-Worcester Polytechnic Institute-
-Natick U.S. Army Soldier Systems Center-

Immobilization of *E.coli* ML35 using Peptides PGQ and Cecropin P1

A Major Qualifying Project report submitted to the faculty of
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Abstract

The Natick U.S. Army Soldier Systems Center seeks to create a durable biosensor to detect pathogenic *E. coli* in the field. The team assisted the center by collecting data on peptides PGQ and cecropin P1 and their binding affinity to *E. coli* ML35 via the whole cell binding assay. They automated the WCBA by transferring it to a robotic platform. Finally, the team determined methods for quantifying peptide on the well-plates and made recommendations for future work in this area.

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Authorship

All team members worked on this project equally. However, due to the scope of the project, team members focused on different aspects to work on. Adam performed bench-top and TECAN assays, ran fluorescence tests on the peptides, contributed to the TECAN's programming, and contributed to the Introduction, Background, Materials and Methods, Results and Discussion, and Conclusions sections of the report. Laurel performed bench-top and TECAN assays, analyzed the collected data points, contributed to the Introduction, Background, Materials and Methods, Results and Discussion, and Conclusions sections of the report, and edited the report. Alex programmed the TECAN, worked with TECAN technical support, wrote a manual on the TECAN for the benefit of the Natick U.S. Army Soldier Systems Center, and contributed to the Background and Materials and Methods sections of the report.

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Introduction

Contaminated food sources pose a potential threat for soldiers in the field, where a single microbial source can incapacitate large numbers of soldiers for days at a time and can sometimes even result in death. The ability to quickly detect a contaminated food source would help alleviate this problem.

Currently, the most common method for detecting contamination is from using antibodies that selectively bind to a specific type of bacteria. Unfortunately, these antibodies are often not durable enough to withstand the harsher conditions that may be found near a battlefield (Blum et al., 1991). Antibodies begin to break down outside of a narrow temperature range, and are unable to detect pathogens at extremely low levels (Blum, et al), which may nonetheless affect soldiers in the field.

An alternative detection method to antibodies are peptides, short chains of amino acids that are heat and solvent stable and have a high affinity for various types of bacteria. Antimicrobial peptides are known for their ability to inhibit bacteria and can be found naturally in a variety of living tissues. Peptides are frequently used as antibiotics in order to kill certain types of bacteria, although it is possible to immobilize the cells at lower concentrations of peptide (Gregory and Mello, 2005). Different peptides may exhibit affinities to different bacteria, and some peptides may be more selective than others. However, the detailed mechanism behind peptide-bacteria binding is not fully understood, and over 880 such peptides are believed to exist. Therefore, the right peptide for use in a biosensor can be difficult to find.

To determine the selectivity of different peptides, a whole cell binding assay has been developed by researchers at Natick Solider Center which can be used to test the relative binding affinity of a specific peptide to bacterial sample. The peptides are synthesized such that their terminus contains a cysteine residue which are immobilized onto a maleimide-coated microwell plate. Any unoccupied active sites are filled with a large protein blocker and whole bacterial cells are added to the well which bind to the

immobilized peptide. Free bacteria are washed from the well and a peroxidase-labeled antibody that is specific to the bacteria is added. A colorimetric assay is then used to determine the amount of bacteria bound to the peptide. The entire whole cell binding assay is conducted on a 96-well plate that requires many different controls to normalize the data. The assay takes approximately eight hours to complete due to the long preparation and incubation times. The large number of samples also increases the risk of contamination, which is not apparent until the assay is completed. The assay also does not incorporate a method for quantifying the amount of peptide bound to the plate wells, which may vary from well to well and make data comparison difficult.

One possible way to increase the reproducibility of the whole cell binding assay is to use an automated assay platform. The use of the TECAN Freedom EVO, a high throughput, robotic assay platform, may enhance reproducibility and productivity while reducing processing time for the whole cell binding assay, as well as decreasing the margin of error. The Freedom EVO is fully automated and operates under a controlled environment, reducing both the preparation time and the chance of human error or contamination. The platform includes a fully automatic, computer controlled microplate reader for the colorimetric assay. Developing a procedure to quantify the amount of peptide bound to the plate wells will also enhance the quality of the data obtained through the whole cell binding assay. Different concentrations of peptides on the plate wells may affect the final concentration of bacteria bound to the peptides. If the concentration of the peptides is known, the amount of bacteria bound can be normalized for that experiment.

The objectives of this project are to transfer the whole cell binding assay to the TECAN Freedom EVO and to develop a method of quantifying the amount of peptide bound to the plate wells. In doing so, the data collection process will be accelerated and more selective peptides will be discovered for use in biosensing applications. Additional data may also help clarify the peptide-bacteria binding mechanism, allowing for customized peptides to be synthesized specifically for certain bacteria.

Background

Bio-Sensors

A biosensor is an analytical device that utilizes a biological sample to detect a chemical species (Kress-Rogers, 1997). There are many types of biosensors used in the world today, and the applications for biosensors are almost limitless. Biosensors consist of two main parts: the biological sample, which reacts with the chemical species, and the transducer, which translates the reaction into a more easily observable form. While not a necessary part of the device, semi-permeable membranes can also be used. While a membrane can augment a biosensor by reducing the risk of contamination and by filtering out unwanted chemicals that could cause a false positive reading, it can also negatively affect the mechanism by reducing its ability to detect chemical species at low concentrations (Kress-Rogers, 1997).

Types of Biosensors

Biosensors can be divided into categories in two ways, according to either their biological component or their detection mechanism. Divided by biological component, there are three main types of biosensor. Metabolism sensors utilize relatively large biological specimens, the most common being enzymes, although bacteria, algae, and plant or animal tissue are also used. Affinity sensors employ antibodies as a biological component, and recombinant sensors use DNA or gene probes (Kress-Rogers, 1997; Eggins, 1996).

There are two major methods of detection used in biosensors: electrochemistry and optical sensing. Electrochemistry includes measurement of the level of current through the biological component, or the measurement of its conductance (opposite of resistance) to detect a reaction. Optical sensing methods detect changes in the cells' ability to absorb or reflect light at a certain wavelength. The majority of biosensors utilize an optical sensing mechanism, as this is often the most simplistic detection method for many applications, and there are a wide variety of methods for displaying photometric change (Eggins, 1996).

Biosensor Applications

Biosensors have many applications in the world today. In health care, they can be used to detect pathogens to diagnose illnesses, as well as to determine general patient information such as metabolic rate. In a related function, they are used in the food processing and biotechnology industries to detect contaminants. Biosensors are also used to detect pollutants in air, water and soil (Eggins, 1996).

The applications of biosensors vary with different biological components, although there is some overlap between biological component types. Metabolism biosensors are most commonly used in environmental monitoring to test for pollutants such as pesticides, herbicides and heavy metals. Affinity sensors can also be used for environmental applications. However, affinity sensors are also used in clinical analyses such as those to detect the presence of carcinogens or drugs in a patient. Recombinant sensors are used in medical applications as well as in the detection of foodborne pathogens (Kress-Rogers, 1997).

Current Issues

While biosensors are a convenient detection method due to their portability and selectivity, they are also extremely fragile. Most biological detection methods, with a few rare exceptions, cannot survive outside a narrow temperature range (15-40°C). In addition, many methods operate optimally inside a narrow pH range; outside of this range, their activity and their effectiveness declines (Blum, et al, 1991). These restrictions may be trivial in a laboratory, but they restrict the usability of biosensors in other settings where conditions are less ideal. Some biological components, such as some enzymes, can also be very costly to prepare and lose effectiveness after a short period of time (Eggins, 1996), making mass-production of a biosensor expensive.

Current Research

In light of the vulnerability of current biosensors outside of narrow temperature and pH ranges, various lines of research have been conducted to find more stable alternative

biological materials. One research area focuses on protein engineering. For example, the replacement of a single amino acid with another can affect a protein's selectivity and affinity for a given material. Experiments in amino acid substitution will hopefully lead to more stable and durable proteins with enhanced utility for biosensing applications (Blum et al, 1991).

Another research area examines the use of peptides as the sensing mechanism, rather than enzymes and antibodies. Not only can peptides withstand conditions that other biosensing mechanisms cannot handle, but the correct peptide can prove more sensitive than its enzyme or antibody counterpart (Soares et al., 2004).

Antimicrobial peptides

Antimicrobial peptides are short chains of amino acids that can range from six to over 59 peptides in length. They are often categorized by their amino acid sequence and structure, which can vary from α-helical to β-sheets to linear, or any combination of those. At this time there are more than 880 different peptides that have been identified or predicted based on an amino acid sequence. These peptides have been isolated from a large variety of sources, including microbes, animal, plant, and invertebrate tissues or cells (Brogden, 2005).

One of the principle properties of these peptides is their antimicrobial activity. It has been known for some time that when isolated, these peptides are able to slow down or kill bacterial infections of tissue. As more strains of bacteria become resistant to current antibiotics, the use of peptides to fight microbial infections may provide an alternative method of treatment (Straus and Hancock, 2006).

How peptides bind and kill bacteria

The exact mechanism of peptide binding and interaction with bacteria is not yet known, but several techniques are being utilized to help develop this knowledge, including microscopy, model membranes, and fluorescent dyes (Brogden, 2005).

Figure 1: Depiction of several possible structures for antimicrobial peptides. The narrow regions represent α-helices and the wider regions represent a β-sheet structure (Brogden, 2005)

One common feature among most peptides is the electrostatic attraction between the net negative charge on the bacterial membrane and the cationic peptide. This negative charge is usually associated with anionic phospholipids and phosphate groups on lipopolysaccharides for Gram-negative bacteria and the anionic teichoic acids found on Gram-positive bacteria. As the peptide approaches the membrane, it conforms to an amphiphilic structure, with the hydrophobic side congregating in the middle and the hydrophilic side facing the solution. As the peptide approaches the cell membrane, it must pass several of the larger membrane constituents found on most cells, such as capsular polysaccharides or teichoic acids, before it can interact with the cytoplasmic membrane and the lipid bilayers (Brogden, 2005; Straus and Hancock, 2006).

Studies have shown that there are two different binding states for peptides once they reach the cytoplasmic membrane of Gram-negative bacteria. At a low ratio of peptide-to-lipid concentration, the peptides will bind parallel to the outer lipid monolayer and remain functionally inactive. This is referred to the surface or S state, and although the peptide has no other function, it serves to stretch and thin the membrane surface by forcing itself between the lipid head chain (Brogden, 2005).

At higher ratios of peptide-to-lipid concentrations, the peptides will orient perpendicular to the membrane, forming pores that stretch across the lipid bilayer. There are several proposed mechanisms for how these pores are formed, each method drawn from the study

of a different peptide. The validity of each model depends on the peptide used and the characteristics of the lipid bilayer. (Brogden, 2005, Straus and Hancock 2006).

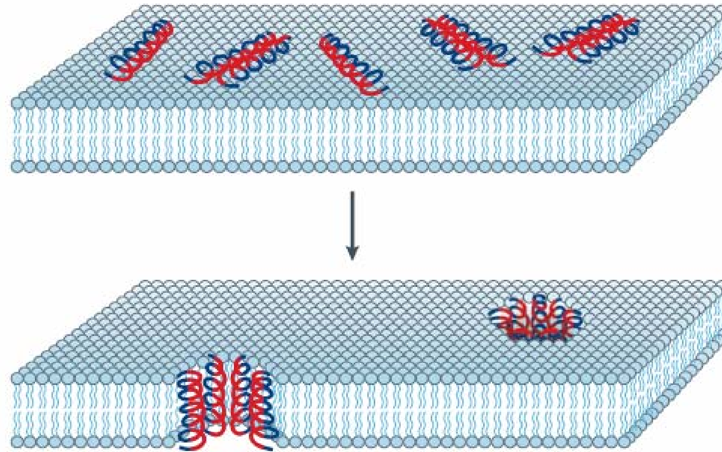

Figure 2: The "barrel-stave" model for peptide insertion into the membrane bilayer. The pore formed is lined with the hydrophilic face of the peptide, shown here in red (Brogden, 2005)

The "barrel-stave model" (Figure 2) suggests that several α-helical peptides aggregate to form a barrel-like structure across the lipid bilayer, with each peptide acting as a single stave in the barrel. In this model, the pore is lined completely with the hydrophilic side of the peptides and the two lipid head monolayers are attached through the hydrophobic section of the peptide (Brogden, 2005; Papo and Shai 2002).

The "carpet model" (Figure 3) suggests that peptides bind parallel to the outer lipid monolayer and accumulate on the surface of the membrane. At high enough concentrations, the peptides cover the membrane in a "carpet", eventually disrupting the membrane to increase the surface area and forming independent micelles out of segments of the lipid bilayer (Brogden, 2005; Papo and Shai 2002).

The "toroidal-pore model" (Figure 4) is similar to the barrel-stave model in that peptides embed into the lipid layers and eventually form transmembrane pores. However, the peptides induce bending of the lipid monolayers until the inner and outer layers connect, creating an opening in the membrane. This model differs from the barrel-stave model in that the pore is lined with both the hydrophilic region of the peptide as well as the lipid

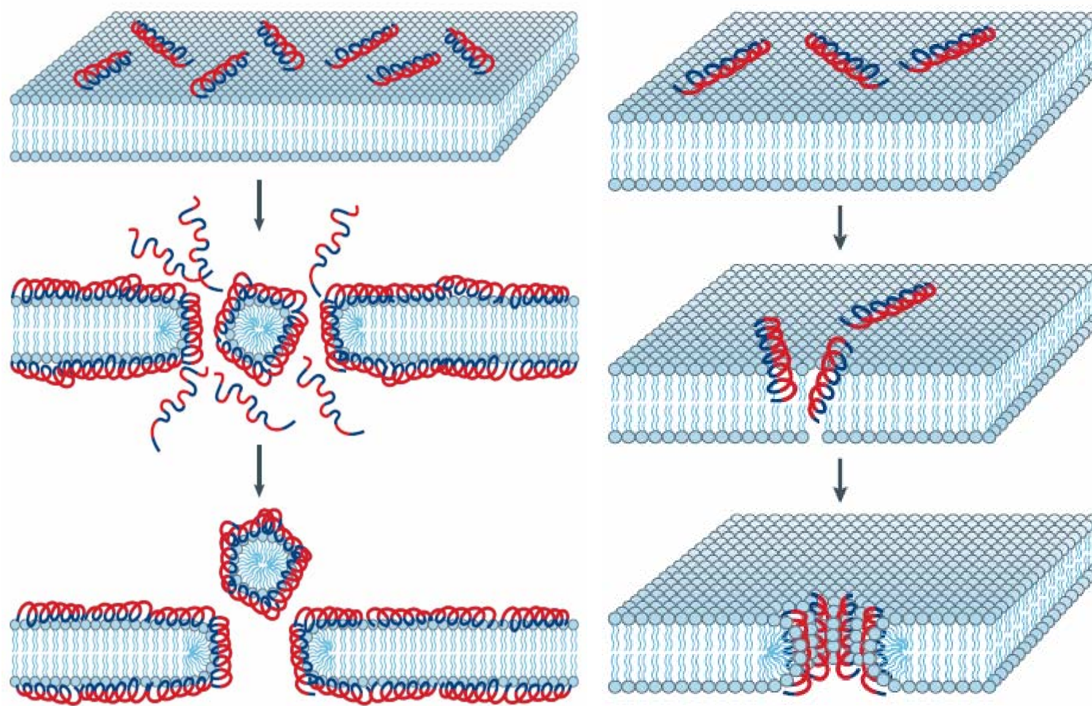

Figure 3 (Left): The "carpet" model. The peptide aggregates on the surface of the membrane, inducing bending in the monolayers until micelles are formed (Brogden, 2005)

Figure 4 (Right): The "toroidal-pore" model. The pore formed is lined with both lipid head groups and the hydrophilic face of the peptide (Brogden, 2005)

head groups. This combination of peptides and lipids lessens the repulsive forces between the cationic peptides and requires less energy for pore formation (Brogden, 2005; Papo and Shai 2002).

In addition to the rupturing of cell membranes, it has been suggested that peptides have additional modes of killing bacterial cells. The positive charge of the cationic peptides can be used to disrupt the transmembrane potential or pH gradient of the cell membrane, interfering with critical cellular respiratory functions. Certain linear peptides have been shown to penetrate the cell membrane through the use of fluorescent dyes. These peptides accumulate within the cell where they can inhibit the synthesis of critical cellular components or other enzymatic activities, eventually leading to cell lysis. Other peptides have been shown to prevent cellular division, either by inhibiting DNA replication or

preventing the formation of certain membrane proteins (Brogden, 2005; Straus and Hancock 2006).

Bacterial resistance

Unlike modern antibiotics, bacterial cells have shown little to no resistance to antimicrobial peptides. The large diversity of peptide sequences and modes of action allow peptides to interact with multiple targets within the cell and may disrupt several critical cellular processes. Multicellular organisms typically rely on several different peptides to fight an infection, further decreasing the chances of the bacteria to develop a resistance to all of the peptides (Straus and Hancock, 2006).

Although there have not been many cases of peptide-resistant bacteria, there are several defensive techniques that cells can use to reduce the activity of peptides, either by preventing attraction, attachment, or insertion of the peptide into the cell (Brogden, 2005). Although these mechanisms reduce the effectiveness of most peptides, it has been shown that in most cases there is only a 2- to 4-fold increase in the minimal amount of peptide required to inhibit bacterial activity (Straus and Hancock, 2006).

Certain cells are capable of transporting positively-charged molecules from within the cell to the surface to reduce the net negative charge that attracts the peptide, or to induce a net positive charge that would repel the peptide (Brogden, 2005). Other forms of resistance can come from reducing the fluidity of the lipid bilayer. The insertion of additional molecules within the bilayer can increase the number of hydrophobic interactions, creating a stronger barrier between the layers and reducing the chance or peptide insertion or penetration. Some cells can also transport antimicrobial peptides across the cellular membrane. These strategies can be utilized to prevent the accumulation of peptides on the cell membrane where they may form pores, or to reduce the number of peptides within the cytoplasm where they may target intracellular organelles (Brogden, 2005).

Peptide Fluorescence

Proteins and peptides that contain the aromatic residues tryptophan, tyrosine, or phenylalanine can fluoresce after excitation and are often used to study protein or peptide conformation and dynamics (Chen and Barkley, 1998). Changes in an electric field can affect the fluorescence intensity of the residue, as well as the wavelength maximum, band shape, anisotrophy, fluorescence lifetimes, and energy transfer. Tryptophan is the most commonly utilized as a fluorescent probe, and various empirical methods have been developed to determine the quantum fluorescence under a variety of environmental conditions (Vivian and Callis, 2001).

Enzyme-Linked Immunosorbant Assay

The Enzyme-Linked Immunosorbant Assay (ELISA) is a popular assay for research related to the immunology field for detecting quantities of antigen-specific antibodies attached to a sample. The main objective behind the assay is to measure the binding strength between an antibody and an antigen. In order to accomplish that, the experiment is carried out within well-plates coated with a particular substance, usually with another antibody known to attach to the antigen (DeCourcey, 2003).

The known antibody first binds to the antigen on the well-plate and then the well-plate is washed to remove excess antigen. The test antibody will than be dropped into the wells, where it attaches to the antigen. An antibody-marker, which tags the test antibody, is used to determine the amount of test antibody attached to the antigen. The marker is usually optical, absorbing different wavelengths, or fluorescent. It shows the quantity of test antibody bound to the antigen (DeCourcey, 2003).

Whole Cell Binding Assay

The whole cell binding assay (WCBA) is a modification of the ELISA. The basic principle of the assay remains the same; however, the WCBA is used to determine the binding strength of a peptide to a specific cell.

Although the WCBA is similar to the ELISA, there are some alterations. Instead of an antibody coated well-plate, a well-plate coated with maleimide, a small organic molecule, is used. The peptides used in the assay have been specially synthesized to end with a cysteine amino acid which forms a carbon-sulfur bond with the maleimide molecule. After a series of washes to remove excess peptide, a blocker protein is added to the well-plate to cover any active maleimide sites left on the wells' surface.

The second step involves adding the bacteria cells, allowing time for the peptides to bind to the cell during the incubation period. Then, another series of washes removes excess cells from the well. An antibody known to bind to the specific cell is then introduced to the well-plate, and lastly, similar to the ELISA, an indicator tag is added to the antibody.

In order to collect reliable results from the WCBA, several controls are tested alongside each assay. By removing one or more elements of the WCBA, these controls test for false positive readings. Blank wells and duplicates also test for contamination of the plate or individual wells.

Materials and Methods

Conducting the Whole Cell Binding Assay (WCBA)

Four buffers were prepared for use in the WCBA: PBS at pH 7.2, PBS at pH 6.5 supplemented with 1mM EDTA and 0.0001mM dithiothreitol (DTT), 0.2% non-fat dried milk (NFDM) in PBS at pH 7.2, and 10% FBS in PBS at pH 7.2. The PBS pH 7.2 buffer was created by diluting PBS stock (a 10M solution) with water to a concentration of 1M, then adjusting the pH. The PBS pH 7.2 buffer was filtered into a non-sterile container and sterilized by autoclaving. The other buffers were filtered into sterile containers. Between assays, the buffers were stored at 4°C.

New peptide solutions for cecropin P1 (CP1) and PGQ were created periodically from stock samples. A Bovive Serum Albumin Colorimetric Assay (BCA) was used to determine the concentration of the solutions, which were created using 2 mg of dry peptide and 1mL of PBS pH 6.5 supplemented with EDTA and DTT. Serial dilutions of bovine serum albumin (BSA) solution at known concentrations were used to develop a calibration curve, which was applied to determine the unknown peptide concentrations in each stock solution. After determining the concentrations, the new peptide solutions were stored at -20°C until needed for the WCBA.

E. coli ML35, a non-pathogenic bacterial strain, was cultured on the day prior to the WCBA. Cells were grown for 3.5 hours at 35°C in lupine broth (LB) with mild agitation before being stored at 4°C overnight. Remaining cell growth was accomplished alongside the first steps of the WCBA. While freshly grown bacterial samples were ideal, due to time constraints, it was not feasible to culture the bacteria and perform the WCBA in the same day.

The WCBA was conducted for the peptides cecropin P1 and PGQ. The peptides were bound to the wells of a 96-well microplate, then a large protein, in this case NFDM, was used to block the remaining active sites on the wells. The cells were bound to the peptide, and an antibody was bound to the immobilized cells. A dye was added which would

qualitatively detect the antibodies in each well. A detailed description of the assay procedure can be found in Appendix A.

The Whole Cell Binding Assay was conducted fifteen times on several different days by two experimenters working in conjunction. When done by hand, the assay took approximately eight hours to complete. This was due to the 4.5 hours of incubation time and several repeated washing steps needed for each assay.

Programming the TECAN Freedom EVO

In an effort to reduce the margin of error for the WCBA and speed up the assay process, Natick Army Base purchased a versatile liquid handling platform with state of the art robotic control work stations called the TECAN Freedom EVO (TECAN). The TECAN is a combination of different assay components such as an incubator-shaker, optical scanner, washer and aspirator, all of which are linked to a computer for easy process control. The TECAN supports accurate measurements; it can be calibrated on a scale of 0.1 microliters for handling liquid, and 0.5 millimeters for measurement.

The original function of the TECAN was to conduct many assays at once for purposes such as such as drug discovery or testing for chemical reactions. For our experiment, the TECAN was programmed to perform the WCBA. Each step of the process needed to be identical between the bench-top assay and the robotic one. The results from both the TECAN and the bench-top assay were compared with standard statistical measurements.

When the WCBA was programmed on the TECAN Freedom EVO, there were several modifications to the original protocol to adjust for the discrepancies between the different pieces of equipment available. The shaking speed of the incubator-shaker on the TECAN was set to the maximum (8.4 Hz maximum), which was still significantly slower than the incubator-shaker used for the bench-top assay (~70 Hz maximum). Several of the solutions containing large biomolecules, which required mixing to prevent settling, were repeatedly aspirated and dispensed using the pipette tips instead. The series of individual

wash steps was replaced with the TECAN's PowerWasher, which used a simultaneous aspirate-dispense step with no intermittent shaking to remove the peptide, cell, and antibody solutions from the wells. Finally, the TECAN utilized eight permanent pipetters rather than disposable pipette tips.

The Whole Cell Binding Assay was conducted eight times using the TECAN Freedom EVO on four different days. Using this robotic platform, the assay time was reduced from approximately eight hours to less than six hours by reducing the duration of the washing steps.

Quantifying the Amount of Peptide Immobilized

Peptides PGQ and Cecropin P1 were tested for fluorescence while suspended in a solution of 1x PBS (pH 6.5) supplemented with 1mM EDTA and 0.0001mM dithiothreitol (DTT) (PED solution). Initial concentrations of 250 µg peptide/mL were prepared and added to a row of wells in triplicate on a clear 96-well microplate. The peptide solution was diluted in series 1:2 six times to observe how the fluorescence corresponded to the peptide concentration. The microplate was read in a spectrofluorometer at an excitation wavelength of 280 nm and a range of wavelengths from 300 nm to 450 nm (Ladohkin et al. 2000). The strongest signal was obtained at 370 nm, so all subsequent fluorescence tests using PGQ and CP1 were read at 370 nm.

The fluorescence of the peptides was also tested while they were immobilized to the well surface using the Corning® 96 Well Clear Polystyrene Sulfhydryl-BIND™ Stripwell™ Microplate. Initial concentrations of 250 µg peptide/mL were prepared in PED solution and added to a row of wells in triplicate on the microplate. The wells containing the peptide solution were then diluted in series 1:2 six times. The microplate was incubated for one hour with mild agitation to allow the peptide to bind to the surface of the well. Once the peptides were immobilized, the microplate was washed three times using 150 µL of 1x PBS (pH 7.2). 100 µL of 1x PBS (pH 7.2) was added to each well containing

peptides as well as several blank wells. The microplate was read in a spectrofluorometer at an excitation wavelength of 280 nm and an absorption wavelength of 370 nm.

Results and Discussion

Bench-top Whole Cell Binding Assay

Table 1 shows the data points collected from the bench-top whole cell binding assay. A total of thirty data points were collected.

Table 1: Absorbance values from the bench-top assays for PGQ and cecropin P1 peptides.

PGQ	CP1
0.004	-0.004
0.002	-0.005
0.005	-0.003
0.044	-0.004
0.06	0.027
0.103	0.059
0.299	0.043
0.07	0.079
0.106	0.209
0.34	0.059
0.066	0.045
0.026	0.047
0.1	0.016
0.065	0.021
0.041	0.047
0.144	0.047
0.093	0.04
0.026	0.05
0.315	0.064
0.429	0.07
0.047	0.086
0.056	0.055
0.081	0.071
0.102	0.102
0.097	0.044
0.129	0.129
0.024	0.022
1.078	0.006
0.108	0.047
0.061	0.049

The validity of the data points was determined first by comparing them to the controls for each assay. If the absorbance value was greater than the control containing *E.coli* ML35 and antibody (theoretical maximum) or less than the control containing antibody only (theoretical minimum), the data point was determined to be invalid. The remaining data points were normalized using a corresponding control, which consisted of the same peptide, blocker and antibody, but no cells. This control was subtracted from the original data point, creating a normalized value. Normalized data points were then examined for possible contamination. Negative values, values greater than two standard deviations from the mean, and erroneous values were deemed contaminated and discarded. Since all controls were performed in duplicate, the average value of each control for a given experiment was used. In cases where a control well was obviously contaminated, this control well was ignored, and only one control value was used in the calculations.

A large percentage (63%) of the data points were determined to be contaminated or invalid through the method described. There was no discernable pattern for the wells contaminated, so it was most likely caused by human error, such as pipetting errors and improper handling of the plate, rather than any systematic errors in the protocol. The layout of the microplate was rearranged several times to determine if the placement of specific wells had an effect on the amount of contamination, but there was no noticeable difference in the results.

The results of the normalized data for the bench-top assays are shown in Figure 5: Mean absorbance values for Cecropin P1 and PGQ from bench-top assays. Standard error shown. The absorbance values from the assay are analogous to the relative binding affinities for each peptide to the bacterial cells. PGQ had a higher affinity for *E.coli* ML35 than Cecropin P1 (CP1), although PGQ had greater variability in the data. The ratio of binding affinities for PGQ and CP1 was 1.34 : 1 for *E.coli* ML35.

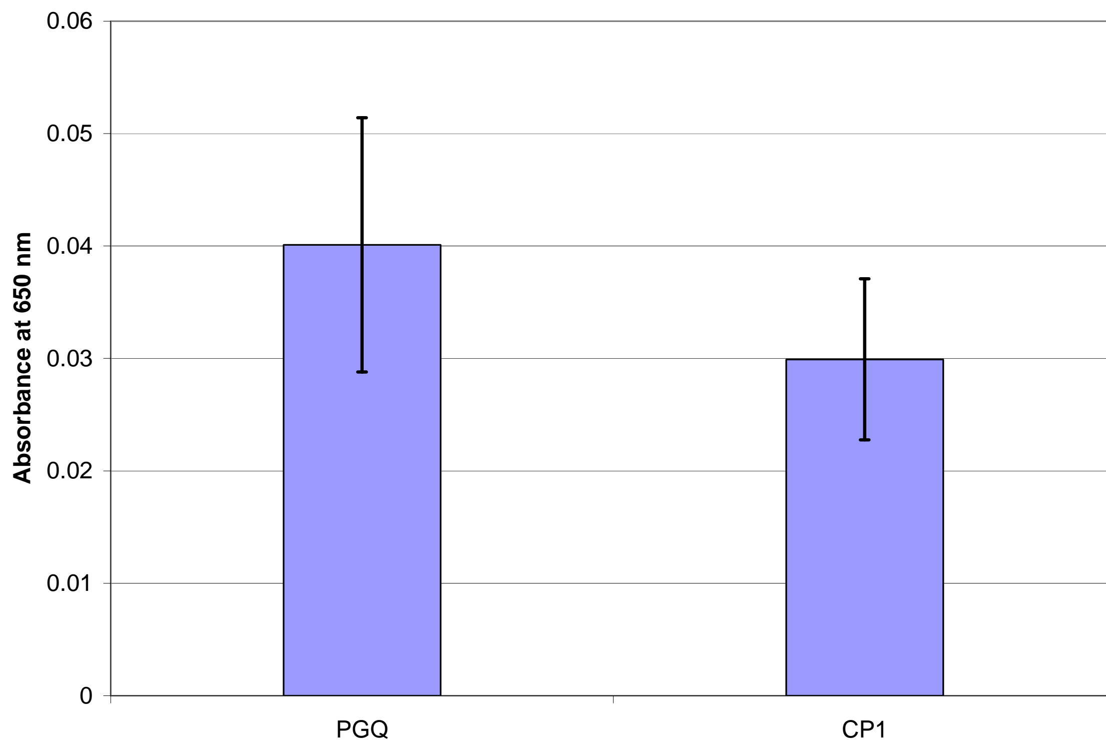

Figure 5: Mean absorbance values for Cecropin P1 and PGQ from bench-top assays. Standard error shown.

Robotic Whole Cell Binding Assay

Table 2 shows the data points for the whole cell binding assay using the TECAN Freedom EVO. A total of sixteen data points were collected.

Table 2: Absorbance values from the TECAN assays for PGQ and cecropin P1 peptides.

PGQ	CP1
0.607	0.4124
0.7206	0.6369
0.5743	0.442
0.7409	0.7096
0.4277	0.3989
0.4145	0.355
0.6338	0.3482
0.371	0.2845
0.4376	0.3119
0.5387	0.4794
0.4906	0.455
0.4756	0.4456
0.7693	0.7633
0.67	0.6312
0.583	0.5466
1.029	0.681

The data from the robotic assays was validated and normalized using the method described above. The percentage of contaminated or invalid data points was less than half of that for the bench-top assays (31%). In addition, the wells that were contaminated were consistent between assays, indicating that the contamination was a result of a systematic error in the protocol rather than random or pipetting error. The use of permanent pipette tips may have increased the likelihood of spreading bacterial cells or antibodies between wells. The changes to the washing steps may also have affected the results. Since there is no agitation involved in the TECAN version of the washing process, the wells may have retained more cells than they did in the bench-top version.

The results of the normalized data for the robotic assays are shown in Figure 6. PGQ had a higher affinity for E.coli ML35 than CP1 with a binding ratio of 1.35 : 1, which was very similar to the previous results from the bench-top assay. Despite this similarity, the absorbance values for the robotic assays were approximately four times greater than those from the bench-top assays. Figure 7 shows a comparison of the two assay methods for both peptides. This is most likely a result of the changes to the washing steps for the robotic assay. Since the PowerWasher does not agitate the wells between washes, less antibody and bacterial cells may be removed from the solution. Since the ratios of

binding affinities for the bench-top and robotic assays were nearly identical, the more rigorous washing steps would be removing antibody uniformly from each well.

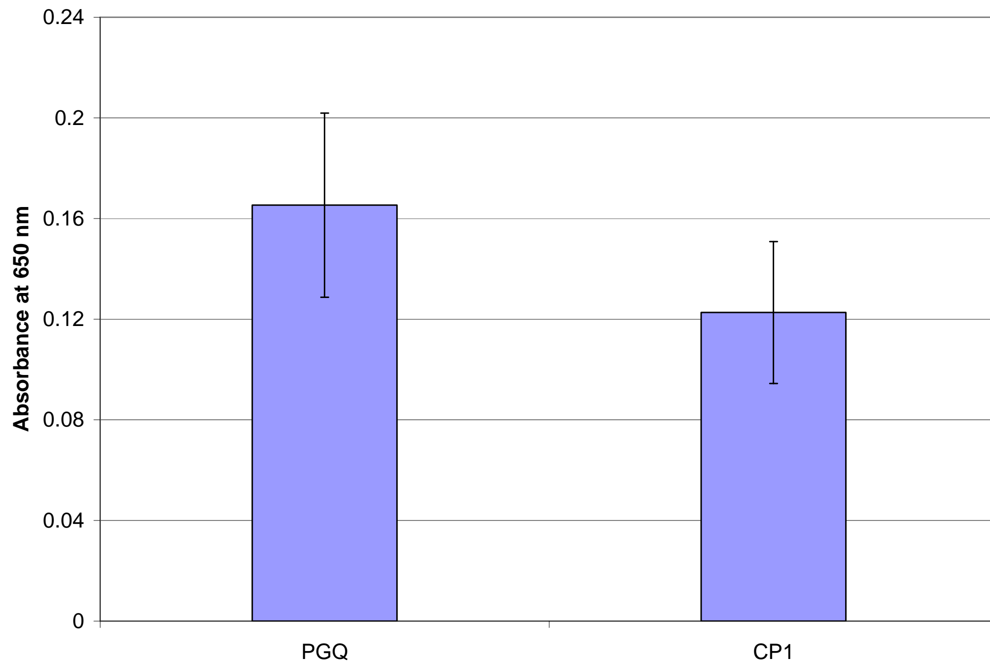

Figure 6: Mean absorbance for Cecropin P1 and PGQ from robotic assays. Standard error shown.

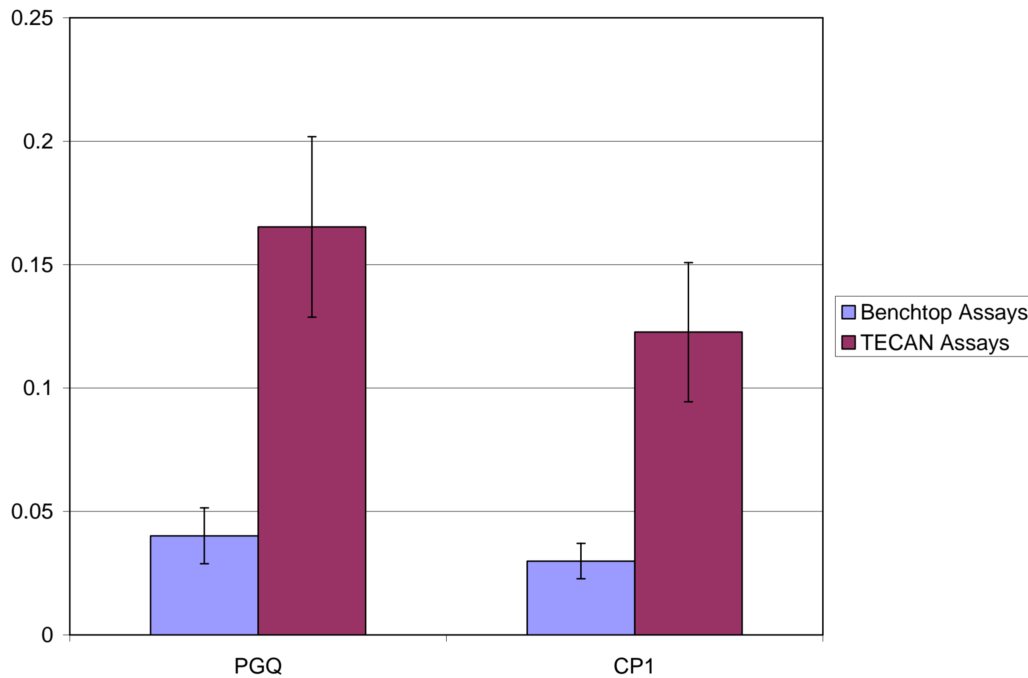

Figure 7: Mean absorbance values for peptides PGQ and cecropin P1 for bench-top vs. robotic assays. Error bars denote standard error.

Peptide quantification

In order to determine the amount of peptide bound to the surface of each well, the fluorescence of both peptides was first tested while suspended in solution. PGQ, containing one Tyrosine residue, did not yield a discernable signal, while CP1, containing one Tryptophan residue, yielded a signal that corresponded to the concentration of the peptide in solution, shown in Figure 8.

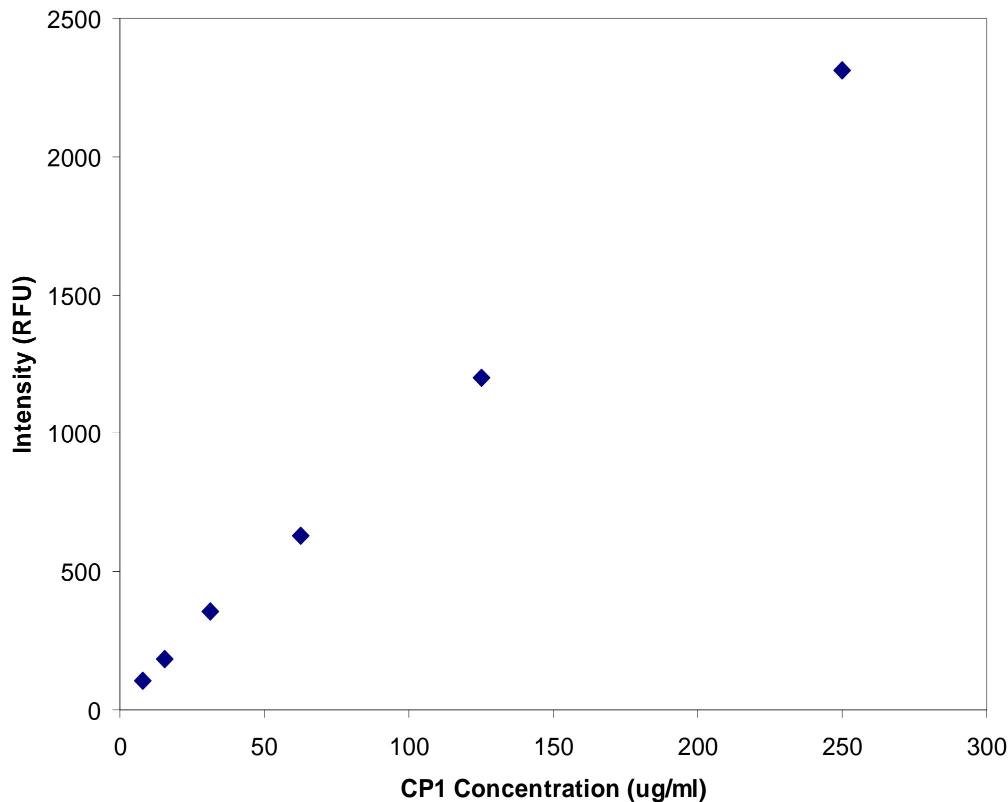

Figure 8: Relationship between cecropin P1 concentration and intensity of flourescence, measured at 370 nm

The linear relationship between the relative fluorescent units (RFU) and the concentration of the peptide in solution suggest that there was no interference from the microplate or the buffer. From these results, the intensity per peptide in solution was calculated to be 4.79×10^{-13} RFU/CP1 peptide.

CP1 was then tested for fluorescence while bound to the surface of the microplate. The WCBA procedure was followed up until the peptide solution was washed from the well.

There was significant amount of interference from the microplate and no signal was detected that corresponded to the peptide concentration. The C-S bonds formed between maleimide and cysteine may be a cause for disrupting the electric potential in the solution and quenching the Tryptophan fluorescence (Callis, 2004).

Conclusions

From the results discussed above, the following points can be concluded from these experiments:

- Based on the results of the Whole Cell Binding Assay, PGQ had a higher binding affinity for *E.coli* ML35 than Cecropin P1, with a binding ratio of 1.34 : 1. Both the bench-top and robotic assays yielded similar results.

- The absorbance values for the robotic assays conducted on the TECAN Freedom EVO were approximately four times greater than those for the bench-top assays. The ratio of binding affinities between PGQ and CP1 was determined to be 1.35 : 1, which is very similar to the bench-top assay results. This indicates that the washing steps for the bench-top assay removed antibody uniformly from the wells with a more rigorous washing procedure than the PowerWasher.

- The amount of contamination of data points was reduced by more than half when the assay was conducted on the TECAN rather than the bench-top. The contaminated data points on the TECAN assays were consistent between assays, suggesting a systematic error in the procedure rather than random error. Using disposable pipette tips may reduce or eliminate this contamination as the tip washing procedure may not be very effective.

- CP1 fluorescence that corresponded with concentration was detected while the peptide was suspended in solution, although there was no discernable signal while the peptide was bound to the surface of the well. The Kaiser test is another possible method for determining relative amounts of peptide immobilized on the surface of the well (Kaiser et al., 1970).

References

1. *Biosensing Principles and Applications;* Blum, L.J.; Coulet, P.R.; Ed.; Marcel Dekker, Inc.: New York, NY, 1991
2. Gregory, K., Mello, C.M. *App. Environ. Microbio.* **2005**, *71(3), 1130-1134*
3. *Handbook of Biosensors and Electronic Noises: Medicine, Food, and the Environment;* Kress-Rogers, E.; Ed.; CRC Press: Boca Raton, FL, 1997
4. *Biosensors: An Introduction;* Eggins, B.; Ed.; Wiley Teubner: New York, NY, 1996
5. Soares, J.W., Morin, K.M., Mello, C.M. *Antimicrobial Peptides for use inBiosensing Applications;* ADA433515; NTIS: Army Natick Research Development and Engineering Center, 2004.
6. Brogden, K.A. *Nature.* **2005**, *3, 238-250*
7. Straus, S.K., Hancock, R.E. *Biochim. et Biophys. A.* **2006**, *1758, 1215-1223*
8. Papo, N., Shai, Y. *Biochem.* **2003**, *42(2), 458-466*
9. Chen, Y., Barkley, M.D. *Biochem.* **1998**, *37(28), 9976-9982*
10. Vivian, J.T., Callis, P.R. *Biophys. J.* **2001**, *80(5), 2093-2109*
11. DeCourcy, K.; *The ELISA Assay: An Immunology Experiment.* Fralin Biotechnology Center, Virginia Tech, 2003
12. Ladokhin, A.S., Jayasinghe, S., and White, S.H. *Analytical Biochem.* **2000**, *285, 235-245*
13. Callis, P.R., Liu, T. *J. Phys. Chem. B* **2004**, *108, 4248-4259*
14. Kaiser, E., Colescott, R.L

Appendices

Appendix A: Whole Cell Binding Assay Bench-top Procedure

Prior to the assay itself, it was necessary to plan the layout of the plate. Determining which wells would receive peptide, bacteria, blocker, and antibody beforehand helped to reduce errors during the experiment. Once an optimal plate layout was determined, it was used in all future experiments and this step was not necessary.

For assays being conducted with a new set of peptide solutions, new dilution calculations were also necessary. 450µL of a 250µg/mL solution of each peptide in PBS pH 6.5 supplemented with EDTA and DTT (PED) was required for the assay, and the concentrations of the stocks changed periodically as old prepared stocks ran out.

100µL of peptide solution was added to the appropriate wells. Experiments were run in duplicate, so a total of four wells contained each peptide solution. The remaining wells being used in the experiment were filled with 100µL of PED to prevent them from drying out. The microplate was incubated at 25°C with constant gentle agitation for one hour, during which time the peptides would bind to the well. Remaining peptide solution and PED were removed through aspiration once the incubation period was complete.

While the microplate was incubating, the E. coli ML35 cells were taken from refrigeration and incubated at 30°C with agitation for approximately thirty minutes. After their incubation period, a small sample of the cells was diluted in LB (100µL cells in 900µL LB) and underwent an optical density (OD) test. Using pure LB, the OD of the sample was measured at 600nm. An OD of slightly more than 0.1 was preferable; if the sample's reading was too low, the remaining cell broth was placed back in the incubator for a short period of time and then tested again. This cycle continued until the desired OD reading was reached.

After the desired amount of cell growth had been achieved, the cell broth was transferred into four 1mL centrifuge tubes and placed in a centrifuge at 12000rpm and 20°C for five

minutes. Once the cells were separated from the broth, the liquid was aspirated out of the tubes by hand while leaving the pellet of cells intact. The cells were re-suspended in 1mL of PBS pH 7.2 per tube. This process of cell separation and re-suspension was repeated twice to clean the cells. Another OD test was taken afterward, using PBS pH 7.2 as a standard, to determine whether any cells were lost during the washes. The cells were recombined into one vial and stored at 4°C until needed.

Following the peptides' incubation period, the remaining liquid was aspirated out of the microplate by machine. Three washes were performed using 150µL of PBS pH 7.2 per well, with an incubation period of 5 minutes at 25°C with agitation in between washes.

After the washes were complete, 150µL of NFDM in PBS pH 7.2 was added to appropriate wells in order to block the remaining active sites in the wells. An equivalent amount of PBS pH 7.2 was added to the remaining control wells. The microplate was then incubated at 25°C for 30 minutes with no agitation.

Following the blocker incubation period, the blocker was aspirated out of the microplate by machine and immediately replaced with 100µL of cell solution for the appropriate wells. The remaining wells were filled with 100µL of PBS pH 7.2. The microplate incubated for 90 minutes under mild agitation at 25°C.

After 90 minutes, the microplate was removed from incubation, and the cell solution was aspirated out by hand. Five washes were performed using 150µL of PBS pH 7.2 per well, with an incubation period of 5 minutes at 25°C with agitation in between washes. Subsequent aspirations were performed by machine.

Between the last two washes, an antibody solution was prepared by diluting 4µL stock of HRP antibody in 4mL of 10% FBS in PBS pH 7.2. Following the last wash and subsequent aspiration, 100µL of the antibody solution was added to each well. The microplate incubated at 25°C for 1 hour with mild agitation.

Following the incubation period, the antibody solution was aspirated out by machine. Six washes were performed using 150µL of PBS pH 7.2 per well, with an incubation period of 5 minutes at 25°C with agitation in between washes. Between the second and third washes, the TMB coloring solution was prepared. 2mL of each of the two reagents were mixed together and set to warm to room temperature.

After the last wash, the liquid was aspirated out by machine, and 100µL of the TMB solution was added to each well. The microplate was placed on a rocker for 20 minutes. Following this time period, an optical density test was performed on each well of the microplate. After recording the results, the plate was placed back on the rocker for an additional 10 minutes before taking another reading.