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Research paper

Novel antibacterial peptides from the skin secretion of the Indian bicoloured frog *Clinotarsus curtipes*☆

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ABSTRACT

HPLC elution profile and MALDI TOF MS analysis of electro-stimulated skin secretion of the Indian Ranid frog *Clinotarsus curtipes* of the Western Ghats confirmed the presence of multiple peptides. Peptides eluted out of the C18 column at higher hydrophobic solvent region showed antibacterial activity against diverse bacterial strains, including the clinical isolates of *V. cholerae* and methicillin resistant *Staphylococcus aureus* (MRSA). Peptidomic analysis of the most potent chromatographic effluent fraction identified five novel peptide amides having sequence homology with brevinin family. These peptides are named as brevinin1CTcu1 (B1CTcu1) to brevinin1CTcu5 (B1CTcu5). Peptide B1CTcu1 is non-haemolytic while the others are haemolytic in nature but all elicited potential antibacterial activity. B1CTcu5 is a twenty-one residue peptide amide having proline hinge region in the middle and the typical C-terminal intramolecular disulfide-bridged hepta peptide domain (Rana box) that is present in most of the brevinin peptides. Analysis of their killing kinetics with *E. coli* and *S. aureus* and the ability to induce membrane depolarization proved that these are two independent events. These novel multifunctional peptides play an important role to protect *C. curtipes* from invading pathogenic microorganisms present in the environment.

1. Introduction

Host defense peptides (HDPs) are endogenous antibiotics secreted by the holocrine glands, play multifunctional role in the innate immunity of vertebrates and other organisms [1]. These effector molecules are rapidly produced ribosomally in response to infections and defend the animal from invading pathogenic microorganisms. Majority of them are cationic in nature, vary in chain length, possess amphipathic structure and exhibit broad-spectrum antibacterial, antiviral, antifungal and anti-inflammatory activities [2,3]. Their wide spread distribution in the animal and plant kingdom suggest that they have served a fundamental role in the evolution of complex multi-cellular organisms. Since majority of these peptides target the microbial membrane, the chance to develop resistance against them is negligible compared to conventional antibiotics [4]. Therefore these molecules can serve as a potential candidate to counter the menace of emerging multidrug resistant strains of pathogenic microorganisms.

Extensive analysis of the frog skin and its secretion identified several bioactive peptides that defend the animals from microbial attack. The genus *Rana* is the most diverse and widely distributed group of anuran amphibians, with more than 250 reported species around world wide [5]. They posses many HDPs with broad spectrum antimicrobial activities like gaegurins [6] and rugosins [7] of *Rana rugosa*, brevinins of *Rana brevipodaporsa* [8], *Rana esculenta* [9] and *Rana sphenocephala* [10], esculentins of *R. esculenta* [11], ranalexin [12] and ranatuerins [13] of *Rana catesbeiana*, and temporins [14] of *Rana temporaria*. Unfortunately this study was limited only to frog species of the temperate region and only a little is known about the nature of molecules present in tropical frogs. The Western Ghats part, Kerala, India is one of the major biodiversity hotspot of the world with high degree of endemism. A detailed exploration of the defensive frog peptides of this region will help to understand their amino acid assembly, secondary structure and valuable information about their mode of action which may help to understand the minimum structural requirement of a peptide to show antibacterial activity. These molecules may also serve as a template to develop novel anti-infectives with predicted bioactivities.

HDPs isolated from Ranid frogs have very high sequential similarity and are grouped under Brevinin family [15]. These peptides

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contain a unique primary structure in which N-terminal is a linear segment and the C-terminal is a seven membered cyclic region formed by a disulfide bond between *i* and *i* + 6th cysteine residues. The present study describes the isolation, characterization and antibacterial activity of five novel skin secreted HDPs from the Indian ranid frog *Clinotarsus curtipes*. Structural analysis showed that these peptides possess very high sequence homology with known brevinin1 family peptides isolated from Ranidae frogs of Eurasian and North American region. A detailed analysis of their primary and secondary structure, biological activity and mechanism of action will be useful to design and develop novel peptides based anti infectives of known activity.

2. Materials and methods

2.1. Collection of frog skin secretions

Skin secretions were collected from twenty adult species of *C. curtipes* (20–30 g; sex unknown) of the Western Ghats region in Kerala, South India as previously described [16]. In brief, a mild electric stimulation was given to the dorsal surface of the frog. The skin secretion was collected by washing the frog skin with small quantities of sterilized, deionized water acidified with trifluoroacetic acid. The frog was released in a healthy state back to field where it was collected. Skin secretions were pooled together, frozen in liquid nitrogen, brought to the laboratory and lyophilized.

2.2. HPLC analysis of peptides

About 1 mg of the lyophilized skin secretion was dissolved in 1 ml of solvent A (water with 30% acetonitrile and 0.1% TFA), injected to C-18 analytical column (Vydac 238 TP, 150 × 4.6 mm) and performed the reverse phase high performance liquid chromatography (RP-HPLC). Peptides were eluted out of the column by slowly increasing the acetonitrile concentration upto 70% over a period of 88 min with 1 ml/min flow rate. Absorbance of the eluted solution was monitored at 214 and 280 nm. After analysing the HPLC profile of the crude skin secretion, the eluted solutions were collected as nine 10 ml fractions. After lyophilisation, the white powder obtained under each fraction was dissolved in water and antibacterial nature was tested against *Escherichia coli* by disc diffusion assay. The fraction eluted between 70 and 80 min that showed highest antibacterial activity was collected and lyophilised. This potential fraction was further purified by RP-HPLC using the same program but starting with 40% acetonitrile concentration in solvent A and the eluted fractions under each peak was collected and lyophilized.

2.3. Sequence analysis

Amino acid sequence in each peptide was established by amino acid analysis, sequencing by automated Edman degradation (Shimadzu CTO-10A) and mass fingerprinting. Mass fingerprint of the purified sample after its reduction and tryptic digestion was carried out using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF/TOF). De novo sequencing was performed by carrying out the peptide fragmentation by MS/MS 2 KV with CID ON method using 4000 laser shots of intensity 4700. Frog protein uniprot data base was incorporated in Protein Pilot 2.0 and the MS/MS spectra was searched against that data base for protein identification. Primary sequence was confirmed by analysing all these results.

2.4. Peptide synthesis

C-terminal amidated peptides were synthesized by the stepwise manual solid phase peptide synthesis (SPPS) technique using CLEAR amide resin as the solid support and following the 9-fluorenylmethoxy carbonyl (Fmoc) chemistry [17]. After the synthesis, peptide amide was cleaved from the resin by adding a cleavage cocktail TFA/EDT/TIS/water (95:1.5:1.5:2 (v/v)) at room temperature. After 4 h, the mixture was filtered and the filtrate was concentrated under vacuum to 1/4th of its volume. The crude peptide amide was precipitated by ice cold ether and collected by centrifugation at 3000 rpm for 15 min. After lyophilisation it was dissolved in solvent A and purified by injecting it into a C-18 column of RP-HPLC. The peptide amide was eluted out of the column by slowly increasing the acetonitrile concentration to 70% in solvent A over a period of 30 min. Homogeneous purification and mass accuracy were confirmed by analytical RP-HPLC profile and MALDI TOF MS analysis. The C-terminal disulphide bond formation in B1CTcu5 was carried out by the oxidation of the two cysteine residues in 50% aqueous DMSO in the absence of light. The extent of cyclisation was monitored by injecting a small volume of the solution to a C18 Vydac column at various time intervals and running a gradient HPLC elution by increasing the acetonitrile concentration in solvent A to 70% over a period of 20 min. The elution profile showed that more than 90% cyclisation was completed within 8 h. B1CTcu5 having C-terminal rana box was recovered by repeating the above purification protocol.

2.5. Antimicrobial activity and killing kinetics

Bacterial strains used for *in vitro* antibacterial assay were *E. coli* (MG1655), clinical isolates of *Vibrio cholerae* collected from Thiruvananthapuram medical college, Kerala, India, *Staphylococcus aureus* (MTCC 9542), *Bacillus subtillis* (ATCC 14416), *Bacillus coagulans* (ATCC 7050), *Methicillin resistant S. aureus* (ATCC 43300) and *Vancomycin resistant enterococcus* (ATCC 29212). Bacterial cultures were grown in Luria bertani broth (Hi-media) by overnight incubation at 37 °C with constant shaking. Microbial cultures having 10^6 CFU/ml were made from OD 0.6 culture. 1 mg/ml stock concentration of peptides were prepared in autoclaved double distilled water and diluted in LB broth to make concentrations ranging from 1 to 200 μg/ml. 50 μl bacterial culture was treated with 50 μl of individual peptide amide solution of various concentrations and incubated for 24 h at 37 °C with constant shaking in a 96 well microtitre plate. Growth control wells had the same amount of bacterial inoculum but without peptide. Absorbance at 600 nm was noted in every 3 h upto 24 h to assess the cell growth and a graph was plotted with time against OD values. The minimum inhibitory concentration (MIC) was taken as the dose at which 100% growth inhibition was observed and it was calculated from the graph.

The killing kinetic analysis of the peptides against Gram-negative *E. coli* (MG1655) and Gram-positive *S. aureus* (MTCC 9542) were carried out at its sub MIC concentrations. Cells in mid-logarithmic growth phase ($OD_{600} = 0.6$) was diluted to get 10^6 CFU/ml and incubated with the peptides at different time intervals 5, 30, 60, 90 and 120 min. Aliquots drawn at these time points were plated on LB agar. The colony number was counted after incubating the plates at 37 °C for 24 h. Cells without treatment with the peptides at the same time intervals as mentioned above were taken as the positive control.

2.6. Haemolytic assay

Haemolytic assay was carried out by following the previously described method [18]. In brief, 10 μg of the peptide was incubated at 37 °C for 30 min with 10% (v/v) fresh suspension of rabbit erythrocytes in phosphate buffered saline (pH 7.2). The cells were centrifuged (3000 × *g* for 5 min), and absorbance of the