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Antimicrobial Activity of a 14-Residue Synthetic Peptide against Foodborne Microorganisms

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ABSTRACT

A chemically synthesized short-chain peptide composed of six leucine and eight lysine (6K8L) residues was demonstrated to be biocidal against several foodborne organisms including *Escherichia coli* O157:H7, *Listeria monocytogenes, Pseudomonas fluorescens,* and *Kluyveromyces marxianus* suspended in phosphate buffer at concentrations of 5 to 50 μg/ml. All strains were reduced by 3 $\log_{10}$ CFU/ml within 10 min at peptide concentrations of <10 μg/ml. The peptide reduced by 3 $\log_{10}$ CFU/ml *E. coli* O157:H7 counts in apple juice and was active over the pH range of 3.5 to 7. Peptide concentrations of 100 μg/ml inhibited the aerobic and anaerobic microorganisms present in meat exudate liquid. However, the peptide was not effective against *E. coli* O157:H7 in skim milk at concentrations up to 100 μg/ml.

Microbial activity is a primary mode of deterioration of many foods and is often responsible for the loss of quality and safety. Concern over pathogenic and spoilage microorganisms in foods is increasing due to increases in foodborne disease outbreaks, new evolving strains of foodborne pathogens *(23),* and an increased demand for long shelf-life minimally processed foods *(17).*

Particular attention has been paid to *Escherichia coli* O157:H7 that has been involved in recent outbreaks associated with consumption of ground beef and milk *(20, 25),* apple cider and juice *(13),* dry fermented sausage, mayonnaise-containing foods *(4),* and yogurt *(15).* This and other pathogens occur with such frequency in many foods that the risk for disease is significant.

One approach to limiting microbial deterioration of foods is the direct addition of antimicrobials such as nitrites, nitrates, sulfur dioxide, and organic and inorganic acids. New sources of antimicrobials have been explored including spices, essential oils, oleoresins, enzymes, and proteins. The use of peptides as food antimicrobials is appealing due to their wide spectra of activity, low mammalian toxicity, and heat stability *(16).* However, with the exception of bacteriocins, few have been tested for food applications *(1, 24).* To date, nisin is the only antimicrobial peptide (bacteriocin) used commercially as a biopreservative in processed cheese, dairy products, milk, and canned foods *(7).* Other bacteriocins such as pediocins and sakacin A could potentially be used in foods because they prevent the growth of *L. monocytogenes* and selected spoilage organisms in fermented meats and ground beef, respectively *(8, 21).*

The potential application of peptides from animal origin to foods has been studied. The effect of magainin and a synthetic magainin analog (mag2 amide) on bacterial strains associated with foodborne disease was investigated in detail *(1).* Mag2 amide exhibited the highest activity causing reductions up to 8.7 $\log_{10}$ CFU/ml. Antimicrobial activity was reduced at lower temperatures and in the presence of bovine serum albumin.

Several studies have demonstrated the enhanced antimicrobial activity of chemically synthesized analogs compared to naturally occurring peptides *(12, 26).* A series of lysine–leucine peptide analogs of 11 to 18 amino acid residues were reported to inhibit *Staphylococcus aureus, E. coli,* and *Pseudomonas aeruginosa.* The analogs composed of 14 or 15 residues exhibited the highest antimicrobial activities, and the 14-amino acid residue analog had the lowest hemolytic activity *(2).* Other leucine–lysine peptides that were 14 amino acid residues long were antimicrobial against selected gram-positive and gram-negative bacteria of clinical significance *(6).* The exact sequence of the leucine and lysine residues influences activity, presumably, by maintaining amphiphilic structure to the α-helix *(12).*

The structure of the 14-residue peptide utilized in this work is based on previous studies *(2),* indicating that this structure imparts high antimicrobial activity and low hemolytic activity. Similar 14-residue peptides have shown activity against clinical isolates prompting us to test this sequence. The objective of this study was to assess the effect of this peptide on a range of foodborne microorganisms. The peptide was tested against several spoilage and pathogenic microorganisms in buffer and against *E. coli* O157:H7 under food-like conditions. Our long-term goal is the use of antimicrobials in food contact surfaces such as packaging materials.

MATERIALS AND METHODS

Peptide synthesis. We synthesized a peptide with the sequence HOOC-L-K-L-L-K-K-L-L-K-L-L-K-K-L-NH_3 (6K8L) by automated solid-phase peptide synthesis using standard F-moc

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chemistry *(5)* on NovaSyn TG resin (0.24 mmol/g; Novabiochem, La Jolla, Calif.) on a MilliGen 9050 (Millipore, Bedford, Mass.) peptide synthesizer utilizing the Cornell University Biotechnology Bioresource Center facilities and equipment. The homogeneity of the peptide was determined by reverse high-pressure liquid chromatography and by capillary electrophoresis. Reverse high-pressure liquid chromatography was performed on a PE Biosystems 130 (Applied Biosystems, Foster City, Calif.) on a Microbore C_{18}, 2- by 100-mm column (YMC Inc., Willmington, N.C.). The peptide was eluted with a linear gradient of 5 to 60% acetonitrile in water containing 0.1% trifluoroacetic acid at a flow rate of 0.24 ml/min at 30°C. UV absorbance was monitored at 215 nm. Capillary electrophoresis was performed in a fused silica capillary of 72 cm long with a 50-μm internal diameter (Applied Biosystems). The carrier electrolyte solution was 50 mM phosphate buffer (pH 2.5). Hydrodynamic injections were made (5 s). Electrophoresis was carried out in the positive mode on an Applied Biosystems model 270 A capillary electrophoresis system (Applied Biosystems) at 25°C with a potential of 20 kV. The electropherogram was monitored at 200 nm.

The peptide molecular mass was confirmed by matrix-assisted laser desorption ionization mass spectrometry on a Lasermat 2000 (Thermo Bioanalysis Corp., Santa Fe, N.M.) mass spectrometer using a matrix of α-cyano-4 hydroxycinnamic acid. The mass was determined relative to an external calibration with bovine pancreatic ribonuclease A of 13,683 Da (Sigma, St. Louis, Mo.).

Inocula preparation. The following strains were used for the antimicrobial assays: *Bacillus subtilis* (wild-type PB2, 168 Marburg strain) *(18), E. coli* O157:H7 (ATCC 33150; Rockville, Md.), *Kluyveromyces marxianus* (cheese isolate, Food Science Dept., Cornell University), *Listeria monocytogenes* (ATCC 689426), *Pseudomonas fluorescence* (milk isolate, Food Science Dept., Cornell University), *Salmonella* Typhimurium H 3380 phage type DT 104 (U.S. Department of Agriculture, Washington, D.C.), *Serratia liquefasciens* (skim milk isolate, Food Science Dept., Cornell University), *Staphylococcus aureus* (ATCC 13566). All cultures were maintained frozen (−40°C) in trypticase soy broth (Becton Dickinson, Cockeyville, Md.) containing 10% glycerol or refrigerated (6°C) in trypticase soy agar (TSA) slants. The cells were grown overnight at 25°C by transferring a loop-full of cell colonies from TSA to 20 ml trypticase soy broth. A 50-μl aliquot from the overnight culture was transferred to fresh trypticase soy broth and grown at 25°C to midexponential phase of growth. Growth was monitored by the Klett–Sommerson photoelectric colorimeter and by standard pour plate count on TSA *(22).* The cells were centrifuged, washed with pH 7.2 phosphate buffer, and serially diluted in phosphate buffer (pH 7.2) to be used as inocula.

Antimicrobial activity of peptide 6K8L: Activity in buffer. We developed concentration–inhibition curves by dissolving and diluting lyophilized synthetic peptide 6K8L in phosphate buffer (pH 7.2) to concentrations of 1, 5, 10, 25, 50, and 100 μg/ml. The peptide solutions were inoculated with midexponential cells to final counts of 10^6 to 10^7 CFU/ml. The cell suspensions were incubated at 25°C. Samples were taken at 10, 30, and 60 min, diluted in buffer, and enumerated on TSA by the pour plate standard method *(22).* Cells suspended in pH 7.2 phosphate buffer without peptide 6K8L served as controls. Individual points on the concentration (i.e., dose) versus colony count curves are single observations.

Activity in apple juice and skim milk. Pasteurized apple juice (pH 3.7) and skim milk were obtained from the Dairy Store at Cornell University. The foods were autoclaved at 121°C and peptide 6K8L was added to final concentrations of 1, 5, 25, 50, and 100 μg/ml. The foods were inoculated with 10^4 to 10^5 CFU/ml of *E. coli* O157:H7 and incubated at 25°C. Samples were taken at 0, 2, 4, and 8 h and enumerated by standard methods on TSA *(22).*

Activity in meat exudate liquid. Meat exudate liquid was obtained from commercially available cut beef packaged in polystyrene foam trays. One-tenth milliliter of buffer with a peptide concentration of 1,000 μg/ml was added to 0.9 ml of meat exudate liquid for a final peptide concentration of 100 μg/ml. This mixture was incubated at 25°C for 6 or 12 h and enumerated for aerobic and anaerobic total plate counts in nutrient agar by standard methods *(22).* The controls were 0.9 ml of meat exudate to which 0.1 ml of sterile buffer was added.

Effect of pH on antimicrobial activity. *E. coli* O157:H7 were inoculated in 0.1 M citrate buffer of pH 3.5 to 7 to final concentrations of 10^6 to 10^7 CFU/ml. Peptide was added to the cell suspensions to give a concentration of 5 μg/ml and the suspensions incubated at 25°C. The controls were cells suspended in pH 3.5 to 7 citrate buffers without peptide 6K8L. After 10 min, samples were taken and enumerated by pour plate on TSA *(22).* The experiment was done in triplicate. Significant differences between the treated cell count means were determined by one-way analysis of variance and the Tukey tests.

RESULTS

Activity of peptide 6K8L against foodborne microorganisms. All strains incubated with peptide, including the yeast *K. marxianus,* demonstrated a reduction in counts compared to controls that showed no reduction in cell counts. In all cases, peptide concentrations of <10 μg/ml caused 3 $\log_{10}$ CFU/ml or more reductions in counts from initial count of approximately 10^7 CFU/ml. Longer incubation times further reduced counts until they reached too few to count (<10^1 CFU/ml).

Reduction was dependent on the concentration of peptide 6K8L: as the concentration increased, fewer CFU/ml were detected in the buffer after 10 min (Fig. 1). Concentrations of 5 μg/ml and higher reduced approximately 10^7 CFU/ml of *B. subtilis, L. monocytogenes,* and *K. marxianus* to <10 CFU/ml in 10 min while *E. coli* O157:H7, *Salmonella* Typhimurium, and *S. aureus* required at least 25 μg/ml for a similar reduction. *S. liquefasciens* and *P. fluorescens* were the most resistant, requiring between 50 and 100 μg/ml, respectively, to be reduced by 6 $\log_{10}$ CFU/ml.

Effect of pH on peptide 6K8L activity. A peptide concentration of 5 μg/ml had little effect on counts at pH 7.0, but counts at pH 3.5 were reduced from 10^7 to < 10^4 CFU/ml. Higher peptide concentrations reduced counts at pH 7.0 as well as at lower pHs. The viable counts of *E. coli* O157:H7 suspended for 10 min in pH 3.5 to 7 buffers without peptide did not decrease and remained ~10^7 CFU/ml (Fig. 2). The number of viable cells remaining after exposure to 5 μg/ml of peptide for 10 min generally decreased as the pH decreased, suggesting that the peptide was more effective at lower pH (Fig. 2). However, significant differences ($P \leq 0.05$) were observed only between the cell reductions at pH 3.5 to 4.0 and pH 6 to 7.

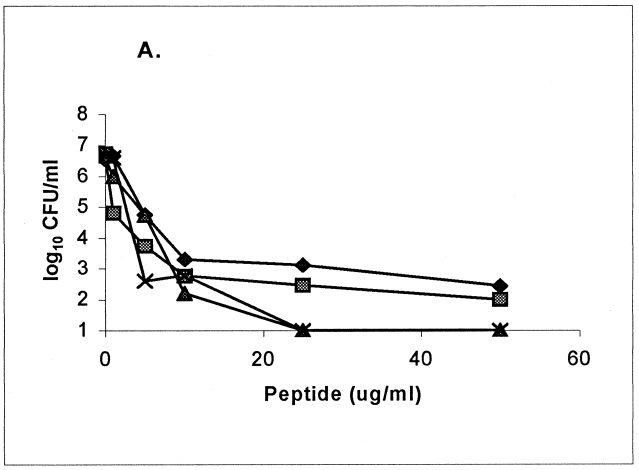

FIGURE 1. *Effect of synthetic peptide 6K8L concentration on the counts of (A)* P. fluorescens *(♦),* S. liquefasciens *(■),* Salmonella *Typhimurium (▲),* E. coli *O157:H7 (×), and (B)* S. aureus *(♦),* L. monocytogenes *(■),* B. subtilis *(▲),* K. marxianus *(×) suspended in phosphate buffer (pH 7.2) at 25°C for 10 min.*

Effect of peptide 6K8L on *E. coli* O157:H7 in apple juice and skim milk. The counts of *E. coli* O157:H7 in control apple juice (pH 3.7) decreased by 1 $\log_{10}$ CFU/ml after 8 h of incubation at 25°C in the absence of peptide. Addition of 5 to 100 μg/ml of peptide resulted in decreased counts in a peptide concentration-dependent manner (Fig. 3). After 8 h, the counts were reduced by 3.5 $\log_{10}$ CFU/ml at a peptide concentration of 100 μg/ml. Peptide concentrations of 25 and 5 μg/ml reduced cell populations by ~1.3 $\log_{10}$ CFU/ml in 8 h. The counts from *E. coli* O157:H7 treated with 1 μg/ml of peptide were not different than the controls.

Peptide concentrations of 100, 50, and 25 μg/ml had no effect on 10^7, 10^5, 10^3, and <10 CFU/ml of *E. coli* O157:H7 growth in skim milk. *E. coli* O157:H7 in skim milk with and without peptide 6K8L grew at the same rate. For example, an initial inoculum of 3.3×10^3 CFU/ml of *E. coli* O157:H7 grew to 2.4×10^5 CFU/ml after 8 h incubation at 25°C. The same inoculum incubated for 8 h

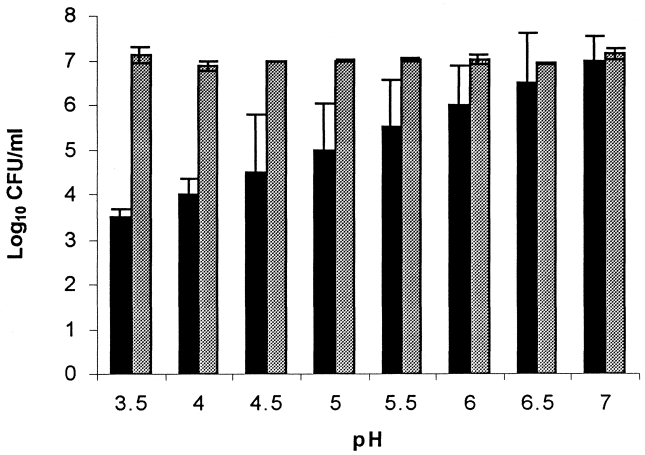

FIGURE 2. *Antimicrobial activity of synthetic peptide 6K8L against* E. coli *O157:H7 suspended in 0.1 M citrate buffers pH 3.5 to 7.0. Midexponential* E. coli *O157:H7 (~10^7 CFU/ml) were incubated for 10 min at 25°C in the presence of 0 μg/ml (□) and 5 μg/ml (■) of peptide.*

with 50 and 100 μg/ml of peptide grew to 2.4×10^5 and 2.2×10^5 CFU/ml, respectively.

Effect of peptide 6K8L on meat exudate microbial flora. The aerobic counts from meat exudate containing 100 μg/ml of peptide were ~2 $\log_{10}$ CFU/ml less than untreated controls after 6 h at 25°C; after 12 h the treated samples rebounded to counts that were similar to the controls. The anaerobic count differences between the controls and the meat exudate liquid treated with 100 μg/ml peptide were ~1 and 0.7 $\log_{10}$ CFU/ml after 6 h and 12 h of incubation, respectively (Table 1).

DISCUSSION

Synthetic peptides composed solely of leucine and lysine have been tested against a limited number of organisms of clinical relevance *(6).* To our knowledge, these or similar short-chain synthetic peptides have not been tested against

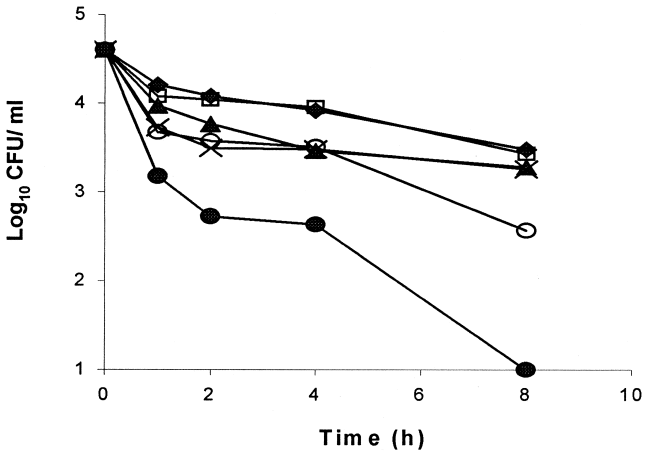

FIGURE 3. *Effect of synthetic peptide 6K8L concentration on the counts for* E. coli *O157:H7 in apple juice (pH 3.7) at 25°C. The concentrations of peptide 6K8L in apple juice were: 0 μg/ml (♦), 1 μg/ml (□), 5 μg/ml (▲), 25 μg/ml (×), 50 μg/ml (○), and 100 μg/ml (●).*

TABLE 1. *Antimicrobial activity of peptide 6K8L on flora present in meat exudate liquid*

Time (h)	Aerobic count (CFU/ml)		Anaerobic count (CFU/ml)	
	Control	Peptide (100 μg/ml)	Control	Peptide (100 μg/ml)
0	1.2×10^5	1.2×10^5	1.2×10^5	1.2×10^5
6	4.8×10^6	1.2×10^4	4.4×10^6	3.2×10^5
12	4.1×10^7	1.4×10^7	7.6×10^7	1.2×10^7

food pathogens and spoilage organisms. The survival assay study in buffer showed that the synthetic 14-amino acid peptide 6K8L exhibited antimicrobial activity against all the foodborne microorganisms tested. The antimicrobial activity was nonspecific for gram-negative or gram-positive bacteria and was effective against yeast. This broad activity has been shown elsewhere for certain larger, more complex peptides of animal origin *(1, 14, 26).* Counts for all strains including *E. coli* O157:H7 were reduced within 10 min of exposure to low μg/ml concentrations of peptide 6K8L.

The observed inhibition of *E. coli* O157:H7 in apple juice suggests that the peptide could potentially inhibit microorganisms present in selected foods. Because foods are complex systems with variables such as pH, ionic strength, temperature of processing/storage, and chemical composition, the effects of these and other parameters on the activity of antimicrobial compounds require testing. pH influences the type of microorganisms that can contaminate foods as well as their growth rate. Changes in pH may also affect the structure (amphiphilic α-helix) of the peptide and thus, its antimicrobial potency. The influence of pH on the activity of some bacteriocins has been previously studied. In some cases bacteriocins were active within a pH range of 2 to 9 *(19),* while in others the activity was decreased near neutral pH due to poor solubility and stability *(16).* In the present study, the 14-amino acid peptide 6K8L was bactericidal against *E. coli* O157:H7 suspended in citrate buffers of pH 3.5 to 7. Cell death was significantly greater at pH 3.5 and 4 compared to 7. Because the theoretical net charge on the peptide at pH 3 to 9 is +6, the stability of the peptide in the citrate buffers at pH 3.5 to 7 was probably not altered. Instead, higher activity at low pH could be the result of a decrease in the resistance of *E. coli* O157:H7 to acidic external pH due to an increased proton conductance caused by the peptide *(10).*

Although these results indicate that the peptide was more effective at acidic pH, it cannot be concluded that the use of peptide 6K8L is more suitable to reduce *E. coli* O157:H7 in high acid foods without further study. *E. coli* O157:H7 cells growing in acidic foods are more likely to adapt to acidic conditions. Acid-adapted cells have different survival rates compared to nonadapted cells exposed to low pH conditions *(11).* In the present study, the cells used in the pH experiments were not previously adapted to acidic conditions.

Survival of *E. coli* O157:H7 in acidic conditions is one of the main concerns in food safety *(11).* Here *E. coli* O157:H7 survived for more than 8 h in apple juice without peptide 6K8L (Fig. 3). Survival was reduced in the presence of peptide 6K8L and less than 10 CFU/ml survived after 8 h at peptide concentrations of 100 μg/ml. The peptide was less effective against *E. coli* O157:H7 in apple juice compared to phosphate buffer (pH 7.2). In the apple juice, $\sim 10^4$ CFU/ml of *E. coli* O157:H7 were reduced by 3 $\log_{10}$ in 8 h with 100 μg/ml of peptide 6K8L (Fig. 3). The same reductions were obtained in 10 min with less than 5 μg/ml of peptide 6K8L in buffer.

When tested in skim milk, no antimicrobial activity of peptide 6K8L on the *E. coli* O157:H7 was found. Neither bacteriostatic nor bactericidal effect was observed in the cells even when the initial inoculum was reduced to 10 CFU/ml and when the concentration of peptide was increased to 100 μg/ml. It is unlikely that the lack of activity of the peptide on the skim milk was related to the growth kinetics of the bacteria, because the growth of *E. coli* O157:H7 in skim milk paralleled that in trypticase soy broth (data not shown).

Some studies have previously shown that the activity of peptides such as magainin, pediocin PA-1, and sakacin A can be decreased in the presence of bovine serum albumin or foods of high protein content *(1, 16).* In addition, the antimicrobial activity of nisin on *L. monocytogenes* in fluid milk decreases as the milk fat content increases *(9).* Therefore, components such as lipids and/or proteins in skim milk could be interacting with the peptide and annulling its activity.

Meat exudates are good substrates for microbial growth *(3).* The reductions in aerobic and anaerobic counts due to peptide addition were less than 1 $\log_{10}$ CFU/ml, suggesting a small degree of inhibition.

These data indicate that peptide 6K8L inhibits several food-related organisms in synthetic media, suggesting that further investigations into efficacy in different foods and mechanisms of inhibition are warranted. In order to gain approval as food preservatives, detailed microbial, nutritional, and toxicological studies on this and other similar peptides would be required, as noted for other antimicrobial peptides *(1).*

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