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# Caerulein-and xenopsin-related peptides with insulin-releasing activities from skin secretions of the clawed frogs, *Xenopus borealis* and *Xenopus amieti* (Pipidae)

Osama K. Zahid [a], Milena Mechkarska [b], Opeolu O. Ojo [c], Yasser H.A. Abdel-Wahab [c], Peter R. Flatt [c], Mohammed A. Meetani [a] J. Michael Conlon [b,*]

[a] Departmentof Chemistry, Faculty of Science, United Arab Emirates University, 17551 Al-Ain, United Arab Emirates
[b] Department of Biochemistry, Faculty of Medicine and Health Sciences, United Arab Emirates University, 17666 Al-Ain, United Arab Emirates
[c] School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine, Northern Ireland BT52 1SA, UK

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ABSTRACT

Caerulein-related peptides were identified in norepinephrine-stimulated skin secretions of the tetraploid frog *Xenopus borealis* and the octoploid frog *Xenopus amieti* using negative ion electrospray mass spectrometry and their primary structures determined by positive ion tandem (MS/MS) mass spectrometry. *X. borealis* caerulein-B1 (pGlu-Gln-Asp-Tyr($SO_3$)-Gly-Thr-Gly-Trp-Met-Asp-Phe.NH2) contains an additional $Gly^5$ residue compared with *X. laevis* caerulein and caerulein-B2 (pGlu-Asp-Tyr($SO_3$)-Thr-Gly-Trp-Met-Asp-Phe.NH2) contains a $Gln^2$ deletion. *X. amieti* caerulein was identical to the *X. laevis* peptide. In addition, xenopsin, identical to the peptide from *X. laevis*, together with xenopsin-AM2 (pGlu-Gly-Arg-Arg-Pro-Trp-Ile- Leu) that contains the substitution $Lys^3 \rightarrow Arg$ were isolated from *X. amieti* secretions. *X. borealis* caerulein-B1, and *X. amieti* xenopsin and xenopsin-AM2 produced significant ($P < 0.05$) and concentration-dependent stimulations of insulin release from the rat BRIN-BD11 clonal β cell line at concentrations $\geqslant$30 nM. The peptides did not stimulate the release of lactate dehydrogenase at concentrations up to 3 μM demonstrating that the integrity of the plasma membrane had been preserved. While their precise biological role is unclear, the caerulein- and xenopsin-related peptides may constitute a component of the animal's chemical defenses against predators.



## 1. Introduction

The skin of the African clawed frog *Xenopus laevis* has proved to be a rich source of biologically active peptides. These include the cholecystokinin (CCK)-like peptide, caerulein [3], the neurotensin-like peptide, xenopsin [5], thyrotropin-releasing hormone [7], the P-domain family peptide, xP2 [20], the activator of dihydropyridine-sensitive $Ca^{2+}$ channels, xenoxin-1 [26,27], and several antimicrobial peptides such as magainin-1 and -2 [19,37], peptide glycine–leucine–amide [4], and peptides derived from the post-translational processing of the biosynthetic precursors of caerulein and xenopsin [18,22]. However, relatively little work has been done to exploit other species belonging to the genus *Xenopus* as a source of peptides with the potential for development into pharmaceutically useful agents.

The clawed frogs comprise 33 species distributed in five genera *Hymenochirus*, *Pipa*, *Pseudhymenochirus*, *Silurana*, and *Xenopus*) within the family Pipidae [17]. The genus *Xenopus* currently contains 19 species although several additional, as yet unnamed, species have been reported [14]. Recent studies have led to the purification and characterization of peptides derived from the N-terminal regions of procaerulein and proxenopsin in norepinephrine-stimulated skin secretions from the Marsabit clawed frog *Xenopus borealis* Parker, 1936 [29] and the Volcano clawed frog *Xenopus amieti* Kobel, du Pasquier, Fischberg, and Gloor, 1980 [12]. These peptides were identified on the basis of their ability to inhibit growth of the microorganisms *Escherichia coli* and *Staphylococcus aureus*. *X. borealis* with $2n = 36$ chromosomes is considered, like *X. laevis*, to be a tetraploid species whereas a further genome duplication event within the tetraploid lineage has given rise to several octoploid species with $2n = 72$ chromosomes that include *X. amieti* [14,25].

In view of the fact that the skin secretions from both *X. amieti* and *X. borealis* contained high concentrations of peptide fragments derived from the processing of procaerulein and proxenopsin, the aim of the present study was to use electrospray ionization mass spectrometry (ES-MS) to identify the caerulein-related and xenopsin-related peptides that are present at the C-termini of the precursors. The strategy recommended by Wabnitz and co-workers [36] was used to identify and characterize the peptides. Caerulein contains a sulfated tyrosine residue so that ES-MS was initially carried out in negative ion mode to obtain the molecular mass of the sulfated $[M-H]^-$ ion followed by tandem MS (MS/MS) in positive ion

* Corresponding author. Fax: +971 37672033.
*E-mail address:* jmconlon@uaeu.ac.ae (J. Michael Conlon).

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mode to obtain the amino acid sequence of the desulfated $[MH-SO_3]^+$ ion. The xenopsin peptides were characterized by tandem MS in positive ion mode. Identification was facilitated by the fact that both caerulein and xenopsin contain a tryptophan residue and so show strong absorbance at 280 nm.

There is no evidence for the existence of either caerulein or xenopsinin mammals and it is unclear whether the peptides circulate in amphibians as well as being released into exocrine secretions. Similarly, there is no indication at this time that the peptides act via specific receptors in amphibians or mammals. The pharmacological actions of caerulein on the pancreas and gastrointestinal tract are mediated primarily through interaction with the CCK-A receptor that differentiates between sulfated cholecystokinin (CCK) and non-sulfated CCK/gastrin [35]. The actions of xenopsin in mammals are mediated through interaction with the NTR1 and/or the NTR2 neurotensin receptors [11].

## 2. Materials and methods

### 2.1. Collection of skin secretions

All procedures with live animals were approved by the Animal Research Ethics committee of U.A.E. University (Protocol No. A21-09) and were carried out by authorized investigators. Specimens of *X. borealis* ($n = 2$; male 23 g, female 33 g) and *X. amieti* ($n = 4$; weight range 4.5–8.0 g; sex not determined) were supplied by Xenopus Express Inc. (Brooksville, FL, USA). Full details of the procedures for stimulation of skin secretions by injection of norepinephrine and partial purification of the peptides on Sep-Pak C-18 cartridges have been provided previously [12,29].

### 2.2. Peptide purification

The skin secretions from each species, after partial purification on Sep-Pak cartridges, were separately redissolved in 0.1% (v/v) TFA/water (4 ml) and injected onto a (2.2 × 25-cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL, USA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 6.0 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 63% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 and 280 nm and fractions (1 min) were collected. Fractions associated with strong absorbance at 280 nm were successively chromatographed on a (1 × 25-cm) Vydac 214TP510 (C-4) column and a (1 × 25-cm) Vydac 208TP510 (C-8) column. The concentration of acetonitrile in the eluting solvent was raised from 14% to 42% over 50 min and the flow rate was 2.0 ml/min.

### 2.3. Structural characterization by electrospray mass spectrometry

Mass spectrometry was carried out using a 6310 ion trap mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) as described [30]. The mass spectrometer was equipped with an electrospray ionization source and operated initially in negative polarity. The scan range was from 50 to 1200 *m*/*z* with maximum accumulation time of 300 ms. Capillary voltage was set to +3500 V, the skimmer voltage was 36.3 V and the trap drive was 74.2 V. The flow of drying gas was set to 10 l/min, the nebulizer gas pressure was set to 70 psi, and the drying temperature was 350 °C. The tandem mass measurements were collected in the auto $MS^n$ mode in positive polarity. Capillary voltage was set to −3500 V. Ions are fragmented by subjecting them to collisions with the background gas, helium. The fragmentation parameters were as follows: the fragmentation cut off was set to 27% of the precursor mass, the fragmentation delay was set to 0 ms, the fragmentation time was set to 40 ms and the fragmentation width was set to 4 *m*/*z*. Amino acid composition analyses were performed by the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE, USA).

### 2.4. Determination of insulin-releasing activity

BRIN-BD11 cells were grown at 37 °C in an atmosphere of 5% $CO_2$ and 95% air in RPMI-1640 tissue culture medium containing 10% (v/v) fetal calf serum, antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin), and 11.1 mM glucose. The origin and characteristics of these cells have been provided in detail previously [28]. The cells were pre-incubated for 40 min at 37 °C in 1.0 ml Krebs-Ringer bicarbonate buffer, pH 7.4 (115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2$,1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 10 mM $NaHCO_3$) supplemented with 5.6 mM glucose and 0.1% (w/v) bovine serum albumin. Incubations with purified endogenous peptides (1–3000 nM; $n = 8$) were performed for 20 min at 37 °C using the same buffer. After incubation, aliquots of cell supernatant were removed for insulin radioimmunoassay [15].

In order to determine cytotoxicity, BRIN-BD11 cells were seeded into 24-multiwell plates and allowed to attach during overnight culture at 37 °C. Before the test, cells were pre-incubated for 40 min at 37 °C in Krebs-Ringer bicarbonate buffer supplemented with 5.6 mM glucose (1.0 ml). Test incubations with endogenous peptides (0.1–3 μM; $n = 4$) were performed for 20 min at 37 °C. Lactate dehydrogenase (LDH) concentrations in the cell supernatants were measured using aCytoTox96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) according to the manufacturer's protocol.

### 2.5. Statistical analysis

Results are expressed as means ± SEM and values were compared using two-way ANOVA followed by Newman–Keuls post hoc test. Groups of data were considered to be significantly different if $P < 0.05$.

## 3. Results

### 3.1. Purification of the peptides

The pooled skin secretions from *X. amieti*, after partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column (Fig. 1). The prominent peaks designated peak 1 (subsequently shown to contain xenopsin) and peak 2 (subsequently shown to contain a mixture of caerulein, desulfated caerulein, and xenopsin-AM2) were associated with strong absorbance at 280 nm and were purified further on semipreparative Vydac C-4 and Vydac C-8 columns. The methodology is illustrated by the partial separation of caerulein/xenopsin-AM2 and desulfated caerulein on the C-4 column (Fig. 2A) and complete separation into well-resolved peaks of xenopsin-AM2 (peak 1) and caerulein (peak 2) on the C-8 column (Fig. 2B). The yields of purified peptides were caerulein 145 nmol, xenopsin 195 nmol, and xenopsin-AM2 345 nmol.

The pooled skin secretions from *X. borealis* were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column under the same conditions used for purification of the *X. amieti* peptides (Fig. 3). The partially resolved peak 1 (subsequently shown to contain caerulein-B1), peak 2 (desulfated caerulein-B1) and peak 3 (caerulein-B2) showed strong absorbance at 280 nm and were purified further. Caerulein-B1 and -B2 were purified to near homogeneity by chromatography on Vydac C-4 and C-8 columns under the same conditions used for purification of the *X. ami-*