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ANTIMICROBIAL PEPTIDES FOR USE IN BIOSENSING APPLICATIONS

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ABSTRACT

Current pathogen detection systems lack the stability, sensitivity, and time-independent functionality required for real-time biosensing in the field. Antibodies exhibit specificity for pathogenic bacteria but lack the sensitivity to detect reduced pathogen levels and the stability needed for detection in harsh environments. We are investigating naturally occurring antimicrobial peptides (AMPs) for pathogen detection due to their intrinsic stability in harsh environments, ease of synthesis, and broad range of activity and affinity towards microorganisms, including gram-negative and gram-positive bacteria. The focus of our research is the tailoring of AMPs not for antimicrobial activity but for selective binding to target pathogenic bacteria. We envision these tailored peptides will replace existing molecular recognition elements in current pathogen detection platforms. Preliminary studies encompassed six full-length peptides (pleurocidin, cecropin P1, PGQ, cecropin A, ceratotoxin A, and SMAP-29) chemically synthesized with the addition of a c-terminal cysteine for site-directed immobilization onto a maleimide reactive plate and subsequent determination of whole cell-binding. Shorter peptide fragments of the native AMPs were also investigated to assess the putative discriminatory binding affinity for the gram-negative food pathogen *Escherichia coli* O157:H7. All six full-length peptides preferentially bound to *E. coli* O157:H7 over gram-positive *Staphylococcus aureus* 27217. In addition, two fragments exhibited equivalent or greater affinity than their corresponding full-length peptides identifying potential *E. coli* O157:H7 bacterial binding domains within the full-length AMPs. The ability to impart selectivity of antimicrobial peptides is an important initial step toward developing selective peptides for use in applications such as homeland security, food safety, drug therapeutics, and water monitoring.

1. INTRODUCTION

Current emphasis on homeland defense, the Army capability requirements for detection of CB agents, and improved food safety has established a need for the development of more durable, robust biological sensors. In particular, hundreds of food-borne pathogens have been isolated and shown to cause illnesses, and in some cases, death (Mead et al., 1999). Contamination of the food supply is an ongoing civilian and military threat with the need for early and real-time detection systems. Antibodies are the predominant recognition elements for current food and environmental detection systems due to their specificity for pathogenic bacteria; however, antibodies lack the sensitivity to detect reduced pathogen levels and the stability needed for detection in harsh environments or complex food matrices. To overcome these inefficiencies, naturally occurring antimicrobial peptides (AMPs) are being investigated for their potential development as molecular recognition elements for real-time biosensing systems.

Hundreds of antimicrobial peptides have been discovered and their structure/activity relationships investigated classifying the majority of the peptides into several groups, all of (Boman, 1995; Maloy and Kari, 1995) which exhibit a broad range of antimicrobial activity and binding affinity toward gram-negative and gram-positive bacteria, fungi, and viruses (Nicolas and Mor, 1995). Linear cationic peptides are of particular interest due to their small molecular size and intrinsic stability generally exhibiting random structure in solution then forming an amphipathic α-helical structure upon interaction with a bacterial membrane (Bevins and Zasloff, 1990; Boman et al., 1991; Tossi et al., 2000). Extensive research has been performed attempting to elucidate the mechanism of interaction of amphipathic helical peptides. Cecropins, in particular, have been a major focus revealing a "carpet" mechanism in which peptide monomer blankets negatively charged lipid headgroups of the bacterial membranes and subsequently lyse the cell through membrane disintegration (Gazit et al., 1995) or pore formation (Christensen et al., 1988). The formation of a surface monolayer does not directly correlate to antimicrobial activity. It has been shown that peptides interact with bacteria in which no activity has been detected (Steiner et al., 1988). This phenomenon is the basis for our investigation into AMPs as molecular recognition elements. We aim to better understand how the physical and chemical properties of both the cell and peptide influence specific binding and selectivity.

Before realizing the benefits of AMPs as molecular recognition elements within pathogen detection platforms, the broad affinity must be tailored for selective binding to target pathogens. We investigated modified full-length amphipathic AMPs for their discriminatory binding behavior to the emerging food

pathogen *Escherichia coli* O157:H7. The ability to impart selectivity of antimicrobial peptides is an important initial step toward developing selective peptides for use in applications such as homeland security, food safety, drug therapeutics, and water monitoring.

2. MATERIALS AND METHODS

2.1 Cell growth

Escherichia coli O157:H7 ATCC 43888 and *Staphyloccocus aureus* ATCC 27217 cells from frozen stocks were grown in luria and nutrient broth respectively to 10^8 cfu/ml. For affinity assays only, cells were pelleted, washed twice and resuspended in 1 ml PBS pH7.2 per ml culture

2.2 Antimicrobial peptide binding affinity

A whole cell binding assay (Fig. 1) was developed utilizing full-length peptides pleurocidin (Cole et al., 1997), cecropin P1 (Lee et al., 1989), PGQ (Moore et al., 1991), cecropin A (Sun et al., 1998), ceratotoxin A (Marchini et al., 1993), and SMAP-29 (Bagella et al., 1995) (Table. 1) and selected peptide fragments synthesized via FMOC solid phase peptide synthesis with the addition of a c-terminal cysteine for site-directed immobilization onto a maleimide reactive plate. A final concentration of 250 µg/ml of each peptide was used for immobilization. Wells were washed with PBS and blocked with 0.2% non-fat dry milk for 30 minutes. PBS washed cells were added to blocked wells containing immobilized peptide for 1.5 hours at 25°C with gentle agitation at 750 rpm. Horseradish peroxidase (HRP)-conjugated polyclonal antibodies for *E. coli* O157:H7 and *S. aureus* (general species) respectively were diluted 1:1000 with 10% fetal bovine serum in PBS pH7.2 and added to washed wells for one hour at 25°C with gentle agitation. Antibody solution was decanted and wells were washed prior to the addition of a 2-component TMB peroxidase substrate system mixed 1:1. Color development was analyzed at an absorbance of 650nm after 30 minute incubation.

2.3 Antimicrobial Activity

Inhibitory assays were performed to determine the minimal inhibitory concentration (MIC) of the full-length peptides and selected peptide fragments. Peptides were serial diluted and added to non-binding sterile microplates in triplicate. *E. coli* O157:H7 culture was diluted to 10^5cfu/ml and added to microplate wells. Growth was measured at t=0 and t=overnight (approx. 18 hours) at an absorbance of 595nm. The MIC is the lowest concentration of peptide at which no bacterial growth was present.

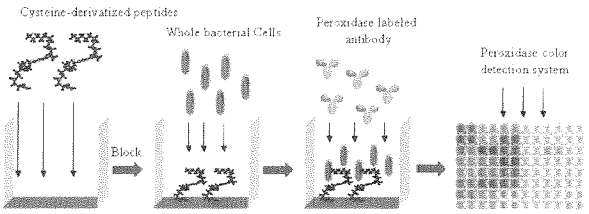

Fig. 1 Schematic of whole cell binding assay. Peptides were diluted to a concentration of 250 ug/ml. *E. coli* serotype O157:H7 (ATCC 43888) and *S. aureus* (ATCC 27217) were grown to mid-log. Horseradish peroxidase (HRP)-conjugated polyclonal antibodies for *E. coli* O157:H7 and *S. aureus* (general species) were diluted 1:1000. Color development was measured at absorbance of 650nm after 30 minutes incubation.

3. RESULTS & DISCUSSION

3.1 Full-length AMP Binding Specificity

A common misconception is that antimicrobial peptide binding affinity and antimicrobial activity are synonymous. In fact, binding is a precursor to antimicrobial activity (Blondelle et al., 1999) but they do not directly correlate. As previously mentioned, antimicrobial peptides bind to bacterial cells in which they show no activity. This suggests that antimicrobial peptides exhibit a broader binding affinity than antimicrobial activity.

The majority of peptide research has focused on mechanism discovery and tailoring AMPs for enhanced potency (Soares and Mello, 2003). Our focus is not on antimicrobial activity but on tailoring AMPs to preferentially bind to target pathogenic bacteria. Cystine-derivatized full-length peptides pleurocidin, cecropin P1, PGQ, cecropin A, ceratotoxin A, and SMAP-29 were analyzed, via a whole cell binding assay, for their discriminatory binding affinity for the pathogenic gram-negative bacteria *E. coli* O157:H7. Cecropin P1 and PGQ exhibited the greatest affinity for *E. coli* O157:H7 while SMAP-29, ceratotoxin A, pleurocidin, and cecropin A exhibited decreasing affinity respectively. All of the full-length peptides exhibited preferential binding of the gram-negative food pathogen *E. coli* O157:H7 versus gram-positive *S. aureus* 27217 (Fig 2). The dissimilar cell wall components of gram-negative and gram-positive bacteria are most likely responsible for the observed binding selectivity. Negatively charged lipopolysaccharide in the outer layer

ANTIMICROBIAL PEPTIDES AND AMINO ACID SEQUENCES

Antimicrobial peptide	Amino Acid Sequence
	1 12 25
pleurocidin	GWGSFFKKAAHVGKHVGKAALTHYL**C**
cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR**C**
PGQ	GVLSNVIGYLKKLGTGALNAVLKQ**C**
ceratotoxin A	SIGSALKKALPVAKKIGKIALPIAKAALP**C**
cecropin A	GGLKKLGKKLEGVGKRVFKASEKALPVAVGIKALG**C**
SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG**C**

Table 1. Full-length antimicrobial peptide (AMP) sequences. Peptides were synthesized with cysteine at the c-terminus for site-directed immobilization onto reactive surfaces.

of a gram-negative cell is thought to be the AMP binding target (Piers et al., 1994; Rana and Blazyk, 1991; Tsubery et al., 2002). Gram-positive bacteria do not contain LPS and thus do not readily bind to the AMPs tested.

The full-length peptides were modified with the addition of a c-terminal cysteine. Although it is unlikely that a single cysteine residue would have a significant impact on structure, the immobilization of the peptide to a surface may constrain the peptide structure. Since structure/function studies have not been conducted relative to binding affinity, some information may be translated to these studies from the extensive analyses performed on enhancing antimicrobial activity. Andreu et al. created a series of n-terminal analogues of insect cecropin A substituting amino acids with helix promoting, helix forming, and helix breaking amino acid residues (Andreu and Merrifield, 1985). Although it was difficult to correlate the degree of helicity with antimicrobial activity, certain amino acid replacements significantly decreased antimicrobial activity. Abler et al. investigated magainin II, a cationic helical AMP, through alanine substitutions allowing for the formation of more stable helices (Abler et al., 1995). The analogue, magainin II amide, displayed enhanced activity showing alanine substitutions had an affect on the secondary structure and thus the behavior of the peptide.

3.2 Fragmental AMP Binding Specificity

An entire AMP sequence may not be required for selective binding to bacterial cells. In fact, several reports suggest a native antimicrobial peptide sequence has multiple domains each contributing in a different manner. In an extensive analysis of insect cecropin B, researchers created n-terminal and c-terminal analogues to investigate the role of amphipathic structure upon antimicrobial activity (Chan et al., 1998). Modification of the n-terminus negated all antimicrobial affects while substitutions within the c-terminus had little to no effect on potency. This suggests the n-terminus is essential for activity while the c-terminus is not. Extensive investigation of insect cecropin A and corresponding analogues elucidated that different bacteria may in fact selectively interact with dissimilar portions of the peptide (Andreu and Merrifield, 1985).

Revealing the bacterial binding domains of full-length AMPs is required to identify regions suitable for tailoring specificity. In addition, development of short peptides with equivalent or enhanced affinity and selectivity for target bacteria, compared to full-length peptides, will lead to ease of commercial production. Full-length PGQ and pleurocidin were fragmented to elucidate portions of the native sequence responsible for

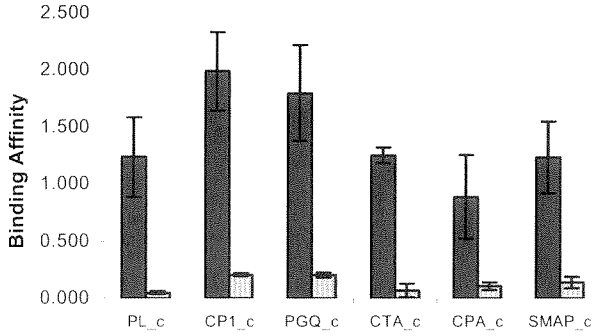

Fig 2. Full length AMP specificity for Gram-negative bacteria. All six full-length peptides tested exhibited preferential binding for the gram-negative *E. coli* O157:H7 versus the gram-positive S. *aureus*. PL_c = pleurocidin; CP1_c = cecropin P1; PGQ_c = PGQ; CTA_c = ceratotoxin A; CPA_c = cecropin A; SMAP_c = SMAP-29. Solid bar = Gram-negative *E. coli* O157:H7; Lined bar = Gram-positive *S. aureus*

binding to *E. coli* O157:H7. The fragments were rationally designed to encompass the entire full-length peptide sequence and overlap at amino acid residues known to be essential in activity analyses. Peptide fragments pleurocidin 1 (PL_1_c) and PGQ 2 (PGQ_2_c) both exhibited affinity equivalent to or greater than their corresponding full-length peptides. When compared to the fragments encompassing the rest of the native sequences, PL_1_c and PGQ_2_c are identified as *E. coli* O157:H7 binding domains (Fig 3). To further verify the gram-negative pathogenic binding domain, the fragments were investigated for their discriminatory binding ability for *E. coli* O157:H7 versus gram-positive *S. aureus*. PL_1_c and PGQ_2_c both displayed preferential binding for *E. coli* O157:H7 (Fig 3). Interestingly, pleurocidin fragment 3 (PL_3_c) and pleurocidin fragment 4 (PL_4_c) both exhibited greater affinity for *S. aureus* than *E. coli* O157:H7 although minimal affinity is observed. Further investigation is needed to determine if sequence modifications may enhance the preference of these respective fragments for *S. aureus*.

It is extremely difficult at this point to elucidate essential amino acids required for binding of a target bacteria since the quantity of data does not yet exist. Although, the extensive structure/function analyses for antimicrobial activity may be leveraged to begin investigating which amino acids may be required for binding. Several amino acids have been elucidated as essential for a peptide to exhibit antimicrobial activity including tryptophan and arginine residues (Andreu and Merrifield, 1985; Kragol et al., 2002). AMPs developed through mimicking the LPS-binding sites of the LPS binding protein family were tailored through arginine substitutions to exhibit preferential activity for a non-pathogenic *E. coli* versus gram-positive *S. aureus* (Muhle and Tam, 2001). In addition, short peptides (5-11 amino acids) consisting of just tryptophan and arginine residues have been designed to show potent antimicrobial activity regardless of order of the residues (Strom et al., 2002). PL_1_c is the only fragment of the native full-length pleurocidin sequence to include a tryptophan residue. PL_3_c, which showed no affinity for *E. coli* O157:H7, is an overlapping fragment of PL_1_c. The fragments overlap at all but the first five n-terminal amino acids, which contains the tryptophan residue at position 2. The enhanced binding results from PL_1_c may be dependent upon just the first five amino acids of that fragment. Analyses are underway to further investigate the importance of tryptophan for cell binding studies.

3.3 Antimicrobial Activity

Our primary focus is the tailoring of antimicrobial peptide binding affinity. However, these peptides may have application as decontaminating agents as well. In order to tailor the binding affinity of the bacteria, modifications will be made to the sequence thus potentially altering the antimicrobial activity effects. Preliminary experiments were conducted to determine if current modifications have adversely affected the known antimicrobial activities.

To demonstrate what affect the sequence modifications had on activity, full-length cysteine-derivatized peptides pleurocidin, cecropin P1, PGQ, and ceratotoxin A were evaluated for their MIC against *E. coli* O157:H7 (Table 2). The cysteine residue had varying affects on the antimicrobial activity although it was difficult to compare since non-derivatized peptide activity against *E. coli* O157:H7 is not readily available. Cecropin P1 analogs, through alteration of amino acid sequence, charge, or helicity, has been shown to affect

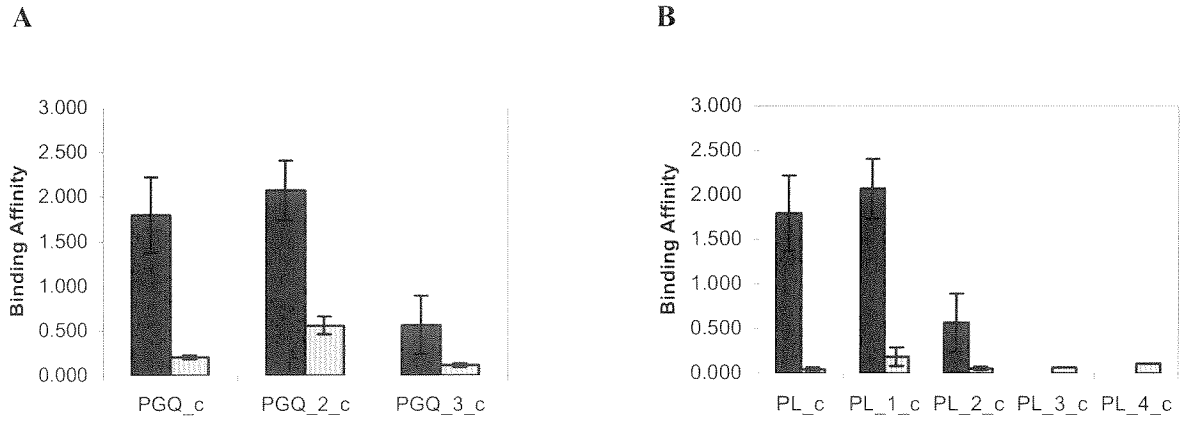

Fig 3. Peptide fragments binding affinity for gram-negative *E. coli* O157:H7 and gram-positive *S. aureus*. A). PGQ, B) pleurocidin. PGQ_2_c and PL_1_c were identified as *E. coli* O157:H7 bacterial binding domains, which were verified after both fragments preferentially bound the gram-negative bacteria versus gram-positive *S. aureus*.

activity against non-pathogenic *E. coli* strains. This is in concurrence with the cysteine-derivatized cecropin P1, which negatively impacted activity as MIC decreased from 1-2 ug/ml (Lee et al., 1989; Vunnam et al., 1997) to 6.5 ug/ml. The cysteine residue had the opposite affect on pleurocidin although minimal increase in activity was seen. Non-derivatized pleurocidin MIC varies from 5.8-8.8 ug/ml (Cole et al., 1997) while the cysteine-derivatized pleurocidin exhibited increased activity to 4.5 ug/ml. This minimal affect could be attributed to the difference in bacterial strains or growth media rather than any sequence or charge induced structural changes although it is currently unknown. Ceratotoxin A and PGQ are known to exhibit poor activity versus all gram-negative bacteria with non-derivatized MIC values of 20 and 62-250 ug/ml respectively (Marri et al., 1996; Moore et al., 1991) against non-pathogenic *E. coli* strains. The cysteine derivatized ceratotoxin A and PGQ exhibited much enhanced activity against *E. coli* O157:H7 as MIC was improved to 5 and 20 ug/ml respectively.

Two peptide fragments were also analyzed for activity against *E. coli* O157:H7 (Table 2). PL_1_c exhibited slightly less activity (MIC = 11 ug/ml) than the corresponding full-length pleurocidin but was still relatively active against *E. coli* O157:H7. These data suggest that the active site of pleurocidin resides in the n-terminus, which includes a tryptophan residue at position 2. PGQ_2_c fragment displayed a substantial decrease in potency as the MIC was reduced from 20 ug/ml for the full-length PGQ to >100 ug/ml for the fragment.

The development of the fragments was expected to potentially effect activity due to the size reduction and the potential separation of essential portions of the sequence for binding and subsequent lysis. Similar to reports regarding short model peptides against *E. coli* O157:H7 (Appendini and Hotchkiss, 1999; Appendini and Hotchkiss, 2000), the sequence length of PL_1_c did not adversely affect activity. It is not known which portions of the native AMP sequences are responsible for cell penetration and lysis but it appears the full-length pleurocidin active site may be at the n-terminus. Non-derivatized PGQ exhibits poor activity against gram-negative bacteria but upon an addition of a c-terminal cysteine residue, activity increases dramatically. Cleary more evidence is needed to verify the effects of charge and amphipathic structure upon these peptides.

The premise of activity and affinity not directly correlating to one another can be seen in the peptides analyzed. Full-length cysteine derivatized ceratotoxin A exhibited reduced affinity for *E. coli* O157:H7 but was more active than PGQ, which exhibited much higher affinity. The most astounding evidence is with PGQ_2_c, which displayed the overall greatest affinity for *E. coli* O157:H7 but by far the worst antimicrobial activity. Clearly there is some relation between affinity and activity as seen with the pleurocidin 1 fragment having enhanced affinity and similar activity to the full-length pleurocidin, but still evidence suggest affinity and activity do not always directly correlate. A peptides' broader affinity opens opportunities for use in applications other than killing microorganisms.

AMP ACTIVITY AGAINST *E. COLI* O157:H7

Cystine-derivatized AMP	MIC (ug/ml)
Full-length	
pleurocidin	4.5
cecropin P1	6.5
PGQ	20
ceratotoxin A	5
Fragments	
PL_1_c	11
PGQ_2_c	>100

Table 2. Cysteine derivatized peptide and peptide fragmental antimicrobial activity against the food pathogen *E. coli* O157:H7 using a microdilution assay

4. CONCLUSION

The majority of research concentrates on tailoring or enhancing antimicrobial activity for development of peptide antibiotics or decontamination. Our focus is on antimicrobial binding affinity, which does not always directly correlate with antimicrobial activity. AMPs broad affinity, environmental stability, and durability make them candidates for use as molecular recognition elements in pathogen detection platforms. Full-length and fragmental AMP preference for the gram-negative food pathogen *E. coli* O157:H7 versus gram-positive *S. aureus* is an initial step in the discovery of peptides for recognition applications. Differentiating between two gram-negative bacteria will be much more challenging due to the cell wall similarities of the bacteria with the difficulty increasing even more upon bacterial strain differentiation (pathogenic vs. non-pathogenic). Investigation of environmental factors such as salt, temperature, and pH upon binding affinity will also be required before realizing peptide advantages in biosensing applications.

Although further investigations of the full-length peptides and the designed fragments are needed, the potential exists for the development of peptides or peptide hybrids for enhanced functions. Identification of crucial amino acids and binding domains is the

beginning steps of developing peptides as molecular recognition elements. The ability to discriminate between bacteria is essential for peptide-based molecular recognition elements in a pathogen detection platform since there is an abundance of interfering bacteria in a soldier's environment. With the current emphasis on homeland defense and the Army capability requirements for detection of CB warfare agents, the durability of these peptides may allow for the development of biological threat detection systems for incorporation as soldier-uniform embedded sensors. In addition, the development of selective peptides may have potential use in applications such as food safety, drug therapeutics, and water monitoring.

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