin. These values were also in terms of bradykinin activity and the values in weight basis of these peptides might be increased about 5 times for Rr–I–1, Rr–I–2 and Rr–I–3, because the activity on the rat uterus contraction of Rr–I–3 was 1/5 of that of bradykinin.

Ranakinin-R (Rr–I–3), the longest peptide among the bradykinin analogous peptide ever isolated from the frog skin, also elongates the peptide chain at the C-terminal end of bradykinin. The amino acid sequence at the elongation part is also different from the others and it seems to be dependent upon the species specificity.

Rr–III was considered to be belonging the ranatensin or bombesin family from its pharmacological properties, but the results from the amino acid analysis and amino acid sequence around the N-terminal region of the peptide suggested that the sequence at the C-terminal region might be different also from both ranatensin and bombesin. Isolation of Rr–III has been started to obtain the more amounts of the peptide.