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Encapsulation of bacteriophages in whey protein films for extended storage and release

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ABSTRACT

The overall motivation of this study was to enable development of pathogen specific antimicrobial packaging materials. These materials are expected to have a significant impact in improving efficacy of antimicrobial treatment and retention of commensal and probiotic microbes on food materials. To achieve these goals, this study was aimed at encapsulation of a model bacteriophage, T4 bacteriophage, in WPI based edible protein films. Phage encapsulated WPI films were characterized for stability and release of encapsulated phages. Antimicrobial efficacy of phage encapsulating edible films was characterized using a microbial growth inhibition assay. Distribution of phages in edible film was measured using a confocal fluorescence microscopy. The results demonstrate that the WPI films are able to stabilize phages at ambient (22 °c and light) and refrigerated (4 °C and dark) conditions without significant loss in phage infectivity over a period of one month. Additionally, the WPI films are able to release a significant concentration of phages in an aqueous environment and leaf surface within 3 h of incubation. Antimicrobial activity measurements demonstrate that the phage encapsulating WPI film can effectively inhibit the microbial growth. The results of microbial growth analysis showed an approximately 5 log difference in microbial levels between the control and the treatment samples. Confocal imaging measurements show that fluorescently labeled phages are homogenously distributed within the WPI film matrix. Overall, this study demonstrates integration of phages with edible packaging materials to develop novel active packaging materials for biocontrol applications.

1. Introduction

Active packaging materials with antimicrobial properties have gained significant interest over the last decade. These material formulations include antimicrobial polymers such as chitosan as well as inert packaging materials with encapsulated antimicrobial agents (Hosseini, Razavi, & Mousavi, 2009; Royo, Fernández-Pan, & Maté, 2010; Seydim & Sarikus, 2006). Whey protein isolate (WPI) edible films with encapsulated antimicrobial materials are a subclass of active packaging materials that have been evaluated for diverse applications in food packaging. The unique advantage of these WPI films is their ability to utilize agri-based biomaterials for packaging applications (Valencia-Chamorro, Palou, del Rio, & Perez-Gago, 2011). Similarly, polysaccharide biomaterials derived from agricultural byproducts have been combined with acids, salts, oils, enzymes, and bacteriocins such as nisin to control and reduce the microbial load on food products (Pintado, Ferreira, & Sousa, 2010; Rossi-Márquez, Han, García-Almendárez, Castaño-Tostado, & Regalado-González, 2009; Zinoviadou, Koutsoumanis, & Biliaderis, 2010). Review of prior studies demonstrate the effectiveness of edible antimicrobial films in reducing the growth of inoculated bacteria by 1–2.5 $\log_{10}$ as compared to the controls without antimicrobial agents (Joerger, 2007).

Currently, most of the antimicrobial active packaging materials are broad spectrum antimicrobials which do not target bacterial pathogenic species specifically. There is a significant need to develop novel antimicrobial packaging materials that have high specificity to target only pathogenic organisms while maintaining commensal bacteria. The need for specificity in antimicrobial activity is important as pathogens may be a small fraction of the total microbial load present in food systems (Payment & Locas, 2011). Thus, developing pathogen specific antimicrobial active packaging materials may improve the antimicrobial efficacy by reducing interactions with non-targeted microbes. Further, non-pathogenic microbes are necessary in the production of some dairy and

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fermented foods. Additionally, the commensal bacteria present may have benefits for human health such is the case with probiotics microbes or even controlling the growth of pathogenic bacteria (García, Martínez, Obeso, & Rodríguez, 2008).

Bacteriophages are viruses of bacteria that have high specificity for their host strain and multiply upon infecting their host for up to 1000 viral progeny per bacteria (Laskin, Gadd, & Sariaslani, 2011). Generating progeny upon infection is markedly different to most antimicrobial agents that are depleted when interacting with microbes. Based on these advantages and increases in antimicrobial resistance among common pathogens, phage therapy is gaining more attention as an alternative approach that can be used to control both human and food borne pathogens (Coffey, Mills, Coffey, McAuliffe, & Ross, 2010; García et al., 2008; Hagens & Loessner, 2007, 2010; Monk, Rees, Barrow, Hagens, & Harper, 2010; Sulakvelidze, Alavidze, & Morris, 2001). For example, phages have been used to control pathogens in the guts of animals. Pathogen specific phages may remain active during the digestive process, allowing for removal of *Escherichia coli* O157:H7 from cows' digestive system (Callaway et al., 2008). Further, *Salmonella* specific phages have been shown to reduce pathogen numbers in poultry (Borie et al., 2008; Higgins et al., 2005). Additionally, phages have been directly applied to fresh foods contaminated with pathogenic microbes such as *E. coli* O157:H7 and *Listeria monocytogenes* and have been shown to be effective in controlling the growth of the pathogens. Phages have been shown to be successful in reducing pathogen numbers by 94%–100% on tomatoes, spinach, broccoli, and ground beef (Abuladze et al., 2008). Similarly, cocktails of phages have been spotted onto contaminated meat surfaces, and a 99%–100% reduction of pathogen level was obtained (O'Flynn, Ross, Fitzgerald, & Coffey, 2004). Also, phages showed a similar capability of reducing pathogen numbers up to 99.9% on fresh cantaloupe slices and lettuce (Sharma, Patel, Conway, Ferguson, & Sulakvelidze, 2009).

In all these prior studies, microbes were inoculated on surface of the food materials, and then the inoculated surface was treated with phages, typically by directly spraying phages on the inoculated surface. In many situations, target pathogens can be localized or spread across the surface of food products and can also be present on either or both surfaces of food products. To expand the use of phages beyond direct spraying of phages on food products, there is a need to develop material formulations that can encapsulate phages for improved stability and release at target sites for delivery. Thus, phages can have significant applications as additives to packaging material formulations.

To develop bacteriophage based antimicrobial edible coatings, this study evaluated the ability of WPI films to encapsulate, stabilize, and release bacteriophages to the surrounding environment. WPI films were selected as a model edible coating material because these films have been used for incorporating diverse antimicrobial agents such as essential oils, lactoperoxidase, and nisin (Min, Harris, & Krochta, 2005; Pintado et al., 2010; Seydim & Sarikus, 2006). In addition, WPI films have excellent mechanical properties and oxygen barrier properties which make them ideal edible packaging materials (Janjarasskul & Krochta, 2010). For this study, *E. coli* and T4 bacteriophage were used as a model bacteria and its corresponding bacteriophage. The stability of encapsulated bacteriophages in WPI based edible films was examined in ambient and refrigerated conditions over an extended period of time. To characterize the release of phages from the films, the activity of released phages from WPI films was measured in water and on lettuce leaves. The antimicrobial activity of WPI films with phages was determined using a microbial growth inhibition assay. In this assay, the microbes were inoculated on surface of phage encapsulating WPI films and the results were compared with control WPI films without phages. Confocal imaging was used for characterizing the distribution of fluorescently labeled encapsulated phages in WPI films.

In summary, this study addresses the need to develop pathogen specific antimicrobial edible films by combining edible films previously used as active packaging materials with phages that are host specific. The specific edible films were evaluated for stability, distribution, release, and efficacy of the phage as the antimicrobial material.

2. Materials and methods

2.1. Bacteriophage T4 and host strain

Coliphage T4 was purchased from Carolina Biological Supply and used without modification. *E. coli DH5α* with a tetracycline resistance selection maker was a gift from Dr. Glenn Young (University of California, Davis) and was used as a model bacterium for all PFU counting experiments. *E. coli BL21* was purchased from ATCC (#BAA-1025) and used in efficacy experiments. Both *E. coli* strains were stored in liquid nitrogen. Prior to experimental use, each liquid nitrogen stored strain was streaked onto an agar plate and grown overnight in a 37 °C incubator. A colony from the plate was used to inoculate Luria Broth (LB) media and allowed to grow overnight in a 37 °C shaking incubator. For use in experiments, each strain was subcultured from the initial culture and grown to log phase.

2.2. Bacteriophage activity assay – plaque forming unit counting

Phage activity was measured with a top agarose overlay plating method and reported in plaque forming units per milliliter (PFU/mL). Briefly, top agar (0.7% agar) was melted, and 3 mL aliquots were kept at 45 °C in a water bath until used. Phage samples were serially diluted into SM buffer, and 100 μl of each sample was combined with 250 μl of fresh log phase bacteria culture with an optical density (O.D.) at 600 nm of 1.5. The samples were incubated for 10 min and then combined with an aliquot of molten agar. The molten agar mixture was poured onto a pre-warmed LB agar plate and allowed to solidify. Once the plates solidified, they were inverted and placed into a 37 °C incubator. The plates were allowed to incubate for 12–18 h and then counted.

2.3. WPI film preparation

Whey Protein Isolate (WPI, Davisco Foods International, Inc. (Eden Prairie, MN)) was a gift from Professor John M. Krochta (University of California, Davis), and glycerol was purchased from Fisher Scientific. WPI films were prepared based on a previously reported method (Yoo & Krochta, 2011). Briefly, 5% w/v WPI was added to deionized (DI) water and stirred vigorously for 20 min. Glycerol at a 2:1 WPI to glycerol weight ratio was added to the solution and then stirred for an additional 20 min. The solution was placed in a water bath at 90 °C for 30 min to denature the whey protein and promote cross linking of the protein to form the film matrix. After cooling to room temperature in an ice bath, the WPI film solution was then degassed with a Welch Vacuum Pump (model 2567B-50 A). Phages in a 1 mL aliquot were then added to the solution and gently mixed to ensure an even distribution into the WPI film solution. The solution was then poured into 16 cm diameter circular cast molds and allowed to dry at 22 °C and 18% relative humidity.

2.4. Phage stability in WPI films

Stability was tested by storing WPI films in the dark under refrigerated conditions (4 °C), and on a lab bench in ambient