Characterization of *Cimex lectularius* (bedbug) defensin peptide and its antimicrobial activity against human skin microflora.

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Abstract

Antimicrobial peptides (AMPs) are components of both vertebrate and invertebrate innate immune systems that are expressed in response to exposure to bacterial antigens. Naturally occurring AMPs from evolutionarily ancient species have been extensively studied and are being developed as potential therapeutics against antibiotic resistant microorganisms. In this study, a putative *Cimex lectularius* (bedbug, CL) defensin is characterized for its effectiveness against human skin flora including Gram-negative and Gram-positive bacteria. The bedbug defensin (CL-defensin), belonging to family of insect defensins, is predicted to have a characteristic N-terminal loop, an α -helix, and an antiparallel β -sheet, which was supported by circular dichroism spectroscopy. The defensin was shown to be antimicrobial against Gram-positive bacteria commonly found on human skin (*Micrococcus luteus*, *Corynebacterium renale*, *Staphylococcus aureus* and *Staphylococcus epidermidis*); however, it was ineffective against common skin Gram-negative bacteria (*Pseudomonas aeruginosa* and *Acinetobacter baumannii*) under low-salt conditions. CL-defensin was also effective against *Micrococcus luteus* and *Corynebacterium renale* in high-salt (MIC) conditions. Our studies indicate that CL-defensin functions by depolarization and pore-formation in the bacterial cytoplasmic membrane.

1. Introduction

Bedbug infestations are a global nuisance and after being nearly eliminated in 1950's through the use of carbamates and organophosphates, they have made a comeback following the ban on these insecticides and resistance to the pesticides [1]. The CDC considers bedbugs a significant public health risk [2]. Bedbugs are obligate feeders on human blood and due to this close interaction, they carry some of the bacteria found on human skin. In previous work, the identified bacteria on bedbugs were found to be mostly Gram-positive bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Micrococcus luteus*, the same bacteria commonly found on human skin [3,4]. Gram-negative organisms were rarely found on bedbugs, but included *Wolbachia*, *Klebsiella* and *Stenotrophomonas maltophilia* [3,4]. The reasons for this disproportionate absence of Gram-negative bacteria are not known.

Antimicrobial peptides have gained significant attention recently as a potential alternative or adjunctive therapy for antibiotics [5]. These peptides are important components of the innate immune system, our first line of defense, and are expressed in response to bacterial, viral or fungal infections. Previously conducted studies have identified such antimicrobial peptides in humans [6], plants [7] and insects [8]. Our group has been studying antimicrobial peptides from

Abbreviations

EC₅₀ = Half maximal effective concentration
MIC= Minimum inhibitory concentration
AMP = Antimicrobial peptide
CFU= Colony forming units
CL-Defensin = *Cimex lectularius* defensin
OM= Outer-membrane
RFU= Relative fluorescence units
DiSC₃(5)= 3,3'- Dipropylthiadicarbocyanine iodide
NaCl= Sodium chloride
EtBr= Ethidium Bromide

various sources including snakes, humans and alligators [9–11]. Invertebrate insect peptides have been previously demonstrated to exhibit activity against Gram-positive [12] and Gram-negative bacteria [13]. These peptides are typically small cationic proteins that are less than 5 kDa [14,15]. The two major categories of antimicrobial peptides found predominantly in insects are cecropins and defensins. Insect defensins are usually 29-43 residues long peptides that are rich in cysteines [15,16]. Much like the mammalian defensins, these peptides have six cysteine residues and three disulfide bonds that stabilize the overall structure, a carboxy-terminal β -sheet that runs anti-parallel and an amino-terminal loop. Two of the three disulfide bridges are between the α -helix to one strand of the β -sheet and the third one is between N-terminal loop and second strand of β -sheet and stabilizes the interaction between N-terminal loop and β -sheet [17,18]. However, unlike mammalian defensin, insect defensins usually have an amphipathic α -helix domain [16]. These defensins are found in fat bodies, hemolymph and thrombocytes in the insects [16].

In this study, we assessed the effectiveness of a bedbug defensin belonging to the Defensin-4 family of insect defensins against skin flora [19,20]. Commonly found on human skin, these bacterial species (harmless otherwise) can cause infections in immunocompromised individuals. To predict the secondary structure of CL-defensin we performed circular dichroism and bioinformatics analysis. To determine the mode of action of CL-defensin peptide, ethidium bromide (EtBr) uptake assay and cytoplasmic membrane depolarization assay were performed

2. Materials and Methods:

2.1 Bacterial strains and growth conditions

Bacterial strains tested in this study are listed in **Table 1** and were obtained from American Type Culture Collection (Manassas, VA). All microorganisms were cultured in nutrient broth (Difco Laboratories, Detroit, MI, USA) at 37°C in shaking incubator at 200 rpm

for 24 hours and were stored in -80°C in 20% glycerol. The bacteria were plated on Mueller Hinton Agar for enumeration.

2.2 Peptides and Bioinformatics Analysis

The sequence of the CL-defensin was obtained from Life Science database archive (“Life science projects in Japan,”;

http://togodb.biosciencedbc.jp/togodb/show/clest_cluster/Cl_contig0261#ja) The following peptide (ATCDLFSFQSKWVTPNHAACAAHCTARGNRGGRCKKAVCHCRK) was

predicted and manufactured by China Peptides (Shanghai, China). The peptide was provided at 85-95% purity and the peptide mass was determined using ESI-mass spectrometry. The

molecular weight of the peptide was confirmed by mass spectrometry to be 4692.45 g/mol and

the purity was confirmed through RP-HPLC. Physiochemical and structural properties of CL-

defensin were calculated using the Antimicrobial Peptide Database (APD2) [21,22]. The

molecular weight of CL-defensin is 4692.45 g/mol. The net charge was calculated to be +7. The

hydrophobicity of this peptide was calculated to be 44%. Swiss model

(<http://swissmodel.expasy.org/interactive>) [28] was used for protein modeling and for generating a Protein Data Bank (pdb) encoding the predicted structure of the protein. A model of the peptide

was generated using Chimera software, an extensible molecular modeling system

(<http://www.cgl.ucsf.edu/chimera/>) [25].

2.4 Minimum inhibitory concentration (MIC)

The MIC for CL-defensin against the microorganisms was determined in triplicate in cation-adjusted Mueller-Hinton broth (CA-MHC). Conditions were in accordance with the

current recommendation of the CLSI broth microdilution protocol [25]. Briefly, a range of

concentrations of the peptides was prepared by serial dilution and added to an equal volume of

exponentially grown bacterial culture (50 μ L) diluted to 1×10^5 CFU. Microtiter plates were incubated for 18-24 h (48 hrs for *M. luteus*) at 37°C. The MIC was defined as the concentration at which no microbial growth was observed spectrophotometrically. Wells containing only growth media was used as the positive control and growth media containing microbial cells without peptide was used as negative control.

2.5 Antimicrobial EC₅₀ assays

The antimicrobial EC₅₀ activity of CL-defensin against skin flora was determined as described previously in low-salt buffer [10]. In a 96 well plate, 1×10^5 CFU of bacteria were incubated with various concentrations of CL-defensin (serially diluted 1:5) in 10 mM sodium phosphate buffer (pH 7.3). After a 3 hour incubation each well was further diluted 10^{-1} , 10^{-2} and 10^{-3} fold in 10 mM sodium phosphate buffer and each dilution was plated in triplicate on Mueller Hinton agar and incubated for 24 hours (48 hours for *M. luteus*). NA-CATH (4175.22 g/mol), a snake cathelicidin was used as a positive control in the EC₅₀ assays [26]. The concentration of peptide required to kill 50% of bacterial population (EC₅₀) was determined by graphing percentage of surviving colonies, after the 24 - 48 hour incubation, as a function of log of peptide concentration and analyzing data using GraphPad Prism 6 (GraphPad Software Inc. San Diego, CA, USA) using the equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{[(\log \text{EC}_{50} - X) \times \text{Hill slope}]})$ where Y corresponds to percentage of surviving bacteria at the given peptide concentration and X corresponds to the concentration of peptide in logarithmic function. The terms “Top” corresponds to upper boundary constrained to <100% and “Bottom” corresponds to lower boundary constrained to >0%. The “no peptide” treatment is graphed at 10^{-2} μ g/ml. The confidence intervals for each experiment are reported in **Table 1** along with the EC₅₀ values.

2.6 Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was performed using a Jasco J-1500 spectropolarimeter. 100 µg/ml of peptide was used in each experiment. Samples were allowed to equilibrate for 3 min prior to data collection at 25 °C in a 1 mm path length cuvette. Spectra were collected from 190 to 240 nm at 0.2-nm intervals, with a data integration time of 4 s and a 1 nm bandwidth. Data presented is an average of four spectra. Peptides were analyzed in 10 mM sodium phosphate and 60 mM sodium dodecyl sulfate (SDS) in phosphate buffer.

2.7 Bacterial cytoplasmic membrane depolarization assay

Membrane depolarization assay was studied using DiSC₃(5) as previously reported [27,28]. Enumerated frozen bacteria were pelleted and washed twice in phosphate buffer (pH 7.2) and then resuspended to 4×10^7 CFU/mL in phosphate buffer containing 50 µg/mL DiSC₃(5). 100 µL of this suspension was added to wells of a black 96 well plate. The plate was incubated in a TECAN infinite F200 spectrofluorometer and monitored until fluorescence leveled off. Various concentrations of peptide in phosphate buffer (pH 7.2) were added to each well. Bacteria without peptide was used as a negative control and Triton X-100 was used as positive control. The plate was immediately returned to the spectrofluorometer. Readings were taken every 2 min for 10 min (excitation = 622 nm; emission = 670 nm).

2.8 Bacterial cytoplasmic membrane permeation

Bacterial cytoplasmic membrane permeation was monitored by using the ethidium bromide uptake assay. The ethidium bromide uptake assay was performed as previously detailed with some modifications [29]. Respective bacteria were grown till log phase in nutrient broth in a shaking incubator (37°C). Bacteria were centrifuged, washed with PBS, and then adjusted to an OD 600 nm of ~ 0.1 in 10 mM Phosphate Buffer. 180 µL of bacteria was added to 10µL ethidium bromide (10 µM final concentration) and 50 µg/mL peptide in various concentrations.

The plate was read in a TECAN infinite F200 spectrofluorometer every 2 min for 30 min at 37°C: (excitation = 540 nm, emission = 590 nm). Δ RFU was calculated by the equation: RFU (desired concentration)-RFU (no peptide control).

2.9 Statistical Analysis

EC₅₀, DiSC₃(5), MIC and EtBr uptake assays were performed in triplicates (n= 3). Standard deviations of the mean of each set are represented by error bars on each graph. Where the error bars cannot be seen, the error is very small. P-values for depolarization assay are calculated using the Two-way ANOVA test. Confidence Interval (CI) (95%) for each EC₅₀ determination is presented in **Table 3**, and represents significance of p<0.05.

3. Results

3.1 Three dimensional structure of CL-defensin

The amino acid sequence of CL-defensin was subjected to Swiss-model for protein homology modeling [23]. Homologues for the CL-defensin sequence were found and sapecin was selected as a template for protein homology modeling since it shared 71% sequence homology with CL-defensin (PDB 1l4v.1.A). The pdb file was then converted to Chimera format (<http://www.cgl.ucsf.edu/chimera/>) to visualize the structure of CL-defensin. As seen in **Figure 1A**, CL-defensin is predicted to contain an N terminal loop, an α - helix and antiparallel β -sheet. The two antiparallel strands are connected by a turn consisting of two amino acids. The structure of CL-defensin is conventional with six cysteine residues at positions 3, 20, 24, 34, 39 and 41 and predicted disulfide bridges (24-41, 20-39, 3-34) (**Figure 1B**). These results are in accordance with other studies conducted in defining the structure of insect defensin [18]. The secondary structure of antimicrobial peptide CL-defensin was further analyzed using circular dichroism

(CD) spectroscopy, with a membrane-mimicking environment which was obtained by adding 60 mM of SDS in 10 mM of sodium phosphate buffer (**Figure 1C**). The CL-defensin spectrum showed signals representing a peptide containing β -hairpin secondary structure and 14% percentage of α -helical contribution (**Figure 1C**). The CD spectra confirmed the structure of CL-defensin obtained from 3-dimensional model of CL-defensin depicted in **Figure 1A**.

3.2 Antimicrobial activity spectrum of CL-defensin

In this study, the antimicrobial effectiveness of CL-defensin was examined against commonly found bacteria on skin. The EC₅₀ values of these microorganisms are listed in **Table 1** along with the 95% confidence interval and their respective μ M values. As seen in **Figure 2** CL-defensin had significant antimicrobial activity against all Gram-positive bacteria tested (**Figure 2 A-D**) in low-salt buffer. CL-defensin had a bacteriocidal effect on both *C. renale* and *M. luteus* at concentrations of 41.66 μ g/ml and 2.60 μ g/ml respectively and a bacteriostatic effect on *S. epidermidis* at concentrations of 41.66 μ g/ml in MIC (high-salt) conditions. However, the MIC values for other tested bacteria were above 300 μ g/ml. These results suggest that CL-defensin has some antimicrobial activity at high salt concentrations such as those found in MIC conditions.

3.3 CL-defensin induces membrane depolarization and pore formation in the cytoplasmic membrane.

To determine the mode of action of CL-defensin against selected microorganisms, we performed a cytoplasmic depolarization assay utilizing the DiSC₃(5) dye, belonging to a family of membrane sensitive fluorescent dyes. Under normal conditions, the dye binds to the bacterial surface, and loses fluorescence. Depolarization releases the dye into the media, therefore increasing the fluorescent signal. Depolarization due to small ruptures in the membrane of

cytoplasm leads to leakage of ions owing to proton flux [30] therefore disrupting the overall homeostasis of the bacteria. **Figure 3A** indicates that significant increase in fluorescence was observed when all microorganisms were treated with various concentration of peptides peptide. CL-defensin dissipates membrane potential in all microorganisms in as less as 0.5 µg/ml. To further investigate the mode of action of CL-defensin, we conducted ethidium bromide uptake assay to assess membrane permeabilization capability of CL-defensin. At high concentration of peptides, membrane permeabilization occurs that result in formation of large pores in the bacterial membrane. When we performed the ethidium bromide uptake assay we found that *C. renale* was most sensitive to membrane permeabilization by CL-defensin at 50 µg/ml as seen in **Figure 3B**. The peptide induces pore formation in other Gram-positive bacteria as well and also causes a small amount of membrane permeabilization in *A. baumannii*. However, *P. aeruginosa* was resistant to pore formation as evidenced by negative ΔRFU. Overall, we conclude that CL-defensin depolarizes membrane of both Gram-positive and Gram-negative bacteria. However, the peptide does not induce pore formation in *P. aeruginosa* membrane and induces a minimal pore formation in *A. baumannii* cytoplasmic membrane.

4. Discussion

In this study, we have identified and tested a defensin-type antimicrobial peptide from the bedbug, *C. lectularius*, called CL-defensin. The aim of the study was to discover the range of activity of this antimicrobial peptide against bacteria that it would normally encounter on its human host. We have demonstrated the antibacterial activity of this peptide under low-salt conditions against a panel of microorganisms that are commonly part of human skin flora. We found that CL-defensin has antimicrobial activity primarily against the panel of Gram-positive

bacteria that are commonly found on human skin. The CL-defensin was ineffective against the two human pathogenic Gram-negative bacteria tested in this study, *P. aeruginosa* and *A. baumannii*. There may still be more antimicrobial peptides produced by bedbugs that would be effective against Gram-negative bacteria.

Antimicrobial insect defensins are more effective against microorganisms in conditions of low ionic strength. High-salt conditions usually negatively impact the activity of these peptides [31]. CL-defensin killed all the tested Gram-positive bacteria at 2 μ M or less in low ionic conditions (10 mM phosphate buffer), yet was not lytic under high ionic conditions against *S. aureus* and Gram-negative microorganisms, *P. aeruginosa* and *A. baumannii*. In this study, however, we found that CL-defensin was bactericidal against *M. luteus* and *C. renale* and bacteriostatic against *S. epidermidis* even in high ionic (MIC) conditions. The *in vivo* physiological conditions consists of high ionic strength (137 mM NaCl) [32]; thus, antimicrobial activity of peptides against bacteria in such conditions is imperative. Other insect defensins that have shown a similar activity against *M. luteus* include defensin from a hemipteran insect *Pyrrhocoris apterus* (MIC < 2 μ M) [33] and termicin from *Pseudacanthotermes spiniger* (MIC 0.8-1.5 μ M) [34].

It has been previously established that insect defensins function by disrupting the polarization of bacterial membrane and creating an ion imbalance across the membrane that eventually kills the targeted bacteria [35]. In this study we have proved a substantial depolarization of both Gram-positive and Gram-negative bacteria in 10 minutes (**Figure 3A:** Data shown for 10 minutes). The relatively fast depletion of cytoplasmic membrane potential in the bacteria indicates that membrane polarization is most likely the primary mode-of-action of CL-defensin in Gram-positive bacteria. The level of depolarization at the concentration of 50

μg/ml was almost equivalent to that of Triton X-100. Another proposed mode for defensins is the formation of pores in the cytoplasmic membrane. These multimeric pores disrupts the intracellular stability of the cell by abnormal influx and efflux of cell contents eventually leading to cell death [36]. We discovered that CL-defensin disrupts Gram-positive membranes by pore formation as depicted in **Figure 3B**. In low ionic condition, we observed some inhibition (~ 70-80%) of Gram-negative bacteria *A. baumannii* and *P. aeruginosa* (**Figure 2F and 2G**) and the membrane depolarization and pore formation results obtained in this study are consistent with the results of EC₅₀ experiments. One of the major components of the Gram-negative bacteria is the presence of an outer membrane (OM). The function of this outer membrane is to restrict the entry of hydrophobic compounds [30]. As seen from the sequence, CL-defensin is 44% hydrophobic and therefore the peptide may not traverse the OM sufficiently in order to access the cytoplasmic membrane. In future studies, additional compounds such Polymyxin B or EDTA could be utilized to increase the permeability of outer membrane before treatment with peptide [37,38].

In conclusion, we have established that CL-defensin primarily kills Gram-positive bacteria and is not very effective against the Gram-negative bacteria tested in our study. We have also demonstrated that the bacterial cytoplasmic membrane is a target of CL-defensin. The discovered peptide can be considered as a template for new synthetic peptides that can function at high salt concentration.

Author contribution

MVH conceived the project. MVH, AK and KG wrote the manuscript. AK and KG performed the experiments.

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Table 1: Bacterial strains tested in this study and EC₅₀ values of CL-defensin against the tested bacteria.

Figure 1: Structure of CL-Defensin. A) Molecular visualization of CL-defensin B) intramolecular disulfide bridges in CL-defensin as predicted by Chimera C) Circular Dichroism of CL-defensin. This figure represents a three dimensional view of the proposed structure of defensin as modeled by Chimera, molecular modeling system. In the figure N-terminal loop, α -helix and antiparallel β -sheets are clearly seen. **B) Diagram of putative CL-defensin sequence.** The predicted N- terminal loop is highlighted in gray, amphipathic α -helix is highlighted in blue and antiparallel β -pleated sheets are highlighted in yellow. The intramolecular disulfide bonding between the six cysteine residues is also shown **C) Circular dichroism of CL-defensin.** The Circular Dichroism spectra (190 – 240 nm) of CL-defensin measured at a peptide concentration of 100 $\mu\text{g/mL}$ dissolved in 60 mM of SDS in 10 mM of sodium phosphate buffer. Baseline of solvent without peptide has been subtracted from sample. Data is representation of three different experiments.

Figure 2: CL-defensin antimicrobial activity against the tested bacteria. Percent survival was calculated after an incubation period of 3 hours in 10 mM phosphate buffer and subsequent plating. **(A)** *C. renale* EC₅₀ = 2.26 $\mu\text{g/mL}$ **(B)** *M. luteus* EC₅₀ = 7.21 $\mu\text{g/mL}$. **(C)** *S. aureus* EC₅₀ = 2.17 $\mu\text{g/mL}$. **(D)** *S. epidermidis* EC₅₀ = 2.45 $\mu\text{g/mL}$. **(E)** *A. baumannii* EC₅₀ > 100 $\mu\text{g/mL}$. **(F)** *P. aeruginosa* EC₅₀ > 100 $\mu\text{g/mL}$.

Figure 3: A) Depolarization and B) Membrane pore formation by CL-defensin of bacterial cytoplasmic membrane. A). *S. aureus*, *C. renale*, *M. luteus*, *S. epidermidis*, *P. aeruginosa* and *A. baumannii* were incubated with a fluorescent dye DiSC₃(5). Depolarization of membrane was observed at the peptide concentrations of 50 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, 10 $\mu\text{g}/\mu\text{l}$ and 0.5 $\mu\text{g}/\mu\text{l}$. Means $\pm$ SD is shown. Error bars indicate the standard deviation from the mean. The asterisk (*) indicates p-value less than 0.0001. **B)** Kinetics of pore formation and binding of ethidium bromide to DNA with 50 $\mu\text{g/mL}$ of peptide. Pore formation is indicated by increase in fluorescence that is a result of DNA binding by ethidium bromide and consequent formation of pores in the membrane of bacteria. Permeabilization of membrane after 30 minutes was compared against no peptide control. Experiments were performed in triplicate (n=3). Means $\pm$ SD is shown.

Table 1: Minimum Inhibitory Concentration and EC₅₀ values of CL-Defensin against various Gram positive and Gram negative bacteria. The EC₅₀ values are also expressed as µM.

Bacteria	ATCC strain no.	MIC (µg/ml) CL-Def	MIC (µM) CL-Def	EC ₅₀ (µg/ml) CL-Def	95% CI (µg/ml) CL-Def	EC ₅₀ (µM) CL-Def	EC ₅₀ (µg/ml) NA-CATH	95% CI (µg/ml) NA-CATH	EC ₅₀ (µM) NA-CATH
<i>C. renale</i>	19412	41.66 ^a	8.88	2.26	1.51- 3.56	0.48	9.57	5.84 - 15.66	2.29
<i>M. luteus</i>	4698	2.60 ^a	0.55	7.21	6.26-8.23	1.53	2.51	2.23 - 2.83	0.60
<i>S. aureus</i>	25293	-	-	2.17	1.72-2.73	0.46	0.29	0.27 - 0.32	0.069
<i>S. epidermidis</i>	14990	41.66 ^b	8.88	2.45	1.87-3.29	0.52	0.52	0.44 -0.61	0.12
<i>A. baumannii</i>	9955	-	-	>100	-	>21.31	1.81	1.41-2.33	0.43
<i>P. aeruginosa</i>	P9027	-	-	>100	-	>21.31	0.63	0.46 -0.86	0.15

a: Represents bactericidal concentration

b: Represents bacteriostatic concentration

Figure 1

Figure 1: Structure of CL-Defensin A) Molecular visualization of CL-defensin B) intramolecular disulfide bridges in CL-defensin as predicted by Chimera and C) Circular Dichroism of CL-defensin and

Figure 2

Figure 2: CL-defensin antimicrobial activity against bacteria tested

Figure 3

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