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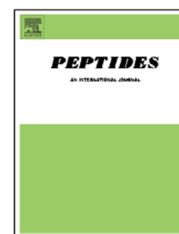

Peptide defenses of the Cascades frog *Rana cascadae*: implications for the evolutionary history of frogs of the *Amerana* species group

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ABSTRACT

The Cascades frog *Rana cascadae* belongs to the *Amerana* (or *Rana boylii*) group that includes six additional species from western North America (*R. aurora*, *R. boylii*, *R. draytonii*, *R. luteiventris*, *R. muscosa*, and *R. pretiosa*). *R. cascadae* is particularly susceptible to pathogenic microorganisms in the environment and populations have declined precipitously in parts of its range so that the protection afforded by dermal antimicrobial peptides may be crucial to survival of the species. Peptidomic analysis of norepinephrine-stimulated skin secretions led to the identification of six peptides with differential cytolytic activities that were present in high abundance. Structural characterization showed that they belonged to the ranatuerin-2 (one peptide), brevinin-1 (one peptide), and temporin (four peptides) families. Ranatuerin-2CSa (GILSSFKGVAKGVAKDLAGKLLETLKCKITGC) and brevinin-1CSa (FLPILAGLAAKIVPKLFCLATKKC) showed broad spectrum antibacterial activity (MIC $\leq 32\ \mu M$ against *Escherichia coli* and *Staphylococcus aureus*) but only brevinin-1CSa was strongly hemolytic against human erythrocytes ($LC_{50} = 5\ \mu M$). The taxonomy of ranid frogs is currently in a considerable state of flux. The ranatuerin-2 gene is expressed in all members of the *Amerana* group studied to-date and cladistic analysis based upon a comparison of the amino acid sequences of this peptide indicates that *R. cascadae*, *R. muscosa* and *R. aurora* form a clade that is distinct from one containing *R. draytonii*, *R. boylii*, and *R. luteiventris*. This conclusion is consistent with previous analyses based upon comparisons of the nucleotide sequences of mitochondrial genes.

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1. Introduction

Frogs belonging to the genus *Rana*, often referred to as ''true frogs'', are a relatively successful group of anurans with more than 250 species distributed worldwide, except for the Polar Regions, southern South America and most of Australia. Approximately one-quarter of known species are found in the New World [12]. On the basis of morphological criteria, five species (*R. aurora*, *R. boylii*, *R. cascadae*, *R. muscosa*, and *R. pretiosa*) that occur in the western states of the U.S.A. were originally classified together in the *Amerana* species group (also known as the *R. boylii* group) [10]. Subsequently, *R. pretiosa* was divided into two species with distinct geographical ranges (*R. pretiosa* in the west and *R. luteiventris* in the east) [18]. More recently, the red-legged frogs, traditionally regarded as a single polytypic species with two subspecies, the northern red-legged frog *R. aurora aurora* and the California red-legged frog *R. aurora draytonii* have also been classified as two separate species, *R. aurora* and *R. draytonii* [33]. Molecular data based upon mitochondrial DNA sequences [21,24] support earlier claims [35] proposing monophyletic status for the *Amerana* and suggest that the group is approximately 8 million years old. However, phylogenetic and evolutionary relationships among members of the group have yet to be fully resolved.

The Cascades frog *R. cascadae* is a medium sized (adult males 5–6 cm, adult females 6–7.5 cm), diurnal frog that inhabits mountainous streams and ponds in forested areas in the Cascade Mountains in a band from Washington to northern California [29]. The species is still fairly abundant in parts of its range but has disappeared completely from other areas, particularly in the southern edge of its range in California [9,13,36]. Multiple factors have been proposed for this decline and include drought [13], destruction of habitat due to logging and agriculture [9,13], introduction of aquatic predators such as trout and the bullfrog *R. catesbeiana* [36], wind-borne agrochemicals [8,9], and sensitivity of oocytes to increased UV-B radiation [22,23]. *R. cascadae* is particularly susceptible to the pathogenic oomycete *Saprolegnia ferax* [22,23] and to the chytrid fungus *Batrachochytrium dendrobatidis* [15]. Consequently, the ability of the animal to mount a defense against these and other microorganisms in the environment may be of critical importance to the survival of the species [27].

Cationic α-helical peptides with broad-spectrum antibacterial and antifungal activities are synthesized in the skins of the majority, but by no means all, species of ranid frogs and represent a component of the animal's system of innate immunity [5,19]. The peptides may be grouped into families on the basis of limited structural similarity but the variation in amino acid sequences of homologous peptides is considerable and it is rare that orthologs have an identical primary structure, even when they are from species that are quite closely related phylogenetically [5]. Previous studies have led to the purification and structural characterization of antimicrobial peptides from the skin secretions of several species belonging to the *Amerana* group: *R. luteiventris* [17], *R. boylii* [7], *R. aurora* [6], *R. draytonii* [3], and *R. muscosa* [32]. The present investigation extends this program by describing the purification and characterization of multiple peptides with antimicrobial activity from skin secretions of *R. cascadae*. The nomenclature used to describe the peptides is the same as that used for other *Rana* skin peptides with CS indicating the species and the isoforms designated by lower case letters.

2. Materials and methods

2.1. Collection of skin secretions

Animals were collected during July in Gumboot Creek, Shasta Trinity National Forest, Siskiyou County, California, under permit (California Department of Fish and Game scientific collection permit 801031-02). Skin secretions were obtained by a procedure previously described [3,32]. In brief, adult and sub-adult specimens of indeterminate sex ($n = 8$; weight 5–15 g) were injected bilaterally with norepinephrine (10 nmol/g body wt.) and individually placed in distilled water (100 ml) for 15 min. The combined secretions and washings were acidified by addition of trifluoroacetic acid (0.5 ml) and immediately frozen. After stimulation, all animals were released unharmed at the exact sites of collection.

2.2. Antimicrobial and hemolytic assays

Purification of the peptides was monitored by incubating lyophilized aliquots of chromatographic effluent in Mueller–Hinton broth (50 μl) with an inoculum (50 μl of 10^6 colony forming units ml^{-1}) from a log-phase culture of reference strains *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25726) 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 were carried out in parallel with increasing concentrations of ampicillin. Minimum inhibitory concentration (MIC) was measured by a standard microdilution method [26] and was taken as the lowest concentration of peptide where no visible growth was observed.

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 ($12{,}000 \times g$ 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 LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in three independent experiments.

2.3. Peptide purification

Skin secretions were passed at a flow rate of 2 ml min^{-1} through 8 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0:29.9:0.1, v/v/v) and freeze-dried. The lyophilisate was redissolved in 0.1% (v/v) trifluoroacetic acid/water (4 ml) and injected onto a (2.2-cm $\times$ 25-cm) Vydac 218TP1022 (C-18) reverse-phase HPLC column (Separations Group, Hesperia, CA) equilibrated with

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