Effect of concentration of glutaraldehyde and glyoxal on binding lysozyme to zein based films to control foodborne pathogens in tomatoes

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Abstract

The demand for biodegradable packaging materials as an alternative to synthetic ones to reduce environmental cost has seen an increase in recent years. In addition, functionalizing the packaging film to provide specific advantages like antimicrobial properties has yet to be explored thoroughly. This study considers adding antimicrobial agents to improve the quality and safety of actively packaged fresh produce using an antimicrobial enzyme (lysozyme) immobilized on a biopolymer based packaging film (corn-zein). The developed packaging material is aimed as an active biodegradable packaging to reduce bacterial contamination on the surface of fresh organic produce, specifically tomatoes. The study uses glutaraldehyde and glyoxal as binding agents to immobilize the enzyme on the packaging film. The effect of concentration of glutaraldehyde and glyoxal on the controlled release of the enzyme was studied. Concentrations of 0.1 and 0.2 g/g lysozyme: cross linking agent had controlled release properties. However, concentrations of 0 or 0.05 are about 20-30% more effective at inactivating bacteria. Antimicrobial activity in the constructed zein films are also tested against selected pathogens (*Salmonella* Newport *and Listeria monocytogenes).* Developed zein based film is tested against inoculated tomatoes to determine the efficacy of the films in reducing the pathogen population. The inoculated tomatoes are stored at room temperature over a storage period of one week. The film was able to reduce *Listeria monocytogenes* population by three logs but was unable to reduce the population of *Salmonella* Newport.

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Chapter 1: Introduction

Background

Active Packaging Technology

Packaging is a balance of science, art, and technology. Packaging is a vital process that works from distribution, storage, sale and use of a product. Food packaging is an important issue that is concerned with better protection and more efficient quality preservation to enhance food safety from harmful bacteria. Biodegradable packaging can be a useful alternative to conventional packaging to reduce waste and create novel applications to improve product stability.

The overall market for organic foods is growing at a rapid rate for multiple reasons. Consumers want healthy foods that are minimally processed to provide a healthy product and a healthy environment. The Organic Trade Association estimates that in 2006 the total sales of organic food and beverage totaled $13.8 billion (Knudon 2007). The organic fruits and vegetable market is the largest in the organic food sector. Sales were expected to increase 71% by the end of 2011 (Knudson 2007). For this reason, biodegradable and antimicrobial films have gained attention in the last few years for organic food packaging. Using eco-friendly materials, food processors can offer new solutions such as biodegradable films (Tharanathan 2003). These films could be a solution to the shifting demands to minimally processed, easily prepared, ready-to-eat food products.

Previously, food packaging has been dominated by petro-chemical based polymers like polyolefins, polyesters, polyamides, and polyethylene. These are preferred because of the low

cost and favorable properties of the plastics. The petro-chemical based films have strong tensile and tear strength, good barrier properties to O_2, and heat sealability. Also, the films technically have a low water vapor transmission rate and they are non-biodegradable (Tharanathan 2003). The biodegradability is a major issue due to the amount of plastics headed to landfills from packaging materials. According to ASTM D 6400-04, a biodegradable plastic is a degradable plastic in which degradation naturally occurs from bacteria, fungi, and algae. However, a time variable for how long until the package degrades was not defined.

One biodegradable type of packaging is corn zein. Corn zein is a protein commercially isolated from corn that can be used as an eco-friendly film. With the growth of the bio-ethanol industry, the amount of zein potentially available has grown considerably, to the extent that techniques are needed to develop new uses for this material (Biswas 2009). Corn zein is commercially available as packaging for confectionery products and nuts because it is a good barrier to oxygen and its water permeability is about 800 times higher than that of typical shrink wrapping film (Aydt 1991). Corn zein can be applied to previous packaging techniques.

Preservation by adding barriers to contaminants focus on using packing techniques such as canning, aseptic packaging, and active packaging. Variables of interest for packaging are presence or absence of oxygen, light, water vapor, bacterial, or other contaminations that can affect the product without protective packaging (Ahvenainen 2003). A more recent trend introduces an element of active packaging. Active packaging has the package interact with the food product for multiple uses. Active packaging can eliminate bacteria, prolong shelf life, improve quality, improve convenience, or even monitor freshness. Antimicrobials or Antioxidants can be infused in the packaging to help achieve these goals.

One example of active packaging is using antimicrobial agents. Antimicrobial agents have been mixed with food formulations and are applied to food surfaces by dusting, dipping, or spraying (Min and Krochta 2005). One of the main causes of spoilage is microbial growth at the food surface, and current methods tend to use chemical additives that can remain in the food matrix. Direct application of antimicrobials (chemical and natural) could also have complex interaction with food components (Rose 1999, Rose 2002). This does not always have a beneficial result for getting new minimally processed foods with fewer chemical additives into the process. The application of antimicrobial agents on food surfaces by different methods have limited beneficial effects because the antimicrobial diffuses from the food surface to the interior parts, which could lead to consumption of the product.

Microbial growth in foods must be controlled to improve shelf life and safety of the product. Other natural compounds such as organic acids, bacteriocins, enzymes, fungicides, and spice extracts have been studied as food preservatives in place of chemical ones because of the ability to prolong shelf life of packed foods and safety for human consumption (Chen et al. 1996 Ha, Kim & Lee 2001, Han 2000).

Antimicrobial packaging is an alternate method to overcome these limitations while minimizing the amount of antimicrobial agent introduced to the food. Antimicrobial packaging design is about controlling how much antimicrobial agent should be released to maintain the inhibitory concentration against pathogenic or spoilage microorganisms during the targeted storage period (Quintavalla and Vicini 2002, Ozdemir and Floros 2003, Buonocore et al 2004). Another approach is to incorporate a cross linking agent to further control the release rate of the antimicrobial agent into the food product. Increasing cross linking agent has improved immobilization of enzymes on the films. The consumption of most antimicrobials are generally

regarded as safe or GRAS. Antimicrobial packaging could extend shelf life or reduce the risk of contamination occurring in food packaging.

Recent Outbreaks

The United States Center for Disease Control (CDC) estimates 48 million Americans are stricken with foodborne illnesses every year. *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* are major sources of illness in the United States and commonly cited for food recalls and health problems. The yearly cost is difficult to estimate but from the five most prevalent food borne diseases, the cost is estimated to be over $6.9 billion. In 2010, *Salmonella*, *E. Coli* O157:H7, and *Listeria* affected about 1.4 million, 73,000, and 2,800 people.

One major interest in antimicrobial food packaging technologies is due to increased food borne microbial outbreaks caused by minimally processed fresh produce (De Roever 1998, Devlieghere 2004). As of September 2011, The CDC has linked cantaloupe to an outbreak that has been contaminated with *Listeria monocytogenes* with 146 illnesses and 30 deaths to the tainted fruits. This is the most deadly outbreak that occurred in many years. *Listeria monocytogenes* tends to attack high risk population such as infants and the elderly and individuals with their immune system compromised (CDC 2011).

Listeria monocytogenes is a short gram positive, nonspore forming rod that is acid tolerant, psychrotolerant, factultatively aerobic, and salt tolerant. *Listeria* can cause listeriosis, a gastrointestinal food infection that may lead to bacteremia and meningitis. *Listeria* is a very tolerant bacterium, that fresh produce can have *Listeria* contamination. Exposure to *Listeria* is common but acute listeriosis is rare. If listeriosis is contacted the mortality rate is nearly 20%. There are about 2,500 cases of listeriosis per year and about 500 cases end up in death. The most

effective treatment is the use of antibiotics for listeriosis. Penicillin, ampicillin, trimethoprimsulfamethoxazole, or combinations of these antibiotics are the most common way to treat listeriosis.

As of June 2011, the CDC was monitoring a large outbreak of *E. coli* in spinach in Germany (CDC 2011). This is one of the most recent outbreaks occurring with ready to eat vegetables. Ready to eat vegetables are typically in your salad like lettuce, tomato, green pepper, or other vegetables that are not thermally treated before use. Most processes use thermal heat to make food bacterially safe, however this is not an option in vegetables because the quality attributes of the vegetables would be altered. Current methods of washing vegetables can decrease but not eliminate contamination.

Escherichia coli are short, gram-negative rods. There are about 200 known pathogenic *E. coli* that can cause life threatening diarrheal disease and urinary tract infections. Enterohemorrhagic *Escherichia coli* (EHEC) produce verotoxin which can cause hemorrhagic (bloody) diarrhea and kidney failure. EHEC strains can grow in the intestine, fecal material, and is a potential for waterborne disease. Treatment of all pathogenic *E. coli* infections involves supportive therapy and in severe cases, antimicrobial drugs to shorten and eliminate infection (Madigan 2006).

Several outbreaks of *Salmonella* in tomatoes have been reported. In September 2011, organic grape tomatoes imported from Mexico were tested and resulted positive for *Salmonella*. Fortunately, this outbreak had no reported illnesses because the recall was handled effectively (Williamson 2011). In June 2008, *Salmonella* was linked to raw red roma and round red tomatoes. About 277 people in 28 states have been affected by Salmonellosis. This also was

well contained and led to zero deaths but it was a nationwide recall. (CDC 2011). *Salmonella* are one of the few bacteria that have not had a significant decrease in occurrences in the past few years.

Salmonella are gram negative facultative aerobic rods. Salmonella are a very common source for foodborne illness because of salmonellosis which is a gastrointestinal disease. Symptoms begin after the pathogen colonizes the intestinal epithelium which can occur 8-48 hours after ingestion. Symptoms include headache, chills, vomiting, and diarrhea but the bacteria usually resolve in 2-3 days. The salmonellosis can cause septicemia (a blood infection) and enteric or typhoid fever. This fever can last several weeks and mortality can approach 15% if typhoid fever is untreated. Salmonella effects about 40,000-45,000 with hospital visits but it is believed that it actually effects about 1.3 million people every year (Madigan 2006).

Justification

The goal of this project is to create a sustainable packaging from biomass that can reduce exposure of food-borne illness associated with ready to eat vegetables. The film is tested against pathogenic bacteria (*Listeria* and *Salmonella*) to determine the activity of the film. The film is tested to see the reduction of bacteria of ready to eat vegetables specifically, fresh cut tomatoes. This film will also use cross linking agents to control release lysozyme to inactivate pathogenic bacteria. The goal is to optimize the release of lysozyme to bind the active ingredient to film rather than allowing it to infiltrate the product.

The packaging that was selected is corn zein based films due to the abundance of corn zein as a by-product from bio ethanol industries. Lysozyme is the enzyme that is immobilized to

the film to reduce pathogenic bacteria. To enhance immobilization, glutaraldehyde and glyoxal are added to the film to link lysozyme to the corn zein film.

The results of the study evaluated the efficacy of using binding agents to improve immobilization of enzymes with antimicrobial properties on biopolymer based packaging films. Successful immobilization and delayed release of antimicrobial activity provides opportunities to develop active packaging films for fresh cut produce.

Hypotheses

Introducing a cross linking agent allows lysozyme to adhere to the film and assist in destroying surface contamination on tomatoes. The increase of crosslinking agent should allow the lysozyme activity to also increase and reduce the gram positive bacteria on the surface of the tomato. Cell population is significantly lowered when using a corn zein film immobilized with lysozyme and is a more effective using a cross linking agent.

Objectives

The objectives of this research are to:

1. Determine which cross linking agent works best: glutaraldehyde or glyoxal.

2. Determine what concentration of glutaraldehyde or glyoxal optimizes the availability of lysozyme to interact with the product while retaining the lysozyme to the corn zein film

3. Determine how much the lysozyme reduces gram positive bacteria (*Listeria innocua*)

4. Examine pathogen reduction of the tomatoes covered with the film

Thesis outline

The thesis consists of 5 chapters. Chapter one provides introduction to the research and background information about the tools being used for this research. Chapter two provides information of current work being done in this area. Recent discoveries and technical background of films, crosslinking agents, and enzymes are discussed here. Chapter 3 works with different concentrations of glutaraldehyde or glyoxal and the effect on the activity of lysozyme. Chapter 4 focuses on the tomato application of the film. The film is applied to fresh cut tomatoes for the most active film established in chapter 3 to determine the log reduction of pathogen bacteria. Chapter 5 is conclusions from this research and which direction future work should take.

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Chapter 2: Literature Review

Introduction

Recent outbreaks in fresh produce and demand for ready to eat vegetables have produced an increasing need to develop methods assuring safety of ready to eat fresh produce. However, traditional methods of eliminating or reducing bacteria such as adding heat are not a possibility. Adding heat affects the physical properties and nutritional quality of the produce. With the high amount of vegetables consumed every year, it is important to have a safe product.

Ready to eat vegetables are a very common food to eat in almost every demographic. Tomatoes are a staple food in the U.S. and around the world. Approximately 5 billion pounds of fresh tomatoes are eaten annually in the United States. One consideration when working with tomatoes is the high risk populations (elderly, pregnant, children) since almost every demographic enjoy tomatoes. These populations have a higher need for antibiotics to treat food borne illnesses.

Salmonella Montevideo is one particular strain of foodborne illness. It has been shown to survive on the surface and the core of the tomatoes at 10°C which suggests a potential for survival during transport and storage preceding ripening and consumption of the tomato (Zhuang 1995). Using water to wash the surface of the tomato could reduce the bacterial content of the tomato but not guarantee elimination of the bacteria.

This has driven the need for the development of new technologies to be used on organic tomatoes that will not affect the product and interact in natural ways. A variety of non-thermal methods have been proposed to reduce bacterial load including chlorine methods, x-ray, and

active packaging. These methods can produce safe for consumption vegetables while maintaining the quality consumers expect.

Current Cleaning Methods

Chlorine cleaning methods are one way of sanitizing vegetables. One major concern from this study was the concern that chlorine is not completely effective in killing the wide range of microorganisms that may be naturally present in or on tomatoes in the packinghouse (Senter 1985). Chlorine methods have been used on tomatoes with varying chlorine and temperature. Zhuang (1995) used a chlorine dipping method to reduce bacterial concentration but was unable to completely inactivate *Salmonella* Montevideo. Chlorine is still a very common organically approved sanitizer, along with ozone and peroxyacetic acid, that is still used today.

Several different chlorine methods have been established to inactivate or destroy pathogenic bacteria. Some of these include using chlorine gas, calcium hypochlorite, sodium hypochlorite, or other chlorine formulae. These methods can eliminate bacteria, fungi, viruses, and cysts but there are concerns for consumption of high concentration of these chlorine methods. However, each chlorine method has associated risk. The major concern is that chlorine will leave undesirable product on the vegetables prior to consumption. Some undesirable products include chloroform or other trihalomethanes that have potential for being carcinogens at high doses. Also at high pH, chlorine reacts with organic nitrogen-based materials to produce mildly toxic chloramines (Suslow 2004). Traditionally, the chlorine methods benefits outweigh the risk. However, alternate methods are being explored to minimize or eliminate the use of chlorine because of the possibility of consumption of carcinogens.

Packaging

Due to consumer demand, biodegradable and active packaging has gained increasing attention. Food packaging protects against external factors, such as humidity and oxygen, to extend shelf life. Recent trends have focused research to incorporate antimicrobial agents into polymeric material and releasing them on the food surface for biopreservation (Devlieghere 2004). By incorporating these antimicrobial agents it is a possibility to reduce the bacterial load on the vegetable without altering characteristics of the vegetable.

When working with edible or biodegradable packaging materials, permeability is the most important variable to maintaining a long shelf life for a product. Permeability is the diffusion controlled molecular enhancement of low molecular solute across a homogenous polymeric material via dissolution and desorption mechanisms. Water vapor, oxygen, and aroma barriers are all very common uses of the kinetics of permeability. Water vapor permeability mostly focuses in inhibiting moisture exchange between the food product and the atmosphere. Water activity can result in microbial growth, texture changes, and deteriorative chemical or enzymatic reactions (Krochta 2010). Oxygen barriers are necessary to reduce oxidation of lipids and food ingredients. Oxygen also allows discoloration of myogolbin in meat, or enzymatic browning of fresh cut produce (Krochta 2010). These variables are necessary for vegetables to be able to breathe in the packaging material.

Mechanical properties of edible films have been studied extensively. The tensile strength, elastic modulus, and glass transition temperature are some of the major variables that affect the mechanical properties of the films. Corn zein films are one example that has been studied for packaging cooked turkey that have worked with elongation, tensile strength, and

Young's modulus of corn zein films with different additives (Herald 1996). Protein and carbohydrate films are often brittle and often use plasticizers to overcome these issues. Most edible films should be biodegradable in nature due to the protein, carbohydrate, or lipid nature of the films. There are also some cellulose based films that may pass through the human system without being absorbed into the body.

Mechanical and thermal properties of protein based films have been studied extensively. In Park et al., (1994) used grain protein based films such as wheat gluten and corn zein. These were tested for characteristics such as water vapor permeability, tensile strength, and elongation.

Antimicrobial packaging is one form of active packaging. Antimicrobial agents can be used in combination with edible or biodegradable packaging to make consumers safe. The use of bacterial enzymes started gaining more attention in the mid 1990s. For example, Herald (1996) examined if bacterial enzymes affected tensile properties of the films. Organic acids were explored to reduce pathogenic bacteria such as *L. monocytogenes, E. Coli*, and *S.* Typhimurium in a whey protein isolate matrix (Cagri 2001). The use of enzymes as antimicrobials significantly increased in the mid 2000's. In 2006, Mecitoglu and others incorporated partially purfied hen egg white lysozyme into zein films (Mecitoglu 2006). Lysozyme has been incorporated into cellulose acetate films (Gemili 2009). Cross linking agents and to improve lysozyme immobilization to films have also been explored (Conte 2007).

Types of Edible Films

Diverse materials can be used in edible packaging. The major categories of film forming materials include polysaccharides, proteins, lipids, and resins. Plasticizers can be used on the

films to increase flexibility. Edible film coatings can be made from food materials as generally recognized as safe.

Edible packaging typically consists of edible films, sheets, coatings, and pouches. Edible packaging is rapidly advancing by utilizing edible compounds such as proteins, polysaccharides, lipids or resins. Edible packaging materials are intended to be integral parts of food products and to be eaten with the products thus they are inherently biodegradable in composting and other biological recycling (Krochta 2010).

Protein Films

Protein films have attracted research attention as potentially the most significant edible packaging material (Krotcha 2010). Protein films come in almost every different shape and size due to the wide range of desirable properties and modifications that can be made to them. Wheat gluten, corn zein, soy protein, and whey protein are all globular proteins that can fold into spheroid structures by combinations of hydrophobic interaction and hydrogen, ionic, and disulfide bonds.

Corn zein is a protein commercially isolated from corn. With the growth of the bio-ethanol industry, the amount of zein potentially available has grown considerably, to the extent that techniques are needed to develop new uses for this material (Biswas 2009). Corn zein is commercially available on confectionery products and nuts because it is a good barrier to oxygen. Also, its water permeability is about 800 times higher than a typical shrink wrapping film (Aydt 1991). Corn zein films have been selected for use on tomatoes. An almost invisible application of corn zein by dipping methods have been used to extend shelf life.

Zein is located in the 'zein bodies' of ~ 1 μm uniformly distributed throughout the cytoplasm of the corn endosperm cells between the starch granules of 5- 35 μm (Duvick 1961). Zein is insoluble in water except in the presence of alcohol, high concentrations of urea, high concentrations of alkali (pH 11 or above) or anionic detergents (Shukla 2000). Zein is rich in glutamic acid, leucine, proline, and alanine, but deficient in basic and acidic amino acids. Mosse (1961) and Pomes (1971) have described the amino acid make-up of zein films. In whole corn, zein occurs as a heterogenoeous mixture of disuflide linked aggregates having a weight average molecular weight of 44000 Da. The structure of zein is a helical wheel model prepared by Argos et al. (1982) where nine homologous repeating units are arranges in an anti-parallel form stabilized by hydrogen bonds resulting in a protein molecule that is slightly asymmetric (Shukla 2000).

Zein occurs in various molecular sizes, solubility, and charge. Alpha (α) and beta (β) zein were first described by McKinney (1958). α - zein was defined as corn soluble in 95% ethanol. Maize α zeins belong to a class of hydrophobic proteins known as prolamines. α-zein has a majority of the zein products in the market before 1957 because corn has a content of about 80% prolamines. β - zein solublity demonstrates different characteristics. In 60% ethanol the β - zein is soluble while in 95% ethanol, the β-zein is insoluble. The solubility characteristics are attributed to the high content of nonpolar amino acids (Gennadios 1990). β -zein tends to be unstable, precipitating and coagulating frequently and was not a good choice for commercial zein preparations (Shukla 2000)

There are some drawbacks to using zein in several applications. There is a lack of amine moieties (lysine amino acids) which limits the number of typical protein derivatizing techniques available. Another drawback is it dissolves in ethanol/water (90:10) mixture, but this mixture

cannot be readily used for chemical modification because alcohol and water may react faster with electrophilic reagents than zein does (Biswas 2009). Recent advancements have been made in the surface modification of zein films. In 2009, Biswas developed a way to modify water absorption and surface wetting behavior of films. This hydrophobic nature could impart desirable properties such as decrease water absorption, increase water repellency, and improve compatibility with organic additives.

Other Protein films

Two other protein sources that appear frequently in literature include wheat gluten (WG) and Whey proteins (WPs). Wheat gluten-based films are traditionally obtained by casting in a thin layer and then drying of aqueous alcoholic solutions (in acidic or basic conditions) in the presence of disruptive agents such as sulfite (Cuq 1998). WG can be dry casted due to the volatiles evaporate from the WG solution. Guilbert (2002) also claims that the moisture, gas, and solute barrier properties of WG based films could be useful for active packaging, drug delivery systems, or modified atmosphere packaging.

Whey proteins remain soluble after casein is precipitated at pH 4.6 during the cheese-making process. Whey protein isolate (WPI) are water soluble without heat denaturation. The WPI films focus on the H-bonding to form the film (Perez-Gago 1999). Whey protein isolate has been used with lactoperoxidase incorporated in the system (Min 2005, Min 2007). These characteristics make it a very good film to work with when making a biodegradable antimicrobial system.

Soy protein isolate, collagen, gelatin, milk proteins, egg white protein, peanut protein, rice protein, pea protein, sorghum protein, fish myofibrillar protein, and keratin are all protein sources that can be used to make biodegradable films. Each source will have unique effects on the properties of the film. Most of these products are by products from another major industry. For example, corn zein was selected by the growth of the bio ethanol industry.

Polysaccharide Films

Polysaccharides have a potential use as edible packaging because they are abundant, low cost, and easy to handle. Polysaccharide films exhibit good mechanical and gas barrier properties (Baldwin 1995). Polysaccharides are long-chain polymers that tend to have hydrophilic moieties present in their structure. Varieties of polysaccharides that exist are cellulose derivatives, chitosan, carrageenans, and varieties of gums.

Chitosan

Chitosan is a polysaccharide derived from chitin combined with alkalis. Currently, chitosan is not approved as a United States food additive (Krochta 2010). This has limited the research done with chitosan but it has proven to have some very useful properties. Chitosan has antimicrobial properties (Dawson et al, 1998, Coma et al 2002). Chitosan films are most effective against yeast and molds. It does have effects on gram-positive and gram-negative bacteria as well. The mechanism of antimicrobial action is not well understood but it is believed that it is the interaction between the positively charged chitosan and the negatively charged microbial cell membrane. Antimicrobial films are produced by dissolving chitosan into hydrochloric, formic, acetic, lactic, and citric acid in solutions (Begin, 1999). Dissolving in each of these organic solvents will affect the viscosity of the chitosan, thus effecting food properties

(Rhim 1998). Chitosan films are flexible but difficult to tear with moderate water vapor permeability and exhibit good barriers to the permeation of oxygen (Rudrapatnam and Farooqahmed 2003).

Chitosan semi-permeable film provides the ability to modify internal atmosphere and decrease transpiration and delay the ripening of fruits and vegetables (El Ghaouth 1992; Jiang 2001, Wang 2007). Chitosan has been found to be an antifungal polysaccharide and it has shown delayed ripening of tomatoes (El Ghaouth 1992). Also the use of chitosan coatings can delay enzymatic browning in fresh produce (Zhang and Quantick 1997).

Film Additives

Film additives are materials that are incorporated into the films to enhance structural, mechanical, handling, or active functions of the films.

Plasticizers

Plasticizers are typically small molecular weight hydrophilic agents added to the film solutions to improve mechanical properties. Selection of plasticizers requires considering plasticizer compatibility, efficiency, permanence, and economics (Sothornvit & Krochta 2005).

Glycerol is a commonly used plasticizer that reduces glass transition temperature of proteins and polysaccharides including zein (Dawson et al 2003) for improved mechanical and barrier properties of biopolymeric films. Glycerol would also facilitate the cell wall structure formation of hydroxy-propyl methyl cellulose and the release properties of encapsulated theophylline. Glycerol can penetrate the protein network and form hydrogen bonds with the

protein molecules and increase the separation between protein chains. This allows movement of the protein molecules to improve flexibility of protein based films

Polyethylene glycol (PEG) is one of the main components that allow edible proteins to overcome film brittleness. PEG causes the zein to have stronger intermolecular forces and increase the mobility of the chains. This reduces cracking and chipping of the film during the handling for the barrier properties. Another major indicator of chipping and cracking is the pH of the film. The most common plasticizer for zein is glycerol, PEG, or a combination of the two. The addition of PEG 400 (molar mass 400g/mol) increases tensile strength and extensibility at higher PEG levels until about 25-30 % weight . After 25-30%, the PEG decreases the tensile strength. Also, the permeability of the film to oxygen starts to increase rapidly at about 25% PEG.

Together, PEG and glycerol have been intensively studied as plasticizers for zein (Parris & Coffin 1997). Krotcha (2010) has provided a comprehensive table that gives values for mechanical properties under certain test conditions for a variety of films. When selecting a film, the table can provide an expected value for mechanical properties using certain ratios of plasticizers or types of films. Park (1994) has studied water vapor permeability, tensile strength, and elongation affected by PEG and glycerol mixtures. Characteristics were altered depending on the amount and the ratio. Mixtures of glycerin and PEG as plasticizers are more effective than glycerin alone and grain protein based films and can reduce the deterioration of mechanical properties during storage.

Antimicrobials

Antimicrobials can be synthetic or organic. Synthetic antimicrobials include sulfonamides that occur in several antibiotics. This is not a natural compound like organic antimicrobials. The common trait organics can either be a type of film, organic acid, enzyme, bacteriocins, or a natural extract. Each antimicrobial has a unique mechanism that is well suited for different situations. At this time, most publications in this area use enzymes such as lysozyme or lactoperoxidase. This could be because they can be isolated from natural sources such as milk or egg whites and are effective.

Lysozyme is active against gram-positive bacteria. Lysozyme can be isolated from egg whites. The lysozyme can destroy the cell wall and have osmotic lysis. Gram negative bacteria, yeasts, and molds are unaffected by lysozyme because lysozyme is structure specific. Lysozyme is a single peptide protein that prefers to hydrolyze the β-1,4 glycosidic linkages between N-acetylmuramic acid and N- acetylglucosamine which occurs in the muco peptide cell wall (peptidoglycan) structure of *Micrococcus lysodeikticus* (Conte 2005). Some factors that could affect the activity of lysozyme are temperature, pH, bacterial load, and bacterial resistance. Lysozyme has an optimal activity between 40-45°C. Lysozyme activity due to pH tends to have the highest activity between 3.5 and 7.0. Lysozyme has had several long term storage tests done on it when activated with film. After 105 days the immobilized activity was very strong (Gimili 2009).

Gram positive and gram negative bacteria have a major distinction because of the cell wall structure. Gram negative cell wall is a multilayered structure that is quite complex whereas the gram-positive cell wall primarily consists of a single type of molecule, and is often much thicker (Madigan 2006). The component to both gram negative and gram positive bacteria is called peptidoglycan which is a polysaccharide composed of two sugar derivatives, N-

actylglucosamine and N-acetylmuramic acid, with a few amino acids. In gram positive bacteria as much as 90% of the cell wall consists of peptidoglycan and can have up to about 25 sheets of peptidoglycan stacked upon one another. While gram negative bacteria is only about 10% peptidoglycan and the majority of the wall consisting of the outer membrane or lippolysaccharide layer (LPS). The LPS consists of ketodeoxyoctonate, heptoses, clucose, galactose, and N-acetylglucosamine (Madigan 2006). Gram negative bacteria that are pathogenic for humans are often due to the toxic outer membrane. The toxic property of the LPS membrane is considered the endotoxin.

Most research uses *Micrococcus lysodeikticus* to establish activity of Lysozyme. Lysozyme affects survival rates of gram positive bacteria and this *Micrococcus* is gram positive structure, spherical, and nonpathogenic bacteria. *Listeria* is also a gram positive bacterium which is the reason it is also affected by the lysozyme. Previous work has been done when creating a cross linked film with polyvinyl alcohol film with lysozyme (Conte 2007). Conte proved that when the antimicrobial efficacy of the film increased, so did the amount of bound enzyme.

Lactoperoxidase works by a completely different mechanism. Lactoperoxidase inhibits microorganisms by oxidizing the sulphydryl groups of microbial enzymes and other proteins. This will cause structural damage to the cytoplasmic membranes that will leak potassium ions, amino acids, and peptides to microbial cells (Kussendrager & can Hooijdonk 2000). Lactopreoxidase has been studied extensively when it is incorporated into a whey protein isolate matrix (Min et. al 2005, Min et. al 2006, Min et. al 2007). Other enzymes have been used such as Ovotransferrin (Seol 2009) and Lactoferrin (Min and Krochta 2005) to reduce pathogenic bacteria.

Cross Linking Agents

Cross linking is the formation of chemical links between molecular chains to form a three-dimensional network of connected molecules. The degree of crosslinking, quantified in terms of cross link density along with molecular structure have a major effect on the characteristics of the cross linked system. Glyoxal, formaldehyde, and glutaraldehyde are cross linking agents capable of reacting with amino acid side chains, particularly, with the lysine to form schiff bases (Marquie 1997). This protein-protein connection allows the lysozyme (protein) to interact with the corn zein (protein) and allow the film to control release the lysozyme. Below are a few cross linking agents with perks and drawbacks listed.

Glutaraldehyde

Glutaraldehyde ($CH_2(CH_2CHO)_2$ is one of the most frequently used technologies for enzyme immobilization in pharmaceutical applications . Since glutaraldehyde (GA) has low-ionic strength, the cationic nature of the surface permits the rapid ionic immobilization of the proteins. GA can permit improvement on enzyme stability by multipoint or multisubunit immobilization. However, if pH is too high glutaraldehyde will not react with the enzyme. Also, incubation at 25 °C is necessary to achieve a higher degree of cross linking.

Glutaraldehyde has been proven to have positive attributes when using glacial acetic acid and glutaraldehyde when reacted with zein (Sessa 2007). The focus of that investigation was to assess the structure properties and correlate it to the thermal and rheological measurements of the GA- modified zein. In concentrations of 4% or higher of GA versus weight on zein it produced films and organo-gels that were insoluble in solvents common for zein.

Swelling characteristics of GA cross linked zein gels can be exposed to air to form films. This is because it is a wet process using solvent/carrier evaporation of dispersed or solubilized proteins. Mechanical and thermal properties of these films were studied (Sessa 2008). However, control release of enzymes using a film matrix using cross linking agents for food purposes has had minimal research explored.

Protein cross linking of GA has been used to stabilize proteins (Marquie 2001). Mosan has explained the reactivity of GA with proteins. In acidic solutions (pH 3.1) the GA is in equilibrium with its cyclical hemiacetal form and cyclical hemiacetal polymers.

In neutral or basic medium the dialdehyde condenses and gives rise to alpha,beta, unsaturated polymers. More polymers form when pH is more basic. Most GA protein cross linking reactions are carried out in high alkaline pH so the α and β unsaturated polymers dominate. The aldehydes tend to react with primary amines to form imines (Schiff bases) stabilized by resonance and resistant to acid hydrolysis (Marquie 2001).

Glyoxal

Glyoxal is another organic compound (OCHCHO) cross linking agent. It is the smallest dialdehyde that can be prepared from the gas phase oxidation of ethylene glycol. Glyoxal does not have the same extensive research compared to glutaraldehyde. Glyoxal prefers alkaline pH so it can have reactions that resemble the formation of Schiff bases (Marquie 2001). Glyoxal primarily works with the arginine guanidyl groups. Glyoxal derivatives such as phenylglyoxal and azidophenylglyoxal are usually inserted in cross-linking agent molecules directed against arginine (Ngo 1981).

Formaldehyde

Formaldehyde has been used over the past century for leather tanning and pharmacological applications. Lysine is an essential protein that formaldehyde can cross link. The reaction of lysine with formaldehyde is a two-step process. First ε-NH2 residues and formaldehydes form aminomethylol derivatives. This is a quick reversible reaction is optimal at high alkaline pH (Means 1968). Unfortunately, formaldehyde is not allowed to be used for food purposes because of Food and Drug Administration (FDA) regulations. It is not allowed to be an additive or indirect additive to any food product so this is not a feasible cross linking agent. Formaldehyde was used in foods for extension of shelf life and in the 1900's it was added to US milk plants to milk bottles as a method of pasteurization. However, its toxicity was discovered after that and now is not a GRAS chemical additive. This eliminates the use of formaldehyde in food uses.

UV/VIS Spectrophotometer for detection

UV/VIS spectrophotometer is absorption or reflectance spectroscopy. It can use wavelengths in the UV-NIR light spectra range. The most common method to quantify concentrations of absorbing bacteria by turbidity is known as Beer Lambert law.

$$A = \log_{10}(I_0/I) = \varepsilon c L$$

where A is the absorbance, I_0 is intensity of the light emitted by the spectrophotometer, I is the transmitted intensity. L is the path length through the sample (size of cuvette) and c is the concentration of the absorbing species. ε is a constant known as molar absorptivity or extinction coefficient(AU/(M*cm)). Beer Lambert law is not a universal relationship for determining

concentration but it works for most first order polynomial relationships. The UV- spectrum cannot be used for lysozyme because it will inactivate the lysozyme (Shugar 1952).

The method to measure the lysozyme activity and the inactivation of *Micrococcus lysodeikticus* has been suggested by Shugar (1952). This is also the current methods from Worthington Biochemical Corporation. The method uses a spectrophotometer to 450 nm at 25 °C. Once 2.9 mL of *Micrococcus lysodeikticus* cell suspension has been placed into the cuvette add 0.1 mL of appropriately diluted enzyme and record the change of A_{450} from the initial linear portion of the curve. This is the same procedure that is reported by Appendini and Hotchkiss (1997). Lysozyme activity can be determined by measuring the decrease in absorbance of *Micrococcus lysodeikticus* incubated with the film in buffer.

The wavelength for 450 was chosen because at larger wavelengths you are capable of seeing larger particles. The assumption is that the measurement is the number of cells because the lysozyme will allow the organelles to be excreted from the cells. These organelles will allow light to penetrate deeper and the smaller particles (organelles) will not be recognized. The activity of lysozyme is expressed in terms of the amount of the lysed cells per minute.

One drawback of using the UV/VIS spectrophotometer is that the corresponding readings between .1-1 on the spectrophotometer range from a 5 - 9 log amount of bacteria. This is consistent with Madigan (2006) in figure 2.1. Some bacteria could affect you with a lower than 10^5 CFU/mL but there has been difficulties finding these numbers. When working with brittle film, it is possible the film will dissolve into chunks and increase the reading of turbidity in the sample.

Other Detection Methods

There are several other methods that can be used to detect bacteria. These methods are far more labor intensive than using the UV/VIS spectrophotometer but they also will have a lower minimum level of detection. Plating the bacteria will be one method to determine bacterial concentration. Using the live/dead staining technique while plating the bacteria establishes which bacteria are still alive and which bacteria were killed by the lysozyme. These techniques can be used in combination with the UV/VIS spectrophotometer in order to establish a calibration curve for absorbance reading versus CFU/mL of microorganism.

The zone of inhibition has been another way to measure the films activity. In short, the procedure for the zone of inhibition is to place the antimicrobial on a full plate of bacteria. When returning a day later, there should be a significant zone where the bacteria will not grow near the film. This is the zone of inhibition or the area that inhibits growth of bacteria. When using a cellulose acetate film, Gemili et al (2009) was able to get a significant zone of inhibition by the film. By measuring the zone of inhibition, activity can be determined of the film.

Instead of always determining the bacteria, there are ways to determine the activity of the lysozyme as well. During the release test, alternate methods of using a high performance liquid chromatography (HPLC) have been used (Conte 2007). A C18 reverse phase column was used and a gradient elution with water acetonitrile gradients containing trifluoacetic acid. This was proven an effective way to determine the lysozyme concentration compared to standard solutions from 6 - 300 ppm.

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Figures (Chapter 2)

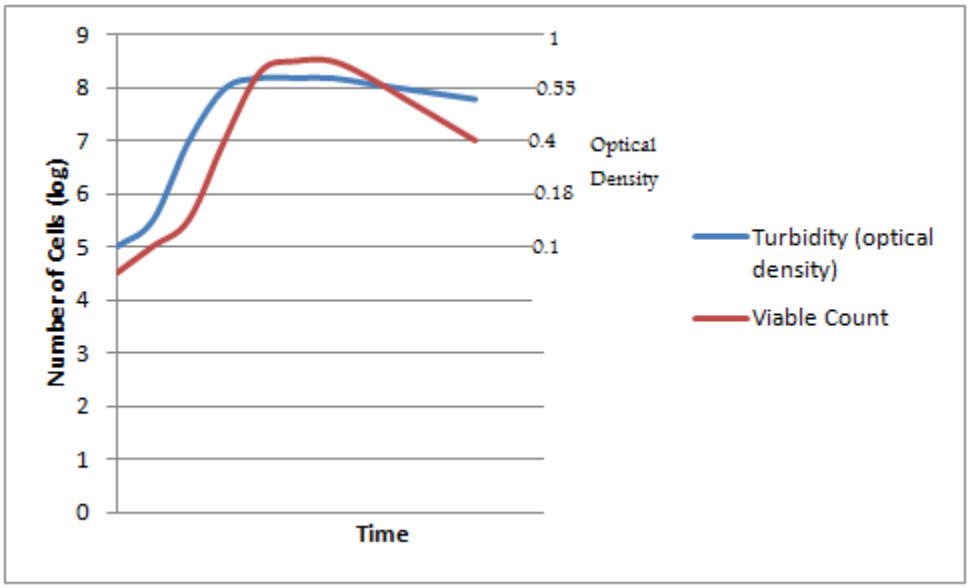

Figure 2. 1. Growth Curve of *Micrococcus lysodeikticus* versus time and comparing it to optical densities that were observed.

Chapter 3: Effect of Concentration of Glutaraldehyde and Glyoxal on the activity of Lysozyme

Abstract

Biodegradable packaging using proteins, polysaccharides, lipids, or resins is rapidly advancing. Research activity has increased, driven by consumer demand for safe, high-quality, convenient foods with long shelf lives. The ability of active biodegradable packaging to carry and control-release active compounds is a promising area of interest.

Extensive efforts have been put into incorporating natural and synthetic antimicrobial agents in edible packaging matrices. When integrating these antimicrobial agents, it is common for the agent to be released from the film matrix but not at a consistent rate. This study aimed to improve the stability of lysozyme on a corn zein matrix by use of different cross linking agents such as glutaraldehyde and glyoxal. With the protein-protein cross linking, the cross linking agents are able to control release lysozyme.

The results of this study show that the addition of glutaraldehyde (GA) or glyoxal (GO) improves immobilization of the enzyme to the film, but it also inactivates the antimicrobial activity at higher concentrations of cross linking agent. The addition of 0 and 0.05 g/g lysozyme: cross linking agent did allow for maximum efficiency of the film. There was also ability for the films to control the release of the lysozyme at concentrations of 0.1 and 0.2 g/g lysozyme: cross linking agent. These concentrations minimized the concentration of *Micrococcus lysodeikticus* detected by the UV/VIS spectrophotometer. Non-pathogenic *Listeria Innocua* was reduced by a 3 logs when the films with lysozyme were added to a broth solution after 24 hours. This proved the film was effective, but only with low concentrations of cross linking agent. Also, GA was more effective than GO by about 40% effectiveness.

Introduction

The ability of biodegradable packaging to incorporate and control-release active compounds is very promising. This allows the reduction of pathogens without altering the physical characteristics of fresh produce. Biodegradable packaging has been incorporated with organic acids, enzymes, bacteriocins, and natural extracts to reduce or eliminate bacteria. One combination of these methods uses lysozyme, nisin, and ethylenediamina tetraacetic acid (EDTA). These have been incorporated with soy protein and corn zein based packaging films (Padgett 1998). Corn zein is an inexpensive option for a biodegradable packaging film due to the prevalence of corn from biofuels and zein is a byproduct in biofuel process. Zhong and Jin (2009) incorporated lysozyme encapsulated in a spray dried zein and the spray dried zein is a good carrier polymer for a system to deliver the enzyme to the product. An effect of pH on the release of lysozyme in the product was also studied by Zhong and Jin (2009).

The highest expense in making the active films is adding the antimicrobial, in the present study it is the addition of lysozyme. Lysozyme is very effective at lysing gram positive bacteria and has proven to maintain a long shelf life under refrigerated conditions for the lysozyme to remain active. To reduce cost of the films a less expensive option is adding a cross linking agent. This could reduce cost because it could control release a cross linking agent making more of the antimicrobial to be attached in the film. Glyoxal (GO) or glutaraldhyde (GA) will increase the amount of lysozyme attached to the film and control release the lysozyme. GO and GA have different mechanisms described in chapter 2 to enhance immobilization on the enzymes. Cross linking agents typically are used to improve mechanical properties of films. An alternative cross linking agent is tetraethyleneglycol dimethacrylate (TEGDMA) and it showed increased lysozyme binding (Lin 2006).

The primary focus of this study was to observe the concentrations of glutaraldehyde and glyoxal on the activity of lysozyme. Lysozyme activity will be measured by the reduction of *Micrococcus Lysodeikticus*. *Micrococcus spp.* are very susceptible to being lysed by lysozyme and can be monitored

by using a UV/VIS spectrophotometer. It is easy to observe the reduction of lysozyme by measuring the absorbance. These results conclude optimal concentration for glutaraldehyde and glyoxal and are used on tomatoes in chapter 4.

Objective

This study establishes the optimal amount of glutaraldehyde and glyoxal to control the release of lysozyme from the film matrix to the product. Lysozyme has been proven effective against gram positive bacteria, especially *Micrococcus lysodeikticus* and is measured using the UV/VIS spectrophotometer. Initially, *Micrococcus lysodeikticus* establishes a control for the films. The goal of these methods is to establish a film, determine the initial and final activity of the film, and to determine how much lysozyme is staying on the film. Lysozyme has been shown to be stable over long periods of time on the film but hopefully the binding agent GA allows more of the lysozyme to be retained to the film. This will help control release the lysozyme rather than to have the lysozyme enter the product.

Materials and Methods

All tests were conducted with a *Micrococcus lysodeikticus* and non-pathogenic *Listeria innocua.* To determine the activity of the lysozyme, the relationship between time and cell concentration was analyzed. The linear portion of the log rhythmic decay determines the activity of the enzyme. The procedure does take careful timing on all parts because the lysozyme is effective at reducing gram positive bacteria.

Film Preparation (Corn Zein)

The following preparation methods were used to make eighteen 5 cm x5 cm films. First, 163 mL of ethanol (95%) was placed into a large glass beaker. The ethanol was placed on a magnetic

heating/stirring pad and elevated in temperature to about 35°C. This allows the corn zein to dissolve more easily. Next, 27g of corn zein powder was slowly added to ethanol until all the corn zein has dissolved. Then, 5 mL of Polyethylene Glycol (PEG) and 5 mL of glycerin is added to the corn zein ethanol solution as a plasticizer. Once dissolved, the beaker was capped and heated the ethanol to a light boil. The solution was allowed to cool for about an hour. This solution was separated into six smaller beakers that have 0, 50, 100, 200, and 500 μL GA and control solution. The respective amount of GA was added to the solution for about two hours and then 1 mL of lysozyme stock (1g lysozyme /10 mL .1 M peptone buffer) was added. The control solution did not contain any of the enzyme or the cross linking agent. The films were recorded as data from the lysozyme: cross linking agent ratios of 0, 0.05, 0.1, 0.2, and 0.5 g/g. The films were slowly poured onto acrylic flat plates with 3M Scotch linerless splicing tape covering the outside of the 5x5 plates. The tape was added to the edge of the plates to make it easier to peel the film from the surface. The samples prior to use are shown in Figure 3.1.

Cell Culture

Micrococcus Lysodeikticus (Sigma - ATCC 4968) stock was initially freeze-dried and needed to be revived prior to experimentation. The frozen stock culture was thawed and a loopful of the stock culture was transferred to BHIB (brain heart infusion broth) and was incubated for 24 hours at 37°C to be brought to stationary phase. One loopful of the culture was transferred thrice at intervals of 24 hours into 25 ml of BHIB and incubated at 37°C for 24 hours in an incubator. The culture was centrifuged at 16,770 g with a ThermoScientific Sorvall T1centrifuge for ten minutes. The pellet was then resuspended in 0.1M peptone buffer.

Initial Activity

The initial activity of the films was collected from the samples of the release test. The films that were created were stored at 4°C and used within a week. For initial activity, 60 mL of 0.3mg/mL

Micrococcus lysodeikticus suspension with a magnetic stirrer is poured into Erlenmeyer flasks. The initial activity film was placed into the suspension and samples were taken at t = 0, 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, and 10 min. The lysozyme activity was measured using a UV/VIS spectrophotometer at 450 nm. This was tested in triplicate.

Release Test

The activity of soluble lysozyme was determined by measuring the decrease in absorbance of *Micrococcus lysodeikticus* suspension (18mg/60mL) prepared in 0.1M Na-phosphate buffer at pH 7.0. A volume of 2.9 mL of the suspension was mixed with 0.1mL of the lysozyme release test solution. The decrease in absorbance was monitored at 450 nm for three minutes by using a UV/VIS spectrophotometer. The sampling times were at t = 0, 15, 30, 60, 90, 120, 150, and 180 seconds. The entire sample was tested at t = 0, 2, 4, 6 9, 12, and 24 hours. Activity was calculated by the initial slope of the linear portion of the logarithmic plot of the absorbance against time. These samples were run in triplicate. The spectrophotometer used in these experiments is shown in Figure 3.2. The procedure ran in triplicate.

Final Activity

The final activity of the films was collected from the samples of the release test. This tested the ability for the lysozyme to remain immobilized to the films. The films tended to crumble in the solution. Centrifuging the sample tubes for two minutes at 8000 rpm avoided the film crumbling. This condensed the film, making it easy to pour out the lysozyme release test solution without losing any film particles. For final activity, 60 mL of 0.3mg/mL *Micrococcus lysodeikticus* suspension with a magnetic stirrer was poured into Erlenmeyer flask. The final activity film are scooped into the suspension and samples are

taken at t = 0, 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, and 10 min. The lysozyme activity was measured using a UV/VIS spectrophotometer, as before at 450 nm. The procedure ran in triplicate.

Listeria Innocua and films

Listeria Innocua was tested by preparing a BHIB inoculating with *Listeria* and placed into seven Erlenmeyer flasks. The microorganism would go through a growth phase during this procedure and it was questioned whether the film would reduce bacterial growth. There were five Erlenmeyer flasks containing a 5x5 cm film with lysozyme and a corresponding level of glutaraldehyde. One flask contained a control film with no lysozyme and no glutaraldehyde. The last sample has no films interacting with the *Listeria*. This is the control for the growth curve of *Listeria*. *Listeria* was proven to be a pure culture prior to experimentation by an API strip. The concentration of bacteria was determined by correlating the data from plating the culture onto plate count agar (PCA) and correlating it with readings for the UV/VIS spectrophotometer. Samples were made to a calibration curve from the PCA agar to the spectrophotometer to compare the readings with the CFU/mL amount of bacteria. PCA agar may have other bacteria growing on the agar but if all the colonies small, uniform, and have a bluish luminescence in florescent light it can be assumed that it is *Listeria*. It can also be confirmed on modified oxford agar. The wavelength of the UV VIS spectrophotometer is established at 600 nm based on the literature data on *Listeria* (Tokarskyy 2008).

Results and Discussion

The most vital part of the experiment was to produce a film that can be easily reproducible and effective. In Figure 3.3, it can be seen that several different concentrations of cross linking agents were added as given in the procedure. Timing was crucial for in making uniform, effective films. Bubbles were an issue when casting the films but they can be easily overcome with carefully casting the film with 5mL from a pipette and spreading it evenly over the acrylic surface. If this was done correctly, the

splicing tape can be removed and the film comes off the plate very easily. Figure 3.4 shows a close up of the corn zein film that was just dry cast.

Glutaraldehyde has been reported to increase cross linking in acidic conditions. Acetic acid (1mL to the current procedure) was added to films to try to increase cross linking of the film. However, the quality of the film severely degraded. As seen in Figure 3.5, the film could not be peeled when acetic acid is added to the film solution. Acetic acid is also considered an antimicrobial that has been used in tomatoes through a chitosan polymer to reduce *Salmonella* Montevideo (Zhuang 1996).

Initial and Final Activity

The procedures for initial and final activity were extremely similar and were a great indicator for determining how much lysozyme was active on the film. Figure 3.6 demonstrates how the film was added to the *Micrococcus* solution to determine initial activity. One 5 x 5 cm piece of film was placed into 60 mL of *Micrococcus* broth. The final activity was evaluated after the film had been submerged in 0.1M phosphate buffer (pH = 7) for 24 hours. Phosphate buffer is used to help regulate the pH in the system. In Figure 3.7, the activity of the film is being determined after being submerged for 24 hours. This helps determine how much of the lysozyme is adhered and the effectiveness of the antimicrobial in the film. The procedure was varied from the initial activity to clean the film prior to testing the final activity of the film. One easy observation to make during the final activity test is the opaqueness of the solution. The solution turned a clear color compared to the other *Micrococcus* solutions. The film was then centrifuged and placed into 60 mL of *Micrococcus* broth and sampled for three minutes. In Figure 3.8, it can be seen that adding glutaraldehyde decreased the initial and final activities of the film. The addition of 0 and 0.05 g/g lysozyme: GA had no statistical significance ($p>.05$) and those were the most effective addition of cross linking agent for the addition of GA. The most effective additions of GO was 0 and 0.05 g/g lysozyme: GO and can be seen in Figure 3.9.

While working with glyoxal, there were very similar trends as using glutaraldehyde. GO was chosen because of the effects of pH. In Figure 3.9, the trend line is very similar to GA. GO is most effective with 0 or 50 g/g lysozyme:GO added. The software used to analyze the p values for statistical analysis was JMP 8. In JMP, using 1 way ANOVA it was proven that choosing a cross linking agent (GA or GO) had statistical significance on the enzyme activity ($p<.05$). However, the levels of 0 and 50 μL of GO or GA were not statistically significant from one another ($p>.05$). In other words, the addition of 0.05 g/g lysozyme: GA or GO did not have a significant increase to be able to say that adding a cross linking agent ever improved the activity of lysozyme. The only thing that can be said is as the concentration of cross linking agent increased, it would inhibit lysozyme in high concentrations. The initial activity in GO is not as high as in GA. However, the final activity did have a similar percent decrease from the initial activity. When producing the films, a variety of methods such as timing for the addition of each ingredient were aimed to improve the activity but none were successful.

In GA, to increase cross linking an acid is needed. However, this had a major effect on the tensile properties of the films. The films became brittle and can be seen in Figure 3.10. Acetic acid was added to the films when the GA was added. Each film tested after this became brittle and unable to be peeled from the acrylic film. The values for activity were very similar to the films with no acetic acid adjusted. In Figure 3.11 shows a film where the pH was not adjusted. This allowed the film to remain cohesive and not break off from one another.

Release Test Activity

The release test has provided some expected results. The release test has provided information that would conclude as concentration of cross linking agent increased, lysozyme released was decreased. The storage conditions for the release test are shown in Figure 3.12 where the film is placed in 30 mL of 0.1M peptone buffer. This is left for a 24 hour period to see how much lysozyme is released into the

peptone buffer. Similar problems occurred when adjusting the pH. In Figure 3.13, it can be shown that even with light shaking of the film prior to sampling, the film would crumble.

Figure 3.14 shows that when the amount of GA increased, less lysozyme is released into the 0.1M peptone buffer. If the enzyme is released, then the lysozyme has been rendered inactive. In Figure 3.15, the same trend is observed for GO. As GO increases, fewer enzymes are released into the peptone buffer. Another unique thing that can be seen from figures 3.14 and 3.15 is the initial increase of activity for both GA and GO for the release of the enzyme until T = 6. At T = 6 the activity level drops down significantly. This is very difficult to explain and it could have something to do with the pH of the system or the lysozyme could be reattaching to the film.

GA and GO both had variability. If there was any inconsistency during film making, the results did not follow the expected results. The higher concentrations of GA and GO tended to have approximately a 2 log reduction of bacteria (confirmed with bacterial cultures that matched the concentrations given in Figure 2.1). The more effective release tests were able to destroy about 3 logs of bacteria in 0 and 0.5 additions of lysozyme to GA or GO. As more cross linking agent was added, this less lysozyme was released in the solution.

Control release was achieved in 0.1 and 0.2 lysozyme: GA. Also, control release was established in 0.1, 0.2, and 0.5 lysozyme: GA solutions. However, the control release must be compared to the activity of the films given from the initial and final activity to select the best films to be used against the pathogenic bacteria on tomatoes in the next chapter.

Again, pH is an issue then the film looks like Figure 3.10. If the acetic acid is not added the film should resemble 3.11. pH was a major indicator for this project and it should be a variable of consideration when building future films.

The release test is not consistent with the initial and final activity. The expected results would be that there would be higher activity from the film when less lysozyme is released from the film. However

if 0.5 g/g lysozyme: GA is used in the film there is no activity for the lysozyme in the release test or on the film. The cross linking agent must be playing a major role in enzyme inhibition at higher concentrations for both of these tests to be true. However, more tests would need to be run to be able to conclude this for a fact.

Listeria Innocua

Listeria was tested to see if lysozyme would be effective for the use on tomatoes. A non-pathogenic *Listeria Innocua* was used to establish the data for Figure 3.16. The interesting result is that each of the films took between 12-24 hours to reduce the growth of *Listeria Innocua.* While in every other experiment the lysozyme acts so quickly and pretty readily leaves the film to interact with the solution. This could be because the BHIB broth is so nutritious that the growth rate of *Listeria* was far higher than the death rate caused by the film until the stationary growth phase. In the stationary phase, the growth and death rate are typically very similar to reach the stationary phase.

The control film with no cross linking agent or lysozyme was able to reduce the amount of *Listeria* by 1-2 logs. It was expected that the lysozyme would reduce *Listeria Innocua* by 3 logs. This could be because the film is ethanol based and the film could be deteriorating in the BHIB. This is further explored in chapter 4.

Conclusion

This production of film provided positive results. There is a 3 log reduction of *Micrococcus Lysodeikticus* and *Listeria Innocua*. The film at 0 and 0.05 g/g lysozyme: cross linking agent were the most effective for both initial and final activity. When the amount of glutaraldehyde increased, it allowed more lysozyme to bind to the film. Control release was established in the 0.1 and 0.2 g/g lysozyme: cross linking agent. However, the film did not allow the lysozyme to interact with the product as well as the lower concentrations. This could be because the lysozyme could be binding the lysozyme to the interior

of the film and not allowing it to interact with the bacteria. Very similar results happened when using glyoxal as a cross linking agent. It was able to be determined that as the concentration of cross linking agent increased, the more lysozyme is being bound to the film. However, the activity for these films are most active for the 0 and 50 mL of glyoxal added.

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Figures Chapter 3

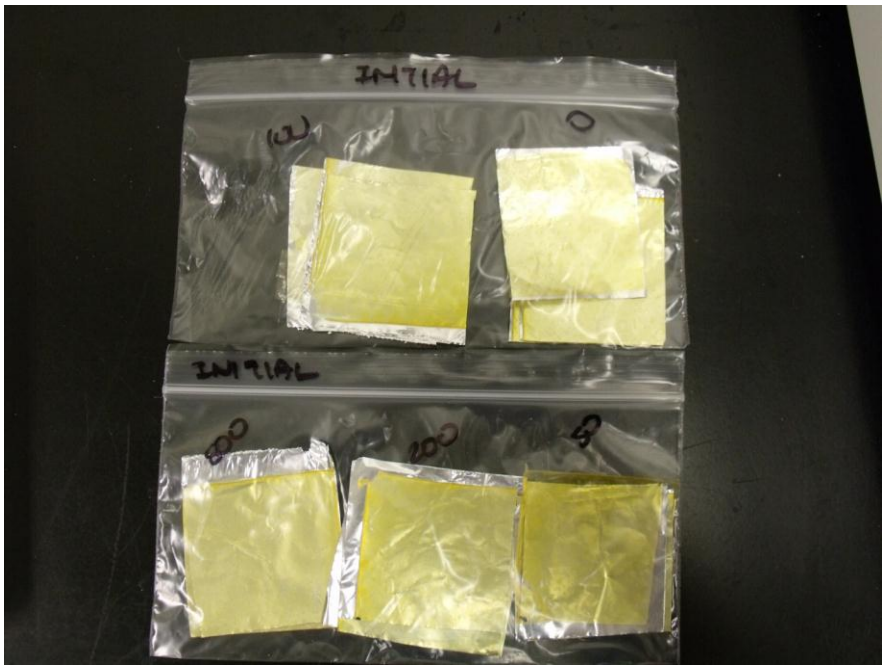

Figure 3. 1. Stored films were kept in 4°C to ensure lysozyme remained stable. Samples were used within a week of production.

Figure 3. 2. The UV/VIS Spectrophotometer and samples during the release test.

Figure 3. 3. Several different concentrations of Glutaraldehyde being mixed prior to casting under uniform conditions.

Figure 3. 4. A close up on the cast corn zein film. Bubbles were eliminated when casting the film to create a uniform film.

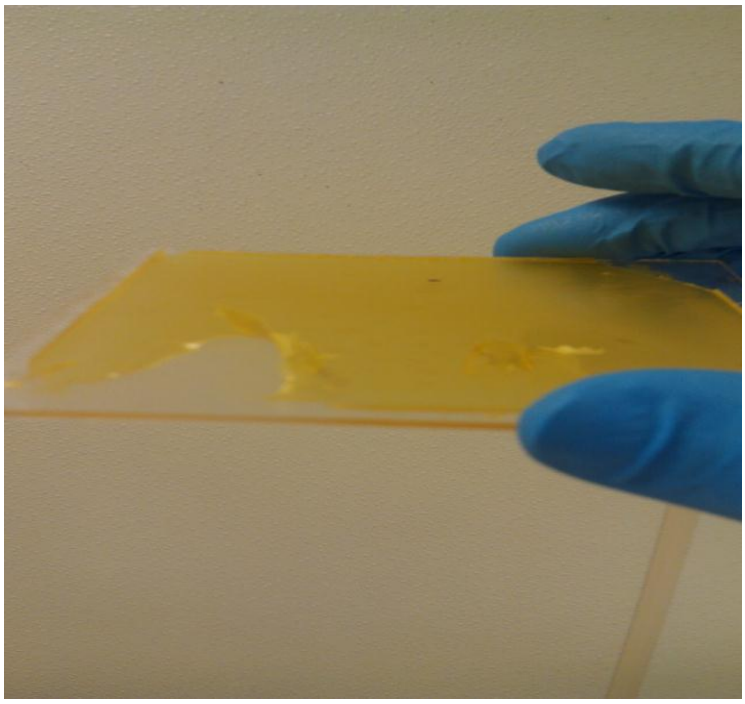

Figure 3. 5. pH was too acidic and caused the film to become brittle and not peel from the acrylic surface.

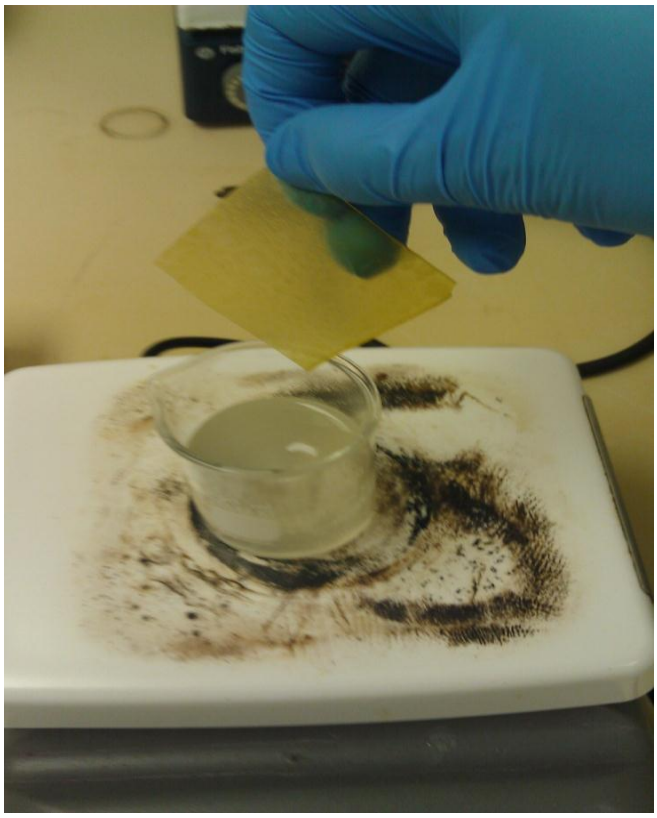

Figure 3. 6. Initial Activity Test is shown here with the film being added to the *Micrococcus Lysodeikticus* solution.

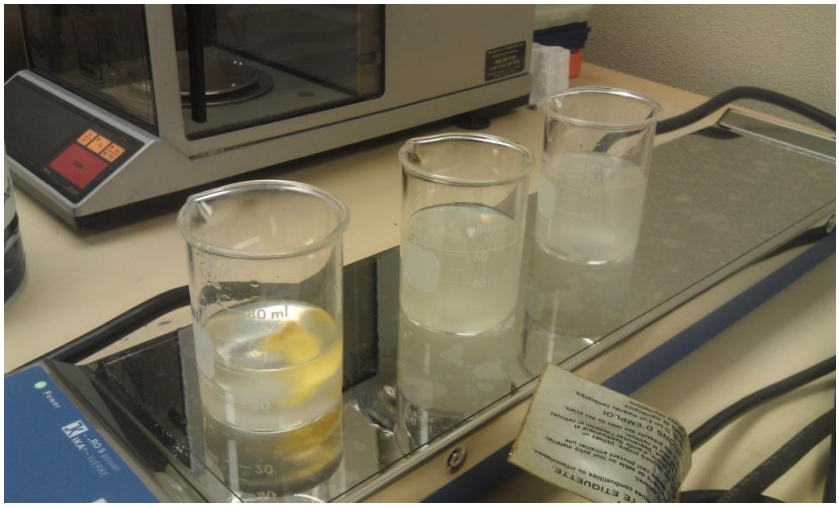

Figure 3. 7. Final Activity Test is being shown against *Micrococcus Lysodeikticus*. The solution will become visibly more clear and this uses the UV/VIS spectrophotometer to put a numerical number with the turbidity.

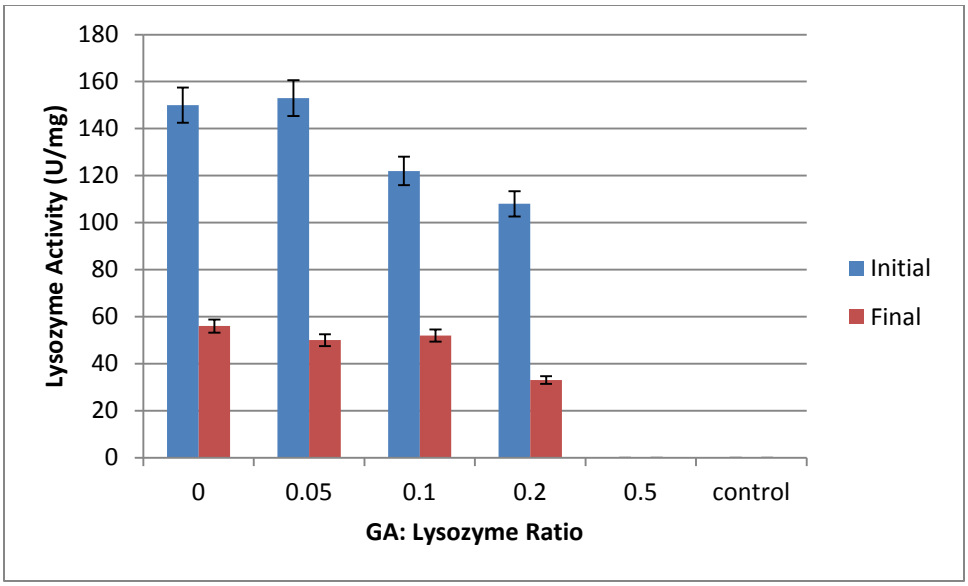

Figure 3. 8. The initial and Final Activity is used for Glutaraldehyde films.

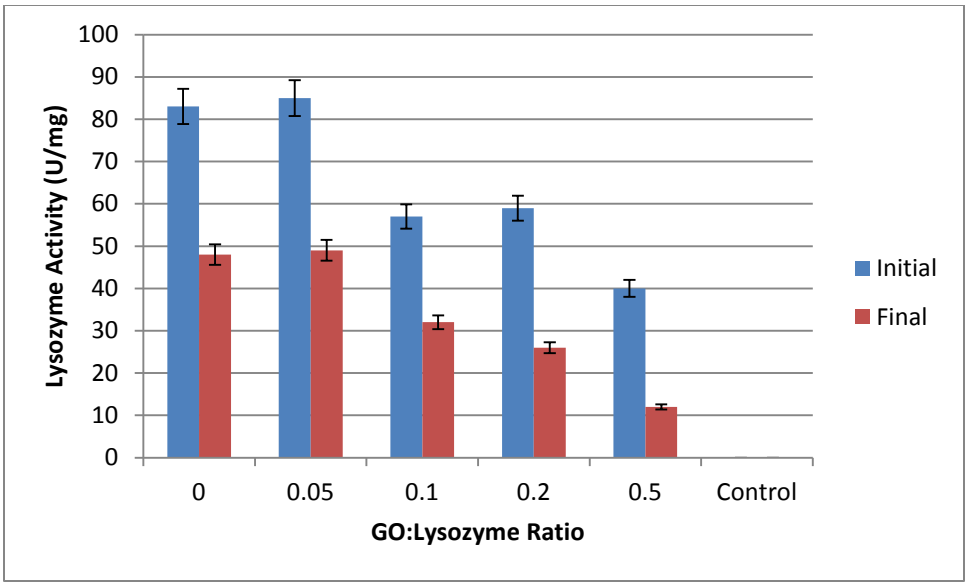

Figure 3. 9. The initial and Final Activity is used for Glyoxal films.

Figure 3. 10. Effect of pH Acidic film increased brittleness during final activity test.

Figure 3. 11. Brittleness of the film decreased if concentration of GA increased or if the pH was not adjusted.

Figure 3. 12. Storage conditions for release test for 24 hours in pH 7 phosphate buffer.

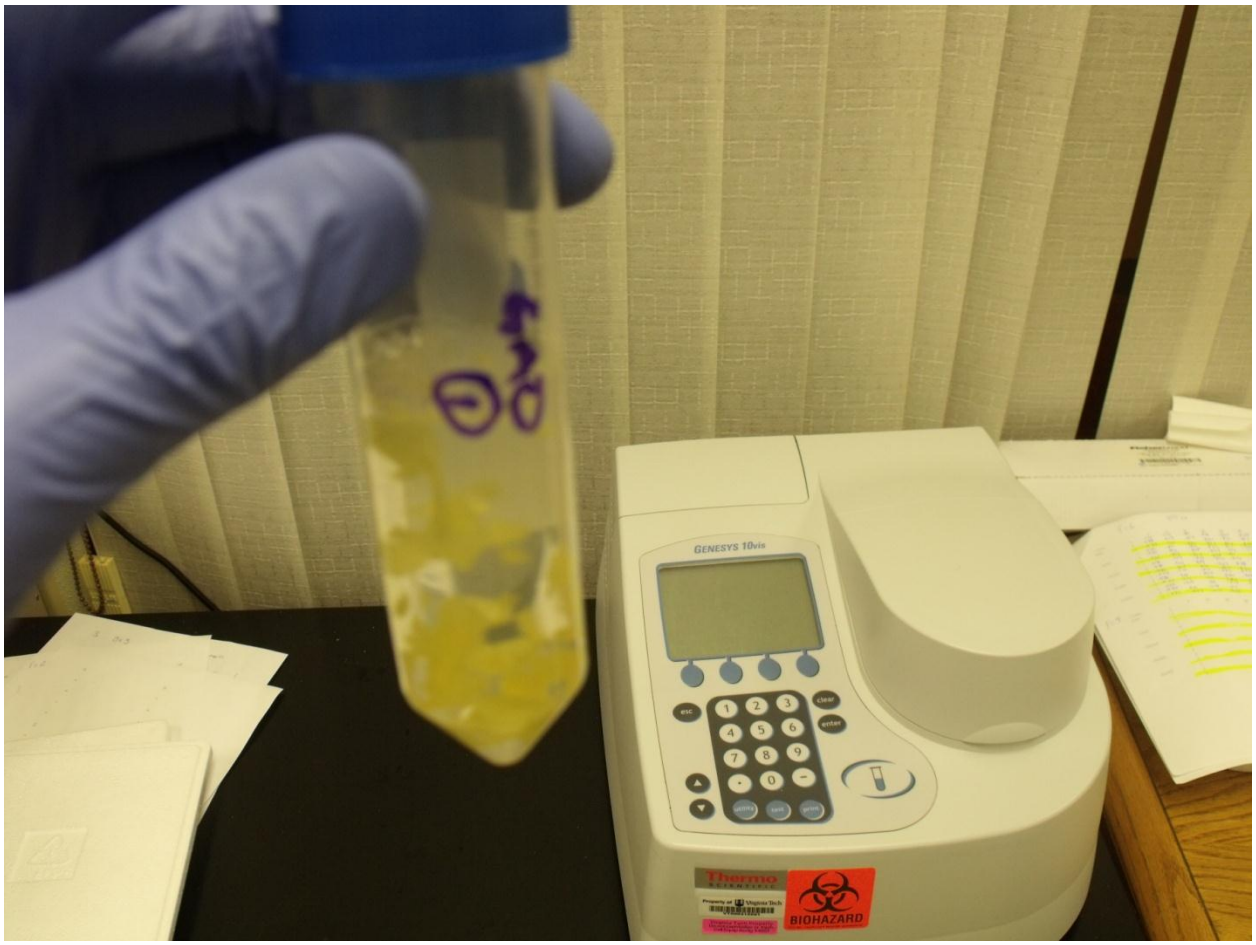

Figure 3. 13. Another example of the adjusted pH caused brittleness for the release test. When the sample was stirred prior to use the film would break down.

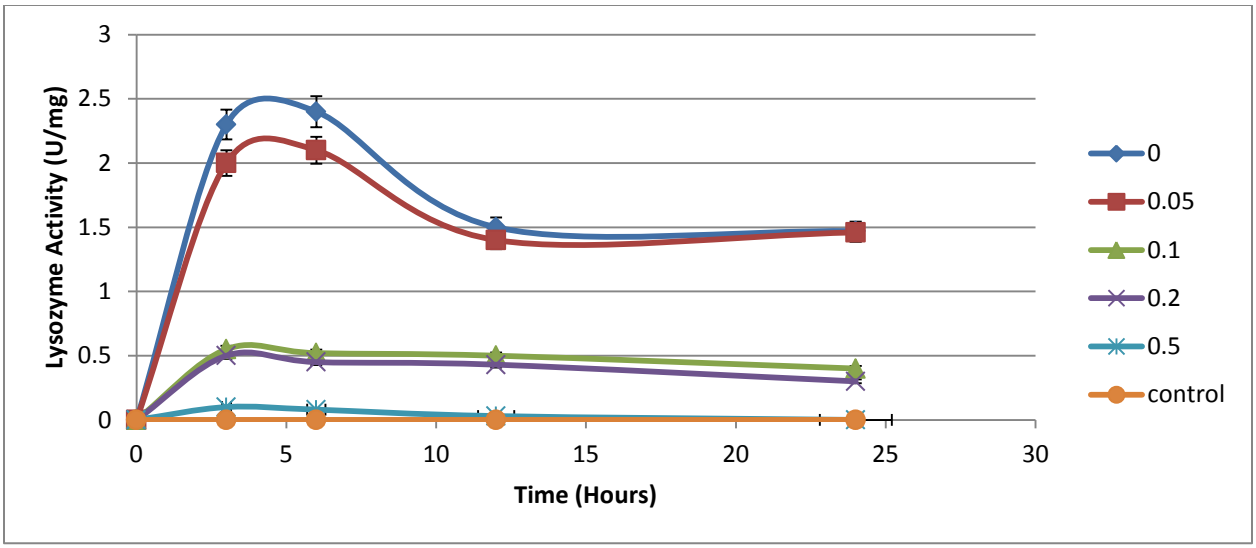

Figure 3. 14. Amount of active lysozyme with GA as a cross linking against *Micrococcus Lysodeitkus* that was released from the film into the 0.1M Peptone buffer solution. The 0.1 and 0.2 demonstrated a control release of lysozyme. However, activity was significantly decreased at higher lysozyme to GA ratio.

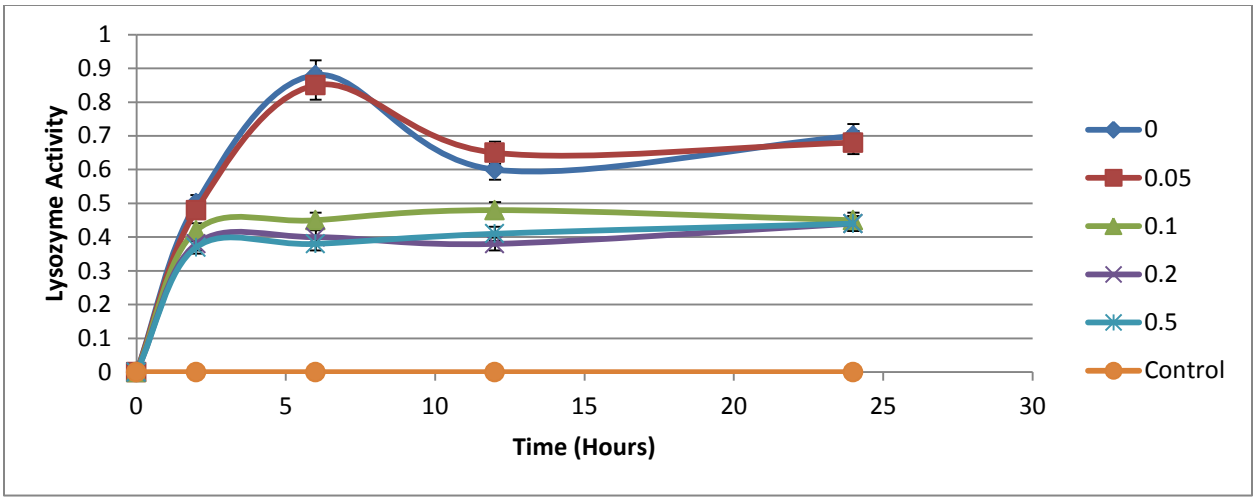

Figure 3. 15. Amount of active lysozyme with GO as a cross linking against *Micrococcus Lysodeikticus* that was released from the film into the 0.1M Peptone buffer solution. 0.1, 0.2, and 0.5 demonstrated a control release of lysozyme.

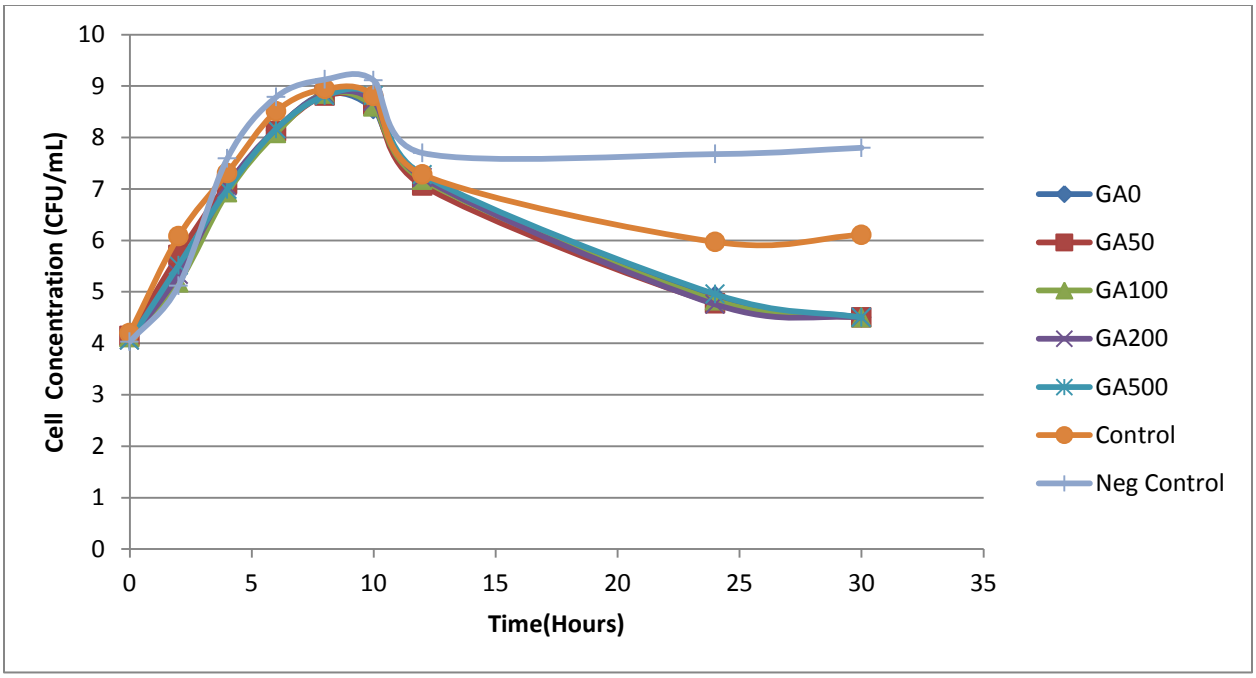

Figure 3.16. Non Pathogenic *Listeria Innocua* was used against the films for testing. Negative control refers to no film was added and the control was a film with no lysozyme or cross linking agent. Control is the film and each of the GA is the amount of GA added to the films.

Chapter 4: Effect of using the film on fresh cut tomatoes.

Abstract

Recent outbreaks in ready to eat vegetables have been well documented. Microbial destruction is necessary for these foods to remain safe, but the use of heat would alter the characteristics of ready to eat vegetables. The use of active biodegradable packaging could be used to reduce bacteria without compromising composition of the vegetable. Lysozyme was selected because of its ability to reduce gram positive bacteria.

The main objective of this study is to study the effect of edible packaging film on pathogen reduction in tomatoes that have been contaminated with *Listeria monocytogenes* and *Salmonella* Newport. The tomatoes were prepared in two ways, skin and sliced (interior of the tomatoes), and stored in room temperature for a week. The samples were prepared with no film, a control film (no lysozyme), 0.05 lysozyme: glutaraldehyde film, and a 0.05 lysozyme: glyoxal film. The addition of cross linking agents glutaraldehyde and glyoxal to lysozyme incorporated corn zein films were effective at reducing the gram positive bacterial contamination by three logs on the *Listeria monocytogenes.* However, the films were unable to significantly reduce the content of *Salmonella* Newport.

Introduction

Approximately 5 billion pounds of fresh tomatoes are eaten annually in the United States. This is a staple food in the U.S. and all over the world. Tomatoes contain a carotenoid called lycopene which has been attributed to several health benefits because of its antioxidant properties. These properties also include some potential anticancer benefits. The main form of lycopene in the tomato is all-trans-lycopene. The lycopene can be more bioavailable in other sources such as tomato paste, spaghetti sauce, tomato soup, salsa, or ketchup (Gartner 1997). With so many uses for tomatoes and so many people consuming tomato products, tomatoes must be looked at from a food safety standpoint.

Currently, there remains high numbers of outbreaks regarding fresh fruits and vegetables. *Salmonella* Newport has caused outbreaks in different varieties of tomatoes. *Listeria monocytogenes* has been associated with a recent outbreak in melons. *Listeria monocytogenes* behavior has been cited in Beuchat (1991) in tomato and tomato products. Prior treatment of the tomatoes with chlorine or packaging did not have an influence on *Listeria'*s growth. The concentration of *Listeria* remained constant for two weeks. *Salmonella* Montevideo was also proven to have stability in tomatoes. Temperature was a major concern during storage and transport to minimize growth of the bacteria (Zhuang 1995). These bacteria are major health hazards as explained in chapter 2.

Recent methods to reduce the amount of bacteria on the skin of the tomatoes are the using X-ray radiation and use of chlorine dioxide gas (Mahmoud 2010, Trinetta 2010). There have been other discoveries such as high pressure processing to reduce *Salmonella enterica* serovars in diced and whole tomatoes (Maitland 2011). This was optimized by placing the tomatoes in a 1% calcium chloride solution and run at 450 MPa to reduce bacteria about seven logs. This did have quality effects and the calcium chloride did reduce the damage caused by the X-ray.

Shelf Life Extension

Edible corn zein film coatings have been proven to extend the shelf life of tomatoes (Park 1994). A six day delay in ripening of the tomatoes (color change to red) occurred for 5 and 15 μm thick corn zein coating without adverse effects. These edible coatings made from food materials are generally recognized as safe (GRAS). These coatings provide good barriers from oxygen and carbon dioxide but a poor barrier to water vapor (Guilbert 1986). Oxygen and carbon dioxide barriers lead to a reduction in respiration by limiting exposure to ambient oxygen and increasing internal CO_2. Water vapor transmission allows movement of water across the coating and prevents water condensation which is a potential source for microbial spoilage (Ben-Yehoshua 1985).

Corn zein is an alcohol soluble protein with excellent film and fiber forming properties. Corn zein has been applied to candies and nuts because it is a good barrier to oxygen and the water vapor permeability is about 800 times higher than typical shrink wrapped film (Aydt 1991). Corn zein is very available due to the increase of the bio ethanol industry, and corn is a major source for producing ethanol.

Several types of tomatoes were looked at because of recent outbreaks in tomatoes. Three tomato types of particular interest are red round, roma, and grape tomatoes. Red round tomatoes are easily skinned and readily available for large sampling. Roma tomatoes tend to be a very hard tomato. It is difficult to get the skin from the roma tomato since the core is so firm. Grape tomatoes tend to be easy to peel the outside. However, the size of grape tomatoes limits the skin that can be peeled. When working with the grape tomatoes the skin can be very thin an also break easily. The tomato that was chosen for the experiments was the red round tomato because that strain of tomato was very easy to get samples for the skin and interior of the tomato.

Objective

Previous methods have applied the corn zein as a dipping method to extend the shelf life of tomatoes. Dipping methods expose the tomato to a liquid film solution and uses two main variables: time dipped and time dried. However, these methods are invalid since the corn zein solution is based from a

95% ethanol. The use of ethanol can cause off flavors and the purpose here is to control release lysozyme from the film. This would cause the bacteria of interest to react to the ethanol based dip and have a major reduction of bacteria. This is why the methods described here are used where the films are dry cast in room temperature for three days, and then these are applied to tomatoes that have been contaminated by *Salmonella* Newport and *Listeria monocytogenes*. The film should be able to reduce the concentration of *Listeria* because of the lysozyme. The only way *Salmonella* is significantly reduced is if there is still some residual activity from the ethanol on the films.

The objective of this study is to create explore the effectiveness of the lysozyme and cross linking agent (GA and GO) against pathogenic bacteria in a tomatoes. This test will be run against gram positive and gram negative bacteria and will be tested over a one week period to establish if the film can reduce the concentration of bacteria and keep the bacteria from growing on the tomatoes.

Materials/Methods

Tomatoes

Organic red round tomatoes were purchased at a local supermarket (Blacksburg, VA) the day of experimentation. The red round tomatoes are washed with distilled water on the exterior of the tomato and prepared in two ways. The first way is to skin the tomatoes. The tomato will be cut in half and the tomato will have the core removed leaving only the skin. The second way will take very thin slice (3-5 mm) of tomato to inoculate the interior of the tomato.

Culture Maintenance

Stock cultures of the *Salmonella* Newport and *Listeria monocytogenes* were obtained from the culture collection at Virginia Polytechnic and State University. The cultures were confirmed through biochemical and serological tests before inoculum preparation. The frozen stock cultures were thawed and

a loopful of each stock culture was transferred to 25 ml of BHIB (Brain Heart Infusion Broth) and incubated for 24 hours at 37°C. One loopful of the culture were transferred three times at intervals of 24 hours into 25 ml of BHIB and incubated at 37°C for 24 hours in a shaking incubator. A loopful of cells from the final transfer into BHIB was streaked onto a modified oxford agar/ xylose lysine tergitol 4 agar for presumptive determination of typical colonies and on tryptic soy yeast extract agar for growth on non-selective medium cultures. The plates were incubated for 24 hours at 37°C. A loopful of culture from the TSA plate was confirmed biochemically using 20E API strips (bioMerieux, Hazelwood, MO) and serologically using latex agglutination tests. Upon confirmation, colonies were streaked on TSA slants and incubated for 24 hours at 37°C, following which the slants were stored in the refrigerator at 4°C for inoculum preparation (Guo 2002).

Inoculum preparation

A loopful of inoculum from the slants was used to inoculate 25 ml of BHI broth. The broth was incubated at 37°C for 24 hours in a shaking incubator. After incubation the culture was centrifuged at 16,770 g with a ThermoScientific Sorvall T1centrifuge for ten minutes. The supernatant was decanted. The pellet was washed twice with sterile distilled water to rid the culture of media. After washing, the pellet was suspended in 10 ml of sterile 0.1% peptone water (PW). The *Salmonella* spp suspension was serially diluted in distilled water to achieve desired concentrations of inoculum (Guo 2002).

Tomato Inoculation

The tomatoes were prepared in two ways using red round tomatoes. A scalpel was used to cut and core the tomato until just the skin remains. If done properly, the skin was very porous and if held up to light it can appear to be an almost yellow, clear color. When slicing the tomatoes, it was important to get the tomato as thin as possible (about 3-5 mm thick) The tomato are sliced, then cut into quarters to

get a 2.5 cm x 2.5 cm slice of tomato. The cultures should begin experimentation at ~10^8 CFU / mL. To inoculate, 100µL of culture media were transferred to the surface of the tomato and spread on the surface of the skin or interior of the tomato.

Films

The films that were used are glutaraldehyde, glyoxal films, and a control film which did not include any glutaraldehyde, glyoxal, or lysozyme. First, 163 mL of ethanol (95%) was placed into a large glass beaker. The ethanol was placed on a magnetic heating/stirring pad and elevated in temperature to about 35°C. This allowed the corn zein to dissolve more easily. Next, 27g of corn zein powder was slowly added to ethanol until all the corn zein had dissolved. Then, 5 mL of polyethylene glycol (PEG) and 5 mL of glycerin were added to the corn zein ethanol solution as a plasticizer. Once dissolved, the beaker was capped and the temperature of the ethanol was increased to a light boil (~80°C). The solution then allowed to cool for about an hour. This solution was separated into three smaller beakers that have 0, 50 GA, and 50 GO. After the GA and GO are added to their respective solutions for about two hours and then 1 mL of lysozyme stock (1g lysozyme /10 mL 0.1 M peptone buffer) was added to the GA and GO solutions. The solutions were slowly poured onto acrylic flat plates with 3M Scotch linerless splicing tape covering the outside of the 5x5 plates. The tape was added to the edge of the plates to make it easier to peel the film from the surface. The films were cut into 2.5 cm x 2.5 cm samples to lay in contact with contaminated area of the tomatoes. Samples were held in stomacher bags until testing. Tests are run in triplicate to ensure reproducible results.

Sampling Times

Samples were prepared by taking the film off of the sample prior to testing. The samples were placed in a stomacher bag with 9 mL of peptone water in the sample and stomached. One mL of the stomached sample was serially diluted in 9mL PW until the proper concentrations were made. If the sample was inoculated with *Salmonella* Newport, it was spread onto XLT4 Agar. If the samples were

contaminated with *Listeria monocytogenes*, modified oxford agar was used. Samples were stored for one week at ambient temperature. Samples were removed on days 0, 1, 3, 5, and 7 for analysis. Samples were run in triplicate using both types of films and using the inner slices of tomatoes and the outside skin of the tomato. Sampling used two types of tomatoes, two types of contamination (*Listeria* and *Salmonella*), four types of films (none, control, GA, and GO), three replications, and samples for five days (one week of sampling time).

Results

When applying the contaminated films with tomatoes, it is important to ensure that the tomato has been skinned as thin as possible prior to testing. If the tomatoes are sliced skinned too thick, the sample being stomached releases pulp into the peptone water (PW) solution. This makes the pipette clog and it is difficult to get a good sample and mostly the supernatant was sampled from. When working with the interior of the tomato, it was extremely difficult to get a good sample. In figure 4.1 the tomato skin is shown to give the approximate shape and thickness of the tomato skin. The skin was chosen to simulate the conditions for the exterior of the tomato.

The variation that could have occurred from this experiment was pipetting from the stomached sample (pulp would clog the pipette) and the contact area of the film (there was about a 95% contact area with each sample). It was a possibility that the tomato or film would slide slightly during storage that did no ensure 100% contact area. Everything else should have been done in uniform and had little to no variation. Also, a few of the samples encountered problems from mold (especially the interior of the tomato). The corn zein films with lysozyme incorporated were not designed to combat molds and yeast.

In the following figures (4.4 – 4.7) it is apparent that the skin versus the inside of the tomato made very little difference on bacterial growth. The skin was much easier to sample on because of the lack of pulp that would be pulled in by the 1 mL pipette for sampling.

The films were stored for a week at ambient temperature. The *Salmonella* Newport was measured after 24 hours on XLT4 agar. When the plates were counted, *Salmonella* appeared as black colonies and would turn the agar a yellow color instead of a red due to a pH indicator on the agar as shown in figure 4.2. Also, on the XLT4 agar occasionally small, dull, white colonies developed when plated. It was determined that these colonies were not *Salmonella* and only appeared if the interior of the tomato was used. Modified oxford agar was used for the determination of *Listeria monocytogenes*. After 48 hours, the modified oxford agar turned black and colonies were counted with no issues as shown in figure 4.3.

Listeria monocytogenes contamination

Listeria is a microorganism that is very susceptible to being lysed by lysozyme because it is gram positive. In chapter 3, it was established that nonpathogenic *Listeria* would be reduced by a three log reduction when the film is introduced to a contaminated broth of nonpathogenic *Listeria*.

In the skin of tomatoes, it was very easy to sample and the results are shown in Figure 4.4. As shown in Figure 4.4, *Listeria monocytogenes* was reduced by the lysozyme added films by 3 logs after 1 day. The results were run in triplicate for each day and the variation always remained in the same dilution as shown by the figure. Lysozyme is expected to hydrolyze the β -1,4 glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine (Madigan 2006).

The interior of the tomato had consistent results with the data from the skin of the tomatoes. The films that incorporated lysozyme (GA and GO) were able to reduce *Listeria* by three logs as well. The results show that when the lysozyme is incorporated into the films it is able to reduce the concentration of bacteria from 10^7 to 10^4 CFU/mL. The results were tested and compared with 1 way ANOVA on each day of the results. The results show statistically significance ($p<.05$) that the GA and GO films were both able to reduce the amount of bacteria for the interior and exterior of the tomato compared using no film or the control film (no lysozyme). The control film (with no enzyme) was not statistically significant from

the no film control ($p > .05$) for the interior or skin of the tomato. This was expected because the control film had no lysozyme on it and therefore nothing else was inhibiting the growth. The results were analyzed with one way ANOVA. This method was chosen because it was important to figure out if the film was effective compared to no-film. The results for the interior of the tomato can be seen on figure 4.5. The *Listeria* is inoculated to a similar concentration as the skin of the tomato and has the same log reduction as figure 4.4. The interior of the tomato would be more acidic, and it had little to no effect on the effectiveness of the film. It is logical to conclude the interior versus the exterior of the tomato has little to no effect on the growth of the bacteria or the effectiveness of the film.

Salmonella Newport contamination

The expected results for *Salmonella* Newport were confirmed during this portion of testing. Lysozyme lyses gram positive bacteria while *Salmonella* is a gram negative microorganism. *Salmonella* Newport was chosen due to the recent outbreaks in tomatoes. Ethanol, glutaraldehyde, and lysozyme that are all incorporated in the film are all not expected to eliminate *Salmonella*. In the following figures it is apparent that the film did not effectively reduce the content of *Salmonella*.

The skin of the tomatoes had demonstrated a clear lack of reduction of the bacteria. When no film is applied to the tomato the *Salmonella* was able to live and grow on the tomato with no issues. In figure 4.6 it is clear that there was no reduction of *Salmonella* on the skin of the tomato. It can be argued that there is one log reduction for the GA result ($p < 0.05$) which was tested by 1 way ANOVA for each individual day. However, the reduction of Salmonella needs to be improved to be practically important. A one log reduction is not enough for this film to be considered effective against *Salmonella*.

Salmonella appears black on the XLT4 agar but dull, small, white colonies would periodically show up on the interior of the tomato. A picture of these would not show up on the camera that was used so a visual is not available. It was confirmed by colleagues in the food science department that it was not a form of *Salmonella* but other bacteria that could grow on the XLT4 agar. This bacteria was not

identified for which strain or species. In figure 4.7, it can be shown that the film had little to no effect on the concentration of *Salmonella*. The results remain consistent that with the skin that the *Salmonella* Newport is not effectively reduced by the film. This was expected because the major mode of destruction of the bacteria was lysozyme, which is only effective on gram positive bacteria.

Conclusions

The films that included lysozyme could successfully reduce the amount of *Listeria monocytogenes* by a three log reduction. However, these films are not designed to combat any other types of bacteria besides gram positive bacteria. Very little difference was observed when using the interior of the tomato versus the skin. The skin took much more preparation to use, however limited errors due to thickness of the slice, mold, yeast, or sampling the tomato after stomached (the pulp clogs the pipette).

The films that had lysozyme (GO and GA) in them had the expected results. The lysozyme was able to reduce the content of *Listeria monocytogenes* by a three log reduction for both the interior and skin of the tomato. Films that did not include lysozyme did not have any effect on the both types of bacteria. *Listeria* and *Salmonella* were able to remain alive on the tomato for five days at room temperature. The tomatoes that were purchased from the local supermarket were sold in an ethylene gassed red color. Mold and yeast played a major part in cutting the experiment off. This could be avoided by using refrigerated conditions but then chilling injury could alter the results.

Salmonella Newport contaminated tomatoes had one log reduction due to the film with GA. GA is used in hospitals to prevent contamination but the concentration in the film is not high. If the concentration of GA is too high, it can be toxic. This was not a significant log reduction but it was unique from the other samples.

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Figures (Chapter 4)

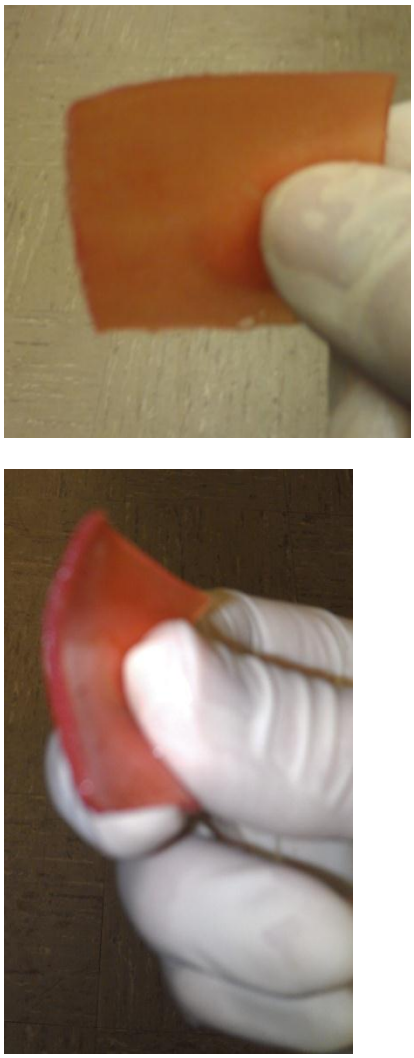

Figure 4. 1. Sliced tomato prior to inoculation.

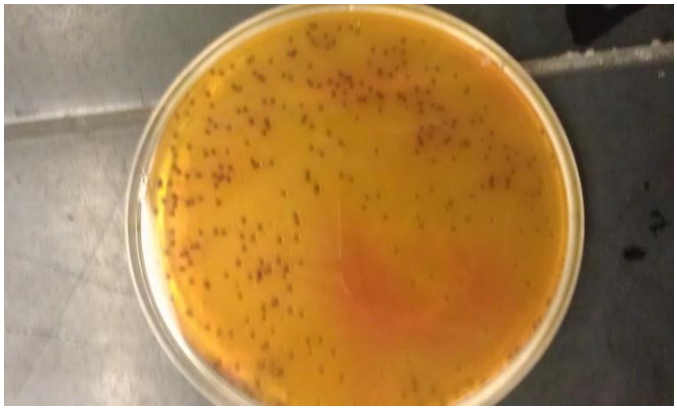

Figure 4. 2. *Salmonella Newport* growth on XLT4 Agar after 24 hours.

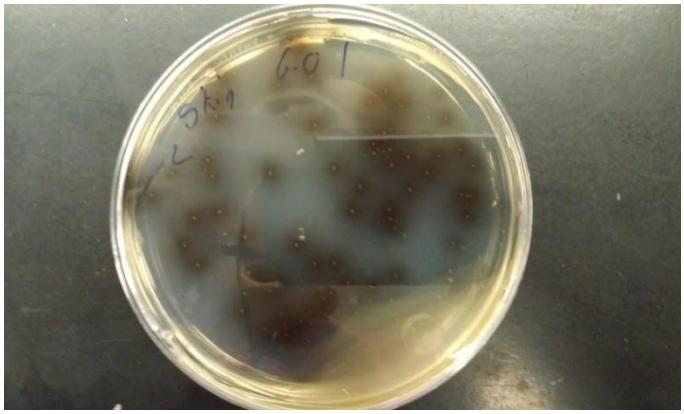

Figure 4. 3. *Listeria monocytogenes* growth on modified oxford media.

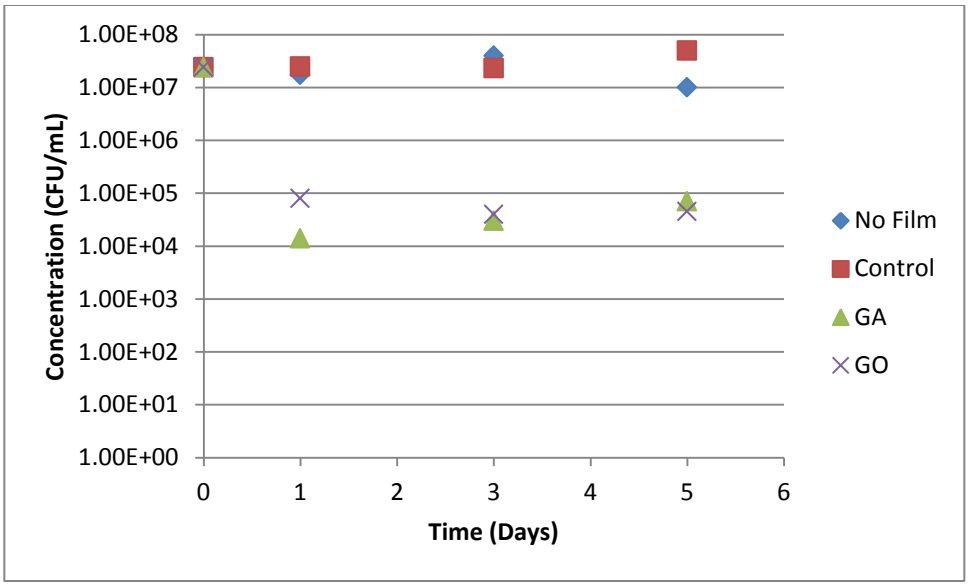

Figure 4. 4. *Listeria Monocytogenes on the skin of the tomato for five days. Significant decrease in GA or GO films.*

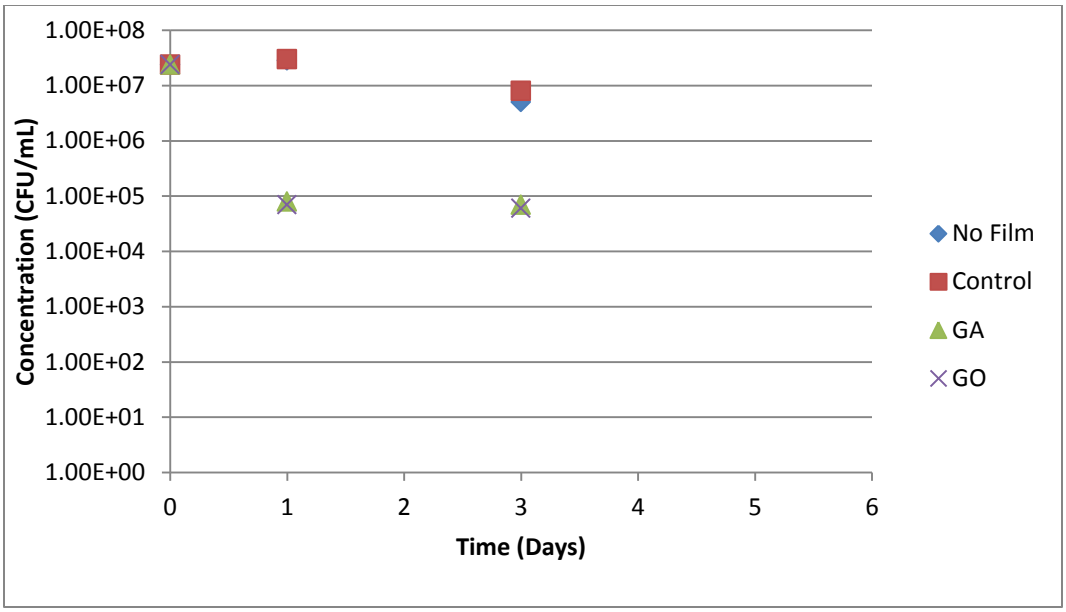

Figure 4. 5. *Listeria Monocytogenes* for the interior of the tomato for three days. The last day was not able to be established because the films had molds grow on them for all samples.

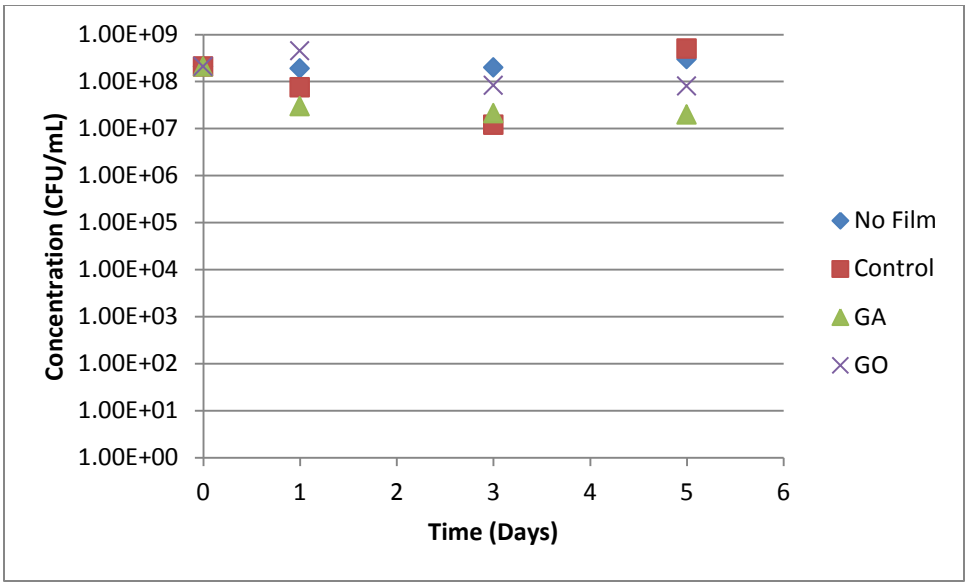

Figure 4. 6. *Salmonella newport* on the skin of tomato for five days. Only a 1 log decrease in the GA film.

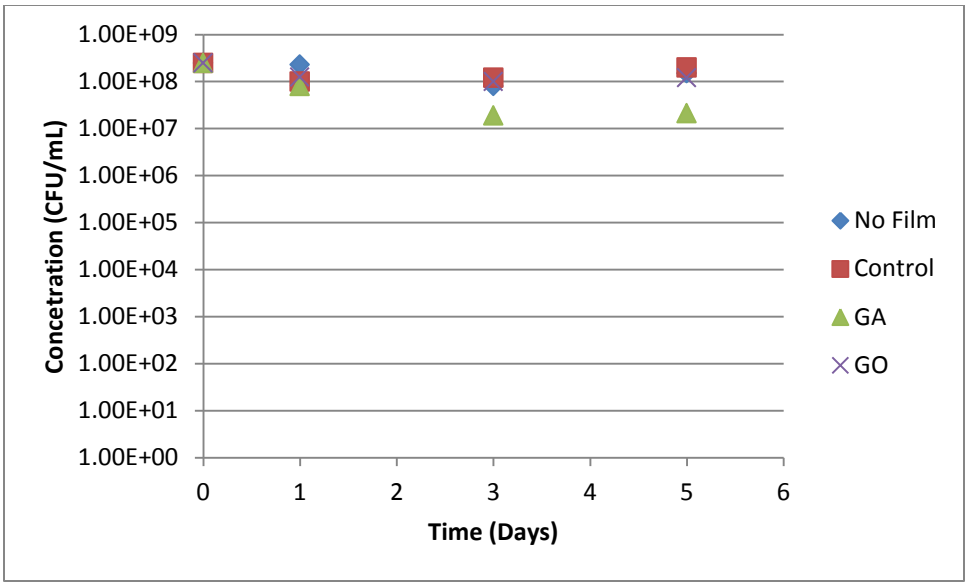

Figure 4. 7. *Salmonella newport* for the interior of the tomato for five days. Only a 1 log decrease after 5 days for the GA film.

Chapter 5: Conclusions/Future Work

Conclusions

The research project investigated the effect of adding a cross linking agent to enhance activity of an antimicrobial agent to extend shelf life of fresh tomatoes. As cross linking agents concentrations increased on the films, the activity of the films decreased as measured by the initial and final activity. Other factors such as pH had a major effect on the activity of the enzyme. If the pH was too basic, the lysozyme would be inactivated. If the pH was too acidic, the film would have weakened tensile properties. On tomatoes, the lysozyme spiked films were able to reduce *Listeria monocytogenes* by a three log reduction which was statistically significant ($p<.05$). Also, it was statistically significant that *Salmonella* Newport was reduced by one log with the film that incorporated GA in chapter 4. However, a one log is not practically important and this would need to be improved to effectively fight *Salmonella*. Overall, the project did provide a good basis for film production and there are plenty of directions that this project could take in the future.

The first study that was run was to establish the optimal concentration of different cross linking agents. For this study, glyoxal and glutaraldehyde were used. When cross linking agent increased, active lysozyme would not be released from the film for one day. This was proven by the release test, which had the film suspended in 0.1 M Peptone buffer for 24 hours. However, high concentrations of cross linking agent inactivated the lysozyme on the film. When cross linking agents was added at 0.5 g/g Lysozyme: GA, there was little to no activity from the film. These are contradicting ideas, and the cross linking agent must have an effect on activity for both of those statements to be true.

Glyoxal and glutaraldehyde at the 0 and 0.05 g/g Lysozyme: GA or Lysozyme: GO were the optimal concentrations of cross linking agent for the initial and final activity. There was no statistical significance between the 0 and 0.05 g/g Lysozyme: Cross linking agent ratio. When 0.1 g/g Lysozyme:

GA or higher concentrations were added for the films, the lysozyme began showing signs of a control release. However, these were not chosen in chapter 4 due to the drop off of 20-30% of the activity. If time was extended prior to use, it could be justified to use the 0.1 or 0.2 Lysozyme: GA ratio.

Also during the first study, pH was also another major indicator. When acetic acid was added to the films the films would become brittle. If a small amount of sodium hydroxide was added to the film, the pH would become too basic and inactivate the lysozyme. However, the film was not brittle in basic conditions. When producing a film the pH should be a characteristic of interest.

The second study used fresh cut tomatoes, inoculated them with *Listeria monocytogenes* or *Salmonella* Newport, and used the film to reduce the bacterial load. The films were prepared in four ways. The first way was to have no film for the tomatoes, establishing a baseline. The second was to prepare a film with no cross linking agent or lysozyme. This film was to ensure there was no other mechanism that was killing the bacteria (such as the film being produced from 95% ethanol). The last two films were prepared at the 0.05 lysozyme: cross linking agent (GO or GA) due to the results from chapter 3. This study proved there was a three log reduction of *Listeria monocytogenes* for any film with lysozyme bound to it. This was part of the expected results because *Listeria* is a gram positive bacterium. *Listeria* has a similar structure as the *Micrococcus Lysodeikticus* since they are both gram positive bacteria. Also, the mechanism for lysozyme to kill is to lyse the cell membrane of gram positive microorganisms. However, an interesting result from this testing is the GA film was able to reduce *Salmonella* Newport by one log which was statistically significant. The practical importance of a one log reduction is minimal. GA, in high enough concentrations, can be used as an antimicrobial. Herruzo-Caberra (1999) tested 2% GA on hospital equipment versus other common disinfectants. The concentration of cross linking agent was minimal because higher concentrations did not allow the lysozyme to remain active. The film would have less than the 2% concentration of GA but it is unique that the GA film was the only film that had a statistically significant result.

Another major conclusion is that the lysozyme does act very quickly with the product. In the release test, most of the lysozyme was released to the solution within six hours. As measured by using the films on the tomato it was clear after one day the *Listeria* was reduced and it remained constant. The film was able to keep the *Listeria* concentration down by three logs for five days or until the ripe tomatoes were contaminated with yeasts and mold.

A side note of this investigation is that the skins of the tomatoes were also far easier to use and yielded very similar results as the interior of the tomato. Contamination (yeast and molds) occurred more frequently in the interior of the tomato because water was expelled from the tomato after storing it at room temperature for a day. This would encourage mold and yeast growth. Another hassle for using the interior of the tomato is the pulp that clogs the pipette during serial dilutions. The preparation work is higher using the skins, but both issues are overcome with using the skin of the tomatoes.

One drawback of this technology that cannot be avoided is the contact area of the film. The film must be in contact with the surface to be contaminated. If whole tomatoes were used, and the interior was infected, it would be impossible to reduce the bacteria on the interior of the tomato. This is a drawback to this technology but there are other uses for this technology. In liquid samples, such as a salsa, ketchup, or liquid product it is possible to raise the contact area if the sample was shaken prior to use. This would increase contact area and reduce gram positive bacteria for the entire sample.

Future Work

New technology drives the future. Biodegradable and active packaging has a wide array of variables that still need to be explored. Some immediate ideas that could be implemented are changing the major characteristics of the film. One change is altering the film matrix from corn zein to chitosan. Chitosan is a natural antimicrobial and a water soluble film. The drawback is using the UV/VIS spectrophotometer would not be a possibility since the film would dissolve in anything water based (Such as a *Micrococcus* solution). Another change is varying the enzyme from lysozyme to lactoperoxidase.

Lactoperoxidase has been proven to reduce the content of *Listeria monocytogenes* when incorporated into whey protein isolate films (Min 2005). Other changes include altering the cross linking agent or change the detection methods that could be used to streamline the process. A new detection method could be using a unique idea that is based on Wang (2003). Several publications explain the development of a biobased sensor to detect immobilization of horseradish peroxidase in chitosan matrix for amperometric determination of hydrogen peroxide. In short, it uses the hydrodynamic response of H_2O_2 to give corresponding concentrations for the range of 5×10^{-9} to 1×10^{-7}. The detection of hydrogen peroxide sounds like a random thing to sample, however it could be applied to films if the films uses lactoperoxidase as the enzyme.

To apply this to film making, a chitosan based film infused with hydrogen peroxide and lactoperoxidase could be a unique novel approach. There are several benefits of making a film like this. Chitosan based films are a natural source for being an antimicrobial (Coma 2002). When lactoperoxidase is also chosen, it could work synergistically with the chitosan to eliminate gram positive bacteria. Lactoperoxidase does need hydrogen peroxide (H_2O_2) for an electron acceptor. This could also allow the use of the biobased sensor to allow a new detection method to be used. It could be determined how quickly the enzyme is working by measuring concentration of hydrogen peroxide. The biobased sensor could be compromised for measuring H_2O_2 if the film includes ascorbic acid or a few other noted compounds (Wang 2005). If a cross linking agent is desired, glutaraldehyde or glyoxal could be used in small concentrations to try to improve the binding of the enzyme to the film. Glyoxal could be used for a cross linking agent. One reason to use glyoxal is there is little research of glyoxal as a cross linking agent (Wang 2005). There are plenty of changes that could be made, and this is just an example for one way.

Another way to adjust the films is to analyze the films pH. To maximize the effectiveness of the film, pH should be an indicator. Glutaraldehyde prefers to have an acidic environment to allow cross linking. However, this acidic pH causes brittleness in the film. Lysozyme prefers pH environments close to 8 or 9 for a pH for maximum activity of the enzyme (Davies 1969). If pH exceeds about 9.2 the

lysozyme will become inactive. Varying the pH of the film could allow the film to increase the activity of the enzyme.

The last major change that needs to be made is dealing with the mold growth. This study was conducted with tomatoes that were ripe and ready to eat. The tomatoes were aimed to have a one week shelf life study on the effects of inoculating with *Listeria monocytogenes* and *Salmonella* Newport. However, even after 3 days a few of the samples began to have mold and yeast growth on the samples. This interfered with some of the data collection process too. After 5 days nearly 80% of all the samples were contaminated and it was impossible to get to the full week for sampling. Fortunately, the corn zein based film immobilized with lysozyme was able to work almost immediately. This study worked on establishing food safety. Mold would be a major concern to extend shelf life of the tomatoes. Refrigerated storage condition could eliminate the mold growth but it could cause chill damage to the fruit.

Overall, there are so many different ways to alter this project to make the system more effective. Altering the enzyme, cross linking agent, film matrix, detection method could drastically alter and improve the process. Also, combining active agents like organic acids, enzymes, baceriocins, or natural extracts could be used synergistically to improve efficiency.

The corn zein films use of a cross linking agent did not improve the effectiveness of the films ($p>0.05$), however the current films were able to reduce *Listeria monocytogenes* by a three log reduction. The films have practical application as long as the film will have contact area with the product. The films are not designed to allow the active agent to disperse into the product.

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