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Purification of peptides with differential cytolytic activities from the skin secretions of the Central American frog, *Lithobates vaillanti* (Ranidae)

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ABSTRACT

Peptide-based defenses of ranid frogs from Mexico and Central America have been studied in much less detail than those from North America. Peptides belonging to the brevinin-1 (5 peptides), palustrin-2 (1 peptide), and ranatuerin-2 (3 peptides) families were isolated from norepinephrine-stimulated skin secretions of the Costa Rican frog, *Lithobates vaillanti* (Ranidae) and characterized structurally. Brevinin-1VLa (FLGAIAG-VAAKFLPKVFCFITKKC) and brevinin-1VLc (FLPVIASVAAKVLPK VFCFITKKC) showed particularly high growth-inhibitory potency (MIC $\leq$3 μM) against a Gram-positive microorganism *Staphylococcus aureus* and the opportunistic yeast pathogen *Candida albicans* and potent cytolytic activity ($LC_{50} \leq 8$ μM) against both human erythrocytes and HepG2 hepatoma-derived cells. The peptides were also active against a Gram-negative microorganism *Escherichia coli* (MIC $\leq$ 50 μM). Substitutions in brevinin-1VLd ($Lys^{11} \rightarrow$ Asn) and brevinin-1VLe ($Lys^{11} \rightarrow$ Ser) that decrease cationicity result in loss of activity against *E. coli*. Ranatuerin-2VLb (GIMDTIKGAAKDLAGQLLDKLKCKITKC) showed relatively weak antimicrobial activity (MIC $\geq$ 75 μM) but selective cytolytic activity against HepG2 tumor cells ($LC_{50} = 30$ μM) compared with erythrocytes ($LC_{50} > 200$ μM). In addition, a dodecapeptide (RICYAMWIPYPC) were isolated from the secretions that were devoid of antimicrobial activity. This component contains an Ala-Met bond that constitutes the scissile bond in the selective elastase inhibitor, elafin but the peptide did not inhibit pancreatic elastase at concentrations up to 100 μM.

1. Introduction

Peptides with broad-spectrum antibacterial and antifungal activities and with the ability to lyse mammalian cells are synthesized in the skins of the majority of species of frogs belonging to the family Ranidae that have been studied to-date (Conlon et al., 2004; in press-b). These peptides probably represent a component of the system of innate immunity that defends the animal against invasion by pathogenic microorganisms (Hancock, 2001; Rinaldi, 2002) and have excited interest as candidates for development into therapeutically valuable anti-infective agents. On the basis of limited similarities in amino acid sequence, the frog skin antimicrobial peptides may be grouped together in families that share a common evolutionary origin (Goraya et al., 2000; Duda et al., 2002) and at least 13 such families have been proposed (Conlon, 2008). 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 (Tennessen and Blouin, 2007). It is speculated that this molecular diversity may provide a broader spectrum of defense against pathogenic microorganisms encountered in the environment although firm evidence to support this hypothesis is lacking.

Skin secretions from frogs belonging to the family Ranidae from North America have been studied in detail and, at the time of writing, antimicrobial peptides have been isolated and characterized from 23 species (reviewed in Conlon et al., in press-b). In contrast, ranid frogs from Central America have been much less extensively investigated and studies have been confined to specimens of *Lithobates tarahumarae* collected in Mexico (Rollins-Smith et al., 2002). We now extend previous studies involving frogs of the genus *Lithobates* by describing the purification of peptides with cytolytic activity against microorganisms, human erythrocytes, and HepG2 hepatoma-derived cells from norepinephrine-stimulated skin secretions from the Costa Rican frog *Lithobates vaillanti*. Nomenclature adopted for antimicrobial peptides from frogs of the Ranidae family follows recent guidelines (Conlon, 2008).

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Vaillant's frog *L. vaillanti* (Brocchi, 1877) is a relatively large, sexually dimorphic animal (males 67–94 mm, females 76–125 mm snout-vent length) that is widely distributed in Central America from Veracruz, Mexico to Northern Columbia and Ecuador and is particularly common in Costa Rica (Savage, 2002). There is, however, both morphological and molecular evidence that the frogs in Columbia and Ecuador may not be conspecific with those in the more northern range (Hillis and Wilcox, 2005). The species is semiaquatic being found at low to moderate elevations associated with pools of stagnant water and slow-moving streams and rivers. Although subject to predation by snakes and introduced invasive species such as the bullfrog, *L. catesbeianus*, the species is not considered to be under threat.

2. Experimental

2.1. Collection of skin secretions

All experiments with live animals were approved by the IACUC committee of the University of Missouri-St Louis and were carried out by authorized investigators. Three specimens of *L. vaillanti* [body masses 18 (male), 84 (female) and 120 (female) g] were collected in Costa Rica near Siquirres in Limon Province under permit from the Ministerio del Ambiente y Energia Sistema Nacional de Àreas de Conservación. Each animal was injected via the dorsal lymph sac with norepinephrine hydrochloride (40 nmol/g body mass; 200 mL) and placed in a solution (100 mL) of composition 50 mM sodium chloride/25 mM sodium acetate, pH 7.0 for 15 min. The frog was removed and the collection buffer was acidified by addition of trifluoroacetic acid (TFA) (1 mL) and immediately frozen for shipment to UAE University. The secretions were pooled and passed at a flow rate of 2 mL/min through 6 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. Bound material was eluted with acetonitrile/water/TFA (70.0:29.9:0.1, v/v/v; 10 mL) and freeze-dried.

2.2. Antimicrobial assays

Purification of the peptides was monitored by incubating lyophilized aliquots of chromatographic effluent in Mueller–Hinton broth (50 mL) with an inoculum (50 mL 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. Incubations with *Candida albicans* (ATCC 90028) were carried out in RPMI 1640 medium for 48 h at 35 °C. In order to monitor the validity and reproducibility of the assays, incubations with bacteria were carried out in parallel with increasing concentrations of ampicillin and incubations with *C. albicans* in parallel with amphotericin B. Minimum inhibitory concentration (MIC) was measured in the concentration range of 1.5–200 mM by standard microdilution methods (Clinical and Laboratory Standards Institute, 2008a,b) and was taken as the lowest concentration of peptide where no visible growth was observed.

2.3. Cytolytic assays

Purified peptides (1–200 mM) 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 mL) 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 LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in three independent experiments.

HepG2 human hepatoma-derived cells (ATCC No. HB-8065) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured as previously described (Conlon et al., in press-a). Cells (2×10^7) were seeded into 96-well microtiter plates and grown to 90% confluence in Minimum Essential Medium, pH 7.25 containing 10% fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM glutamine (100 µL) in a humidified atmosphere of 5% CO_2 in air at 37 °C. One hour before incubation, the medium in each well was replaced with Minimum Essential Medium containing 5% fetal calf serum (100 mL). Peptides in the concentration range 1–100 mM were incubated with the cells for 1 h at 37 °C in duplicate. Cytolytic activity was determined by the measurement of release of lactate dehydrogenase into the medium using a CytoTox96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The LC_{50} value was taken as the mean concentration of peptide producing 50% cytolysis in three independent experiments.

2.4. Enzymatic assay

The ability of peptide RC-12 (20 µM and 100 µM) to inhibit the activity of porcine pancreatic elastase (Calbiochem, San Diego, CA, USA) was determined using succinyl-Ala-Ala-Pro-Abu-*p*-nitroanilide [Abu = L-α-aminobutyric acid] (Calbiochem) as substrate as described (Largman, 1983).

2.5. Peptide purification

The pooled skin secretions, after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) TFA/water (2 mL) and injected onto a (2.2×25-cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Separations Group, Hesperia, CA, USA) 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 mL) 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 (1×25-cm) Vydac 214TP510 (C-4) column and a (1×25-cm) Vydac

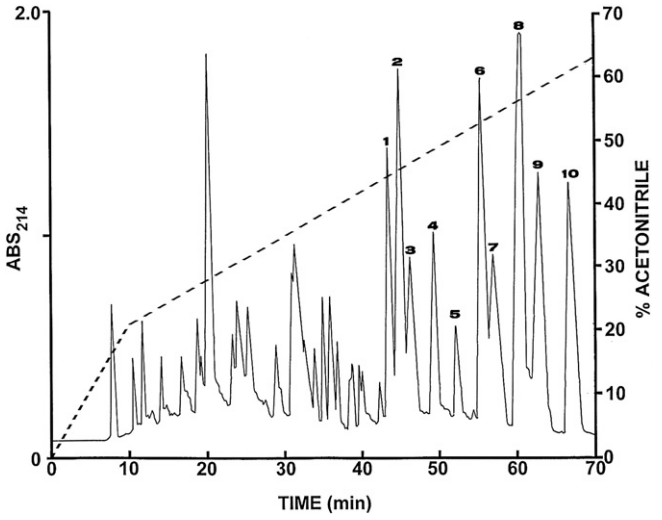

Fig. 1. Reversed-phase HPLC on a preparative Vydac C-18 column of skin secretions from *L. vaillanti* after partial purification on Sep-Pak cartridges. The peaks designated 1–3 and 5–10 displayed antimicrobial and hemolytic activities and were purified further. Peak 4 contained peptide RC-12. The dashed line shows the concentration of acetonitrile in the eluting solvent.