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Isolation of peptides of the brevinin-1 family with potent candidacidal activity from the skin secretions of the frog *Rana boylii*

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Abstract: The emergence of strains of the human pathogen *Candida albicans* with resistance to commonly used antibiotics has necessitated a search for new types of antifungal agents. Six peptides with antimicrobial activity were isolated from norepinephrine-stimulated skin secretions from the foothill yellow-legged frog *Rana boylii*. Brevinin-1BYa (FLPILASLAA^{10}KFGPKLF CLV^{20}TKKC) was particularly potent against *C. albicans* [minimal inhibitory concentration (MIC) = 3 μM] and also active against *Escherichia coli* (MIC = 17 μM) and *Staphylococcus aureus* (MIC = 2 μM), but its therapeutic potential for systemic use is limited by its strong hemolytic activity (HC_{50} = 4 μM). The single amino acid substitution (Phe^{12} → Leu) in brevinin-1BYb resulted in a fourfold lower potency against *C. albicans* and the additional amino acid substitutions (Lys^{11} → Thr, Phe^{17} → Leu and Val^{20} → Ile) in brevinin-1BYc resulted in a ninefold decrease in activity. Two members of the ranatuerin-2 family and one member of the temporin family were also isolated from the secretions but showed relatively low potency against the three microorganisms tested.

Introduction

Candidiasis, usually caused by *Candida albicans* but also by other *Candida* spp. is the most common of the opportunistic fungal infections (1). Prevalence is particularly high in a hospital environment (nosocomial infection) (2) and in individuals with immune deficiencies such as human immunodeficiency virus-infected patients and bone marrow transplant recipients (3,4). A variety of antifungal agents (fluconazole and related azoles, amphotericin B) are

available for treatment of *Candida* spp. infections but resistance to these drugs is rapidly increasing (5,6). Consequently, there is a clear need to develop new types of antifungal agents to which the pathogens have not been exposed in order to control infections caused by drug-resistant strains.

The skins of frogs of the genus *Rana* have proved to be a rich source of peptides with broad spectrum antibacterial activity, some of which show antifungal properties [reviewed in (7,8)]. The peptides are synthesized as a complex array of structurally related components that may be grouped into families on the basis of structural similarity. Among the North American ranid frogs, nine families of peptides have been identified: esculentin-1, esculentin-2, brevinin-1, ranatuerin-1, ranatuerin-2, temporin, palustrin-1, palustrin-2, and palustrin-3 [reviewed in (9)]. Among ranid frogs of Eurasian origin, additional families include brevinin-2 (10), tigerinin (11), japonicin-1 (12) and japonicin-2 (12). It has been speculated that this molecular diversity is important in protecting the animals against invasion by a wide range of microbial species (13).

The foothill yellow-legged frog *Rana boylii* is a relatively small (4–7 cm), strongly aquatic amphibian that inhabits streams in coastal and mountain areas of California and Oregon. Recent years have seen marked declines in population in several regions of their range that have been ascribed to disruptions of habitat, such as alteration of stream flows, pollution of the environment by wind-borne pesticides (14), and displacement by invasions of the bullfrog, *R. catesbeiana* (15). It is currently being petitioned that the species should be listed for Federal protection. The present study describes the isolation and characterization of six antimicrobial peptides from the skin secretions of *R. boylii*, one of which shows high potency against *C. albicans*.

Experimental

Collection of skin secretions

A single adult male specimen of *R. boylii* (body weight 18 g) was collected in Shasta County, Northern California under California Department of Fish and Game permit number 801023-05 issued to Carlos Davidson. The animal was transported to Vanderbilt University and housed in a polycarbonate vivarium containing wet and dry areas. The frog was fed live crickets three times weekly. All experimental procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committtee. Skin secretions were collected at monthly intervals for 5 months as previously described (9). In brief, the animal was injected bilaterally with norepinephrine (2 nmol/g body wt) into the dorsal lymph sac and placed in a solution (100 mL) of composition 25 mM sodium chloride/25 mM ammonium acetate, pH 7.0 for 15 min. The animal appeared to suffer no discomfort and is still alive more than 1 year after the series of experiments began.

The combined secretions and washings were acidified by addition of trifluoroacetic acid (1 mL) and passed at a flow rate of 2 mL/min through 2 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0 : 29.9 : 0.1, v/v/v) and freeze-dried.

Antimicrobial and hemolytic assays

Aliquots (50 μL) of the fractions of chromatographic effluent (Fig. 1) were tested for their ability to inhibit the growth of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (NCTC 8325). Lyophilized aliquots of chromatographic effluent, reconstituted in Mueller–Hinton broth (50 μL) were incubated with an inoculum (50 μL of 5×10^5 colony forming units/mL) from an overnight culture of the bacteria in 96-well microtiter cell-culture plates for 18 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. Incubations with *C. albicans* (ATCC 90028) were carried out in RPMI 1640 medium for 48 h at 35 °C. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. Minimal inhibitory concentrations (MICs), measured by a standard microdilution method (16), were taken as the lowest concentration of peptide where no visible growth was observed. In order to monitor the validity of the assays, incubations with *E. coli* and *S. aureus* were carried out in parallel with increasing concentrations of the broad-spectrum antibiotic, bacitracin and incubations with *C. albicans* in parallel with amphotericin B.

Peptides in the concentration range 1–200 μM were incubated with washed human erythrocytes (2×10^7 cells) from a healthy donor in Dulbecco's phosphate-buffered saline, pH 7.4 (100 μL) for 1 h at 37 °C. After centrifugation (12 000 × ***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 HC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis.

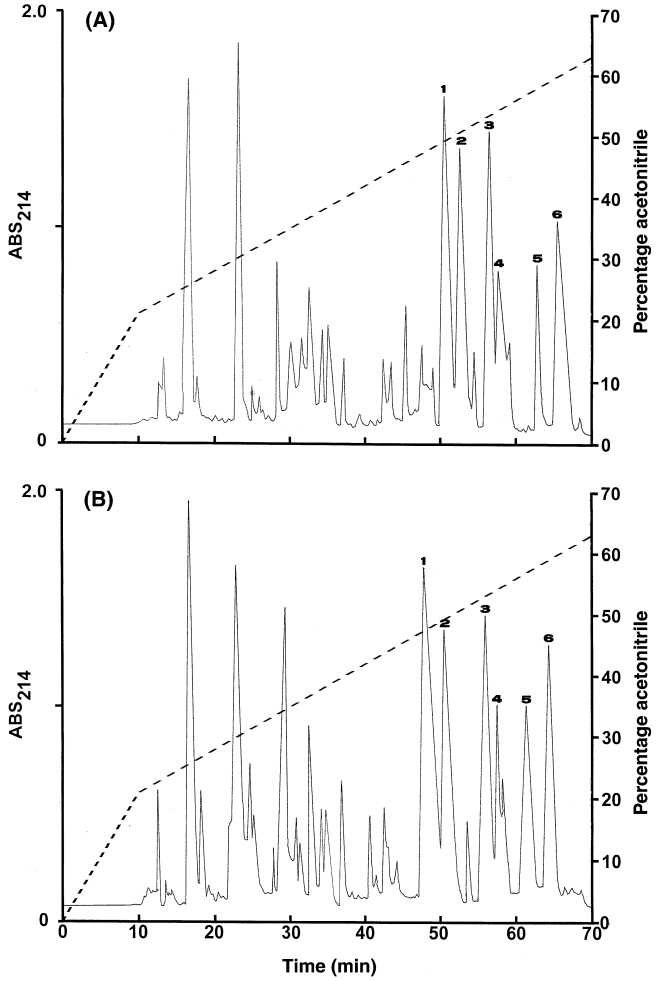

Figure 1. Reverse-phase high performance liquid chromatography on a semipreparative Vydac C-18 column of (A) a pooled sample of *Rana boylii* skin secretions collected over a 3-month period and (B) a pooled sample collected under the same conditions over a subsequent 2-month period. The peaks containing antimicrobial activity are designated as (1) ranatuerin-2BYa, (2) ranatuerin-2BYb (3) brevinin-1BYa, (4) brevinin-1BYb, (5) temporin-1BYa and (6) brevinin-1BYc. The dashed line shows the concentration of acetonitrile in the eluting solvent.

Purification of the peptides

The skin secretions, after concentration and partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) trifluoroacetic acid/water (2 mL). Secretions from months 1 to 3 were pooled together (sample A) and from months 4 and 5 were pooled together (sample B). The two samples were separately injected onto a (25 × 1 cm) Vydac 218TP510 (C-18) reverse-phase high performance liquid chromatography (HPLC) column (Separations Group) equilibrated with 0.1% trifluoroacetic acid/water at a flow rate of 2 mL/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and 63% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 and 280 nm and fractions (1 min) were collected. The fractions containing antimicrobial activity were rechromatographed on (25 × 1 cm) Vydac 214TP510 (C-4) column. The concentration of acetonitrile in the eluting solvent was raised from 35 to 63% over 40 min and the flow rate was 2 mL/min.

Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems (Foster City, CA, USA) procise 494 sequenator. MALDI mass spectrometry was carried out using a Voyager RP MALDI-TOF instrument (Perspective Biosystems Inc., Framingham, MA, USA) equipped with a nitrogen laser (337 nm). The instrument was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 25 kV. The instrument was calibrated with a range of synthetic peptides and the accuracy of mass determinations was within 0.02%.

Results

Purification of the peptides

The two pooled samples of concentrated secretions (samples A and B) were tested for their ability to inhibit growth of the fungus *C. albicans*, the Gram-negative bacterium *E. coli* and the Gram-positive bacterium *S. aureus*. Antimicrobial activities against the three microorganisms were detected in both crude extracts even at high sample dilution. Samples A and B were separately chromatographed on a semi-preparative Vydac C-18 column. The elution profiles, shown in Fig. 1A and B, are remarkably similar particularly in the region of the chromatogram associated with antimicrobial activity (retention times between 47 and 66 min). Five prominent, well resolved peaks, subsequently shown to contain ranatuerin-2BYa (peak 1), brevinin-1BYa (peak 3), brevinin-1BYb (peak 4), temporin-1BYa (peak 5), and brevinin-1BYc (peak 6) were associated with growth-inhibitory activity towards *S. aureus*. Peaks 3, 4, and 6 inhibited growth of *C. albicans*. Peak 2 (Fig. 1) contained a peptide, subsequently shown to be ranatuerin-2Byb, that inhibited the growth of *E. coli* only. The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape, by further chromatography on a semipreparative Vydac C-4 column. Purification of brevinin-1BYa is shown in Fig. 2. The final yields (nmol) of the pure peptides,

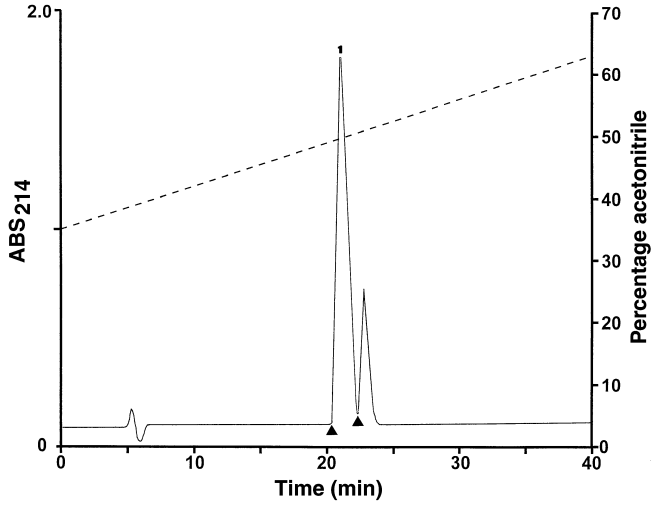

Figure 2. Purification of brevinin-1BYa (peak 1) on a semi-preparative Vydac C-4 column. The arrowheads show where peak collection began and ended.

from both purifications combined, were brevinin-1BYa (194), brevinin-1BYb (152), brevinin-1BYc (107), ranatuerin-2BYa (223), ranatuerin-2Byb (225), temporin-1BYa (70).

Peptide characterization

The primary structures of the antimicrobial peptides were established by automated Edman degradation and their amino acid sequences are shown in Fig. 3. All sequences were obtained without ambiguity except for uncertainty regarding the C-terminal residue of temporin-1BYa. Mass spectrometry demonstrated that this amino acid was either Leu amide or Ile amide but could not distinguish between the two. However, of 30 temporin peptides that had been characterized by the year 2000 (17), 27 terminate in Leu.NH_2 and none terminates with Ile.NH_2. Mass spectrometry demonstrated the presence of a cystine bridge in the brevinin-1 and ranatuerin-2 peptides and the absence of post-translational modifications to individual amino acids (Fig. 3).

Antimicrobial and hemolytic properties

The abilities of the peptides isolated in this study to inhibit the growth of *C. albicans*, *E. coli* and *S. aureus* and to produce hemolysis of human erythrocytes are compared in Table 1.

Discussion

This article describes the purification and characterization of six previously undescribed antimicrobial peptides from the skin secretions of *R. boylii*. The primary structure of three of the peptides identifies them as members of the brevinin-1 family, first isolated from the skin of the Asian frog *Rana brevipoda porsa* (10) and subsequently from several species of ranid frogs of North American (9,18–23) and European (24) origin. A comparison of the amino acid sequences of the peptides (Fig. 3) provides insight into structure-activity relationships within the brevinin family. The ability of a peptide to lyse cells is the result of a complex interelationship of factors involving conformation, charge, hydrophobicity and amphipathicity (25). The brevinin-1 peptides generally comprise a N-terminal hydrophobic region, a proline containing hinge region in the central portion and a C-terminal disulfide-bonded loop (26). Circular dichroism studies have shown that the brevinins adopt an amphipathic α-helical conformation in a

Peptide	Primary Structure	Mass (a.m.u.)
Brevinin-1BYa	FLPILASLAAKFGPKLFCLVTKKC	2605.4 (2605.6)
Brevinin-1BYb	FLPILASLAAKLGPKLFCLVTKKC	2571.5 (2571.5)
Brevinin-1BYc	FLPILASLAATLGPKLLCLITKKC	2524.5 (2524.5)
Ranatuerin-2BYa	GILSTFKGLAKGVAKDLAGNLLDKFKCKITGC	3306.8 (3306.8)
Ranatuerin-2BYb	GIMDSVKGLAKNLAGKLLDSLKCKITGC	2873.3 (2873.6)
Temporin-1BYa	FLPIIAKVLSGLL.NH_2	1381.9 (1381.9)

Figure 3. Amino acid sequences of the antimicrobial peptides isolated from *Rana boylii* skin secretions.

Table 1. **Minimal inhibitory concentrations (MICs) and hemolytic activities (HC_{50}) of peptides isolated from *Rana boylii* skin secretions**

	MICs (μM)			
	Candida albicans	*Escherichia coli*	*Staphylococcus aureus*	HC_{50} (μM)
Brevinin-1BYa	3	17	2	4
Brevinin-1BYb	16	16	4	4
Brevinin-1BYc	35	NA	8	ND
Ranatuerin-2BYa	NA	7	27	120
Ranatuerin-2BYb	NA	17	NA	>200
Temporin-1BYa	NA	NA	15	ND

NA, not active at 50 μM; ND, not determined.

membrane-mimetic environment (26). As shown in Table 1, the substitution ($Phe^{12} \rightarrow Leu$) in brevinin-1BYa results in a fourfold reduction in potency against *C. albicans* and a twofold reduction against the gram-positive bacterium *S. aureus* without significant change in potency against the Gram-negative bacterium *E. coli* or in hemolytic activity. This selective reduction in potency may be ascribed to a decrease in hydrophobicity. In a series of analogs related to magainin 2, it was shown that increasing overall hydrophobicity and hydrophobic moment (27) had relatively little influence on activity against Gram-negative bacteria but enhanced activity against Gram-positive bacteria (28). The appreciably lower potency of brevinin-1BYc against microorganisms, particularly *E. coli*, may be ascribed to the substitution $Lys^{11} \rightarrow$ Thr. The cationic residues in an antimicrobial peptide are considered to be important in the initial binding to the negatively charged phospholipids in the cell membranes of microorganisms (25) and, up to a certain limit, there is a good correlation between peptide cationicity and antimicrobial potency in a range of systems (29).

Brevinin-1BYa is the most potent peptide against *C. albicans* to be isolated from frog skin by the present investigators which, taken together with its broad spectrum antibacterial activity, make it a candidate for drug development. However, its therapeutic potential is seriously limited by its strong hemolytic activity. A helical wheel representation of brevinin-1BYa indicates that the peptide has the propensity to form a strongly amphipathic α-helix. Several studies have demonstrated a direct correlation between degree of amphipathicity and hemolytic activity, not only in peptides that form an α-helix (28,30,31), but also those that adopt a β-sheet conformation (32). It has been suggested that increasing the hydrophobic moment of an antimicrobial peptide has a relatively modest effect on the ability to permeabilize the negatively charged cell membrane of microorganisms but a marked effect on the more zwitterionic phospholipid membrane of the erythrocyte (30). Further studies, therefore, will focus upon the synthesis of brevinin-1BYa analogs with increased cationicity but reduced amphipathicity.

Peptides of the ranatuerin-2 family were first isolated from extracts of the skin of the bullfrog, *R. catesbeiana* (18) but are widely distributed in ranid frogs of North American origin (9,20,21,23). Consistent with properties of ranatuerin-2BYa and ranatuerin-2BYb, the ranatuerins generally show low potencies against human pathogens including *C. albicans* but ranatuerin-2P, isolated from the skin of *R. pipiens* (20), has been shown to be active against frog virus 3, a pathogenic iridovirus infecting anurans (33) and three members of the family are active against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines (34). Peptides of the temporin family were first isolated from the skin of the European frog, *Rana temporaria* (35) but are also widely distributed among ranid species (17). Consistent with previous data from the laboratory (20,21,23), temporin-1BYa selectively inhibited the growth of *S. aureus*.

The reproducible reverse-phase HPLC elution profiles of samples of *R. boylii* skin secretions taken at different times several months apart (Fig. 1) illustrate the potential of this methodology for taxonomic identification of closely-related species or sub-populations of a particular species. Skin secretions can be obtained from the animal under non-invasive conditions, even from endangered species in the wild, so that such a chromatogram provides a 'fingerprint' that can be used for species characterization. Combining HPLC with mass spectrometric analysis permits unambiguous assignment of species. On the basis of morphological and biochemical criteria (36,37), *R. boylii* is regarded as belonging to a monophyletic group comprising *R. boylii*, *R. aurora*, *R. cascadae*, *R. muscosa*, *R. pretiosa* and

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Brevinin-1BYa                  FLPILASLAAKFGPKLFCLVTKKC
Brevinin-1BYb                  -----------L------------
Brevinin-1BYc                  ----------TL----L--I----
Brevinin-1La                   ---M--G---SMV---V--I----
Brevinin-1Lb                   ---M--G---SMV--FV--I----

Ranatuerin-2BYa       GILSTFKGLAKGVAKDLAGNLLDKFKCKITGC
Ranatuerin-2BYb       --MDSV-----    N---K---SL-------
Ranatuerin-2La        ---DS---V----------K----L-------
Ranatuerin-2Lb        ----SI--V------NV-AQ---TL-------

Temporin-1BYa          FLPIIA KVLSGLL.NH2
Temporin-1La           V--L-S MA-GK--.NH2
Temporin-1Lb         N--GTLINLAKK IM.NH2
Temporin-1Lc           ----LINLIHK---.NH2
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Figure 4. A comparison of the primary structures of the brevinin-1 and ranatuerin-2 peptides isolated from *Rana boylii* and *R. luteiventris*. (–) denotes residue identity. Peptides designated –BY are from *R. boylii* and designated –L are from *R. luteiventris*. Gaps have been introduced into the sequences to maximize structural similarity.

R. luteiventris. Although this classification has been questioned (38), recent data involving the comparison of the nucleotide sequences of several mitochondrial genes support monophyletic status (39). As shown in Fig. 4, the high degree of amino acid sequence similarity between the brevinin-1 and ranatuerin-2 peptides isolated from the skins of *R. boylii* and the Columbia spotted frog *R. luteiventris* (20) supports a close phylogentic relationship between the species. However, the amino acid sequences of the temporin peptides have been much less well conserved between the two frogs. The primary structure of temporin-1BYa (FLPIIAKVLSGLL.NH_2) resembles much more closely the consensus sequence (FLPLIASLLSKLL.NH_2) that has been derived by Wade (17) from an analysis of the known temporin sequences than do the primary structures of the temporin-1La, -1Lb, and -1Lc. Assignment of these *R. luteiventris* peptides to the temporin family is somewhat arbitrary.

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