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Identification and structural insights of three novel antimicrobial peptides isolated from green coconut water

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ABSTRACT

Infections caused by pathogenic bacteria could cause an expressive negative impact on human health. A significant enhance in resistance to commercial antibiotics has been observed in all kinds of pathogenic bacteria. In order to find novel approaches to control such common infections, a wide number of defense peptides with bactericidal properties have been characterized. In this report, three peptides lower than 3 kDa were purified and identified from green coconut (*Cocos nucifera* L.) water by using reversed phase-high performance liquid chromatography (HPLC), showing molecular masses of 858 Da, 1249 Da and 950 Da. First one, named *Cn*-AMP1, was extremely efficient against both Gram-positive and Gram-negative bacteria, being MICs calculated for three peptides. All complete sequences were determined by MALDI-ToF analysis showing no identity in databanks. Moreover, peptide net charge and hydrophobicity of each peptide was *in silico* evaluated. Finally molecular modeling and dynamics were also applied generating peptides three-dimensional structures, indicating a better explanation to probable mechanisms of action. *Cn*-AMPs here reported show remarkable potential to contribute in the development of novel antibiotics from natural sources.

1. Introduction

In the last two decades, a significant increase in pathogenic bacteria with enhanced resistance to commercial antibiotics has been observed. The increasing tendency of microorganisms to develop resistance to currently used antibiotic drugs continuously demands multiple screenings for new classes of antimicrobial agents [4,8,19]. In order to help to solve the bacterial infection problem, a wide number of proteinaceous compounds, known as antimicrobial peptides or AMPs, with deleterious effects toward microorganisms have been isolated from numerous sources such as plants, animals and microorganisms [4,8,21,27–29]. These peptides make part of an ancient, non-specific innate immune system, which is considered the first main defense system for the majority of living organisms, acting against pathogenic organisms invasion [13].

Despite of AMPs have been commonly isolated from different plant tissues, which included flowers, tubers, leaves, roots and seeds [9,20,22], several other plant sources still need to be explored. In this view, this report focuses the study of antimicrobial peptides in green coconut water (GCW). GCW is a popular drink in the tropics, especially in Tropical Asia and Latin America. Moreover, GCW is commonly used for nutritional formulation beverages and food products due to the presence of sugars, vitamins, minerals and proteins [23,24]. Their compositions also make GCW successfully used in the treatment of child and adult diarrhea in poor regions of the world, hydrating the individual and protecting gastrointestinal tract against different infections [25]. In folk medicine GCW is used as a remedy for gastroenteritis, urinary stone dissolution and coronary heart disease [1,3,16]. Additionally it was also reported that GCW have cardio protective action [5].

In spite of several medical properties described for GWC, few reports showed the presence of biological active peptides in GCW and only recently, Wang and Ng [30] have been isolated an antifungal peptide from coconut fibers. For all these reasons, the present study was carried out in order to investigate the presence of antibacterial peptides in green coconut water, elucidating their

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Abbreviations: AMPs, antimicrobial peptides; CHCA, α-cyano-4-hydroxycinnamic acid; *Cn*-AMPs, *Cocos nucifera* antimicrobial peptides; Cp-thionin2, cowpea-thionin2; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; GCW, green coconut water; MALDI-ToF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometry; Pg-AMP1, *Psidium guajava* antimicrobial peptide; PSD, post-source decay; TFA, trifluoroacetic acid; TLC, thin layer chromatography.

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primary and tertiary structures. Inhibitory activity was observed against Gram-negative and Gram-positive bacterial pathogens, responsible for nosocomial and urinary infections in immunosuppressed patients. This is the first report of AMPs isolated from GCW with inhibitory activity against human bacterial pathogens.

2. Materials and methods

2.1. Sample preparation

Green coconuts were collected from local market of Kharagpur, West Bengal, India. 500 ml of GCW was collected and centrifuged for 20 min at 4000 × *g* at 4 °C. The supernatant was dialyzed for three days in tubing (cut-off of 3 kDa) (Biorad, Germany). Dialysis was performed at 15 °C against 1 l of distilled water at pH 2.0, adjusted with acetic acid. The diffusates were changed every 24 h and further lyophilized. Dry samples were resuspended in 1 ml of 5% (v/v) acetonitrile solution containing 0.01% (v/v) trifluoroacetic acid.

2.2. Peptides purification

Resuspended samples were fractionated onto reverse phase-HPLC (Agilent 1100 series) with a ZORBAX-Eclipse XDB-C18 column (4.6 mm × 150 mm, particle size 5 μm), at a flow rate of 600 μl min^{-1}, by using a linear acetonitrile gradient (5–60%, v/v) during 45 min at 30 °C. 0.04% (v/v) trifluoroacetic acid was used as ion pairing agent. The elution was monitored at 220 nm with a UV-DAD detector (DAD, G1315B). Selected peaks of the HPLC chromatogram were collected using a coupled fraction collector (GILSON, France). Individual fractions were concentrated by Speed-Vac. Target fraction, which showed antibacterial activity, was re-chromatographed in the same column, at a flow rate of 600 μl min^{-1}, under isocratic elution with 55% (v/v) acetonitrile containing 0.04% (v/v) trifluoroacetic acid for 30 min at 30 °C. The elution was monitored at 220 nm. Selected peaks were collected and processed as previously described. Subsequently, each fraction was dissolved in water and runner on a silica based TLC (thin layer chromatography) plate by using as mobile phase n-butanol:acetic acid:water (60:20:20). Plate was sprayed with a developing reagent (ninhydrin in acetone) in order to confirm peptide purity degree.

2.3. Bacterial bioassays

Purified peptides were dissolved in 0.1% DMSO (dimethyl sulfoxide) and antibacterial activities were tested by the growth-inhibition-zone assay [12]. The minimal inhibitory concentrations MICs were also obtained according to Park et al. [18]. The microorganisms used for MIC bactericidal assay were *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis*. All strains were obtained from the stock culture of Department of Microbiology, Vidyasagar University, India. The lowest concentration of the peptide in which no growth occurred was defined as the MIC.

2.4. Mass spectrometry analysis

The lyophilized dried peptides were reduced by dithiothreitol (DTT) in 50 mM NH_4HCO_3 at 37 °C for 2 h followed by acidification with 1% TFA. Two microliter of reduced peptide solution was mixed to 24 μl of CHCA (α-cyano-4-hydroxycinnamic acid) 10 mg ml^{-1}, which as used as matrix. Then, 1.0 μl sample was spotted onto the MALDI 100 well stainless steel sample plate and allowed to air dried prior to the MALDI analysis. A Voyager time-of-flight mass spectrometer (Applied Biosystem, USA) was used for obtaining MALDI mass spectra equipped with 337 nm N_2 laser and operated in an accelerating voltage 20 kV. The spectra were recorded in the post-source decay (PSD) ion mode as average of 100 laser shots with a grid voltage of 75%. The reflector voltage was reduced in 25% steps and guide wire was reduced 0.02–0.01% with an extraction delay time 100 ns. Reproducibility of each spectrum was checked 20 times from duplicate samples.

2.5. In silico analyses and molecular modeling

Initially, a threading method was carried in order to find the best template for homology modeling. Bioinfo Meta Server [10] was utilized, indicating an absence of structures with similarity to three available coconut peptides. Consequently, Gromacs for molecular dynamics [15] was utilized for prediction of protein structures using *ab-initio* procedures. A primary structure was constructed using Modeller 9.4 [17] program. This structure was then placed in a cubic box, with water molecules. A partial simulation for energy minimization was executed using 2000 steps from Steepest Descent in order to remove possible stereochemical disturbances. Complete simulation was performed using temperature and coupling pressure of 300 k and 1 atm, respectively, and Newton's movement equations (MD) as a dynamic method in 60,000 ps. The final model was visualized using Pymol [6] and SPDB-Viewer [11]. All simulations were performed on a Sun AMD Opteron bio-processor workstation.

3. Results and discussion

3.1. Peptides isolation and characterization

In order to isolate low molecular (<3 kDa) antimicrobial peptides from *C. nucifera*, GCW was dialyzed with a MW cut-off tubing of 3 kDa and exudates were further applied onto a reversed phase chromatography (HPLC), revealing a major peak eluted with approximately 36% acetonitrile and two minority peaks (Fig. 1A). Furthermore, three peaks were evaluated by TLC, showing a single band in each lane (Fig. 1B). The antimicrobial activity of each fraction was *in vitro* challenged against four bacterial strains (*E. coli, B. subtilis, S. aureus* and *P. aeruginosa*). *E. coli* is a multidrug resistant bacterium, universal inhabitant of human digestive tract. *S. aureus* and *B. subtilis* cause food spoilage and poisoning. Finally *P. aeruginosa* is a potential human pathogen found inhabiting milk products, vegetables and meat [25]. Fraction 1 displayed broad

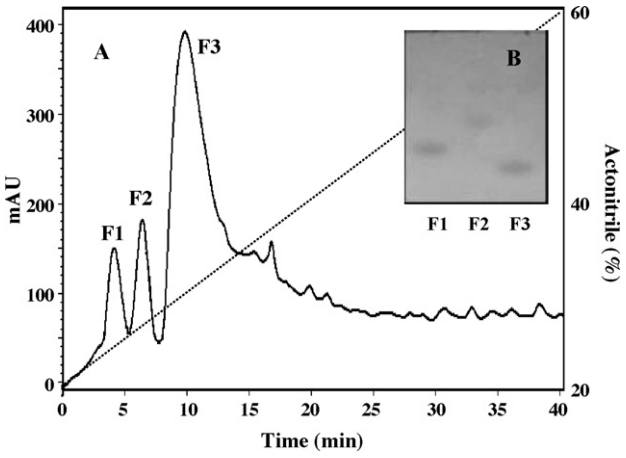

Fig. 1. (A) HPLC reversed-phase chromatogram profile (ZORBAX-Eclipse XDB-C18 column) of lower ≤3 kDa fraction from green coconut water. Diagonal line indicates a linear acetonitrile gradient (5–60%). (B) Thin layer chromatogram of HPLC fractions (F1, F2 and F3) from green coconut water.

Table 1
Antibacterial activities of isolated peptides from green coconut water. The growth-inhibition-zone assay was performed on assay plates in nutrient medium to determine the minimum inhibitory concentration (MIC). *Cn*-AMP1 corresponds to F1; *Cn*-AMP2 to F2 and *Cn*-AMP3 to F3. Data are the mean of triplicates do not differing more than 12%.

Pathogenic bacteria	*Cn*-AMP1 MIC ($\mu g\ ml^{-1}$)	*Cn*-AMP2 MIC ($\mu g\ ml^{-1}$)	*Cn*-AMP3 MIC ($\mu g\ ml^{-1}$)
E. coli	82	170	302
B. subtilis	76	150	257
P. aeruginosa	79	169	259
S. aureus	80	170	274

spectrum of antibacterial activity against all the tested strains, with lower MICs (Table 1). Fraction 2 and 3 showed less activity than that of fraction 1 at the tested concentration against all bacterial strains. The microorganisms here studied showed obvious differences in their susceptibility to peptides. The minimum inhibitory concentration (MIC) for the tested strain was 75–85 $\mu g\ ml^{-1}$ of fraction 1, which is almost two and four fold lower than fraction 2 and 3, respectively. Other AMPs isolated from plants also showed similar MICs. For example a glycine-rich peptide, named Pg-AMP1, isolated from *Psidium guajava* seeds showed a clear bactericidal activity, where MIC values obtained for this peptide were 72 $\mu g\ ml^{-1}$ for *E. coli* and 32 $\mu g\ ml^{-1}$ for *K. pneumoniae*. None activity was obtained towards *S. typhimurium* [22]. Moreover a defensin named Cp-thionin2, isolated from *Vigna unguiculata* seeds also showed similar MICs when evaluated against Gram-positive and Gram-negative bacteria [9].

3.2. Amino acid sequence analysis of HPLC purified fractions

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) have been used for molecular weight determinations of proteins and peptides for almost a decade [2]. Recently, the fragment ions that are produced after leaving the ion source during the flight in the field-free region were used for peptide sequencing. These ions are called "post-source decay" or PSD ions [26]. Peptide sequencing by PSD MALDI-ToF MS has been demonstrated and revealed that reduced peptides produced a series of y and b ions [28]. PSD MALDI-ToF MS analysis was applied and the resulting pattern of fragmentation obtained for fraction 1 revealed a series of the complementary y- and b-type ion-fragments, which were very informative about the primary sequence of the peptide. The spectrum obtained from fraction 1, observed a series of y-type ions: *m/z* 858.31 (y9), *m/z* 758.99 (y8), *m/z* 688.71 (y7), *m/z* 404.09 (y4), *m/z* 275.41 (y3), *m/z* 218.93 (y2), *m/z* 87.01 (y1), and a series of b-type ions: *m/z* 850.85 (b9), *m/z* 719.71 (b8), *m/z* 662.17 (b7), *m/z* 533.60 (b6), *m/z* 462.25 (b5), *m/z* 306.93 (b4), *m/z* 249.11 (b3) and *m/z* 178.24 (b2). The subtraction of these successive *m/z* values have permitted to assign the sequence as showed at the top of Fig. 2A. The calculated mass of the peptide is *m/z* 876, whereas the observed mass value is *m/z* 858 this may be due to the loss of one water molecule. The other two peptides were sequenced by the same way (Fig. 2B and C) and it was found that their total charge was negative (Table 2). After sequencing, F1, F2 and F3 were named *Cn*-AMP1, *Cn*-AMP2 and *Cn*-AMP3 respectively.

The *Cn*-AMPs amino acids residues sequence were compared to NCBI databank by Blast showing no matches. Moreover, when *Cn*-AMP1 was compared to AMP database (http://aps.unmc.edu/AP/main.html), was observed a lower homology (30%) to temporin G (AP00099) and uperin 2.1 (AP00316). *Cn*-AMP2 and 3 showed no identity in that databank. The total net charge of *Cn*-AMP1 is +1, exhibiting a hydrophobic ratio of 44% and a boman index of 1.32 kcal/mol. These properties, associated to the ability to form a

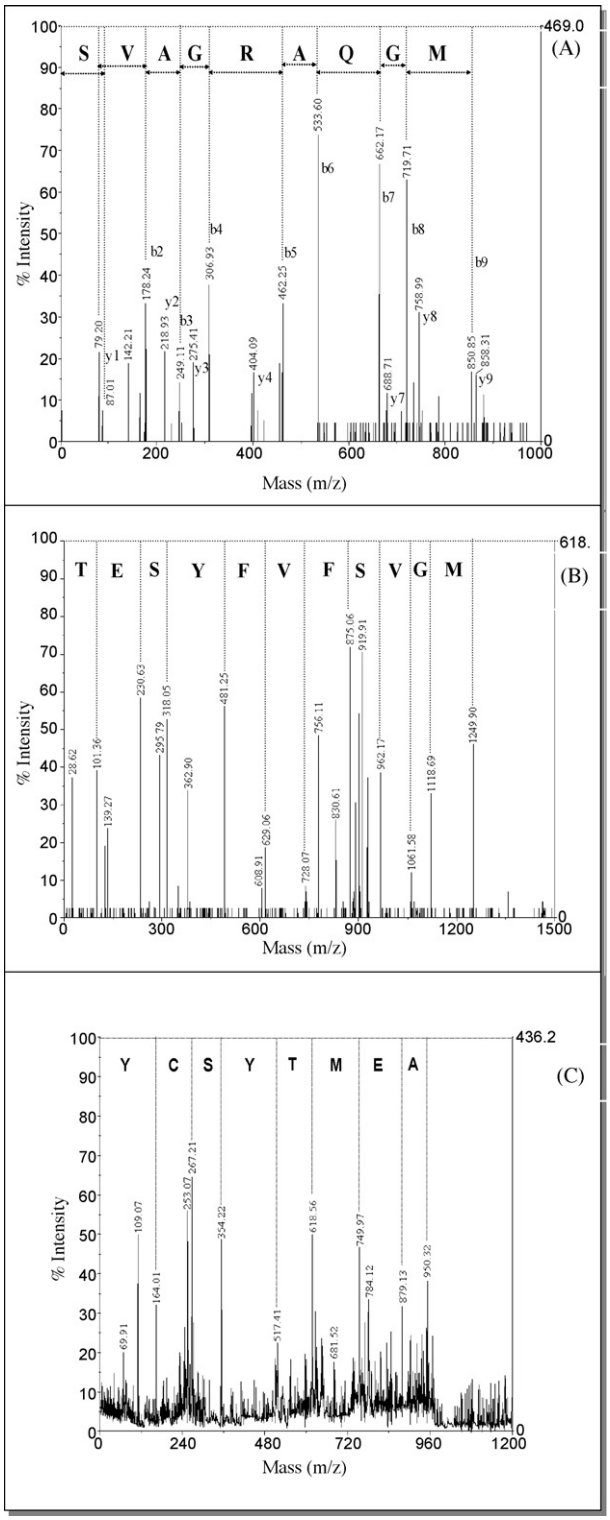

Fig. 2. MALDI-ToF MS spectrum of reduced *Cn*-AMP1 (A), *Cn*-AMP2 (B) and *Cn*-AMP3 (C) in the post-source decay (PSD) ion mode as average of 100 laser shots with a grid voltage of 75%. The reflector voltage was reduced in 25% steps and guide wire reduced 0.02–0.01% with an extraction delay time 100 ns.

helix, as observed by helix wheel predictor (data not shown) and molecular modeling (Fig. 3) explain why this peptide was able to cause deleterious effects in bacteria. Moreover, antibacterial activity of cationic peptides can also be modulated through modification in peptide's hydrophobicity or net charge [14,32]. Moreover, *Cn*-AMP2 and 3 showed acidic properties (Table 2) but

Table 2
Amino acid residues and molecular masses of the purified peptide from green coconut water.

Peptide	Sequence	Calculated mass (Da)	Observed mass (Da)	Hydrophobic ratio (%)	Charge
Cn-AMP1	SVAGRAQGM	876	858	44	+1
Cn-AMP2	TESYFVFSVGM	1266	1249	45	−1
Cn-AMP3	YCSYTMEA	967	950	37	−1

similar hydrophobic rate suggesting that cationic charges found in *Cn*-AMP1 is the main cause for higher antibacterial activity when compared to *Cn*-AMP2 and 3. Ionic interaction probably is the initial attraction between AMPs and target cell, which occur through an electrostatic bonding between cationic peptide and negatively charged components present on the outer bacterial envelope, such as phosphate groups from lipopolysaccharides of Gram-negative bacteria or lipoteichoic acids exposed on Gram-positive bacteria surfaces. In the case of Gram-negative bacteria, this peptide may also insert into the outer membrane structure in a process driven by hydrophobic interactions and possibly involving refolding of the peptides into a membrane-associated structure [13]. However, the antibacterial activity caused by acidic peptides such as *Cn*-AMP2 and 3 was a surprisingly data, since no basic exposed residue was observed. This result indicates that basic residues are truly important for bactericidal activity, since *Cn*-AMP1 showed a clear higher activity. Nevertheless these residues seems to be not essential, being the antimicrobial activity of *Cn*-AMP2 and 3 more related to hydrophobic amino acid residues as described above.

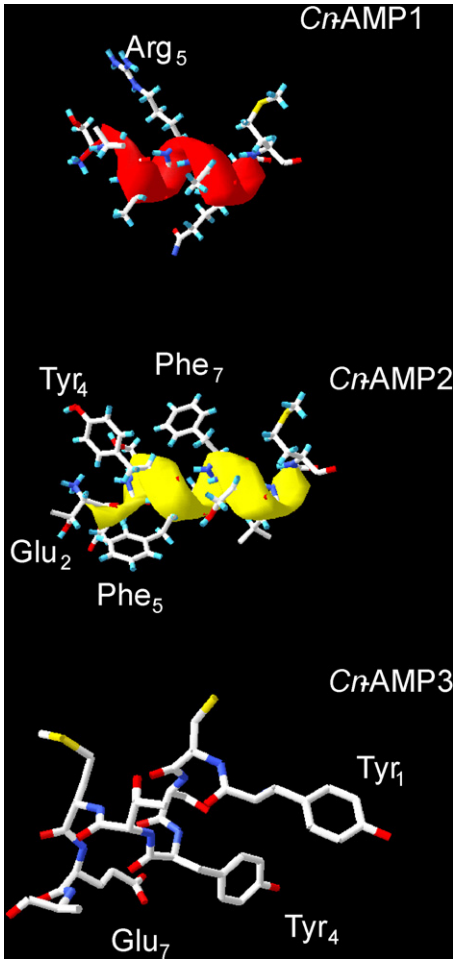

Fig. 3. Cartoon representation of *Cn*-AMP1, *Cn*-AMP2 and *Cn*-AMP3 obtained from GWC. Structures was constructed by using Modeller [6] and visualized by Deep View Swiss PDB Viewer programs [10]. Yellow and red colors indicate α-helices. Stick representation indicated residues involved in a possible interaction to bacterial membranes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

3.3. Peptides molecular modeling

In order to elucidate tertiary structure of *Cn*-AMPs, *ab-initio* molecular modeling associated to dynamics was carried out. *Cn*-AMP1 final model is composed of a reliable α-helix (Fig. 3). Furthermore these data were corroborated by the helical wheel applet software (http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html), which also indicates that four non-polar amino acids residues on the same hydrophobic surface (Fig. 3). At *Cn*-AMP1 structure two glycine residues are present (Gly_4 and Gly_8), conferring greater flexibility to this structure. At the α-helices center a single extended arginine (Arg5) residue is observed, which seem to be important for providing the positive charge to this region. Arginine residues seem to be important in antimicrobial activity, interacting with the pathogen cell surface, as previously observed in peptides isolated from guava [22] and cowpea seeds [9]. *Cn*-AMP2 also showed a single and small α-helix (Fig. 3). In this case the negatively charged region is formed predominantly of glutamic acid residues and can be found at the external α-helix (Glu2, Fig. 3). This peptide is also rich in aromatic residues (Tyr_4, Phe_5 and Phe_7) indicating a possible membrane interaction by hydrophobic attraction. Similar properties were also observed in *Cn*-AMP3, while no helix is observed (Fig. 3). *Cn*-AMP3 also showed an acidic property, caused by an extended Glu_7 and a non-polar surface, characterized by two aromatic residues (Tyr_1 and Tyr_4). Despite of most antimicrobial peptides found in plants have been characterized as cationic [19], few organisms have shown the presence of acidic bactericidal peptides with included chilli peppers [7], snakes [31] and several others.

In summary, data here reported clearly shows that coconut is able to synthesize different antimicrobial peptides in their water, with diverse properties and mechanisms of actions. This report is the first description of GCW antimicrobial peptides with activity against human pathogenic bacteria, including *C. nucifera* as a potent source of biotechnological products. Application in pathogen inhibition will likely include genetic engineering-derived antibiotic production or development of novel disinfectants for hospital environments.

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