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The Studies on the Active Peptide in the Skin of *Rana rugosa*. II. The Structure of Ranatensin-R, the New Ranatensin Analogue, and Granuliberin-R, the New Mast Cell Degranulating Peptide[1)]

TADASHI YASUHARA, OSAMU ISHIKAWA, and TERUMI NAKAJIMA

Institute of Pharmaceutical Sciences, Hiroshima University, School of Medicine[2)]

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The skin of *Rana rugosa* contained ranatensin-R, a new analogous peptide to ranatensin. Ranatensin-R is heptadecapeptide amide which is a longest analogue of ranatensin ever isolated from the frog skin.

Granuliberin-R, dodecapeptide amide, was obtained from the same ranatensin-R fraction during the separation process. The peptide is a new type of amphibian skin peptide which acts on the rat peritoneal mast cell to liberate granules and histamine from the cells.

Keywords——ranatensin-R; granuliberin-R; *Rana rugosa*; active peptide; amphibian skin; sequence analysis; mast cell degranulation

In the previous paper,[1)] we have isolated the various kinds of vasoactive peptides in the skin of *Rana rugosa* (Japanese name: Tsuchigaeru) and identified these peptides chemically as 4 kinds of bradykinin analogues and the new peptide analogous to ranatensin[3)] or bombesin,[4)] although this new peptide has not been elucidated in full sequence.

This paper describes the chemical characterization and the full sequence of this peptide. The peptide was called "ranatensin-R" based upon both the similarity of the amino acid sequence to ranatensin and the species name of the frog, *rugosa*.

The paper also deals with the structure of granuliberin-R,[5)] the new mast cell degranulating peptide which accompanied to ranatensin-R fraction during the separation process.

Experimental

Assay Methods——Pressor and oxytocic activities were assayed by the methods described in the previous paper.[1)]

The mast cell degranulating activity was assayed as follows; the rat peritoneal mast cells were collected according to the method of Bloom and Haegermark.[6)] The mast cells of 10^6 were suspended in 500 μl of 0.9% NaCl solution. Ten μl of the suspension was added to the sample solution of the same volume and the mixture was incubated at 25° for 10 min. The degradation of the mast cells was examined under the phase contrast microscope.

The Enzyme Solution——TPCK-trypsin (Worthington Biochemicals, 185 units/mg protein) and ∂-chymotrypsin (Worthington Biochemicals, 53 units/mg protein) were dissolved to 100 μg/ml with 0.1 N triethylamine bicarbonate buffer (pH 8.0). Leucine amino peptidase (Worthington Biochemicals, 43 units/mg protein) was dissolved in 100 μg/ml in 0.125 M $MgCl_2$ solution. Carboxypeptidase A (Sigma, 54 units/mg protein) was dissolved in 500 μg/ml in 10% LiCl solution.

The procedure for enzymatic digestion, dansylation, Edman degradation, and the thin-layer chromatography were performed by the methods in the previous paper,[1)] and otherwise described in the text.

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2) Location: *Kasuki Hiroshima, 734, Japan.*
3) T. Nakajima, T. Tanimura, and J.J. Pisano, *Fed. Proc.*, **29**, 282 (1970).
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5) Preliminary communication: T. Nakajima and T. Yasuhara, *Chem. Pharm. Bull.* (Tokyo), **25**, 2464 (1977).
6) G.D. Bloom and O. Haegermark, *Exp. Cell Research*, **40**, 637 (1965).

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Results and Discussion

Purification of Ranatensin-R and Granuliberin-R

Some preferential separative procedures were investigated to obtain ranatensin-R effectively. Ranatensin-R in the residue of the crude methanol extract was more soluble in the lower layer of *n*-butanol: acetic acid: water (4: 1: 5) than in the dilute formic acid solution which was employed in the previous paper. Ranatensin-R was eluted at the concentration of 1.0 N ammonium formate from the SE-Sephadex column and was considered to be the most basic peptide among the vasoactive principles in the skin of *Rana rugosa*. Therefore, it might be possible to separate ranatensin-R from the other materials when the SE-Sephadex column is eluted with the more sloped concentration gradient of ammonium formate buffer.

Ranatensin-R in the aqueous solution was adsorbed effectively on porous polystyrene polymer such as XAD–II or Hitachi gel 3010 and was eluted from these resins by addition of methanol, but the method was not practical because the recovery was less than 30%.

In the droplet counter current chromatography, ranatensin-R distributed in the upper layer of *n*-butanol: acetic acid: water (4: 1: 5) and distributed almost 1: 1 for the upper and the lower layer of *sec*-butanol: trifluoroacetic acid: water (120: 1: 160), but the activity of ranatensin was not stable in this solvent system which might be due to the decomposition of tryptophyl residue in the peptide with trifluoroacetic acid.

The recovery of ranatensin-R in the QAE-Sephadex column chromatography with the week basic buffer as an eluent, was also poor which might be due to the oxidation of me thionyl residue in the peptide.

skin of *Rana rugosa* (100 frogs)
↓ droplet counter current chromatography
tube number: 50 tubes
moving phase: lower layer of *n*-BuOH: AcOH: H_2O (4: 1: 5)
stationary phase: upper layer of the same solvent system

active fraction
↓ droplet counter current chromatography
tube number: 75 tubes
the same solvent system was used

active fraction
↓ SE-Sephadex chromatography
column size: 7 × 500 mm
elution: linear gradient
H_2O 120 ml
1.0 N $HCOONH_4$ (pH 6.5), 120 ml

active fraction
↓ Sephadex G-15 chromatography
column size: 9 × 700 mm
elution: 0.05 N $HCOONH_4$ (pH 6.5)

active fraction
↓ recromatography with Sephadex G-15
column size: 9 × 700 mm
elution: 0.01 N $HCOONH_4$ (pH 6.5)

active fraction
↓ SE-Sephadex chromatography
column size: 5 × 480 mm
elution: 0.2 N $HCOONH_4$ (pH 6.5)

active fraction
(Rr–III-1, Rr–III-2, Rr–III-3)

Chart 1. Purification of the Active Principles in the Skin

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Under the consideration of these preferential results, ranatensin-R was isolated by the method as shown in Chart 1.

The crude extract obtained from the skin of 100 frogs was dissolved in 20 ml of the lower layer of *n*-butanol: acetic acid: water (4: 1: 5) and separated by the droplet counter current chromatography. The activities of ranatensin on the long-lasting rat uterus contraction and on the rat arterial hypertensive action were measured in each separation process to distinguish ranatensin-R from the other active principles. The droplet counter current chromatography was repeated with the same solvent system. The active fraction was chromatographed on an SE-Sephadex column by the linear gradient elution from water to 1.0 N ammonium formate (pH 6.5). Two major active fractions revealed oxytocic activity which were corresponded to Rr–II and Rr–III (ranatensin-R) respectively in the previous paper.[1] The latter fraction was purified for three times with gel permeation chromatography on Sephadex G-15 and finally purified again with SE-Sephadex column by flat elution of 0.2 N ammonium formate (pH 6.5). The eluate was monitored by UV absorbance at 280 nm. The chromatogram at this step of separation was shown in Fig. 1. The substance was separated into three peaks by UV absorbance (Rr–III–1, Rr–III–2 and Rr–III–3). The fraction Rr–III–1 and –2 revealed oxytocic and pressor activity. These peaks showed the UV spectra of a λ_{max} at 280 nm and a shoulder at 295 nm. The fraction Rr–III–3, which had no oxytocic nor pressor activity practically, showed UV spectrum of a λ_{max} at 280 nm.

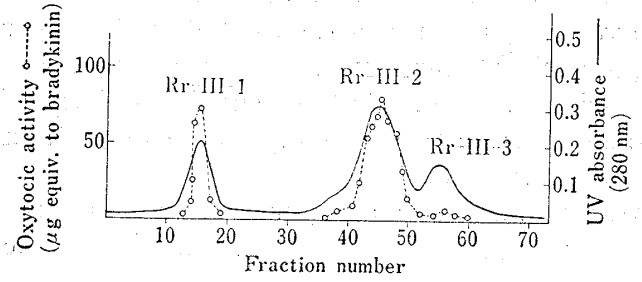

Fig. 1. SE-Sephadex Chromatogram of Rr-III-1, Rr-III-2, Rr-III-3

The column (5×480 mm) was eluted with 0.2 N $HCOONH_4$ (pH 6.5). 2.5 ml each of the eluate was collected.

An aliquot of each fraction was hydrolysed with 6 N hydrochloric acid at 110° for 24 hrs and the amino acid compositions were determined by an amino acid analyser (JEOL 5-AH) and the following data were obtained;

Rr-III-1; $His_{1.26}$, $Arg_{5.66}$, $Asp_{2.33}$, $Thr_{1.89}$, $Ser_{2.22}$, $Glu_{1.26}$, $Pro_{3.98}$, $Gly_{3.02}$, $Ala_{4.53}$, $Met_{0.72}$, $Ile_{2.06}$, $Leu_{2.46}$, $Tyr_{1.90}$, $Phe_{4.18}$, Trp_1(UV).

Rr-III-2; $His_{1.14}$, $Arg_{2.18}$, $Asp_{2.16}$, $Thr_{2.03}$, $Ser_{1.03}$, $Glu_{1.44}$, —, $Gly_{1.00}$, $Ala_{2.03}$, $Met_{0.61}$, —, $Leu_{1.07}$, $Tyr_{0.86}$, $Phe_{1.13}$, Trp_1(UV).

Rr-III-3; $Arg_{1.93}$, —, —, $Ser_{0.80}$, —, $Pro_{3.00}$, $Gly_{1.00}$, $Ala_{1.19}$, —, $Ile_{0.90}$, $Leu_{0.88}$, $Tyr_{0.94}$, $Phe_{2.00}$.

The amount of tryptophyl residue was calculated from the UV absorbance. The composition of Rr–III–2 was the same ranatensin-R in the previous paper,[1] and that in Rr–III–1 suggested that the peptide might be a long chain peptide locating the peptide Rr–III–2 or Rr–III–3 at the head or tail, or ranatensin-R and Rr–III–3 might form the complex to be eluted together in this fraction.

The dansyl procedure in these fraction, however, supported the latter possibility. After the dansylation of the fraction Rr–III–1, two major yellow fluorescent bands were observed on a thin layer of Silica gel H with the solvent system of *n*-butanol: acetic acid: water (4: 1: 5) and the both fluorescent bands were coincided to each dansyl product of ranatensin-R and Rr–III–3 respectively. This result suggested that the peptide in Rr–III–3 was likely accompanied with ranatensin-R.

Sequence Analysis of Ranatensin-R

The peptide of 50 n mol was analysed by dansyl Edman procedure to comfirm the preliminary results and the sequence of Ser–Asx–Ala–Thr–Leu–Arg–Arg–Tyr–Asx–Glx–Xxx–Ala–

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resulted from this experiment. In the degradation step 10, the eleventh position of amino acid could not be recovered after the acid hydrolysis with 6 N hydrochloric acid at 90° for 16 hr and this position was considered to be tryptophan.

The complete enzymatic hydrolysis of the peptide was carried out with the combination of chymotrypsin and leucine amino peptidase. The peptide of 6 n mol was dissolved in 100 μl of 0.1 N triethylamine bicarbonate buffer (pH 8.0) and 10 μl of chymotrypsin solution was added and incubated at 25° for 24 hr. Leucine amino peptidase of 10 μl was added to the reaction mixture and incubated further for 20 hr at 25°. The amino acid composition in this enzymatic hydrolysate was, Trp_1, His_1, $Thr(Thr+Asn+Glu)_5$, Ser_1, Gly_1, Ala_2, Met_1, Leu_1, Tyr_1, and Phe_1. This result indicated that the aspartic acid and glutamic acid residues in the peptide were asparagine and glutamine respectively.

The peptide of 80 n mol was dissolved in 100 μl of 0.1 N triethylamine bicarbonate buffer (pH 8.0) and digested by adding 10 μl of chymotrypsin solution. An aliquot of the reaction mixture was dansylated and chromatographed on a thin layer of Silica gel H. The chromatogram was shown in Fig. 2, and 7 fluorescent bands were obtained. Each dansylated peptide was purified by preparative thin-layer chromatography, and the amino acid compositions and their N-terminal amino acids were determined after acid hydrolysis with 6 N hydrochloric acid at 90° for 16 hr. The following amino acid compositions were given from the hydrolysate of C-2, C-3, C-4 and C-7.

C-2; DNS-Ser,[7] Arg_2, Asp_1, Thr_1, Ala_1, Leu_1, O-DNS-Tyr
C-3; DNS-Ser, Asp_1, Thr_1, Ala_1, Leu_1
C-4; DNS-Asp, His_1, Thr_1, Glu_1, Gly_1, Ala_1, Phe_1
C-7; DNS-Met

Other fluorescent bands were the mixtures of minor fragments and did not show the clear-cut results. The dansyl fragment obtained from C-7 was coincided to dansyl methionine amide when chromatographed on Silica gel H plate with the solvent system of benzene: acetone (7:3) or ether: acetone (9:1). This fragment should be at the C-terminal end of the peptide.

At the same time, the another aliquot of the chymotryptic digest was digested further with carboxypeptidase A. The major liberated amino acids were leucine, tyrosin and phenylalanine which was analysed by an amino acid analyser. The result indicated that the

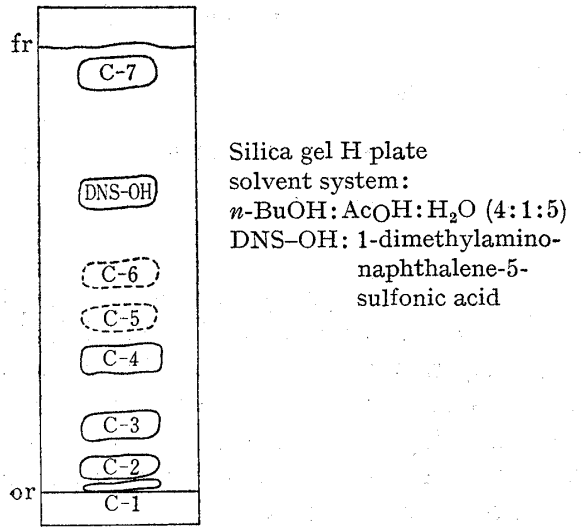

Fig. 2. Thin layer Chromatogram of Dansylated Chymotryptic Fragments of Ranatensin-R

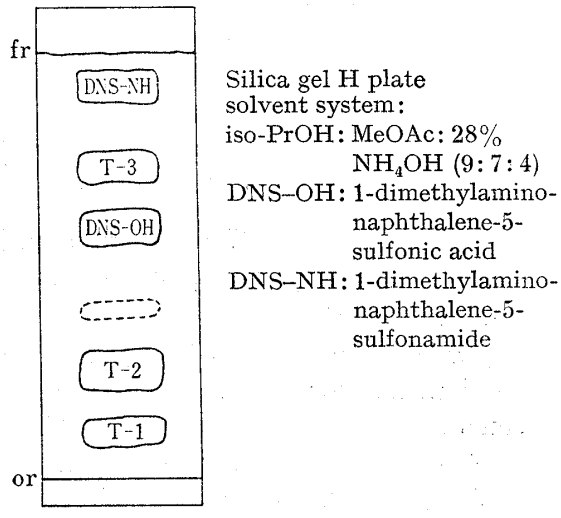

Fig. 3. Thin Layer Chromatogram of Dansylated Tryptic Fragments of Ranaten sin-R

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

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tryptophyl bond in the peptide was less sensitive for chymotrypsin digestion in this reaction condition.

These experimental data so far suggested the following combination of the peptide;

Ser–Asn–Thr–Ala–Leu–Arg–Arg–Tyr–Asn–Gln–(Trp)–Ala–(His, Thr, Gly)–Phe–Met–NH_2

|————————C-2————————| |————————C-4————————| |—C-7—|

|————C-3————|

The trypsin solution of 10 μl was added to 40 n mol of the intact peptide dissolved in 100 μl of 0.1 N triethyaline bicarbonate buffer (pH 8.5) and the mixture was incubated at 25° for 48 hr. 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 chromatogram was shown in Fig. 3 and the peptide was cleft into 3 fragments (T-1, T-2 and T-3) by trypsin digestion.

The dansylated fragments were isolated, hydrolysed and their amino acid compositions were determined as in the similar manner to chymotrypsin digestion experiment. The amino acid composition of each fragment was as follows;

T-1; DNS-Ser, Arg_1, Asp_1, Thr_1, Ala_1, Leu_1

T-2; DNS-Arg

T-3; DNS-Tyr, His_1, Asp_1, Thr_1, Glu_1, Gly_1, Ala_1, Met_1, Phe_1

The another aliquot of dansylated T-3 fragment was dissolved in 100 μl of 0.1 N triethylamine bicarbonate buffer and 10 μl of chymotrypsin solution (1 mg/ml) was added and incubated at 30° for 24 hr. The dansyl Edman procedure was performed directly to this incubation mixture. In the basal step, dansyl alanine and dansyl methionine were identified, and the sequence of Thr–Gly–His was identified in the successive steps by polyamide layer chromatography using the solvent system of benzene: acetic acid (9: 1) and 1.5% formic acid.

As the results of these experiments, the full sequence of ranatensin-R was deduced as shown in Fig. 4.

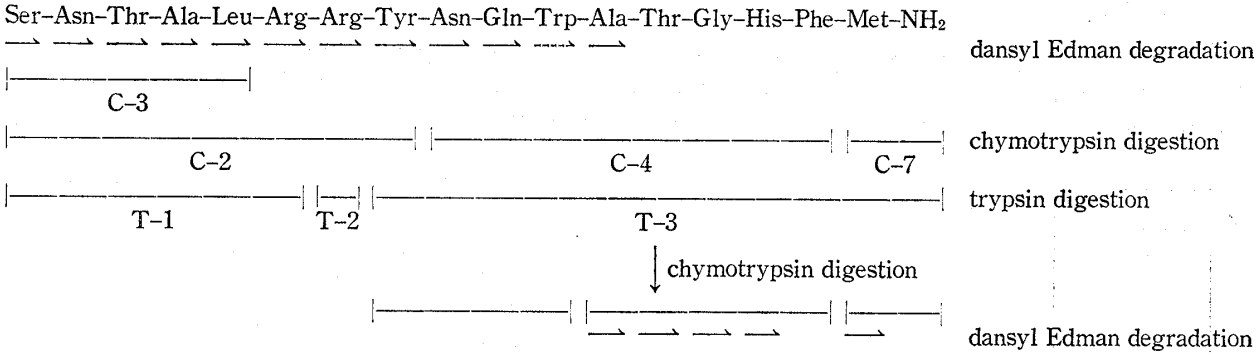

Fig. 4. Major Profile of Sequence Analysis of Ranatensin-R

Sequence Analysis of Rr-III-3 (Granuliberin-R)

The N-terminal amino acid of the peptide was detected as phenylalanine by dansyl method. The peptide of 10 n mol was dissolved in 100 μl of 0.1 N triethylamine bicarbonate buffer (pH 8.0) and 15 μl of trypsin solution (1 mg/ml) was added. The mixture was incubated at 25° for 24 hr. The reaction product was evaporated to dryness under the nitrogen stream and was dansylated. The two dansylated tryptic fragments were observed by thin-layer chromatography of Silica gel H with the solvent system of *n*-butanol: acetic acid: water (4: 1: 5). The amino acid compositions of these fragments were DNS–Arg (Ser_1, Pro_1, Ala_1) for the lower *Rf* value fragment and DNS–Phe (Arg_1, Pro_1, Gly_1, Ile_1, Leu_1, O–DNS–Tyr, Phe_1) for the upper *Rf* value fragment.

The intact peptide of 10 nmol in 50 μl of 0.1 N triethylamine bicarbonate buffer (pH 8.0) was similarly treated by adding with 5 μl of chymotrypsin solution (1 mg/ml) and incubated

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at 25° for 16 hr. The reaction mixture was dansylated and chromatographed on a thin layer of Silica gel H as in the case of trypsin digestion experiment. Chymotrypsin also split the peptide into two fragments, DNS–Phe (Pro_1, Gly_1, Ile_1, Leu_1, O–DNS–Tyr, Phe_1) and DNS–Arg (Arg_1, Ser_1, Pro_1, Ala_1). The dansyl derivative of the chymotryptic N-terminal fragment was not cleft further repeated treatment of chymotryptic digestion because of its poor solubility for this derivative.

The peptide of 20 n mol was dissolved in 50 μl of 0.1 N triethylamine bicarbonate buffer (pH 8.0) and 50 μl of carboxypeptidase A solution was added and incubated at 35° for 1 hr. The peptide was not susceptible for this enzyme and did not liberate any amino acid for further incubation.

The peptide of 50 n mol was analysed by dansyl Edman degradation and the sequence of Phe–Gly–Phe–Leu–Pro–Ile–Tyr–Arg–Arg–Pro–Ala–Ser was obtained. In the last sequence of the peptide in dansyl Edman degradation, dansyl serine amide was detected when the product was developed without acid hydrolysis with acetone on a thin layer of Silica gel H, and the C-terminal position of the peptide was considered to be amidated.

The full sequence of the peptide was deduced to;

Phe–Gly–Phe–Leu–Pro–Ile–Tyr–Arg–Arg–Pro–Ala–Ser–NH_2

The peptide possesses the notable sequence, in which hydrophobic amino acid residues are located in the N-terminal region and the C-terminal region is hydrophilic and basic. The peptide seems to be a property of the natural detergent. The peptide was tested for the rat peritoneal mast cells and bathophils on degranulating action and showed the degranulation in the concentration of 5×10^{-9} mol/ml. The peptide was named granuliberin-R and has been synthesized chemically.[8]

The skin of *Rana rugosa* contained four types of biological active principles. These are the bradykinin analogue, ranakinin-R, the ranatensin analogue, ranatensin-R, the new type of peptide, granuliberin-R and serotonin.

Some of the frog contained ranatensin or bombesin family in the skin.[9] Both of these peptides contain the common active site region of Trp–Ala–Val–Gly–His–Xxx–Met–NH_2 at the C-terminal end. The amino acid residue in the second position from the C-terminal of ranatensin group is phenylalanine. Ranatensin, ranatensin-C and litorin belong to this group. While in bombesin group, this position is replaced by leucine. Bombesin and alytesin are known in this group. Ranatensin-R is the longest one among these groups and in addition, the fifth position of amino acid residue from the C-terminal end is different from both ranatensin or bombesin, and this position is replaced by threonin, although the second position from the C-terminal end is the same to ranatensin.

Recently the similarity of amino acid sequence between bombesin and vasoactive intestinal polypeptide in mammals has been pointed out[10] and the presence of immunoreactive peptide against bombesin antibody has also been demonstrated in the dog gut.[11] This seems to be also the case of the sequence similarity between mammalian physiological active peptide and the amphibian skin peptide.

On the other hand, granuliberin-R is the completely different type of active peptide which might act directly for mast cell membrane and release the granules from the cell. The

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peptide also liberates histamine from mast cell which was detected by high performance liquid chromatography. The presence of this type of active peptide in animal origin has been reported in bee venom, and wasp venom under the name of mast cell degranulating peptide (MCD-peptide),[12] and mastparan[13] respectively. But the sequence of these and granuliberin-R are completely different from each other. The mechanism of these peptides for mast cell degranulation has not been known. Further physiological and pharmacological studies using these peptides as the tool may reveal the mechanism between the cell membrane and the peptide interaction.

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