THE ANTIBACTERIAL MODE OF ACTION AND PROPERTIES OF IB-AMP1, A PLANT-DERIVED ANTIMICROBIAL PEPTIDE, AGAINST *ESCHERICHIA COLI* O157:H7

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A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Food Science

Written under the direction of

Dr. Karl R. Matthews and Dr. Rong Di

And approved by

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New Brunswick, New Jersey

May, 2013

ABSTRACT OF THE DISSERTATION

The Antibacterial Mode of Action and Properties of Ib-AMP1, a Plant-Derived Antimicrobial Peptide, Against *Escherichia coli* O157:H7

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The continual occurrence of foodborne outbreaks along with the consumer demand for use of fewer traditional antimicrobial agents in foods has driven research interests in development of plant-derived antimicrobial agents (pAMPs) for use in food and food processing. Ib-AMP1 is a pAMP isolated from seeds of *Impatiens balsamina*. Previous studies indicated that it is a broad spectrum pAMP and the therapeutic index against eight human pathogens was 23.5; however, for future utilization, other antibacterial properties and mode of action must be elucidated. The purpose of this dissertation was to investigate the antibacterial properties and mode of action of Ib-AMP1 against *Escherichia coli* O157:H7, a foodborne pathogen that has been continually associated with foodborne outbreaks. The study design provided insight on the implantation and potential application of Ib-AMP1; a specific docking site or ligand-receptor relationship was not studied. The results demonstrated that Ib-AMP1 exhibited bactericidal activity against *E. coli* O157:H7, *Salmonella enterica* serovar Newport, *Pseudomonas aeruginosa*

and *Staphylococcus aureus*. Ib-AMP1 at lethal concentrations (1X and 2X MIC) resulted in 1.46 to 2.69 log reduction of viable cells and prevented outgrowth when tested against low (10^3 CFU/mL) and medium (10^6 CFU/mL) *E. coli* O157:H7 populations. Ib-AMP1 at 2X MIC failed to inhibit and prevent outgrowth when cell numbers were 10^9 CFU/mL. No residual activity of Ib-AMP1 was apparent following interaction of the peptide with bacteria or the medium. Ib-AMP1 concentration less than 100 µg/mL showed little or no inhibition of human cell proliferation including human small intestine, colon and liver cells, which are associated with oral consumption of an AMP. The mode of action study demonstrated that a concentration dependent effect of Ib-AMP1 on the *E. coli* O157:H7 cell membrane occurred. Ib-AMP1 treatments resulted in efflux of K^+ and ATP, suggesting pores of sufficient size to allow efflux of large molecules. The efflux of intracellular components may be associated with damage to the outer membrane and dissipation of cytoplasmic membrane potential. Results of this study suggest Ib-AMP1 is bactericidal interfering within outer and inner membrane integrity permitting efflux of ATP and interfering with intracellular biosynthesis of DNA, RNA, and protein.

ACKNOWLEDGMENT

I would like to express my deepest appreciation to my advisor Dr. Karl R. Matthews for giving me the opportunity and encouraging me to pursue my Ph.D. I could not have done my research and dissertation without his generous and helpful guidance. His intelligence and diligence has not only inspired me in my studies but has also made an impact in my life at large. My appreciation also goes to my co-advisor Dr. Rong Di, for starting the project on plant antimicrobial peptide and her help on finding career opportunity for me.

I would like to thank my committee members: Dr. Mikhael L. Chikindas and Dr. John A. Renye. I always appreciate your valuable questions which indeed help me think more thoroughly and critically. My work would never be fully completed without the help from these men of wisdom.

Furthermore, I would like to thank my lab mates for their companion: Seungwook Seo, Chang-Hsin Liu, Yangjing Jang, Hyein Jang, Germain Tsui and Yan Nee Tan. I am thankful for their company and kind help on my experiments. Hoan-Jen Pang (Eunice Yim), thank you for guiding me through my qualifying exam.

My work would not be finished without the support and encouragement from all my friends: Yi-Chieh Tsai, Ya-Ling Shih, Hui-Hsuan Cheng, Wei-Tsen Chien and Wan-Ru Cin. You show me how true friendships are. En-Ming Chang, thank you for your support and sharing both the upside and downside of my Ph.D. career and my life; thank you for

helping me get through all the difficult times. I also would like to thank your parents for encouraging me and taking care of me.

Lastly, and most importantly, I wish to thank my parents. The words *thank you* do not adequately express my appreciation for the love, support and encouragement that my parents have showered on me. Without your generous support and encouragement, I couldn't put my dream of pursuing my graduate study in the US into practice. My parents have always provided me the freedom to make my own choices in life. Moreover, I would like to also thank my sister, for being a companion to share my journey through my life. I love you all.

DEDICATION

To my parents,

Ding-Jung Wu and Shen-Chih Chang

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

Chapter 4

Appendix

LIST OF ILLUSTRATIONS

Appendix

CHAPTER 1

LITERATURE REVIEW

I. Rational and significance

Foodborne diseases represent a significant public health concern not only in the United State but globally. The latest epidemiological estimation reported by CDC indicated during the past almost 10 years, in the United States, there were 47.8 million cases per year of foodborne illnesses, which included 127,839/year hospitalizations and 3,037 deaths/year (Scallan et al., 2011a, b). The initial epidemiological estimation done by CDC in 1999 estimated there were76 million foodborne illnesses, 325,000 cases of hospitalization and 5,000 deaths annually (Mead et al., 1999). Although the difference in the estimation methods and the advance in detection technology prevent us to conclude that there were significant decreases in the incidence of foodborne illness, the reports suggest that foodborne illnesses are a persistent public health issue. Antimicrobial agents are continually on demand to inhibit the growth of human pathogens and further reduce the incidence of foodborne illness, as well as efficient sanitizers to clean processing facilities and equipment. Bacteria and virus are the major causative agents. Foods, a nutrient environment, are optimal vehicles to transfer bacteria to humans. Improper handling of foods and poor personal and environmental hygiene increase the incidence of food related illness. Foodborne pathogens, such as *Listeria*, *Escherichia coli*, *Salmonella*, *Campylobacter*, *Shigella*, *Vibrio* and *Yersinia* are

under CDC surveillance (FoodNet, http://www.cdc.gov/foodnet/). Outbreaks associated with food products, such as fresh produce, deli meat, dairy and or even dehydrated vegetable protein in which the low water activity is supposedly not a favorable condition for most of microorganism, have been reported.

In the food industry, sanitizers with greater efficacy are in demand. Currently, for certain non-thermal food processing, it is often aimed at controlling commensal microbial load to extend shelf life, but not the specific control of foodborne pathogens. For instance, in produce processing facilities, chlorinated wash water is used to control the microbial load in wash water; however, it has limitation in bacteria reduction and is sensitive to organic and inorganic matter. Except for subsequent refrigeration, usually, no other controls are implemented, and this may pose a potential risk for food products that have undergone no thermal process and may be contaminated with foodborne pathogens, such as *E. coli* O157:H7 which has low infective dose. Moreover microbiological spoilage is often the major cause of economic loss for the food industry. Furthermore, consumers are now more aware of the benefit of a healthy diet leading to the demand for natural food preservatives. A good natural food preservative has to possess a broad antimicrobial spectrum of activity, but yet not be toxic to humans. It has to have no effect on the flavor and color of food products; and has to be cost effective for commercial use.

Antibiotic resistance is one of the major problems the pharmaceutical industry encounters leading to explore AMPs as alternatives to traditional antibiotics. With the

extended use of antibiotics, antibiotic resistant microorganisms have been isolated from humans, animals, and even food products. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) are two of the most well characterized antibiotic resistant bacteria. Those strains pose a significant burden on treatment of infection. Multiple antibiotic-resistant *E. coli* have also been isolated; those stains were found to be co-resistance to four or more unrelated families of antibiotics (Cohen et al., 1989; Ariza et al., 1994; Maynard et al., 2003). AMPs have been researched for their potential clinical use, as they can be antibiotic alternatives.

In the agriculture industry, transgenic plants with insertion of gene encoding antimicrobial peptide could be a way to decrease the incidence of plant diseases and potentially the contamination of foodborne pathogens. The approaches could be anti-inset, antimicrobial transgenic plants. They could also be used to control the post-harvest decay caused by pathogens or bacteria that spoil agricultural products. Scientists believe that antimicrobial peptides are promising and potential agents to overcome all these problems.

We believed that Ib-AMP1 can be a potential natural food preservative and has therapeutic potential. The aim of the present study was to determine the antimicrobial properties of Ib-AMP1 against *E. coli* O157:H7 and evaluate the cytotoxicity of Ib-AMP1 upon oral consumption. We also investigated the mode of action of Ib-AMP1 against *E. coli* O157:H7. The significance of the present study is to give further insight on the potential application of Ib-AMP1 based on their mode of action, as finding novel AMPs

are one of the strategies to control antimicrobial resistance. To the best of our knowledge, this is the first report on the mode of action of Ib-AMP1 on a Gram-negative bacterium. A specific docking site or ligand-receptor relationship was not studied in this dissertation.

II. Antimicrobial peptides (AMPs)

Antimicrobial peptides are small proteins that show inhibition on bacteria, fungi and other microorganisms; most of them are cationic and amphipathic (van't Hof et al., 2001; Wang and Wang, 2004; Barbosa Pelegrini et al., 2011). They have caught researchers' attention for their varied prospective applications, including their therapeutic potential. Those peptides are produced throughout the kingdom of life, from bacteria, fungi, plants, insect, vertebrate and mammalian. They are products of innate or adaptive immunity to protect their host from infection. AMPs such as Nisin from *Lactococcus lactis*, defensin from human or plants, and magainins from Xenopus skin have been studied extensively.

Plant-derived AMPs (pAMPs) capture our interest due to their potential future application in the agriculture industry, such as plant disease control and genetically modify crops which might lead to decreasing of food-borne illnesses. Majority of pAMPs are small cationic cysteine-rich proteins containing less than 50 amino acid residues (Hammami et al., 2009; Cândido et al., 2011). Plants are constantly exposed to harsh environments and a broad range of pathogens; therefore, plants produce

antimicrobial substances as their primary defense while those substances cause no damage to the plant. Those pAMPs could be constitutively expressed or expressed upon infection; almost every plant structure (i.e., leaf, root, and stem) produces at least one pAMP (Garcia-Olmedo et al., 1998; Thomma et al., 2002; Lay and Anderson, 2005). Due to their diversity in source, pAMPs are diverse in size, amino acid composition and structure. Nuclear magnetic resonance spectroscopy (NMR) was used to determine the 3-dimentional structure of pAMPs. Results indicate that pAMPs may contain α-helices, β-sheet, cyclic or cyclic structures (Hammami et al., 2009; Cândido et al., 2011). Those structures render pAMPs amphipathicity which may facilitate the interaction of pAMPs with their target microorganisms.

It is difficult to classify AMPs generally, because they each may act differently biologically, chemically, and physically. Classification based on secondary structure is commonly used (van't Hof et al., 2001). AMPs are grouped into 1) linear peptide with α-helical structure, 2) linear peptides with an extended structure, 3) peptides with a looped structure and 4) peptides with β-strand structure. The relationship between AMPs' secondary structure and antimicrobial activity is still not completely delineated; however, it is known that antimicrobial activity is not solely dictated by secondary structure but also other factors, such environmental conditions, other properties of AMPs and type of target microorganisms.

Due to the lack of structural information of many pAMPs, the most extensively used classification is according to their amino acid composition, conformation, their

mechanism of action and other characteristics in common; however, the classification is not absolute. The PhytAMP database classified pAMPs as cyclotides, defensins, Hevein-like, Impatiens, knottins, thionin or vicilin-like, MBP-1 and beta-barellin (Hammami et al., 2009; Cândido et al., 2011).

Most of the pAMPs have been shown to have a broad spectrum of antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria and fungi including many plant pathogens, but also, based on limited research, foodborne and human pathogen. (Fernandez de Caleya et al., 1972; Kelemu et al., 2004; Pelegrini et al., 2009; Wang et al., 2009). According to PhytAMP database, although only 35 % of the recorded pAMPs were tested for biological activity; 51 % of pAMPs possess antifungal activity, 35 % of pAMP are antibacterial, and 10 % are antiviral and around 5 % are insecticidal and anti-yeast (Hammami et al., 2009).

III. Antibacterial mode of action

Elucidating the mode of action of a given AMP is critical and essential for any future application. It also facilitates design and development of novel AMPs with higher efficacy. The research on the interaction between AMPs and their target bacterial cells demonstrates they target the cell membrane. Recent studies indicate AMPs can also inhibit intracellular macromolecule synthesis, such as DNA, RNA and protein (Epand and Vogel, 1999; van't Hof et al., 2001; Cudic and Otvos, 2002; Brogden, 2003; Yeaman and Yount, 2003; Jenssen, 2006; Nicolas, 2009). Briefly, AMP antibacterial mode of action

involves the disruption of membrane integrity and/or affects synthesis of intracellular components, which subsequently leads to membrane dysfunction, and/or metabolism malfunction and eventually results in cell death.

The entire antibacterial mode of action of AMPs is still not clearly understood yet for many of them; it is possible that it varies for different peptides against different microorganisms. Several factors may influence antibacterial activity, including intrinsic and extrinsic factors. Intrinsic factors include but are not limited to peptide charge, hydrophobicity, amino acid composition, conformation. Extrinsic factors include membrane charge, membrane lipid composition, and membrane fluidity of target microorganisms (Yeaman and Yount, 2003). Those factors affect affinity between AMPs and target microorganisms. According to the AMPs database, a high content of cysteine or glycine residue is important to antibacterial activity (Wang and Wang, 2004; Hammami et al., 2009). The presences of disulfide bridges formed by cysteine residues enhance structural stability, and those peptides have the tendency to form β-sheet structure. In contrast, peptides rich in glycine residues tend to form α-helices and are more structurally flexible (Jenssen et al., 2006; Pelegrini et al., 2008; Wang and Wang, 2004; Hammami et al., 2009). Structures or conformational difference of AMPs may affect the mode of action and activity; however, the correlation between AMPs structure and antimicrobial activity may not be absolute. Likely, α-helices provide structure flexibility while β-sheets provide structure stability (Barbosa Pelegrini et al., 2011). Furthermore, if all-D peptides are equipotent to the naturally occurring all-L peptides then it is unlikely that a highly stereospecific target, such as a membrane-

bound protein receptor or a cytoplasmic enzyme, would be required to mediate their bacteriostatic effects (Epand and Vogel, 1999). This may suggest the non-specific mode of action of AMPs. The whole bacterial inhibition or killing process can be separated step by step as the initial membrane attraction, membrane binding, membrane disruption/traverse, intracellular targets and cell death (Fig. 1).

The interaction between AMPs and their target microorganism cells are proposed to initially interact with bacterial cell membrane, due to the electrostatic affinity between cationic AMPs and anionic bacterial cell membrane components. This electrostatic affinity also renders AMPs more selective to the bacterial cell membrane than to the mammalian cell membrane. The details of selectivity will be elaborated later in this dissertation. In Gram-positive bacteria, the cell envelop consists of the cytoplasmic membrane in contact with cytosol and a thick multilayer of peptidoglycan in contact with the extracellular environment. Gram-negative bacteria cell envelop consists of the cytoplasmic membrane, a thin layer of peptidoglycan and an additional layer of outer membrane in contact with the extracellular environment. The anionic components on both Gram-positive and Gram-negative cell envelop surface strengthen the electrostatic affinity. Those anionic cell surface components are teichoic acid, lipotechoic acid on Gram-positive bacteria peptidoglycan layer; lipopolisaccharide, phosphate groups of phospholipid in the outer membrane in Gram-negative bacteria. The electrostatic force attracts AMPs to the bacterial cell envelop. Moreover, the bacterial cytoplasmic membrane is abundantly composed of negatively charged phospholipid, such as hydroxylated phospholipids phosphatidylethanolamine (PG), phosphatidylserine (PS)

and cardiolipin (CL). The negatively charged phosphate groups of cytoplasmic lipid bilayer further strengthen the electrostatic interaction with positive charges of AMPs. After being attracted to the bacterial cell membrane via electrostatic attraction, AMPs contact the cell membrane by hydrophobic interaction with the hydrophobic regions of AMPs binding to hydrophobic regions of cytoplasmic membrane, and hydrophilic regions of AMPs binding to hydrophilic regions of cytoplasmic membrane. The amphipathic nature of lipid bilayer in cytoplasmic membrane favors the permeabilization or traversing of amphipathic AMPs via hydrophobic interaction.

Due to the presence of a more rigid outer membrane, Gram-negative bacteria compared to Gram-positive bacteria are more resistant to antibacterial agents. Three uptake pathways have been proposed: hydrophilic-uptake pathway, hydrophobic-uptake pathway and self-promoted pathway (Nikaido and Nakae, 1979; Hancock et al., 1981; Hancock and Wong, 1984). The hydrophilic-uptake pathway involves the uptake of hydrophilic antibiotics through porins to cross the outer membrane of Gram-negative bacteria. In the hydrophobic-uptake pathway, hydrophobic antimicrobial agents diffuse across the outer membrane lipid bilayer; however, it seems to occur less prevalent in Gram-negative bacteria and some bacteria are resistant to hydrophobic antibiotics (Nikaido, 1976; Hancock, 1984). The self-promoted uptake pathway involves in the uptake of polycationic antibiotics. The outer membrane structures are held by binding cations, such as Ca^{2+} and Mg^{2+}. Loss of those cations destabilizes LPS structure. Polycationic antibiotics are demonstrated to replace cations associated with lipid A of

LPS, and this replacement destabilizes the outer membrane structure and subsequently promotes uptake of the polycationic antibiotics (Hancock, 1997).

Efflux of intracellular components or markers has been used widely to study the membrane permeation effect of AMPs. Studies showed that AMPs induce leakage of artificial model membrane (Hall et al., 2003; Zhang et al., 2001). Leakage of fluorescent probes from model membrane has been used widely to determine the membrane permeability of AMPs. However, in vitro model membrane cannot predict precisely the in vivo mechanism. Assays such as leakage of intracellular components are used to further determine the mechanism(s) on bacteria in vivo (Orlov et al., 2002; Yasuda et al., 2003). Other studies demonstrated that AMP-cell membrane interaction may also involve cell surface receptors. Nisin, a well-studied bacteriocin, has been shown to specifically bind to bacterial lipid II which is involved in peptidoglycan synthesis. This further supports the fact that Gram-positive bacteria are more susceptible to nisin than Gram-negative bacteria (Breukink and de Kruijff, 1999).

Once AMPs are attracted to the bacterial membrane, AMPs start to bind to the bacterial cytoplasmic membrane by either permeabilizing or traversing the cytoplasmic membrane. As mentioned previously the amphipathic nature of AMPs favors the interaction with amphipathic bacterial cytoplasm membrane via hydrophobic interaction. Several membrane permeabilization models have been proposed; barrel-stave model, carpet model, toroidal pore model and aggregate model; the details and comparison will be described later. Several factors are required for AMPs to approach

bacterial cell membranes. First of all, studies have showed that a threshold concentration needs to be reached to start the membrane permeabilization action (Yang et al., 2000). The threshold involves an AMP/lipid ratio. AMPs are parallel to the lipid bilayer at a low AMP/lipid ratio; however AMPs become perpendicularly and insert into the lipid bilayer at a high ratio (Yang et al., 2001a).

Conformational changes of AMPs also have been observed when they interact with the cell membrane. AMPs may form a random structure in an aqueous environment, but form a more ordered structure when in contact with a target membrane. Those changes are mainly seen in AMPs having a bare α-helical structure. Circular dichroism (CD) and NMR examination showed that magainins formed α-helical structure only when interacting with negatively charged artificial vesicles or monolayer material lipid bilayer (Matsuzaki et al., 1989&1991, Bechinger et al., 1993; Hirsh et al., 1996). Other AMPs, such as melittin and synthetic cecropin A(1-8)-melittin(1-18) hybrid peptide, have also been shown to act in the same manner (Bello et al., 1982; Dathe and Wieprecht, 1999; Mancheño et al., 1996). AMPs baring β-sheet structures are less likely to undergo conformational change upon contact with a cell membrane, due to the presence of disulfide bonds that constrain the structure. Tachyplasin, a cationic peptides purified from horseshoe crab, contains a type II β-turn and possess the same β-turn structure both in an aqueous and membrane-mimetic environment (Oishi et al., 1997; Nakamura et al., 1988). Exception may be seen when the quaternary structure dissociates upon interacting with the cell membrane (Yeaman and Yount, 2003).

Several models have been proposed to illustrate the membrane permeabilization mechanism of AMPs as mentioned previously in this section. In the barrel-stave model, AMPs are initially parallel to the plane of the membrane; once the threshold concentration is achieved, AMPs reorient to become perpendicular to the plane of the membrane, and results in the insertion of AMPs. Subsequently, the AMPs form a bundle in the membrane with hydrophobic regions facing the membrane and hydrophilic regions facing the aqueous environment. In the carpet model, AMPs accumulate parallel to the membrane surface as a carpet; once a threshold concentration has been reached, the membrane collapses and eventually leads to the formation of micelles. In the toroidal pore model, AMPs adapt an orientation perpendicular to the membrane which results in the membrane bend inward to form a pore which results in a positive curvature strain. The pores are lined by both AMPs and phospholipid head groups. In the aggregate model, similar to the toroidal pore model, AMPs adopt no particular orientation and span the membrane as an aggregate with micelle-like AMP-lipid complex.

Both the aggregate and toroidal pore models are able to explain membrane permeabilization and translocation across the cell membrane without damaging cell membrane integrity, however, in the aggregate model informal pores are formed, but in the toroidal pore model formal pores are formed. Both the barrel-stave model and carpet model cause dissipation of membrane potential and loss of membrane integrity; however the dynamic of the pores is different, where there are positive curvature strain of the membrane in the carpet model but not in barrel-stave model. Both the toroidal

pore and carpet model cause a positive curvature strain which was shown to facilitate the formation of a torus-type pore. In contrast, the presence of negative curvature-inducing lipids inhibits pore formation (Matsuzaki et al., 1998).

A growing number of AMPs have been shown to inactivate microorganisms without affecting bacterial membrane integrity. An alternative antibacterial mode of action has also been demonstrated where AMPs target intracellular macromolecules, such as DNA, RNA, protein or other cell components such as peptidoglycan. The majority of studies elucidate the affinity to those molecules; however, the specific ligand-receptor relationship is still unknown for most AMPs. AMPs that inhibit the synthesis of intracellular macromolecules have been reported. Buforin II binds to both DNA and RNA without permeabilizing the *E. coli* cytoplasmic membrane (Park et al., 2000). PR-39 inactivates *E. coli* by inhibition of DNA and protein synthesis (Boman et al., 1993). Finally, cell membrane permeation and inhibition of intracellular macromolecule synthesis may directly or indirectly result in cell death.

In summary, AMPs approach and bind to the bacterial membrane via electrostatic and hydrophobic interaction. Bacterial membrane composition and surface charge favor the permeabilization and traverse of AMPs across bacterial cell membranes. This interaction may cause the formation of permanent pores which may be lethal or the formation of transient pores that allow AMPs to enter the intracellular domain; once AMPs enter cells, they may target intracellular macromolecules through inhibition of their synthesis. AMPs are also thought to target multiple sites rather than a single site.

Questions have been raised concerning whether AMPs are cytotoxic to humans. The affinity of AMPs to membranes is influenced by factors including membrane charge and membrane curvature. Studies utilizing artificial lipid membrane indicated that AMPs possess higher affinity to bacterial cell membrane than to mammalian cell membrane (Matsuzaki et al., 1995; Matsuzaki, 2009). The composition and charge of the membrane account for the difference (Fig.2). Bacterial cell membranes are composed of a high abundance of acidic phospholipids, such as phosphatidylglycerol (PG), phosphatidylserine (PS), cardiolipin (CL), which are negatively charged. Those negatively charged of phospholipids interact with positively charged AMPs through electrostatic interaction. Plant and mammalian membranes contain a higher level of zwitterioinc phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM). Therefore the electrostatic interaction between AMPs and the mammalian cell membrane is relative week. The presence of cholesterol in the mammalian cell membrane decreases membrane fluidity and hinders translocation across plant and mammalian membranes. Zhang et al. (2001) demonstrated that peptide, regardless of structure and conformation, have higher binding affinity to a negatively charged lipid monolayer than to a lipid monolayer with a neutral charge. Many studies have demonstrated that AMPs have no cytotoxicity or hemolytic activity on erythrocyte or other mammalian cell lines even at concentration a lot higher than MIC to target microorganisms. Results may indicate the potential application in pharmaceutical or food industry.

IV. Ib-AMP1

Ib-AMP1 is one of four highly homologous peptides and was isolated from seeds of *Impatiens balsamina* (Tailor et al., 1997). *Impatiens balsamina* has been used in traditional Chinese medicine for centuries to treat infection, inflammation, and other ailments. Extracts from different parts of the plant exhibited anti-tumor, antimicrobial, and antioxidant activity (Yang et al., 2001b; Ding et al., 2008; Wang et al., 2011; Su et al., 2012). Tailor et al. (1997) reported that Ib-AMP1 was expressed in mature seeds during the course of seed development. However, whether Ib-AMP1 is expressed in other plant tissues has yet to be determined. Ib-AMP1 is a 20-mer small cationic peptide containing four cysteine residues, which form two intra-molecular disulfide bridges (Lee et al., 1999; Thevissen et al., 2005). The amino acid sequence from N-terminus to C-terminus is QWGRRCCGWGPGRRYCVRWC (Tailor et al., 1997). Studies had focused on Ib-AMP1 and Ib-AMP4 since they showed higher antifungal and antimicrobial activity than Ib-AMP2 and Ib-AMP3, the homologous peptides (Tailor et al., 1997). Standard solid phase synthesis and purification from the seeds had been used in studies to produce Ib-AMP1. Research to determine the antimicrobial activity Ib-AMP1 has been conducted using both natural and synthetic forms of the peptide generating a range of results (Tailor et al., 1997; Lee et al., 1999; Thevissen et al., 2005; Wang et al., 2009). The source and method of production of Ib-AMP1 may affect its antimicrobial activity. The naturally purified Ib-AMP1 from seeds of *Impatiens balsamina* was active against only Gram-positive bacteria; however the synthetic Ib-AMP1 showed comparable antimicrobial activity against both Gram-positive and Gram-negative bacteria. The

synthetic Ib-AMP1 showed comparable antifungal activity as their naturally purified counterpart. This indicates solid phase synthesis is a potential way for large-scale production of a bioactive form of Ib-AMP1. Moreover, the small size (20-mer) makes Ib-AMP1 solid-phase synthesis/production straightforward and cost-effective.

Studies have shown that Ib-AMP1 inhibits the growth of a range of bacteria and fungi at the micro molar level (Tailor et al., 1997; Lee et al., 1999; Thevissen et al., 2005; Wang et al., 2009). The microorganisms tested were mainly plant pathogens. Table 1 summarizes the antimicrobial activity of Ib-AMP1 against various fungi, yeast, and bacteria. For antifungal activity, synthetic Ib-AMP1 was more efficacious against yeast than fungi. Besides determining the minimum inhibitory concentrations, other antibacterial properties (e.g., bactericidal, bacteriostatic, residual activity) of Ib-AMP1 were not elucidated in those studies.

Structural studies by CD analysis showed that Ib-AMP1 possessed no α-helix and β-sheet structure, but instead β-turn structure (Fig.3) (Tailor et al., 1997; Patel et al., 1998). These β-turns result in one hydrophobic region flanked by two hydrophilic regions (Patel et al., 1998), and render Ib-AMP1 amphipathic property. Other research groups also confirmed its β-turns structure. NMR results showed that the intracellular disulfide bridges adopt a loop structure, however, a random coil conformation is formed when no disulfide bridge linkage (Lee et al. 1999, Thevissen et al. 2005). Wang et al. (2009) showed that Ib-AMP1 formed a random coil structure in an aqueous solution; however, it formed β-turn structure when in contact with negatively charged prokaryotic

membrane-mimetic environment, and a partially folded structure when in contact with zwitterionic eukaryotic membrane-mimetic environment. This indicated Ib-AMP1 may have different affinity or interact differently to prokaryotic and eukaryotic membrane. Studies also showed that Ib-AMP1 become less active in high ionic strength solution, and this may indicate the presence of cations may decrease the antimicrobial activity. Additionally, it was reported that Ib-AMP1 tended to precipitate at concentrations more than 3μM (Tailor et al., 1997).

Limited information is available on mechanism of action of its antimicrobial and antifungal activities. Model membrane which mimics the prokaryotic and eukaryotic membrane environments have been used to determine the interaction of Ib-AMP1 with bacterial and fungal membranes, respectively (Lee et al., 1999; Wang et al., 2009). By determining the degree of efflux of large fluorescent compounds, the studies evaluated the ability of Ib-AMP1 to permeabilize bacterial or fungal mimetic membrane. Results showed that Ib-AMP1 caused little leakage on bacterial model membrane, but caused almost 80% leakage on fungal model membrane at MIC levels. Moreover, Ib-AMP1 failed to depolarize *S. aureus* membrane. Lee et al. (1999) showed that when fungal cells are intact, Ib-AMP1 located at the cell surface or penetrated into the cells, and that Ib-AMP1 tended to localize in certain intracellular areas in permeabilized cells. Altogether Ib-AMP1 may not only target the bacterial cell membrane, but intracellular processes; in contrast Ib-AMP1 may have multiple targets on the fungal cell membrane. The mode of action of Ib-AMP1 on Gram-negative bacteria has not yet been elucidated.

Toxicity and hemolytic activity tests showed that synthetic Ib-AMP1 was not cytotoxic to human erythrocytes or tumor cells, such as K-562 (human bone marrow lymphoblast cells), A549 (human lung epithelial cells) and NDA-MB-361 (human mammary gland epithelial cells), at 100 μM (250 μg/mL) (Lee et al., 1999). Thevissen et al. (2005) showed that synthetic Ib-AMP1 did not exhibit any hemolytic activity against rabbit erythrocytes at 200 μM, which is 12.5 - 400-fold higher than its IC_{50} against plant fungal pathogens (table 1); and was not toxic to mouse myeloma cells at 100 μM. Wang et al. (2009) also proved that synthetic Ib-AMP1 and all other linear analogues did not cause lysis of human red blood cells at 400 μM, which is almost 12 times higher than its MICs against human bacterial pathogens (table 1). Previous research suggests that synthetic Ib-AMP1 has greater antibacterial and antifungal activity, but exhibits no increased hemolytic and toxic activity. Results suggest that chemically synthesized Ib-AMP1 is more potent and commercially prudent way to produce Ib-AMP1 compared to extraction from the seeds.

V. Snakin-1

Snakin-1 is a plant antimicrobial peptide isolated from potato tuber (*Solanum tuberosum*) (Segura et al., 1999). Nahirñak et al. (2012) have demonstrated that snakin-1 plays roles in plant growth, such as cell division, cell wall composition, and leaf metabolism. Using *Arabidopsis* as a model plant, Almasia et al. (2010) concluded that snakin-1 is induced by temperature and wounding. It has been shown that snakin-1 is

active against plant pathogens both in vitro and in vivo (Segura et al., 1999; Almasia et al., 2008). Altogether, these studies demonstrated that snakin-1 plays roles in protection and normal growth of potato plant. Utilization of snakin-1 as antimicrobial treatments on plants to control plant disease or as antimicrobial agents in food systems to control food spoilage and foodborne pathogens may decrease production loss associated with plant disease, and to enhance microbial safety of food products. However, studies on snakin-1 are limited to its application in plant disease control, especially in transgenic plants overexpressing snakin-1; its application in other areas including food preservation and as sanitizers have not been explored. Cytotoxic and hemolytic activities have not been studied.

Snakin-1 is a 63-mer peptide containing 12 cysteine residues which form six disulfide bridges. Detail structural analysis has not been determined, therefore, formation of natural disulfide bridges remains speculative. The amino acid sequences from N-terminus to C-terminus are GSNF**C**DSK**C**KLR**C**SKAGLADR**C**LKY**C**GV**CC**EE**C**K**C**VPSGTYGNKHE**C**P**C**YRDKKNSKGKSK**C**P (Segura et al., 1999). Based on its amino acid sequence, snakin-1 is highly basic and has a central hydrophobic stretch which is flanked by highly polar, long N-terminal and C-terminal domains. There are no evident amphipathic helices in the structure.

Studies indicated that natural snakin-1 was active against to fungi and Gram-positive bacteria (Segura et al., 1999; López-Solanilla et al., 2003). Limited number of bacterial genus was tested and antibacterial activity screening against more genera will be

required to determine its overall antibacterial activity. Its antimicrobial mode of action is still not known. Segura et al. (1999) indicated that snakin-1 caused aggregation of both Gram-positive (*Clavibacter michiganensis* subsp. *sepedonicus*) and Gram-negative (*Ralstonic solanacearum*) bacteria; however the aggregation does not appear to correlate with it antimicrobial effect. The aggregation of both bacteria did not lead to inactivation of those bacteria. The expression of genes (*StSN1*) encoding snakin-1 protein was detected in tubers, stems, patels, sepals and some other storage and reproductive organs, but not in root, stolons or leaves (Segura et al., 1999). Based on the expression pattern, the author concluded that snakin-1 protein may play roles in pre-existing defense barriers. Transgenic potato plants overexpressing snakin-1 gene were shown to have enhanced resistance to plant pathogens (Almasia et al., 2008). Susceptibility study and overexpression in transgenic plants study may suggest that snakin-1 is active both in vitro and in vivo.

VI. *E. coli* expression system

Large-scale production of AMPs is one of the obstacles for their implementation. Purity, cost and bioactivity are other factors to be considered that affect feasibility for large-scale production. The difficulties in industrial-scale production also impede the comprehensive screening of antimicrobial activity, studies on mode of action and eventually clinical trials. Most AMPs are isolated from plants, mammalian and microorganisms. Isolation and purification of plant AMPs (pAMPs) directly from natural

plant tissue usually result in low peptide yield and the whole process is usually time-consuming. Mammalian AMPs are typically produced by chemical synthesis. However, chemical synthesis is not cost-effective for peptides larger than 30 amino acid residues or peptides that require other post-translational modification. Cost for such manufacture may be in the range of US$ 100 to 600 per gram of peptide (Hancock and Sahl, 2006). Bacteriocin, a type of antimicrobial peptide produced by bacteria, is relatively easy to mass-produce, since bacterial replication time is relatively short. Heterologous expression system, based on the fermentation technology, is currently considered as a potential method for mass production of AMPs. Among heterologous expression systems, *E. coli* has been used widely as the expression host due to its rapid growth rate and its extensive understanding of the system. The system involves using a plasmid encoding antimicrobial peptide(s) that is transformed into an *E. coli* host. The antimicrobial peptides are isolated from bacterial culture. Plasmid systems, such as pET and pQE system, are highly recognized and are commonly used in research, in which sets of fusion tags, such as HIS-tag and S-tag, are designed to facilitate purification or expression. Carrier proteins, such as thioredoxin, GST (glutathione transferase) are used to stabilize target protein expression. A set of well-developed *E. coli* expression hosts are also available to increase expression level and protect AMPs from proteolytic cleavage. *E. coli* BL21 (DE3) strain is devoid of *lon* and *omp* proteases; protecting target protein(s) from cleavage by the *E. coli* host. However, large-production of AMPs by *E. coli* expression has only been reported for a limited number of AMPs. The remaining drawback is still the relative low yield for commercial use.

VII. Application

AMPs have a potential spectrum of application, for example as therapeutic agents (Marshall and Arenas, 2003; Altman et al., 2006; Sang and Blecha, 2008; Keymanesh et al., 2009; López-Meza et al., 2011). AMPs are also found to exert antioxidant activity and immunomodulatory effect (De et al., 2000; Memarpoor-Yazdi et al., 2012).

A wealth of research on AMPs and the major application of AMPs have been championed by the pharmaceutical industry, due to their therapeutic potential. Besides their rapid killing of target microorganisms, AMPs, compared to conventional antibiotics, inactivate a broad spectrum of microorganisms, such as bacteria, fungi, parasites and virus. Some of them are able to kill cancer cells (Hoskin and Ramamoorthy, 2008). Conventional antibiotics inactivate predominantly bacteria and fungi. In addition, the targets of antibiotics are generally a metabolic enzyme which results in a relatively easy route for microbes to develop resistance. AMPs generally target the cell membrane or have multiple targets making it inherently difficult for microbes to develop resistance (Sang and Blecha, 2008).

The therapeutic potential of AMPs is broad spectrum. They can potentially be used to treat a variety of microbial-related diseases, such as bacterial infection, topical infection and ulcer using topical to systemic application. They are also shown to neutralize bacterial endotoxin. Lacticin 3147 and nisin, two lantibiotic bacteriocin from *Lactococcus lactis*, were showed to be active against drug-resistant *Staph. aureus* and

Enterococcus, including MRSA and VRE (Piper et al., 2009). Treatment of drug-resistant pathogens caused by extensive use of antibiotics has been difficult due to the lack of effective drugs. Research also demonstrated that in vivo and in vitro AMPs can neutralize bacterial endotoxin (Gough et al., 1996). AMPs, such as human and rabbit α-defensin, have been shown to control sexually transmitted pathogens, such as HIV and *Treponema pallidum* (Borenstein et al., 1991; Zhang et al., 2002; Sinha et al., 2003). Nisin, magainins were shown to have contraceptive potential by immobilization of sperm both in vivo and in vitro (Reddy et al., 1996; Reddy and Manjramkar, 2000; Aranha et al., 2004). Due to the safety uncertainty of the systemic application of AMPs, many pharmaceutical companies are developing novel AMPs aiming for topical use and some are in clinical trials. MSI-78, an α-helical peptide derived from magainin, is in phase-III clinical trials to treat foot-ulcer infection in diabetics (http://clinicaltrials.gov/show/NCT00563433).

Transgenic expression in plants may help to reduce plant diseases. Transgenic rice overexpressing the wasabi defensin gene exhibited a reduction of disease lesions caused by blast fungus (Kanzaki et al., 2002). Zainal et al. (2009) demonstrated that transgenic tomato overexpressing chili defensin conferred resistant to plant pathogens both in vivo and in vitro. However, when investigating the disease control effect, potential adverse effects, such as toxicity toward certain tissue, inhibition of normal growth, should be considered and investigated (Allen et al., 2008).

Thermal processing of food has been used extensively to inactive microorganisms to ensure food safety and prevent growth of spoilage microorganisms. The disadvantages of thermal processing are changes in organoleptic properties and loss of nutritive value due to high temperature. Non-thermal processing of food has emerged as alternative to thermal processing or as part of hurdle technology approach to ensure the safety of food products. Biopreservation is one of the research focuses of areas of academia and the food industry to fulfill consumers' demand and achieve food safety. A wealth of the research and development of application strategies have focused on bacteriocins, such as nisin, due to their application in fermented dairy and meat products (Cleveland et al., 2001; Chen and Hoover, 2003). However, one of the drawbacks of bacteriocins is their narrow spectrum of antimicrobial activity. Bacteriocins are active against mostly Gram-positive bacteria but not very effective against Gram-negative bacteria and fungi including mold, one of the spoilage microorganisms. Bacteriocins require chelating agents, such as food grade EDTA, to inactive Gram-negative bacteria. Eukaryotic AMPs usually exhibit broad-spectrum activity against both Gram-positive and Gram-negative bacteria and fungi, and they are promising biopreseratives. So far, nisin is the only FDA-approved antimicrobial peptide used in specific food products, including dietary products. The in vivo activity of AMPs is usually lower than that in vitro due to the presence of ions, salts, proteins and lipids, and the effect of pH and temperature (Rydlo et al., 2006). Therefore, higher dosage will be required in vivo, so safety issues must be considered when investigating novel food bio-preservatives.

VIII. Tables and figures

Table 1. Antimicrobial activity of Ib-AMP1.

Ref.	Source	Activity	Microorganisms	MIC or IC_{50}	Test conditions
Tailor et al., 1997	native	antifungal	*Alternaria longipes,* spore	IC_{50}: 3 µg/mL (1.2 µM); 50 µg/mL (20 µM)*	1. Incubated at 24 ˚C for 48 h in potato dextrose broth.
			Botrytis cinerea, spore	IC_{50}: 12 µg/mL (4.8 µM); > 200 µg/mL (80.4 µM)*	
			Cladosporium spharerospermum, spore	IC_{50}: 1 µg/mL (0.4 µM); 50 µg/mL (20 µM)*	
			Fusarium culmorum, spore	IC_{50}: 1 µg/mL (0.4 µM); 50 µg/mL(20 µM)*	
			Penicillium digitatum, spore	IC_{50}: 3 µg/mL (1.2 µM); 200 µg/mL (80.4 µM)*	

Table 1. Antimicrobial activity of Ib-AMP1 (continued).

	Trichoderma viride, spore	IC_{50}: 6 µg/mL (2.4 µM); > 200 µg/mL (80.4 µM)*	
	Verticillium alboatrum, spore	IC_{50}: 3 µg/mL; >200 µg/mL (80.4 µM)*	
Antibacterial Gram-positive	*Bacillus subtilis*	IC_{50}: 10 µg/mL (4.0 µM)	1. Incubated at 28 ˚C for 24 h in 1 % trypton (or 1 % peptone) + 0.5 % low melting point agarose.
	Micrococcus luteus	IC_{50}: 10 µg/mL (4.0 µM)	
	Staph. aureus	IC_{50}: 30 µg/mL (12.1 µM)	
	Strep. faecalis	IC_{50}: 6 µg/mL (2.4 µM)	
	Erwinia amylovora¶	IC_{50}: N.D	
Antibacterial Gram-negative	*E. coli* HB101	IC_{50}: > 500 µg/mL (201.1 µM)	
	Proteus vulgaris	IC_{50}: > 500 µg/mL (201.1 µM)	

Table 1. Antimicrobial activity of Ib-AMP1 (continued).

			Pseudomonas. solanacearum[¶]	IC_{50}: > 500 µg/mL (201.1 µM)	
			Xanthomonas campestris[¶]	IC_{50}: N.D	
			Xanthomonas oryzue[¶]	IC_{50}: N.D	
Lee et al., 1999	synthetic	antifungal	*Candida albicans*, spore	MIC: 5.0 µM (oxidized)*/ 20 µM (reduced)	1. Incubated in YM medium[‡] at 28 ˚C for 24 h. 2. Final spore number: $2x10^3$ spores/well. 3. MIC according to MTT[a] analysis
			Aspergillus flavus	MIC: 2.5 µM (oxidized)*/ 10 µM (reduced)	

Table 1. Antimicrobial activity of Ib-AMP1 (continued).

Thevissen et al., 2005	native	antifungal	*Neurospora crassa* *Botrytis cinerea* *Fusarium culmorum*	IC_{50}: 0.5 µM (-IS)[§]/ 8 µM (+IS)[§] IC_{50}: 1.5 µM(-IS)[§]/ 50 µM (+IS)[§] IC_{50}: 1.4 µM(-IS)[§]/ > 50 µM (+IS)[§]	1. Incubated in ½ potato dextrose broth (PDB) + 50 mM HEPES-NaOH (pH 7.0) for 72 h.
	native	Anti-yeast	*Saccharomyces cerevisiae* *Pichia pastoris*	IC_{50}: 15 µM (-IS)[§]/ 50 µM (+IS)[§] IC_{50}: 16 µM(-IS)[§]/ > 50 µM (+IS)[§]	1. Incubated in PDB + 50 mM HEPES-NaOH (pH 7.0) and 5 mM $CaCl_2$ for 72 h.
Wang et al., 2009	synthetic	Antibacterial Gram-negative	*E. coli* KCTC1682 *P. aeruginosa* KCTC1637	MIC: 16 µM MIC: >32 µM	1. Incubated in 1 % peptone at 37 ˚C for18 - 20 h.

Table 1. Antimicrobial activity of Ib-AMP1 (continued).

		S. typhimurium KCTC1926	MIC: >32 μM	2. Final cell number:
	Antibacterial	*B. subtilis* KCTC3068	MIC: 16 μM	$2x10^5$ CFU/well.
	Gram-	*Staph. epidermidis* KCTC1917	MIC: 16 μM	
	positive	*Staph. aureus* KCTC1621	MIC: 16 μM	
		MRSA (methicillin-resistant *S. aureus*) CCARM 3543	MIC: 16 μM	
		MDRPA (multidrug-resistant *P. aeruginosa*) CCARM 2095	MIC: >32 μM	

IC_{50} is the protein concentration that achieves 50% inhibition of microbial growth.
MIC is the lowest protein concentration that achieves 100% inhibition of microbial growth.
[*] Medium supplemented with 1mM $CaCl_2$ and 50mM KCl
[†] Oxidized: oxidized form (with disulfide bridges). Reduced: reduced form (no dilsulfide-bridged)
[‡] YM medium: 1 % glucose, 0.3 % malt extract, 0.5 % peptone, 0.3 % yeast extract
[§] -IS: low ionic strength medium. +IS: high ionic strength medium
[¶] cells were incubated in 1 % peptone + 0.5 % low melting point agarose with Ib-AMP1.
[a] 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) solution.

Table 2. Antimicrobial activity of snakin-1.

Reference	Source	Activity	Microorganisms	MIC or IC_{50}	Test condition
Segura et al., 1999	natural	antifungal	*Fusarium solani*	IC_{50} = 5 µM (34.6 µg/mL)	1. potato dextrose broth
			Botrytis cinerea	IC_{50} = 3 µM (20.8 µg/mL)	2. SN1 in sterile water (SW)
			Colletotrichum lagenarium	IC_{50} = 20 µM (138.4 µg/mL)	
			Bipolaris maydis	IC_{50} = 5 µM (34.6 µg/mL)	
			Aspergillus flavus	IC_{50} > 100 µM 691.9 µg/mL)	

Table 2. Antimicrobial activity of Snakin-1 (continued).

		Antibacterial Gram-positive	*Clavibacter michiganensis* subsp. *sepedonicus*	IC_{50} = 1 µM (6.9 µg/mL)	1. 50 µL bacteria in nutrient broth + 100 µL SN1 in SW
		Antibacterial Gram-negative	*Ralstonic solanacearum*	IC_{50}> 100 µM 691.9 µg/mL)	2. Final cell number = $1.5x10^3$ CFU/well
López-Solanilla et al., 2003	natural	Antibacterial Gram-positive	*Listeria monocytogenes*	MIC: 10 µg/mL (69.2 µg/mL)	
			Listeria innocua	MIC: 10 µg/mL (69.2 µg/mL)	
			Listeria ivanovii	MIC: 10 µg/mL (69.2 µg/mL)	

IC_{50} is the protein concentration that achieves 50% inhibition of microbial growth.

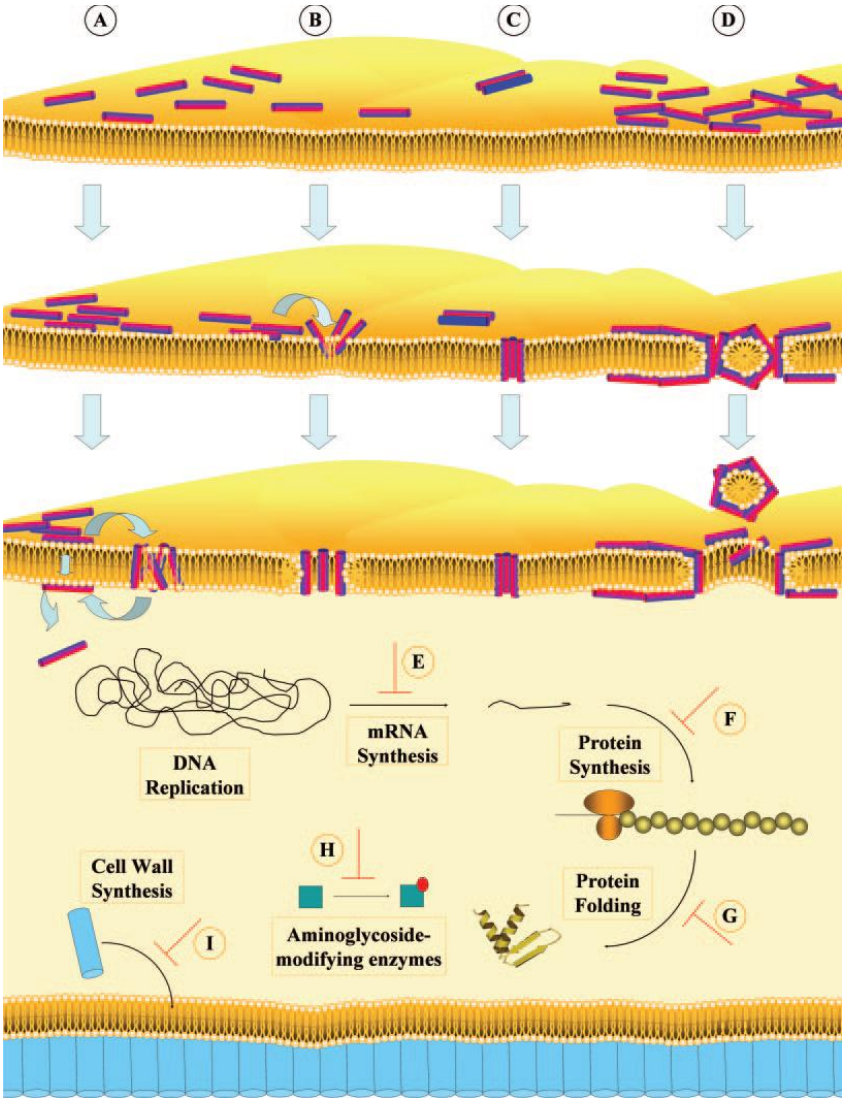

Figure 1. Mechanism of antimicrobial action of AMPs. The bacterial membrane lipid bilayer is represented as a yellow lipid bilayer. Amphipathic AMPs are showed in red for the hydrophilic regions are blue for the hydrophobic regions. (A) Aggregate model. (B) Toroidal model. (C) Barrel-stave model. (D) Carpet model. (E) Inhibition of mRNA synthesis. (F) Inhibition of protein synthesis. (G) Interference with protein folding. (H) Inhibition of aminoglycosides production. (I) Inhibition of cell wall synthesis. Adopted from Jenssen et al., 2006.

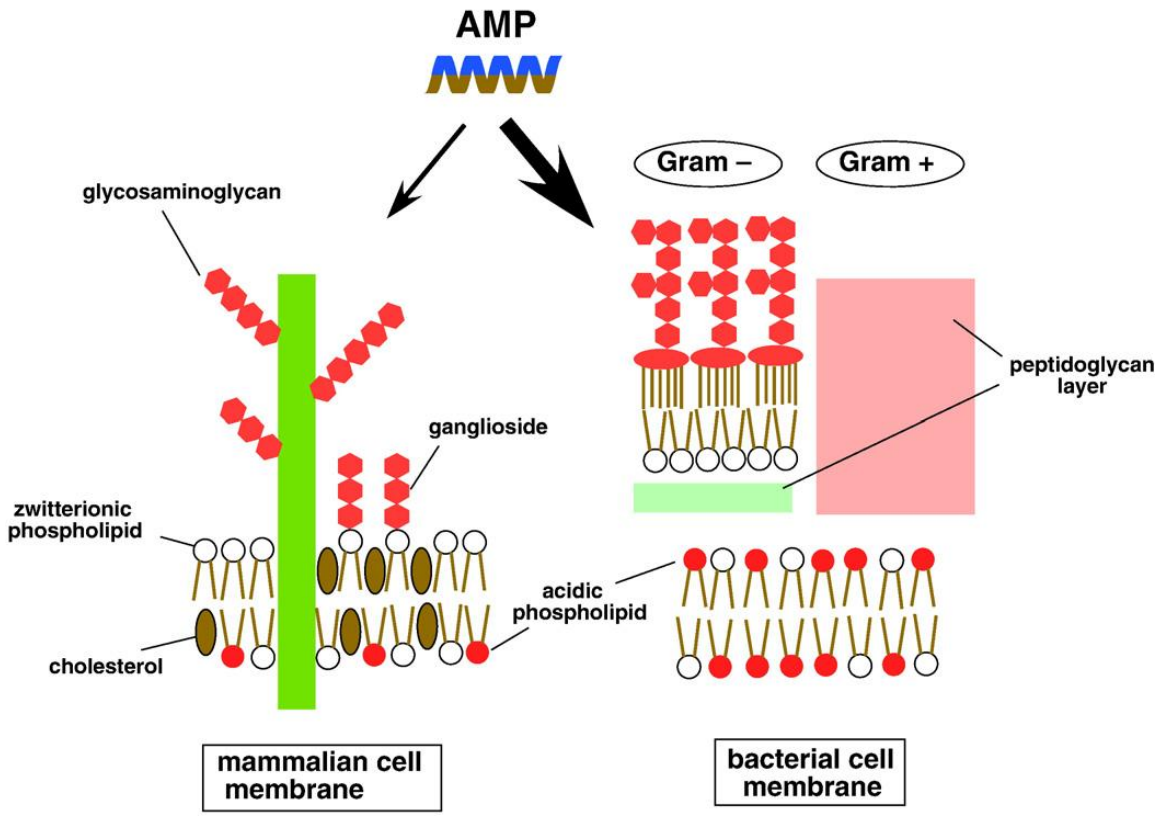

Figure 2. Molecular basis of cell selectivity of AMPs. The amiphipathic AMPs with hydrophilic (positively charged) region (blue) and hydrophobic region (brown) have stronger electrostatic attraction to bacterial cell membrane (right) than to mammalian cell membrane (left). The electrostatic interaction occurs between the cationic region of AMPs (blue) and the anionic regions (red) of cell membrane. Bacterial cell membranes have anionic phosphilids in both inner and outer leaflet; however the outer leaflet of mammalian cell membrane is mainly zwitterionic phospholipid. Cholesterol (brown) in the mammalian cell membrane also prevents the traverse of AMPs across mammalian cell membrane. Adopted from Matsuzaki, 2009.

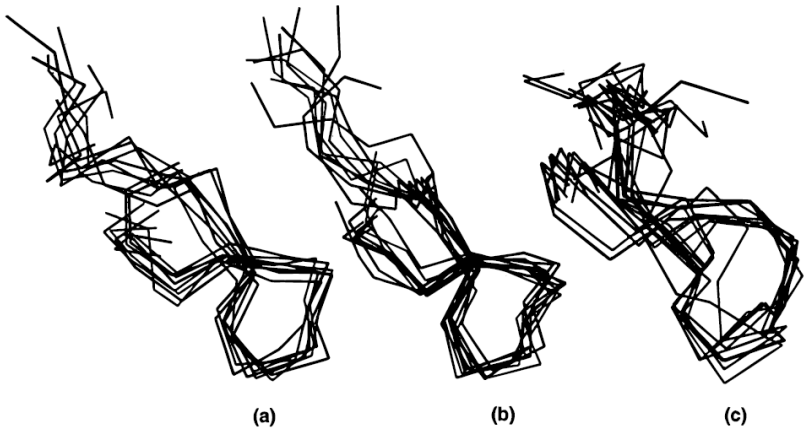

Figure 3. Secondary structure of Ib-AMP1. Superposition of the Cα trace for the top 10 structures obtained from DIANA for Ib-AMP1. (A) No disulfide connectivity. (B) Disulfide connectivity at C_6-$C1_6$ and C_7-C_{20}. (C) Disulfide connectivity at C_6-C_{20} and C_7-C_{17}. The structures were from 26 solutions of Ib-AMP1). Adopted from Patel et al., 1998.

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CHAPTER 2

HYPOTHESIS AND OBJECTIVES

The broad spectrum antimicrobial activity of Ib-AMP1 against plant pathogens, both pathogenic bacteria and fungi, let us believe in its potential to control foodborne pathogens. Indeed, Wang et al. (2009) demonstrated the antibacterial effect of Ib-AMP1 against foodborne pathogens, *S.* Typhimurium*, B. subtilis* and *Staph. aureus,* and provided limited mode of action of Ib-AMP1 on *Staph. aureus*. Besides MICs, other antibacterial properties of Ib-AMP1 on Gram-negative bacteria have not been studied. The purpose of the present study was to further determine the antibacterial activity of Ib-AMP1 against *E. coli* O157:H7. We hypothesized that the mode of action of Ib-AMP1 targets at both the bacterial cell membrane and intracellular macromolecules.

Hypothesis – Ib-AMP1 targets both the bacterial cell membrane and intracellular macromolecules

Chapter 4: bacterial susceptibility to Ib-AMP1 and cytotoxicity of Ib-AMP1

A. To determine the antibacterial activity of Ib-AMP1: evaluate antibacterial activity of Ib-AMP1 against foodborne pathogens.
B. To determine the bactericidal activity of Ib-AMP1: determine the survival of *E. coli* O157:H7 after Ib-AMP1 treatment.

C. To determine the sustainability of Ib-AMP1: determine the residual antibacterial activity of Ib-AMP1 against *E. coli* O157:H7.

D. To determine the cytotoxicity of Ib-AMP1: determining cytotoxic activity against human cells that would be influenced following oral ingestion and metabolism.

Chapter 5: mode of action of Ib-AMP1 on *E. coli* O157:H7

A. To determine the effect of Ib-AMP1 on the cell membranes: determine the efflux of intracellular components and membrane damage to *E. coli* O157:H7 by Ib-AMP1.

B. To determine the effect of Ib-AMP1 on intracellular macromolecules: determine the inhibition of DNA, RNA and protein synthesis in *E. coli* O157:H7 by Ib-AMP1.

CHAPTER 3

COMPREHENSIVE MATERIALS AND METHODOLOGIES

I. Chemicals and reagents

Media and buffers purchased from BD, Franklin Lakes, NJ: Muller Hinter Broth (MHB, Difco™), Tryptic Soy Agar (TSA, Difco™), Tryptic Soy Broth (TSB, Difco), phosphate buffer saline (PBS, BBL™). Media and chemicals purchased from ATCC, Manassas, VA: Hybri-care medium, Dulbecco's Modified Eagle's Medium (DMEM), Eagle's Minimum Essential Medium (EMEM), epidermal growth factor (EPF). Chemicals purchased from Life Technologies, Grand Island, NY: fetal bovine serum (FBS), Penicillin-streptomycin stock solution. Chemicals purchased from Sigma-Aldrich Corp,. St. Louis, MO: dimethyl sulfoxide (DMSO), HEPES buffer, N-Phenyl-1-naphthylamine (NPN), trichloroacetic acid (TCA), Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit, HEPES potassium salt, K_2-EDTA, EDTA. Chemicals purchased from Fisher Scientific, Pittsburg, PA: glucose, KH_2PO_4, HEPES, glycerol (Acros Organics), ScintiSafe 30%, glycin, NaCl. Chemicals purchased from MD Biomedicals, Santa Ana, CA: Valinomycin, Nigericin, tritium-labeled precursors: [methyl-^{3}H] Thymidine, [5,6-^{3}H] Uracil and [3,4,5-^{3}H] L-leucine. MTS (tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt) reagent was purchased from Promega, Madison, WI. Standard potassium solutions 1000ppm (KCl) and Tris-acetate were purchased from Research organics, Cleveland, Ohio. NaCl ionic strength adjuster was purchased from Jenco Instruments, Inc., San Diego, CA. LIVE/DEAD BacLight™ Bacterial

Viability Kit was purchased from Invitrogen Molecular Probes, Eugene, Oregon. Fluorescent probe $DiSC_3(5)$ (3,3-dipropylthiadicarbocyanine iodide) was purchased from AnaSpec, Fremont, CA.

II. Bacterial strains

Escherichia coli O157:H7 ATCC43895, *Salmonella enterica* serovar Newport, *Staphylococcus aureus* ATCC10832 and *Pseudomonas aeruginosa* ATCC15442, *Bacillus cereus* ATCC9818 were cultured in TSB for at least 16 h at 37 ˚C with agitation at 200 rpm. Frozen stocks were kept at -80 ˚C in TSB containing 20 % glycerol. Cells were sub-cultured twice in TSB and streaked onto TSA plates; plates were incubated at 37 ˚C overnight. Cultures were prepared by inoculating MHB with a single well-separated colony and incubate at 37 ˚C for more than 18 h with agitation at 200 rpm.

III. Ib-AMP1 peptide preparation

Ib-AMP1 was chemically synthesized by GenScript (Piscataway, NJ) based on solid phase synthesis. The amino acid sequences were according to Tailor et al. (1997) (QWGRRCCGWGPGRRYCVRWC). Lyophilized Ib-AMP1 was analyzed by mass spectrometer, HPLC and SDS-PAGE to confirm the purity. Ib-AMP1 was dissolved in sterile distilled de-ionized water (SDDW) to the final concentration of 4 mg/mL as the

stock solution. The stock solution was kept at -80 ˚C. The working solution was diluted from the stock solution with SDDW.

IV. Antimicrobial activity of Ib-AMP1 - minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The microdilution assay was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Ib-AMP1 against target bacteria. The assay was conducted according to methods outlined by the Clinical and Laboratory Standards Institute with some modifications (CLSI, 2003).

All five bacteria were cultured individually in 5 mL MHB and incubated at 35 ˚C with agitation for more than 18 h. Cells were collected by centrifugation at 3,500 rpm for 15 min at 4 ˚C, and resuspended in 5 mL of fresh MHB. Inoculum was prepared by making serial ten-fold dilutions in fresh MHB to approx. 10^5 CFU/mL. Ib-AMP1 was serial diluted in SDDW to a final volume of 100 µL at 2X concentration in wells of a 96-well plate, and each well was inoculated with 100 µL of inoculum, which resulted in a two-fold dilution of the Ib-AMP1 concentration in each well. A bacterial growth control and negative controls (medium alone and Ib-AMP1 alone) were included. Plates were incubated at 35 ˚C in a Dynex 96-well plate reader MRX with Revelation software to monitor optical density at λ = 630 nm for 24 h. All assays were performed in triplicate and repeated. The MIC is the minimum concentration of Ib-AMP1 that inhibits 80 % growth by the optical density of target bacteria at 16 h. After 24 h incubation, an aliquot (20 µL in

duplicate) of each well was plated onto a TSA plate in duplicate to determine the viability of bacterial cells. MBC is the minimum concentration of Ib-AMP1 that shows absence of viable cell.

V. Bactericidal activity of Ib-AMP1

The assay determined whether Ib-AMP1 is effective in killing of *E. coli* O157:H7. *E. coli* O157:H7 was grown in 10 mL of MHB at 37 ˚C for more than 18 h. Overnight cultures were diluted in ½X MHB to a desired cell number, low (10^3 CFU/mL), medium (10^6 CFU/mL) and high (10^9 CFU/mL). Ib-AMP1 at final concentrations of 25 (½X MIC), 50 (1X MIC) and 100 µg/mL (2X MIC) were mixed with each cell suspension and incubated at 37 ˚C. Aliquots were removed at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 20, and 24 h from each of the reaction tubes and immediately diluted in PBS to minimize the antibacterial effect. Serial ten-fold dilutions were made as required and 100 µL aliquots were plated onto TSA plates in duplicate. Plates were incubated at 37 ˚C for more than 18 h. Viable counts were expressed as CFU/mL.

VI. Residual antibacterial activity of Ib-AMP1

The residual antibacterial activity was studied to determine the sustainable and residual efficacy of Ib-AMP1. *E. coli* O157:H7 was used as the bacterium model. *E. coli* O157:H7 was grown in MHB at 37 ˚C for more than 18 h. The overnight culture was then diluted

to 10^3 CFU/mL (low cell number) and 10^6 CFU/mL (high cell number) in ½X MHB and used as the inoculum. Cells at each concentration were treated with Ib-AMP1 at final concentrations of 1X (50 µg/mL) and 2X MIC (100 µg/mL), independently, and incubated at 37 ˚C with agitation at 200 rpm for 24 h. A cell-free control (CF, Ib-AMP1 at 50 and 100 µg/mL alone), and negative control (NC, cells alone) were included. After 24 h incubation, to collect residual Ib-AMP1 in the supernatant, samples were centrifuged at 5,000 rpm for 10 min and the supernatant was collected and passed through a 0.2 µm filter to remove cells. The antibacterial activities of the resulting supernatant were then determined using the microdilution assay with some modifications. In brief, an overnight culture of *E. coli* O157:H7 was diluted to 10^6 CFU/mL in 10X MHB and used immediately as the inoculum. One hundred and ninety microliters of filtered supernatant samples (NC SN, 1X SN, 2X SN, CF 1X SN and CF 2X SN) were added into each well in triplicate and 10 µL of inoculum was inoculated into each well, which resulted in final cell concentration at 10^5 CFU/mL. Synthetic Ib-AMP1 at 1X and 2X MIC were included as the positive controls. Plates were incubated at 35 ˚C in Dynex 96-well plate reader MRX with Revelation software to monitor optical density at λ = 630 nm for 24 h. After 24 h incubation, aliquots of each well were serial diluted and plated onto TSA plates in duplicate to determine the cell survival.

VII. Mammalian cell cytotoxicity

The cytotoxicity of Ib-AMP1 toward human cells was investigated using MTS reagent (Hayes and Markovic, 2002; Massodi et al., 2010; Meot-Duros et al., 2010). The assay includes the reduction of MTS tetrazolium compound to a colored formazan product by NADPH or NADH by dehydrogenase in metabolically active cells.

FHs 74 Int, HT-29 and Hep G2 cells were purchased from ATCC (Manassas, VA, USA). FHs 74 Int cells were grown in 10 mL complete medium containing Hybri-care medium supplemented with 30 ng/mL epidermal growth factor (EPF), 10 % fetal bovine serum (FBS), 200 units/mL penicillin and 200 µg/mL streptomycin in a 75 cm^2 tissue culture flask. HT-29 and Hep G2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and Eagle's Minimum Essential Medium (EMEM), respectively, containing FBS, penicillin and streptomycin in a 75 cm^2 tissue culture flask. Exponentially growing cells were harvested using 0.05 % trypsin in EDTA/PBS/phenol-red solution for 10 min at 37 ˚C with 5 % CO_2. Cells were then washed, resuspended in complete medium to achieve a final concentration of $1X10^5$ cells/mL. One hundred microliters of cells at $1x10^5$ cells/mL was seeded into a 96-well tissue culture plate to make the final concentration at $1x10^4$ cells per well. The plates were incubated at 37 ˚C with 5 % CO_2 until confluence was achieved. When cells were confluent, cells were washed with 100 µL complete medium and then incubated with 100 µL complete medium containing Ib-AMP1 from 25 to 1,000 µg/mL. Cells alone, medium alone, and Ib-AMP1 alone were included as controls. Plates were incubated at 37 ˚C with 5 % CO_2 for 24 h. After 24 h incubation, 20 µL MTS reagent was added into each well. MTS was prepared per manufacturer's instruction. Each plate was incubated at 37 ˚C with 5 % CO_2 for 2 - 4 h and absorbance

at λ = 490 nm were measured using Synergy HT plate reader (Biotek, Winooski, VT, USA). All concentrations were tested in triplicate and the assay conducted twice.

VIII. Membrane permeability assay

Membrane permeability was determined using the LIVE/DEAD BacLight™ Bacterial Viability Kit (Swe et al., 2009; Murdock et al., 2010). This assay is designed to differentiate permeable and intact cells, where permeable cells are stained red and intact cells are stained green. *E. coli* O157:H7 was grown to log phase in MHB. Four milliliters of cell culture were centrifuged (14,000 x g, 1 min) and washed twice in 1 mL of SDDW and resupended in 1 mL of SDDW. Ten microliters of the cell suspension was transferred to a new tube and centrifuged again; the resulting pellet was incubated with 10 µL of SDDW or SDDW containing Ib-AMP1 at final concentrations of 25, 50, or 100 µg/mL for 30 min at room temperature. Double strength STYO9 and propidium iodine stains stock solutions were prepared according to the manufacturer's instruction. Ten microliters of treated cells were incubated with 5 µL of 2X SYTO9 stock and 5 µL of 2X propidium iodine stocks for 15 min, in the dark at room temperature (RT). A 0.5 µL volume of the stained cells was dispensed on a microscope slide and covered with a glass coverslip. Slides were observed using an Olympus BH2-RFCA fluorescence microscope fitted with a Pixera camera. Five random fields were counted; the assay was conducted three times.

IX. K^+ efflux assay

Potassium (K^+) efflux assay was conducted to determine whether Ib-AMP1 changes *E. coli* O157:H7 cell membrane permeability to potassium ions. Cells maintain a certain level of K^+ when growing and K^+ ion was used as a marker to determine the efflux of intracellular components (Schultz and Solomon, 1961). Potassium ion selective probes have been used widely to investigate the efflux of intracellular potassium by cells after treatment with antimicrobial agents (Matsuzaki et al., 1997; Katsu et al., 2002; Orlov et al., 2002; Yasuda et al., 2003; Murdock et al., 2010). A potassium combination electrode K001508 (Jenco Instruments, Inc., San Diego, CA) connected to a Jenco pH/mV/Temp./ION bench meter 6219 (Jenco Instruments, Inc., San Diego, CA) was used. The ion potential response (mV) was monitored and recorded. Various concentrations of standard potassium solutions (KCl) containing 5 M NaCl ionic strength adjuster and the corresponding mV readings were plotted to generate a standard curve. The resulting mV readings from experiments were then converted to concentration based on a standard curve. Prior to each experiment, the probe was calibrated with potassium standard solution (KCl at 1000, 100 and 10 ppm) with 5 M NaCl ionic strength adjuster.

E. coli O157:H7 was grown to log phase in MHB. Cells were centrifuged at 4,500 rpm, 10 min at 4 ˚C and washed twice in 10 mM Tris-acetate, pH 7.4; cells were then resuspended in an eighth of the original volume and ready to use as concentrated cells. A total of 4 mL of solution containing 1 mL of concentrated cells, 2.9 mL of 10 mM Tris-acetate buffer (pH 7.4), and 100 µL of Ib-AMP1 to achieve final concentrations of 25 (½X

MIC), 50 (1X MIC) and 100 µg/mL (2X MIC); the reaction suspension was mixed with a magnetic stir bar duirng the course of experiment. Non-treated cells were included as baseline, and cells treated with DMSO (at final concentration of 50 % were also included to determine the maximum efflux of K^+ ions. The mV readings were recording for 30 min with 1 min interval at RT. The assay was conducted twice.

X. ATP efflux assay

ATP efflux assay was determined using Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit. The assay includes a coupled enzyme reaction in which ATP is reduced to adenyl-luciferin by luciferase with the presence of luciferin. The resulting adenyl-luciferin then interacts with oxygen and produces light. The amount of light emitted is proportional to the amount of ATP present.

The assay was conducted as described previously (McEntire et al., 2004; Suzuki et al., 2005; Murdock et al., 2010) with some modifications. The efflux of ATP was determined by comparing the level of extracellular and total ATP concentration of cells treated with Ib-AMP1. Cells generate and maintain a certain level of ATP for energy and ATP was used as a marker to determine the efflux of an intracellular component, a relative large molecule compared to K^+ ion.

E. coli O157:H7 was grown to log phase in MHB. Four milliliters of cells were centrifuged (14,000 rpm, 1 min) and washed twice in 1 mL of 50 mM HEPES buffer, pH 7.0. Cells

were resupended in 1 mL of buffer with glucose (50 µM HEPES with 0.2% glucose, pH 7.0) to energize the cells for 20 min at room temperature. Two hundred microliters of energized cells were incubated with 200 µL of buffer or buffer containingIb-AMP1 at final concentrations of 25 (½X MIC), 50 (1X MIC) and 100 µg/mL (2X MIC) at room temperature. An aliquot (20 µL) of each treatment was removed at 0, 1, 10, 20, 30, 45 and 60 min to determine the levels of extracellular and total ATP.

Extracellular ATP level was determined by adding 20 µL aliquots of each reaction into 980 µL of fresh buffer, mix by gentle inversion. A 100 µL aliquot was removed and mixed with 100 µL of diluted ATP assay mix and the light intensity was measured by a spectrophotometer (Luminoskan TL Plus luminometer, Labsystems Oy, Helsinki, Finland).

Total ATP concentration was determined by adding 20 µL aliquots of each treatment into 40 µL of DMSO; samples were held at RT for 1 min to enable DMSO to permeate cell membrane. Nine hundred and forty microliters of fresh buffer was added and swirled gently. A 100 µL aliquot was then removed and mixed with 100 µL of diluted ATP assay mix and the light intensity was measured by a spectrophotometer.

Diluted ATP assay mix (1/25X) was prepared according to manufacturer's instruction. Various concentrations of ATP solution were prepared. One hundred microliters of ATP solution was mixed with 100 µL of diluted ATP assay mix, and gentle swirling. Light intensity was measured by a spectrophotometer. The amount of light emitted and the ATP concentration were plotted to generate an ATP standard curve. The total and

extracellular ATP concentration at each time point was calculated according to the ATP standard curve. The assay was conducted twice.

XI. Membrane potential dissipation assay (Δψ)

A cytoplasmic membrane potential dissipation assay was conducted in order to determine whether Ib-AMP1 causes dissipation of the cytoplasmic electrical membrane potential (Δψ). The cell membrane produces proton motive force (pmf) to store energy. This energy is stored in two forms, electrical potential (Δψ) and chemical proton gradient (ΔpH). Fluorescent probe $DiSC_3(5)$ was used as a marker to monitor the change of electrical membrane potential (Δψ) upon interaction with Ib-AMP1. It is a cationic membrane potential sensitive dye which accumulates on a negative inside membrane potential cell membrane where they form aggregates involved in self-quenching; therefore, the fluorescence intensity decreases. Cytoplasmic membrane potential dissipation results in the release of $DiSC_3(5)$ into the medium where it is no longer self-quenched and the fluorescence intensity increases.

The assay was based on methods described previously with some modification (Breeuwer and Abee, 2004; Turovskiy et al., 2009; Murdock et al., 2010). *E. coli* O157:H7 cells were grown to log phase in MHB at 37 ˚C. Cells were washed twice with wash buffer containing 50 mM K-HEPES, pH7.0 and resuspended in 1/100 of the original volume in respiration buffer containing 5 mM HEPES, 100 mM KH_2PO_4, 20 % glucose, 1 mM K_2-EDTA, pH 7.1. Cells were held on ice before use. A total of 1980 µL assay buffer

containing 50 mM K-HEPES , 1 mM EDTA, pH 7.1 and 3 µL of $DiSC_3(5)$ (stock: 5 mM) was added and mixed in a quartz cuvette. Once the signal stabilized, 20 µL of cell suspension was added. Nigericin at final concentration of 20 µM was added to convert ΔpH of PMF to Δψ. Nigericin promotes the antiport transport of H^+ and K^+ and results in dissipation of the pH gradient. Ib-AMP1 at final concentrations of 25 (½X MIC), 50 (1X MIC) or 100 µg/mL (2X MIC) was then added into the cuvettes. SDDW at a volume equal to Ib-AMP1 was added to untreated cells as a control. Valinomycin at a final concentration of 20 µM was added to dissipate any remaining Δψ. Valinomycin promotes the uniport of K^+ and dissipates Δψ of PMF. An increase of fluorescent intensity after addition of Ib-AMP1 and valinomycin indicates the dissipation of the cytoplasmic membrane potential. Real-time fluorescence intensity was monitored using a spectrofluorometer (Perkin Elmer, luminescence Spectrometer, LS50B) with excitation and emission wavelength of 643 nm and 666 nm, respectively, and with 10 nm split wavelength. Total duration of the assay was 900 sec with a 0.1 sec interval. The assay was conducted twice.

XII. NPN uptake assay

NPN (N-Phenyl-1-naphthylamine) was used as a fluorescent probe to determine the permeability of the outer membrane after Ib-AMP1 treatment (Hancock and Wong, 1984; Helander et al., 2001). NPN is a hydrophobic and neutral probe which is weakly fluorescent under aqueous conditions. When the outer membrane is damaged, NPN

binds to the glycerophospholipid milieu where it becomes fluorescent (Wu and Hancock, 1999).

The assay was conducted according to Loh et al. (1984) with some modifications. *E. coli* O157:H7 cells were grown to early-log phase in MHB at 37 ˚C and then diluted to OD_{600nm} = 0.6 in half of the original volume in 5 mM HEPES buffer, pH 7.2. NPN at a final concentration of 10 µM and buffer were added into a cuvette and then 1mL of cells was added to bring the final volume of 2 mL. The resulting suspension was incubated at room temperature (RT) for 3 min to allow the fluorescence to become stable. After 3min, the fluorescence intensity was read using a spectrofluorometer (Perkin Elmer, luminescence Spectrometer, LS50B) with excitation and emission wavelength of 350 nm and 420 nm, respectively, and with 5nm split wavelength. Immediately after reading, Ib-AMP1 at final concentrations of 25 (½X MIC), 50 (1X MIC) and 100 µg/mL (2XMIC) or EDTA at final concentrations of 0.5, 1 and 2 mM were added into the cuvette independently and incubated for an additional 10 min at RT; the fluorescence intensity was then read after 10 min incubation at RT. Treatment containing SDDW was included as negative; cells only, Ib-AMP1 only, NPN only and cells with NPN were included as controls. EDTA binds to divalent cations that are required to stabilize the outer membrane structure resulting in destabilization of the outer membrane. Studies show that EDTA released up to 40 % of LPS from the outer membrane (Leive, 1965 and 1974; Hukari et al., 1986; Alakomi et al., 2000)

XIII. Macromolecular synthesis inhibition assay

The ability of Ib-AMP1 to inhibit bacterial DNA, RNA and protein synthesis was investigated using radio-labeled DNA, RNA and protein precursors (Cherrington et al., 1990; Oliva et al., 1993; Patrzykat et al., 2002). The decrease of radio-labeled precursor incorporation indicates the inhibition of synthesis of the corresponding macromolecules. The assay was conducted according to Cotsonas King and Wu (2009) and Xiong et al. (2002) with some modifications. In brief, *E. coli* O157:H7 were grown to early-log phase and then diluted to OD_{600nm} = 0.04 with fresh half strength MHB. Ib-AMP1 was added to half strength MHB at final concentrations of 25 (½X MIC), 50 (1X MIC) and 100 µg/mL (2XMIC), and cells were then added to each reaction at a final concentration at OD_{600nm} = 0.02. Tritium-labeled precursors: [methyl-^{3}H] Thymidine, [5,6-^{3}H] Uracil and [3,4,5-^{3}H] L-leucine were then added immediately to a final concentration of 20, 20 and 10 µCi/mL to determine inhibition of DNA, RNA and protein synthesis, respectively. All reaction tubes were incubated at 37 ˚C and a 100 µL aliquot was removed at 0, 20, 40, 60 min for DNA and RNA analysis and 0, 20, 40, 60, 80, 100 min for protein analysisfrom each tube. Each aliquot was mixed with 1mL of 10 % ice-cold TCA solution and kept on ice for at least 1h to precipitate incorporated radio-labeled precursors. Samples were then passed through Whatman GF/C glass fiber filters (GE Healthcare, Buckinghamshire, UK) using a vacuum filtering system to collect the precipitate. Filters were washed twice with 5 mL of 5 % ice-cold TCA and then twice with 3 mL of ice-cold 75 % ethanol, and then dried for 10 min. The unincorporated and free radio-labeled precursors are soluble in TCA and are passed through the filter membrane; the incorporated radio-labeled

precursors are not soluble in TCA and precipitate on the filter membrane. The dry filters were placed in scintillation vials with 5 mL of scintillation fluid (ScintiSafe 30 %). Radioactivity was quantified using a liquid scintillation counter (LS6500 Scintillation Counter, Beckman coulter, USA). The assay was conducted twice.

Radio-labeled precursor free samples were included in parallel to determine the viable cell counts at each time point. Aliquots of samples were removed and serial diluted in PBS. Viable counts of each sample at each time point were enumerated by plating on TSA plate. Plates were incubated at 37 ˚C for overnight.

XIV. References

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CHAPTER 4

The following data were published online in Journal, Food Control on 26 February, 2013.

Wen-Hsuan Wu, Rong Di and Karl R. Matthews. 2013. Activity of the plant-derived peptide Ib-AMP1 and the control of enteric foodborne pathogens. Food Control. 33(1) 142-147. DOI: 10.1016/j.foodcont.2013.02.013.

Objectives:

I. To determine the minimum inhibitory concentration and minimum bactericidal concentration of Ib-AMP1 against foodborne and humane pathogens.
II. To determine the bactericidal activity of Ib-AMP1 on *E. coli* O157:H7
III. To determine the residual antibacterial activity of Ib-AMP1 against *E. coli* O157:H7
IV. To determine the cytotoxicity of Ib-AMP1 against human small intestine, colon and liver cell lines.

Activity of the Plant-Derived Peptide Ib-AMP1 and the Control of Enteric Foodborne Pathogens

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Key Words:

Antimicrobial peptide, Ib-AMP1, foodborne pathogens, *E. coli* O157:H7.

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I. Abstract

Consumer demand for use of fewer traditional antimicrobial agents in foods has driven research interest in development of plant based antimicrobial agents for use in food and food processing. The purpose of the present study was to investigate Ib-AMP1, a plant antimicrobial peptide (pAMP), isolated from seeds of *Impatiens balsamina*. Activity against foodborne pathogens, cytotoxicity to select human cells, and residual activity were investigated. Results of these experiments aid in determining the feasibility of using Ib-AMP1 as an antimicrobial agent to control foodborne pathogens. Ib-AMP1 exhibited bactericidal activity against *Staphylococcus aureus, Escherichia coli* O157:H7, *Salmonella enterica* serovar Newport, *Pseudomonas aeruginosa*, and *Bacillus cereus*. When tested using low (10^3 CFU mL^{-1}) and intermediate (10^6 CFU mL^{-1}) *E. coli* O157:H7 cell numbers, an approximately 1.46-2.69 log reduction in cell numbers occurred at the 1X and 2X minimum inhibitory concentration (MIC) of Ib-AMP1. The results suggest that a concentration of Ib-AMP1 several fold greater than the MIC would be required in foods with high levels of commensal bacteria. A separate experiment showed no residual activity of Ib-AMP1 was apparent following interaction of the peptide with bacteria. Results of the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] cell proliferation assay indicated that Ib-AMP1 at 200, 400 and 600 μg mL^{-1} inhibited by 50% cell proliferation activity of Hep G2, FHs 74 Int and HT29 cells, respectively. Taken together, these data suggest that Ib-AMP1 has potential application as an antimicrobial agent in food systems.

Highlights:

- Ib-AMP1 is a antimicrobial agent produced by seeds of *Impatiens balsamina*
- Ib-AMP1 exhibits bactericidal activity against foodborne pathogens.
- Ib-AMP1 at 4X MIC showed less than 50 % inhibition of proliferation of human liver, small intestine, and colon cells.
- Ib-AMP1 activity is influenced by binding to bacterial cells or components in the extracellular environment.

II. Introduction

Antimicrobial peptides (AMPs) are a group of small proteins that exert antibacterial, antifungal or antiviral activity; some AMPs also exhibit anti-parasite activity (Epand & Vogel, 1999; Jenssen, Hamill, & Hancock, 2006). These peptides are produced throughout the prokaryote and eukaryote kingdoms as products of innate or adaptive immunity to protect their host from infection. They exhibit a broad spectrum of activity and are promising alternatives to antibiotics and food preservatives (Marshall, & Arenas, 2003; Altman et al., 2006; Sang & Blecha, 2008; Keymanesh, Soltani, & Sardari, 2009; Butua, 2011; López-Meza, Ochoa-Zarzzosa, Aguilar, & Loeza-Lara, 2011). Prior to considering potential areas of application of antimicrobial peptides, properties that may limit the spectrum of use must be delineated.

Research suggests that AMPs are less toxic to human cells than to bacterial cells. Membrane perturbation has been demonstrated to be the antibacterial mode of action of many AMPs (Epand & Vogel, 1999; Yeaman & Yount, 2003). Studies utilizing artificial lipid membranes demonstrated that AMPs possess higher affinity to bacterial cell membranes than to mammalian cell membranes (Matsuzaki, Sugishita, Fujii, & Miyajima, 1995; Matsuzaki, 2009). The composition and charge of the membrane accounted for the difference. Most of the AMPs are cationic and amphipathic in biological conditions. Bacterial cell membranes have a high abundance of acidic phospholipids, such as phosphatidylglycerol (PG), phosphatidylserine (PS), and cardiolipin (CL), which are negatively charged. The negative charged phospholipids interact with the positive charged AMPs through electrostatic interaction. Zhang, Rozek, & Hancock (2001) demonstrated that peptides, regardless of structure and conformation, have higher binding affinity to a negatively charged lipid monolayer than to a lipid monolayer with neutral charge. Mammalian membranes contain a higher level of zwitterionic phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM). Therefore, the electrostatic interaction between AMPs and the mammalian cell membrane is relatively weak. The presence of cholesterol in the mammalian cell membrane decreases membrane fluidity, and hinders the translocation across mammalian membranes. The cytotoxicity of each AMPs should be evaluated against a range of cells (intestine, liver, skin squamous epithelium) to determine extend of utility (e.g., in food, oral care products, personal skin care products).

Most plant derived antimicrobial peptides (pAMPs) contain cysteine forming disulfide bonds for stability; however, there is no direct link of stability to activity (Pelegrini et al, 2011; van 't Hof, Veerman, Helmerhorst, & Amerongen, 2001). Ib-AMP1 is one of four highly homologous peptides and was isolated from seeds of *Impatiens balsamina* (Tailor et al., 1997). *Impatiens balsamina* has been used in traditional Chinese medicine for centuries to treat infection, inflammation, and other aliments. Extracts from different parts of the plant exhibited anti-tumor, antimicrobial, and antioxidant activity (Yang et al., 2001; Ding, Jiang, Chen, Lv, & Zhu, 2008; Wang et al., 2011; Su et al., 2012). Tailor et al. (1997) reported that Ib-AMP1 was expressed in mature seeds during the course of seed development. However, whether Ib-AMP1 is expressed in other plant tissues has yet to be determined. Ib-AMP1 is a 20-mer peptide containing four cysteine-residues, which form two intra-molecular disulfide bonds (Patel, Osborn, Rees, & Thornton, 1988; Lee et al., 1999; Thevissen et al., 2005). Research to determine the antimicrobial activity Ib-AMP1 has been conducted using both natural and synthetic forms of the peptide generating a range of results (Tailor et al., 1997; Lee et al., 1999; Thevissen et al., 2005; Wang et al., 2009). Besides determining minimum inhibitory concentrations, other antibacterial properties (e.g., bactericidal, bacteriostatic, residual activity) of Ib-AMP1 were not elucidated in those studies.

The source and method of production of Ib-AMP1 may affect its antimicrobial activity. Since the synthetic form demonstrates a greater range of antimicrobial activity; subsequent commercial application is more feasible (Tailor et al., 1997, Lee et al., 1999, Thevissen et al., 2005, Wang et al., 2009). Solid phase synthesis becomes a potential

method for large-scale production of a bioactive form of Ib-AMP1. Moreover, the small size (20-mer) makes Ib-AMP1 solid-phase synthesis/production straightforward and cost-effective. Regardless of whether it can be produced economically a major drawback to its use may be off-odor associated with the multiple disulfide bonds. Researchers have demonstrated that Ib-AMP1 analogs lacking disulfide bonds were equally effective as the native molecule (Wang et al., 2009).

The aims of the present study were to determine whether bacterial cell number within a matrix would affect application, whether Ib-AMP1 exhibited residual antibacterial activity after interaction with a bacterial cell, and to determine cytotoxic activity against cells that would be influenced following oral ingestion. Experiments were conducted using *E. coli* O157:H7, a foodborne pathogen that has been linked to many multistate foodborne outbreaks in the United States (Mead & Griffin, 1998; Center for Disease Control and Prevention, 2012). In the present study, desalted synthetic Ib-AMP1 was used since formation of disulfide bridges is not required for activity.

III. Material and methods

A. Bacteria

E. coli O157:H7 ATCC 43895, *Salmonella enterica* serovar Newport, *Staphylococcus aureus* ATCC 10832, *Pseudomonas aeruginosa* ATCC 15442, and *Bacillus cereus* ATCC 9818 were cultured in Muller Hinter Broth (MHB) (BD Difco™, Franklin Lakes, NJ) for at least 18 h at 37 ˚C with agitation. Frozen stocks were kept at -80 ˚C in medium

containing 20 % glycerol. Cells were sub-cultured twice and then streaked onto agar plates and incubated at 37 ˚C for at least 18 h. Cultures used in experiments were prepared by inoculating MHB with a single well-separated colony and cultured as described above.

B. Ib-AMP1 peptide preparation

Ib-AMP1 was chemically synthesized by GenScript (Piscataway, NJ) based on solid phase synthesis. The amino acid sequence was synthesized according to the published sequence, QWGRRCCGWGPGRRYCVRWC (Tailor et al., 1997). Lyophilized Ib-AMP1 was analyzed using mass spectrometry and HPLC. SDS-PAGE analysis revealed a single band, confirming the purity. Ib-AMP1 was dissolved in sterile distilled de-ionized water (SDDW) to the final concentration of 4 mg mL^{-1} as the stock solution. The stock solution was kept at -80 ˚C. The working solution was diluted from the stock solution with SDDW.

C. Screening of antimicrobial activity - minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The microdilution assay was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Ib-AMP1 against target bacteria. The assay was conducted according to standards set forth from the Clinical and Laboratory Standards Institute (CLSI, 2003a). All five bacteria were cultured individually

in 5 mL MHB and incubated at 35 ˚C with agitation for 18 h. Cells were collected by centrifugation at 3,500 rpm for 15 min at 4 ˚C, and resuspended in 5 mL of fresh MHB. Inoculum was prepared by making serial 1:10 dilutions in fresh MHB to achieve approximately 10^5 CFU mL^{-1}. Ib-AMP1 was serially diluted in SDDW with a final volume of 100 µL $well^{-1}$ in a 96-well plate; and 100 µL of inoculum was dispensed into each well. A bacterial growth control, Ib-AMP1 only control, and negative control (medium only) were included. Plates were incubated at 35 ˚C in a Dynex 96-well plate reader MRX with Revelation software to monitor optical density at λ = 630 nm for 24 h. All assays were performed in triplicate and repeated twice. The MIC is the minimum concentration of Ib-AMP1 that inhibits 80 % growth of target bacteria at 16 h based on the optical density. After 24 h incubation, an aliquot of each well was plated onto an agar plate, in duplicate, to determine cell viability. The MBC is the minimum concentration of Ib-AMP1 that shows absence of viable cells.

D. Bactericidal activity of Ib-AMP1 against *E. coli* O157:H7

The bacterial viability assay was conducted to determine whether Ib-AMP1 effectively inactivates cells of *E. coli* O157:H7. *E. coli* O157:H7 was grown in 10 mL of MHB at 37 ˚C for approximately 18 h. Overnight cultures were diluted in ½X MHB to a desired cell number: 10^3, 10^6 and 10^9 CFU mL^{-1}. Ib-AMP1 at final concentrations of ½X, 1X and 2X MIC were mixed with each cell suspension and incubated at 37 ˚C. Aliquots (100 µL) were removed at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 20, and 24 h from each of the reaction tubes

and immediately diluted (1:10) in phosphate buffer saline (PBS; BD BBL™, Franklin Lakes, NJ) to minimize continued antibacterial activity. Serial 1:10 dilutions were made as required, and 100 µL aliquots were plated onto Tryptic Soy Agar (TSA) plate (BD Difco™, Franklin Lakes, NJ) plates in duplicate. Plates were incubated at 37 ˚C for more than 18 h. Viable counts were expressed as CFU mL^{-1}.

E. Residual antibacterial activity of Ib-AMP1

The residue effect was studied to determine the sustainable and residual efficacy of Ib-AMP1. *E. coli* O157:H7 was used as the model organism. *E. coli* O157:H7 was grown in MHB at 37 ˚C for approximately 18 h. The overnight culture was then diluted to 10^3 CFU mL^{-1} (low cell number) and 10^6 CFU mL^{-1} (high cell number) in ½X MHB and used as the inoculum. Cells were treated with Ib-AMP1 at final concentrations of 1X and 2X MIC of Ib-AMP1 and incubated at 37 ˚C with agitation for 24 h. A cell-free control (CF), containing only Ib-AMP1 at 1X and 2X MIC, and cells only control (NC) were included. After 24 h incubation, cells were centrifuged at 5,000 rpm for 10 min and the supernatant was collected and passed through a 0.2 µm filter to remove cells. The antibacterial activities of the resulting supernatants were then determined by microdilution assay with some modifications. In brief, an overnight culture of *E. coli* O157:H7 was diluted to 10^6 CFU mL^{-1} in 10X MHB and used immediately as the inoculum. One hundred and ninety microliters of filtered supernatant (SN) samples (NC SN, 1X SN, 2X SN, CF 1X SN and CF 2X SN) were added into each well in triplicate and 10 µL of

inoculum were dispensed into each well. Synthetic Ib-AMP1 at 1X and 2X MIC were included as the positive controls. Plates were incubated at 35 ˚C in Dynex 96-well plate reader MRX with Revelation software to monitor optical density at λ = 630 nm for 24 h. After 24 h incubation, aliquots of each well were serial diluted and plated onto TSA plates in duplicate to determine cell survival.

F. Mammalian cell toxicity studies

FHs 74 Int, HT29 and Hep G2 cells were purchased from ATCC (Manassas, VA, USA). FHs 74 Int cells were grown in 10 mL complete medium containing Hybri-care medium supplemented with 30 ng mL^{-1} epidermal growth factor (EPF), 10 % fetal bovine serum (FBS), 200 units mL^{-1} penicillin and 200 µg mL^{-1} streptomycin in a 75 cm^2 tissue culture flask (Invitrogen, Grand Island, NY). HT29 and Hep G2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and Eagle's Minimum Essential Medium (EMEM), respectively, containing FBS, penicillin and streptomycin as mentioned previously in a 75 cm^2 tissue culture flask. All mediums and EPF were purchased from ATCC (Manassas, VA, USA); all other supplements were purchased from Life Technologies (Grand Island, NY). Exponentially growing cells were harvested using 0.05 % trypsin in EDTA/PBS/phenol red solution for 10 min at 37 ˚C with 5 % CO_2. Cells were then washed, resuspended in complete medium, and seeded into wells of a 96-well tissue culture plate at 1 X 10^4 cells per well. The plates were incubated at 37 ˚C with 5 % CO_2 until confluence was achieved. When cells were confluent, cells were washed with complete medium and

then incubated with complete medium containing Ib-AMP1 from 25 to 1,000 μg mL^{-1}. Cells only, medium only, and Ib-AMP1 only were included as controls. Plates were incubated at 37 ˚C with 5 % CO_2 for 24 h. After 24h incubation, 20 μL MTS (tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt) reagent (Promega, Madison, WI, USA) were added into each well. The assay includes the reduction of MTS tetrazolium compound to a colored formazan product by NADPH or NADH produced by dehydrogenase in metabolically active cells. Each plate was incubated at 37 ˚C with 5 % CO_2 for 2-4 h and absorbance at λ = 490 nm were measured using Synergy HT plate reader (Biotek, Winooski, VT, USA). All samples were tested in triplicate and repeated twice.

IV. Results

A. MICs and MBCs

MICs and MBCs of Ib-AMP1 against human and foodborne pathogens are shown in Table 1. Ib-AMP1 inactivated all tested bacteria within a range of 50 - 200 μg mL^{-1}. Greatest activity was exhibited against *E. coli* O157:H7 and *Staph. aureus* at 50 μg mL^{-1}. The MBC was either equivalent to or up to 4-fold greater than the corresponding MIC under conditions evaluated. For example, the MIC and MBC for *B. cereus* was 50 μg mL^{-1} and >200 μg mL^{-1}, respectively.

B. Bacterial viability assay

Bacterial viability after Ib-AMP1 treatments is shown in Fig. 1. At low cell numbers (10^3 CFU mL^{-1}) Ib-AMP1 at 1X and 2X MIC was bactericidal for *E. coli* O157:H7 cells exhibiting a 1.46 and 2.69 log reduction on viable cell number after a 6 h incubation, respectively. Untreated cells and Ib-AMP1 at ½X MIC showed 2.83 and 1.73 log cell growth after a 6 h incubation, respectively. At the medium cell number (10^6 CFU mL^{-1}) evaluated, Ib-AMP1 at 1X and 2X MIC resulted in a 1.89 and 2.68 log reduction on viable cell number after a 6 h incubation, respectively. However, untreated cells and cells treated with Ib-AMP1 at ½X MIC showed 2.93 and 2.19 log cell growth after a 6 h incubation, respectively. Treated and untreated cells, except at the low cell number of *E. coli* O157:H7 treated with Ib-AMP1 at 1X and 2X MIC, and at the medium cell number of *E. coli* O157:H7 treated with Ib-AMP1 at 2X MIC, increased to > 10^8 CFU mL^{-1} after 24 h incubation.

C. Residual antibacterial activity of Ib-AMP1

The viable cell numbers of each *E. coli* O157:H7 treated SN samples are presented in log CFU mL^{-1} and shown in Table 2. All SN samples exhibited no inhibitory effect against *E. coli* O157:H7; there was no difference in cell number of untreated cells and SN-treated cells at 24 h incubation. Freshly prepared synthetic Ib-AMP1 at 1X and 2X MIC was included as positive controls and they showed zero viable cell counts.

D. Cytotoxicity assay

MTS was used to determine the ability of Ib-AMP1 to inhibit cell proliferation. IC_{50} of Ib-AMP1 on HT29, FHs 74 Int and Hep G2 cells was 600, 400 and 200 µg mL^{-1}, respectively (Fig. 2). IC_{80} of Ib-AMP1 is > 1000, > 1000 and 800 µg mL^{-1} for HT29, FHs 74 Int and Hep G2 cells, respectively. IC_{50} and IC_{80} is the concentration of Ib-AMP1 that inhibits the production of NADPH or DADH in metabolically active cells by 50 % and 80 %, respectively.

V. Discussion

Plant antimicrobial peptides, including Ib-AMP1, may have potential broad application from use in foods to personal care products. Previous studies on Ib-AMP1 did not investigate properties of the antimicrobial that would specifically influence its use in food. The purpose of the present study was to determine the bactericidal activities, residual properties, and cytotoxicity of Ib-AMP1. Initial experiments demonstrated that Ib-AMP1 was bactericidal against *Staph. aureus, B. cereus, E. coli* O157:H7, *S*. Newport, and *P. aeruginosa*. Gram-positive and Gram-negative bacterial growth was inhibited at concentrations from 50 to 200 µg mL^{-1} which is equivalent to 40.2 to 80.5 µM. According to the CLSI standard, the MIC ranges are similar to that of conventional antibiotics. MICs of conventional antibiotics, such as kanamycin and ampicillin against *E. coli* ranged from 1 - 128 µg mL^{-1} (CLSI, 2003b). The MIC of Nisaplin, a commercial brand of nisin, against *Listeria monocytogenes*, tested in our lab, was 150 µg mL^{-1}. Our results

are in agreement with a previous study demonstrating that synthetic Ib-AMP1 is active against *Staph. aureus, Salmonella*, and *P. aeruginosa* (Wang et al., 2009). In the present study, we demonstrated activity against the foodborne pathogens *B. cereus* and *E. coli* O157:H7. Results of cytotoxicity assays suggest that concentrations 4-fold greater than the MIC for *E. coli* O157:H7 could potentially be used in products intended for oral consumption. The activity of Ib-AMP1 is neutralized once it interacts with a bacterium, similar to other antimicrobial agents.

Initial experiments were conducted using some of the pathogens included in previous studies to facilitate comparison of results. Based on results of MIC and MBC experiments *E. coli* O157:H7 was chosen as the model bacterium since it had one of the lowest MICs and the antibacterial effect of Ib-AMP1 against it had not been evaluated. The ability of Ib-AMP1 to inactivate the foodborne pathogen, *E. coli* O157:H7 was further investigated. The results demonstrated that the peptide is bactericidal for *E. coli* O157:H7. A comparable decrease in cell numbers occurred when *E. coli* O157:H7 at low and medium cell numbers was exposed to 1X MIC or 2X MIC Ib-AMP1. Ib-AMP1 at 2X MIC inactivated *E. coli* O157:H7 and prevented its outgrowth at both low and medium cell numbers. However, when the cell population was increased to 10^9 CFU mL^{-1}, cell numbers initially decreased at the 2X MIC of Ib-AMP1; by 6 h incubation bacterial populations were similar regardless of the Ib-AMP1 concentration. These results suggest that once a critical level of molecules of Ib-AMP1 has interacted with bacteria, activity of the compound has been neutralized and viable cells are free to grow. This could potentially limit the use of Ib-AMP1, particularly in foods that have high

populations of commensal bacteria. Increased concentrations of Ib-AMP1 could be used, but cost effectiveness would become an even greater issue.

In the present study, we observed that cells exposed to Ib-AMP1 at a sublethal concentration (½X MIC) exhibited longer lag times and higher OD_{630nm} after 16 h incubation. Appendini and Hotchkiss (1999) suggested that the increase in optical density may be due to plasmolysis of *E. coli* cells. Plasmolysis is associated with potassium leakage (Cabral, 1990). Unpublished studies conducted in our laboratory showed that Ib-AMP1 caused efflux of potassium ions further suggesting that the higher optical density may be associated with plasmolysis. Moreover, Subbalakshmi and Sitaram (1998) suggested that after treating with indolicidin, an AMP from cytoplasmic granules of bovine neutrophils, the increased OD_{550nm} of *E. coli* cells was associated with the elongation and filamentation. The author further suggested that filamentation may have resulted from the inhibition of DNA synthesis (Lutkenhaus, 1990). Research in our laboratory demonstrated that Ib-AMP1 at a sublethal concentration (½XMIC) inhibited DNA synthesis based on decrease in the incorporation of tritium-labeled thymidine (unpublished data). Altogether, the sublethal concentration of Ib-AMP1 may not affect cell proliferation in terms of viable cell number, but may affect cell morphology and metabolism.

In the residual antibacterial test, the results indicated that Ib-AMP1 is finite in activity which may be the result of irreversible reaction(s) with bacterial cell components. Ib-AMP1 incubated in media at 37 ˚C for 24 h and then tested failed to exert any

antibacterial activity. Given this, the application of Ib-AMP1 may be limited. Indeed, in agreement with other studies (Tailor et al., 1997, Patel et al., 1998, Thevissen et al., 2005), we observed that Ib-AMP1 precipitates at extreme concentrations (>10 mg mL^{-1}) and it is sensitive to high ionic strength medium (data not shown). In our observations, Ib-AMP1 tended to precipitate in TSB compared to MHB; the antibacterial activity was also decreased when tested in TSB compared to MHB. TSB is a rich general purpose nutrient medium which contains ions. MHB is a minimum nutrient medium, containing no ions, which eliminates the effect of ions Ib-AMP1 activity.

Ib-AMP1 at 200 (80 µM), 400 (161 µM) and 600 (241 µM) µg mL^{-1} inhibited the proliferation of Hep G2 (human liver epithelial cell), FHs 74 Int (human fetal small intestine epithelial cell), and HT29 cells (human colon epithelial cell), respectively. Ib-AMP1 was cytotoxic at concentrations 4 to 12-times higher than the MIC against *E. coli* O157:H7.

Synthetic Ib-AMP1 was not cytotoxic to human erythrocytes or tumor cells, such as K-562 (human bone marrow lymphoblast cells), A549 (human lung epithelial cells) and NDA-MB-361 (human mammary gland epithelial cells), at 100 µM (250 µg mL^{-1}) (Lee et al., 1999). The results of the present study compared to those published underscores the need to evaluate cytotoxicity to appropriate human cells before potential application strategies are considered. Food application of Ib-AMP1 would ultimately result in exposure of intestinal cells to the compound. Thevissen et al. (2005) showed that synthetic Ib-AMP1 did not exhibit any hemolytic activity against rabbit erythrocytes

at a concentration of 200 µM and was not toxic to mouse myeloma cells at 100 µM. Wang et al. (2009) also demonstrated that synthetic Ib-AMP1 and all other linear analogs did not cause lysis of human red blood cells at 400 µM.

Now that Ib-AMP1s activity, cytotoxicity, and residual activity have been investigated, future research will focus on evaluating potential synergies with other antimicrobials. Model studies must be conducted in food systems to determine efficacy and effect on organoleptic properties.

VI. Conclusion

The present study demonstrated that Ib-AMP1 was bactericidal against foodborne pathogens at MIC from 50 – 200 µg mL^{-1}. Ib-AMP1 at 2X MIC inactivated *E. coli* O157:H7 when the cell population was less than 10^6 CFU mL^{-1} and prevented its outgrowth. Irreversible interaction of Ib-AMP1 with bacteria cell components, cations, or other components in the medium inactivated the peptide making it no longer biologically active. Concentrations less than 200 µg mL^{-1} of Ib-AMP1 generally showed less than 50 % inhibition on the proliferation of human liver, small intestine, and colon cells. Intelligent design and formulation are required for the development of a novel and safe product(s) containing Ib-AMP1 for the use in food and food systems that improves microbial safety of those products.

VII. Tables and figures

Table 1. Antimicrobial activity of Ib-AMP1 against pathogens evaluated.

Classification	Bacterial Species	[a] MIC (μg mL^{-1})	[†]MBC (μg mL^{-1})
Gram negative	*Escherichia coli* O157:H7 ATCC43895	50	50
	Salmonella Newport	100	400
	Pseudomonas aeruginosa ATCC15442	100	200
Gram positive	*Staphylococus aureus* ATCC10832	50	100
	Bacillus cereus ATCC9818	200	>400

Peptides were dissolved in MHB and added to approx. 10^5 CFU mL^{-1} of bacterial suspension, incubated at 35˚C for 16 h. OD_{630nm} were recorded every 15 min for 16 h in a temperature-controlled micro-plate reader. Data were from two independent assays and each Ib-AMP1 concentration was tested in triplicate in each experiment.

[a] MIC is determined by comparing the OD_{630nm} of untreated cells where there are more than 80 % reductions with respect to visible turbidity.

[†] MBC is determined by absence of viable cells after 24 h incubation at 37˚C.

Table 2. Residual antibacterial activity of Ib-AMP1.

Samples [a]		Log CFU mL^{-1} ±STDEV
[b] With low cell number	NC SN	8.43±0.2
	1X SN	8.97±0.1
	2X SN	8.56±0.3
[c] With high cell number	NC SN	8.72±0.0
	1X SN	8.75±0.0
	2X SN	8.69±0.3
Without cells (Ib-AMP1 only)	1X SN	8.91±0.1
	2X SN	8.20±0.9
Synthetic Ib-AMP1	1X MIC	0±0
	2X MIC	0±0
Untreated cells		8.85±0.1

[a] All samples were centrifuged and filtered through 0.22 µm filter to remove cells and incubated with an average of $9.3X10^4$ CFU mL^{-1} *E. coli* O157:H7 cells at 35˚C for 24 h.
[b] Ib-AMP1 at 0 µg mL^{-1} (NC), 1X and 2X MIC were treated with approx. $4.41X10^3$ CFU mL^{-1} of *E. coli* O157:H7 for 24 h at 37˚C before centrifugation and filtration.
[c] Ib-AMP1 at 0 µg mL^{-1} (NC), 1X and 2X MIC were treated with approx. $4.61X10^6$ CFU mL^{-1} of *E. coli* O157:H7 for 24 h at 37˚C before centrifugation and filtration.
Data are presented as average Log CFU mL^{-1} ± STDEV from two independent assays.

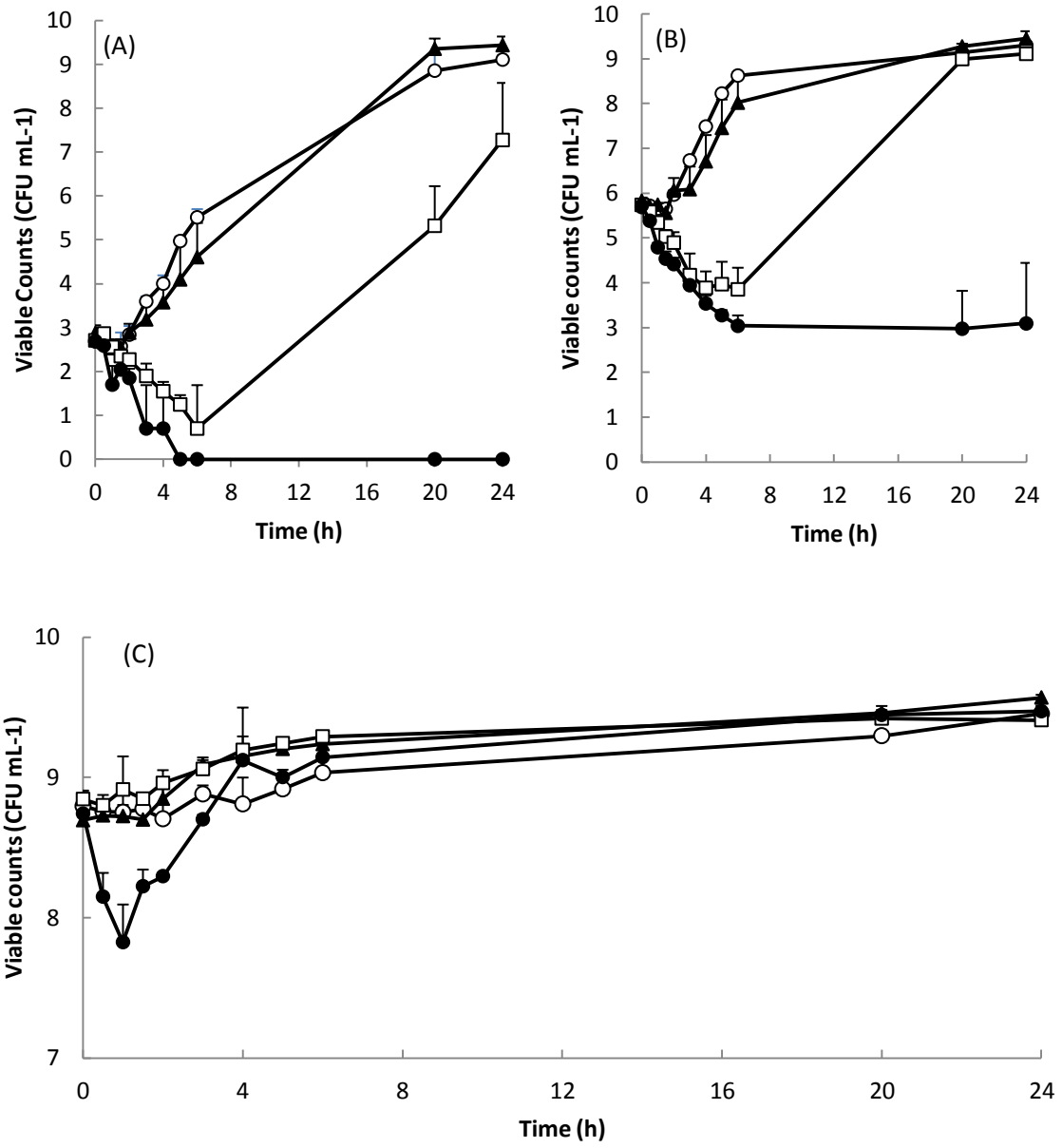

Figure 1. Cell viability of Ib-AMP1-treated *E. coli* O157:H7. Cell viability of treated *E. coli* was determined with different initial cell numbers: (A) low (B) medium (C) high initial cell numbers. Data are presented as average Log CFU mL^{-1} ± STDEV from two independent assays. Ib-AMP1 concentration: (○) untreated cells, (▲) 25 µg mL^{-1}, (□) 50 µg mL^{-1} and (●) 100 µg mL.

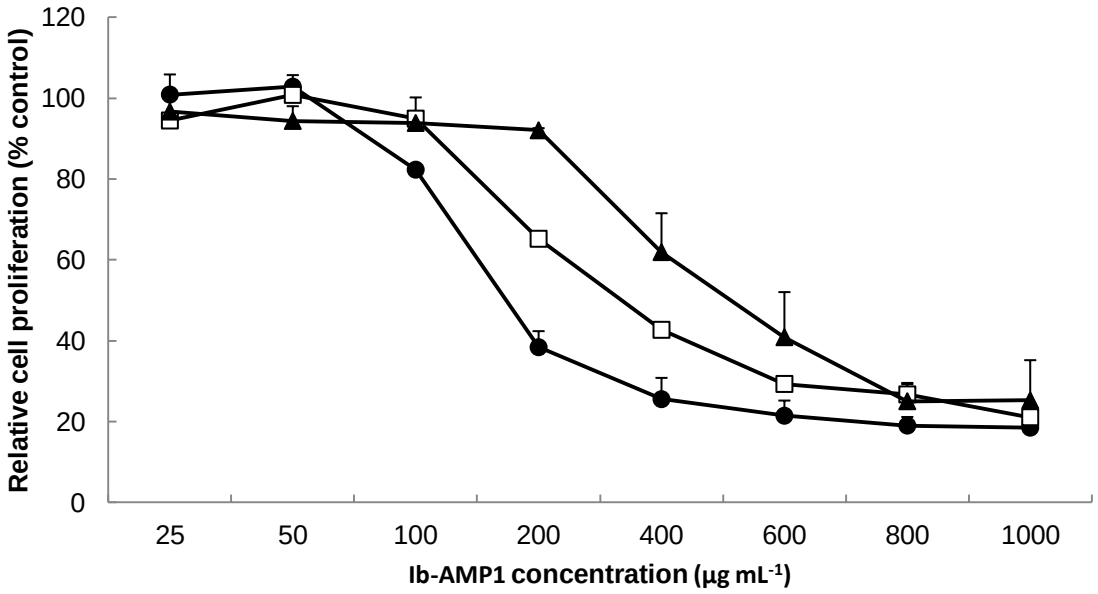

Figure 2. Relative cell proliferation of HepG2, FHs 74 Int and HT 29 cell by Ib-AMP1. Relative cell proliferation was calculated by following formula: OD_{490nm} of treated cells / OD_{490nm} of untreated cells X100. Untreated cell is considered 100% cell proliferation. Data are presented as average ± STDEV from two independent assays. Each Ib-AMP1 concentration was tested triplicate in each experiment. Cell types: (▲) HT-29, (□) FHs 74 Int and (●) Hep G2

VIII. References

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CHAPTER 5

The following data were published online in Journal, Probiotics and Antimicrobial Proteins at 15 February 2013. In press, accepted manuscript.

Wen-Hsuan Wu, Rong Di and Karl R. Matthews. 2013. Antibacterial mode of action of Ib-AMP1against *Escherichia coli* O157:H7. Probiotics Antimicro Prot. DOI: 10.1007/s12602-013-9127-1.

Objectives:

I. To determine the ability of Ib-AMP1 to permeabilize *E. coli* O157:H7 cell membrane.
II. To determine the ability of Ib-AMP1 to cause efflux of intracellular potassium ions (K^+) and ATP on *E. coli* O157:H7
III. To determine the ability of Ib-AMP1 to dissipate *E. coli* O157:H7 cytoplasmic membrane and damage the outer membrane.
IV. To determine the ability of Ib-AMP1 to inhibit DNA, RNA and protein synthesis in *E. coli* O157:H7

Antibacterial Mode of Action of Ib-AMP1 against *Escherichia coli* O157:H7

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Key Words:

Antimicrobial peptide, Ib-AMP1, *E. coli* O157:H7, mode of action.

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Abbreviations: AMPs: antimicrobial peptides. pAMPs: plant-derived antimicrobial peptides. SDDW: sterile de-ionized distilled water. NPN: N-Phenyl-1-naphthylamine. $DiSC_3(5)$: 3,3-dipropylthiadicarbocyanine iodide. RT: room temperature.

I. Abstract

Continual occurrence of foodborne outbreaks, along with the increase of antibiotic resistance which burdens clinical treatments has urged scientists to search for other potential promising antimicrobial agents. Antimicrobial peptides are emerging as one of the potential alternatives. The mode of action of a given AMP is critical and essential for future application; however, it is still not completely known for many of these compounds. Ib-AMP1 is a plant-derived AMP, purified from seeds of *Impatiens balsamina* and has been shown to exert antibacterial and antifungal activity at the micromolar level. A study had shown that the therapeutic index of Ib-AMP1 against eight human pathogens is 23.5. The objective of the present study was to determine the *in vivo* mode of action of Ib-AMP1 against *Escherichia coli* O157:H7. A concentration dependent effect of Ib-AMP1 on the *E. coli* O157:H7 cell membrane occurred. Ib-AMP1 treatments resulted in efflux of K^+ and ATP, suggesting pores of sufficient size to allow efflux of large molecules. Ib-AMP1 at sublethal concentrations exerts a greater effect at the intracellular level. In contrast Ib-AMP1 at a lethal concentration permeabilizes cell membranes and may directly or indirectly inhibit intracellular macromolecule synthesis. Collectively, results of this study suggest Ib-AMP1 is bactericidal interfering within outer and inner membrane integrity permitting efflux of ATP and interfering with intracellular biosynthesis of DNA, RNA, and protein.

II. Introduction

Foodborne diseases are a significant public health concern not only in the United States but globally. Based on epidemiological data, the CDC suggested that during the past 10 years, in the United States, there were 47.8 million cases of foodborne illnesses per year including 127,839 hospitalizations and nearly 3,000 deaths [1, 2]. These data coupled with the 1999 CDC report on foodborne disease [3] suggest that foodborne illnesses are a continual public health issue. Infected person, contaminated raw food or ingredient, and cross-contamination are among the top ten causes of foodborne outbreaks [4]. Treatments of foodborne illness will be difficult if the stains became resistant to conventional antibiotics. Antibiotic resistant *E. coli* O157:H7 and *Salmonella* have been isolated from humans, foods and animals [5, 6]. This leads to the demand for antibiotic alternatives for clinical treatments of foodborne diseases, as well as novel food preservatives and hand sanitizers with greater efficacy. Antimicrobial peptides (AMPs) have caught researchers' attention for their broad spectrum of applications, including their therapeutic potential. The AMPs are produced throughout the Prokaryotic and Eukaryotic Kingdoms, from bacteria, fungi, plants, insects, invertebrates and mammals. Some are products of innate and adaptive immunity designed to protect the host from infection [7-10]. AMPs such as defensin from humans and plants, and magainins from *Xenopus* skin have been studied extensively. Plant-derived AMPs (pAMPs) are of our interest due to their potential future application in the agriculture industry, such as plant disease control and production of crops that confer control of foodborne bacteria and have potential as natural preservatives. Plant AMPs (pAMPs) are generally small

cysteine-rich proteins containing less than 50 amino acid residues [11, 12]. Plants are constantly exposed to harsh environmental conditions and a wide range of pathogens; therefore, they produce antimicrobial substances as their primary defense while causing no damage to themselves. Plant AMPs may be constitutively expressed or expressed upon infection. Almost every plant structure (i.e., leaf, root, and stem) produces at least one pAMP [8, 13, 14]. Due to their diversity in source, pAMPs vary in size, amino acid composition and structure. Nuclear magnetic resonance spectroscopy was used to elucidate the 3-dimensional structures of pAMPs. Results indicate that pAMPs may contain α-helices, β-sheet or cyclic structures [11, 15]. Most of pAMPs have been shown to have a broad spectrum of antimicrobial activity against Gram-positive, Gram-negative bacteria and fungi, including many plant pathogens and foodborne pathogens. According to PhytAMP database, although only 35 % of the recorded pAMPs were tested for biological activity, 51 % of evaluated pAMPs possess antifungal activity, 35 % are antibacterial 10 % are antiviral, and around 3 % are insecticidal [11].

Ib-AMP1 is a 20-mer pAMP, purified from seeds of *Impatiens balsamina* [16]. It is cationic and forms two intra-molecular disulfide bonds for stability. Research has demonstrated that Ib-AMP1 analogs lacking disulfide bonds retained antibacterial activity equal to or greater than the parent molecule [17]. Studies suggest that the structure of Ib-AMP1 is temperature and pH stable [18]; however, there may be no direct link of structural stability to biological activity. It has been shown that Ib-AMP1 is active against fungi, Gram-positive and Gram-negative bacteria at micromolar levels [16, 19-21]. Studies also demonstrated that Ib-AMP1 has no cytotoxicity or hemolytic

activity on erythrocytes or other mammalian cell lines at concentrations 2 - 400 times greater than the IC_{50} or MICs against target microorganisms [17, 19, 20]. Wang et al. [17] showed that the therapeutic index of Ib-AMP1 against eight human pathogens, including methicillin-resistant *Staphylococcus aureus*, was 23.5. Results may indicate the potential application in the pharmaceutical or food industries. However, the mode of action of Ib-AMP1 has not been completely elucidated.

Understanding the mode of action of a given AMP is critical and essential for any future application. Studies indicate that most AMPs are cationic in physiological environments which render affinity to the negatively charged bacterial surface, and are amphipathic which allow them to transfer across the bacterial hydrophobic lipid bilayer [21, 22]. The affinity of AMPs to bacterial cell membranes suggests a pore-forming mode-of-action. Recent studies indicate AMPs can also inhibit intracellular macromolecule synthesis of DNA, RNA, and protein [10, 23-25].

The present study was conducted to determine the mode of action of Ib-AMP1, focusing on the foodborne pathogen *E. coli* O157:H7. *E. coli* O157:H7 is responsible for foodborne illness linked to the consumption of contaminated foods including ground beef, lettuce, spinach, apple cider, and raw milk [26]. The *in vivo* effects of Ib-AMP1 on disruption of the membrane and intracellular macromolecules, DNA, RNA, and protein of *E. coli* O157:H7 were studied.

III. Material and Methods

A. Bacteria

E. coli O157:H7 ATCC43895 was cultured in Mueller Hinton broth (MHB) for 16 h at 37 ˚C with agitation. Frozen stocks were kept at -80 ˚C with medium containing 20 % glycerol. Cells were sub-cultured twice and then streaked onto agar plates and incubated at 37 ˚C overnight. Cultures were prepared by inoculating MHB with a single well-separated colony and incubated as indicated above.

B. Ib-AMP1 peptide preparation

Ib-AMP1 was chemically synthesized by GenScript (Piscataway, NJ) by solid phase synthesis based on the published amino acid sequence, QWGRRCCGWGPGRRYCVRWC [16]. Lyophilized Ib-AMP1 was analyzed using mass spectrometry, HPLC and SDS-PAGE to confirm the purity. Ib-AMP1 was dissolved in sterile distilled de-ionized water (SDDW) to the final concentration of 4 mg/mL as the stock solution. The stock solution was kept at -80 ˚C. The working solution was diluted from the stock solution with SDDW.

C. Membrane permeability assay

Membrane permeability was determined using the LIVE/DEAD BacLight^{TM} Bacterial Viability Kit (Invitrogen Molecular Probes, Eugene, OR). This assay permits differentiation of permeable and intact cells, where permeable cells stain red and intact cells stain green. *E. coli* O157:H7 was grown to log phase in MHB at 37 ˚C. The cell

pellet was collected, washed twice in SDDW and resuspended in SDDW to one-quarter the initial volume. Ten microliters of the cells were transferred to a second tube and centrifuged again; the resulting pellet was incubated with 10 µL of SDDW or SDDW containing Ib-AMP1 at a final concentration of 25, 50 and 100 µg/mL for 30 min at room temperature (RT). STYO9 and propidium iodine stains stock solutions were prepared according to manufacturer's instruction. All cells were incubated with both stains for 15 min in the dark. A 0.5 µL volume of the stained cells were loaded on a microscope slide, covered with a cover slide, and observed using an Olympus BH2-RFCA fluorescence microscope (Olympus Corporation, Lake Success, NY) fitted with a Pixera camera (Pixera, Santa Clara, CA). Five random fields were counted. The assay was conducted three times. Numbers of permeable and intact cells were counted and percent of permeable cells were calculated according to the following formula: (numbers of permeable cells / numbers of total cells) X 100.

D. K^+ efflux assay

Potassium (K^+) efflux assay was conducted to determine change in permeability of *E. coli* O157:H7 cell membrane after exposure to Ib-AMP1. Potassium ion selective probes have been used widely to investigate the efflux of intracellular potassium by cells after treatment with antimicrobial agents [27-29]. Potassium combination electrode K001508 (Jenco Instruments, Inc., San Diego, CA) connected to a Jenco pH/mV/Temp./ION bench meter 6219 (Jenco Instruments, Inc., San Diego, CA) were

used. The ion potential response (mV) was monitored and recorded. Various concentrations of standard potassium solutions (KCl) (Research organics, Cleveland, Ohio) containing 5 M NaCl ionic strength adjuster (Jenco Instruments, Inc., San Diego, CA) and the corresponding mV readings were plotted to generate a standard curve. The resulting mV readings from each experiment were then converted to potassium concentration based on the standard curve.

E. coli O157:H7 was grown to log phase in MHB at 37 ˚C. Cells were centrifuged, washed twice in 10 mM Tris-acetate, pH 7.4 (Research Organics, Cleveland, OH) and resuspended in an eighth of the original volume and ready to use as concentrated cells. A total of 4 mL of solution containing 1 mL of concentrated cells, 2.9 mL of 10 mM Tris-acetate buffer (pH 7.4), and 100 µL of Ib-AMP1 to achieve final concentrations of 25, 50 and 100 µg/mL were added to a flask. The reaction suspension was mixed with a magnetic stir bar in the course of experiment. Untreated cells were included as baseline, and cells treated with DMSO (Sigma-Aldrich Corp., St. Louis, MO) at final concentration of 50 % were also included to determine the maximum efflux of K^+ ions. The mV readings were recorded for 30 min with 1min interval at room temperature (RT). Assay was conducted in triplicates. Percent of K^+ efflux was calculated using the following formula: (concentration of K^+ efflux of treated cells / Total K^+ concentration of untreated cells) X 100. Total K^+ concentration was determined by cells treated with 50 % DMSO.

E. ATP efflux assay

The efflux of ATP from Ib-AMP1 treated *E. coli* O157:H7 was determined using Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma-Aldrich Corp., St. Louis, MO). The assay includes a coupled enzyme reaction in which ATP is reduced to adenyl-luciferin by luciferase with the presence of luciferin. The resulting adenyl-luciferin then interacts with oxygen and produces light. The amount of light emitted is proportional to the amount of ATP present. The assay was conducted as described previously [29, 30] with some modifications. Efflux of ATP was used as a marker to determine exit of an intracellular component, a large molecule compared to K^+ ion.

E. coli O157:H7 was grown to log phase in MHB at 37 ˚C. Cells were collected, washed twice in 50 mM HEPES buffer, pH 7.0 (sigma-Aldeich Corp., St. Louis, MO) and resuspensed in one quarter of the original volume of the same buffer containing 0.2 % glucose to energize the cells for 20 min at RT. Energized cells were incubated with buffer or buffer containing Ib-AMP1 at the final concentrations of 25, 50 and 100 µg/mL at RT. An aliquot of each treatment was removed at 0, 1, 10, 20, 30, 45 and 60 min to determine the levels of extracellular and total ATP using a spectrophotometer (Luminoskan TL Plus luminometer, Labsystems Oy, Helsinki, Finland). Samples used for determining total ATP concentration were treated with DMSO to permeabilize the cell membrane. The amount of light emitted and the ATP concentration were plotted to generate an ATP standard curve. The total and extracellular ATP concentration at each time point was calculated according to the ATP standard curve. Percent of total and extracellular ATP concentration were calculated according to the following formula: percent total ATP concentration = (total ATP concentration of treated cells / total ATP

concentration of untreated cells) X 100. Percent of extracellular ATP concentration = (Extracellular ATP concentration of treated cells / total ATP concentration of untreated cells) X 100.

F. Membrane potential dissipation assay (Δψ)

Disruption of the cytoplasmic electrical membrane potential (Δψ) was determined on cells exposed to Ib-AMP1. The cell membrane produces proton motive force (PMF) to store energy, which is stored in two forms, electrical potential (Δψ) and chemical proton gradient (ΔpH). Fluorescent probe $DiSC_3(5)$ (3,3-dipropylthiadicarbocyanine iodide) was used as a marker to monitor the change of electrical membrane potential (Δψ) upon interaction with Ib-AMP1. It is a cationic membrane potential sensitive dye which accumulates on the negatively charged inside membrane forming aggregates resulting in self-quenching and subsequent decrease in fluorescence intensity. Cytoplasmic membrane potential dissipation results in the release of $DiSC_3(5)$ into the medium where they are no longer self-quenched and the fluorescence intensity increases.

The assay was based on methods described previously with some modifications [29, 31]. *E. coli* O157:H7 cells were grown to log phase in MHB at 37 ˚C. Cells were washed with wash buffer containing 50 mM K-HEPES (sigma-Aldeich Corp., St. Louis, MO), pH 7.0 twice and resuspended in 1/100 of original volume in respiration buffer containing 5 mM HEPES, 100 mM KH_2PO_4, 20 % glucose, 1 mM K_2-EDTA, pH 7.1. Cells were kept on ice before use. A total of 1980 µL assay buffer containing 50 mM HEPES, 1 mM K_2-EDTA,

pH 7.1 and 3 µL of $DiSC_3(5)$ (stock: 5 mM) was added and mixed in a quartz cuvette. Once the signal became stable, 20 µL of cell suspension was added. Nigericin at a final concentration of 20 µM was added to convert chemical proton gradient (ΔpH) of proton-motive force (pmf) to electrical potential (Δψ). Nigericin promotes the antiport transport of H^+ and K^+ and results in dissipation of pH gradient. Ib-AMP1 at final concentration of 25, 50 and 100 µg/mL was then added into the cuvette, independently. SDDW at a volume equal to Ib-AMP1 added was added to untreated cell as a control. Valinomycin at a final concentration of 20 µM was added to dissipate any remaining Δψ. Valinomycin promotes uniport of K^+ and dissipates Δψ of PMF. Increase of fluorescence intensity after addition of Ib-AMP1 and valinomycin indicates the dissipation of cytoplasmic membrane potential. Real-time fluorescence intensity was detected using a spectrofluorometer (Perkin Elmer, luminescence Spectrometer, LS50B, Waltham, MA) with excitation and emission wavelength of λ = 643 nm and 666 nm, respectively, and with 10 nm split wavelength. Total duration of assay was 900 s with a 0.1 s interval.

G. Outer membrane permeability assay

The permeability of the outer membrane in *E. coli* O157:H7 treated with Ib-AMP1 was determined using the fluorescent probe, NPN (N-Phenyl-1-naphthylamine) (Sigma-Aldrich Corp., St. Louis, MO). NPN is a hydrophobic and neutral probe, which is weakly fluorescent in an aqueous environment. When the outer membrane is damaged, NPN

partitions into the glycerophospholipid milieu, a hydrophobic environment, where it becomes fluorescent.

The assay was a modification of the method described previously [32]. *E. coli* O157:H7 cells were grown to log phase in MHB at 37 ˚C and then resuspended in half of original volume in 5 mM HEPES buffer, pH 7.2. NPN at a final concentration of 10 µM and 5 mM HEPES buffer were added into the cuvette and mixed well. One milliliter of cells was then added to make the final volume of 2 mL. The resulting suspension was incubated at RT for 3 min to allow the fluorescence to become stable. The cells were exposed with Ib-AMP1 at final concentration of 25, 50 and 50 µg/mL or EDTA at final concentration of 0.5, 1 and 2 mM. The fluorescence intensity before and 10min after addition of Ib-AMP1 was read using a spectrofluorometer (Perkin Elmer, luminescence Spectrometer, LS50B) with excitation and emission wavelength of λ = 350 nm and 420 nm, respectively, and with 5 nm split wavelength. Treatment containing SDDW was included as negative control; cells alone, Ib-AMP1 alone, NPN alone and cells plus NPN were also included as controls.

H. Macromolecular synthesis inhibition assay

The ability of Ib-AMP1 to affect synthesis of DNA, RNA, and protein was determined using radio-labeled precursors [33, 34]. The decrease of radio-labeled precursor incorporation indicates the inhibition of synthesis of the corresponding macromolecules. In brief, *E. coli* O157:H7 were grown to early-log phase. Ib-AMP1 was dissolved in half

strength MHB at a final concentration of 25, 50 and 100 µg/mL, and cells were then added to each reaction at a final concentration of OD_{600nm} = 0.02. Tritium-labeled precursors: [methyl-^{3}H] Thymidine, [5,6-^{3}H] Uracil and [3,4,5-^{3}H] L-leucine (MP Biomedicals, LLC., Santa Ana, CA, USA) were then added immediately at final concentrations of 20, 20 and 10 µCi/mL to determine inhibition of DNA, RNA and protein synthesis, respectively. All reaction tubes were incubated at 37 ˚C and a 100 µL aliquot was removed at pre-designated time points. Each aliquot was mixed with 1 mL of 10 % ice-cold trichloroacetic acid (TCA, Sigma-Aldrich Corp,. St. Louis, MO) solution and kept on ice for at least 1 h to precipitate incorporated radio-labeled precursors. Samples were then passed through Whatman GF/C glass fiber filters (GE Healthcare, Buckinghamshire, UK) using a vacuum filtering system to collect the precipitate. Filters was washed twice with 5 mL of 5 % ice-cold TCA and then twice with 3 mL of ice-cold 75 % ethanol, and then dried for 10 min. The unincorporated and free radio-labeled precursors are soluble in TCA and are passed through the filter membrane; the incorporated radio-labeled precursors are not soluble in TCA and precipitate on the filter membrane. The dry filters were placed in scintillation vials with 5 mL of scintillation fluid (ScintiSafe 30 %, Fisher BioReagent). Radioactivity was quantified using liquid scintillation counter (LS6500 Scintillation Counter, Beckman coulter, USA).

Radio-labeled precursor free samples were included in order to determine the viable cell counts at each time point. Aliquots of samples were removed and serial diluted in phosphate buffer saline (PBS) (BD BBL^{TM}, Franklin Lakes, NJ). Viable counts of each

sample at each time point were enumerated by plating on Tryptic Soy Agar (TSA) plate (BD Difco™, Franklin Lakes, NJ) Plates were incubated at 37 ˚C for overnight.

IV. Results

A. Ib-AMP1 peptide preparation

The purity of synthetic Ib-AMP1 was confirmed by mass spectrometer and HPLC analysis of the synthetic Ib-AMP1. SDS-PAGE analysis showed only one band further demonstrating the purity of synthetic Ib-AMP1 (data not shown). Disulfide bridge formation was not investigated since they likely have a limited role in activity [17].

B. Membrane permeability assay

Numbers of permeable and intact cells were determined (Fig. 1). Untreated cell preparations contained 1.68 % naturally permeable cells. Cell suspensions treated with Ib-AMP1 at 25, 50 and 100 µg/mL contained 8.35 %, 30.89 % and 56.18 % permeable cells, respectively.

C. K^+ efflux assay

Leakage of K^+ was observed from Ib-AMP1 treated cells (Fig. 2). The reaction was rapid and within 5 minutes 3.77 %, 9.69 % and 11.74 % efflux of K^+ from cells treated with Ib-

AMP1 at 25, 50 and 100 µg/mL, respectively, occurred. In untreated cells K^+ was taken up.

D. ATP efflux assay

Extracellular ATP concentration at one minute after addition of Ib-AMP1 to cells increased from 4 % to 5.17 %, 7.18 % and 32.31 % at 25, 50 and 100 µg/mL, respectively (Fig.3a). After the initial efflux, extracellular ATP concentrations remained constant until 60 minutes after Ib-AMP1 addition. A similar trend was observed in percent total ATP concentration which decreased from 100 % to 52.42 %, 34.17 % and 27.63 % for cells treated with Ib-AMP1 at 25, 50 and 100 µg/mL, respectively, at one minute after addition of Ib-AMP1 (Fig. 3b). Total ATP of the untreated cells represents 100% of ATP of the cell suspension. A rapid increase of extracellular ATP concentration indicated the efflux of intracellular ATP to the extracellular environment.

E. Cytoplasmic membrane potential dissipation assay

Real-time changes in membrane potential after treatment of cells with Ib-AMP1 were observed and expressed as fluorescence intensity (absolute unit, A.U.) (Fig. 4a). The initial fluorescence intensity of $DiSC_3(5)$ was approximately 170 A.U. (not shown); the intensity dropped to approximately 10 A.U. indicating the uptake of $DiSC_3(5)$ by the cells. Ib-AMP1 dissipated membrane potential in a concentration dependent manner. At 25

µg/mL, sublethal concentration, Ib-AMP1 caused no or little membrane potential dissipation; however, Ib-AMP1 at 50 and 100 µg/mL dissipated membrane potential completely as indicated by the increase of fluorescence intensity and no further increase after addition of valinomycin. No change in fluorescence intensity was observed in untreated cells, which were treated with SDDW. Addition of valinomycin dissipated the remaining membrane potential in untreated cells and cells treated with Ib-AMP1 at 25 µg/mL.

F. Outer membrane permeability assay

Ib-AMP1 resulted in outer membrane permeability, demonstrated by the increase in NPN associated with the outer membrane (Fig. 4b). The results are presented as the change of fluorescence intensity ($A.U._{after} - A.U._{before}$). Ib-AMP1 caused outer membrane damage in a negative concentration dependent manner; the higher the Ib-AMP1 concentration, the lower the NPN uptake by the cells. No major change in the fluorescence intensity was observed for cells treated with SDDW (negative control). The positive control, cells treated with EDTA at 0.5, 1 and 2 mM, showed an increase in fluorescence. Ib-AMP1 resulted in greater NPN uptake than EDTA. Incubation of Ib-AMP1 at all concentrations with NPN resulted in no change in fluorescence demonstrating that the fluorescence increases were due to the interaction of Ib-AMP1 and *E. coli* O157:H7 cells.

G. Macromolecular synthesis inhibition assay

Inhibition of *E. coli* O157:H7 DNA, RNA and protein synthesis by Ib-AMP1 are shown in Fig. 5. A concentration dependent inhibition of DNA, RNA and protein were observed; however, Ib-AMP1 at 50 and 100 µg/mL showed a similar degree of inhibition. After 5 min inhibition, Ib-AMP1 at 25, 50 and 100 µg/mL reduced DNA synthesis by 23.1 %, 55.5 % and 41 %, respectively. After 60 min incubation, DNA synthesis was inhibited by more than 90 % by Ib-AMP1 at both 50 and 100 µg/mL. Ib-AMP1 at 25 µg/mL, sub-lethal concentration, inhibited DNA synthesis by 64.6 % at 40 min incubation, but only by 33.6 % at 60 min incubation. RNA synthesis was inhibited by more than 97 % by Ib-AMP1 at all three concentrations after 60 min incubation. Ib-AMP1 at 50 and 100 µg/mL inhibited protein synthesis by 88.2 % and 88.5 %, respectively, after 100 min incubation. Ib-AMP1 at 25 µg/mL resulted in a 53.6 % reduction of protein synthesis at 80 min and a 49.5 % reduction at 100 min incubation. Percent of [methyl-^{3}H] Thymidine, [5,6-^{3}H] Uracil and [3,4,5-^{3}H] L-leucine incorporation inhibition were calculated by the following formula: [1 - (counts per minute of Ib-AMP1 treated cells / counts per minute of untreated cells)] X 100. Untreated cells were considered as 0 % of inhibition.

V. Discussion

The mode of action of Ib-AMP1 against the target organism *E. coli* O157:H7 was investigated. Results of the present study suggest that Ib-AMP1 destabilizes or increases permeability of the cell membrane and inhibits intracellular molecular

processes. Based on review of the published literature the antibacterial mode of action of Ib-AMP1 had not been elucidated completely. Model membrane systems designed to mimic bacterial cell membrane composition rather than live cells were used to determine the mode of action in previous study [17].

In this study, *E. coli* O157:H7 was selected as the target bacterium, since it showed the greatest sensitivity to Ib-AMP1 among the bacterial strains tested. The minimum inhibitory concentration (MIC) of Ib-AMP1 against several foodborne pathogens was determined (data not shown). The MIC is defined as the lowest concentration of a compound that inhibits 80 % of bacterial growth based on optical density. The MIC of Ib-AMP1 for *E coli* O157:H7 was 50 µg/mL. Concentrations of half (25 µg/mL) and 2X (100 µg/mL) MIC were also used to gauge the mode of action of Ib-AMP1.

The efflux of intracellular components, such as potassium ion and ATP, has been used widely to determine the extent of damage to the cell membrane after exposure to noxious agents [27-30, 35]. The results demonstrate that Ib-AMP1 exhibits a concentration dependent effect on *E. coli* O157:H7 cell membrane permeability based on efflux of potassium ions and ATPs. The level of efflux of K^+ ion correlates with the levels of efflux of ATP. The effect of Ib-AMP1 on the *E. coli* O157:H7 cell membrane permeability was very rapid, occurring within 1 min of exposure (Fig. 2 and Fig. 3). The membrane permeability assay further demonstrated that not all cells were affected, even at 2X MIC. Results demonstrate that as the concentration of Ib-AMP1 increases a greater number of cells were affected. The change in membrane permeation may be

associated with outer membrane damage and cytoplasmic membrane potential ($\Delta\psi$) dissipation caused by Ib-AMP1. Whether the disruption of membrane integrity was the lethal event was not conclusive.

The decrease in the total ATP of treated cells could not be accounted for by the increase of extracellular ATP (Fig. 3). The depletion of intracellular ATP caused by Ib-AMP1 cannot be wholly attributed to cellular ATP efflux and may indicate the hydrolysis of ATP or the reduction of ATP synthesis in *E. coli* O157:H7. This may suggest that the proton motive force was reenergized under a stress condition through utilization of ATP [36-38]. Loss of membrane potential also results in loss of energy source for ATP production. Moreover, the decrease in intracellular ATP may be the result of the positively charged Ib-AMP1 binding to ATP, a negatively charged molecule [39].

Ib-AMP1 inhibited DNA, RNA and protein synthesis of *E. coli* O157:H7. Inhibition was rapid for DNA and RNA, occurring within 5 minutes of exposure. The inhibition may be either direct or indirect since disruption of membrane integrity impairs cell homeostasis, which may further inhibit macromolecular synthesis. However, Ib-AMP1 is highly positively charged (no negatively charged residue) and it is not surprising that it has affinity to bind negatively charged nucleotide chains, interrupting their synthesis. Ib-AMP1 showed a greater effect on RNA synthesis. The results may also suggest that the inhibition of DNA and RNA synthesis in cells treated with 25 µg/mL Ib-AMP1 may not be lethal to the cell since at 5 and 20 min cells remained viable, even though 23 % - 86 % inhibition of DNA and RNA occurred (Fig. 5). Since Ib-AMP1 affects cell membrane

integrity and loss of membrane integrity may cause disruption of normal metabolism, the effect of Ib-AMP1 on macromolecular synthesis may be overestimated. Therefore, Ib-AMP1 at a sublethal concentration may be the most representative for evaluating the effect on macromolecular synthesis. Ib-AMP1 at 25 μg/mL (half MIC) resulted in minimal disruption of cell membrane integrity (Fig. 1, 2 and 3) and decrease in cell number while inhibiting RNA, DNA, and protein synthesis by 90.91 %, 64.56 % and 39.80 %, respectively. Therefore, we concluded that at sublethal concentrations, the inhibition of cell function by Ib-AMP1 is associated with inhibition of RNA, DNA and protein synthesis rather than cell membrane disruption.

Wang et al. [17] showed that Ib-AMP1 at the MIC concentration failed to dissipate *S. aureus* membrane potential. The study also showed that there was no leakage of negatively charged bacterial membrane-mimicking lipid vesicles exposed to the MIC level of Ib-AMP1. However, our study showed that Ib-AMP1 resulted in membrane permeation in *E. coli* O157:H7. This may suggest that Ib-AMP1 targets specific proteins or cell surface components on the *E. coli* O157:H7 cell membrane, such as porins. With affinity binding to the specific components on the outer membrane, Ib-AMP1 permeabilized the Gram-negative cell membrane. Studies have shown that porin-deficient bacteria are more resistant to antimicrobial agents. A *Mycobacterium smegmatis* mutant deficient in a major porin, MspA, exhibited a higher MIC than the wild type [40]. The antimicrobial activity of the quinolone, KB-5246, against *E. coli* was shown to require the porin, OmpF [41]. In the study conducted by Wang et al. [17], both *S. aureus* and the artificial lipid vesicles were devoid of outer membrane. The

results also demonstrate that Ib-AMP1 may exhibit different antibacterial effects on Gram-positive and Gram-negative bacterial membranes. In the present study, activity of Ib-AMP1 was evaluated rather than Ib-AMP2 or Ib-AMP3 since it showed greater antifungal and antibacterial activity in previous studies [16].

Gram-negative bacteria compared to Gram-positive bacteria are more resistant to antimicrobial agents due to the presence of an outer membrane. Three uptake pathways have been proposed: hydrophilic-uptake pathway, hydrophobic-uptake pathway and self-promoted pathway [42-44]. The hydrophilic-uptake pathway involves the uptake of hydrophilic antimicrobial agents through porins to cross the outer membrane of Gram-negative bacteria. Ib-AMP1 exhibited a greater potential to cross the outer membrane through this pathway. Structural analysis conducted by Patel et al. [18] indicated that Ib-AMP1 exhibits a β-turn structure, which results in hydrophilic regions at two sides and hydrophobic region in the middle. The hydrophilic region may be the interaction site of Ib-AMP1 that initially makes contact with the bacterial cell membrane. This may explain why the negative charge of the hydrophilic region of Ib-AMP1 prevents the disruption of negatively charged liposomes and the Gram-positive bacteria cell membrane, which contains negatively charged cell surface moieties, including teichoic acid and lipoteichoic acid [17].

Ib-AMP1 caused more extensive outer membrane damage than EDTA, based on greater NPN uptake by Ib-AMP1 treated cells compared to EDTA treated cells. The EDTA

concentrations used in the present study were in the range used in other studies and resulted in comparable levels of NPN uptake [45, 46]. The results may suggest that Ib-AMP1 and EDTA exert different modes of action on the *E. coli* O157:H7 outer membrane. EDTA binds to divalent cations that are required to stabilize the outer membrane structure, therefore, resulting in destabilizing outer membrane. Studies showed that EDTA released up to 40 % of LPS from outer membrane [46-49]. The results may further support our speculation that Ib-AMP1 may affect *E. coli* O157:H7 outer membrane through hydrophilic-uptake pathway, possibly affinity binding to specific sites on outer membrane, rather than self-promoted pathway which involves in the destabilization of LPS. An all D-form of Ib-AMP1 may be helpful to determine the whether the interaction with *E. coli* O157:H7 is stereo-specific and a specific site is involved.

Research in our laboratory suggests that the sublethal concentration of Ib-AMP1, 25 µg/mL (½X MIC), has limited effect on cell viability, but appears to affect cell morphology and metabolism (unpublished data). In this study, the sublethal concentration of Ib-AMP1 damaged the *E. coli* O157:H7 outer membrane, had little or no effect on dissipation of the cytoplasmic membrane potential, and affected predominantly intracellular macromolecular synthesis. The results may suggest that at the sublethal concentration Ib-AMP1 dispersed on the cell surface causing greater outer membrane damage due to the affinity binding to certain outer membrane moieties. Ib-AMP1then traversed or caused small pores in the cytoplasmic membrane based on the low percentage of permeable cells after treatment and limited efflux of ATP. Once Ib-AMP1 entered the cytosol DNA, RNA, and protein synthesis was affected through an

unknown mechanism(s). Ib-AMP1 at lethal concentrations, 1X and 2X MIC, caused small and large pores in the *E. coli* O157:H7 cell membrane which resulted in complete dissipation of cytoplasmic membrane potential. It then directly or indirectly affected DNA, RNA, and protein synthesis. The threshold concentration for influencing macromolecular synthesis may have been reached since Ib-AMP1 at 1X and 2X MIC showed a similar degree of inhibition. The higher concentration (2X MIC) of Ib-AMP1 caused formation of larger pores since greater ATP efflux occurred compared to cells treated 1X MIC. The results may suggest that Ib-AMP1, at different concentrations, exhibit different modes of action on *E. coli* O157:H7. At a sublethal concentration Ib-AMP1 may cross the cell membrane through according to the aggregate or toroidal pore model; both models explain membrane permeabilization and translocation across cell membranes without damaging cell membrane integrity. In contrast, at a lethal concentration Ib-AMP1 may behave according to the barrel-stave model and carpet model; both models are associated with dissipation of membrane potential and loss of membrane integrity. Collectively, results of the present study suggest that Ib-AMP1 at a sublethal concentration forms transient pores and exerts a greater effect on intracellular components resulting in limit inactivation of cells. At a lethal concentration Ib-AMP1 produced large pores and extensive disruption of cell membranes resulting in cell death.

The present study suggests that Ib-AMP1 is bactericidal to *E. coli* O157:H7 causing membrane disruption, pores in the cell membrane and inhibition of DNA, RNA, and protein synthesis. The research suggests that at sublethal concentrations Ib-AMP1

affects exerts a greater effect at the intracellular level, whereas at a lethal concentration it permeabilizes cell membranes and may directly or indirectly inhibits intracellular macromolecule synthesis. Studies were not conducted to determine specific docking molecules. The present study provides novel observations and insights to the antibacterial mode of action of Ib-AMP1 on *E. coli* O157:H7. The results will serve as the basis for determining docking sites and strategies for application of Ib-AMP1 as an antimicrobial agent.

VI. Acknowledgments

The work was supported by a research grant from the Center for Advanced Food Technology, Rutgers, The State University of New Jersey, New Brunswick, New Jersey.

VII. Figures

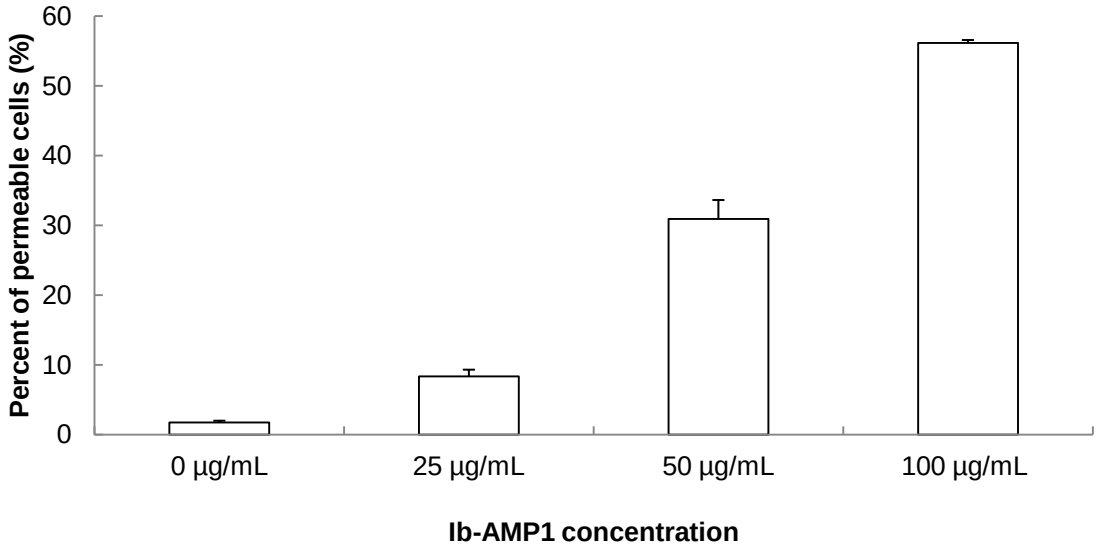

Figure 1. Change in membrane permeability of *E. coli* O157:H7 after Ib-AMP1 treatment. Numbers of permeable and intact cells were counted and percent of permeable cell was calculated according to the following formula: (numbers of permeable cells / numbers of total cells) X 100. Results were presented as average ± standard deviation (STDEV) from two independent experiments. Ib-AMP1 at 0 µg/mL is the untreated cells.

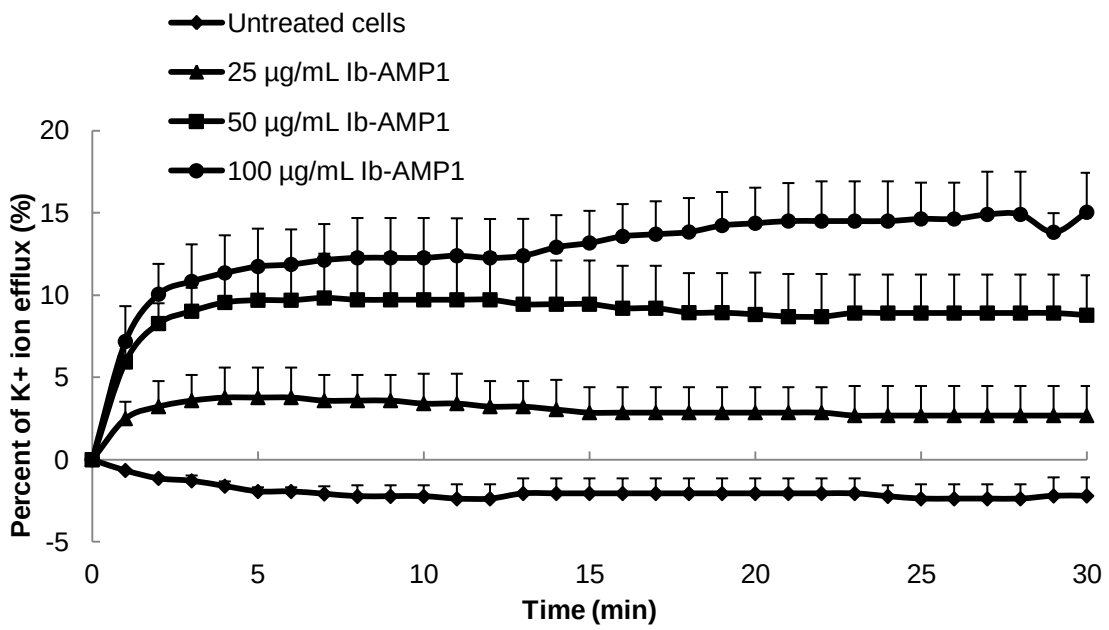

Figure 2. Potassium ion (K^+) efflux (%) from *E. coli* O157:H7 treated with Ib-AMP1. K^+ efflux was calculated using the following formula: (concentration of K^+ efflux of treated cells / total K^+ concentration of untreated cells) X 100. Total K^+ concentration was based on cells treated with 50 % DMSO. Data presented as average ± STDEV from three independent experiments.

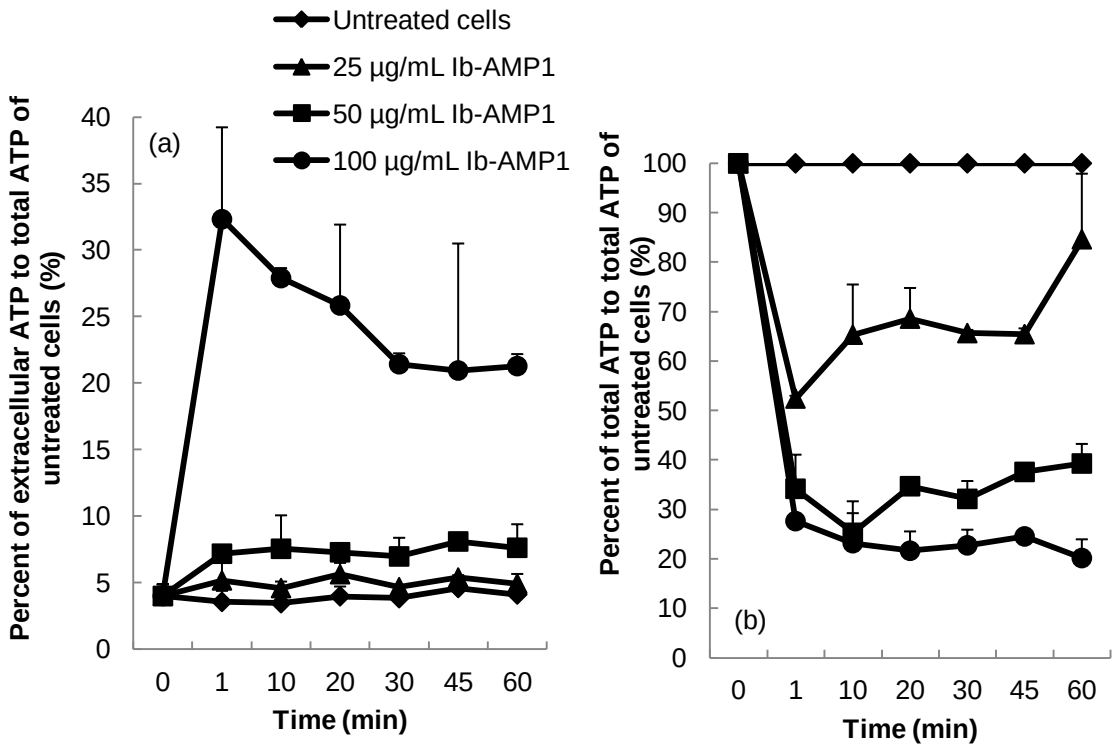

Figure 3. Change in extracellular and total ATP concentrations following treatment of *E. coli* O157:H7 with Ib-AMP1. (a) Percent of extracellular ATP concentration. (b) Percent of extracellular total concentration. Data was presented as average ± STDEV. The total ATP concentration of untreated cells was considered as 100 % which was used to calculate the percentage of both total and extracellular ATP concentration for all treatments.

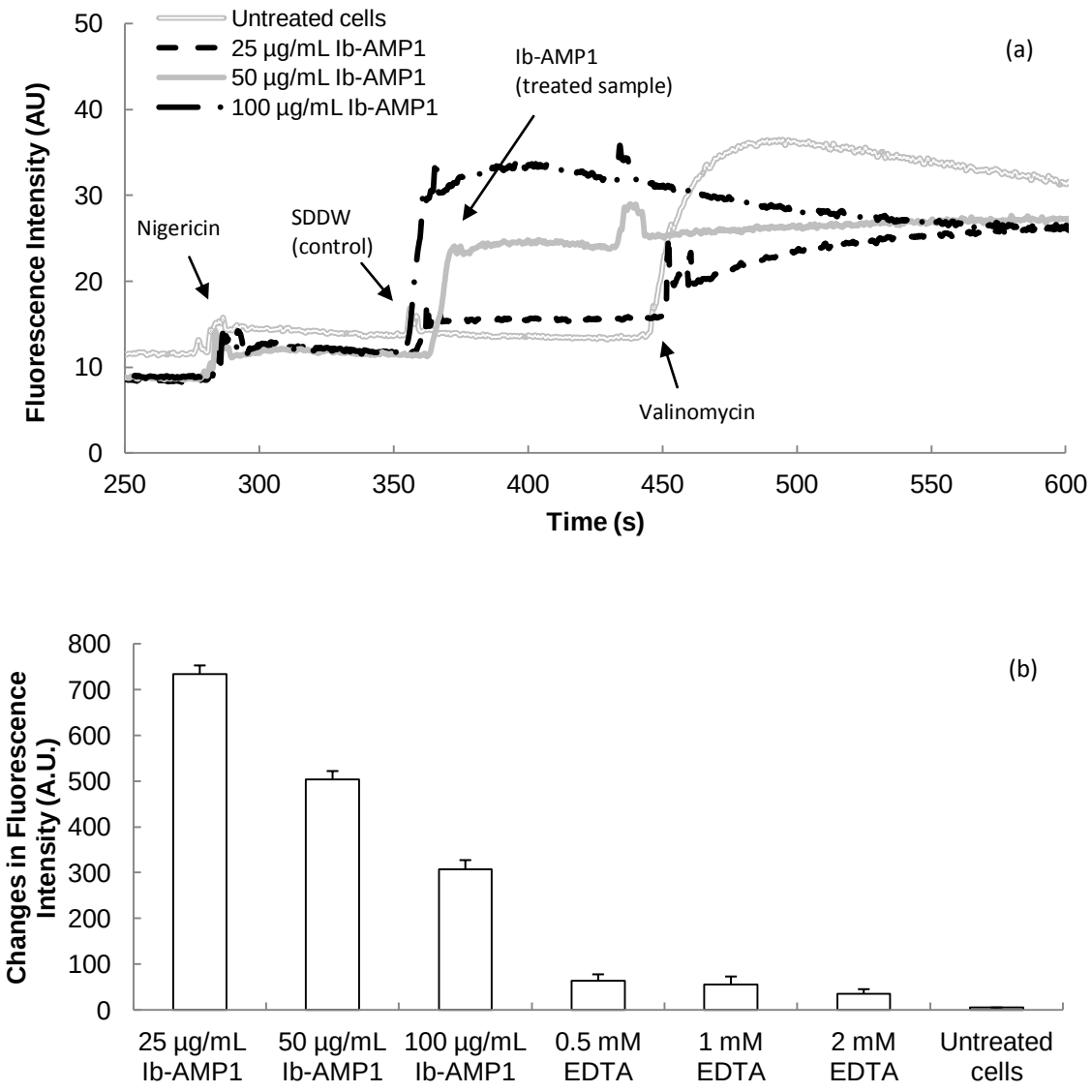

Figure 4. Effect of Ib-AMP1 on (a) dissipation of cytoplasmic membrane potential (Δψ) and (b) outer membrane permeability of *E. coli* O157:H7. (a) Nigercin was added around 280 s to convert ΔpH to Δψ. Ib-AMP1 and SDDW (as the untreated cells) was added around 350 s. Valinomycin was then added around 450 s to dissipate any remaining membrane potential. (b) Changes of fluorescence intensity represent the fluorescence difference before and after treatments. SDDW was added as the negative control. Untreated cells were included to ensure no interaction between cells and NPN.

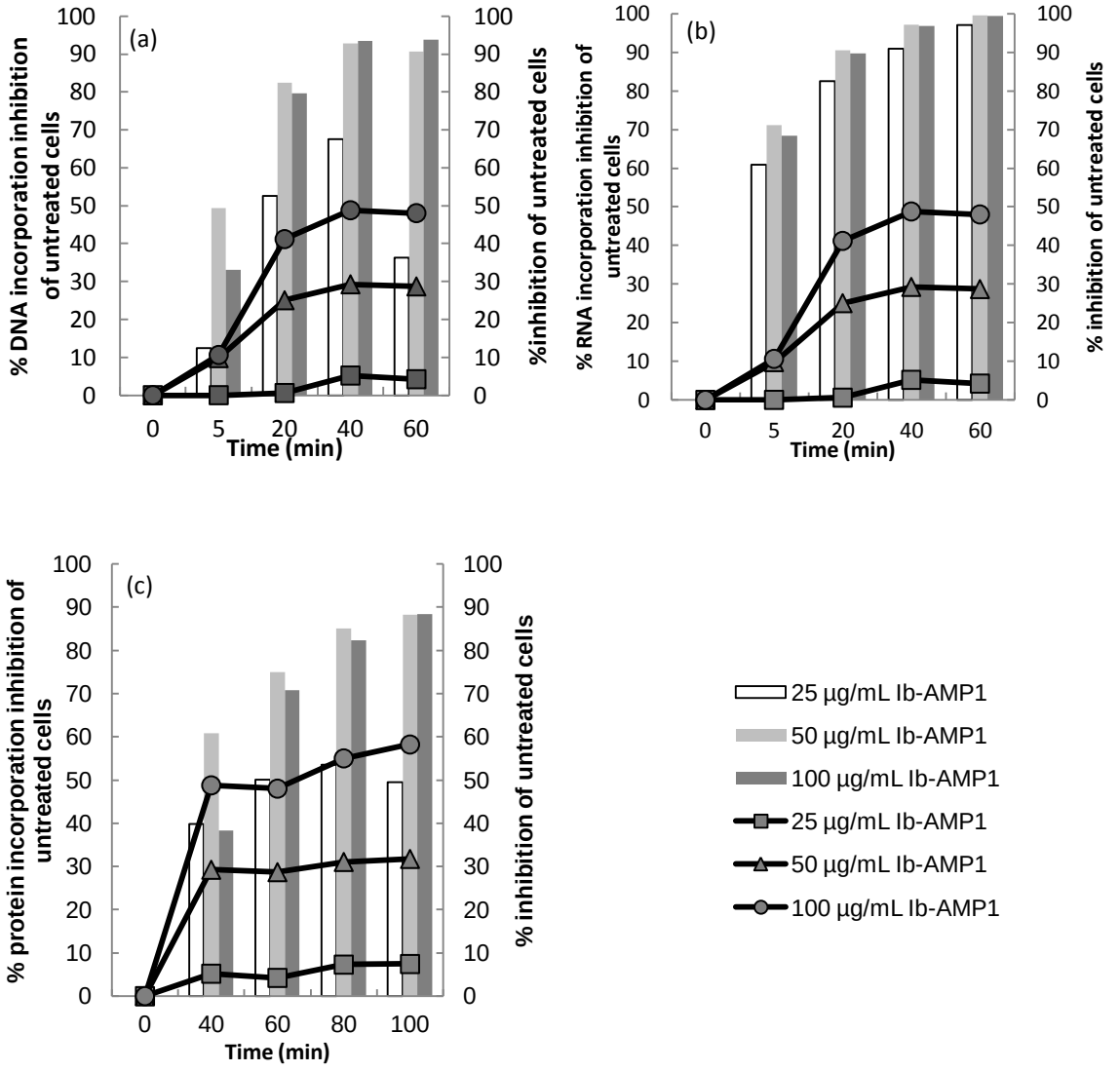

Figure 5. The effect of Ib-AMP1 on intracellular (a) DNA, (b) RNA and (c) protein synthesis in *E. coli* O157:H7. The bar graph represents the percent inhibition of macromolecular precursor incorporation in *E. coli* O157:H7 treated by Ib-AMP1. The percentage was calculated based on untreated cells, which was considered 0% inhibition. The line graph represents the percent inhibition of *E. coli* O157:H7 viable cells treated by Ib-AMP1. The untreated cells were considered 0% inhibition.

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CHAPTER 6

COMPREHENSIVE DISCUSSION AND CONCLUSIONS

I. Discussion

The aim of the present study was to investigate the antibacterial mechanism and properties of Ib-AMP1 on *E. coli* O157:H7, a foodborne pathogen that has been continuously linked to foodborne outbreaks. The studies were designed to provide a general understanding on the antibacterial effect rather than identify a specific ligand-receptor mechanism. The goal was to provide novel information of antibacterial mode of action of a pAMP and to determine its efficacy in controlling the foodborne pathogen, *E. coli* O157:H7. The results serve as the basis for determining docking sites, strategies for application of Ib-AMP1 as an antimicrobial, and future novel drug development. Desalted synthetic Ib-AMP1 was used since formation of disulfide bridges is not required for activity, and the synthetic form demonstrated a greater range of antimicrobial activity (Tailor et al, 1997; Wang et al., 2009).

Three concentrations of Ib-AMP1 ½X (25 µg/mL), 1X (50 µg/mL) and 2X MIC (100 µg/mL) were tested to understanding the antibacterial effect with respect to peptide concentration. The first part of the dissertation discusses the antibacterial effect of Ib-AMP1 against *E. coli* O157:H7. The initial screening demonstrated that Ib-AMP1 was bactericidal to *E. coli* O157:H7, *S.* Newport, *Staph. aureus*, *B. cereus* and *P. aeruginosa*.

The MICs were 50 - 200 µg/mL and MBCs ranged from 1-4 fold higher than the corresponding MIC; greatest activity was against *E. coli* and *Staph. aureus*. The results agree with previous studies that Ib-AMP1 is a broad spectrum antibacterial peptide (Tailor et al., 1997; Wang et al., 2009). The MICs were comparable to other conventional antibiotics and Nisaplin (a crude commercial nisin) indicating the potential suitability as a commercial antibacterial agent (CLSI, 2003).

The bactericidal activity against *E. coli* O157:H7 was affected by, but not limited to, peptide and cell concentration. Three cell concentrations (10^3, 10^6 and 10^9 CFU/mL) were included to represent a range of cell concentrations that may be associated with a product. Ib-AMP1 at lethal concentrations (1X and 2X MIC) showed a maximum 2.68 log reduction in viable cell numbers and was able to control *E. coli* O157:H7 growth when initial cell numbers were less than 10^6 CFU/mL; however, it failed to prevent outgrowth when cell numbers reached 10^9 CFU/mL. Ib-AMP1 at sublethal concentrations did not cause cell death. The results may suggest that Ib-AMP1 at 2X MIC is suitable for as a food preservative in food products that contain medium to low levels of commensal bacteria. However, food commodities such as produce that typically contain high levels of commensal bacterial may require the use of Ib-AMP1at concentrations several fold greater than the MIC; safety issue will have to be considered. The cytotoxicity of Ib-AMP1 against human small intestine (FHs 74 Int), colon (HT-29) and liver (Hep G2) cells showed that greater than 20% inhibition of cell proliferation occurred when the Ib-AMP1 concentration was greater than 200 µg/mL. Greater than 800 µg Ib-AMP1 /mL were required for 80% inhibition of cell proliferation.

The Ib-AMP1 residues, after treating with *E. coli* O157:H7, showed no antibacterial activity. The activity of Ib-AMP1 may have been neutralized once interacting with the bacterial cell, similar to other antimicrobial agents. The results may suggest the possibility as therapeutic agent, since there will be no or little toxicity to human cells after reaction with target bacteria. However, it may be not beneficial to use as a food preservative in food products having a long shelf-life and that require a long term antibacterial effect. Ib-AMP1 may have potential application in active packaging systems by sustaining release and therefore antibacterial activity during a prolonged period. Additionally, Ib-AMP1 could be part of a hurdle technology approach to control undesired microbial growth.

The second part of the dissertation focuses on the antibacterial mode of action against *E. coli* O157:H7. The results indicated that the antibacterial effect of Ib-AMP1 showed a concentration dependent effect on permeation of the *E. coli* O157H7 cell membrane, number of permeable cells in a population, and efflux of K^+ and ATP. Ib-AMP1 at 2X MIC resulted in 56.2 % permeable cells, 15 % of K^+ efflux and 21.4 % ATP efflux after 30 min. Cell numbers in these assays were around 10^8 - 10^9 CFU/mL. The permeation of the *E. coli* cell membrane resulted in large pore formation and it may be associated with damage to the outer membrane and dissipation of cytoplasmic membrane potential. Ib-AMP1 also showed inhibition of DNA, RNA, and protein synthesis; the inhibition may be direct due to affinity binding or indirect due to disruption of the cell membrane. AMPs, such as tachyplesin I was shown to bind DNA, and buforin II has been reported to bind to RNA (Yonezawa et al., 1992; Park et al., 1998).

In the present study, we observed that the decrease in the total ATP of treated cells could not be accounted for by the increase of extracellular ATP. The depletion of intracellular ATP caused by Ib-AMP1 cannot be wholly attributed to cellular ATP efflux and may indicate the hydrolysis of ATP or the reduction of ATP synthesis in *E. coli* O157:H7. This may suggest that the proton motive force was dissipated and had to reenergize under stress conditions through utilization of ATP (Guihard et al., 1993; Ultee et al., 1999; Pol et al., 2002). Loss of membrane potential also results in loss of energy source for ATP production. Moreover, the decrease in intracellular ATP may also be the result of the positively charged Ib-AMP1 binding to ATP, a negatively charged molecule (Hilpert et al., 2010).

E. coli O157:H7 cells exposed to Ib-AMP1 at a sublethal concentration (½X MIC) exhibited longer lag times and higher OD_{630nm} after 16 h incubation. Appendini & Hotchkiss (1999) suggested that the increase in optical density may be due to plasmolysis of *E. coli* cells. Plasmolysis is associated with potassium leakage (Cabral, 1990). The mode of action study showed that Ib-AMP1 caused efflux of potassium ions further suggesting that the higher optical density may be associated with plasmolysis. Moreover, Subbalakshmi & Sitaram (1998) suggested that after treating with indolicidin, an AMP from cytoplasmic granules of bovine neutrophils, the increase of OD_{550nm} of *E. coli* cells was associated with cell elongation and filamentation. The author further suggested that the filamentation may have resulted from the inhibition of DNA synthesis (Lutkenhaus, 1990). The mode of action study also demonstrated that Ib-AMP1 at sublethal concentration (½X MIC) inhibited DNA synthesis based on decrease in

the incorporation of tritium-labeled thymidine. Altogether, the sublethal concentration of Ib-AMP1 may not affect cell proliferation in terms of viable cell number, but may affect cell morphology and metabolism.

The mode of action results may suggest that Ib-AMP1 permeabilizes the *E. coli* O157:H7 outer membrane though a hydrophilic uptake pathway which involves the uptake of hydrophilic antimicrobial agents through porins to cross the outer membrane of Gram-negative bacteria. The structural analysis conducted by Patel et al. (1998) indicated that Ib-AMP1 exhibits a β-turn structure which results in hydrophilic regions at two sides and hydrophobic region in the middle. The hydrophilic region may be the interaction site of Ib-AMP1 that initially makes contact with the bacterial cell membrane. This may explain why the negative charge of the hydrophilic region of Ib-AMP1 prevents the disruption of negatively charged liposomes and the Gram-positive bacteria cell membrane which contains negatively charged cell surface moieties, including teichoic acid and lipoteichoic acid (Wang et al., 2009). The speculation may also be supported by our results for the NPN uptake assay. Ib-AMP1 caused greater outer membrane damage than EDTA, based on higher NPN uptake in Ib-AMP1 treated cells than in EDTA treated cells. The EDTA concentrations tested were commonly used in other studies which resulted in NPN uptake (Alakomi et al., 2000; Helander et al., 2001). The results suggest that Ib-AMP1 and EDTA exert different mode of action on the *E. coli* O157:H7 outer membrane. EDTA binds to divalent cations, which are required to stabilize the outer membrane structure, resulting in destabilization of the outer membrane. Studies showed that EDTA released up to 40 % of LPS from the outer membrane (Leive, 1965 &

1974; Hukari et al., 1986; Alakomi et al., 2000). The results suggest that Ib-AMP1 may affect the *E. coli* O157:H7 outer membrane through the hydrophilic-uptake pathway, possibly through affinity binding to specific sites on outer membrane, rather than a self-promoted pathway which involves the destabilization of LPS as EDTA. An all D-form of Ib-AMP1 may be helpful to determine the whether the interaction with *E. coli* O157:H7 is stereo-specific and if a specific site is involved. Our results along with the study conducted by Wang et al. (2009) suggest that Ib-AMP1 exerts a different mode of action against Gram-positive and Gram-negative.

II. Conclusions

Taken together, Ib-AMP1 at sublethal concentration (½X MIC, 25 μg/mL) may not affect cell proliferation in terms of viable cell numbers, but may affect cell morphology and metabolism. In the present study, a sublethal concentration of Ib-AMP1 resulted in greater disruption to the *E. coli* O157:H7 outer membrane, but exhibited little or no effect on dissipation of the cytoplasmic membrane potential. However, Ib-AMP1 at half MIC had a substantial affect on the intracellular macromolecular synthesis. The results suggest that, at a sublethal concentration, Ib-AMP1 dispersed on the cell surface which resulted in more extensive membrane damage, due to the affinity binding to certain outer membrane components. Results suggest that it traversed through or caused small pores in the cytoplasmic membrane based on the low percentage of permeable cells after treatment and minimal ATP efflux. It subsequently entered the cytosol and

affected DNA, RNA, and protein synthesis through unknown mechanism(s). Ib-AMP1 at lethal concentrations, 1X and 2X MIC, caused small and large pores in the *E. coli* O157:H7 cell membrane which resulted in complete dissipation of cytoplasmic membrane potential. It then directly or indirectly affected DNA, RNA and protein synthesis. The threshold concentration for macromolecular synthesis may have been reached, since Ib-AMP1 at 1X and 2X MIC showed a similar degree of inhibition. The higher concentration (2X MIC) caused larger pore formation since greater ATP efflux occurred in cells treated with 2XMIC Ib-AMP1 than with 1X MIC Ib-AMP1. The results suggest that Ib-AMP1, at different concentrations, showed different mode of actions against *E. coli* O157:H7. At sublethal concentrations, Ib-AMP1 may cross the cell membrane through the aggregate or toroidal pore model; both models explain membrane permeabilization and translocation across the cell membrane without damaging cell membrane integrity. However, at lethal concentrations, Ib-AMP1 may affect the cell membrane based on the barrel-stave model and the carpet model; both models cause dissipation of membrane potential and loss of membrane integrity. Altogether, the present study demonstrated that Ib-AMP1 at sublethal concentrations predominantly affects intracellular processes; however, these reactions may not be lethal or only a low number of cells were affected or inactivated. At lethal concentrations, Ib-AMP1 caused formation of large pores which may subsequently affect intracellular component(s) synthesis and eventually resulted in cell death.

III. Future studies

Now that antibacterial properties and mode of action of Ib-AMP1s have been clarified, future research will focus on evaluating potential synergies with other antimicrobials. Model studies must be conducted in food systems to determine efficacy and effect on organoleptic properties. Intelligent design and formulation of a novel and safe product containing Ib-AMP1 for the use in food and food systems that improve microbial safety of those products can be developed.

The application of AMPs is diverse, however, only limited AMPs are now available and approved by governmental agencies for commercial use. The major reason is cost for industrial-scale production and the uncertainty in terms of safety. More research is required to overcome these limitations.

Finally, a standard method that is suitable to screen the mode of action of varied AMPs should be developed. The current inherit problem of the mode of action studies is the different cell numbers used in each assay which may make the comparison difficult even among assays. Around 10^5 CFU/mL cells is usually used to determine MIC and MBC; however other assays, such as K^+ and ATP efflux, may require a higher cell number in order to show effect or difference.

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APPENDIX

The following appendix is the report of the current progress of the study on the development of E. coli expression system for snakin-1 production.

Wen-Hsuan Wu, Karl R. Matthews and Rong Di. Development of an *Escherichia coli* Expression System for Snakin-1 Production

Objectives:

I. To develop an E. coli expression with proper bacterial host and vector to produce snakin-1
II. To purify and concentrate recombinant snakin-1
III. To test the antibacterial activity of purified recombinant snakin-1

Development of an *Escherichia coli* Expression System for Snakin-1 Production

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Key Words:

E. coli expression, snakin-1,

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I. Abstract

Industrial-scale production of potent antimicrobial agents is one of the obstacles that impede the subsequent application and implementation. The cost for solid phase chemical synthesis of peptide is still expensive and can be technically difficult if the peptide is large in size or requires post-translational modification. Direct extraction of target antimicrobial agents from the producing host usually is time consuming and results in low yield. Alternative approaches are being studied aiming to lower the production cost and achieve higher yield. Snakin-1 is a plant antimicrobial peptide from *Solanum tuberosum*. It has been shown to inhibit plant and foodborne pathogens at the micromolar level. The purpose of the present study was a proof of concept that we can produce at a large scale snakin-1 using *Escherichia coli* as a bacterial host. The objective was to develop an *E. coli* expression system that allows production of bio-active snakin-1 in a cost-effective way. Two constructs were developed, designated RD21 and RD74. RD21 is snakin-1 cloned into pET32a and transformed into Rosetta-gami B (RGB) cells; RD74 is snakin-1 cloned into pJexpress and transformed into BL21 (DE3) pLysS. The final purified RD21 resulted in sankin-1 with no additional tags and RD74 resulted in snakin-1 carrying six Histidine tags at the C-terminus. RD21 and RD74 were purified from the soluble fractions of bacterial cells. The antimicrobial activity of RD21 against *Listeria monocytogenes* and RD74 against *L. monocytogenes* and *Salmonella entrica* serovar Newport were determined and results indicated that we failed to produce bio-active snakin-1 based on the conditions evaluated. Future analysis to determine the correct

folding of snakin-1 containing six disulfide bridges is required to facilitate the production and purification.

II. Introduction

Antimicrobial peptides (AMPs) are a group of small proteins that possess the ability to inhibit a broad spectrum of microorganism including bacteria, fungi, parasites and viruses (van't Hof et al., 2001; Wang and Wang, 2004; Barbosa Pelegrini et al., 2011). Literally, every single organism evaluated produces AMPs which give the diversity to AMPs. Most AMPs are positively charged and amphipathic which may render stronger affinity to microbial cell membrane than to mammalian cell membranes (Matsuzaki, Sugishita, Fujii, & Miyajima, 1995; Matsuzaki, 2009). Due to these properties, microorganisms are less prone to develop resistance compared to conventional antibiotics; therefore, AMPs are promising alternatives to antibiotics and can inactive multidrug resistant pathogens. Mass production of AMPs remains one of the crucial factors for the future application of any efficient AMPs.

Isolation and purification of plant AMPs (pAMPs) directly from the corresponding plant tissue usually result in low peptide yield. Mammalian AMPs are usually produced by chemical synthesis. However, chemical synthesis will not be cost-effective for peptides larger than 30 amino acid residues or peptide that requires other post-translational modification. Bacteriocins, antimicrobial peptides produced from bacteria, are relatively easy to mass produce, since bacterial replication time is relatively short. Utilization of *E. coli* as a bacterial host to produce eukaryotic peptides has been developed. A plasmid encoding the antimicrobial peptides is transformed into an *E. coli* host and the antimicrobial peptides are then isolated from the bacterial culture.

Plasmid systems, such as the pET and pQE systems have been well developed, in which sets of fusion tags, such as HIS-tag and S-tag, are designed to facilitate purification or expression. However, its yield is not robust enough for commercial scale production to supply quantities required for use in food systems.

Snakin-1 is a plant antimicrobial peptide isolated from potato tuber (*Solanum tuberosum*) (Segura et al., 1999). Nahirñak et al. (2012) have demonstrated that snakin-1 plays roles in plant growth, such as cell division, cell wall composition, and leaf metabolism. Using *Arabdopsis* as a model plant, Almasia et al. (2010) concluded that snakin-1 is induced by temperature and wounding. It has been shown that snakin-1 is active against plant pathogens both *in vitro* and *in vivo* (Segura et al., 1999; Almasia et al., 2008). Collectively, these studies demonstrated that snakin-1 plays roles in protection and normal growth of potato plant. Utilization of snakin-1 as an antimicrobial treatment on plants to control plant disease or as an antimicrobial agent in food systems to control food spoilage and foodborne pathogen contamination may decrease the production loss due to plant disease, and ensure the safety of food products. However, studies on snakin-1 are limited to its application in plant disease control, especially in transgenic plant over-expressing snakin-1. Its application in other areas, such as an antibiotic alternative, food preservative and sanitizer, has not been completed. Cytotoxic and hemolytic activity has not been studied.

Snakin-1 is a 63-mer peptide possessing 12 cysteine residues to form six disulfide bridges. The amino acid sequence from N-terminus to C-terminus is

GSNF**C**DSK**C**KLR**C**SKAGLADR**C**LKY**C**GV**CC**EE**CKC**VPSGTYGNKHE**C**P**C**YRDKKNSKGKSK**C**P. Previous studies isolated natural snakin-1 from potato tuber; however, the yield was low. According to Segura et al. (1999) one kilogram of potato tube produced 2.08 g of snakin-1. The purification was time-consuming. Other approaches to mass produce snakin-1 are required, especially for the cys-rich small peptide. As mentioned previously, the relative large size and presence of many disulfide bonds make snakin-1 difficult and not cost-efficient to chemically synthesize. An *E. coli* expression system may be a potential means to produce snakin-1.

The purpose of the present study was a proof of concept that we can mass produce snakin-1 using *E. coli* as a bacterial host. The objective is to develop an *E. coli* expression system that allows production of snakin-1 in a cost-effective way.

III. Materials and Methods

A. Cloning of snakin-1 cDNA and expression of snakin-1 in *E. coli* cells

Two constructs were developed, designated pRD21 and pRD74. For pRD21, cDNA encoding snakin-1 (*sn1*) was synthesized (table 1.), cloned into pUC57 vector by GenScript, Piscataway, NJ. It was then transformed into *E. coli* DH5α. Plasmids from DH5α cells containing pUC57+sn1 were extracted using a commercial plasmid purification kit, respectively, and then digested at *NcoI* and *HindIII* restriction sites. The extract was run on a 2 % agarose gel at 100 V and the bands with corresponding size of sn1 were cut and extracted using GeneJET gel extraction kit (Thermo Fisher Scientific,

Waltham, MA). The resulting snakin-1 fragment was then cloned into pET32a vector (Novagen) at *NcoI* and *HindIII* sites using T4 ligase, and transformed into to *E. coli* Rosetta-gami B (RGB) competent cells (Novagen, Billerica, MA), which enhances both the expression of eukaryotic protein and the formation of target protein disulfide bonds. This resulted in recombinant RD21 as Trx-6xHIS-S-rEK-SN1. Thioredoxin-tag (Trx-tag), S-tag (S protein), HIS-tag (6xHIS) and enterokinase (rEK) were used to increase the expression of cysteine-rich proteins and facilitate purification and detection.

For pRD74, cDNA encoding snakin-1 with six histidine codons at the 3'-end was synthesized (table 1.), cloned into pJexpress 401 bacterial expression vector by DNA2.0., Inc. (Fig.1B; Menlo Park, CA, USA), resulting in pRD74 which was then transformed into BL21(DE3) pLysS bacterial cells, which helps to stabilize the expression of toxic target protein. This resulted in recombinant RD74 as SN1-6xHIS. PCR was used to confirm the correct size of fragments of both construct cassettes and both purified plasmids were sent out for sequencing to confirm the correct nucleotide sequences.

B. Target peptide induction

RGB cells harboring pRD21 was grown in Luria-Bertani broth (LB) containing tetracycline, chloramphenicol, kanamycin and ampicillin at final concentrations of 12.5, 34, 50 and 100 µg/mL, respectively at 37 ˚C with agitation at 200 rpm till OD_{600nm} reaches 0.6. Isopropyl-beta-D-thiogalactopyranoside (IPTG; Growcells, Irvine, CA) was added at final concentration of 1 mM and the culture was incubated at 37 ˚C for 4 h with agitation at

200 rpm to induce the expression of RD21. Cells were collected by centrifugation at 3,500 rpm for 15 min, and kept -20 ˚C until further use for purification.

BL21(DE3) pLysS cells harboring pRD74 was grown in LB containing kanamycin and chloramphenicol at final concentration of 50 and 34 μg/mL, respectively, at 37 ˚C with agitation at 200 rpm till OD_{600nm} reaches 0.6. IPTG was added at a final concentration of 1 mM and the culture was incubated at room temperature for more than 14 h with agitation at 200 rpm to induce the expression of RD74. Cells were collected by centrifugation at 5,000 rpm for 15 min, and kept at -20 ˚C until use for purification.

C. Target protein extraction

For RD21, pellet from one liter culture was collected and resuspended in 5mL HIS-cartridge wash buffer (50 mM NaH_2PO_4, pH 8.0, 0.3 M NaCl and 20 mM imidazole) in order for subsequent HIS-tag affinity chromatography. The cell suspension was passed through a FRENCH press (Thermo Electron Cooperation, Needham Heights, MA) at 1260 psi for 3 times to break up cells. The resulting cell milieu was then centrifuged at 3,500 rpm for 15 min to separate supernatant and pellet. The supernatant as crude cell extract was then purified by HIS-tag affinity chromatography using HIS-select High Flow cartridge (Sigma-Aldrich Corp., St. Louis, MO). A total of two liters of culture were collected and processed.

For RD74, pellet from 100 mL culture was resuspended in 4 mL B-PER cell lysis solution (Thermo Science, Rockford, IL) supplemented with DNaseI (Boehringer Manheim, Pleasanton, CA) at final concentration of 5 U/mL and lysozyme (Sigma-Aldrich Corp., St. Louis, MO) at final concentration of 0.1 mg/mL. The suspension was incubated at RT for 20 min. Sonication was used to assist breaking up of cells. Soluble (supernatant) and insoluble (pellet) proteins were separated by centrifugation at 14,000 rpm for 4 min and kept at -80 ˚C for purification. The soluble fraction (supernatant) was then purified by HIS-tag affinity chromatography by HIS-select Nickel affinity gel (self-packed, Sigma-Aldrich Corp., St. Louis, MO). A total of five liters of culture were collected and processed.

D. Target protein purification

Tagged RD21 (Trx-6xHIS-S-SN1-rEK), in the crude cell extract, was isolated by HIS-select High Flow cartridge (Sigma-Aldrich Corp., St. Louis, MO) to separate tagged RD21 and other cellular components. The pre-packed cartridge contained 1.25 ml of HIS-Select High Flow (HF) Nickel affinity gel. The conditions were according to manufacturer's instruction. The crude cell extract was passed through the cartridge, washed and the tagged RD21 was eluted with 5mL elution buffer (50 mM sodium phosphate pH 8.0, 0.3 M NaCl, 500 mM imidazole). The eluant was dialyzed with S-tag wash buffer (1.5 M NaCl, 200 mM Tris-HCl, 1 % Triton X-100, pH 7.5) for subsequent S-tag/rEK purification using S-tag rEK purification kit (Novagen, Billerica, MA). The kit includes S-tag affinity

resin (0.5 mL) to extract S-tagged target protein (resuspend in 1 mL of wash buffer), followed by the rEK treatment to cleave S-tag and rEK from target protein. The rEK (5 units) was further added to remove S-tag and rEK by EKapture agarose (125 µL) which resulted in purified RD21 without tags. The S-tag/rEK purification resulted in approx. 1 mL of purified RD21.

Tagged RD74 (SN1-6xHIS), in the soluble fraction, was purified using HIS-select Nickel affinity gel (Sigma-Aldrich Corp., St. Louis, MO) according to manufacturer's instruction. A column was packed with 2 mL HIS-select Nickel affinity gel and then equilibrated according to manufacturer's instruction. The supernatant (soluble fraction) was passed through the column, washed with wash buffer (50 mM sodium phosphate pH 6.0, 0.3 M NaCl and 20 mM imidazole) and eluted with 20 mL of elution buffer (50 mM sodium phosphate pH 6.0, 0.3 M NaCl and 250 mM imidazole). The column was washed with wash buffer until the A_{280nm} was the same as the wash buffer before elution. Eluant (20 mL) was kept at -80 ˚C before future process. Protein concentration was monitored and measured using a Nanodrop device (Thermo Fisher Scientific, Wilmington, DE).

E. Concentration and dialysis of target protein

Purified RD21 (1 mL) was dialyzed against sterile de-ionized distilled water (SDDW) using Midi Trap G10 (GE Healthcare, Piscataway, NJ) and resulted in 1.2 mL of purified RD21.

RD74 eluant (HIS-tagged purified RD74, 15 mL) was centrifuged at 4,000 x g at RT by a centrifugal filter (EMD Millipore, Billerica, MA**)** with a MWCO at 3 kDa. The eluant was concentrated from 15 mL to 0.5 mL and then dialyzed in another 3 kDa MWCO unit. Fifty milliliter of SDDW was added twice for dialysis to remove salt and imidazole. The purified and concentrated RD74 was aliquoted and kept at 4 ˚C for future analysis.

F. SDS-PAGE and Western Blot

Purified RD21 (SN1) sample and purified RD74 (SN1+6xHIS) samples were visualized by 15 % SDS-PAGE and 16.5 % Tris-Tricin SDS-PAGE with 6M urea, respectively. Tris-tricine SDS-PAGE was performed according to Schagger and von Jagow (1987) to analyze protein in size smaller than 100 kDa. Gels were either stained with Coomassie blue or silver staining.

RD74 was further analyzed by western blot. SDS-PAGE gel was transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) and blotted for 1 h at 100 V. The membrane was then blocked with phosphate buffer saline (PBS; Fisher Scientific, Pittsburg, PA) with 5 % not-fat dry milk at 37 ˚C for 2 h. After blocking, the membrane was probed with primary antibody, anti-HIS mouse antibody (1:2500, GenScript, Piscataway, NJ) in PBS with 5 % not-fat dry milk at RT for more 14 h following by the secondary antibody, anti-mouse rabbit IgG antibody-alkaline phosphotase conjugate (1:5000, Sigma-Aldrich Corp., St. Louis, MO) in PBS with 5 % non-fat dry milk at RT for 1.5 h. Between antibody incubation, the membrane was washed with TBST. The

membrane was then developed using the BCIP/NBT phosphatase substrate system (Sigma-Aldrich Corp., St. Louis, MO) performed according to manufacturers' instruction.

G. Antimicrobial activity

The micro-dilution assay was used to determine the antimicrobial activity of recombinant HIS-tagged snakin-1 (RD21 and RD74) against target bacteria. The assay was conducted according to standards outlined by Clinical and Laboratory Standards Institute (CLSI 2003). The antimicrobial activity of RD21 was tested against *L. monocytogenes* J1-110, and that of RD74 was tested against *L. monocytogenes* J1-110 and *S.* Newport. *L. monocytogenes* J1-110 was cultured in Brain Heart Infusion (BHI) Broth; *S.* Newport was cultured in Muller Hinton Broth (MHB). All cultures were incubated at 37 ˚C with agitation at 200 rpm for more than 18 h. Cells were collected by centrifugation at 4,000 x g for 10 min at 4 ˚C, and resuspended in 5 mL of fresh 10X BHI (for *L. monocytogenes*) or 10X MHB (for *S.* Newport). Inoculum was prepared by making serial tenfold dilution in corresponding 10X medium to approx. 10^5 CFU/mL and used immediately. Purified RD21 and RD74 was filter sterilized by passing through a 0.2 µm filter. Ninety microliter of purified RD21 and RD74 were serial twofold diluted in SDDW in separated 96-well plate and incubated with 10 µL of inoculum. A bacterial growth control and negative controls (medium alone, RD74 alone) were included. Plates were incubated at 37 ˚C in Dynex 96-well plate reader MRX with Revelation software to monitor optical density at λ = 630 nm for 16 h. Inoculum cell numbers were confirmed

by plating aliquots on BHI or Muller Hinton agar plate. The plates were incubated overnight at 37 ˚C, colonies counted, and the cell numbers were expressed as CFU/mL. Antibacterial activity of recombinant HIS-tagged snakin-1 (RD21 and RD74) was determined based on the optical density.

IV. Results

A. Cloning of snakin-1 cDNA and expression of snakin-1 in *E. coli* cells

The cDNA of pRD21 and pRD74 were sent out for sequencing, and the results indicated the correct nucleotide sequence of snakin-1 was present (data not shown). RD74 was designed to optimize tRAN codon to increase target protein expression. The presence of rare mRNA codons impedes transcription and results in low protein expression.

B. Expression of snakin-1 protein in *E. coli* cells

Two constructs, pET32a-SN1 (RD21), pJexpress-SN1-6xHIS (RD74), were designed and transformed into *E. coli* RGB and BL21 (DE3) pPLyS host cells, respectively. Cell cultures were collected at pre-designated time points, and the pellet was collected and analyzed by a 15 % SDS-PAGE and 16.5 % Tris-Tricine SDS-PAGE for RD21 and RD74, respectively (Fig. 2.). Expression of RD21 resulted in a 24.6 kDa snakin-1 including all fusion tags; expression of RD74 resulted in a 7.7 kDa snakin-1 including C-terminal 6xHIS. SDS-PAGE gel showed a clear and sharp band around 25 kDa indicated the success expression of

RD21 (Fig. 2A). The empty vector showed an expression of a 17.4 kDa peptide indicating the basal expression of all fusion tags (data not shown). This further indicated the correct expression of RD21 in RGB cells. For RD74 expression, the 14 h induction at RT resulted in expression of a peptide with size close to 10 kDa (Fig. 2B).

C. Target protein purification and dialysis

RD21 and RD74 were purified in naïve condition. HIS-tagged purified RD21 and RD74 elution fractions were analyzed using 15 % SDS-PAGE and 16.5 % Tris-Trice SDS-PAGE, respectively, to visualize the presence of target RD21 and RD74 (Fig. 3A, 3C). The results indicate the presence of 24.6 kDa HIS-tagged RD21 (Trx-6xHIS-S-rEK-SN1) and 7.7 kDa HIS-tagged RD74 (SN1-6xHIS).

HIS-tagged RD21 eluant was then collected and dialyzed using Midi Trap G-10 with MWCO at 700 Da to change buffer for consequent S-tag purification. SDA-PAGE analysis of dialyzed HIS-tagged RD21 is shown in Figure 3B. The results showed that HIS-tagged RD21 aggregated into di-mer, tri-mer and tetra-mer forms based on bands at 50, 75 and 100 kDa, respectively. The aggregation was not evident after HIS-tag affinity chromatography (before dialysis). A major sharp band was showed on SDS-PAGE after HIS-tagged purification (Fig. 3A). The results indicated dialysis promoted protein folding. The resulting dialyzed HIS-tagged RD21 was then subjected to S-tag/rEK purification to remove S-tag and enterokinase. The final purified RD21 was then dialyzed against SDDW and analyzed by 16 % tris-tricine SDS-PAGE (Fig. 4A). Coomassie blue stained gel

showed a sharp protein band at approximately 15 kDa. The results suggest that the majority of purified RD21 was in the di-mer form, which is 14.2 kDa.

RD74 eluted from HIS-tag affinity chromatography was concentrated using a MWCO 3 kDa centrifugal filter. The >3 kDa fractions were then dialyzed against SDDW and analyzed by 16.5% Tris-tricine SDS-PAGE and western blot (Fig. 4B, C). The results show the majority of RD74 was close to 10 kDa and 20 kDa in size. Pellets of BL21 (DE3) pPyLs cells harboring RD74 showed similar pattern of HIS-tagged RD74.

D. Protein concentration

Protein concentration was determined by Nanodrop. The maximum concentration that resulted in enough of the sample for antimicrobial activity analysis was 1431 µg/mL and 68.76 µg /mL for RD74 and RD21, respectively. The protein yield of RD21 and RD74 was 48 and 176.4 µg/L of culture, respectively. RD74 resulted in 3.68 fold higher in protein yield than RD21.

E. Antimicrobial activity

The antimicrobial activities of the purified RD21 and RD74 were tested against selected foodborne pathogens. RD74 was tested at concentrations from 1431 µg/mL to 11.18 µg /mL using serial two log dilution method. RD21 was tested at concentrations from 68.76 µg /mL to 0.54 µg /mL. The results indicate that RD21 exhibited no antibacterial activity

against *L. monocytogenes* J1-110 and RD74 showed no antimicrobial activity against *L. monocytogenes* J1-110 and *S*. Newport (Fig. 5.).

V. Discussion

Based on results of the present study, under the conditions used, production of bio-active snakin-1, using *E. coli* as expression host was not achieved. The construct of RD21 using pET32a and RBG cells seems to be able to protect snakin-1 and showed better folding based on the protein laddering (Fig. 3B). The result showed evidence of induced expression based on SDS-PAGE (Fig.1); however, the yield of purified recombinant snakin-1 (both RD21 and RD74) was low and no antibacterial activity was detected which may be due to low peptide concentration or the peptide was not in bio-active conformation. Recombinant snakin-1 may have been lost during purification procedures since multiple steps of purification were required in order to remove all the fusion tags. Therefore, a higher level of expression may be needed to compensate for the loss during purification steps. Moreover, a total of six disulfide bonds are formed in sankin-1, the presence of disulfide bonds may affect the antibacterial activity. Unfortunately, the structure of snakin-1 has not been elucidated. Further protein/peptide structure and folding information may be required to better determine correct protein folding.

The other construct, RD74, was designed to increase the peptide yield and minimize the purification steps. Although the yield of RD74 was 3.68 fold greater than that of RD21, it

was still relatively low for industrial-scale production. The yield of RD21 was 0.048 mg/L of culture and that of RD74 was 0.1764 mg/L of culture. The improved expression of recombinant snakin-1 may due to the nucleotide sequence DNA 2.0 design that avoids the rare eukaryotic codons but also due to the fact that fewer purification steps were involved in RD74 purification.

Both the recombinant RD21 and RD74 were low yield and showed no antibacterial activity. For RD74, we observed that it tended to form aggregates which may bury the HIS-tag and were not captured by the HIS-affinity resin. The soluble fractions after HIS-tag purification contained predominantly monomer in which the HIS-tags are exposed and can be purified using the column.

The next step of the present study is to purify RD74 inclusion body since it tends to aggregate. However, a proper solubilization and refolding process will need to be determined. The goal of the solubilization and refolding process will be designed to fit future industrial-scale production. Other expression systems may be studied for recombinant snakin-1 production, since it forms aggregates easily. Studies using recombinant baculovirus systems successfully purified correct folded and bioactive β-defensins which is also a cysteine-rich peptide (Vogel et al., 2004; Galesi et al., 2007).

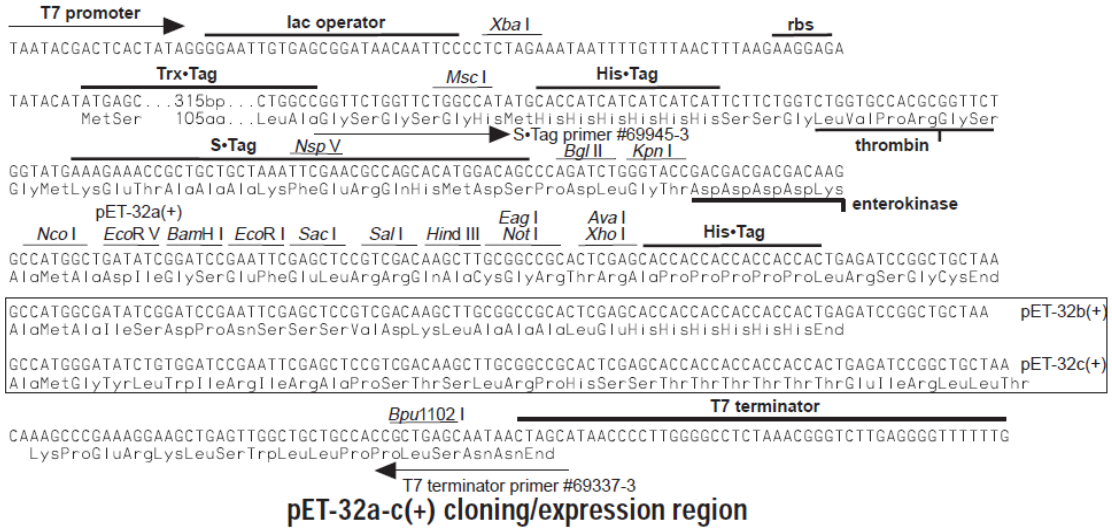
VI. Table and figures

Table 1. Nucleotide sequence of pRD21 and pRD74 cDNA.

RD21	1	ATGGCTGGTTCAAATTTT**TGT**GATTCAAAG**TGC**AAGCTGAGA**TGT**TCAAA
		M A G S N F **C** D S K **C** K L R **C** S K
	51	GGCAGGACTTGCAGACAGA**TGC**TTAAAGTAC**TGT**GGAGTT**TGTTGT**GAAG
		A G L A D R **C** L K Y **C** G V **C** **C** E
	101	AA**TGC**AAA**TGT**GTGCCTTCTGGAACTTATGGTAACAAACATGAA**TGT**CCT
		E **C** K **C** V P S G T Y G N K H E **C** P
	151	**TGT**TATAGGGACAAGAAGAACTCTAAGGGCAAGTCTAAA**TGC**CCTTGA
		C Y R D K K N S K G K S K **C** P *
RD74	1	ATGGGTAGCAACTTC**TGC**GACAGCAAA**TGT**AAACTGAGA**TGC**AGCAAAGC
		M G S N F **C** D S K **C** K L R **C** S K A
	51	GGGCCTGGCGGACCGC**TGT**TTGAAGTAT**TGC**GGTGTT**TGTTGT**GAAGAG**T**
		G L A D R **C** L K Y **C** G V **C** **C** E E
	101	**GC**AAA**TGC**GTGCCGTCCGGTACCTACGGTAATAAGCACGAG**TGT**CCG**TGC**
		C K **C** V P S G T Y G N K H E **C** P **C**
	151	TACCGTGATAAGAAAAACTCTAAGGGCAAGAGCAAA**TGC**CCGCATCACCA
		Y R D K K N S K G K S K **C** P H H H
	201	CCACCATCATTAA
		H H H *

* is the stop codon.

(A)



pET-32a-c(+) cloning/expression region

(B)

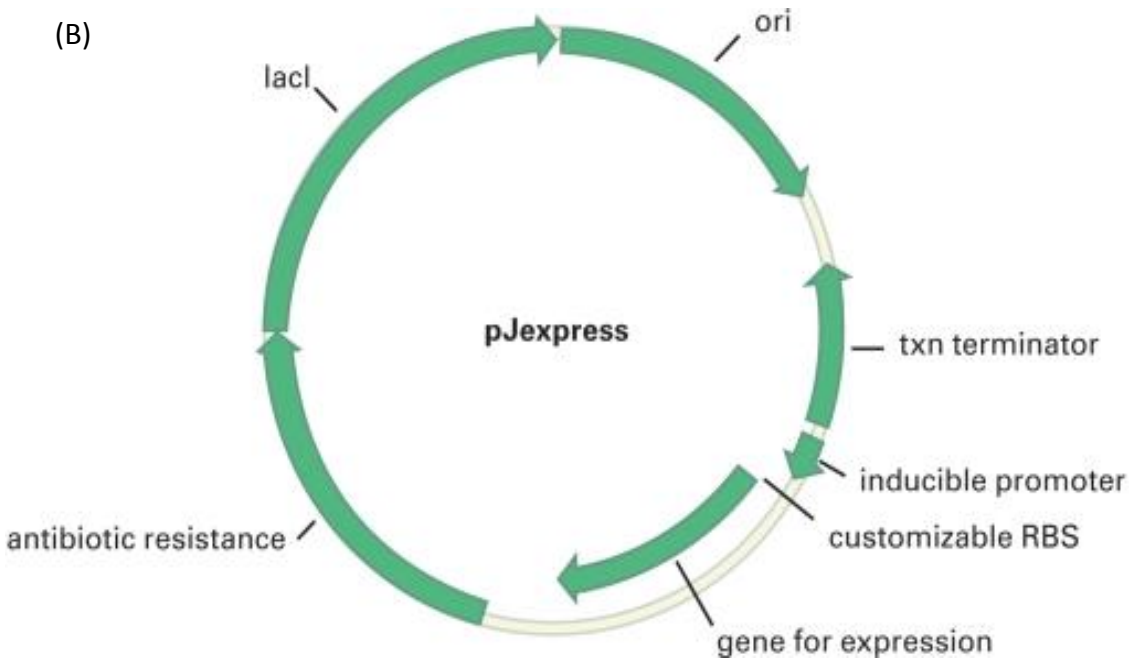


Figure 1. Plasmid map of (A) pET32a and (B) pJexpresss. The illustrations were adopted from Novagen and DNA 2.0, respectively.

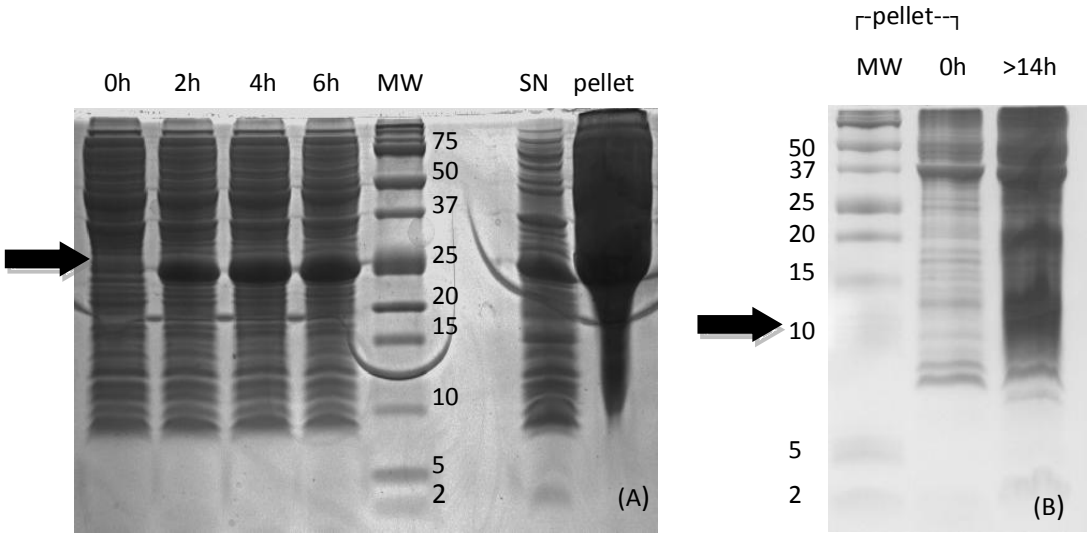


Figure 2. Detection of induction of RD21 and RD74. (A) Pellets from pre-designed time points were analyzed by 15 % SDS-PAGE. (B) Pellets before and after 14 h induction were analyzed by 16.5 % Tris-tricine SDS-PAGE. Gels were coomassie blue stained.

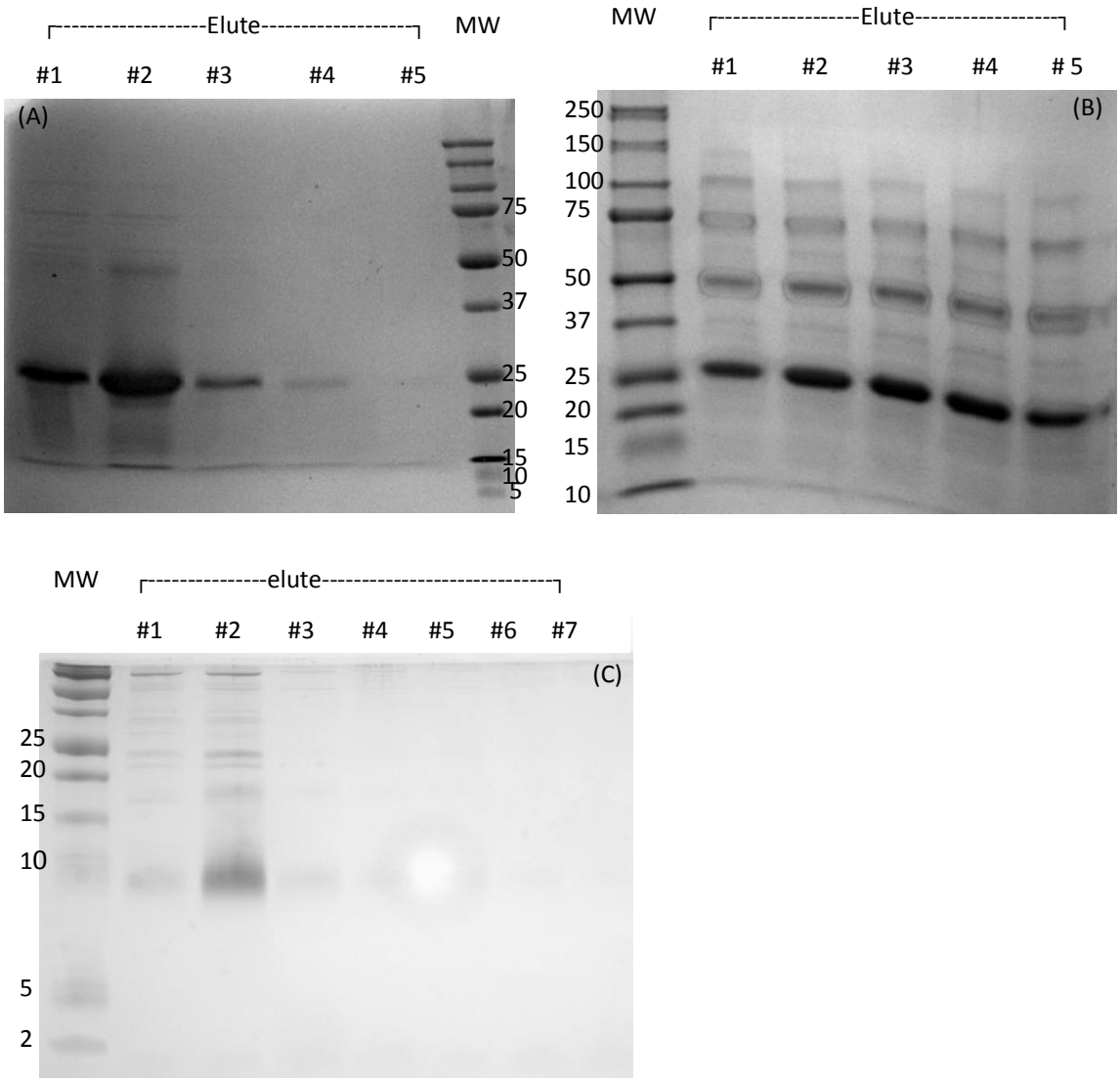


Figure 3.HIS-tag purified RD21, dialyzed HIS-tag purified RD21 and HIS-tag purified RD74. HIS-tag purified RD21 after (A) HIS-tag affinity purification and after (B) subsequent dialysis was analyzed by 15 % SDS-PAGE. (C) HIS-tag purified RD74 was analyzed by 16.5 % Tris-tricine SDS-PAGE. All gels were coomassie blue stained.

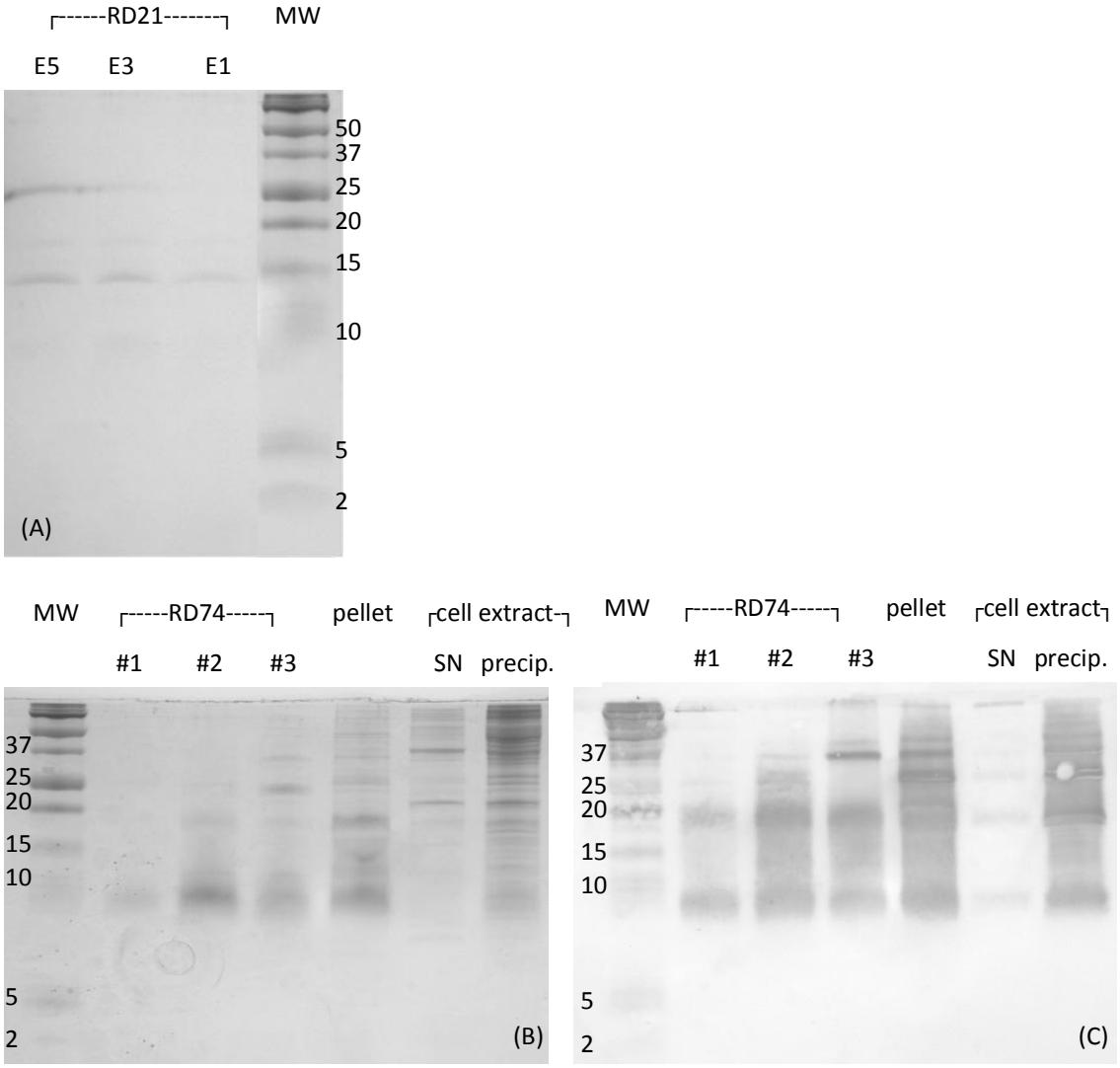


Figure 4. Purified RD21 and RD74. Purified (A) RD21 on 16.5 % Tris-Tricine SDS-PAGE, (B) RD74 on 16.5 % Tris-Tricine SDS-PAGE and (C) RD74 analyzed by Western blot. Gels were coomassie blue stained.

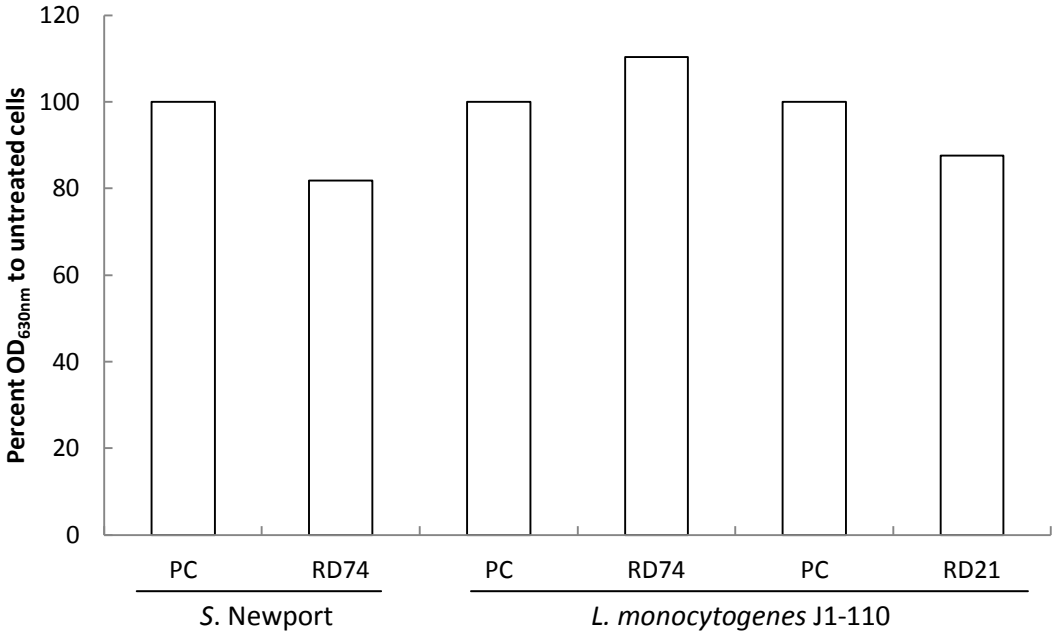


Figure 5. The antibacterial activity of purified RD74 and RD21. The results were presented as the percent of OD_{630nm} of treated cells to untreated cells (PC, positive control) at 16 h incubation. The date was from three replicates.

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