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Induction of synthesis of an antimicrobial peptide in the skin of the freeze-tolerant frog, *Rana sylvatica*, in response to environmental stimuli

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Abstract An extract of skin taken from specimens of the freeze-tolerant wood frog, *Rana sylvatica*, that were collected from cold (<7°C) ponds and maintained at 5°C lacked detectable antimicrobial activity. In contrast, an extract of skin taken from specimens maintained at 30°C for 3 weeks under laboratory conditions contained a high concentration (approximately 4 nmol/g) of a single antimicrobial peptide of the brevinin-1 family (FLPVVAGLAAKVLPSIICAVTKKC). The peptide inhibited growth of *Escherichia coli* (minimum inhibitory concentration 45 μM) and *Staphylococcus aureus* (minimum inhibitory concentration 7 μM). The data suggest that synthesis of the peptide is induced when the animal is in an environment that promotes the growth of microorganisms consistent with a role in the animal's defense strategy. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Brevinin-1; Antimicrobial peptide; Amphibian skin

1. Introduction

The wood frog *Rana sylvatica* is the most extensively studied of a small group of terrestrially hibernating amphibians that can withstand the freezing of extracellular body fluids during winter hibernation [1]. The onset of freezing is accompanied by physiological changes that include accumulation of high concentrations of glucose, derived primarily from hepatic glycogen stores, that acts as a cryoprotectant, cardioacceleration to distribute the glucose throughout the body, ischemia due to the plasma freezing, and reduction of cell volume in all organs due to water loss into extracellular ice masses [1–3]. Freezing is also associated with the up-regulation of expression of several genes, including those encoding ice-nucleation proteins [4], the α- and γ-subunits of fibrinogen [5], mitochondrial ADP/ATP translocase [6], a novel 10 kDa protein (Fr10) containing a nuclear exporting sequence [7] and several, as yet uncharacterized, proteins in liver, skeletal muscle, heart and brain [8]. The skin is clearly the first organ in the animal to experience changes in the environment but the effects of freezing and/or thawing on the regulation of gene expression in *R. sylvatica* skin have not been reported.

The skins of all frogs of the genus *Rana* yet studied (reviewed in [9–11]) contain granular glands that synthesize a wide range of antimicrobial peptides that are released into skin secretions as a result of contraction of myocytes surrounding the glands [9]. These peptides have been recently classified into eight families on the basis of amino acid sequence similarities and comprise the brevinin-1, brevinin-2, esculentin-1, esculentin-2, ranalexin, ranatuerin-1, ranatuerin-2 and temporin families [10]. It has been proposed that these peptides represent an important component of the animal's defense strategy protecting it against the range of pathogenic microorganisms that might be encountered in nature [12] but firm evidence to support this hypothesis is lacking. The present study demonstrates for the first time induction of the synthesis of an antimicrobial peptide of the brevinin-1 family in the skin of a Ranid frog in response to environmental change. The data provide support for the proposal that such peptides are important in protecting the animal against microbial invasion.

2. Materials and methods

2.1. Animals

Adult male specimens of wood frog *R. sylvatica* (weight range 3.9–5.1 g) were collected during the time of the spring thaw from breeding ponds in the Ottawa region. The temperature of the water was always <7°C. The animals were transported to the laboratory and housed in plastic boxes containing wet moss at 5°C. Skin (21 g) was taken from these cold-acclimated specimens ($n=40$) and immediately frozen in liquid nitrogen. Other specimens ($n=23$) were shipped to Creighton University on ice and were acclimated gradually over a period of 48 h to a temperature of 30±2°C. The animals were housed at this temperature and after 1 week began feeding. A meal of crickets was given every 3 days. After 3 weeks, the animals were sacrificed by pithing and skin (12 g) was removed and immediately frozen on dry-ice.

2.2. Tissue extraction

The frozen skins from 5 and 30°C-acclimated frogs were separately extracted by homogenization in ethanol/0.7 M HCl (3:1 v/v; 10 ml g^{-1}) at 0°C using a Waring blender. The homogenates were stirred for 2 h at 0°C and centrifuged (4000×g for 30 min at 4°C). Ethanol was removed from the supernatants under reduced pressure and, after further centrifugation (4000×g for 30 min at 4°C), the extracts were separately pumped onto 4 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series at a flow rate of 2 ml/min. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0:29.9:0.1, v/v/v) and freeze-dried.

2.3. Antimicrobial assays

Minimal inhibitory concentrations (MICs) of the peptides were determined by a standard microdilution method using 96-well microtiter cell-culture plates as previously described [10]. Serial dilutions of the peptides in Mueller–Hinton broth (50 μl) were incubated with an inoculum (50 μl of 10^3 cfu/ml) from an overnight culture of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (NCTC 8325), for 18 h at 37°C in a humidified atmosphere of 5% CO_2 in air. After

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incubation, the absorbance at 550 nm of each well was determined using an M.A. Bioproducts model MA308 microtiter plate reader. The MIC of each peptide was taken as the lowest concentration where no visible growth was observed. In order to monitor the validity of the assay, incubations with *E. coli* and *S. aureus* were carried out in parallel with increasing concentrations of the broad-spectrum antibiotic, bacitracin.

2.4. Peptide purification

Each skin extract, after partial purification on Sep-Pak cartridges, was redissolved in 1% (v/v) trifluoroacetic acid/water (5 ml) and injected onto a (25×1 cm) Vydac 218TP510 C-18 reverse-phase high performance liquid chromatography (HPLC) column (Separations Group, Hesperia, CA, USA) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2 ml min^{-1}. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 49% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 and 280 nm and fractions (1 min) were collected. The ability of aliquots (50 μl) of the fractions to inhibit the growth of *S. aureus* and *E. coli* was determined as described in Section 2.3. Fractions containing maximum activity (denoted by the bar in Fig. 1) were pooled and were successively rechromatographed on (25×1 cm) Vydac 214TP510 (C-4) and Vydac 219TP510 (phenyl) reverse-phase HPLC columns. In both cases, the concentration of acetonitrile in the eluting solvent was raised from 35 to 65% over 40 min.

2.5. Structural analysis

Hydrolysis in 5.7 M HCl (24 h at 110°C) of approximately 1 nmol of peptide was carried out. Amino acid compositions were determined by precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate using a Waters AccQ Tag system with fluorescence detection and separation of the amino acid derivatives by reverse-phase HPLC. The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 491 sequenator. Electrospray mass spectrometry was carried out using a Perkin Elmer Sciex API 150EX single quadrupole instrument. The accuracy of mass determinations was ±0.02%.

3. Results

3.1. Peptide purification

The elution profile on a semi-preparative Vydac C-18 reverse-phase HPLC column of the extract of *R. sylvatica* skin from 30°C-acclimated animals, after partial purification on Sep-Pak cartridges, is shown in Fig. 1A. Growth-inhibiting activity against both *S. aureus* and *E. coli* was eluted from the column in a single broad fraction, denoted by the bar. Further purification on Vydac C-4 (Fig. 2A) and Vydac phenyl (Fig. 2B) columns demonstrated that the antimicrobial activity was associated with a single component, subsequently identified as brevinin-1SY, that was purified to near homogeneity. The final yield of pure peptide was 48 nmol. In contrast, no fraction from chromatography of the skin extract from 5°C-acclimated frogs on the Vydac C-18 column (Fig. 1B) inhibited the growth of either *S. aureus* or *E. coli* when tested under the same assay conditions using up to 200 μl aliquots of each fraction.

3.2. Peptide characterization

The primary structure of the antimicrobial peptide (brevinin-1SY) was established by automated Edman degradation and is shown in Fig. 3. The amino acid composition of the peptide in residues/mol peptide was determined as: [Ser 1.0 (1), Gly 1.1 (1), Thr 1.0 (1), Ala 3.7 (4), Pro 2.0 (2), Val 3.4 (4), Lys 2.8 (3). Ile 1.4 (2), Leu 2.8 (3), Phe 1.0 (1)] which was consistent with the sequence analysis data and demonstrated that the full sequence had been obtained. The values in parentheses are the number of residues predicted from the proposed structure. The presence of a cystine bridge in the peptide was shown by mass spectrometry (M_r observed 2440.2; M_r calculated for the reduced peptide 2440.1).

The MIC of brevinin-1SY was 7 μM against the Gram-positive bacterium *S. aureus* and 45 μM against the Gram-negative bacterium *E. coli*.

4. Discussion

The present study, by demonstrating that synthesis of an antimicrobial peptide in frog skin is induced in response to environmental influences, has provided evidence to support the hypothesis that such peptides play a physiologically important role in defending the organism against invasion by microorganisms. Fasting animals, collected immediately after hibernation in the frozen state and maintained at 5°C did not have detectable concentrations of antimicrobial components in the skin. Under these circumstances, the bacterial challenge to the animal was weak. In contrast, animals maintained at

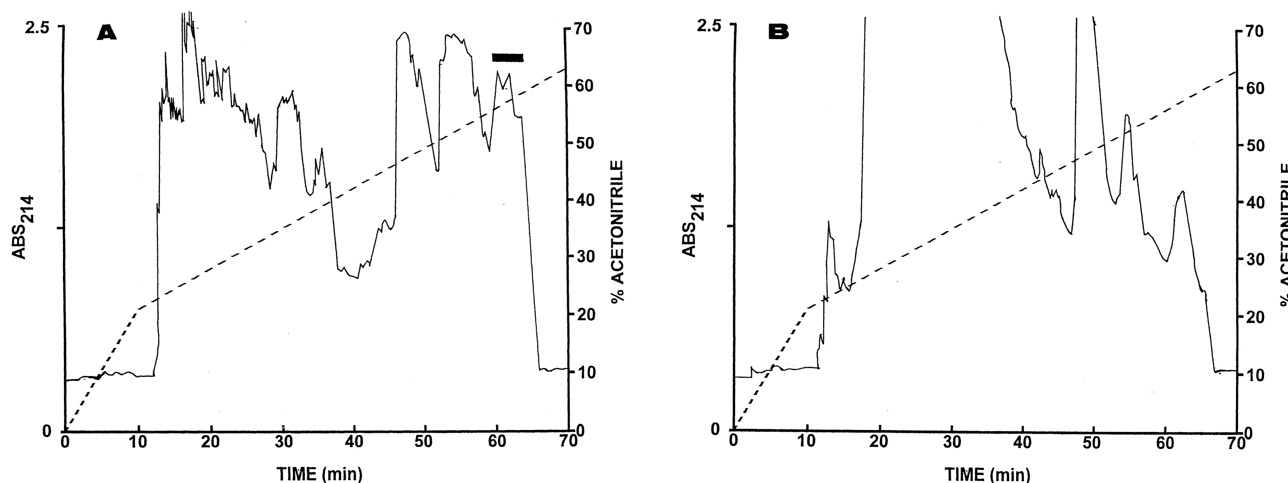

Fig. 1. Reverse-phase HPLC on a semipreparative Vydac C-18 column of an extract of *R. sylvatica* skin from animals maintained at (A) 30°C and (B) 5°C. The bar designates those fractions with growth-inhibitory activity towards *S. aureus*. The dashed line shows the concentration of acetonitrile in the eluting solvent.

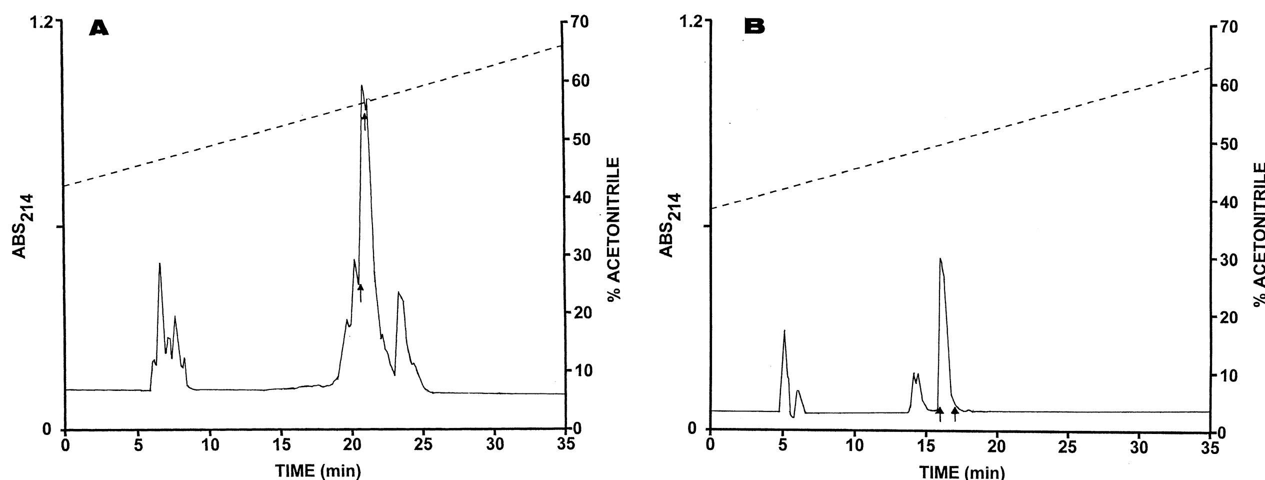

Fig. 2. Purification to near homogeneity of brevinin-1SY on (A) Vydac C-4 and (B) Vydac phenyl columns. The arrows show where peak collection began and ended.

30°C that were feeding and defecating vigorously synthesized relatively high concentrations of an antimicrobial peptide of the brevinin-1 family that was active against both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria. These circumstances mimic the conditions in the wild in late spring and are conducive to the growth of bacteria. Adopting the terminology of Simmaco et al. [13], the antimicrobial peptide is described as brevinin-1SY to indicate its species of origin (S has already been used for *Rana sphenocephala* [14]). The regulation of the synthesis of antimicrobial peptides in frog skin has not been studied extensively but a previous study has shown that treatment of the frog *Rana esculenta* with glucocorticoids inhibits synthesis of antimicrobial peptides by a mechanism that involves up-regulation of the inhibitor of transcription IκBα [15].

Peptides of the brevinin-1 family were first isolated from the skin of the Asian frog, *Rana brevipoda porsa* [16] and subsequently from a wide range of European (*R. esculenta*), Asian (*Rana rugosa*) and North American (*Rana catesbeiana*, *Rana luteiventris*, *Rana berlandieri*, *Rana pipiens*, *R. sphenocephala*, *Rana palustris*) species (reviewed in [9,10,17]). All members of the family characterized to date comprise 24 amino acid residues and contain a C-terminal cystine-bridged, heptapeptide ring. The primary structures of selected brevinin-1 peptides are shown in Fig. 3 in order to demonstrate that conservation of amino acid sequence in the family has been very poor. Only four residues (Pro[3], Ala[9], Cys[18], Lys[23], Cys[24]) are invariant in the known peptides. However, all brevinin-1 peptides contain

Brevinin-1SY	FLPVVAGLAA KVLPSIICAV TKKC	[This study]
Brevinin-1	----L--I-- --V-ALF-KI ----	[16]
Brevinin-1Ea	---AIFRM-- --V-T---SI ----	[13]
Brevinin-1Eb	VI-F--SV-- EMMQHVY--A SR--	[13]
Brevinin-1Sc	-F-I---V-G Q--KK-Y-TI S---	[14]

Fig. 3. A comparison of the primary structures of peptides of the brevinin-1 family that were selected to illustrate lack of conservation of amino acid sequence. Brevinin-1SY is from *R. sylvatica*, brevinin-1 is from *R. brevipoda porsa*, brevinin-1Ea and -1Eb are from *R. esculenta* and brevinin-1Sc is from *R. sphenocephala*. (–) denotes residue identity and the residues underlined in brevinin-1SY are invariant in all members of the family.

a preponderance of basic residues (primarily lysine) and have the ability to adopt amphipathic α-helical conformations upon interaction with the lipid bilayer of the bacterial cell membrane [18]. The family belongs, therefore, to a wider group of cationic peptide antibiotics whose common mechanism of action has been discussed [19].

An unexpected feature of the study was the observation that the skin of *R. sylvatica* produced only a single detectable antimicrobial peptide. In general, a particular amphibian species synthesizes a range of antimicrobial peptides belonging to different families with each family containing several isoforms. For example, 22 antimicrobial peptides belonging to eight different families have been isolated from skin secretion of the pickerel frog, *R. palustris* [17]. Despite the sequence similarities, the members of a particular family have a distinct spectra of antimicrobial activity and it has been speculated that this molecular diversity is important in protecting the animal from invasion by a wide array of different microorganisms [20]. In view of the pronounced decline in frog populations throughout North America, studies are clearly warranted to assess the ability of brevinin-1SY to inhibit the growth of those pathogenic microorganisms that the wood frog may encounter in the wild.

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