Identification of Novel Antimicrobial Peptides to be used in Functional Foods

Sean Doering[1], Michael Thomas[1], Jennifer Grant[2] Ph.D., Eun Joo Lee[3] Ph.D.

1. The Applied Science Program, University of Wisconsin-Stout, Menomonie, WI 54751 2. Biology Department, University of Wisconsin-Stout, Menomonie WI 54751 3. Food and Nutrition Department, University of Wisconsin-Stout, Menomonie WI 54751

Introduction

Phosvitin, an egg yolk protein similar to casein in milk, is the most phosphorylated protein found in nature. The resulting anionic charge allows phosvitin to bind important bivalent ions such as zinc, iron, magnesium, and calcium[1]. Because of its metal binding properties, phosvitin shows intriguing potential as a source of peptides that could be used in functional foods. Anti-bacterial properties of phosvitin have come to light as well. In this abstract we report on the antibacterial properties associated with phosvitin, and also discuss work to identify potential peptides that could be used in functional foods as either calcium delivery vehicles or antibacterial agents.

In previous studies, phosvitin has only been shown to be antimicrobial alone against *Escherichia coli* under thermal stress, and against *E. coli* and *Staphylococcus aureus* when combined with chitosan[2-3]. We have found that when bound to a bivalent cation such as zinc, phosvitin has previously been shown to elicit antimicrobial activity against certain bacterial species isolated from human hands (unpublished results). Since *Micrococcus luterus* is a hand bacteria associated with human pathology, an isolate of this bacteria was utilized in antibacterial tests of phosvitin activity. Both Kirby Bauer and broth dilution antimicrobial tests were utilized to determine any potential antimicrobial activity of phosvitin alone, and in the presence of zinc or calcium.

Work is also underway to identify potential calcium-binding peptides derived from phosvitin and identify potential antibacterial activity that may be associated with them. We synthesized two peptides consisting of residues 2-13 of phosvitin (Figure 1). Peptide 1 was unchanged while peptide 2 included a phosphorylation post-translational modification on residue 12 resulting in the peptide of AEFGTEPDAKpTS. This peptide was chosen based on an analysis of calcium binding literature[4-5]. The binding capacity and antimicrobial activity of this peptide will be compared to phosvitin as a potential bioavailable replacement.

Figure 1A. Illustrates the peptide where red represents a negative charge, blue represents a positive charge, and white represents a neutral charge. Threonine will be phosphorylated to produce a negative charge that enhances the peptide's charge pattern for calcium binding and antimicrobial activity. The phosphorylated threonine and the resulting negative charge is marked with an asterisk. **B:** Illustrates the peptide without the phosphorylated threonine. Both peptides were synthesized and used in the research to determine the optimum charges and charge spacing on a small peptide.

Materials and Methods

Kirby Bauer and broth dilution antimicrobial tests were used to determine antimicrobial effects in all of the experiments performed in this research. Bacteria grown at 37˚C in LB broth for 24 hours in shakers at 250 rpm were used to inoculate the Kirby Bauer and serial dilution tests. For Kirby Bauer tests, a lawn of bacterial solution was spread across the plate immediately before treatment. Broth dilution treatments were inoculated with 10 µl of bacterial solution. Experimental treatments were cultivated at 37˚C for 48 hours (at 250 rpm for broth dilutions). Discs used in Kirby Bauer tests were created from autoclaved filter paper and dipped into the treatment solution immediately before being placed on the plate inoculated with bacteria.

Experiment A

25 mg/ml phosvitin alone, with 1 mM $ZnCl_2$, or with 1 mM $CaCl_2$ was used to treat Kirby Bauer tests inoculated with *S. marsescens, Bacillus cereus, Micrococcus luteus, Staphyloccoccus epidermidis, Escherichia coli, Proteus mirabilis,* and *Pseudomonas aeruginosa.* Discs treated with 1 mM $ZnCl_2$ or 1 mM $CaCl_2$ were used as controls in their respective experimental group against plates inoculated with the tested bacteria.

Experiment B

25 mg/ml, 10 mg/ml, and 1 mg/ml phosvitin with 1 mM $CaCl_2$ LB broth dilutions were created and inoculated with either *B. cereus* or *M. luteus.* A follow up two-fold serial dilution experiment of 1000-3.9 µg/ml phosvitin in 1 mM $CaCl_2$ was performed against *M. luteus*. Bacteria alone and in 1 mM $CaCl_2$ LB broth was used as controls.

Experiment C

Peptide 1 and peptide 2 were used in two-fold serial broth dilutions from 1000-3.9 µg/ml in 1 mM $CaCl_2$ LB broth against *M. luteus*. A non-phosvitin derived peptide GPTRHLG was used as a control at the same concentrations in 1 mM $CaCl_2$ LB broth against *M. luteus*.

Results

Experiment A

Bacteria	Treatment Antimicrobial?
Bacillus cereus	No
Micrococcus luteus	Yes
Staphylococcus epidermidis	Reduced Growth
Escherichia coli	No
Proteus mirabilis	No
Pseudomonas aeruginosa	No
Serratia marsescens	No

Bacteria	Treatment Antimicrobial?
Bacillus cereus	Yes (little)
Micrococcus luteus	Yes
Staphylococcus epidermidis	No
Escherichia coli	No
Proteus mirabilis	No
Pseudomonas aeruginosa	No
Serratia marsescens	No

Bacteria	Treatment Antimicrobial?
Bacillus cereus	Yes (little)
Micrococcus luteus	Yes
Staphylococcus epidermidis	No
Escherichia coli	Reduced Growth
Proteus mirabilis	Reduced Growth
Pseudomonas aeruginosa	No
Serratia marsescens	No

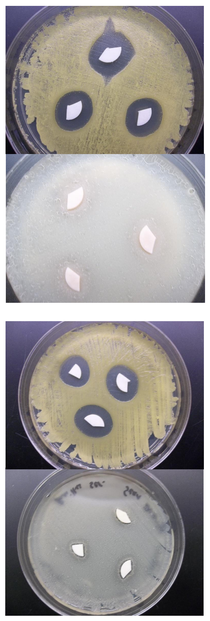

Figure 2. Table portraying whether or not treatments were antimicrobial against bacterial strains. **Top:** 25 mg/ml phosvitin alone. **Middle:** 25 mg/ml phosvitin in 1mM $ZnCl_2$ solution. **Bottom:** 25 mg/ml phosvitin in 1 mM $CaCl_2$ solution. Pictures show Kirby Bauer tests against the two bacterial strains affected most by the antimicrobial tests: *Micrococcus luteus* (top) and *Bacillus cereus* (bottom).

Experiment B

B. cereus was not notably inhibited at any concentration while *M. luteus* was inhibited at the 25, 10, and 1 mg/ml concentrations in 1 mM $CaCl_2$. Two-fold serial dilution of the treatment yielded a minimum inhibitory concentration (MIC) between 31 and 62 ug/ml (~0.733 uM) against *M. luteus*.

Experiment C

Neither peptide 1, peptide 2, or the control peptide was able to inhibit *M. luteus* at 1 mg/ml (~798.5 uM) in a 1 mM $CaCl_2$ solution.

Discussion

Phosvitin

The novel antimicrobial responses phosvitin elicits in this study alone and in the presence of bivalent cations continues to demonstrate the potential phosvitin has as a source of antimicrobial peptides. At less than 1 uM, the antimicrobial effect phosvitin has against *M. luteus* is intriguing. Although there were many bacteria that appeared resistant to phosvitin, changing the cation solution phosvitin was in had an affect against some of the bacteria.

Peptides

Both peptides of interest were synthesized for their calcium binding potential. With no antimicrobial activity as of yet at almost 1 mM, their antimicrobial potential is limited. However, their calcium binding and delivery potential remains to be tested.

Future Directions

As phosvitin continues to show potential as a source of bioactive peptides, more peptides will be synthesized in the future. The aim of these peptides will be to have antimicrobial as well as calcium binding/delivery capabilities. A peptide with this capacity could be placed in gum and rebuild tooth enamel while protecting against harmful bacteria in the mouth.

- Synthesize novel peptides geared toward both antimicrobial and calcium-binding/delivery.
- Test phosvitin's antimicrobial effects when paired with different metals and/or compounds.
- Experiment with more species of bacteria.
- Create gum, dietary supplement, or soap product that utilizes phosvitin's antimicrobial and/or metal binding potential.

Acknowledgments

This project was supported by a UW-Stout research and dissemination grant to present at the 2016 conference of the American Society of Biochemistry & Molecular Biology. A special thanks to Dr. Burritt for materials and expertise in the field of microbiology.

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UW-Stout has partnered with WiSys Technology Foundation for this project. WiSys holds intellectual property rights covering phosvitin-derived peptides for a variety of therapeutic uses, and further experimental validation is currently being performed. For more information and to learn about partnering opportunities, contact Jennifer Cook (jennifer@wisys.org) or Kristen Ruka (kruka@wisys.org).