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Characterization of antimicrobial activity against *Listeria* and cytotoxicity of native melittin and its mutant variants

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ABSTRACT

Antimicrobial peptides (AMPs) are relatively short peptides that have the ability to penetrate the cell membrane, form pores leading to cell death. This study compares both antimicrobial activity and cytotoxicity of native melittin and its two mutants, namely, melittin I17K (GIGAVLKVLTTGLPALKSWIKRKRQQ) with a higher charge and lower hydrophobicity and mutant G1I (IIGAVLKVLTTGLPALISWIKRKRQQ) of higher hydrophobicity. The antimicrobial activity against different strains of *Listeria* was investigated by bioassay, viability studies, fluorescence and transmission electron microscopy. Cytotoxicity was examined by lactate dehydrogenase (LDH) assay on mammalian Caco-2 cells. The minimum inhibitory concentration of native, mutant I17K, mutant G1I against *Listeria monocytogenes* F4244 was 0.315 ± 0.008, 0.814 ± 0.006 and 0.494 ± 0.037 μg/ml respectively, whereas the minimum bactericidal concentration values were 3.263 ± 0.0034, 7.412 ± 0.017 and 5.366 ± 0.019 μg/ml respectively. Lag time for inactivation of *L. monocytogenes* F4244 was observed at concentrations below 0.20 and 0.78 μg/ml for native and mutant melittin I17K respectively. The antimicrobial activity against *L. monocytogenes* F4244 was in the order native > G1I > I17K. Native melittin was cytotoxic to mammalian Caco-2 cells above concentration of 2 μg/ml, whereas the two mutants exhibited negligible cytotoxicity up to a concentration of 8 μg/ml. Pore formation in cell wall/membrane was observed by transmission electron microscopy. Molecular dynamics (MD) simulation of native and its mutants indicated that (i) surface native melittin and G1I exhibited higher tendency to penetrate a mimic of bacterial cell membrane and (ii) transmembrane native and I17K formed water channel in mimics of bacterial and mammalian cell membranes.

1. Introduction

Antimicrobial peptides (AMPs) are a diverse and vast group of peptides variously active against bacteria, enveloped viruses, protozoa and fungi and can be produced in all multi-cellular organisms [1–3]. These AMPs do share some common features, such as usually being electropositive and mostly amphiphilic as a result of the spatial segregation of hydrophobic and hydrophilic amino acid residues [2,4]. AMPs are generally considered to kill their microbial targets through insertion, damage/permeabilization or even solubilization of the cytoplasmic membranes of the target microorganisms [5–10]. They have been broadly studied for potential applications as anti-tumor agents, food preservatives, antiseptic agents, and potential replacement for antibiotics due to little resistance microorganisms generate [1,2,11–14].

The demand for the discovery and development of novel antimicrobial compounds defeating the known mechanisms of bacterial resistance due to their novel modes of action is becoming increasingly important due to the dramatic increase in bacterial resistance to numerous conventional antibiotics [15]. In the past decades, there has therefore been an increased interest in the investigation of AMPs that are found in several organisms and for which the structural and functional characteristics make them very promising therapeutic agents [16–18] even though the full-scale mechanisms of action by which these AMPs kill bacteria are still not clearly understood. Several general mechanisms, namely the 'barrel-stave', 'carpet-like' and 'toroidal' models have been proposed for pore formation in membranes by AMPs [1,5,19]. After initial electrostatic binding to the outer leaflet of the bacterial membrane, the hydrophobic peptide regions align with the lipid core region and the hydrophilic peptide regions form the interior

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region of pore. Pore formation by AMP as well as the structure of the pore have been investigated by various experimental techniques based on fluorescence [20], oriented circular dichroism [21], NMR spectroscopy [5], X-ray crystallography [21] and quartz crystal microbalance [22,23]. However, at higher AMP concentration, the peptides can disrupt lipid bilayers by orienting parallel to the surface of membrane and forming a layer or carpet outside the membrane. The toroidal model is applicable when the attached peptides aggregate and induce charged phospholipid heads to bend thereby lining the pore with both inserted peptides as well as phospholipid heads [19]. In an earlier investigation, we proposed a toroidal model for pore formation by aggregates of peptides in a lipid bilayer to evaluate the free energy of pore formation in terms of intermolecular interactions [24].

Net charge and hydrophobicity are major biophysical parameters that influence AMPs antimicrobial activities [25]. According to more recent studies, cationicity is believed to chiefly determine the initial interaction of AMP with the negatively charged bacterial membrane [26,27], whereas hydrophobicity appears to act with other structural parameters [26,28–30]. The antimicrobial efficiency and specificity of individual α-helical AMPs can be regulated by hydrophobicity, which influences the extent to which AMP interacts with the lipid bilayers [31–34]. However, optimal hydrophobicity level is essential for maintaining antimicrobial activity. A decrease of antimicrobial activity [26], along with a corresponding increase in mammalian cell toxicity have been proved above the optimal hydrophobicity level [30,35–37].

Melittin is a well-studied antimicrobial and hemolytic peptide from the venom of European honey bee (Apis mellifera). It contains 26 amino acid residues (GIGAVLKVLTTGLPALISWIKRKRQQ, molecular weight M_W = 2846.46 g/mol), which are primarily hydrophobic in the amino-terminal region and there are four out of the total number of six positively charged residues in the hydrophilic carboxy-terminal region [6,38,39]. In aqueous solutions, monomeric melittin with a random coil conformation will be predominantly present at low concentrations. Thus, melittin molecules is able to induce themselves parallel to the membrane surface. However, at higher ionic strength and pH, higher peptide concentrations lead to aggregation of melittin to tetramers [39,40], which can promote aggregation of melittin in the vicinity of negatively charged bilayers thus leading to local accumulations that are two orders of magnitude higher than the bulk concentration [41,42]. Several studies have also supported the α-helical conformation [38,43,44] and aggregation of membrane-associated melittin is more pronounced at high peptide concentration and high ionic strength [39,40,45]. Furthermore, increasing peptide concentration enhances the fraction of molecules in transmembrane or pseudo-transmembrane orientation [39,46,47]. The fraction tends to form pores resulting in the permeation of the membrane and indicates the toroidal pore model appears to be the most possible model for the melittin-induced pores [47,48]. Diameters of melittin-induced pores have been estimated in the range of 1–6 nm [47,49].

Though melittin possesses high antimicrobial activity, it exhibits high cytotoxicity to mammalian cells [50]. It is of interest to design analogues of melittin that possess high antibacterial activity but with reduced or no hemolytic activity. D-amino acid containing analogues of melittin (diastereomers), a CA-melittin hybrid peptide consisting of cecropin A and melittin sequences, and an analogue designed from the C-terminal 15-residue fragment (residues 12–26) each exhibited similar antibacterial activity to melittin but markedly less hemolytic activity [51]. Besides, a melittin analogue in which the Trp-19 residue of melittin was replaced by a single Trp mutation with a peptide residue (Nhtrp) was shown to exhibit much lower cytotoxicity against human red blood cells, HeLa (Human cervical carcinoma) and NIH-3T3 (mouse fibroblast) cells than native melittin [52].

Molecular dynamics (MD) simulation has been employed to characterize the interaction of peptides with lipid bilayers [53] and their influence on peptide conformation [54], deformation of lipids and peptide penetration [55]. MD simulation study shows an energy barrier for penetration of melittin into lipid bilayer [56]. Placement of transmembrane melittin into bilayer is found to result in bending of phospholipids consistent with toroidal pore structure as well as formation of water channel [57–59]. In a previous study, we have shown that there is a critical peptide/lipid ratio necessary for pore formation as demonstrated by water channel formation by transmembrane melittin [60].

In the present study, we combine different techniques to characterize the effects of melittin on different species of *Listeria* including *Listeria monocytogenes*, since it has a mortality rate of 19% affecting immunocompromised individuals; the elderly, pregnant women and their fetuses, and AIDS patients [61]. To the best of our knowledge, this is the first such report examining antilisterial effect of melittin.

Moreover, in order to investigate the effects of charge and hydrophobicity on antimicrobial activity, we synthesized two melittin variants (mutant melittin). In the first variant, we replaced Ile residue at position 17 of native melittin with Lys residue (Table S1). In the second, we replaced Gly with Ile in position 1 (Table S1). The lytic activity of melittin mutants against several bacterial cells, and mammalian cells was investigated. We also monitored the kinetics of deactivation of several microorganisms and show the existence of lag time for microbial deactivation at sufficiently low melittin concentrations.

Table 1
Listeria species used in the present study.

Listeria	Strain
Wild-type *Listeria*	
Listeria grayi	ATCC 19120
Listeria innocua	F4248
Listeria ivanovii	ATCC 19119
Listeria marthii	ATCC BAA-1595
Listeria monocytogenes	F4244
Listeria rocourtiae	CIP−109804[a]
Listeria seeligiri	ATCC 35967
Listeria welshimeri	ATCC 35897
Recombinant *Listeria*	
Listeria monocytogenes gfp[+b]	F4244 (parent strain)

Listeria strains were procured from American Type culture collection (ATCC), Manassas, VA, USA and Center for Disease Control and Prevention (CDC) strain from our lab culture collection.

[a] Culture provided by Dr. Haley Oliver, Department of Food Science, Purdue University, West Lafayette, IN, United States.

[b] Culture provided by Dr. Bruce Applegate, Department of Food Science, Purdue University, West Lafayette, IN, United States.

2. Materials and methods

2.1. Bacteria, growth media and melittin

All *Listeria* cultures (Table 1) were stored as 10% frozen glycerol stocks in deep-freezer at −80 °C. Prior to experiments, frozen stocks were streaked on brain heart infusion (BHI; Accumedia, Neogen) agar plate and incubated at 37 °C for 16 h to obtain a single colony, which was propagated in BHI broth at 37 °C for 16 h prior to experiments. Green fluorescent protein (GFP) expressing *L. monocytogenes* F4244 (provided by Dr. Bruce Applegate at Purdue University) was grown in Luria Bertani (LB; BD Biosciences) broth in the presence of 10 μg/ml of erythromycin for 24 h at 37 °C.