Biochimie 95 (2013) 1288–1296

Contents lists available at SciVerse ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Medusins: A new class of antimicrobial peptides from the skin secretions of phyllomedusine frogs

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ARTICLE INFO

Article history:
Received 13 November 2012
Accepted 4 February 2013
Available online 14 February 2013

Keywords:
Amphibian
Antimicrobial
Peptide
Molecular cloning

ABSTRACT

Natural drug discovery represents an area of research with vast potential. The investigation into the use of naturally-occurring peptides as potential therapeutic agents provides a new "chemical space" for the procurement of drug leads. Intensive and systematic studies on the broad-spectrum antimicrobial peptides found in amphibian skin secretions are of particular interest in the quest for new antibiotics to treat multiple drug-resistant bacterial infections. Here we report the molecular cloning of the biosynthetic precursor-encoding cDNAs and respective mature peptides representing a novel group of antimicrobial peptides from the skin secretions of representative species of phyllomedusine leaf frogs: the Central American red-eyed leaf frog (*Agalychnis callidryas*), the South American orange-legged leaf frog (*Phyllomedusa hypochondrialis*) and the Giant Mexican leaf frog (*Pachymedusa dacnicolor*). Each novel peptide possessed the highly-conserved sequence, LGMIPL/VAISAISA/SLSKLamide, and each exhibited activity against the Gram-positive bacterium, *Staphylococcus aureus* and the yeast, *Candida albicans*, but all were devoid of haemolytic effects at concentrations up to and including the MICs for both organisms. The novel peptide group was named medusins, derived from the name of the hylid frog sub-family, Phyllomedusinae, to which all species investigated belong. These data clearly demonstrate that comparative studies of the skin secretions of phyllomedusine frogs can continue to produce novel peptides that have the potential to be leads in the development of new and effective antimicrobials.

1. Introduction

Antimicrobial peptides are considered to be one of the most important components of the innate defence system of a variety of eukaryotic organisms including humans, plants and insects [1]. They are considered to be primitive as they are one of the first immune defence mechanisms to evolve in Nature that lack specific targets [2]. Their biosynthesis and secretion can be induced by the entry of foreign invading agents and some may be expressed constitutively [3]. Over the past 35 years, the therapeutic potential of antimicrobial peptides as putative novel antibiotics to arrest the global health crisis in antibiotic resistance has been critically-evaluated [4,5]. To survive in a microbe-rich environment, the innate immune response is the first line of defence against pathogen invasion and endogenous antimicrobial peptides are important natural compounds that act rapidly and effectively against microbes [1–5]. One of the major groups of agents in this respect is the low-molecular mass amphipathic cationic peptides [5].

Although amphibian skin secretions contain a broad range of biologically-active molecules, peptides are predominant in many species [6]. Those with antimicrobial activity are often the most abundant class of these with many hundreds of peptides of unique sequences having been isolated from this source to date [7]. With such a large number of molecular structures, it is possible to classify individual peptides into structurally-related families, most of which are taxon-specific. For example, brevinins, esculentins and temporins are found in ranid frogs [8], dermaseptins and phylloseptins from phyllomedusine leaf frogs and bombinins from bombinid toads [9,10]. With an ever-increasing interest in this area of research and an ever-increasing number of species being subjected to study, it is not surprising that antimicrobial peptides with unique structures that do not readily fit into existing families, are being reported with an increased frequency, examples being kassinatuerins [11,12], limnonectins [13] and kassorins [14]. It is, however, more unusual to discover a novel class of antimicrobial peptide in the skin secretions from multiple species within a taxon that has been extensively-studied for many years and in which

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http://dx.doi.org/10.1016/j.biochi.2013.02.005

several discrete families have long been established [15,16]. Here we report three highly-conserved representatives of a novel antimicrobial peptide family, one each from the skin secretions of representative species from three genera of phyllomedusine leaf frogs, *Agalychnis (callidryas)*, *Phyllomedusa (hypochondrialis)* and *Pachymedusa (dacnicolor)*. The peptides were given the name of medusins and were designated medusin AC, medusin PH and medusin PD, respectively. All three peptides were C-terminally amidated and consisted of 17 amino acid residues with the highly-conserved sequence: LGMIPL/VAISAISA/SLSKLamide. Each peptide was synthesised by solid-phase Fmoc chemistry and each possessed inhibitory activity against the model Gram-positive bacterium, *Staphylococcus aureus* and the model yeast, *Candida albicans*. Little haemolytic activity was demonstrable at peptide concentrations up to and including their respective MICs.

2. Materials and methods

2.1. Acquisition of skin secretions

Specimens of the Mexican leaf frog (*P. dacnicolor*), were obtained as captive-bred metamorphs (1.5 cm in length) and were raised to maturity over a period of 18 months. Specimens of the Central American red-eyed leaf frog (*A. callidryas*) (4–5 cm in length) and the South American orange-legged leaf frog (*P. hypochondrialis*) (2–3 cm in length) were obtained as captive-bred adults and were settled into their new surroundings for least three months prior to experimentation. All species were housed separately in purpose-designed terraria under 12 h/12 h light/dark cycles and were fed multivitamin-loaded crickets three times per week. Skin secretions were obtained by transdermal electrical stimulation after the method of Tyler et al. [17] and these were washed from the skin using de-ionized water, snap-frozen in liquid nitrogen, lyophilized and stored at −20 °C prior to analysis.

2.2. "Shotgun" cloning of cDNAs encoding novel peptide biosynthetic precursors

Five milligrams of each lyophilised skin secretion were separately dissolved in 1 ml of cell lysis/mRNA stabilization solution (Dynal, UK). Polyadenylated mRNA was isolated using magnetic oligo-dT beads as described by the manufacturer (Dynal Biotech, UK). The isolated mRNA was subjected to 3′-RACE procedures to obtain full-length prepromedusin nucleic acid sequence data using a SMART-RACE kit (Clontech UK) essentially as described by the manufacturer. Briefly, the 3′-RACE reactions employed a UPM primer (supplied with the kit) and a sense primer (S1; 5′- ACTTTCYGAWTTRYAAGMCCAAABATG-3′) (Y=C + T, W = A + T, R = A + G, M = A + C, B = T + C + G) that was designed to a highly-conserved segment of the 5′-untranslated region of phylloxin cDNA from *Phyllomedusa bicolor* (EMBL accession no. AJ251876) and the opioid peptide cDNA from *P. dacnicolor* (EMBL accession no. AJ005443). The PCR cycling procedure was carried out as follows: initial denaturation step: 90 s at 94 °C; 35 cycles: denaturation 30 s at 94 °C, primer annealing for 30 s at 58 °C; extension for 180 s at 72 °C. PCR products were gel-purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequencer.

2.3. Identification and structural analysis of the novel cDNA-deduced mature peptides

A further 5 mg of lyophilised skin secretion from each species, were dissolved separately in 0.5 ml of trifluoroacetic acid (TFA)/water (0.05:99.95, v/v) and clarified of microparticulates by centrifugation (2500 × g for 5 min). The clear supernatants were carefully decanted and separately pumped directly onto an analytical reverse phase HPLC column (Jupiter C_5; 250 mm × 4.6 mm, Phenomenex, UK) and peptides were eluted using a gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 80 min at a flow rate of 0.5 ml/min. A Cecil CE4200 Adept (Cambridge, UK) gradient reverse phase HPLC system was employed and fractions were collected automatically at 1 min intervals. The computed molecular masses of predicted novel mature peptides from open-reading frames within the cloned cDNAs were used to interrogate a mass spectral library of skin secretion peptides derived from sequential analysis of each reverse phase HPLC fraction using matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE mass spectrometer (Perseptive Biosystems, MA, USA) in positive detection mode using alpha-cyano-4-hydroxycinnamic acid as the matrix. Internal mass calibration of the instrument was achieved using standard peptides of established molecular mass providing a determined accuracy of ±0.1%. The peptides with masses coincident with those of putative novel cDNA-encoded peptides were each subjected to primary structural analysis by MS/MS fragmentation sequencing using an LCQ-Fleet electrospray ion-trap mass spectrometer (Thermo Fisher Scientific).

2.4. Solid-phase peptide synthesis

Following establishment of the unequivocal primary structures of the novel peptides, each was chemically-synthesized by solid-phase Fmoc chemistry using a PS3 automated solid-phase synthesizer (Protein Technologies, Inc., AZ, USA). Following cleavage from the resin and deprotection, each peptide was analyzed by both reverse-phase HPLC and electrospray mass spectrometry to establish both degree of purity and authenticity of structure.

2.5. Antimicrobial activity assays

Antimicrobial activity of each synthetic peptide was assessed by determination of minimal inhibitory concentration (MIC) using a standard Gram-positive bacterium (*S. aureus*—NCTC 10788), a standard Gram-negative bacterium (*Escherichia coli*—NCTC 10418) and a standard pathogenic yeast (*C. albicans*—NCYC 1467). Peptides were tested within the concentration range of 1–512 mg/L and these were made in Mueller-Hinton broth (MHB). Peptide solutions were inoculated with microorganism cultures (10^5 colony forming units (CFU)/ml) and placed into 96-well microtitre cell culture plates. Plates were incubated for 18 h at 37 °C in a humidified atmosphere. Following this, the growth of bacteria/yeast was determined by means of measuring optical density (OD) at λ 550 nm using an ELISA plate reader (Biolise BioTek EL808). MIC was defined as the lowest concentrations of peptide at which no growth was detectable.

2.6. Haemolysis assay

A 2% (v/v) suspension of red blood cells was prepared from defibrinated horse blood (TCS Biosciences Ltd, UK). Peptide solutions at different concentrations were prepared as described in the previous section. Red blood cell suspension samples (200 μl) were incubated with a range of peptide concentrations, similar to those employed for antimicrobial activity assays, at 37 °C for 60 min and 120 min. Lysis of red cells was assessed by measurement of optical density at λ = 550 nm using an ELISA plate reader (Biolise BioTek EL808). Negative controls employed consisted of a 2% (v/v) red cell suspension and sodium phosphate-buffered saline in equal volumes and positive controls consisted of a 2% (v/v) red cell suspension and an equal volume of sodium phosphate-buffered saline containing 2% (v/v) of the non-ionic detergent, Triton X-100 (Sigma–Aldrich).