[Chem. Pharm. Bull. 23(12)3132—3140(1975)]

UDC 547.965.02.05.09 : 542.98

Isolation and Structure of a New Active Peptide Xenopsin on Rat Stomach Strip and Some Biogenic Amines in the Skin of *Xenopus laevis*[1]

Kengo Araki, Shinro Tachibana, Mikio Uchiyama,[2a] Terumi Nakajima, and Tadashi Yasuhara[2b]

Eisai Research Laboratories[2a] and Institute of Pharmaceutical Sciences, School of Medicine, Hiroshima University[2b]

(Received April 8, 1975)

A new active peptide xenopsin was obtained from the skin of *Xenopus laevis*, possessing a potent contractile activity on rat stomach strip *in vitro*. The amino acid sequence of xenopsin has been found to be pyrGlu–Gly–Lys–Arg–Pro–Trp–Ile–Leu–OH. The contractile responses on a rat stomach strip were proportional to the peptide doses. This peptide also had a moderate contractile activity on guinea-pig ileum but the response was not reproducible. Intravenous injection of this peptide lowered arterial blood pressure of the anesthetized rat but a prompt and intense tachyphylaxis was observed. Xenopsin and several of its analogous peptides were synthesized and their biological activities were determined. Essential sequence that displayed the activity was Arg–Pro–Trp–Ile–Leu–OH.

In addition to xenopsin and caerulein, it was confirmed that the skin of *Xenopus laevis* contained biological active amines, serotonin, buffotenidine, and N-methylserotonin.

From the skin of an amphibian frog, many biologically active and pharmacologically interesting peptides have been extracted, such as caerulein,[3] ranatensin,[4] bombesin,[5] bradykinin,[6] and its derivatives.[7] We have also made systematic screening of active peptides in the amphibian skin by some biological tests and reported new active peptides.[1,8]

Erspamer[9] divided these peptides into five groups from their structural features; eledoisin-like, bradykinin-like, caerulein-like, alytesin-like, and miscellaneous polypeptides. The present report concerns the isolation and determination of a new active peptide, xenopsin, from the skin of a frog, *Xenopus laevis*, which was structurally different from previously discovered peptides.

Experimental

Amphibian Material——Thirty frogs (*Xenopus laevis*) were obtained from Kusatsu Laboratory of Amphibian Biology, Kusatsu, Gunma Prefecture.

Materials used for the purification and degradative experiments of xenopsin were obtained from the following sources: Trypsin (TPCK), chymotrypsin, carboxypeptidase A, and carboxypeptidase B from Worthington Biochemical, Freehold, N.J., U.S.A., papain from Sigma Chemical Co., St. Louis, Mo., U.S.A., SE-, SP-, and QAE-Sephadex, and Sephadex G-25 from Pharmacia, Uppsala, Sweden, alkaline alumina (activity grade 1) from M. Woelm, Eshwege, Germany; silica gel H from Merck, AG., Darmstat. Cellulose powder-coated plate "Abicel" from Funakoshi Chemical Co., Tokyo.

1) Communications to the Editor: K. Araki, S. Tachibana, M. Uchiyama, T. Nakajima, and T. Yasuhara, *Chem. Pharm. Bull.* (Tokyo), **12**, 2801 (1973).
2) Location: *a) Koishikawa-4, Bunkyo-ku, Tokyo*; *b) Kasumi, Hiroshima.*
3) A. Anastasi, V. Erspamer, and R. Endean, *Arch. Biochem. Biophys.*, **125**, 57 (1968).
4) T. Nakajima, T. Tanimura, and J.J. Pisano, *Fed. Proc.*, **29**, 282 (1970).
5) V. Erspamer, G.F. Erspamer, M. Inselvini, and L. Negri, *Brit. J. Pharmacol.*, **45**, 333 (1972).
6) A. Anastasi, V. Erspamer, and G. Bertaccini, *Comp. Biochem. Physiol.*, **14**, 43 (1964).
7) T. Nakajima, *Chem. Pharm. Bull.* (Tokyo), **16**, 2088 (1968).
8) T. Yasuhara, M. Hira, T. Nakajima, N. Yanaihara, C. Yanaihara, T. Hashimoto, N. Sakura, S. Tachibana, K. Araki, M. Bessho, and T. Yamanaka, *Chem. Pharm. Bull.* (Tokyo), **21**, 1388 (1973).
9) V. Erspamer, *Ann. Rev. Pharmacol.*, **7**, 327 (1970).

Bioassay——Biological activity of each of isolated products was determined by the contractility on the isolated strip of fundus from a rat stomach.[10] Activity of pure peptide xenopsin was assayed on the blood pressure of a rat anesthetized with urethan (1.42 g/kg, intraperitoneally) and contractility on isolated guinea-pig ileum.[11]

Amino Acid Sequence Analysis——N-terminal amino acid was determined by the dansyl (DNS) method of Gray and Hartley.[12] DNS-amino acid was identified by thin-layer chromatography (TLC) on silica gel H at room temperature. The solvents used were BuOH: AcOH: H_2O (4: 1: 5) and AcOMe: iso-PrOH: NH_4OH (9: 7: 4). DNS-xenopsin (DNS-Xe) and DNS-fragment produced by enzymic digestion of DNS-Xe were isolated by TLC on silica gel H.

Quantitative Amino Acid Analysis——Xenopsin and its enzymic DNS-fragments were hydrolyzed with 6N HCl and the amino acids were determined by an automatic amino acid analyzer (Nihon Denshi JLC-5AH).

Paper Chromatography——The ascending one-dimensional technique on Whatman No. 1 paper was routinely employed for the identification of biogenic amines using the following solvent systems[13–15]; BuOH: AcOH: H_2O (4: 1: 5), BuOH: 30%$MeNH_2$ (8: 3), 20% KCl in H_2O, MeCOEt: 2N NH_4OH (2: 1) and BuOH: 14% NH_4OH (2: 1). The detecting reagents used for biogenic amines were 0.3% solution of Heinrich and Schuler NNCD reagent (2-chloro-4-nitrobenzenediazonium naphthalene-2-sulfonate) and Ehrlich reagent (2% *p*-dimethylaminobenzaldehyde in 2N HCl).[13]

Results

Separation and Purification

Separation process was reported briefly previously.[1] Fresh skin from 30 frogs was extracted with 1000 ml of methanol containing 10 ml of 6% trichloroacetic acid. The extract was evaporated under reduced pressure. The residue was dissolved in 30 ml of diluted formic acid, pH 3, and centrifuged. The supernatant was applied to a column of SP-Sephadex C-25 (2.5×50 cm), which was previously equilibrated with 0.05 M ammonium formate, pH 6.5, and developed initially with the same buffer. Then the gradient elution with 1500 ml of the same buffer to 1500 ml of 0.5 M ammonium formate, pH 6.5, starting at the tube No. 90. Bioassay pattern revealed that the contractile activities on the rat stomach strip were present in three different peaks of the chromatogram (Fig. 1).

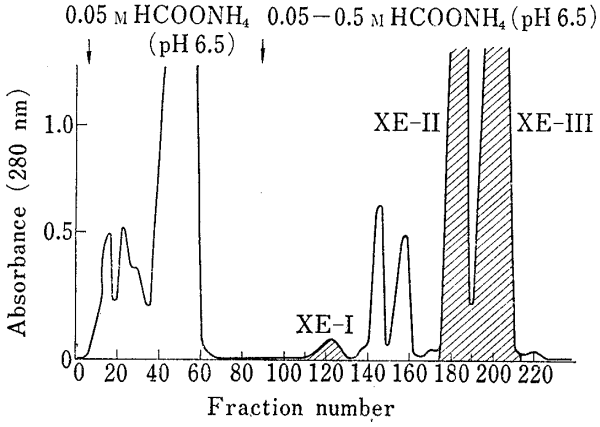

Fig. 1. Chromatography of the Methanol Extracts on SP-Sephadex C-25 Column

The column (2.5×50 cm) was eluted with 0.05M $HCOONH_4$ (pH 6.5) and a linear gradient elution from 0.05 to 0.5M $HCOONH_4$ (1500—1500 ml) was started at tube No. 90. The fraction volume was 15 ml each. The contractile activities on rat stomach strip were located in the shaded areas, XE-I, -II, and -III.

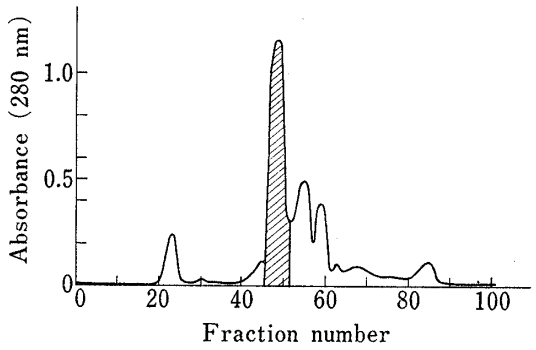

Fig. 2. Chromatography of XE-I (Fig. 1) on Sephadex G-25

The column (2.5×40 cm) was eluted with 0.1M AcOH and the fraction volume was 3 ml each. Shaded area was pooled.

10) J.R. Vane, *Brit. J. Pharmacol.*, **12**, 344 (1957).
11) D.E. Elliot and G.P. Lewis, *Biochem. J.*, **95**, 437 (1965).
12) W.R. Gray and B.S. Hartley, *Biochem. J.*, **89**, 59 (1963).
13) V. Erspamer, V. Vitali, M. Rosenghini, and J.M. Cei, *Biochem. Pharmacol.*, **16**, 1149 (1967).
14) W.M. McIssac and I.H. Page, *J. Biol. Chem.*, **234**, 858 (1959).
15) F.M. Bumpus and I.H. Page, *J. Biol. Chem.*, **212**, 111 (1955).

Tube Nos. 120—130, 175—190, and 191—210 were pooled, lyophilized, and designated respectively as fractions XE-1, XE-II, and XE-III. Among these 3 fractions, activity of XE-I was lost by treatment with pronase and chymotrypsin, while its activity diminished partially with trypsin. Fraction XE-I was further purified by gel filtration through Sephadex G-25 in 0.1 M acetic acid (Fig. 2).

The active fractions were pooled and applied to a column of QAE-Sephadex A-25 in 0.001 M ammonia (Fig. 3).

The active material obtained from the QAE-Sephadex column was lyophilized and submitted to droplet countercurrent distribution, the inner volume of which was 240 ml, by using the solvent system of butanol: acetic acid: water (4: 1: 5). Fractions of 3 ml each were collected at 30-min intervals with an automatic fraction collector. The active material was recovered from the tube Nos. 90 to 120.

Finally, it was purified by a column of SE-Sephadex C-25 (0.8×40 cm) by elution with 0.05M ammonium formate, pH 6.5. Fractions of 3 ml each were collected, and bioassay showed that the activity appeared in tube Nos. 31—42. The purity of lyophilized substance of the active fractions obtained from the SE-Sephadex column was checked by DNS method. Its DNS-derivative gave a single band on TLC plate of silica gel H developed with the solvent systems of butanol: acetic acid: water (4: 1: 5) and methyl acetate: isopropanol: ammonia (9: 7: 4).

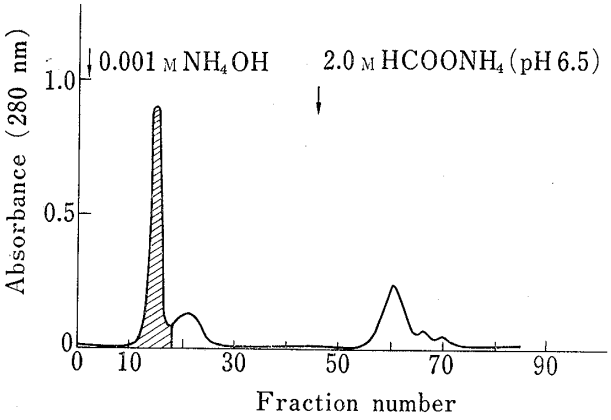

Fig. 3. Chromatography of Active Material obtained from Sephadex G-25 on QAE-Sephadex A-25

The column size was 0.9×50 cm and the fraction volume was 3 ml each. The activity was located in the shaded area.

Table I. Amino Acid Analysis of Xenopsin

Amino acid	Molar ratio
Lysine	0.95
Arginine	0.94
Glutamic acid	0.98
Proline	0.91
Glycine	1.18
Isoleucine	1.12
Leucine	0.94
Tryptophan	1.25[a]

a) UV absorbance at 280 nm

About 0.8 mg of pure peptide was isolated from the skin of 30 frogs. The name xenopsin was proposed for this peptide.

Amino Acid Composition

Amino acid composition in acid hydrolysate is shown in Table I. Tryptophan was determined by ultraviolet (UV) absorbance at 280 nm. The amino acid sequence was determined mainly by enzymic degradation of DNS-Xe. N-terminal amino acid was not detected by DNS-method but ε-DNS-lysine was detected at this procedure.

Chymotryptic Digestion

Incubation of xenopsin with chymotrypsin completely destroyed its activity. Xenopsin (2.5×10^{-8} mol) was dansylated as usual and DNS–Xe was separated from DNS–OH and DNS–NH_2 by TLC on silica gel H. DNS–Xe was dissolved in 1 ml of 0.1 M triethylamine-carbonate buffer, pH 8.0, and 5 μl of chymotrypsin solution (1 mg/ml in the same buffer) was added. The mixture was kept in a water bath at 26° for 6 hr. Then reaction mixture was lyophilized, and the residue was dansylated. TLC of the dansylated mixture revealed the presence of two DNS-derivatives of fragment, C-1 and C-2, different from DNS–Xe (Fig. 4).

C-1 and C-2 were separately extracted from the respective spots on TLC plate with the solvent mixture of acetone: water: pyridine: acetic acid (50: 50: 1: 3). N-terminal amino acid was not detected in C-1 but DNS-isoleucine was detected in C-2 by hydrolysis with 6N HCl for 16 hr at 100°. Therefore C-1 is the N-terminal residue of xenopsin and the amino acid composition of C-1 in acid hydrolysate was (Lys, Glu, Gly, Arg, Pro)-Trp (Table II).

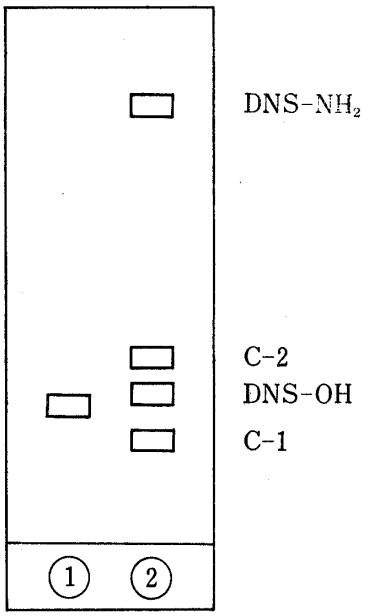

Fig. 4. TLC of DNS-Xe and Chymotryptic Fragments

1. DNS-Xe
2. dansylated products of chymotryptic hydrolysate of xenopsin solvent: BuOH: AcOH: H_2O=4:1:5

Table II. Amino Acid Analysis of Chymotryptic DNS-Fragment C-1

Amino acid	Molar ratio
Lysine	0.26[a]
Arginine	0.89
Glutamic acid	1.06
Proline	1.00
Glycine	1.02

a) Molar ratio is low because of dansylation of ε-NH_2 residue of lysine.

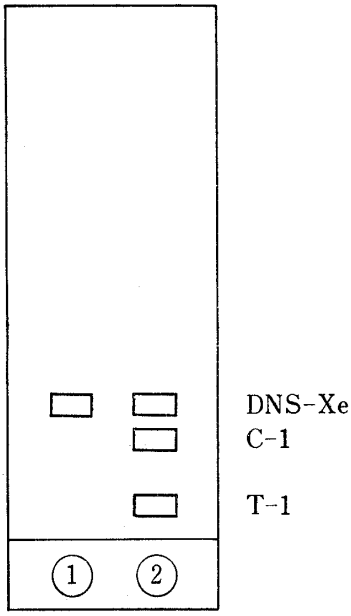

Fig. 5. TLC of Tryptic Fragments

1. DNS-Xe
2. tryptic hydrolysate of DNS-Xe solvent: BuOH: AcOH: H_2O =4:1:5

The C-terminal position of tryptophan in fragment C-1 was deduced from the specificity of the enzyme. To 5×10^{-8} mol of C-1 dissolved in 1 ml of 0.1 M triethylamine-carbonate (pH 7.6), 5 μl of carboxypeptidase A solution (50 μg/ml in the same buffer) was added, and the mixture was digested at room temperature. Aliquots of 200 μl were taken at 10 min, 5 hr, and 19 hr after the start of the experiment. To 200 μl of the residue from the reaction mixture 5 μl of carboxypeptidase B (50 μg/ml in the same buffer) was added and the mixture was digested in a water bath at 26° for 5 hr.

The each aliquot was immediately lyophilized and the residues were dissolved in 1 ml of 0.001 N HCl and the solution was applied to the amino acid analyzer. Only tryptophan was always detected as released amino acid by treatment with carboxypeptidase A and B. Therefore the sequence of prolyltryptophan was assumed.

To determine the C-terminal amino acid of xenopsin, 2×10^{-8} mol of xenopsin was dissolved in 500 μl of 0.1 M triethylamine-carbonate (pH 7.6) and 5 μl of carboxypeptidase A (50 μg/ml in same buffer) was added. The mixture was digested at room temperature for 10 min and was applied to the amino acid analyser in the same manner as above. Leucine (1.4×10^{-9} mole) and isoleucine (trace) were detected as released amino acids.

Therefore, C-2 was deduced to be –Ile–Leu–OH and the C-terminal sequence of xenopsin was suggested to be –Pro–Trp–Ile–Leu–OH.

Tryptic Digestion of DNS-Xe

DNS-Xe was more resistant to digestion with trypsin than native peptide and DNS-Xe was not digested by trypsin in the same condition as described in chymotrypsin. To 1×10^{-7} mol of DNS-Xe dissolved in 1 ml of 0.1 M triethylamine-carbonate (pH 8.0), 50 μl of trypsin

solution (1 mg/ml in the same buffer) was added and the mixture was digested for 15 hr at room temperature. The reaction mixture was then lyophilized.

In addition to original DNS-Xe, two DNS-fragments were detected by TLC (Fig. 5). One of them was identical to C-1 and the other was a new fragment T-1.

N-terminal amino acid was not detected in T-1 and the amino acid composition of T-1 in acid hydrolysate showed (Lys, Glu, Gly)-Arg. The C-terminal position of arginine in fragment T-1 was deduced from the specificity of the enzyme. From the results of chymotrypsin and trypsin digestion, partial amino acid sequence of xenopsin was deduced to be (Lys, Glu, Gly)-Arg-Pro-Trp-Ile-Leu-OH. This suggested the tryptic fission of arginylproline bond and tryptophanylisoleucine bond. The possibility of arginylproline bond was also supported by following Birch reduction.

Birch Reduction

Reductive cleavage of N-proline-peptide was made with sodium in liquid ammonia, in the presence of a proton donor.[16,17] Xenopsin (2×10^{-8} mol) was dissolved in 2 ml of liquid ammonia containing 2 μl of methanol at 50°, and a small piece of sodium was added to the solution, and the reaction mixture was allowed to stand at room temperature. After the reaction was completed, the residue neutralized with dilute acetic acid was hydrolyzed with 6 N HCl for 24 hr and analyzed amino acid composition (Table III).

Arginine was reduced about one-fourth of the other constituent amino acid. This suggested that the peptide had a sequence of arginylproline bond.

Table III. Amino Acid Analysis of Xenopsin after Birch Reduction

Amino acid	Molar ratio
Lysine	0.96
Arginine	0.23
Glutamic acid	1.11
Proline	0.74
Glycine	1.07
Isoleucine	1.06
Leucine	1.06

Table IV. Radioactivities of Constituent Amino Acids of P-1 after Tritium Labeling Reaction

Amino acid	Radioactivity (count/min)
Lysine	529
Glycine	1156
Glutamic acid	1316
ε-DNS-lysine	9882

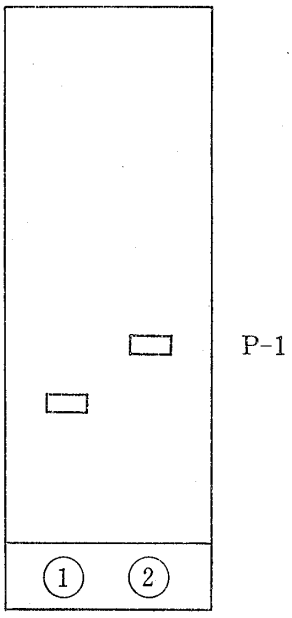

Fig. 6. TLC of Papainic Fragment
1. DNS-Xe
2. papainic hydrolysate of DNS-Xe
solvent: BuOH: AcOH: H_2O=4:1:5

Papainic Digestion of DNS-Xe

DNS-Xe was digested with papain in the same way as described for chymotrypsin and produced a new DNS-derivative of fragment P-1 as detected by TLC (Fig. 6). Amino acid composition of P-1 in acid hydrolysate was (Lys, Glu, Gly), which was a N-terminal fragment.

16) A.J. Birch, J. Cymerman-Craig, and M. Slaytor, *Aust. J. Chem.*, **8**, 512 (1955).
17) M. Wilchech, S. Sarid, and A. Patchornik, *Biochim. Biophys. Acta*, **104**, 616 (1965).

To determine the C-terminal amino acid of P-1, the selective tritium labeling reaction was performed by the method of Matsuo, *et al.*[18] P-1, isolated from TLC plate of silica gel H, equivalent to 4.1 μg of ε-DNS-lysine was dissolved in a mixture of redistilled pyridine (10 μl) and ^3H_2O (5 μl) with specific radioactivity of 5 Ci/ml. To the solution was added 10 μl of redistilled acetic anhydride and the mixture was kept in an ice bath for 5 min and further for 15 min at room temperature. Again the mixture was cooled in an ice bath and 20 μl each of pyridine and acetic anhydride were added, and the mixture was allowed to stand for 5 min at 0° and then for 1 hr at room temperature. After 5 μl of ^3H_2O was added, it was kept for 1 hr at room temperature for decomposition of unreacted acetic anhydride. After evaporation of the mixture *in vacuo* and complete removal of the washable isotope as usual, the residue was hydrolyzed with 100 μl of 6N HCl at 105° for 17 hr. The hydrolysate was chromatographed on a plate coated with cellulose powder to separate the constituent amino acids (lysine, glutamic acid, glycine and ε-DNS-lysine) using the solvent system[19] of isopropanol: formic acid: water (40: 2: 10).

On a half of line of origin, the standard cold amino acid mixture of its constituents was superimposed and then chromatogram were run at room temperature for 4 hr. ε-DNS-lysine was detected by UV lamp (365 nm) and the position of other amino acids was detected by spraying the Ninhydrin reagent, and radioactivity of the corresponding amino acid and ε-DNS-lysine was measured by a liquid scintillation counter (Beckman LS-230) (Table IV).

The radioactivity of the constituent amino acid showed that ε-DNS-lysine was the C-terminal amino acid of P-1.

N-terminal Amino Acid

From the result of tritium labeling reaction, the N-terminal amino acid of xenopsin was either N-terminal blocked glutamic acid or glycine. Xenopsin (5×10^{-9} mol) was treated with 5 μl of 1 N NaOH for 24 hr at 26°, the reaction mixture was neutralized with 50 μl of 0.1 N acetic acid, and evaporated *in vacuo*. The residue was dansylated and hydrolyzed with 50 μl of 6 N HCl for 16 hr at 100°. In addition to ε-DNS-lysine, DNS-glutamic acid was detected by TLC on silica gel H. The control acetylglutamic acid was not hydrolyzed and control pyroglutamic acid was completely hydrolyzed with 1 N NaOH.

This suggested the N-terminal amino acid to be pyroglutamic acid (pyrGlu). The amino acid sequence of P-1 was deduced to be pyrGlu-Gly-Lys. From the results of enzymic digestion of DNS-Xe and other experiments, the complete sequence of xenopsin is shown in Fig. 7.

This proposed structure of xenopsin was confirmed by the synthesis but its details will be represent elsewhere.

Biological Activity of Xenopsin

Xenopsin had a very strong contractile activity on rat stomach strip *in vitro* and it was proportional to the doses. Average dose response curves of xenopsin, serotonin, and acetylcholine are presented in Fig. 8. In low doses, xenopsin produced the strongest response but a maximum contractile response was about one-half of that of acetylcholine.

This peptide also had a moderate contractile activity on quinea-pig ileum but the response was not reproducible. Intravenous injection of the peptide lowered the arterial blood pressure of the anesthetized rat but a prompt and intense tachyphylaxis was observed.

Activity of Fragments and Analogs of Xenopsin

Some fragments and analogs of xenopsin were synthesized and their contractile activity on rat stomach strip was assayed (Table V).

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

19) G.N. Holcoml, S.A. James, and D.N. Ward, *Biochemistry*, **7**, 1291 (1968).

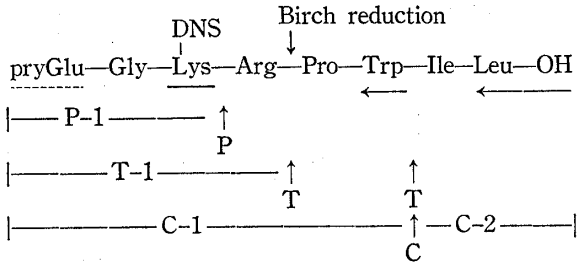

Fig. 7. Summary of Approaches used in Establishing the Amino Acid Sequence of Xenopsin

P: papain, T: trypsin, C: chymotrypsin, ← carboxypeptidase A, ------ alkali-treatment, —— tritium labeling reaction

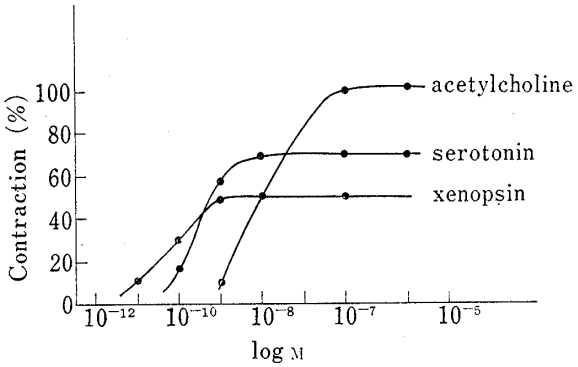

Fig. 8. log Dose Response Curves of Xenopsin, Serotonin and Acetylcholine on Rat Stomach Strip

Each point represents the average of three estimates.

Lys^2–Gly^3–xenopsin had 70% activity of xenopsin and Lys^2–Pro^3–Gly^5–xenopsin had a very weak activity. The activity was also reduced to one-fifth of the original activity by modification of C-terminal leucine to leucine amide. The essential loss of activity by removal of C-terminal amino acid suggested that leucine seems to be extremely important in this peptide.

C-terminal tetrapeptide, Pro–Trp–Ile–Leu–OH, had no activity but pentapeptide, Arg–Pro–Trp–Ile–Leu–OH, had a weak activity, suggesting that this pentapeptide was the essential amino acid sequence which displayed the activity of xenopsin.

Table V. Contractile Activities of Xenopsin Analogs on Rat Stomach Strip

Compound	Activity
Xenopsin: pyrGlu–Gly–Lys–Arg–Pro–Trp–Ile–Leu–OH	100
H–Pro–Trp–Ile–Leu–OH	0
H–Arg–Pro–Trp–Ile–Leu–OH	0.5
pyrGlu–Gly–Lys–Arg–Pro–Trp–Ile–OH	0.002
pyrGlu–Lys–Gly–Arg–Pro–Trp–Ile–Leu–OH	70
pyrGlu–Lys–Pro–Arg–Gly–Trp–Ile–Leu–OH	0.02
pyrGlu–Gly–Lys–Arg–Pro–Trp–Ile–Leu–NH_2	20
pyrGlu–Gly–Lys–Arg–Pro–Trp–Ile–NH_2	0

Fraction XE-II and XE-III

For further purification, XE-II and XE-III were passed through a column of alkaline alumina which was eluted with a gradient of 99% ethanol to 50% ethanol. Figs. 9 and 10 shows the respective alkaline alumina chromatographic pattern of fractions XE-II and XE-III.

Bioassay showed that the contractile activity on rat stomach strip appeared in about 80% ethanol eluate from the alkaline alumina column loaded with fraction XE-II, and that it appeared in about 90% and 70% ethanol eluates in the case of XE-III (respectively designated as fraction XE-II-1, XE-III-1, and XE-III-2).

Ultraviolet absorption spectra of three active fractions showed similar to that of serotonin. Aliquots of these active eluates were concentrated under reduced pressure and then chromatographed on Whatman No. 1 paper using five kinds of solvent systems. Indole spots were detected by NNCD reagent and Ehrlich reagent on the chromatogram. Paper chromatograms revealed that fraction XE-II-1 had the same positive indole spot with the same *Rf* value as N-methylserotonin, and fractions XE-III-1 and XE-III-2 had *Rf* values identical with that of bufotenidine and serotonin, respectively (Table VI).[13–15,20,21]

20) G. Falconieri, V. Erspamer, and J.M. Cei, *Biochem. Pharmacol.*, **19**, 321 (1970).
21) V. Erspamer, M. Rosenghini, and J.M. Cei, *Biochem. Pharmacol.*, **13**, 1083 (1964).

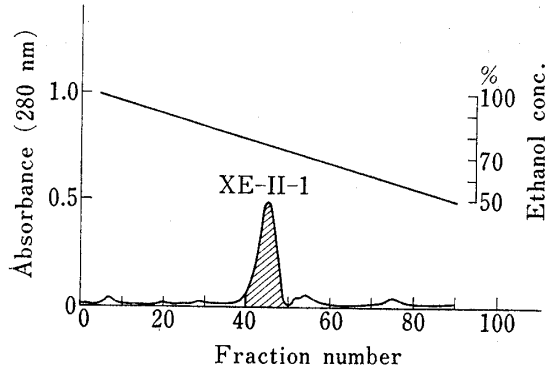

Fig. 9. Chromatography of Fraction XE-II on Alkaline Alumina Column

The column (1×70 cm) was eluted with a linear gradient elution of 99% EtOH—50% EtOH (300—300 ml). The fraction volume was 7 ml each. Contractile activity on rat stomach strip was located in the shaded area.

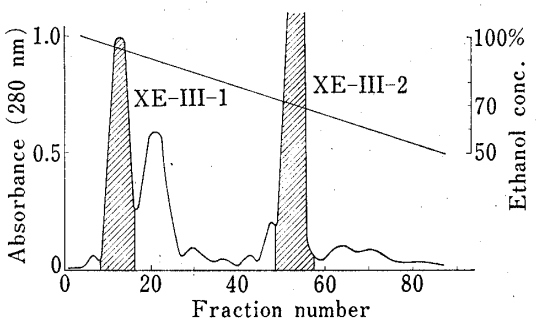

Fig. 10. Chromatography of Fraction XE-III on Alkaline Alumina Column

The column (1×70 cm) was eluted with a linear gradient elution of 99% EtOH—50% EtOH (300—300 ml). The fraction volume was 7 ml each. Contractile activities on rat stomach strip were located in the shaded areas.

TABLE VI. *Rf* Values of Indole Derivatives Occuring in the Skin of *Xenopus lacvis* and of Pure Natural or Synthetic Indole Compounds

	A	B	C	D	E
XE-II-1	0.48	0.83	0.39	—	—
N-methylserotonin	0.48	0.82	0.38	—	—
XE-III-1	0.47	—	—	0.17	0.14
Buffotenidine	0.49	—	—	0.17	0.15
XE-III-2	0.42	0.68	0.34	—	—
Serotonin	0.43	0.66	0.34	—	—

The ascending unidimensional technique on Whatman No. 1 paper was employed.
solvent systems: A, BuOH: AcOH: H_2O (4: 1: 5), B, BuOH: 30%$MeNH_2$ (8: 3), C, 20% KCl in H_2O, D, BuOH: 14% NH_4OH (2: 1), E, MeCOEt: 2N NH_4OH (2: 1).

Discussion

Anastasi, *et al.*[22] have reported the presence of caerulein, serotonin and buffotenidine in the skin of *Xenopus laevis*.

In Fig. 1, showing the SP-Sephadex chromatography of methanol extracts of the skin of *Xenopus laevis*, the fraction of tubes 20—30 had a stimulating action on the contraction of guinea-pig gall bladder, on rat pancreatic secretion, and on rat gastric secretion, which suggested the presence of caerulein in this fraction. As biogenic amines in the skin of *Xenopus laevis*, N-methylserotonin was identified in addition to serotonin and buffotenidine.

The new active peptide, xenopsin, had a very strong contractile activity on rat stomach strip *in vitro* (Fig. 8) and the response was not suppressed by atropine, diphenhydramine, or dibenzyline which are the inhibitor, respectively, of acetylcholine, histamine, and serotonine. It was suggested that the mode of action of xenopsin was direct stimulation on smooth muscle at a receptor site different from acetylcholine and serotonin.

Pyroglutamyl residue, the N-terminal amino acid of xenopsin, was determined by the hydrolysis of pyrrolidone ring to glutamic residue by treatment with 1 N NaOH. It was suggested that the alkaline hydrolysis of pyrolidone ring was effectively used as the method for the determination of the N-terminal pyroglutamyl peptide.[23]

22) A. Anastasi, G. Bertaccini, J.M. Cei, G. De Caro, V. Erspamer, M. Impicciatore, and M. Rosenghini, *Brit. J. Pharmacol.*, **38**, 221 (1970).
23) A. Anastasi, J. Erspamer, and R. Endean, *Arch. Biochem. Biophys.*, **125**, 57 (1968).

We also investigated the ring opening condition using some model compounds. Pyroglutamic acid was completely hydrolyzed to glutamic acid with 1 N NaOH for 24 hr at room temperature, while acetylglutamic acid was not cleaved to glutamic acid. In some model peptides, this alkaline treatment sometimes produced hydrolysis of peptide bond in addition to the ring opening.

For example, in the case of TRH (pyrGlu–His–Pro–NH_2) and caerulein (pyrGlu–Gln–Asp–Tyr (SO_3H)–Thr–Gly–Trp–Met–Asp–Phe–NH_2), in addition to DNS-glutamic acid, DNS-histidine and DNS-glycine were respectively detected by treatment with 1 N NaOH at room temperature for 24 hr, dansylation and the following acid hydrolysis.

Therefore, alkaline ring opening for the determination of pyroglutamyl residue as N-terminal amino acid is said to be entirely definite method.

Digestion of xenopsin with trypsin did not completely destroy its activity. It was assumed that trypsin probably attacks the site of lysylarginine bond of xenopsin and produced a still biologically active pentapeptide, Arg–Pro–Trp–Ile–Leu–OH, which was the essential amino acid residue for the activity. Tryptic digestion of DNS-Xe was more resistant than other enzymic digestion, because of dansylation of ε–NH_2 residue of lysine. By addition of excess trypsin, about one-third of DNS-Xe was hydrolyzed at the site of arginylproline bond from the result of the amino acid composition of tryptic fragment T-1. The cleavage of DNS-Xe by trypsin at the site of arginylproline bond was also supported by the result of Birch reduction. It seemed likely that tryptophanyl isoleucine bond was cleavaged by excessive trypsin.

In 1973 Carraway and Leeman,[24] on the way of the purification of substance P from bovine hypothalamic extracts, found the presence of a new hypotensive peptide, neurotensin, and reported the amino acid sequence of this peptide as pyrGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Tyr–Ile–Leu–OH.[25] Neurotensin induces hypotension in the rat and stimulates the contraction of guinea-pig ileum and rat uterus.

However, it produces relaxation of the rat duodenum. The most interesting fact which has been emerged from the present study is that there is a chemical resemblance existing between xenopsin and neurotensin at the C-terminal pentapeptide, which is the essential fragment for the activity of xenopsin. In biological effects, the two polypeptides have the contractile activity on guinea-pig ileum and the hypotensive activity on a rat with acute tachyphylaxis.

It is very interesting to study comparatively the biological and the pharmacological effects of the two peptides.

24) R. Carraway and S.E. Leeman, *J. Biol. Chem.*, **248**, 6854 (1973).
25) R. Carraway and S.E. Leeman, *Fed. Proc.*, 33, 548 (1974).