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Development of an assay for testing the antimicrobial activity of skin peptides against the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) using *Xenopus laevis* ☆

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Abstract

This report describes the preliminary characterization of a bioassay for testing the antimicrobial activity of amphibian skin peptides against the chytrid fungus, *Batrachochytrium dendrobatidis*. Peptide secretions from *Xenopus laevis* were induced by subcutaneous injections of norepinephrine. Partially purified secretions were quantified and incubated at various dilutions with 10^7 cells/mL of freshly isolated zoospores for 7 days. Peptide bioactivity was measured as cell growth inhibition over the incubation period. The concentration that inhibited growth by 80% or greater (IC_{80}), based on the linear portion of the growth curve, averaged 457 ± 158 μg/mL. Growth curve slopes of best-fit line equations for individual samples were less variable within control groups than the average IC_{80} value, and are viewed as a more reliable indicator of peptide mixture bioactivity. This assay may be useful for evaluating the impact of environmental chemicals on amphibian host resistance to potentially lethal skin infections.
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Keywords: *Xenopus laevis*; Amphibian; *Batrachochytrium dendrobatidis*; Chytrid; Antimicrobial peptides; Bioassay; Amphibian declines

1. Introduction

Current downward trends in global amphibian populations are a major concern for amphibian conservation and have serious implications for environmental quality. Amphibian declines appear to be associated, in part, with a decrease in resistance to opportunistic or new pathogens that cause disease or deformities (Daszak et al., 1999; Carey et al., 1999; Kiesecker, 2002). Strong evidence suggests that pathogens, such as the chytrid fungus *Batrachochytrium dendrobatidis*, are the cause of many localized mass mortality events of anuran species. *B. dendrobatidis* zoospores infect the keratinized epithelia of amphibians where they mature into sessile, spherical zoosporangia equipped with discharge papillae for disbursement of newly formed zoospores. Some amphibians subsequently develop the infectious disease chytridiomycosis, which is characterized by dermal hyperkeratosis and hyperplasia. These physiological changes are believed to lead to host death through disruption of the respiratory and osmotic regulating functions of amphibian skin (Berger et al., 1998; Pessier et al., 1999). Neurological effects of *B. dendrobatidis* infection, which may also contribute to the host mortality, have been described in some amphibians (Berger et al., 1998). These effects are thought to be the result of putative toxins secreted by the pathogen (Berger et al., 1998).

B. dendrobatidis has only recently been recognized as an amphibian pathogen (Berger et al., 1998). The rapid spread of the pathogenic chytrid fungus in amphibian species (Rollins-Smith and Conlon, 2005) may suggest

☆ *Animal Welfare Statement*: This study was conducted in accordance with national and institutional guidelines for the protection of animal welfare as outlined in the NIH Office of Laboratory Animal Welfare Public Health Service Policy on Humane Care and Use of Laboratory Animals (2002) and the University of Louisiana at Monroe Handbook for the Care & Use of Laboratory Animals.

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compromised amphibian immunity (Carey et al., 1999; Rollins-Smith, 2001). However, a link between contaminant exposure and emerging diseases in amphibian species remains speculative. Although many chytrid-induced mass mortality events have occurred in protected areas that lack direct anthropogenic input, traces of persistent agricultural and industrial chemicals are likely present in protected/pristine areas where mass mortality events have occurred (Carey et al., 2001). Several pesticide mixtures have been shown to modulate the amphibian immune system (Christin et al., 2004; Gendron et al., 2003; Gilbertson et al., 2003; Taylor et al., 1999). Recent evidence suggests a correlation between wind patterns, pesticide application and amphibian declines in California (Davidson et al., 2001, 2002). One of these pesticides, carbaryl, inhibits the release of protective antimicrobial skin peptides in young frogs (Davidson et al., 2007). Therefore, further studies of the impact of low-level environmental contamination on amphibian host-defense mechanisms are needed.

Each amphibian species secretes a unique suite of peptides that are individually and synergistically bioactive against a variety of pathogens (Erspamer, 1994; Simmaco et al., 1998; Rinaldi, 2002; Conlon et al., 2004; Apponyi et al., 2004; Pukala et al., 2006). In *Xenopus laevis*, bioactive peptides include the cationic peptides magainin, caerulein precursor fragment (CPF), and PGLa (Bevins and Zasloff, 1990). Most amphibian antimicrobial peptides are amphipathic in nature, which allows them to form α-helices in the phospholipid bilayers of target cells (Simmaco et al., 1998). The inter-lipid formation of α-helices disrupts membrane function and ultimately leads to cell death. Although peptide secretion is known to be regulated by the sympathetic nervous system, little is known about stimulation of secretions in indigenous populations. In the laboratory, secretions can be stimulated via injection or immersion in solutions containing agents that activate adrenergic receptors (epinephrine and norepinephrine) (Gibson et al., 1986; Giovanni et al., 1987).

The lack of evidence for a cell-mediated immune response to chytrid infection suggests that secreted skin peptides may be a critical amphibian defense against the chytrid pathogen (Berger et al., 1998; Pessier et al., 1999). Recent studies indicate that two declining chytrid-sensitive amphibian species in Australia have less secretions with lower potency than non-declining species (Woodhams et al., 2006). Experimental infection studies of four Australian species using *B. dendrobatidis* showed a significant correlation between skin peptide effectiveness against this pathogen *in vitro* and resistance of infected animals when exposed (Woodhams et al., 2007). These observations support the hypothesis that secreted peptides have a crucial role in amphibian resistance to lethal chytrid infection. Therefore, inhibition of the production or release of these antimicrobial peptides by xenobiotic agents may have significant consequences for amphibian pathogen resistance and subsequent survival. In an effort to facilitate such investigations, a growth inhibition assay developed by Rollins-Smith et al. (2002a) was evaluated as an amphibian bioassay. This assay utilizes infectious *B. dendrobatidis* zoospores and amphibian secretions released from granular skin glands. These glands produce, store, and secrete complex mixtures of bioactive peptides that are individually and synergistically bioactive against several pathogens *in vitro*, including *B. dendrobatidis* (Soravia et al., 1988; Zasloff, 1987; Rollins-Smith et al., 2002a). Therefore, the objective of the present study was to develop an amphibian bioassay for evaluation of the impact of environmental chemicals on amphibian antimicrobial peptide production and activity. *X. laevis* was selected as the amphibian model, in part, because this species has well-characterized peptide secretions, are commercially available, and are easily maintained in the laboratory. The lack of chytrid-induced mortality among *X. laevis* in the wild suggests the suite of peptides secreted by this taxon provide crucial protection from the chytrid pathogen. If this is the case, disruption of the synthesis/secretion of peptides would interfere with this defense, leaving the animals more susceptible to fatal infections. Once developed, this bioassay can be applied to other species that have varying degrees of susceptibility to the chytrid fungus, including species that are currently in decline.

2. Materials and methods

2.1. Ethics statement

This study was conducted in accordance with national and institutional guidelines for the protection of animal welfare as outlined in the NIH Office of Laboratory Animal Welfare Public Health Service Policy on Humane Care and Use of Laboratory Animals (2002) and the University of Louisiana at Monroe Handbook for the Care & Use of Laboratory Animals.

2.2. Maintenance of animal and pathogen cultures

X. laevis embryos and juveniles were purchased from Nasco (Fort Atkinson, WI). Animals were housed in a 520 L Min-O-Cool tank containing aerated, charcoal-filtered tap water maintained at $20\pm2\,^{\circ}C$. A 12-h light and 12-h dark photoperiod was automatically maintained with cool-white fluorescent lights that provided a light intensity ranging from 200 to 300 lx. Nieuwkoop and Faber Stages 49/50 embryos (Nieuwkoop and Faber, 1967) were acclimated for approximately 21 days before assignment to treatment groups and juveniles were acclimated for at least 14 days before assignment to treatment groups. All animals were maintained in flow-through systems that were monitored weekly for temperature, pH, dissolved oxygen, conductivity, and hardness. All water quality measurements were within generally acceptable limits: temperature, $21\pm1\,^{\circ}C$; pH, 7.4 ± 0.2; dissolved oxygen, 6.4 ± 1.0 mg/L; conductivity, $195\pm4\,\mu\text{ʊ}$; hardness, 55 ± 1 mg/L as $CaCO_3$. Larvae were maintained at a density of 1 tadpole/5 L of water and fed Nasco tadpole powder three times per week at a rate of 2 g/10 L water. Postmetamorphic froglets and juveniles were fed Nasco frog brittle at a rate of 1 g/animal.

B. dendrobatidis cultures were received from stocks maintained by Joyce Longcore at the University of Maine. Cultures were grown on TGhL agar (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, and 10 g agar per 1 L milliQ water) or in H broth (10 g tryptone and 3.2 g glucose per 1 L milliQ water) at $20\pm1\,^{\circ}C$. Broth cultures were passaged every 4 days to ensure cells were in active growth phases. *B. dendrobatidis* zoospores were harvested from agar plates inoculated with H broth