Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto
HARUAKI YAJIMA
KOUKI KITAGAWA

Institute of Pharmaceutical Sciences, School of Medicine, Hiroshima University Kasumi, Hiroshima
TOMIO SEGAWA
MASUMI NAKANO

School of Medicine, Ehime University Matsuyama, Ehime
KIYOSHI KATAOKA

Received September 18, 1975

[Chem. Pharm. Bull. 23(12)3301—3303(1975)]

UDC 547.965.05 : 591.141 : 597.6

Occurrence of Pyr-His-Pro-NH_2 in the Frog Skin

The tripeptide obtained from the skin of *Bombina orientalis* BOULENGER was coincided to mammalian thyrotropin releasing hormone by chemical characterization of the peptide. The amount of the peptide in the skin was much higher in cocentration than in mammalian hypothalamus.

The tripeptide, Pyr–His–Pro–NH_2, has been recognized as the thyrotropin releasing hormone (TRH) in various mammals.[1] Occurrence of this peptide in the brain of many poikilotherms has also been demonstrated.[2] However, administration of synthetic TRH to these animals does not activate thyroid gland function.[3] Recently many kinds of gastropod which do not produce thyroid hormones, have been observed to contain the immunoreactive TRH in the circumesophageal ganglia.[4] Thus it has been proposed that in these animals TRH modulates synaptic transmission rather than releasing thyrotropin. Further supports for a role of TRH in synaptic transmission are the finding that administration of the synthetic TRH leads to an increase in noradrenaline turnover in rat brain,[5] and that hypothermia produced in individual cats by the intravascular injection of TRH is mediated by the release of noradrenaline in the brain.[6]

We have found the occurrence of a lot of amounts of this peptide in the skin of korean frog, *Bombina orientalis* BOULENGER, during the separation of vasoactive peptides in the skin. A role of this peptide in the amphibian skin is not yet clear, but this finding may imply the additional support of another function of TRH.

This report deals with the isolation and chemical characterization of this peptide in the skin of *Bombina orientalis*.

Isolation of TRH in the Skin

The fresh skin of 13 frogs was dripped with 15 ml of 1% trichloroacetic acid and extracted by adding 150 ml of methanol. The skin was extracted further with adding 100 ml of methanol twice. The extracts were combined and evaporated under a reduced pressure. The dilute

1) A.V. Schally, A. Arimura, C.Y. Bowers, I. Wakabayashi, A.J. Kastin, T.W. Redding, J.C. Mittler, R.M.G. Nair, P. Pizzolato, and J. Segal, *J. Clin. Endocrinol.*, **31**, 291 (1970).
2) I.M.D. Jackson and S. Reichlin, *Endocrinol.*, **95**, 816 (1974); C. Oliver, R.L. Eskay, N.Ben-Jonathan, and J.C. Porter, *ibid.*, **95**, 540 (1974).
3) A.G. Gona and O. Gona, *Gen. Comp. Endocrinol.*, **24**, 223 (1974).
4) Y. Grimm-Jørgensen, J.F. MacKelvy, and I.M.D. Jackson, *Nature*, **254**, 620 (1975).
5) H.H. Keller, G. Bartholini, and A. Pletshner, *Nature*, **248**, 528 (1974).
6) G. Metcalf, *Nature*, **252**, 310 (1974).

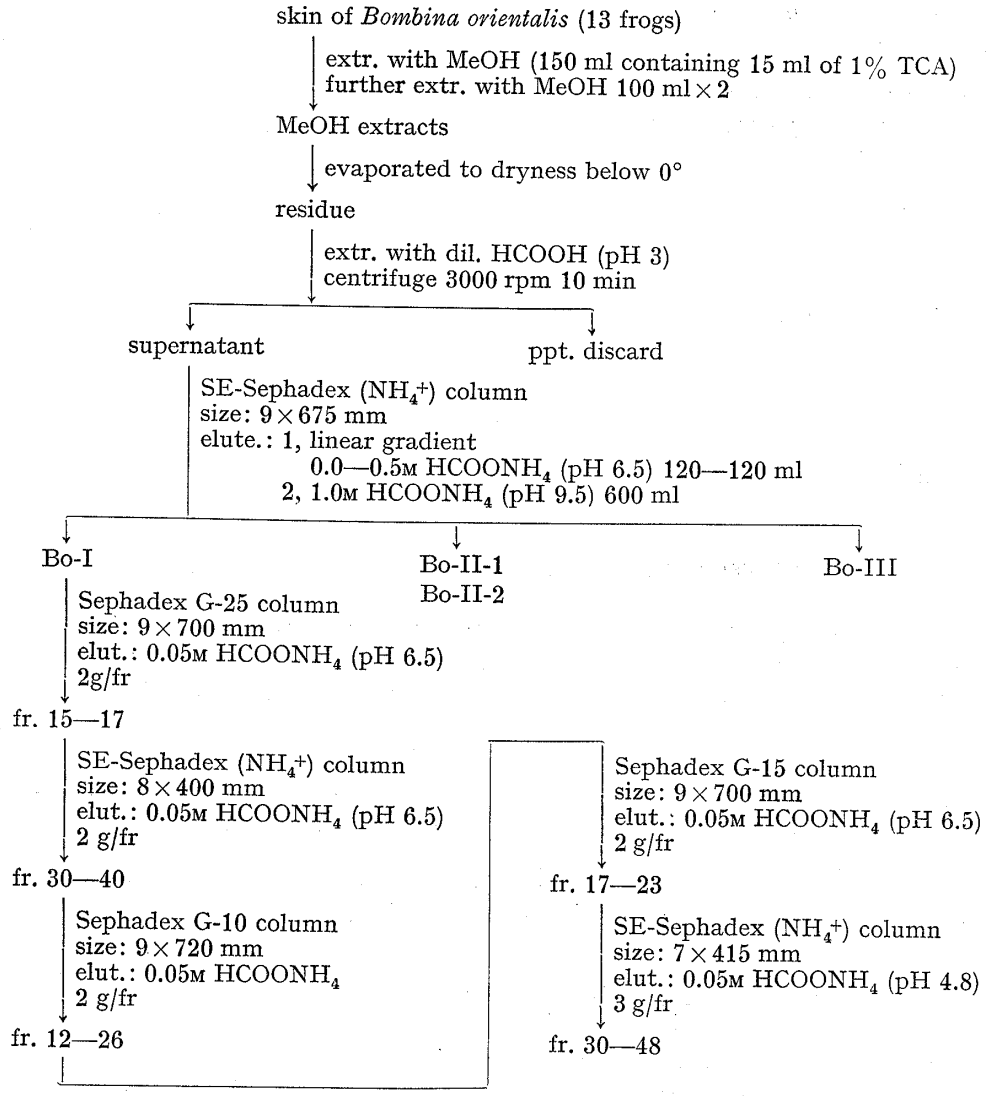

Chart 1. Separation of Pyr–His–Pro–NH_2 in the Frog Skin

formic acid solution (pH 3) of 10 ml was added to the residue and the residue was stirred and centrifuged for 10 min with 3000 rpm. The supernatant was adsorbed and chromatographed on a SE-Sephadex column, as we described previously on the separation of smooth muscle contractile materials in this frog.[7] The separatory process was summarized in Chart 1. As shown in the chart, Bo–II–1, Bo–II–2 and Bo–III contained bombesin, bradykinin and bradykinyl–Gly–Lys–Phe–His respectively, and the fraction Bo–I, weekly adsorbed on the column, was further separated by column chromatography with Sephadex G-25, SE-Sephadex, Sephdex G-10 and G-15. Finally the fraction was separated by SE-Sephadex column chromatography using 0.05M ammonium formate (pH 4.8) as the eluent. Fractions 30 to 48 showed the contractile activity on rat uterus. After an aliquot of every three fraction was hydrolysed with 6N hydrochloric acid at 110° for 24 hr, amino acids in each fraction were assayed by an amino acid analyser (JEOL 5AH). Amino acid composition was varied in each fraction, except in fractions 40 and 44, in which equimolar amount of histidine, glutamic acid and proline were identified. Other amino acids in these fractions were aspartic acid, glycine and leucine. The amount of these amino acids were less than 10 to 20% comparing with those of histidine, glutamic acid and proline. This indicates that the rat uterus contractile

7) 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).

material in the fractions 30 to 48 is not yet purified but some kind of tripeptide is contaminated in the fractions 40 to 44.

Characterization of the Peptide in the Fraction 44

The peptide did not react with dansyl chloride by our procedure.[8] Birch reduction[9] was carried out to split the N-peptide bond of prolyl sequence of the peptide. After the reduction, the reaction mixture was dansylated, and dansyl proline and dansyl proline amide were identified on a thin-layer chromatography of Silica gel H by overlapping with the authentic samples. On the oxidation of N-bromosuccinimide according to the method of Shaltiel, *et al.*,[10] which cleaves the C-peptide bond of histidyl residue, similar results were obtained, *i.e.* dansyl proline and dansyl proline amide were identified after dansylation of the reaction mixture. Both in the Birch reduction and N-bromosuccinimide oxidation, histidine was disappeared from the hydrolysate of these reaction mixtures. The same results were obtained by these procedures for synthetic TRH.

The peptide and synthetic TRH were chromatographed together on a thin-layer of Silica gel H with the solvent systems for TRH.[11] Both peptides moved as the single spot and showed the same *Rf* value as stained by Pauly reagent.

These results indicate that the peptide in the fraction 44 is TRH itself. Although the smooth muscle contractile principle in the fractions 30 to 48 has not been identified, we find unexpectedly TRH in these fractions. The amount of this peptide containing in one frog skin (*ca.* 0.6 g wet weight) was approximately 23 μg calculating from the results of amino acid analysis. This amount seems to be much higher in concentration in mammalian hypothalamus.[12] Sequential similarities or resemblance between mammalian neuropeptides and amphibian cutaneous peptides, such as substance P and physalaemin, neurotensin and xenopsin, this case, *etc.*, raises an interest of comparative studies on a role of biological active peptides.

Acknowledgement We are deeply grateful to Dr. N. Yanaihara, Shizuoka College of Pharmacy for generous gift of synthetic TRH, and Dr. S. Sakakibara, Protein Research Foundation for gift of proline amide. We also acknowledge the support of our research by Grant-in-Aid for Scientific Research from the Ministry of Education.

Institute of Pharmaceutical Sciences,
School of Medicine, Hiroshima University
Kasumi, Hiroshima

Tadashi Yasuhara
Terumi Nakajima

Received September 25, 1975

8) Z. Tamura, T. Nakajima, T. Nakayama, J.J. Pisano, and S. Udenfriend, *Anal. Biochem.*, **52**, 595 (1973).
9) A.J. Birch, J. Caymerman-Craig, and M. Slaytor, *Austr. J. Chem.*, **8**, 512 (1955).
10) S. Shaltiel and A. Patchornik, *J. Am. Chem. Soc.*, **85**, 2799 (1963).
11) R. Burgus, T.F. Dunn, D. Desiderio, D.N. Ward, W. Vale, and R. Guillemin, *Nature*, **226**, 321 (1970).
12) A.V. Schally, T.W. Redding, C.Y. Bowers, and J.F. Barrett, *J. Biol. Chem.*, **244**, 4077 (1969).