INCORPORATION OF ANTIMICROBIAL PEPTIDES INTO POLYMERIC FILMS AND COATINGS

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ABSTRACT

Microbial degradation occurs in a variety of materials including polymer composites, metal alloys, paints and textiles, resulting in reduction in physical and mechanical properties. Traditional biocides for protection against materials degradation, such as metal and phenolic compounds, are not environmentally friendly or selective toward the target organisms. Antimicrobial peptides, however, have biocidal activity against bacteria and fungi in microgram quantities and are safe for the environment. To leverage their functionality, innate stability, and durability, antimicrobial peptides are being investigated for biocidal activity in polymeric films and coatings The antimicrobial peptide cecropin P1 was tested for antimicrobial activity against *E. coli* using a semi-quantitative plate overlay assay. Peptides were shown to active in thin films and coatings. Activity was retained after exposure to organic solvents and high temperature, demonstrating that peptides are robust and processible. The use of naturally occurring antimicrobial peptides for material preservation may be possible and has certain advantages to biocides currently in use.

INTRODUCTION

Microbial degradation of materials causes a loss of functionality and decreases product shelf-life. Almost all materials are susceptible to microbial attack, including wood, polymer resins, composites, steel, paints, and textiles. Peptides offer the potential to be used in material preservation applications. The biocidal and biostatic effect of these peptides against bacteria and fungi is well known in the literature.(4, 5, 7, 9)

Antimicrobial peptides are naturally occurring antibiotics that have activity against broad classes of microorganisms. They are cationic, composed typically of 20-50 amino acids and have specific biocidal activity against bacteria and fungi in microgram quantities. Although the precise mechanism of action is not well defined, they are thought to bind to the cell wall, inducing pore formation or membrane destabilization and causing loss of essential cellular metabolites. The specificity of the peptide to a target microbe varies; in general, classes of organisms are targeted. For example, the peptide might be very active against gram-negative bacteria, but exhibit reduced or no activity against gram-positive bacteria or fungi.

Antimicrobial treatments are a popular research area that addresses an ever-growing need to protect soldiers and material from biological attack. The current chemical biocides used for individual soldier protection are primarily non-covalently attached metal-based coatings adsorbed onto solid substrates. They function by leaching to disrupt a microorganism's biochemical processes (e.g. blocking necessary nutrients, inhibiting

pathways). Concentrations will decrease over time causing loss of biocidal activity and require reapplication. Additionally, target microorganisms may develop biochemical resistance to metabolize or inactivate the biocide. Currently used biocides also suffer from other disadvantages including inadequate durability, loss of activity upon surface treatments (i.e. uniform waterproofing) or environmental exposure, and hazards to users and their surroundings.

Both silver and copper based systems have been extensively studied due to the promise that they are effective and that bacterial resistance would not occur. Recent reports, however, demonstrate the evolution of such resistance. Of emerging concern are several strains of *Acinetobacter baumannii* identified with resistance to nine metal ions, including silver.(8) With the recent increase of infections in Iraq from multi-drug resistant (MDR) strains of *A. baumannii* in wounded soldiers, this resistance to silver is of particular concern.(2) The use of metal-based antimicrobial agents also presents environmental issues due to the leaching of ions from the material (a requirement for killing microorganisms). Once having leached, copper ions can be very toxic and for this reason other antimicrobials are being investigated. Antimicrobial peptides offer an environmentally friendly alternative to existing technologies. These naturally occurring compounds kill in a physical manner by binding to and destabilizing the microbial cell membrane. Since they do not disrupt biochemical processes, resistance commonly seen with chemical biocides would not be expected.

Due to the promise that antimicrobial peptides hold, the ability to inhibit bacterial growth was investigated by incorporation of cecropin into polymer films and coatings**.** Cecropin P1, a 31 amino acid residue peptide isolated from nematodes found in porcine intestine(7), has activity against gram-negative bacteria and was the focus of this work. By blending cecropin P1 with polyethylene oxide and polyurethane polymers and casting films, we demonstrate in this report the inhibition of growth of *E. coli*. Activity has been assessed semi-quantitatively using a plate overlay assay in which zones of clearing indicate inhibition of growth of the target organism. Antimicrobial peptides offer an alternative to the disinfectants and biocides currently in use. Unlike antibiotics and other biocides, there have been no reports of microorganisms developing resistance to peptides. Biocides and disinfectants currently in use are hazardous materials that are not environmentally friendly. There are many potential applications for the use of peptides in materials, including preservation of composites and textiles, inhibition of biofouling, and use as filters and wound dressings.

METHODS

The antimicrobial activity of the peptides in solution and in polymeric systems was determined using a plate overlay assay. Peptides were obtained from Sigma-Aldrich Corp. (St. Louis, MO) and solubilized in sterile water at 1 ug/ul. Peptides were applied to an M9 minimal medium agar plate (per liter: 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g

NH_4Cl, 10 ml 10 mM $CaCl_2$, 10 ml 20% glucose, 1 ml 1M $MgSO_4$, 1 ml 10 mg/ml thiamine; 15 g agar), followed by M9 molten agarose containing 10^8 CFU/ml of the test organism. Plates were incubated at 37°C overnight. A zone of clearing around the sample indicated activity. Test organism *E. coli* (ATCC 43827) was grown to log phase in M9 minimal salts medium. Cecropin P1 (5 ug) in water was spotted as a positive control.

Two polymer solutions, polyethylene oxide (Aldrich Chemical, St. Loius, MO) and estane 58277 (Noveon, Inc., Cleveland, OH), were doped with the peptide to generate cast films and coated filters. Polyethylene oxide was solubilized in 50:50 ethanol: water at 5% (w/w), and estane in DMF at 10% (w/w). The desired amount of peptide from the stock solution (1 ug/ul in H_2O) was dried under vacuum. The lyophilized peptide was dissolved in 20 ul of polymer solution. For cast films, the sample was applied to an aluminum pan and dried under vacuum for 1 h at 40°C. The films were transferred to M9 agar plates for analysis by overlay assay. Coated filters were prepared on aluminum pans under the same conditions as the films by adding polymer solution to 6 mm diameter Whatman GF/C glass fiber filters. The activity of peptide in 20 ul of the appropriate solvent was tested as a control. 20 ul of the solvent alone was tested for possible antimicrobial activity (Figure 1).

Cecropin P1 was also immobilized on a microtiter plate to test for of activity. The peptide was synthesized with a C-terminal cysteine allowing for directional attachment to a sulfhydryl reactive surface (Corning Inc., Corning, NY). Dilute peptide to desired concentration in a final volume of 100 ul PED reducing buffer (PBS, 1 mM EDTA, 10^{-4} mM DTT, pH 6.5). Incubate 1 h with mixing. Remove unbound peptide. Wash wells 3x with 150 ul PBS, pH 7.2. Remove wash solution. Add *E. coli* test organism in M9 medium as above. Incubate at 37°C. Measure optical density at A_{595}.

The effect of solvents (methylene chloride, DMF, DMF/THF/dioxane, chloroform) and heat on peptide activity (minimal inhibitory concentration) was performed in a sterile microtiter plate by microdilution assay.(6) *E. coli* cells at 10^5 CFU/ml were incubated in M9 broth in the presence of serial 2-fold dilutions of peptide. The desired quantity of peptide from a 1 ug/ul stock solution in water was dried under vacuum and resuspended in 20 ul of solvent (final concentration 0.625-1 ug). The solvent was removed by drying under vacuum and the peptide solubilized in 20 ul water. The 2-fold dilutions were done by addition of 10 ul peptide with 10 ul water. 90 ul of cells was added to a final volume of 100 ul. Peptide subjected to heat was autoclaved 20 min at 121°C. The solution volume was adjusted to 20 ul with water and diluted as above. Inhibition of growth was determined by measuring optical density at A_{595} after an incubation time of 17 h at 37°C. Peptide in water was used as a positive control.

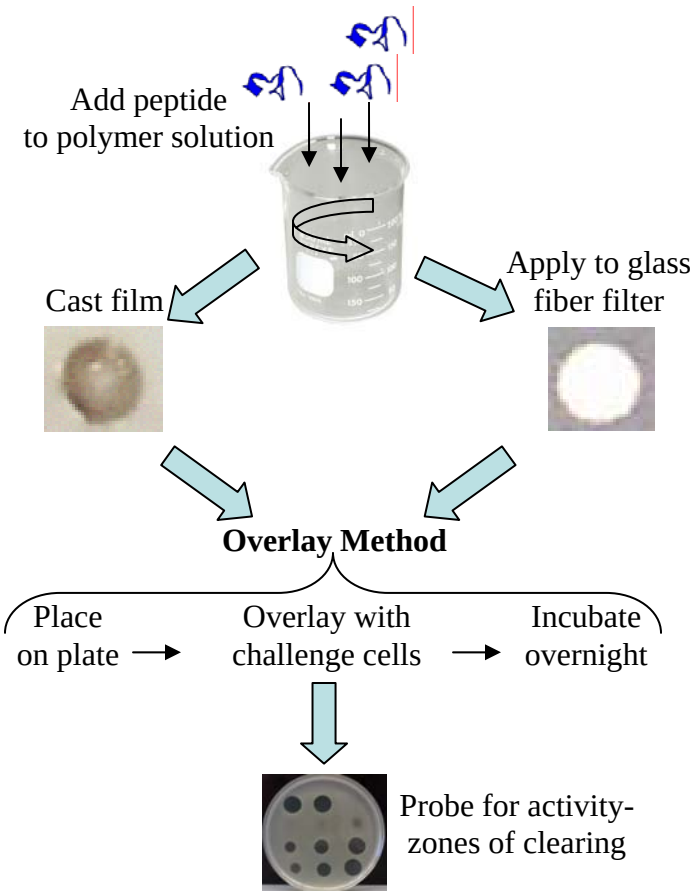

Figure 1. Overlay method for determining antimicrobial activity of films and coatings.

RESULTS

Antimicrobial activity in polymeric systems. Cecropin P1 incorporated into cast films exhibited antimicrobial activity (Figure 2). Peptides exhibited higher activity in PEO than in estane. Since PEO is water soluble the film essentially dissolves, allowing more cecropin P1 to inhibit bacterial growth. No activity is seen below 0.5 ug on PEO or 5 ug in estane (data not shown).

Cecropin also demonstrated activity in polymers used as a coating, in this case to coat glass fiber filters (Figure 3). The results were similar to the cast films; less cecropin P1 was required to exhibit antimicrobial activity in PEO relative to estane, presumably due to PEO's solubility in water. The zone of clearing for 5 ug peptide in an estane coated filter was larger than the cast film, indicating a greater amount is available for inhibiting growth. The coated filters may have larger surface areas than the films, which would increase peptide bioavailability.

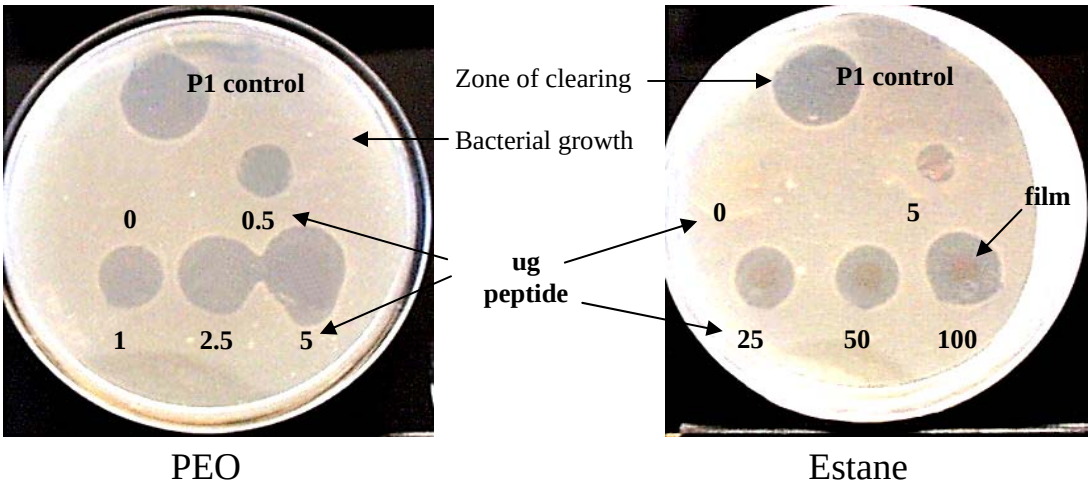

Figure 2. Incorporation of cecropin P1 into polymer films. Activity seen in both polymers, but much more peptide needed for activity in water insoluble estane. Zone of clearing shows inhibition of bacterial growth. 5 ug peptide in water used as a control.

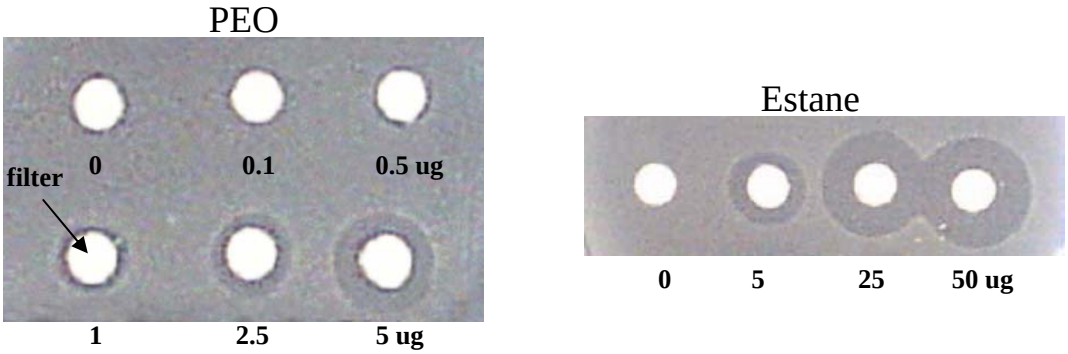

Figure 3. Incorporation of cecropin P1 into polymer coatings on glass fiber filters. Activity seen in both polymers, but much more peptide needed for activity in water insoluble estane. Zone of clearing shows inhibition of bacterial growth.

Immobilization. Immobilization of cecropin P1 showed possible activity before cells continued to grow (data not shown). It is possible that the amount of peptide immobilized was below the minimal inhibitory concentration needed for activity (theoretical maximum binding capacity is about 1.5 ug cecropin P1 per well). If the amount of peptide is less than the minimum concentration needed, some cells survive to continue growth.

Activity in solvents. Activities of several peptides have been tested. Cecropin P1 activity after exposure to extreme environments is not inhibited. Exposure to solvents and high temperature did not significantly decrease antibacterial activity (Figure 4). Three other peptides, dermaseptin, indolicidin and PGQ also showed little loss of activity under these conditions (data not shown).

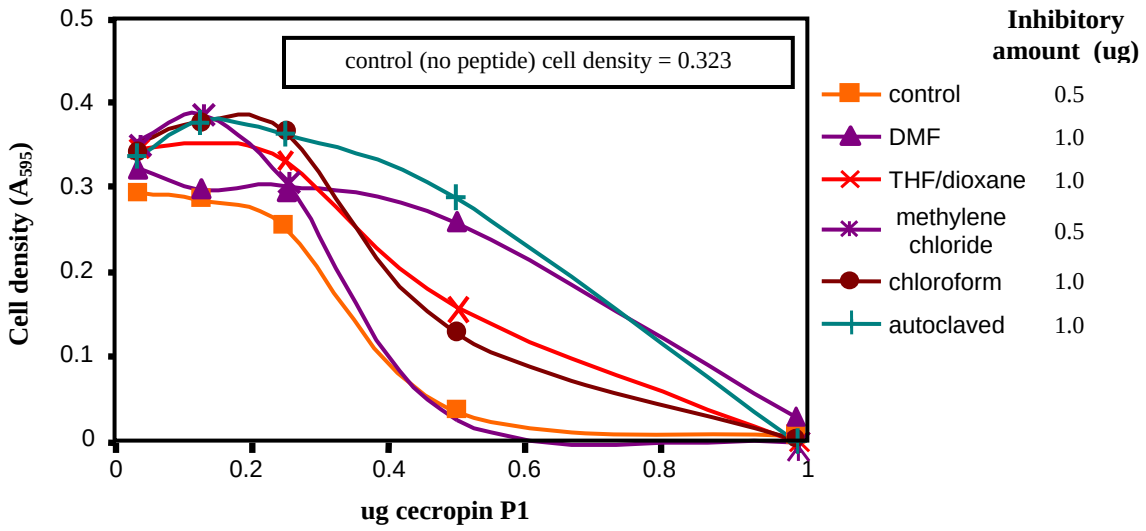

Figure 4. Cecropin P1 activity after exposure to solvents and heat determined by microplate assay. Peptide retains activity although is slightly reduced by some treatments. Cells were grown overnight at 37°C.

DISCUSSION

The antimicrobial peptide cecropin P1 demonstrated activity in polymer films and coatings toward gram-negative bacteria using a plate overlay assay. Table 1 summarizes these results.

TABLE 1. Antibacterial activity of cecropin P1 in polymeric systems. More peptide required in estane because the polymer is not water soluble.

	ug peptide in:	
	PEO (water:ethanol)	Estane (DMF)
Solution	0.5	5
Cast film	0.5	5
Coated filter	1	5

In PEO the activity of cecropin was similar to that of the peptide in water, requiring only 0.5 ug of peptide. This was not completely unexpected; since PEO is water soluble, the polymer essentially dissolved in the overlay assay, leaving most or all of the peptide available to inhibit bacterial growth. This is consistent with results from incorporation of the antimicrobial peptide nisin into polymer films(1), where higher activity was observed with PEO relative to polyethylene. A slightly larger amount of peptide (1 ug) was needed in the coated filter. Estane, however, is a water insoluble polyurethane that required more cecropin P1 to demonstrate activity; 5 ug was needed for minimal activity in both cast films and coated filters. While investigating this result, it was noted that peptide added to glass fiber filters without an estane coating also exhibited activity and retained it even after washing. This may result from the peptide being physically entrapped within the filter or bound to the filter via an electrostatic interaction.

One of the more interesting results was that antimicrobial peptides are robust, retaining activity even after exposure to solvents and heat/pressure. The solvents without peptide were checked with the overlay assay and no inhibition of bacterial growth was found. Cecropin P1 in DMF solvent remained active. This observation was not expected; typically biological molecules must be kept at or near physiological conditions to remain active. The ability to withstand extreme conditions is presumably due to the peptides' short length and lack of secondary structure that otherwise might undergo denaturation under harsh conditions. The peptide only assumes the active α-helical conformation when it contacts and binds to the cell wall of the target organism.(3) These characteristics make the peptides processible, potentially able to withstand the conditions used in manufacturing of materials that many other biological molecules could not.

While the peptides show activity when simply added to the polymer, immobilization of peptides on the surface of a material might improve its antimicrobial activity. A greater proportion of the peptide would be available to bind to and kill the target organisms and not be trapped below the surface. Immobilization of peptides has been attempted on solid supports and there are indications of activity. The peptide was randomly immobilized, such that only a portion would be expected to be in the correct orientation to possess activity. The use of an immobilization chemistry that controls peptide orientation may result in an increase in activity.

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