



The alyteserins: Two families of antimicrobial peptides from the skin secretions of the midwife toad *Alytes obstetricans* (Alytidae)

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ABSTRACT

Two families of structurally related C-terminally α -amidated antimicrobial peptides have been identified in norepinephrine-stimulated skin secretions of the midwife toad *Alytes obstetricans* (Alytidae). The alyteserin-1 peptides (Gly-Leu-Lys-(Asp/Glu)-Ile-Phe-Lys-Ala-Gly-Leu-Gly-Ser-Leu-Val-Lys-(Gly/Asn)-Ile-Ala-Ala-His-Val-Ala-(Asn/Ser).NH₂) show limited structural similarity to the ascapins from the skins of frogs of the family Leiopelmatidae. Alyteserin-2a (Ile-Leu-Gly-Lys-Leu-Leu-Ser-Thr-Ala-Ala-Gly-Leu-Leu-Ser-Asn-Leu.NH₂) and alyteserin-2b and -2c (Ile-Leu-Gly-Ala-Ile-Leu-Pro-Leu-Val-Ser-Gly-Leu-Leu-Ser-(Asn/Ser)-Lys-Leu.NH₂) show limited sequence identity with bombinin H6, present in the skins of frogs of the family Bombinatoridae. The alyteserin-1 peptides show selective growth inhibitory activity against the Gram-negative bacteria *Escherichia coli* (MIC = 25 μ M) whereas alyteserin-2a is more potent against the Gram-positive bacteria *Staphylococcus aureus* (MIC = 50 μ M). The hemolytic activity against human erythrocytes of all peptides tested is relatively weak (LC₅₀ > 100 μ M). The data demonstrate that the frogs belonging to the family Alytidae are among those producing dermal antimicrobial peptides that may represent a component of the animal's system of innate immunity.

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1. Introduction

Peptides with broad-spectrum antibacterial and antifungal activities and with the ability to lyse mammalian cells are synthesized in the skins of species from certain families of Anura (frogs and toads). These peptides probably represent a component of the system of innate immunity that defends the animal against invasion by pathogenic microorganisms [21] and have excited interest as candidates for development into therapeutically valuable anti-infective agents [27]. Structural characterization of the peptides has shown that they comprise between 10 and 48 amino acid residues and a comparison of their amino acid sequences reveals the lack of any conserved domains that are associated with biological activity. However, the peptides, with few exceptions, are cationic and contain at least 50% hydrophobic amino acids. Circular dichroism and NMR studies have shown that they generally lack stable secondary structure in aqueous solutions but have the propensity to form an amphipathic α -helix in the

environment of a phospholipid vesicle or in a membrane-mimetic solvent such as 50% trifluoroethanol–water [26]. On the basis of limited similarities in amino acid sequence, the frog skin antimicrobial peptides may be grouped together in families, many of which share a common evolutionary origin [15]. Skin secretions from a single species frequently contain several members of a particular peptide family with varying degrees of antimicrobial potency and selectivity that are presumed to have arisen from multiple duplications of an ancestral gene [32]. It is speculated that this molecular diversity may provide a broader spectrum of defense against pathogenic microorganisms although firm evidence to support this hypothesis is lacking.

It is a common misconception that antimicrobial peptides are synthesized in the skins of all frog species. At this time, cationic α -helical antimicrobial peptides have been identified in the skins of frogs from species belonging to the Bombinatoridae [23], Hylidae [1,3], Hyperoliidae [24], Leiopelmatidae [11,13], Leptodactylidae [8], Myobatrachidae [6], Pipidae [35], and Ranidae [9] families but several well studied species from the Bufonidae, Ceratophryidae, Dicroglossidae, Microhylidae, Pelobatidae, Pyxicephalidae, Rhacophoridae, and Scaphiropodidae families do not appear to synthesize these peptides (reviewed in [12]). Prior to this study, no species

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belonging the family Alytidae had been investigated for the presence of dermal antimicrobial peptides.

The midwife toad *Alytes obstetricans* (Laurenti, 1768) is a small (snout-vent length of 55 mm) predominantly nocturnal and terrestrial anuran that is best known for its male parental care behavior. Once the oocytes have been fertilized, the male attaches the egg strings to his hindlegs which are then kept moist and protected against predators until the newly hatched tadpoles are released into water. The species was once widely distributed in western Europe in woodland areas at altitudes between 200 and 700 m [19]. However, populations in many regions have declined precipitously due to habitat loss, introduction of predator fish into their breeding waters, and diseases such as chytridiomycosis arising from infection by the chytrid fungus *Batrachochytrium dendrobatidis* [4,5,14]. The present study describes the purification and characterization of two families of structurally related peptides with differential antimicrobial activity from norepinephrine-stimulated skin secretions from *A. obstetricans* that have been termed alyteserin-1 and alyteserin-2.

2. Experimental

2.1. Collection of skin secretions

All experiments with live animals conform to the ethical and animal care guidelines issued by the Swiss Academy of Natural Sciences and were approved by the Veterinary Office of the Canton Zurich (permit 221/07). Adult specimens of *A. obstetricans* ($n = 12$; weight range 4.1–6.3 g; sex not determined) were collected under permit in Germany, allowed to reproduce in captivity at the University of Zurich, and skin secretions were obtained after breeding in August, 2008. Each animal was injected with norepinephrine bitartrate (40 nmol/g frog weight) and immersed in water for 15 min. The solution containing the secretions was acidified and passed at a flow rate of 2 ml/min through 2 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (TFA) (70.0:29.9:0.1, v/v/v) and dried under of a stream of nitrogen for shipment to U.A.E. University.

2.2. Antimicrobial assays

Purification of the peptides was monitored by incubating lyophilized aliquots of chromatographic effluent in Mueller-Hinton broth (50 μ l) with an inoculum (50 μ l of 10^6 colony forming units/ml) from a log-phase culture of reference strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) in 96-well microtiter cell-culture plates for 18 h at 37 °C in a humidified atmosphere of air. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. In order to monitor the validity and reproducibility of the assays, incubations with bacteria were carried out in parallel with increasing concentrations of ampicillin. Minimum inhibitory concentration (MIC) of the peptides was measured the concentration range of 3–200 μ M by a standard double dilution method [7] and was taken as the lowest concentration of peptide where no visible growth was observed. This value was confirmed by measurement of absorbance at 630 nm.

2.3. Hemolysis assay

Purified peptides (1–200 μ M) were incubated in duplicate with washed human erythrocytes (2×10^7 cells) from a healthy donor in Dulbecco's phosphate-buffered saline, pH 7.4 (100 μ l) for 1 h at 37 °C. After centrifugation (12,000 \times g for 15 s), the absorbance at 450 nm of the supernatant was measured. A parallel incubation in

the presence of 1% (v/v) Tween-20 was carried out to determine the absorbance associated with 100% hemolysis. The LC₅₀ value was taken as the mean concentration of peptide producing 50% hemolysis in three independent experiments.

2.4. Peptide purification

The dried skin secretions (total weight 68.2 mg), after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) TFA/water (2 ml) and injected onto a (2.2 cm \times 25 cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Separations Group, Hesperia, CA) 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 nm and 280 nm, and fractions (1 min) were collected. The abilities of freeze-dried aliquots (50 μ l) of the fractions to inhibit the growth of *S. aureus* and *E. coli* were determined as described in the previous section. Fractions associated with antimicrobial activity were successively chromatographed on a (1 cm \times 25 cm) Vydac 214TP510 (C-4) column and a (1 cm \times 25 cm) Vydac 219TP510 (phenyl) column. The concentration of acetonitrile in the eluting solvent was raised from 21% to 49% over 50 min for purification of the alyteserin-1 peptides and from 28% to 56% over 50 min for purification of the alyteserin-2 peptides. The flow rate was 2.0 ml/min.

2.5. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 494 Procise sequenator (Foster City, CA). MALDI-TOF mass spectrometry was carried out using a Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2000–4000 Da range. The accuracy of mass determinations was $\pm 0.02\%$.

3. Results

3.1. Purification of the peptides

The pooled skin secretions from *A. obstetricans*, 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 1–7 were collected and subjected to further purification. Under the conditions of assay, peaks 1–4 were associated with the ability to inhibit the growth of *E. coli* only and peaks 5–7 inhibited the growth of *S. aureus* only. Subsequent structural analysis demonstrated that peak 1 contained alyteserin-1a, peak 2: alyteserin-1b, peak 3: alyteserin-1c, peak 4: alyteserin-1d, peak 5: alyteserin-2a, peak 6: alyteserin-2b, and peak 7: alyteserin-2c. The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by further chromatography on semipreparative Vydac C-4 and Vydac phenyl columns. The methodology is illustrated by partial purification of alyteserin-1c on a Vydac C-4 column (Fig. 2a) and a final purification on a Vydac phenyl column (Fig. 2b). The final yields of purified peptides (nmol) were alyteserin-1a 230, alyteserin-1b 350, alyteserin-1c 170, alyteserin-1d 75, alyteserin-2a 390, alyteserin-2b 65, and alyteserin-2c 75.

3.2. Structural characterization

The primary structures of the antimicrobial peptides isolated from *A. obstetricans* skin secretions were established by automated

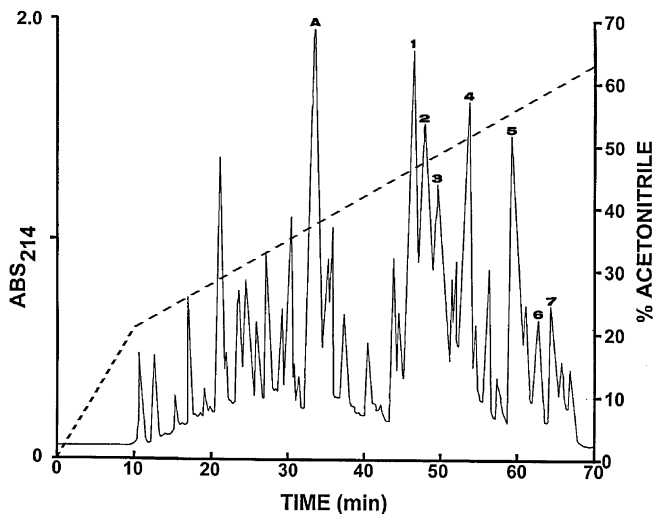


Fig. 1. Reversed-phase HPLC on a preparative Vydac C-18 column of skin secretions from *A. obstetricans* after partial purification on Sep-Pak cartridges. The peaks designated 1–7 displayed antimicrobial activity and were purified further. The peak designated A contained alytesin [2]. The dashed line shows the concentration of acetonitrile in the eluting solvent.

Edman degradation and their amino acid sequences are shown in Fig. 3. MALDI-TOF mass spectrometry was used to confirm the proposed sequences and to demonstrate that all peptides were C-terminally α -amidated. As there was some ambiguity with regard to identification of the C-terminal amino acid residue in the alyteserin-2 peptides, the sequences were confirmed by amino acid composition analysis [alyteserin-2a: Asx 1.1 (1), Thr 1.1 (1), Ser 2.1 (2), Gly 2.2 (2), Ala 2.2 (2), Ile 0.8 (1), Leu 5.7 (6), Lys 1.1 (1); alyteserin-2b: Asx 1.1 (1), Ser 1.1 (1), Pro 1.1 (1), Gly 2.2 (2), Ala 1.1 (1), Ile 1.8 (2), Leu 5.8 (6), Lys 1.2 (1); alyteserin-2c: Ser 2.1 (2), Pro 1.1 (1), Gly 2.1 (2), Ala 1.1 (1), Ile 1.8 (2), Leu 5.9 (6), Lys 1.1 (1) residues/mol peptide]. Figures in parentheses show the number of residues predicted from the proposed sequences. The molecular mass of the peptide in the prominent peak designated A in Fig. 1 (1534.8 Da) identified it as the bombesin-like myotropic peptide, alytesin (<EGR LGTQWAVGHLM.NH₂) [2].

3.3. Antimicrobial and hemolytic activities

The abilities of the most abundant peptides in the skin secretions (alyteserin-1a, -1b, and -1c, and alyteserin-2a) to inhibit the growth of reference strains of *S. aureus* and *E. coli* and to lyse human erythrocytes from a healthy donor are compared in Table 1.

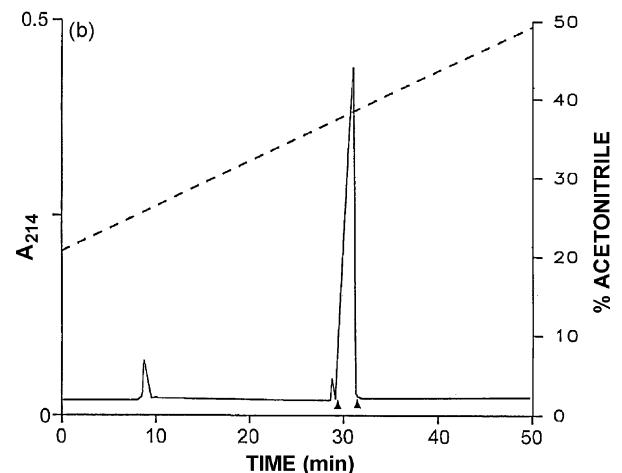
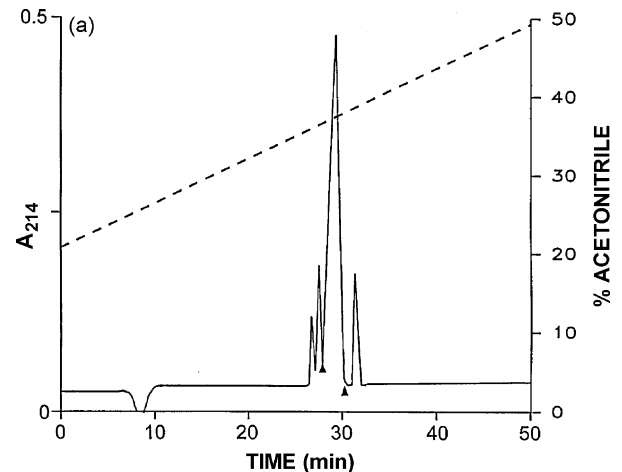


Fig. 2. Purification to near homogeneity of alyteserin-1c on semipreparative (a) Vydac C-4, and (b) Vydac phenyl columns. The arrowheads show where peak collection began and ended.

4. Discussion

In common with most anuran species, the taxonomy of the frogs of the Alytidae family has undergone major revision in recent years. Traditionally, *A. obstetricans* was included in the family Discoglossidae which was divided into the four genera *Alytes*, *Discoglossus*, *Bombina*, and *Barbourula* [16]. However, the monophyletic status of the Discoglossidae has been called into question [18] and current taxonomic recommendations restrict the taxon Alytidae to two genera *Alytes* (5 species) and *Discoglossus* (6 species). *Bombina* (6

		M_r obs	M_r calc
Alyteserin-1a	GLKDI FKAGL GSLVKGIAAHVAN.NH ₂	2277.3	2277.3
Alyteserin-1b	GLKEI FKAGL GSLVKGIAAHVAN.NH ₂	2291.4	2291.4
Alyteserin-1c	GLKEI FKAGL GSLVKGIAAHVAS.NH ₂	2263.5	2264.3
Alyteserin-1d	GLKDI FKAGL GSLVKNIAAHVAN.NH ₂	2334.5	2334.4
Alyteserin-2a	I LGKLLSTAAGLLSNL.NH ₂	1582.1	1582.0
Alyteserin-2b	I LGAILPLVSGLLSNKL.NH ₂	1632.1	1632.1
Alyteserin-2c	I LGAILPLVSGLLSSKL.NH ₂	1605.0	1605.1

Fig. 3. Amino acid sequences, observed molecular masses (M_r obs), and calculated molecular masses (M_r calc) of the antimicrobial peptides isolated from skin secretions of *A. obstetricans*.

Table 1

Minimum inhibitory concentrations (μM) against microorganisms and LC_{50} values (μM) against human erythrocytes of the endogenous peptides isolated from skin secretions of *A. obstetricans*.

	<i>E. coli</i>	<i>S. aureus</i>	LC_{50}
Alyteserin-1a	25	200	>100 (11%)
Alyteserin-1b	25	200	210
Alyteserin-1c	25	100	145
Alyteserin-2a	150	50	135

The value in parentheses shows the % hemolysis at a concentration of 100 μM .

species) is united with *Barbourula* (2 species) in the family Bombinatoridae [17]. Cladistic analysis based upon the complete nucleotide sequence of the mitochondrial genomes strongly supports a sister group relationship between *A. obstetricans* and *Discoglossus galganoi* to the exclusion of *Bombina orientalis* [30].

The Alytidae are regarded as a phylogenetically ancient group of frogs and are traditionally placed along with the other “primitive” frogs belonging to the Discoglossidae, Leiopelmatidae, Pelobatoidae, and Pipoidea within the Archaeobatrachia in order to distinguish them from the more highly derived Neobatrachia, such as the Hylidae and Ranidae [16]. The primary structures of the alyteserin peptides determined in this study are consistent with this placement. As shown in Fig. 4, the alyteserin-1 peptides show limited structural similarity (between 37 and 39%) to the C-terminally α -amidated antimicrobial peptides ascaphin-1 and ascaphin-8, previously isolated from *Ascaphus truei* [13] and *Ascaphus montanus* [11] belonging to the family Leiopelmatidae. The tailed frogs *Ascaphus* spp. (Stejneger, 1899) occupy a uniquely important position in amphibian phylogeny as the most primitive extant anurans [20]. Originally classified alone in the family Ascaphidae as the sister group to the clade of all other living frogs, *Ascaphus* is now united with the New Zealand frogs of the genus *Leiopelma* (Fitzinger, 1861) in the Leiopelmatidae family [17]. Similarly, the alyteserin-2 peptides show limited structural similarity (between 41 and 65%) to bombinin H6, previously identified in the skin secretions of the toads *Bombina bombina*, *B. orientalis*, and *B. variegata* belonging to the family Bombinatoridae [23] (Fig. 4). Bombinin H6 is unusual in that isoforms have been found to contain either D-leucine or D-alloisoleucine instead of L-leucine at position 2 in the molecule [25] and the enzyme responsible for effecting this transformation has been isolated from frog skin [22]. Further work is clearly warranted to determine whether peptides containing D-amino acids are present in the skin secretions of *A. obstetricans*.

Alyteserin-1a	GLKDI F KAGLGS L V K G I A A H V A N . NH ₂
Ascaphin-1	GFRDVL K G A A K A F V K T V A G H I A N . NH ₂
Ascaphin-1M	GFRDVL K G A A K E F V K T V A G H I A N . NH ₂
Ascaphin-8	GFKDLL K G A A K A L V K T V L F . NH ₂
Alyteserin-2a	ILGKL L S T A A * * * * G L L S N L . NH ₂
Alyteserin-2b	ILG A I L P L V S * * * * G L L S N K L . NH ₂
Bombinin-H6	ILG P I L G L V S N A L G S L L . NH ₂

Fig. 4. A comparison of the primary structures of (a) alyteserin-1a with ascaphin-1 and ascaphin-8 from *Ascaphus truei* [12] and ascaphin-1 M from *Ascaphus montanus* [10], and (b) alyteserin-2a and -2b with bombinin H6 from *Bombina* spp. [22]. Structural similarity is emphasized by the shading.

In common with the vast majority of frog skin antimicrobial peptides, the alyteserins are cationic with a charge of +3 (alyteserin-1) and +2 (alyteserin-2) at neutral pH. It has been proposed that the positive charge facilitates transport of the peptide across the bacterial cell wall and promotes interaction with the negatively charged bacterial cell membrane [21]. Secondary structure prediction by the method of Rost and Sander [29] indicates that the (2–21) region of alyteserin-1a and the (2–13) region of alyteserin-2b have a high probability of forming a stable α -helical conformation. A Schiffer-Edmundson wheel representation [31] of the alyteserin-1a structure illustrates the amphipathic nature of the helix with the polar residues Lys³, Asp⁴, Lys⁷, and Lys¹⁵ segregating together on the hydrophilic face of the helix (polar angle $\theta = 140^\circ$) (Fig. 5). However, the presence of the more hydrophobic Val¹⁴ and Ala¹⁸ on this hydrophilic face results in a decrease in the hydrophobic moment (a semi-quantitative measure of the amphipathicity of a α -helical peptide) [33]. The alyteserin-1 peptides show relatively potent growth inhibitory activity against the Gram-negative bacteria *E. coli* (MIC = 25 μM) together with weak hemolytic activity against human erythrocytes ($\text{LC}_{50} > 100 \mu\text{M}$) (Table 1) so that these peptides show potential for development into therapeutically useful anti-infective agents [10]. Hemolytic activity is strongly dependent upon amphipathicity and small increases in the hydrophobic moment may produce major changes in potency [33]. It is suggested, therefore, that the low hemolytic activity of the alyteserin-1 peptides is a consequence of the decreased amphipathic character of the α -helical conformation compared with that of many frog skin antimicrobial peptides. The alyteserin-2 peptides are comparable in size to the α -helical temporin peptides present in skin secretions of the majority frogs of the Ranidae family [23]. In common with those temporin peptides with a charge +2 at pH 7, alyteserin-2a shows more potent growth inhibitory activity against Gram-positive bacteria (Table 1).

Although this study has demonstrated that the skin secretions of *A. obstetricans* contain peptides with antimicrobial activity against two bacteria with clinical relevance, it is unclear to what extent the alyteserins provide the animal with protection against pathogens it may encounter in the wild. The emergence in almost all parts of the world of the pathogenic chytrid fungus *B. dendrobatidis* has led, or has contributed, to widespread declines in frog populations [14,28]. The fungus parasitizes the mouthparts of larvae and the keratinized epidermis of post-metamorphic amphibians which may produce electrolyte depletion and osmotic imbalance leading to death. Studies *in vitro* have shown that a wide

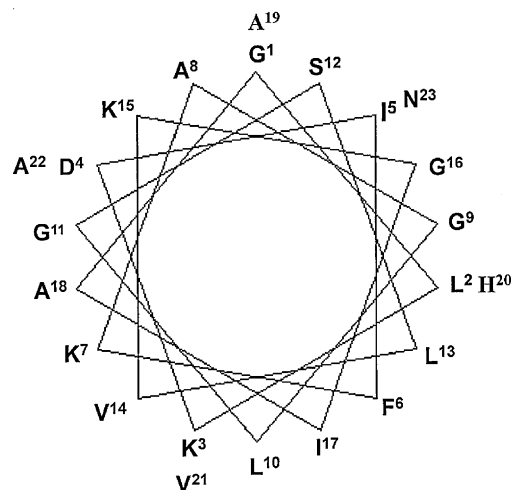


Fig. 5. A Schiffer-Edmundson helical wheel representation of the alyteserin-1a structure illustrating the amphipathic nature of the α -helical conformation.

range of frog skin peptides will inhibit the growth of *B. dendrobatidis* [28,34]. However, chytridiomycosis has largely extirpated *A. obstetricans* in mountainous areas of central Spain whereas populations of *Bufo bufo*, whose skin secretions do not contain cationic α -helical antimicrobial peptides (unpublished data) had not declined in the same region [4]. However, chytridiomycosis-related mortalities of *B. bufo* in other regions of Spain have been reported [5]. Consequently, it is not possible at this time to draw a firm conclusion regarding the importance of the alyteserins in the animal's system of innate immunity. Similarly, the smooth muscle-stimulating peptide alytesin [2] represented the most abundant peptide in the Sep-Pak concentrated skin secretions (Fig. 1) but it is unclear to what extent, if any, this peptide provides a deterrent to ingestion by predators. It is tempting to speculate that alytesin may activate gastrointestinal smooth muscle and cause the prey to be vomited from the mouths of predators but evidence to support this idea is lacking.

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References

- [1] Amiche M, Ladram A, Nicolas P. A consistent nomenclature of antimicrobial peptides isolated from frogs of the subfamily Phyllomedusinae. *Peptides* 2008;29:2074–82.
- [2] Anastasi A, Erspamer V, Bucci M. Isolation and amino acid sequences of alytesin and bombesin, two analogous active tetradecapeptides from the skin of European discoglossid frogs. *Arch Biochem Biophys* 1972;148:443–6.
- [3] Apponyi MA, Pukala TL, Brinkworth CS, Maselli VM, Bowie JH, Tyler MJ, et al. Host-defence peptides of Australian anurans: structure, mechanism of action and evolutionary significance. *Peptides* 2004;25:1035–54.
- [4] Bosch J, Rincón PA. Chytridiomycosis-mediated expansion of *Bufo bufo* in a montane area of central Spain: an indirect effect of the disease. *Diversity Distrib* 2008;14:637–43.
- [5] Bosch J, Martínez-Solano I. Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Peñalara Natural Park, Spain. *Oryx* 2006;40:84–9.
- [6] Chia BC, Carver JA, Mulhern TD, Bowie JH. The solution structure of uperin 3.6, an antibiotic peptide from the granular dorsal glands of the Australian toadlet, *Uperoleia mjobergii*. *J Pept Res* 1999;54:137–45.
- [7] Clinical Laboratory and Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard M07-A8. CLSI, Wayne PA, 2008.
- [8] Conlon JM. A proposed nomenclature for antimicrobial peptides from frogs of the genus *Leptodactylus*. *Peptides* 2008;29:1631–2.
- [9] Conlon JM. Reflections on a systematic nomenclature for antimicrobial peptides from the skins of frogs of the family Ranidae. *Peptides* 2008;29:1815–9.
- [10] Conlon JM, Al-Ghaferi N, Abraham B, Leprince J. Strategies for transformation of naturally-occurring amphibian antimicrobial peptides into therapeutically valuable anti-infective agents. *Methods* 2007;42:349–57.
- [11] Conlon JM, Bevier CR, Coquet L, Leprince J, Jouenne T, Vaudry H, et al. Peptidomic analysis of skin secretions supports separate species status for the tailed frogs, *Ascaphus truei* and *Ascaphus montanus*. *Comp Biochem Physiol* 2007;2D:121–5.
- [12] Conlon JM, Iwamuro S, King JD. Dermal cytolytic peptides and the system of innate immunity in anurans. *Ann NY Acad Sci* 2009; in press.
- [13] Conlon JM, Sonnevend A, Davidson C, Smith DD, Nielsen PF. The ascaphins: a family of antimicrobial peptides from the skin secretions of the most primitive extant frog, *Ascaphus truei*. *Biochem Biophys Res Commun* 2004;320:170–5.
- [14] Dasak P, Cunningham AA, Hyatt AD. Infectious disease and amphibian population declines. *Diversity Distrib* 2003;9:141–50.
- [15] Duda Jr TF, Vanhoye D, Nicolas P. Roles of diversifying selection and coordinated evolution in the evolution of amphibian antimicrobial peptides. *Mol Biol Evol* 2002;19:858–64.
- [16] Ford LS, Cannatella DC. The major clades of frogs. *Herpetol Monogr* 1993;7:94–117.
- [17] Frost DR. Amphibian species of the world: an online reference. Version 5.2. American Museum of Natural History, New York, USA. Electronic database accessible at <http://research.amnh.org/herpetology/amphibia/index.php>, 2008.
- [18] Frost DR, Grant T, Faivovich J, Bain RH, Haas A, Haddad CFB, et al. The amphibian tree of life. *Bull Am Mus Nat Hist* 2006;297:1–370.
- [19] Gasc J-P. Atlas of amphibians and reptiles in Europe. Societas Europaea Herpetologica, Bonn Germany; 1997.
- [20] Gissi C, San Mauro D, Pesole G, Zardoya R. Mitochondrial phylogeny of Anura (Amphibia): a case study of congruent phylogenetic reconstruction using amino acid and nucleotide characters. *Gene* 2006;366:228–37.
- [21] Hancock RE. Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet Infect Dis* 2001;1:156–64.
- [22] Jilek A, Mollay C, Tippelt C, Grassi J, Mignogna G, Müllegger J, et al. Biosynthesis of a D-amino acid in peptide linkage by an enzyme from frog skin secretions. *Proc Natl Acad Sci USA* 2005;102:4235–9.
- [23] Mangoni ML, Marcellini HG, Simmaco M. Biological characterization and modes of action of temporins and bombinins H, multiple forms of short and mildly cationic anti-microbial peptides from amphibian skin. *J Pept Sci* 2007;13:603–13.
- [24] Mattute B, Knoop FC, Conlon JM. Kassinatuerin-1: a peptide with broad-spectrum antimicrobial activity isolated from the skin of the hyperoliid frog, *Kassina senegalensis*. *Biochem Biophys Res Commun* 2000;268:433–6.
- [25] Mignogna G, Simmaco M, Kreil G, Barra D. Antibacterial and haemolytic peptides containing D-alloisoleucine from the skin of *Bombina variegata*. *EMBO J* 1993;12:4829–32.
- [26] Powers JP, Hancock RE. The relationship between peptide structure and antibacterial activity. *Peptides* 2003;24:1681–91.
- [27] Rinaldi AC. Antimicrobial peptides from amphibian skin: an expanding scenario. *Curr Opin Chem Biol* 2002;6:799–804.
- [28] Rollins-Smith LA, Conlon JM. Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. *Dev Comp Immunol* 2005;29:589–98.
- [29] Rost B, Sander C. Prediction of protein secondary structure at better than 70% accuracy. *J Mol Biol* 1993;232:584–99.
- [30] San Mauro D, García-Paris M, Zardoya R. Phylogenetic relationships of discoglossid frogs (Amphibia:Anura:Discoglossidae) based on complete mitochondrial genomes and nuclear genes. *Gene* 2004;343:357–66.
- [31] Schiffer M, Edmundson AB. Use of helical wheels to represent the structures of proteins and to identify segments with helical potential. *Biophys J* 1967;7:121–35.
- [32] Tennessen JA, Blouin MS. Selection for antimicrobial peptide diversity in frogs leads to gene duplication and low allelic variation. *J Mol Evol* 2007;65:605–15.
- [33] Wieprecht T, Dathe M, Krause E, Beyermann M, Maloy WL, MacDonald DL, et al. Modulation of membrane activity of amphipathic, antibacterial peptides by slight modifications of the hydrophobic moment. *FEBS Lett* 1997;417:135–40.
- [34] Woodhams DC, Rollins-Smith LA, Carey C, Reinert L, Tyler MJ, Alford RA. Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs. *Oecologia* 2006;146:531–40.
- [35] Zasloff M. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms and partial cDNA sequence of a precursor. *Proc Natl Acad Sci USA* 1987;84:5449–53.