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Development of Antimicrobial Peptides (AMPs) for Use in Self-Decontaminating Coatings

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ABSTRACT Antimicrobial peptides (AMPs) are a class of short polypeptides usually associated with the host organism's innate immune system. AMPs have been identified in a wide range of host organisms, including plants, amphibians, fish, and humans. These peptides usually consist of 30−100 amino acids and are most often cationic. In addition to a net positive charge, AMPs often are amphipathic, containing both hydrophobic and hydrophilic domains. This property allows for increased interaction with and insertion into negatively charged cell walls and membranes of microbes. Because of the prevalence of antibiotic resistance among common human pathogens, recent research into AMPs has revolved around the attempt to increase the availability of drugs to which microbes are susceptible. Because the mechanism of kill for AMPs is different from that of most conventional antibiotics, which tend to be very specific in their targets, AMPs are thought to be a very attractive future substitute for traditional antibiotics. The development of novel self-decontaminating surfaces containing two AMPs previously isolated from *Chrysophrys major* is reported. These AMPs, Chrysophsin-1 and -3, demonstrated 1−4 logs kill of both Gram-positive and Gram-negative bacteria when incorporated into control acrylic coating systems.

KEYWORDS: peptides • coatings • self-decontaminating surfaces • antimicrobial • surfaces

INTRODUCTION

With the increase in antibiotic resistant microbes, interest in the production of self-decontaminating surfaces has become an area of research that has seen a surge of interest in recent years (1, 2). Such surfaces, when incorporated into commercial products such as children's toys (3), medical devices (4−6) and hospital surfaces (7, 8) could reduce the number of infections caused by pathogenic bacteria. A number of active components for self-decontaminating surfaces have been investigated, including common antibiotics (9, 10), silver ions (4−6), quaternary ammonium salts (11−13), and antimicrobial peptides (AMPs) (14, 15).

Antimicrobial peptides (AMPs) are a class of short polypeptides usually associated with the host organisms innate immune system (16). AMPs have been identified in a wide range of host organisms, including plants, amphibians, fish and humans (17−20). AMPs usually consist of 30−100 amino acids and are most often cationic. In addition to a net positive charge, AMPs are often α-helical and amphipathic, containing both hydrophobic and hydrophilic domains. These properties allow for increased interaction with and insertion into negatively charged cell walls and membranes of microbes (21). Additionally, the amphipathic nature of AMPs makes them an excellent candidate for incorporation into self-decontaminating surfaces comprised of a hydrophilic resin. Amphipathic molecules have been shown to surface segregate within such coatings (11), allowing for increased bioavailability of the antimicrobial component.

Because of the prevalence of antibiotic resistance among common human pathogens, recent research into AMPs has revolved around the attempt to increase the availability of drugs to which microbes are susceptible. Because the mechanism of kill for AMPs is different from that of most conventional antibiotics, which tend to be very specific in their targets, AMPs are thought to be a very attractive future substitute for traditional antibiotics. However, in addition to their antimicrobial properties, many of the currently known AMPs exhibit toxicity to human cells as well (16). One possible strategy for reducing the human toxicity of AMPs is the incorporation of amino acid isomers. Nearly all amino acids found in proteins are the L-isomer. The mirror image D-isomers are almost exclusively found in the cell walls of bacteria. However, recent studies suggest that incorporation of D-isomers alongside L-isomers in previously toxic AMPs reduce their toxicity without greatly reducing their antimicrobial activity (22).

Several applications for AMPs have been investigated, including therapeutic antibiotics (16, 23), medical devices (14, 15, 24, 25), and preservatives (26, 27). Studies regarding the use of AMPs as active ingredients in the form of surface tethered peptides (14, 28, 29), as well as their use as preservatives in latex coatings as an in-can preservative (30, 31) have been reported. However, no investigations of the addition of AMPs to a coating system as a bulk additive and subsequent postcure screening for retention of antimicrobial activity have been reported.

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Of particular interest to this study are two AMPs recently isolated from the gills of the red sea bream, *Chrysophrys major*. These AMPs, Chrysophsin-1 and -3 (Chr-1 and -3) have demonstrated antimicrobial activity in the low micromolar concentrations against both Gram-positive and Gram-negative bacteria (19). However, these compounds have also shown toxicity to human cells, although this toxicity can be reduced by removal of a carboxy-terminal Arg-Arg-Arg-His amino acid sequence not found in other AMPs (32). The effect that removal of this motif will have on antimicrobial activity has not been investigated.

The mechanisms by which AMPs exert their antimicrobial activity likely vary from peptide to peptide and are thought to be determined by such factors as structure, charge, and lipid composition of the target organism (33, 34). AMPs with similar structure and charge characteristics to those found in Chr-1 and Chr-3 are thought to act on bacterial membranes via the "carpet" mechanism, wherein cationic peptides coat the cell membrane via electrostatic interactions with the negatively charged phospholipid head groups present in the membrane. This leads to the formation of transient membrane pores, and eventually membrane disintegration (35).

Herein, we report the development of a novel self-decontaminating surface containing AMPs added as a bulk additive into a commercial acrylate coating system. These coatings demonstrate the ability to kill both Gram-positive and Gram-negative bacteria that come into contact with the surface. In addition, the removal of the carboxy-terminal Arg-Arg-Arg-His motif either greatly reduced (Chr-1) or completely abrogated (Chr-3) the antimicrobial activity of the peptides both in solution and in a coating.

EXPERIMENTAL SECTION

Bacteria and Media. Bacterial strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA.) *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 11229) were used for bacterial Gram-positive and Gram-negative challenges, respectively. Luria–Bertani (LB) media (Difco Laboratories, Detroit, MI), prepared as per the manufacturer's specifications, was used as a bacterial growth and dilution medium for preparation of bacteria for Gram-positive and Gram-negative challenges.

Peptides. Peptides Chr-1, Chr-3, Chr-1 truncated, and Chr-3 truncated were obtained from Biosynthesis, Inc. (Lewisville, TX). Peptides were synthesized then purified by HPLC. Peptides arrived lyophilized and were resuspended in sterile H_2O. For minimum inhibitory concentration (MIC) testing, this suspension was added to media as described below. For surface challenges, this peptide mixture was added to a commercial acrylate coating resin system at 1% (w/w) solids.

Coating Preparation. Films were prepared by combining 25 mg of the respective peptide with 4.95 g of acrylate resin (50% w/w solids) with agitation. The final solution was allowed to stir for an additional 30 min and films were cast by brush onto a precleaned aluminum foil. The coatings were allowed to cure at ambient conditions for 24 h prior to microbial testing. Average thickness of films was determined by a QuaNix 4500 (Automation) paint thickness gauge.

Bacterial Challenge. ***MIC Testing.*** To determine the minimum inhibitory concentration (MIC) of peptides, compounds were weighed and dissolved in sterile water. Each compound was then added to Luria–Bertani (LB) media at concentrations ranging from 0.5 to 0.000667 mg/mL. Bacteria were grown at 37 °C. Log phase cells were harvested by centrifugation, counted on a hemocytometer using bright field microscopy, pelleted by centrifugation at 4000 × g for 10 min, and resuspended in a 0.5% NaCl solution at a concentration of 1×10^7 cfu/mL. To the mixture of LB and biocide was added a 10 μL aliquot containing 1×10^5 colony forming units (CFUs) of either *Staphylococcus aureus* (ATCC 25923) for Gram-positive or *Escherichia coli* (ATCC 11105) for Gram-negative. Cultures were then incubated for 18 h at 37 °C with agitation and examined for turbidity. MIC was determined to be the lowest concentration of biocide that prevented visible bacterial growth at 18 h.

Surface Testing. Bacteria were grown at 37 °C. Log phase cells were harvested by centrifugation, counted on a hemocytometer using bright field microscopy, pelleted by centrifugation at 4000 *g* for 10 min, and resuspended in a 0.5% NaCl solution at a concentration of 1×10^7 cfu/mL. To prevent desiccation of the bacteria during testing, we prepared a hydration chamber. The chamber consisted of a sterile 3 × 3 in gauze pad placed in the bottom of a sterile 150 × 15 mm Petri dish. The gauze pad was saturated with 5 mL of sterile water and the test samples placed on top. A 10 μL aliquot containing 1×10^5 bacteria was added to each test coating (280 mm^2), and then placed in a hydration chamber at room temperature. After 2 h of incubation, the remaining bacteria were recovered by placing the coating in a tube containing 5 mL of sterile LB media, followed by 30 s of vortexing. Serial dilutions were carried out, and incubated for 18 h at 37 °C with agitation. Following incubation, the cultures were examined for the presence of turbidity, indicating bacterial growth. Each coating was tested in triplicate. Log kill was determined by the following: Log kill =5 − highest dilution exhibiting bacterial growth. All bacterial challenge procedures were conducted using standard aseptic techniques in a BSL-2 hood.

Surface Energy and Contact Angle. Surface energy and contact-angle measurements were performed using a VCA 2500 video contact angle system by AST Products, Inc. Contact-angle measurements were taken using the sessile drop technique. An image was captured of the interface 15 s after application and contact angle was measured.

Surface Segregation of Peptide. Each 280 mm^2 section of the test coating was submerged in 0.5% bromophenol blue for 1 min. Coatings were then removed and washed three times with 20 mL of di-H_2O. Bromophenol blue was then recovered from the test samples using 0.05 M HCl in ethanol. Absorbance at 600 nm was determined using a Cary 5E UV–vis–NIR (Varian, Walnut Creek, CA). Beer's Law was used to determine concentration of bromophenol blue and thus concentration of peptides at the surface of the coatings.

RESULTS AND DISCUSSION

Peptides. Antimicrobial peptides have been shown to be effective against a broad spectrum of bacteria, viruses and fungi (36, 37). However, many of these peptides, including Chr-1 and Chr-3, also exhibit varying levels of toxicity to eukaryotic cells (19, 38). Chr-1 and -3 contain a Carboxy-terminal Arg-Arg-Arg-His (RRRH) motif not commonly seen in antimicrobial peptides that when removed greatly reduces the hemolytic activity of the peptides. To determine the effect that the removal of this motif on antimicrobial activity, we studied both full length peptides as well as truncated peptides lacking the RRRH motifs (Table 1).

Minimum Inhibitory Concentration. Minimum inhibitory concentrations for peptides in solution were determined and are summarized in Table 2. MIC for the full

Table 1. PeptideSequences

peptide	sequence[a]
Chr-1	FFWLI***K***GAI***H***AG***K***AI***H***GLI***HRRRH***
Chr-1 truncated	FFWLI***K***GAI***H***AG***K***AI***H***GLI***H***
Chr-3	FIGLLISAG***K***AI***H***DLI***RRRH***
Chr-3 truncated	FIGLLISAG***K***AI***H***DLI

[a] Cationic amino acid residues in bold italics.

Table 2. MIC Results for Peptides in Solution (mg of peptide/mL of culture media)

peptide	Gram +	Gram −
Chr-1	0.00667	0.00667
Chr-1 truncated	0.0133	>0.5
Chr-3	0.00667	0.0667
Chr-3 truncated	>0.5	>0.5

length peptides have been reported previously (19), and our results are similar. Many antimicrobial compounds thought to act on the exterior structures of organisms (cell wall and/or cell membrane) show varying effective concentrations with compounds frequently showing greater effectiveness against Gram-positive strains than Gram-negative (39−41). However, full length peptides show similar results for both Gram-positive and Gram-negative challenges. Removal of the RRRH motif reduced antimicrobial activity for both Chr-1 truncated and Chr-3 truncated, with Chr-3 truncated showing no measurable antimicrobial activity.

Surface Testing. Although the activity of antimicrobial peptides has been the subject of much recent study, little is known about their activity when incorporated as a bulk additive into a resin system. To determine the activity of peptides in the context of a coating, we added 1% (w/w) of each peptide to a commercial acrylic resin system and then allowed to cure for 24 h before testing. Tested coatings had an average thickness of 0.01 mm as measured by a QuaNix 4500 paint thickness gauge. Similar to the MIC results, Chr-1 showed high activity against both Gram-positive and Gram-negative bacteria, Chr-1 truncated and Chr-3 showed moderate activity, whereas Chr-3 Truncated showed none (Figure 1). As with the MIC testing, removal of the C-terminal RRRH motif greatly reduced (Chr-1 truncated) or completely removed (Chr-3 truncated) antimicrobial activity against both Gram-positive and Gram-negative. Unfortunately, the similarity in activity of peptides against Gram-positive and Gram-negative bacteria seen in the MIC results is not present when the peptides are incorporated into a coating.

Surface Energy and Contact Angle. Because of the complex nature of coating and difficulty in ascertaining the surface segregating ability of the peptide, we elected to use the surface contact-angle method and subsequent critical surface energy calculations based on the data obtained. In all cases the surface became more hydrophobic as indicated by the significant increase in contact angle for water. The data generated from ethylene glycol were not significantly different and thus no trend could be drawn, and methylene iodide was consistent with the water data. The critical surface energy was also significantly altered upon addition of all peptides described (Table 3). Upon subsequent analysis, a correlation was observed between critical surface energy and surface antimicrobial properties, especially Gram-positive, and to a much lesser extent Gram-negative. This result is consistent with past reports of surface segregation of amphipathic antimicrobial compounds within hydrophilic resin systems (11).

Surface Segregation of Peptide. Bromophenol blue has been used extensively to quantify the amount of protein or peptide in a solution (42, 43). A test method to visualize surface peptide using bromophenol blue was adapted from these solution test methods. To determine the extent to which peptides were segregated to the surface of test coatings, each coating was reacted with an aqueous solution containing 0.5% bromophenol blue. This solution provided a molar excess of bromophenol blue that was able to undergo an acid−base reaction with the amines of the peptide at a 1:1 ratio. The reaction was then reversed, and the bromophenol blue recovered with 0.05 M HCl in ethanol. The absorbance of this solution at 600 nm was determined and Beer's Law was used to determine the concentration of bromophenol blue (Figure 2). This could be directly converted into number of peptides at the surface and correlated directly to the effectiveness of each coating. We were able to demonstrate an approximate 9 fold increase in the

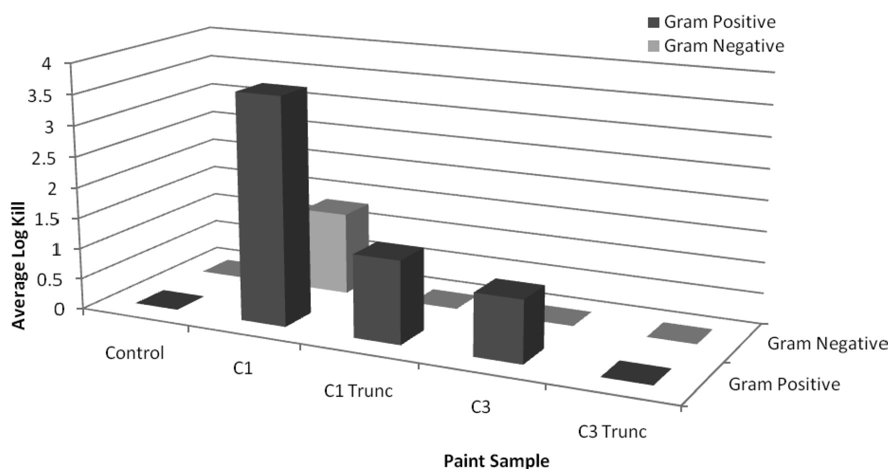

FIGURE 1. Log kill of peptides in commercial acrylic coating.

Table 3. Contact Angle Measurements and Critical Surface Energy

peptide	H_2O	CH_2OHCH_2OH	CH_2I_2	critical surface energy (dyn/cm)
control	38.27 (± 3.05)	60.62 (± 2.00)	58.72 (± 5.30)	90.67
Chr -1	53.05 (± 4.04)	60.77 (± 3.35)	69.45 (± 4.81)	131.6
Chr -1 truncated	50.00 (± 2.66)	65.80 (± 3.43)	62.73 (± 4.20)	111.9
Chr -3	43.88 (± 5.01)	63.02 (± 3.51)	64.57 (± 3.52)	97.22
Chr -3 truncated	46.90 (± 2.96)	62.67 (± 1.90)	70.28 (± 3.80)	101.7

Table 4. Molecules of Peptide at Surface of Coating, With Percentage of Total Peptide Shown in Parentheses

sample	molecules peptide per mm^2 (% of total)
Chr-1	6.8 × 10(13) (~4.5%)
Chr-1 truncated	3.5 × 10(13) (~2%)
Chr-3	1.9 × 10(13) (~1%)
Chr-3 truncated	1.0 × 10(13) (~0.5%)

amount of surface peptide present in the best coating (Chr-1) as compared to the surface peptide present in the least effective coating (Chr-3 truncated) (Table 4).

CONCLUSIONS

The results presented herein represent the first report of a coating containing antimicrobial peptides (AMPs) as an additive for the killing of microbes on its surface. We have demonstrated the ability to produce a novel self-decontaminating surface consisting of an acrylic resin system and AMPs which self-orient and surface segregate as the resin cures. While the MIC results reported here and previously (19) indicate that these peptides are equally effective against Gram-positive and Gram-negative bacteria, this equality was lost when the peptides were incorporated into the coating, perhaps an indication that residues involved in Gram-negative toxicity have become buried within the resin itself. We observed a loose correlation between the MIC data and surface activity, with the most efficacious of the tested peptide containing coatings (Chr-1) able to reduce Gram-positive and Gram-negative bacteria by 99.9% and 90% respectively after two hours. In addition, removal of the C-terminal RRRH amino acid motif from the peptides either greatly reduced (Chr-1 Truncated) or completely destroyed (Chr-3 Truncated) the antimicrobial activity of the peptides. While this strategy is effective in reducing the toxicity of these peptides toward eukaryotic cells (32), the subsequent reduction in antimicrobial activity observed with these truncated peptides makes them unusable as an active antimicrobial additive.

We have also demonstrated a direct correlation between an increase in surface contact angle, indicating an increase in hydrophobic moieties, and antimicrobial activity. While an increase in hydrophobicity at the coating surface does indicate less contact between the coating and bacteria, many previous studies have indicated that the hydrophobic residues present in AMPs are integral to their antimicrobial activity (21, 33−35). Thus, in the case of these coatings, less contact equates to more kill due to the mode of action of the antimicrobial additive. Experiments to determine surface concentration of AMPs utilized a method derived from a

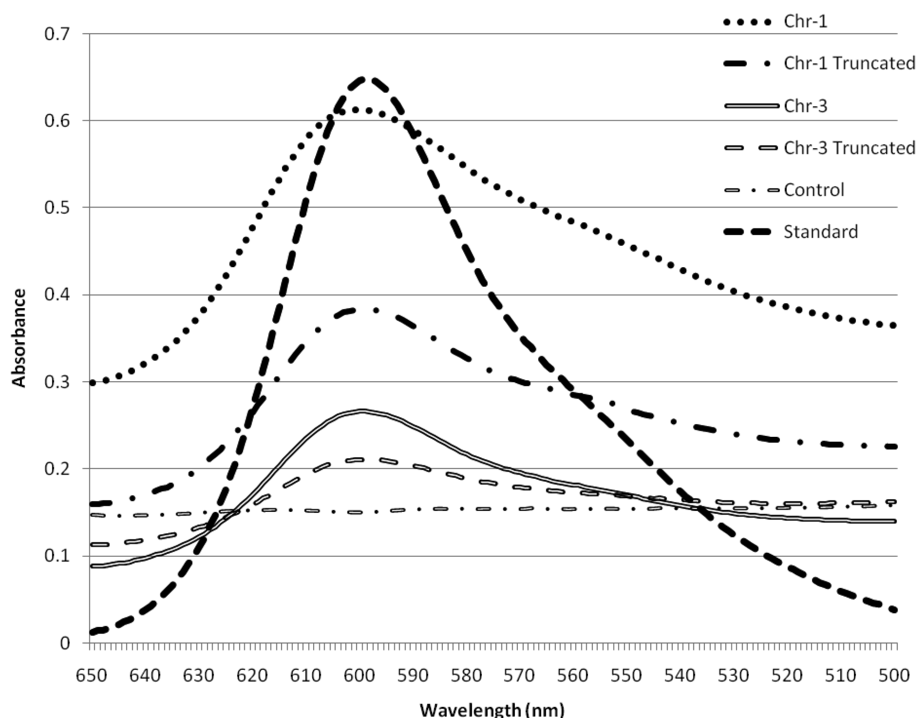

FIGURE 2. Absorbance at 600 nm of bromophenol blue reacted with peptide coatings.

ARTICLE

commonly used method for quantification of proteins and peptides by bromophenol blue staining (42, 43). By utilizing this method to quantify the amount of bromophenol blue bound the surface of our coatings, we were able to demonstrate that amphipathic AMPs possess the ability to surface segregate, and results showed a direct correlation between surface concentration and antimicrobial activity. The coating with the highest activity, Chr-1, showed a 9-fold increase of surface peptide when compared to the least effective coating. Chr-1 was able to reduce bacterial loads by at least 90% while having only 4.5% of available peptides at the surface. Additional studies to determine superior methods of peptide incorporation into a resin system, including tethering, could serve to increase the antimicrobial activity of self-decontaminating surfaces containing AMPs and are currently underway.

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