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Isolation and Structure of a New Active Peptide "Xenopsin" on the Smooth Muscle, especially on a Strip of Fundus from a Rat Stomach, from the Skin of *Xenopus laevis*

We have examined some biological active peptides in the skin of *Xenopus laevis* and obtained a new active peptide possesing more potent contractile activity than serotonin and acetylcholine on rat stomach strip *in vitro*. The contractile responses were proportional to the peptide doses (threshold dose; 0.05—0.1 ng/ml). This had also a moderate contractile activity on guinea pig ileum but the response was not reproducible (threshold dose; 10—50 ng/ml). Intravenous injection of the peptide lowered the arterial blood pressure of the anesthetized rat but a prompt and intense tachyphylaxis was observed. The name "Xenopsin" is proposed. We report here on the separation and chemical characterization of Xenopsin.

Separation of Active Peptide

Fresh skins of 30 frogs were extracted with 1000 ml of methanol containing 10 ml of 6% trichloroacetic acid and were further extracted with 500 ml of 80% aqueous methanol.

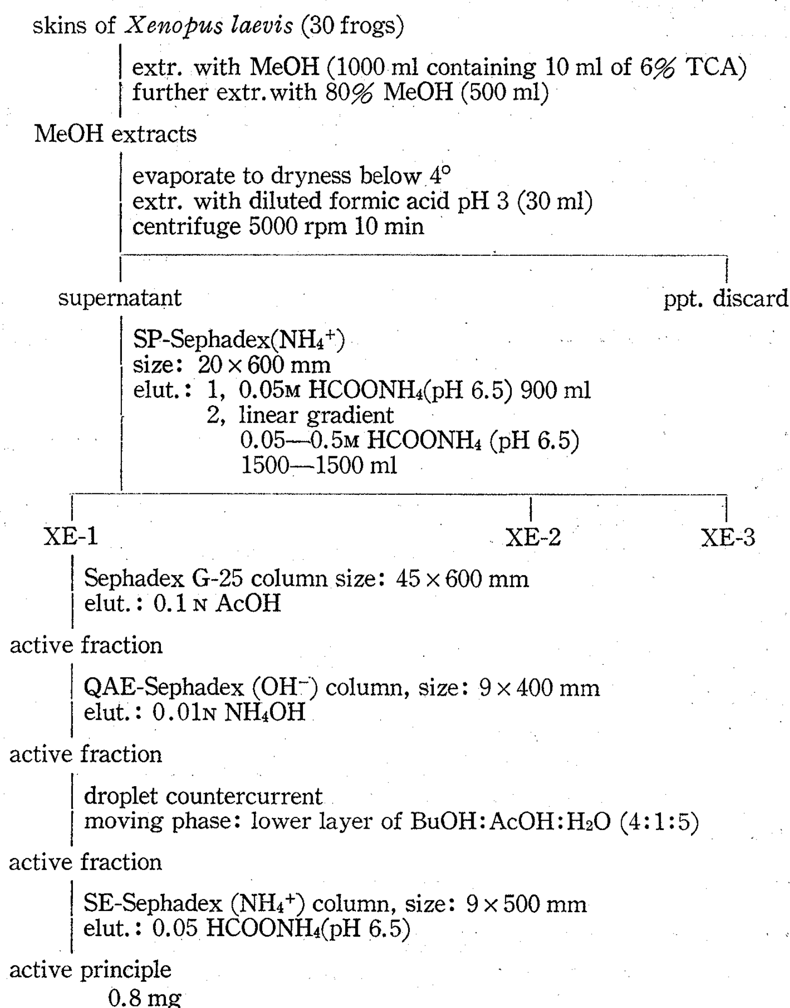

Chart 1. Separation and Purification of Xenopsin

1) J.R. Vane, *Brit. J. Pharmacol.*, **12**, 344 (1957).

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The first and second extracts were combined and undissolved materials were filtered off. The filtrate was evaporated under reduced pressure. The residue was dissolved in 30 ml of diluted formic acid, pH 3, and centrifuged. The supernatant was used for further purification. Separatory process of Xenopsin was summarized in Chart 1. Activity in each fraction was assayed by contraction of rat stomach strip *in vitro*.[1] Contractile activity on stomach strip was found in three fractions (XE-1, XE-2 and XE-3) in the first SP-Sephadex chromatography. Except XE-2 and XE-3, activity of XE-1 was lost by treatment with chymotrypsin, trypsin and pronase. XE-1 was applied for further purification to a column of Sephadex G-25, QAE-Sephadex A-25 and droplet countercurrent distribution. Finally it was purified by a column of SE-Sephadex C-25. Lyophilized active fractions from the eluate of SE-Sephadex showed a single DNS (dimethylaminonaphthalenesulfonyl)derivative with thin–layer chromatography on Silica gel H.

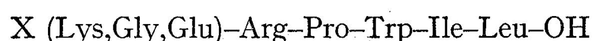
Characterization of the Peptide in XE-1

Xenopsin was lost its activity by treatment with chymotrypsin, carboxypeptidase A and papain but it was rather survived by treatment with trypsin. Amino acid composition in the acid hydrolysate determined by an amino acid analyser was as follows: Lys(1), Arg(1), Gly(1), Glu(1), Pro(1), Ile(1), Leu(1), Trp(1) (determined by ultraviolet (UV) absorbance at 280 nm). The amino acid sequence was determined mainly by the enzymatic degradation of its DNS-derivative. N-Terminal amino acids were not detected by DNS-method.

Digestion with Chymotrypsin——Digested with chymotrypsin and then dansylated, DNS-Xenopsin (DNS-Xe) yielded two DNS-derivatives of the fragments, C-1 and C-2, on thin–layer chromatography. N-Terminal amino acid of C-1 was not detected, so C-1 was N-terminal peptide and amino acid composition of C-1 in acid hydrolysate was as follows: (Lys, Glu, Gly, Arg, Pro) Trp. When C-1 was treated with carboxypeptidase A and following with carboxypeptidase B, only tryptophan was detected as released amino acid by an amino acid analyser. So the sequence of Pro–Trp was assumed. DNS-isoleucine was detected as the N-terminal amino acid of C-2. To determine the C-terminal amino acid, Xenopsin was treated with carboxypeptidase A for 10 min at 26° and the reaction mixture was directly applied to an amino acid analyser. Leucine and a little isoleucine were detected as released amino acids. Regarded to the compositive amino acids of C-2, C-terminal sequences of the peptide was suggested to be –Pro–Trp–Ile–Leu–OH.

Digestion with Trypsin——Digestion of DNS–Xe with trypsin was more resistant than the native peptide. When large amount of enzyme was added to the solution of DNS–Xe, in addition to the original DNS–Xe, two DNS-fragments were detected by thin–layer chromatography. One of them was identical to C-1 and the other was the new fragment T-1. N-terminal amino acid of T-1 was not detected and the amino acid composition of T-1 was (Lys, Gly, Glu) Arg.[2]

Birch Reduction[3]——When Xenopsin was reduced by sodium in liquid ammonia with methanol as proton donor (Birch reduction) and analysed amino acid composition after acid hydrolysis, arginine was reduced about one third of the each other compositive amino acid. This fact suggested that the peptide had a sequence of Arg–Pro. From the results of Birch reduction and the digestions with chymotrypsin and trypsin, we deduced the sequence of the peptide as follows:


X (Lys,Gly,Glu)–Arg–Pro–Trp–Ile–Leu–OH

Digestion with Papain——When DNS–Xe was treated with papain, DNS-derivative of the fragment (P-1) was isolated by thin–layer chromatography. Its amino acid composition

2) The unusual bond fission of Arg-Pro took place by trypsin, which phenomenon was reported in the studies on Viscotoxin (G. Samuelsson, L. Seger, and T. Olson, *Acta Chem. Scand.*, **22**, 2624 (1968).
3) A.J. Birch, J. Cymerman-Craig and M. Slaytor, *Aust. J. Chem.*, **8**, 512 (1955).

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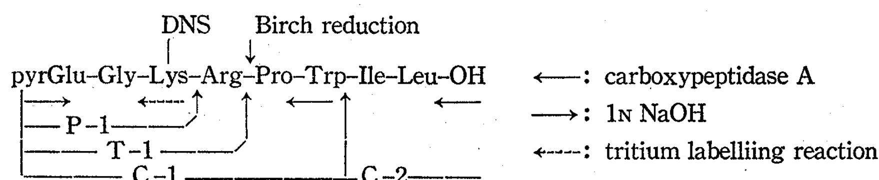
in acid hydrolysate was (Lys, Gly, Glu) which was N-terminal peptide. ε-DNS–Lys was detected as C-terminal amino acid of P-1 by the method of tritium labelling reaction through the formation of C-terminal peptide oxazolone.[4]

To determine the N-terminal amino acid, Xenopsin was treated with 1N sodium hydroxide at 27° for 24 hr, then dansylated and hydrolysed with 6N hydrochloric acid, in addition to ε-DNS-lysine, DNS-glutamic acid was detected by thin-layer chromatography. This suggested the N-terminal amino acid to be pyroglutamic acid (pyrGlu). The amino acid sequence of P-1 was pyrGlu–Gly–Lys.

The sequential analysis of Xenopsin was deduced as follows:



Synthesis of the New Peptide, Xenopsin[5]

The synthesis of above the new octapeptide was performed by the conventional chain elongation procedure as shown in Chart 2.

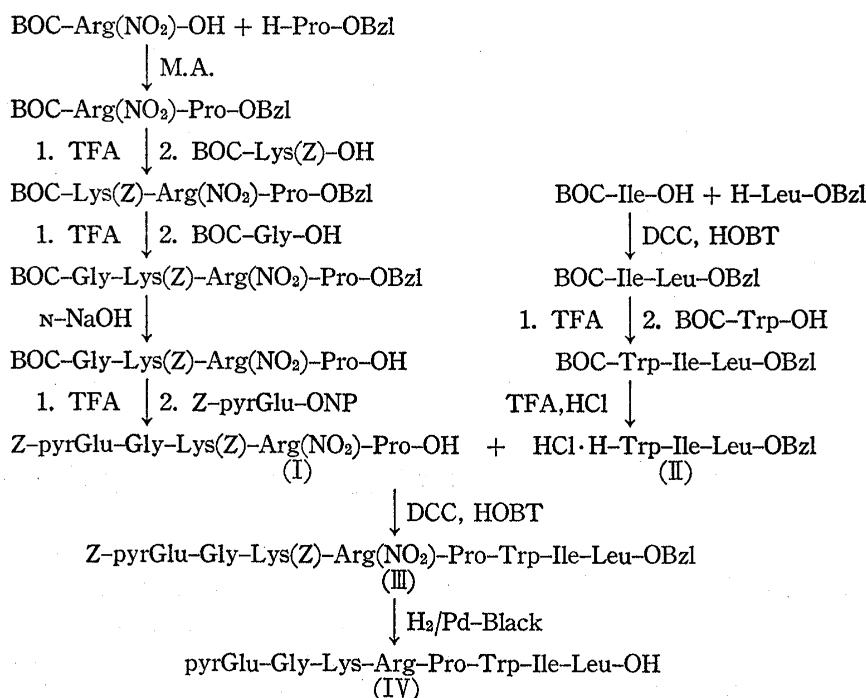


Chart 2. Synthetic Route of Xenopsin

For the final coupling reaction above, the protected N-terminal pentapeptide Z–pyrGlu–Gly–Lys(Z)–Arg(NO_2)–Pro–OH[I] [*Anal.* Calcd. for $C_{40}H_{52}O_{13}N_{10} \cdot H_2O$: C, 53.43; H, 6.06; N, 15.58. Found: C, 53.65; H, 5.99; N, 15.36. mp 134—135° (decomp.); $[\alpha]_D^{26}$ −54° (c=0.5,

4) S. Matsuo, Y. Baba, R.M.G. Nair, A. Arimura, and A.V. Schally, *Biochem. Biophys. Res. Commun.*, **43**, 1334 (1971).
5) The peptides and peptide derivatives mentioned in this communication are of the L-configuration. Abbreviations: Z=benzyloxycarbonyl; BOC=*tert*-butyloxycarbonyl; M.A.=mixed anhydride method; OBzl=benzyl ester; TFA=trifluoroacetic acid; DCC=dicyclohexylcarbodiimide; HOBT=N-hydroxybenztriazole; DMF=dimethylformamide; ONP=*p*-nitrophenyl ester.
6) Designations of solvent systems for thin-layer chromatography on Silica gel G (Merck) are: Rf^1 *n*-BuOH–AcOH–H_2O (4:1:5) upperlayer, Rf^{11} *n*-BuOH–pyridine–AcOH–H_2O (30:20:6:24).
7) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).

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MeOH); Rf^1 0.49, Rf^{11} 0.72[6] was condensed by the DCC plus HOBT procedure[7] in DMF with the C-terminal tripeptide ester hydrochloride HCl·H–Trp–Ile–Leu–OBzl[II] [*Anal.* Calcd. for $C_{30}H_{41}O_4N_4Cl \cdot H_2O$: C, 62.63; H, 7.55; N, 9.74. Found: C, 62.62; H, 7.26; N, 9.84. mp 219—220°; $[\alpha]_D^{27}$ −25° (c=0.75, 50% MeOH); Rf^1 0.83, Rf^{11} 0.87], after neutralization with N-methylmorpholine, to give the fully protected octapeptide ester Z–pyrGlu–Gly–Lys(Z)–Arg–(NO_2)–Pro–Trp–Ile–Leu–OBzl [III] [*Anal.* Calcd. for $C_{70}H_{90}O_{16}N_{14} \cdot H_2O$: C, 59.97; H, 6.62; N, 13.99. Found: C, 59.68; H, 6.49; N, 13.98. mp 155—160° (decomp.), $[\alpha]_D^{23}$ −61° (c=1, EtOH), Rf^1 0.82, Rf^{11} 0.88]. This was then dissloved in MeOH containing a little amount of acetic acid and hydrogenated in the presence of Pd-black to remove all the protecting groups. The deblocked octapeptide thus obtained was purified by column chromatography on SP-Sephadex using ammonium formate buffer to give homogeneous pyrGlu–Gly–Lys–Arg–Pro–Trp–Ile–Leu–OH [IV] [diformate tetrahydrate, *Anal.* Calcd. for $C_{47}H_{73}O_{10}N_{13} \cdot 2$ HCOOH. 4 H_2O: C, 51.42; H, 7.50; N, 15.91. Found: C, 51.11; H, 7.43; N, 16.30. Amino acid ratios in acid hydrolysate: Lys 0.95, Arg 0.99, Glu 1.06, Pro 1.05, Gly 1.05, Ile 0.96, Leu 0.96: $[\alpha]_D^{27}$ −96° (c=0.5, H_2O); Rf^1 0.25, Rf^{11} 0.54].

This synthetic octapeptide was identiacl with the natural origin "Xenopsin" in multiple criteria; chromatographic behaviors, enzymatic digestion patterns and biological activities.

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Spectrofluorometric Titrants for Trypsin and Chymotrypsin[1]

In order to determine the absolute concentration of active sites in a given enzyme solution, one requires a stoichiometric reaction which can be observed simply and accurately. Several spectrophotometric methods for this purpose have been described.[2] However, spectrophotometric titrations with the *p*-nitrophenyl esters so far developed require at least 1—2 nmole of an enzyme. In this respect, spectrofluorometric titration techniques seem fruitful in that the amount of enzyme required for assay should be much less than for conventional spectrophotometric assays. We have previously reported that *p*-nitrophenyl *p*-amidinobenzoate (NPAB) is a good titrant for determining operational normality of trypsin.[3] By changing the leaving group of NPAB from *p*-nitrophenol to 4-methylumbel-

1) Proteolytic Enzymes. VII. Part VI: K. Tanizawa, S. Ishii, K. Hamaguchi, and Y. Kanaoka, *J. Biochem.*, **69**, 893 (1971).
2) *a*) F.J. Kézdy and E.T. Kaiser, "Methods in Enzymology," vol. 19, Academic Press, New York, 1970, p. 3, and papers cited; *b*) T. Chase, Jr. and E. Shaw, *ibid.*, vol. 19, p. 20, and papers cited; *c*) G.R. Schonbaum, B. Zerner, and M.L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961); *d*) M.L. Bender, M.L. Bequé-Cantón, R.L. Blakeley, L.J. Brubacher, J. Feder, C.R. Gunter, F.J. Kézdy, J.V. Kilheffer, T.H. Marshall, C.G. Miller, R.W. Roeske, and J.K. Stoope, *J. Am. Chem. Soc.*, **88**, 5890 (1966).
3) *a*) K. Tanizawa, S. Ishii, and Y. Kanaoka, *Biochem. Biophys. Res. Comm.*, 32, 893 (1968); *b*) *Idem*, *Chem. Pharm. Bull.* (Tokyo), **18**, 2247 (1970); *c*) K. Tanizawa, S. Ishii, K. Hamaguchi, Y. Kanaoka, and T. Ikenaka, *ibid.*, **18**, 2571 (1970).

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