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I am submitting herewith a thesis written by Jeffrey Smith Boland entitled "Influence of Chelators on the Antimicrobial Activity of Lysozyme Against *Escherichia coli* O157:H7." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

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Influence of Chelators on the Antimicrobial Activity of Lysozyme Against *Escherichia coli* O157:H7

A Thesis

Presented for the

Master of Science Degree

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Jeffrey Smith Boland

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Dedication

I would like to dedicate this dissertation in loving memory of my grandfather,

Mr. Charles Edwin Smith

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Abstract

The objectives of these studies were to determine the influence of chelator combinations on the effectiveness of the food antimicrobial lysozyme against the foodborne pathogenic bacterium, *Escherichia coli* O157:H7. By itself, lysozyme has little or no activity against Gram-negative bacteria, however, in the presence of chelating agents, the antimicrobial spectrum of lysozyme can be increased to include Gram-negative bacteria such as *E. coli* O157:H7. Experiments were three-fold. The first series of experiments examined the effects of lysozyme and three chelating agents (ethylenediamine tetraacetic acid (EDTA), disodium pyrophosphate (DSPP), and pentasodium tripolyphosphate (PSTPP)) on growth inhibition of *E. coli* O157:H7. The second study determined how pH, cation size and strain of *E. coli* O157:H7 influenced neutralization of lysozyme-chelator combinations. The third experiment used zeta potential measurements to observe the effects of lysozyme and EDTA on surface charge properties of *E. coli* O157:H7 strain 932.

In the first study, the inhibitory effect of 300-1500 µg/mL EDTA and 3,000-15,000 µg/mL DSPP and PSTPP in combination with 200-600 µg/mL lysozyme was studied at pH 6, 7, and 8 on four strains of *Escherichia coli* O157:H7 for 48 hours, using a microplate dilution assay. Addition of EDTA enhanced the inhibitory effect of lysozyme against strains of E. coli O157:H7. EDTA concentrations ≥ 300 µg/mL combined with 200 µg/mL lsyzoyme were sufficient to inhibit the strains at pH 6 and 8. At pH 7, 200 µg/mL lysozyme and EDTA concentrations ≥ 1,000 µg /mL were effective in inhibiting three of four strains. DSPP at pH 6, was effective at ≥ 10,000 µg/mL when

combined with 200 µg/mL lysozyme. In contrast, PSTPP increased the inhibitory activity of lysozyme more effectively at pH 8. 200 µg/mL lysozyme was effective against two strains of *E. coli* O157:H7 when used in conjunction with ≥ 5,000 µg/mL PSTPP. Remaining strains were inhibited by ≥ 10,000 µg/mL PSTPP. Results indicated that inhibition occurred with each lysozyme-chelator combination, however, greater concentrations of polyphosphates were required, compared to EDTA, to increase the antimicrobial spectrum of lysozyme against *E. coli* O157:H7.

The second study analyzed the influence of seven mineral salts on neutralization of the inhibitory properties of lysozyme-chelator combinations. Lysozyme-chelators used were 1500 µg/mL EDTA and 15,000 µg/mL DSPP or PSTPP in the presence of 600 µg/mL lysozyme. Mineral salts were added to lysozyme-chelator combinations at 1.0, 4.0, 7.5, and 10 mM at pH 6.0, 7.0, 8.0 on *E. coli* O157:H7 strains 932 and H1730. For strain, 932 Ca^{++} and Mg^{++} neutralized inhibition by lysozyme and EDTA except for EDTA at pH 6.0. Inhibition of strain H1730 was eliminated by Ca^{++} and Mg^{++} except for EDTA at pH 6.0 and Mg^{++} with DSPP at pH 6.0. Concentrations ≥ 4.0 mM Fe^{++} neutralized all lysozyme-chelator combinations. Addition of Na^{+} and K^{+} to lysozyme-chelator combinations produced minimal to no reverse inhibition of strain 932. However, Na^{+} and K^{+} concentrations were effective in reversing lysozyme-chelator inhibition for strain H1730. The addition of ≥ 1.0 mM Fe^{+++} or Al^{+++} was effective in overcoming inhibition of lysozyme and EDTA at pH 6.0, 7.0, and 8.0. It was concluded that calcium and magnesium are important in the reversal of lysozyme-chelator inhibition, whereas the reversal by sodium and potassium were variable and strain dependent. Iron was

additionally effective in neutralizing lysozyme-chelator inhibition, probably because of its wide range of functions and binding constants to chelators.

In the third, zeta potential values were obtained for pure lysozyme and lysozyme-EDTA combinations. EDTA and lysozyme-EDTA combinations were additionally examined to determine the effects of the antimicrobial-chelator combinations on *E. coli* O157:H7 surface charge. Over a pH range of 5-13, zeta potential measurements for lysozyme ranged from 61.26 to – 42.14 mV, respectively. The presence of EDTA reduced lysozyme zeta potential to 38.13 at pH 5.0 to – 33.45 mV at pH 13.0. Native *E. coli* O157:H7 possessed a negative charge over a pH range of 5-13. Values ranged from – 11.67 at pH 5.0 to – 36.95 mV at pH 13.0. EDTA and lysozyme-EDTA combinations lowered the zeta potential of *E. coli* O157:H7. Lysozyme and EDTA together significantly reduced the zeta potential of *E. coli* O157:H7 strain 932, from – 3.76 at pH 5.0 to – 32.85 mV at pH 13.0. Treatments with EDTA alone further reduced zeta potential values to – 1.28 to – 27.47 mV for pH 5 and 13, respectively. It was concluded that the presence of lysozyme-EDTA combinations and EDTA alone significantly lowered the zeta potential values of *E. coli* O157:H7 strain 932.

The addition of chelators successfully enhanced the antimicrobial spectrum of lysozyme to inhibit *E. coli* O157:H7. It was found that certain metal ions are responsible for stabilizing the LPS layer and protecting the bacterial cell from the inhibitory activity of lysozyme. Additionally, environmental factors such as pH, cation size/charge, and *E. coli* O157:H7 strain type influence the chelation of these ions. Furthermore, it was concluded that the synergistic properties of lysozyme and EDTA reduce bacterial surface charge, allowing lysozyme and EDTA to inhibit *E. coli* O157:H7.

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Chapter 1: Review of Literature

1.1 Introduction

Foodborne illnesses have caused a major health impact in the United States and around the world. The Centers for Disease Control and Prevention (CDC) estimates that foodborne disease is responsible for approximately 76 million illnesses, 325,000 hospitalizations and 5,000 deaths each year in the United States alone (*77*). The symptoms of foodborne illness may range from mild gastroenteritis to life-threatening neurological, hepatic, or renal syndromes. Of these illnesses, 38.6 million infections occur by known pathogens and 30% of all foodborne illnesses via foodborne transmission are caused by bacteria (*8*). Since its discovery in the early 1980's, *Escherichia coli* O157:H7 has gained particular interest in the food industry due to its evolution from a clinical novelty to a global public-health concern (*54*).

1.2 *Escherichia coli* O157:H7

1.2.1 Introduction

In the United States alone, *Escherichia coli* O157:H7 has been documented as the source of over 100 outbreaks and estimated to cause more than 20,000 infections and up to 250 deaths per year. *E. coli* O157:H7 was first identified in a hemorrhagic colitis outbreak in the United States linked to the consumption of undercooked hamburgers distributed by a single fast-food restaurant chain in 1982 (*14*, *42*). Since then, *E. coli* O157:H7 has been linked to major global outbreaks and product recalls. The consumption of undercooked ground beef affected several states in the western United States in 1993 and over 5700 cases of *E. coli* O157:H7 infection were reported in Japan from contaminated radish sprouts in 1996 (*42*). More recently, a United States beef company recalled over 25 million pounds of ground beef due to possible contamination

by *E. coli* O157:H7 (*26*). Additionally, *E. coli* O157:H7 outbreaks have been known to occur from the consumption of unpasteurized milk and apple cider, fresh vegetables, unchlorinated water and via person-to-person contact (*14*). *E. coli* O157:H7 became the first of several strains to be referred to as enterohemorrhagic *E. coli* (EHEC). EHEC distinguishes itself from other pathogenic strains of *E. coli* by secreting a Shiga toxin that can result in the development of hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and even death. Over the last two decades, *E. coli* O157:H7 has evolved into a global public health concern that is challenging physicians, alarming food producers, and has influenced the public's perception about the safety of their food (*14*, *54*).

1.2.2 Emergence of *E. coli* O157:H7

Starting with the first recognized outbreak, the number of *E. coli* O157:H7 foodborne outbreaks has increased steadily since 1982 (*1*). Several factors have led to the emergence of this pathogen. Although *E. coli* O157:H7 was first recognized as a pathogen in 1982, it may have arisen 10-20 years earlier (*54*). Many scientists believe that Shiga toxin was introduced to *E. coli* by a genetic mutation, possibly phage mediation, between *Shigella* spp. and *E. coli* isolates (*1*, *62*). Evolution can produce pathogens that create and establish new characteristics that enable them to cause disease. Many microorganisms, including *E. coli* O157:H7, have dynamic and sophisticated genetic responses to harsh or stressful environments that allows them to survive. Under stressful conditions, the environment may allow a microorganism to select a specific mutation that offers a competitive, beneficial advantage to that organism. These mutations may create an organism that is "cross-protected" to a variety of different stresses (*62*).

E. coli O157:H7 is generally found in the feces of healthy cattle and can be transmitted to humans through contaminated food, water, and direct contact between infected animals or people. Introduction of *E. coli* O157:H7 into human populations may have possibly been influenced by the vertical integration of the livestock industry (*1*). Additionally, demographics could possibly be linked to the spread of *E. coli* O157:H7 throughout the world population. As the world's population grows at a significant rate, globalization of the world's food supply has contributed to changing patterns of food consumption (*62*). Furthermore, countries including the United States import a large portion of their food from less developed countries. These countries sometimes lack proper sanitary conditions and proper regulations for further processing facilities. Furthermore, as the world's food supply becomes more dependent upon trade, the importation of contaminated food carries the potential to transport pathogens, such as *E. coli* O157:H7 to areas where they have never existed (*1*, *62*).

1.2.3 Virulence and Pathogenesis

Genetic responses to stress(es) can activate certain virulence components to serve as a "contingency plan" for microbial survival in hostile environments (*62*). *E. coli* O157:H7 has several distinct virulence factors which appear to play a significant role in its pathogenesis. The first and most important virulence factor is the production of the cytotoxic Shiga-like toxins. *E. coli* O157:H7 can produce two Shiga-like toxins, Stx_1 and Stx_2. Stx_1 has been determined to have the same molecular structure of the Shiga toxin produced by *Shigella dysenteriae* type I (*54*). Stx_2 is a more divergent molecule, with only 56% of the nuceotide sequence homology to Stx_1 (*42*, *54*). Of the two Shiga-like

toxins produced, Stx_2 is most commonly secreted by *E. coli* O157:H7 and is associated with most cases of HUS (*15*, *42*).

The proposed mechanism for the pathogenesis of HC and HUS in humans is contributed to the release of Stx from *E. coli* O157:H7 cells (*15*). These toxins adhere to intestinal epithelial surfaces causing colonic vascular damage and exposing the toxin to the circulatory system (*15*, *42*). The toxin binds to the glycolipid receptor (Gb_3) of red bloods cells, therefore allowing the toxin to internalize within the cell and cause death (*15*). Additionally, the Shiga toxins of *E. coli* O157:H7 contain enzymatic activity to block new protein synthesis in cells and prevent the production of critical host proteins needed for cell survival (*13*, *15*).

A second virulence factor of *E. coli* O157:H7 is its ability to produce hemolysin via a 60 mDa plasmid (*42*). *E. coli* O157:H7 infection increases the production of cytokines, i.e., interleukin 1-beta (IL-1-beta), from human monocytes (*13*). The secretion of IL-1-beta partially damages vascular epithelial cells, therefore allowing the hemolysin to further destroy these cells and increase the toxicity of the Shiga toxin (*13*, *42*). It is also believed that the production of the hemolysin allows *E. coli* O157:H7 to use blood released into the intestines as a source of iron (*54*). The correlation between production of hemolysin and Stx_1 and Stx_2 is high, suggesting a possible link between these antigens and the pathogenesis of *E. coli* O157:H7 (*42*).

A third virulence factor is the ability of *E. coli* O157:H7 to adhere to and colonize onto intestinal surfaces. The attachment to intestinal walls prevents the loss of bacterial cells to peristalsis (*42*). *E. coli* O157:H7 contains an attaching and effacing chromosomal gene (*eae* A) that encodes the intimin protein. The protein facilitates actin

polymerization and cytoskeletal rearrangement of the intestinal cells, resulting in the development of characteristic attaching and effacing lesions (*15*, *42*). Furthermore, adherence aids in delivery of the toxin to the cell surface so that it can be distributed within the intestinal tract. Studies performed by Paton et al. (1997) indicated that *E. coli* O157:H7 isolates were three times more adherent to human intestinal cells than regular, non-pathogenic *E. coli*.

1.2.4 Structural Properties and Cell Wall Composition

E. coli is classified as Gram-negative rod that represents a majority of the natural microflora in the gut. This enteric organism is a facultative anaerobe which can colonize in the gastrointestinal tract via its P-pili and type I fimbriae. Additionally, the presence of a flagella allows *E. coli* to mobilize (*41*). The flagella is comprised of polymerized flagellum proteins which make up the H-antigen (*54*). Gram-negative bacteria, such as *E. coli* O157:H7, have a lipolysaccharide (LPS) layer that consists of a long carbohydrate chain that provides protection to the bacterial cell in the presence of bile salts and other hydrophobic compounds. The LPS is antigenic and is known as the "O" antigen. The O-antigen is highly variable which leads to antigenic variation between *E. coli* isolates. The O157:H7 serotype is designated by its somatic O and H antigen (*42*). Therefore, *E. coli* O157:H7 expresses the 157th somatic O-antigen and the 7th flagellar H-antigen (*54*).

The bacterial cell envelope plays an important role in the adaptation and growth of microorganisms in different environments. Although bacterial cell envelopes are comprised of many organized and distinctive features, one of the most important structures of the envelope is the bacterial cell wall (*66*). The cell wall acts as a functional and physical barrier between the inside of the cell and its environment, allowing bacterial

resistance to acids, antimicrobials, and other environmental factors (*49*, *66*). Bacteria can be classified based on their cell wall composition and its response to the crystal violet Gram stain (*49*). Bacteria are separated into either Gram-positive or Gram-negative by the retention of a crystal violet dye by their cell walls. Although their cell walls perform similar, protective functions, the structural architecture of Gram-positive and Gram-negative cell walls is very different and unique (*66*).

Gram-positive bacteria protect their cytoplasmic membrane with a thick cell wall comprised sugars and amino acids called peptidoglycan (Fig. 1.1). Although it varies among Gram-positive microorganisms, peptidoglycan can represent up to 90% of total cell composition (*78*). Additionally, the cell wall of Gram-positive bacteria contains many layers of murein. Murein is composed of glycan sugar chains that are cross-linked by peptides and is a critical component in maintaining the shape and rigidity of Gram-

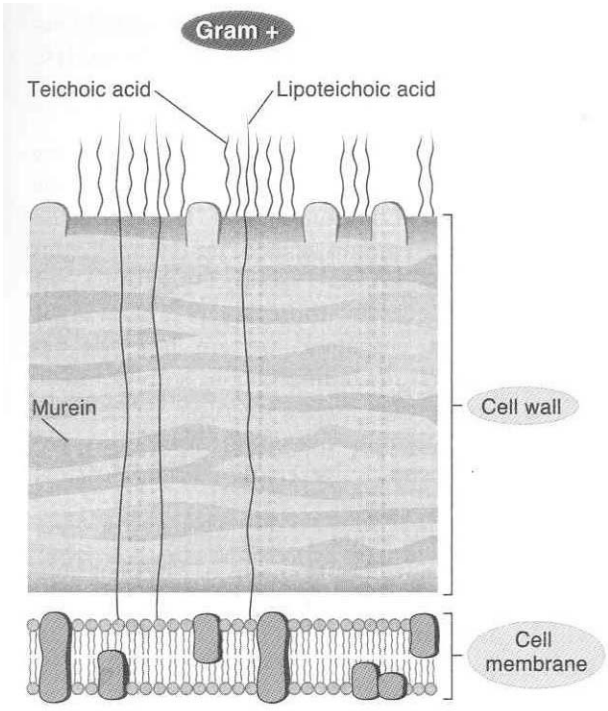


Fig. 1.1: Diagram of Gram-positive cell wall (*66*)

positive bacteria. Because of the abundance and highly polar nature of murein, it can impede the passage of hydrophobic compounds through the cell wall. Gram positive walls also contain unique polymers, such as teichoic acids (*66*). Teichoic acids are chains of ribitol or glycerol linked by phosphodiester bonds that attach to the peptidoglycan framework and aid in protective function of the Gram-positive cell wall (*58*, *68*).

By contrast, the cell envelope of Gram-negative bacteria is more complex (Fig. 1.2). The Gram-negative cell envelope consists of two membranes separated by a layer of peptidoglycan and a cellular compartment known as the periplasm. Peptidoglycan found in Gram-negative bacteria is 20-fold less than amounts present in Gram-positive bacteria and is generally found in the periplasmic space (*66*). In addition, a bilayered cell membrane is present on the outside of the peptidoglycan layer preventing the layer from interacting with the extracellular environment (*31*, *78*). This bilayered structure contains an outer leaflet that contains a unique component known as the LPS layer. The presence of the LPS layer in Gram-negative bacteria makes the membrane so chemically distinctive that it is not found elsewhere in nature (*66*).

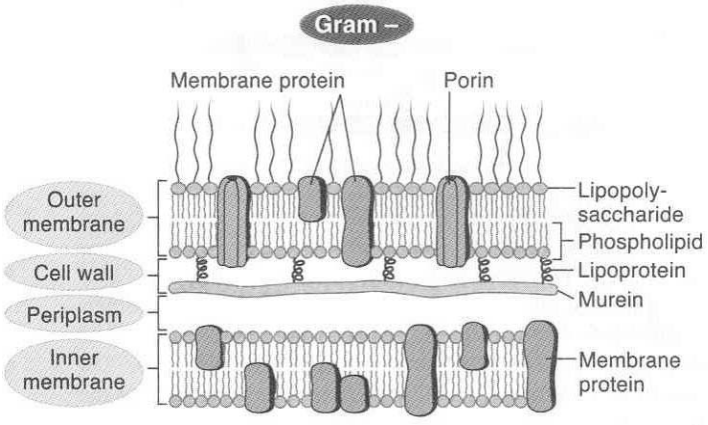


Fig 1.2: Diagram of Gram-negative cell wall (*66*)

The LPS layer is comprised of three components: the Lipid A, the core, and the O-antigen (*31*, *66*). Lipid A is a glycolipid made of disaccharides which are attached to short-chain fatty acids and phosphate groups. The Lipid A structure anchors the LPS in the outer leaflet of the membrane (*66*). Attached to the Lipid A region is a rough oligosaccharide core containing keto-deoxyoctanoic acid, heptose and hexose residues. These sugar units help to connect the Lipid A and O-antigen components (*31*, *66*). The final component of the LPS layer is the O-antigen, which is a long carbohydrate chain comprised of up to 40 sugars in length. The hydrophilic chains of the O-antigen cover the bacterial surface and prevent penetration of hydrophobic compounds through the cell wall (*66*). Furthermore, the LPS layer carries a negative charge resulting in the strong negative surface charge of Gram-negative bacteria (*66*, *78*). The LPS is anchored in the outer membrane by binding to outer membrane proteins through hydrophobic interactions with Lipid A. Additionally, the presence of divalent cations, such as magnesium and calcium, form non-covalent cross bridges within the LPS to reinforce and maintain the structural stability of the LPS layer (*31*).

1.2.5 Tolerance

Characteristics specific to *E. coli* O157:H7 lend to its ease of transmission and survival in various environmental conditions (*13*, *42*). These characteristics include the microorganism's acid tolerance, low infectious dose and survival under refrigeration and/or freezing conditions (*42*). Outbreaks associated with unpasteurized apple cider, yogurt and salami can be linked to the acid tolerance of the microorganism. Studies performed by Duffy et al. (1999) indicated that in the presence of competitive microflora, *E. coli* O157:H7 was not inhibited by an acidic environment. Tolerance to low pH

facilitates passage through the stomach, making it possible for *E. coli* O157:H7 to cause disease (*62*). Additionally, the acid tolerance of *E. coli* O157:H7 allows the microorganism to cause disease with low infectious doses (*42*). Consumption of less than 50 organisms and possibly as few as 10 have been reported to cause *E. coli* O157:H7 infection (*14*, *42*). *E. coli* O157:H7 is also very resistant to cold temperatures, surviving in temperature lower than 5° C. Studies performed by Doyle and Schoeni (1984) concluded that the organism survived well in ground beef when frozen below 0°C.

1.2.6 Preservation

Production of safe food includes suppressing microbial growth and reducing or eliminating the microbial load by processing and preventing post-processing contamination (*43*). Heat treatments are traditionally used to pasteurize or sterilize food. However, excessive heat treatment applied by traditional means may create undesirable sensory changes and loss of nutrients (*39*, *55*). As consumers increasingly perceive fresh food as healthier than heat-treated food, the food industry is seeking alternative techniques to maintain the fresh attributes, safety and storage stability of food. These non-thermal alternative methods include high pressure processing, irradiation, ohmic heating, ultrasound, pulsed electric field, and ultraviolet radiation (7, *54*). These technologies can inactivate foodborne microorganisms while preserving the sensory and nutritional values of food products (*43*, *55*). However, most non-thermal heat techniques are expensive and can only achieve the equivalent of pasteurization, not sterilization, in foods (*43*). As industry explores alternative ways to improve or replace thermal and non-thermal processing methods, increasing consumer demand for fresher, minimally

processed, and extended shelf-life foods have renewed interest in the use of naturally occurring food antimicrobials as a means of food preservation (*11*).

1.3 Naturally Occurring Antimicrobials

1.3.1 Introduction

Food antimicrobials have been used as a preservation method to inhibit the growth of microorganisms, including foodborne pathogens, for a number of years. However, in the United States, there is a widespread trend for the development of more "natural" food products. In an effort to meet this growing consumer demand, the food industry has focused attention on the development and use of more naturally occurring antimicrobial preservatives (*5*).

1.3.2 Lysozyme

Lysozyme is a naturally occurring enzyme that is found abundantly in nature. Lysozyme is present in milk, egg white, plant tissues and human tears (*11*, *69*). However, commercial uses for lysozyme are extracted from egg whites, where it comprises 3.5% of egg white solids. In addition, the enzymatic activity of lysozyme is known to be bactericidal towards many microorganisms, especially Gram-positive bacteria (*51*).

Chemical and Physical Properties

Lysozyme is a single polypeptide chain that consists of 129 amino acids with a molecular weight between 14,300-14,600 Daltons. The protein is highly basic with an isoelectric point of 10.7 and maintains enzymatic activity over a wide pH range of 4-10. (9, *38*). The lysozyme molecule is cross-linked with four disulfide bonds, which are significant for its enzymatic activity. In order for lysozyme to remain enzymatically

active, at least two disulfide bonds must remain intact. However, if three or four disulfide bonds are broken within the lysozyme structure, lysozyme loses its activity (*38*). The 3-dimensional structure of the lysozyme molecule is in the form of a prolate ellipsoid. The structure consists of a deep cleft on one side that divides the molecule into lobes. The first lobe consists of the two ends of the chain (amino acid residues 1-39 and 85-129) that are comprised of four helices. The second lobe (residues 40-84) contains three-stranded anti-parallel β-pleated sheets (*53*). Non-polar residues are found in the interior of the molecule while mostly polar residues are found on the exterior of the protein (*51*, *53*).

Mechanism

The suggested mechanism by which lysozyme inactivates bacteria is based on the hydrolysis of the β 1-4 glycosidic bonds between N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) in the peptidoglycan layer of the bacteria cell wall (*51*). Phillips (1973) studied hydrolysis of peptidoglycan by lysozyme. He hypothesized that lysozyme attaches to the bacterial cell wall by interacting with six of the residues connected to NAM. The attachment changes the conformation of the NAM structure, allowing the Glu 35 residue of the lysozyme molecule to donate a hydrogen atom to the oxygen atom in the glycosidic bond, resulting in cleavage of the bond.

Spectrum of Activity

Since its discovery in 1922 by Sir Alexander Fleming, the antimicrobial activity of lysozyme has been studied extensively (*53*, *64*). The specific antimicrobial role of lysozyme was interpreted by Salton and Pavilik in 1964. The authors evaluated the effects of lysozyme on Gram-positive strains of *Bacillus*, *Corynebacterium*,

Lactobacillus, *Micrococcus*, *Sarcina*, *Sporosarcina*, *Staphylococcus*, and *Streptococcus*. It was concluded that isolated walls from the microorganisms were sensitive to lysozyme. However, in comparsion with the other microorganisms, *Staphylococcus aureus* cell walls were more resistant to lysozyme. In addition, the authors further hypothesized that bacterial cell walls with a higher proportion of 1,4 β glycosidic bonds between NAM and NAG would make the cell wall more sensitive to lysozyme. In contrast, the cell wall of *S. aureus* may contain fewer contents of 1,4 bonds and higher amounts of 1,6 or 1,3 linkages between NAM and NAG allowing it to be more resistant to lysozyme.

The work of Wasserfall and Teuber (1979) studied the antimicrobial spectrum of lysozyme against proteolytic Gram-positive microorganisms, including *Bacillus cereus* and *Clostridium tyrobutyricum*. The authors reported that 500 U/mL (one unit produces a change in the absorbance of a *Micrococcus lysodeikticus* suspension at 450 nm of 0.001 per min at pH 6.24, 25°C) were effective in inhibiting vegetative *B. cereus* and *C. tyrobutyricum* cells.

Peterson and Hartsell (1955) tested the antimicrobial effects of lysozyme against 135 strains of Gram-negative bacteria. Viability studies performed on agar plates showed that lysozyme had no or minimal inhibitory activity against species of *Salmonella*, *Brucella*, *Klebsiella*, *Shigella*, *Neisseria*, *Pseudomonas*, *Pasteurella*, *Erwinina*, *Escherichia*, *Vibrio*, and *Proteus*. In addition, Vedmina et al. (1979) tested lysozyme against 476 strains of Gram-negative bacteria. The authors found *Vibrio cholerae Eltor* and *Pseudomonas* to be resistant to lysozyme sensitivity. Additionally, little or no inhibition occurred with *Aeromonas*, enteropathogenic *E. coli*, and NAG-vibrios in the presence of lysozyme. The authors concluded that a lipopolysaccharide layer found

within the cell wall of Gram-negative bacteria prevents exposure of lysozyme to the peptidoglycan layer.

Hughey and Johnson (1987) studied the effects of lysozyme on a number of foodborne spoilage and pathogenic bacteria, including *B. cereus*, *Bacillus stearothermophilus*, *C. botuninum types* A, B, and C, *Clostridium thermosaccharolyticum*, *C. butyricum*, *Clostridium perfringens*, *Clostridium sporogenes*, *Clostridium thermosaccharolyticum*, *Clostridium tyrobutyricum*, *Campylobacter jejuni*, *E. coli* O157:H7, *Klebsiella pneumoniea*, *L. monocytogenes*, *Salmonella typhimurium*, *Staphylococcus*. *aureus*, *Vibrio chloerae* and *Yersinia enterocolitica*. Strains of *C. botunlinum*, C. *thermosaccharolyticum*, *C. tyrobutyricum*, *B. stearothermophilus*, and *L. monocytogenes* were effectively or partially inhibited in the presence of lysozyme. However, lysozyme concentrations were unable inhibit growth of *S. typhimurium*, *V. cholerae, E. coli* O157:H7 and *Y. enterocolitica*. Chemical and physical influences, such as EDTA, temperature, and changes to the organism's growth phase and medium, were needed to enhance the antimicrobial activity of lysozyme to the Gram-negative bacteria.

Lysozyme is known to be bactericidal to Gram-positive microorganisms. Lysozyme alone is essentially ineffective against Gram-negative bacteria due to the presence of a lipopolysaccharide (LPS) layer in the outer membrane of the cell. The LPS layer acts as a permeability barrier to prevent lysozyme from penetrating the cell interior (*21*). Lysozyme does not lyse or inhibit the growth of Gram-negative bacteria unless the outer membrane is permeabilized by physical or chemical means. One way to increase cell sensitivity of Gram-negative bacteria to lysozyme is with the addition of chelating

compounds. Chelators can work synergistically with food antimicrobials to inhibit Gram-negative bacteria (*21*, *57*).

Applications and Regulations

Lysozyme currently has a small number of applications in the food industry. The most common use is to prevention of a defect known as "late blowing" in Edam and Gouda cheeses. This defect is characterized by the formation of holes in the cheese and the generation of unacceptable off flavors due to the growth of *Clostriudium tyrobutyricum* (*9*, *29*). Additionally, patents have been issued for the application of lysozyme to oysters, shrimp, sushi, sake, kimchi, potato salad, and several types of sausage in Japan (*64*).

Egg white lysozyme did not receive generally recognized as safe (GRAS) status in the United States until the FDA issued a tentative final rule in 1998 for the allowing lysozyme to prevent late blowing in cheese (*23*). Cheese manufactured in the United States with the addition of lysozyme may contain up to a maximum of 400 µg/mL lysozyme. Egg white lysozyme is considered safe because its source, egg white, has been a regular source of protein in the human diet throughout history. However, egg whites can cause allergic reactions in sensitive individuals, therefore requiring cheeses with added lysozyme to carry a label stating that this product may contain "egg white lysozyme" (*23*).

1.4 Influence of Chelators on Antimicrobial Activity

1.4.1 Introduction

It has been recognized since the 1960's that the susceptibility of Gram-negative microorganisms to lysis by food antimicrobials can be increased by the use of membrane

disrupting agents, such as chelators. Chelating compounds are normally added to food products to sequester trace metals that act as pro-oxidants (*40*). However, chelators can function synergistically with food antimicrobials and increase the antimicrobial spectrum of compounds such as lysozyme (*21*, *57*). It has been well documented that chelators, such as EDTA, can effectively enhance the activity spectrum of food antimicrobials. However, little is known about the interactions of other GRAS chelators, such as polyphosphates, with food antimicrobials in inhibiting growth of Gram-negative bacteria.

1.4.2 Factors Influencing Chelation

A metal complex is formed with the association of a metal ion and another species, known as a ligand, which can be either an anion or a polar molecule (*3*). The metal-chelate complex forms as a result of a competitive reaction for the metal ion between a sequestrant anion and a precipitating anion in the same solution (*22*). For chelation of any metal ion to occur, two general principles must be fulfilled. The chelating agent must have the proper steric and electrochemical configuration in relation to the metal being sequestered. Secondly, environmental conditions, such as pH, ionic strength, and solubility, must favor the binding of metal ions (*27*, *37*).

1.4.3 EDTA

Chemical and Physical Properties

EDTA is a polyaminocarboxylic acid with a molecular weight of 292.1 (*75*). It is one of the most effective and widely used chelating agents currently used in foods (*80*). EDTA is produced commercially by the reaction of ethylene diamine with sodium cyanide and formaldehyde under alkaline conditions at elevated temperatures (*80*). EDTA primarily chelates alkaline earth metals by forming noncovalent bonds through

shared electrons from nitrogen or carboxylic acid groups. Metals that are effectively chelated by EDTA include Al, Ni, Fe, Cu, Co, Zn, Ba, Ca, Mg, Mn, and Sr (*67*).

Mechanism

EDTA increases the sensitivity of Gram-negative bacteria to food antimicrobials such as lysozyme. Although the mechanism of EDTA is not fully understood, it likely involves the chelation of membrane cations from the LPS layer of Gram-negative bacteria (6, *21*). Furthermore, the reversal of EDTA activity by divalent cations suggests that EDTA acts by a chelation mechanism (*25*, *46*) EDTA contains six donor atoms and acts as a hexadentate chelator. It can form a maximum of five chelate rings. In most instances, EDTA forms an octahedral complex in which the metal ion and EDTA have a coordination number of six (*6*). EDTA can effectively sequester metal ions from the LPS layer of Gram-negative bacteria (*21*, *25*). The removal of essential cations destabilizes the LPS layer and increases cell sensitivity and permeability to lysozyme (*68*). Leive (1965) showed that EDTA released 50% of the lipopolysaccharide from *E. coli* cells, allowing inhibition to occur by several antimicrobials. In a follow-up experiment, Leive (1968) hypothesized that the action of EDTA occurred by the chelation of divalent cations such as magnesium or calcium. Studies performed by Gray and Wilkinson (1965) proposed that cations such as calcium and magnesium were responsible for the formation of salt bridges that reinforce the structural integrity of the LPS layer. The authors postulated that EDTA disrupts the salt bridge formation by the chelation of magnesium and calcium, resulting in LPS release.

Interaction with Lysozyme

Although EDTA has been shown to possess antimicrobial activity alone, the inhibitory effects may be increased when combined with antimicrobials such as lysozyme. As mentioned previously, EDTA can enhance the antimicrobial spectrum of lysozyme to Gram-negative bacteria. Repaske (1956) first reported that EDTA (0.4-1.6 mg) and lysozyme (30-50 µg/mL) lysed cells of *E. coli*, *Azotobacter vinelandii*, and *P. aeruginosa*. In addition, EDTA alone caused appreciable inhibition of *P. aeruginosa* cells, but not to the extent of the lysozyme-EDTA combination. Eagon and Carson (1965) reported results simliar to Repaske (1956) regarding the inhibition of *P. aeruginosa* by EDTA and lysozyme. However, Eagon and Carson found that EDTA alone was just as effective as lysozyme-EDTA combinations in killing of *P. aeruginosa*. Their studies indicated that EDTA chelated calcium, magnesium, and zinc from *P. aeruginosa* cells.

The effect of Mg^{2+} on lysis of *P. aeruginosa* cells by lysozyme in the presence of EDTA was examined by Ayres et al. (1998). Their results indicated that in the presence of Mg^{2+}, lysis of *P. aeruginosa* by lysozyme and EDTA was greatly reduced. The authors stated that Mg^{2+} may replace cations removed from the outer membrane and/or effectively remove the permeabilizing effect of EDTA.

Branen and Davidson (2003) investigated lysozyme and EDTA against two strains of enterohemorrhagic *E. coli*. Studies indicated that the EDTA minimal inhibitory concentration (MIC) was lowered by 50% in the presence of 25 and 125 $\mu g \cdot mL^{-1}$ lysozyme. The authors concluded that EDTA acted synergistically with lysozyme by

increasing the permeability of the *E. coli* outer membrane to lysozyme, due to the binding of divalent cations.

The effects EDTA alone and lysozyme-EDTA combinations were examined on *Pseudomonas fragi* in lactose-yeast extract, half-and-half milk and, skim milk EDTA (1 mg/mL) and EDTA combined with lysozyme (0.2 mg/mL) were shown to inhibit *Pseudomonas fragi* in lactose-yeast extract broth, but not half-and-half milk or skim milk (Moustafa and Collins, 1968). The authors hypothesized that lack of antimicrobial activity in milk was attributed to the excess of metal cations in the dairy products.

Hughey and Johnson (1987) investigated the activity of lysozyme and EDTA against *L. monocytogenes* in vegetables, meat, and dairy foods. The authors found that lysozyme (100 mg/kg) plus EDTA (5 mM) caused a $\geq$ 4 log reduction in viable *L. monocytogenes* cells on fresh corn, fresh green beans, shredded cabbage and lettuce, and carrots stored at 5° C. Lysozyme and EDTA combinations increased the lag phase of *L. monocytogenes* in bratwurst and Camembert cheese. However, the microorganism was eventually able to grow to 10^6 CFU/g. The authors concluded the lysozyme in combination with EDTA could be a useful preservative for foods such as fresh vegetables.

Gill and Holley (2002) inoculated processed ham and bologna with *E. coli*, *Salmonella enterica* serovar Typhimurium, *Serratia grimesii* and *Shewanella putrefaciens*. EDTA (1000 mg/l) with 500 mg/l lysozyme was successful in inhibiting *E. coli*. EDTA at 500 mg/l in combination with 500 mg/l lysozyme inhibited *Salmonella* Typhimurium and *Serratia grimesii*. However, S*hewanella putrefaciens* was not inhibited by the selected concentrations. Greater than 2000 mg/l EDTA with lysozyme was

required for inhibition. As previous lysozyme-EDTA studies had indicated, the authors concluded that EDTA enhanced the antimicrobial spectrum of lysozyme to Gram-negative bacteria due to the chelation of metal ions.

Applications and Regulatory Aspects

EDTA is allowed for use in foods in either the calcium or disodium salt form (21 CFR 172.120 and 172.135). EDTA is commonly used in several foods including canned peas and beans, cooked sausage, strawberry pie filling, salad dressings, sauces, and shelf-stable mayonaise (*28*). It is used as a preservative, to promote color retention, and used as a cure accelerator in cooked sausages. The allowable amount of EDTA in foods ranges from 0.025 to 0.5 mg/mL, depending on the food.

While EDTA is commonly used in foods as a chelating agent, its applications and concentrations are limited. EDTA should only be used in foods to chelate cations, where no excess of the unbound form of EDTA remains. The presence of unbound EDTA inside the body can interfere with adsorption of essential minerals (*67)*.

1.4.4 Polyphosphates

The application of other, novel generally recognized as safe (GRAS) chelators could expand the antimicrobial activity of lysozyme and possibly replace EDTA in foods. Due to their widespread use, GRAS status, and antimicrobial activity, polyphosphates could serve as replacement chelators for EDTA

Chemical and Physical Properties

Phosphates are a group of diverse compounds. The group contains ortho-, poly-, tetra-, hexa-, and metaphosphates, with each phosphate group exhibiting different structures and physiochemical properties. The name “polyphosphate” refers to a group of

compounds having two or more phosphorous atoms joined by an oxygen bridge in a chain structure. Although most phosphates possess some chelating ability, disodium pyrophosphate ($Na_2H_2P_2O_7$) and pentasodium tripolyphosphate ($Na_5P_3O_{10}$) are two polyphosphates that are among the most effective in chelation of metal ions (*12*, *22*). Pyrophosphates are the simplest of the condensed or polymeric phosphates. The pyrophosphate and tripolyphosphate anions contain two and three phosphorus atoms, respectively, that are linked by a shared oxygen atom(s). The addition of sodium replaces two hydrogens groups in the disodium pyrophosphate allowing for four potential metal binding sites. In contrast, five sodium atoms are present in the pentasodium tripolyphosphate structure, creating five potential binding sites for metal cations (*22*).

Mechanism

Polyphosphates are able to readily form metal complexes with cations such as copper, calcium, magnesium, iron, and zinc due to their strong negatively charged nature. It was proposed by Van Wazer and Callis (1958) that the phosphate tetrahedron within polyphosphates structures are geometricallly arranged in a way so that oxygen atoms from each of the neighboring phosphate groups can be in close proximity with the complexed metal ion (*22*). The sequestering of cations by polyphosphates occurs with the formation of a metal-chelate ring that is formed between oxygen atoms found in the phosphate structure and metal ions.

Studies performed by Van Wazer and Campanella in 1950 concluded that polyphosphates form strong complexes with alkaline-earth and heavy metal ions, such as copper, zinc, magnesium, and iron. Additionally, they further concluded that polyphosphates are strong complexing agents, whereas ring phosphates are weaker

complexing agents and orthophosphates form complexes at very low concentration and can precipitate at higher concentrations.

Irani and Callis (1962) determine the effect of temperature and pH on polyphosphate chelation of metal ions. The authors determined that as temperature increased, the ability of sodium polyphosphates to sequester calcium and magnesium increased. Furthermore, they determined that the amount of calcium or magnesium sequestered by any of the polyphosphates was affected by the pH.

Antimicrobial Activity

Studies performed by Jen and Shelef (1986) suggested that the chelation of essential metal ions from *Staphylococcus aureus* 196E by sodium tripolyphosphate (STPP) could be reversed with the supplementation of cations. Mg^{2+} was effective in overcoming inhibition of *S. aureus* 196E in the presence of 0.5% STPP. However, inhibition was partly eliminated by the addition of Ca^{2+} and Fe^{2+}, but not by Zn^{2+} or Mn^{2+}.

Knabel et al., (1991) examined the effects of polyphosphate chelation on the inhibition of *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus*. Results indicated that the addition of monosodium phosphate (MSP), tetrasodium pyrophosphate (TSPP), sodium acid pyrophosphate (SAPP), sodium tripolyphosphate (STPP), and sodium hexametaphosphate (SHMP) were effective in inhibiting the growth of the three Gram-positive microorganisms. The authors proposed that the inhibition of the microorganisms was due to chelation of essential metal ions from the cell membrane. When magnesium and iron were added to the medium, the inhibitory properties of the polyphosphates were reversed. The addition of magnesium stimulated inhibition of all

microorganisms in the presence of the polyphosphate chelators. Iron was effective in neutralizing the inhibition of *S. aureus* and *L. monocytogenes* in the presence of TSPP and STPP.

Lee et. al (1993) studied the effects of sodium tripolyphosphate (STPP) and tetrasodium pyrophosphate (TSPP) on the early exponential phase cells of *Staphylocccus aureus*. STPP (0.1%) and TSPP (0.5%) were bactericidal to the *S. aureus* cells. The addition of 0.01 M Mg^{2+} or Ca^{2+} reversed the inhibitiory effects of STPP and TSPP to *S. aureus*.

Several studies have indicated that polyphosphates can effectively inhibit Gram-positive microorganisms through chelation of essential membrane cations. However, no experiments have evaluated the inhibitory properties of these compounds against Gram-negative bacteria. In addition, few studies have examined if polyphosphates can increase the antimicrobial spectrum of lysozyme to Gram-negative bacteria.

1.5 Zeta potential

1.5.1 Introduction

Interfaces can carry an electrical charge due to the adsorption of molecules that either are ionic or are capable of being ionized (e.g., proteins, certain polysaccharides, or ionic surfactants). In interfacial chemistry, the presence of an interfacial layer that carries a charge is of utmost importance since many reactive species or compounds that are capable of catalyzing a reaction can carry a charge as well (*18*). The interaction of reactant and catalyst will thus influence the overall reaction. If, for example, the catalyzing agent and interfacial layer carry a similar charge, then the repulsive electrostatic interaction will prevent the catalyst from coming into close contact with the

substrate, and the kinetics of the chemical reaction will be greatly reduced (*76*). On the other hand, if the catalyst and the interfacial layer carry an opposite charge, then the catalyst can actually be physically bound to the layer and the concentration within the vicinity of the interface is therefore much greater than one would ordinarily expect. As a result, the rate of the reaction will be increased (*18*, *76*). In the case of this study, the interaction of a charged antimicrobial with the charged surface of a microorganism may be impacted by the zeta potential.

1.5.2 The Electrical Double Layer

As previously mentioned, molecules that are present within the interface may be able to bind or release electrons from the outer electron hull that surrounds the positively charged proton-neutron core—i.e., they can be ionized. In systems with interfacial boundaries containing ions that carry a charge, a spatial distribution of counter ions surrounding the interface will develop (*52*). The number of counter ions will decrease as the distance from the interface increases. The counter ion atmosphere is also referred to as the ion cloud. The two regions, charged interface and surrounding ion cloud, are known as the electrical double layer. Since the overall charge of the system has to be zero, the charge at the interface itself must balance the combined charge of the ion atmosphere. The area-specific excess charge at the interface is called the interfacial charge density (*34*, *52*).

1.5.3 Methods for Measuring Bacterial Surface Charge

Assessment of bacteria cell surface charge has been of interest since the 1920's, which has led to the evolution of various analytical methods capable of characterizing bacterial cell surfaces with regard to their electrostatic properties (*78*).

Microelectrophoresis, electrostatic interaction chromatography, aqueous two-phase partitioning, and isoelectric equilibrium analysis are methods that have been employed to assess the measurable electrostatic charge as it relates to bacterial surface physiology (*78*).

Recent applications used electrophoretic light scattering (ELS) as an effective alternative to characterize bacterial cell surface charge properties. ELS measures the electrophoretic mobility of particles in an electric field by determining frequency shifts of the laser light beam that is scattered by the particles (*4*, *78*). Using the appropriate model, the mobility can be used to calculate the surface charge of particles (*78*).

1.5.4 Measuring Zeta Potential

The electrical potential is a continuous function that depends on the distance from the interface. The electrical potential at the plane of shear is called the zeta potential (*76*). Zeta potential, based on the previously introduced theoretical models, can be used to assess the net bacterial surface charge (*78*).

To determine the surface charge of bacterial cells from the zeta potential, one must select the appropriate double layer model suitable for a bacterial cell surface (Fig. 1.3). Generally, one can distinguish between three regions, the so-called Stern layer, the plane of shear, and the diffuse layer. The Stern layer is the area where ions are firmly attached to the bacterial surface. In contrast, the diffuse layer is further away from the bacterial surface and ions are not bound to the bacterial surface (*63*). The plane of shear, also known as the plane of slip, is located at a specific distance from the cell surface where solvent molecules are no longer firmly bound to the surface and thus do not move as a unit with the particle. The electric potential of a charged particle at the plane of

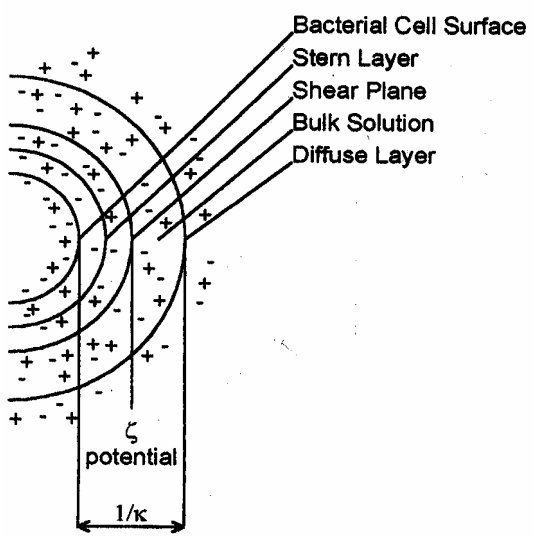


Fig. 1.3: Cross-section representation depicting the various solvent layers surrounding a bacterial cell (*78*)

shear is known as the zeta potential (*78*).

1.5.5. Microbial Cell Studies

Many of the recent applications of ELS have focused on investigating the role of bacterial cell surface charge on adhesion to disparate solid surfaces. (*70*, *78*). Studies performed by van Loosdrecht et al. (1987) found that cell surface charge was an important feature in the adhesion of bacterial cells to negatively-charged polystyrene. In contrast, Li and McLandsborough (1999) reported no influence of electrostatic interactions on binding of *E. coli* to beef muscle. Experiments by Vanhaecke et al. (1990) also showed that cell surface charge did not impact binding of *P. aeruginosa* to stainless steel.

Zeta potential studies have been used to obtain a better understanding of the outer cell envelope physiology in pathogenic Gram-negative bacteria (*78*). Harkes et. al, (1993) studied the zeta potential of eight *E. coli* strains as a function of pH. Zeta

potential measurements for *E. coli* over a pH range of 2-11 were electronegative and electronegativity increased as the pH became more alkaline. In response to Harkes work with *E. coli*, Mangia et al (1994) measured the zeta potential of five enteropathogenic *E. coli* serogroups (O111:H2; O111:H12; O125:H9; 0119:H6, and O26:H11). Their results indicated that all bacterial surfaces were negatively charged with mean zeta potential values ranging from –9.0 (O26:H11) to –11.9 mV (O11:H2) at pH 7.0. Zeta potential measurements were also examined for enzymatic treatments of the bacterial cell surface. The zeta potential for *E. coli* strains was measured in the presence of trypsin, phospholipase C, and neuraminidase. Treatment with trypsin and phospholipase C significantly decreased the zeta potential of each strain. Addition of neuraminidase did not reduce the zeta potential of the five enteropathogenic *E. coli* serogroups.

Hart and Champlin (1988) speculated that *Pasteurell multocida* was resistant to the daptomycin, a lipopeptide antibiotic, due to electrostatic repulsion between the polyanionic compound and the negatively charged outer membrane surface. This notion was tested by Morris et al. (1995) who used B nonpeptide (PMBN), a polycationic outer membreane permeabilizer, to increase the sensitivity of *Pasteurella multocida*, *E. coli*, and *Pseudomona aeruginosa* to the antibiotic daptomycin. The authors hypothesized that PMBN would reduce electronegativity of the cell surface by disorganizing the outer membrane, therefore allowing the antibiotic to associate with and destabilize the outer cell membrane. Zeta potential was used to measure the electrophoretic mobility of control and PMBN-treated cells. The zeta potential of PMBN-treated cells was lower in comparison to cells without PMBN treatment, therefore explaining the sensitizing of cell walls of *P. aeruginosa*, *E. coli*, and *P. multocida* to daptomycin. The authors

hypothesized that the permeability of the cell wall to daptomycin was dependent on PMBN's ability to decrease the overall cell surface electronegativity.

References

1. Armstrong, G.L., J. Hollisworth, and J.G. Morris. 1996. Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. Epidemiol. Rev. 18: 29-51.

2. Ayres, H.M., J.R. Furr and A.D. Russell. 1998. Effect of divalent cations on permeabilizer-induced lysozyme lysis of *Pseudomonas aeruginosa*. Lett. Appl. Microbiol. 27: 372-374.

3. Bell, C.F. 1977. Principles and applications of metal chelation. Clarendon Press, Oxford.

4. Blake, R.C., E.A. Shute, and G.T. Howard. 1994. Solubilization of minerals by bacteria: electrophoretic mobility of *Thiobacillus ferrooxidans* in the presence of iron, pyrite, and sulfur. Appl. Environ. Microbiol. 60: 3349-3357.

5. Branen, J.K. and P.M. Davidson. 2003. Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. Intl. J. of Food Micro. In Press.

6. Bucheli-Witschel, M. and T. Egli. 2001. Environmental fate and microbial degradation of aminopolycarboxylic acids. FEMS Microbiol. Rev. 25: 69-106.

7. Butz, P. and B. Tauscher. 2002. Emerging technologies: Chemical aspects. Food Res. Intl. 35: 279-284.

8. Centers for Disease Control. 2000. Foodborne Infections. From *Division of Bacterial and Mycotic Diseases.* http://www.cdc.gov/ncidod/dbmd/diseaseinfo/foodborneinfections_t.htm

9. Chung, W. and R.E.W. Hancock. 2000. Action of lysozyme and nisin mixture against lactic acid bacteria. Intl J. of Food Micro. 60: 25-32.

10. Chung, M.C.J. and L.A. Shelef. 1986. Factors affecting sensitivity of *Staphylococcus aureus* 196E to polyphosphates. Appl. Environ. Micro. 52: 842-846.

11. Conner, 1993. Naturally occurring antimicrobials. In: Antimicrobials in Foods, 2nd edition. P.M. Davidson and A.L. Branen (Eds). Marcel Dekker, Inc., New York.

12. Deman, J.M. and P. Melnychyn (Eds). 1970. Symposium: Phosphates in Food Processing. AVI Publishing Company, Inc., Westport, Connecticut.

13. Donnenberg, M.S. (Ed.). 2002. *Escherichia coli*: Virulence mechanisms of a versatile pathogen. Elsevier Science. Orlando, Florida.

14. Doyle, M.P. 1991. *Escherichia coli* O157:H7 and its significance in foods. Intl. J. of Food Micro. 12: 289-302.

15. Doyle, M.P. 2003. Keeping *E. coli*

16. Doyle, M.P. and J.L. Schoeni. 1984. Survival and growth characteristics of *Escherichia coli* associated with hemorrhagic colitis. Appl. Environ. Microbiol. 48: 855-856.

17. Duffy, G., R.C. Whiting and J.J. Sheridan. 1999. The effect of a competitive microflora, pH, and temperature on the growth kinetics of *Escherichia coli* O157:H7. Food Microbiol. 16: 299-307.

18. Dukhin, S.S, G. Kretzschmar and R. Miller. Dynamics of adsorption at liquid interfaces: Theory, experiment, applications. Elsevier Science, Amsterdam.

19. Dykes, G.A. 2000. The effect of freezing on the survival of *Escherichia coli* O157:H7 on beef trimmings. Food Res. Intl. 33: 387-392.

20. Eagon, R.G. and K.J. Carson. 1965. Lysis of cell walls and intact cells of *Pseudomonas aeruginosa* by ethylenediamine tetraacetic acid and by lysozyme. Can. J. Microbiol. 11: 193-201.

21. Elliason, D.J. and S.R. Tatini. 1999. Enhanced inactivation of *Salmonella typhimurium* and verotoxigenic *Escherichia coli* by nisin at 65°C. Food Microbiol. 16: 257-267.

22. Ellinger, R.H. 1972. Phosphates as Food Ingredients. CRC Press, Cleveland, Ohio.

23. FDA, 1998. Direct food substances affirmed as generally recognized as safe: Egg white lysozyme. Fed. Reg. 63(4): 12421-12426.

24. Ferris, F.G. 1989. Metallic ion interactions with the outer membrane of Gram-negative bacteria. In: Metal ions and Bacteria. John Wiley and Sons, New York.

25. Ferris, F.G. and T.J. Beveridge. 1986. Physiochemical roles of soluble metal cations in the outer membrane of *Escherichia coli* K-12. Can. J. Microbiology. 32: 594-605.

26. FSIS, 2002. Colorado Firm Recalls Beef Trim and Ground Beef Products for possible *E. coli* O157:H7. Recall Release. http://www.fsis.usda.gov/OA/recalls/prelease/pr055-200

27. Furia, T.E. 1968. Sequestrants in Food. In: Handbook of Food Additives. The Chemical Rubber Company.

28. Gill, A.O. and R.A. Holley. 2002. Interactive inhibition of meat spoilage and pathogenic bacteria by lysozyme, nisin and EDTA in the presence of nitrite and sodium chloride at 24°C. Intl. J. of Food Microbiol. 80: 251-259.

29. Gould, G.W. Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. J. Food Prot. 1996 supplement: 82-86.

30. Gray, G.W. and S.G. Wilkinson. 1965. The action of ethylenediaminetetraacetic acid on *Pseudomonas aeruginosa*. J. Appl. Microbiol. 28: 153-164.

31. Hancock, R.E.W. 1984. Alterations in outer membrane permeability. Ann. Rev. Microbiol. 38: 237-264.

32. Harkes, G., H.C. van der Mei, P.G. Rouxhet, J. Dankert, H.J. Busscher, and J. Feijen. 1993. Physiochemical Characterization of *Escherichia coli*: A comparison with Gram-positive bacteria. Cell Biophys. 20: 17-32.

33. Hart, G., H.C. van der Mei, P.G. Rouxhet, J. Dankert, H.J. Busscher, and J. Feijen. 1992. Physiocochemical characterization of *Escherichia coli*. A comparison with Gram-positive bacteria. Cell Biophys. 20: 17-32.

34. Hiemenz, P.C. and R. Rajagopalan. 1997. Principles of Colloid and Surface Chemistry, 3rd edition. Marcel Dekker, New York.

35. Hughey, V.L. and E.A. Johnson. 1987. Antimcirobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. Appl. Environ. Microbiol. 55: 531-638.

36. Irani, R.R. and C.F. Callis. 1962. Calcium and magnesium sequestration by sodium and potassium polyphosphates. J. Am. Oil Chemists' Soc. 39: 156-159.

37. Jeffery, G.H., J.Basset, J. Medham, and R.C. Denny (Eds). 1989. Vogel's Textbook of Quantitative Chemical Analysis, 5th edition. Longman, London.

38. Jolles, J., J. Jaurequi-Adell, I. Bernier, and P. Jolles. 1963. The chemical structure of hen's egg white lysozyme: the detailed study. Biochim. Biophys. Acta. 78: 668.

39. Kilcast, D. 1995. Food Irradiation: Current problems and future potential. Intl. Biodeter. and Biodegrad. 36: 279-296

40. Klaassen, C.D. 1980. Absorption, distribution, and excretion of toxicants. In: Toxicology, The Basic Science of Poisons, 2nd edition. Macmillian, New York.

41. Knabel, S.J., H.W. Walker and P.A. Hartman. 1991. Inhibition of *Aspergillus flavus* and selected Gram-positive bacteria by chelation of essential metal cations by polyphosphates. J. Food Prot. 54: 360-365.

42. Kuntz, T.B. and S.T. Kuntz. 1999. Enterohemorrhagic *E. coli* Infection. Prim. Care Update Ob/Gyns. 6: 192-196.

43. Lado, B.H. and A.E. Yousef. 2002. Alternative food-preservation technologies: efficacy and mechanisms. Microbes and Infection. 4: 433-440.

44. Lee, R.M., P.A. Hartman, D.G. Olson and F.D. Williams. 1993. Metal ions reverse the inhibitory effects of selected food grade phosphates in *Staphylococcus aureus*. J. Food Prot. 57: 284-288.

45. Leive, L. 1965. Release of lipopolysaccharide by EDTA treatment of *E. coli*. Biochem. Biophy. Res. Commun. 21: 290-296.

46. Leive, L. 1968. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetic acid. J. Biol. Chem. 2373-2375.

47. Li, J. and L.A. McLandsborough. 1999. The effects of the surface charge and hydrophobicity of *Escherichia coli* O157:H7 on its adhesion to beef muscle. Intl. J. Food Microbiol. 53: 185-193.

48. Liang, J. Lindsay, J. Pestka, M. Pierson, P. Slade, R.B. Tompkin, and M.L. Tortorello. 2000. IFT Expert Report on Emerging Microbiological Food Safety Issues.

49. Lugtenberg, B. and L. Van Alphen. 1983. Molecular architecture and functioning of the outer membrane of *Escherichia* coli and other Gram-negative bacteria. Biochim. Biophys. Acta. 737: 51-115.

50. Mangia, A.H.R., L.M. Teixeira and F.C.S. Filho. 1995. The electrokinetic surface of five enteropathogenic *Escherichia coli* serogroups. Cell Biophys. 26: 45-55.

51. Masuda, T., Y. Ueno, and N. Kitabatake. 2001. Sweetness and enzymatic activity of lysozyme. J. Agric. Food Chem. 49: 4937-4941.

52. McClements, D.J. and E.A. Decker. 2000. Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. J. Food Sci. 65: 1270-1282.

53. McKenzie, H.A. and F.H. White Jr. 1991. Lysozyme and α-Lactalbumin: Structure, Function, and Interrelationships. In: *Advanced Protein Chemistry*. C.B. Anfinsen, F.M. Richards, J.T. Edsall, and D.S. Eisenberg (Eds). 41, Academic Press, San Diego, California.

54. Mead, P.S. and P.M. Griffin. 1998. *Escherichia coli* O157:H7. The Lancet, 352: 1207-1212.

55. Meng, J. and M.P. Doyle. 2003. Introduction: Microbiological food safety. Microbes and Infection. 4: 395-397.

56. Morris, C.M., A. George, W.W. Wilson and F.R. Champlin. 1995. Effect of polymyxin B nonapeptide on daptomycin permeability and cell surface properties in *Pseudomonas aeruginosa*, *Escherichia coli*, and *Pasteurella multocida.* J. Antibiotics. 48: 67-72.

57. Moustafa, H.H. and E.B. Collins. 1969. Effects of selected food additives on growth of *Pseudomonas fragi*. J. Dairy Sci. 52: 335-339.

58. Ngwenya, B.T., I.W. Sutherland and L. Kennedy. 2003. Comparison of the acid-base behaviour and metal adsorption characteristics of a Gram-negative bacterium with other strains. App. Geochem. 18: 527-538.

59. Paton, A.W., E. Voss, P.A. Manning and J.C. Paton. 1997. Shiga toxin-producing *Escherichia coli* isolates from cases of human disease show enhanced adherence to intestinal epithelial (Henle 407) cells. Infection and Immunity. 65: 3799-3805.

60. Peterson, R.G. and S.E. Hartsell. 1955. The lysozyme spectrum of the Gram-negative bacteria.

61. Phillips, D.C. 1973. The three-dimensional structure of an enzyme molecule. In. *Organic Chemistry of Life: Readings from Scientific American*. M. Calvin and W.A. Pryor (Eds.). W.H. Freeman and Co., San Francisco, California.

62. Potter, M. D. Archer, A. Benson, F. Busta, J.S. Dickson, M.P. Doyle, J. Farber, B.B. Finlay, M. Goldblatt, C. Hedberg, D. Hoover, M. Jahncke, L. Jaykus, C. Kaspar, A.P. Powrie, W.D. 1977. Chemistry of eggs and egg products. In *Egg Science and Technology*, 2nd edition. W.J. Stadelman and O.J. Cotterille (Ed), 75. AVI Publishing, Westport, CT.

63. Poortinga, A.T., R. Bos, W. Norde, and H.J. Busscher. 2002. Electric double layer interactions in bacterial adhesion to surfaces. Surface Sci Reports. 47: 1-32.

64. Proctor, V.A. and F.E. Cunningham. 1988. The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. CRC Crit. Rev. Food Sci. Nut. 26: 359-395.

65. Repaske, R. 1956. Lysis of Gram-negative bacteria by lysozyme. Biochim. Biophys. Acta. 22: 189-191.

66. Schaechter, M., N.C. Engleberg, B.I. Eisenstein and G. Medoff (Eds). 1998. Mechanisms of Microbial Disease, 3rd edition. Williams and Wilkins, Baltimore, Maryland.

67. Shelef, L.A. and Seiter. 1993. Indirect Antimicrobials. In: Antimicrobials in Foods, 2nd edition. P.M. Davidson and A.L. Branen (Eds). Marcel Dekker, Inc., New York.

68. Stevens, K.A., B.W. Sheldon, N.A. Klapes, and T.R. Klaenhammer. 1991. Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. Appl. Environ. Microbiol. 57: 3613-3615.

69. Tranter, H.S. 1994. Lysozyme, ovotransferrin, and avidin. In: *Antimicrobials and Food Preservation*, V.M. Dillon and R.G. Board (Eds). CAB International, Wallingford, UK.

70. van Loosdrecht, M.C.M., J. Lyklema, W. Norde, G. Schraa and A.J.B. Zehnder. 1987. Electrophoretic mobility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion. Appl. Environ. Microbiol. 53: 1898-1901.

71. Van Wazer, J.R. and C.F. Callis. 1958. Metal complexing by phosphates. Chem. Rev. 58: 1009-1017.

72. Van Wazer, J.R. and D.A. Campanella. 1950. Chelation of metals by polyphosphates. J. Am. Chem. Soc. 72: 655-658.

73. Vedmina, E.A., N.A. Pasternak, V.A. Shenderovich, T.P. Zhuravleva, I.T. Andrusenko, I.A. Aleksandrova, I.A. Stepanove, and V.R. Sobolev. 1979. Sensitivity of Gram-negative microflora to lysozyme. Antibiotiki. 24: 746-752.

74. Wasserfall, F. and M. Teuber. 1979. Action of egg white lysozyme on *Clostridium tyrobutyricum*. Appl. Environ. Microbiol. 38: 197-199.

75. Welcher, F.J. 1958. The analytical uses of ethylenediamine tetraacetic acid. Van Nostrand Company, Inc. Princeton, New Jersey.

76. Weiss, J. 2002. Concepts of interfacial properties in food chemistry. In: Current Protocols in Food Analytical Chemistry. Marcel-Dekkar Inc., New York.

77. White, D.G., S. Zhao, S. Simjee, D.D. Wagner, P.F. McDermott. 2002. Antimicrobial resistance of foodborne pathogens. Microbes and Infection. 4: 405-412.

78. Wilson, W.W., M.M. Wade, S.C. Holman and F.R. Champlin. 2001. Status of methods for assessing bacterial cell surface charge properties based on zeta potential measurements. J. Microbiol. Meths. 43: 153-163.

79. Witschel, M.B. and T. Egli. 2001. Environmental fate and microbial degradation of aminopolycarboxylic acids. FEMS Microbiol. Rev. 25: 69-106.

80. Wolf, K. and P.A. Gilbert. 1992. EDTA-ethylenediaminetetraacetic acid. In: The Handbook of Environmental Chemistry, vol. 3. Springer-Verlag, Berlin.

Chapter 2:

Enhanced Inactivation of *Escherichia coli* O157:H7 by Lysozyme and Chelators

2.1 Abstract

This study examined the effect of three chelating agents (EDTA, disodium pyrophosphate (DSPP), pentasodium tripolyphosphate (PSTPP)) on growth inhibition of *Escherichia coli* O157:H7 by lysozyme. The objective of this study was to identify replacement chelators that exhibit synergistic properties similar to EDTA. The inhibitory effect of 300-1500 μg/mL EDTA and 3,000-15,000 μg/mL DSPP and PSTPP in combination with 200-600 μg/mL lysozyme was studied at pH 6, 7, and 8 on four strains of *Escherichia coli* O157:H7 for 48 hours, using a microplate dilution assay. Addition of EDTA enhanced the inhibitory effect of lysozyme against strains of E. coli O157:H7. EDTA concentrations ≥ 300 μg/mL combined with 200-600 μg/mL lsyzoyme were sufficient in inhibiting the strains at pH 6 and 8. At pH 7, 200-600 μg/mL lysozyme and EDTA concentrations ≥ 1,000 μg /mL were effective in inhibiting three out of four strains. DSPP at pH 6, was effective at ≥ 10,000 μg/mL when combined with 200-300 μg/mL lysozyme. In contrast, PSTPP increased the inhibitory activity of lysozyme more effectively at pH 8. 200-600 μg/mL lysozyme was effective against two strains of *E. coli* O157:H7 when used in conjunction with ≥ 5,000 μg/mL PSTPP. Remaining strains were inhibited by ≥ 10,000 μg/mL PSTPP. Our results indicate that inhibition occurred with each lysozyme-chelator combination, but larger concentrations of polyphospates were required, compared to EDTA, to increase the antimicrobial spectrum of lysozyme against *E. coli* O157:H7.

2.2 Introduction

Some food antimicrobials, such as lysozyme and nisin, have enhanced inhibitory activity against Gram-negative bacteria when used in the presence of chelating agents *(3)*.

This inactivation occurs by a mechanism that likely involves the chelation of membrane cations. The chelation of cations destabilizes the outer cell membrane allowing the migration of antimicrobials to the interior of the cell *(4)*. Interest continues to grow in the food industry in developing compounds that can be added to food systems to substantially reduce or inhibit growth of Gram-negative bacteria by food antimicrobials *(3)*.

Food antimicrobials have been used as a preservation method to inhibit the growth of microorganisms, including foodborne pathogens, for a number of years. Naturally occurring antimicrobials, such as lysozyme, have received renewed attention because of their abundance in nature. In addition, the enzymatic activity of lysozyme is known to be bactericidal to Gram-positive microorganisms. The suggested mechanism by which lysozyme inactivates bacteria is based on the hydrolysis of the 1-4 glycosidic bonds between N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) in the peptidoglycan layer of the bacterial cell wall (17).

However, the antimicrobial activity of lysozyme in foods is limited to specific classes of microorganisms. Lysozyme alone is essentially ineffective against Gram-negative bacteria due to the presence of a lipopolysaccharide (LPS) layer in the outer membrane of the cell. The LPS layer acts as a permeability barrier to prevent lysozyme from penetrating the cell interior *(4)*.

Ethylene diaminetetraacetic acid (EDTA) is a chelating compound added to food products to sequester trace metals that act as pro-oxidants *(10)*. Additionally, EDTA can function synergistically with food antimicrobials to inhibit Gram-negative bacteria. The chelation of metal ions from microorganisms, specifically Gram-negative bacteria, can

increase the antimicrobial spectrum of compounds such as lysozyme *(4, 15, 19)*. Chelation of these metal ions destabilizes the LPS layer and increases cell sensitivity and permeability allowing lysozyme to penetrate the LPS layer, resulting in lysis of the cell *(21)*. While EDTA is used in foods as a chelating agent, its applications are limited to selected food products. The application of other, novel generally recognized as safe (GRAS) chelators could expand the antimicrobial activity of food antimicrobials and serve as a replacement for EDTA.

The use of polyphosphates as food additives has increased in recent years. This can be attributed to changes in regulations permitting their use in a variety of meat products and the need to reduce the sodium chloride (NaCl) levels used in meat processing *(11)*. Polyphosphates are added to food products, to increase water and meat-binding, reduce rancidity and color deterioration, enhance emulsification, and amplify color development during curing *(5, 14)*. Polyphosphates are negatively charged polyanions that readily form complexes with cations such as copper, calcium, iron, zinc, and magnesium *(18)*. Sequestering of metal ions by phosphates occurs with the formation of a metal-chelate ring produced between oxygen atoms found in the polyphosphate structure, and metal ions (24). Although polyphosphates have antimicrobial activity, few experiments have evaluated the interactive antimicrobial effects of these compounds on Gram-negative bacteria such as *E. coli* O157:H7 *(13)*.

The objective of this study was to evaluate the efficiency of EDTA, disodium pyrophosphate (DSPP) and pentasodium tripolyphosphate (DSTPP) to increase the activity of lysozyme against *E. coli* O157:H7. The GRAS polyphosphates were selected based on similar binding constants when compared to EDTA *(6)*. Although relatively

ineffective against Gram-positive bacteria, studies have shown that polyphosphates can expand the activity spectrum of food antimicrobials *(1)*.

2.3 Materials and Methods

2.3.1 Culture Preparation

Escherichia coli O157:H7 strains 932, F4546, E0019 and H1730 were obtained from Dr. Larry R. Beuchat of the University of Georgia. All cultures were grown in tryptic soy broth (TSB; Difco, Sparks, MD) and transferred every two weeks to maintain viability. Working cultures were obtained by inoculating a loopful of culture into TSB and incubating for 24 hr at 35°C. After incubation, the cultures were diluted to 4.0 log CFU/mL.

2.3.2 Antimicrobial Preparation

Lysozyme (58,100 units/mg protein [one unit produces a change in the absorbance of a *Micrococcus lysodeikticus* suspension at 450 nm of 0.001 per minute at pH 6.24, 25° C] Sigma, St. Louis MO.) was dissolved in distilled water and passed through a 0.45 μm sterile filter (Corning Incorporated, Corning, NY). Lysozyme solutions were prepared to contain 200, 300, 400, 500, and 600 μg/mL. Disodium ethylene diamine tetra acetic acid (EDTA; Sigma) was dissolved in distilled water and tested at 300, 500, 1000, 1250, and 1500 μg/mL, while aqueous solutions of anhydrous disodium pyrophosphate (DSPP; Sigma) and anhydrous pentasodium tripolyphosphate (PSTPP; Sigma) were evaluated at 3,000, 5,000, 10,000, 12,500, and 15,000 μg/mL. Concentrations of DSPP and PSTPP were greater than EDTA because preliminary

experiments demonstrated that higher concentrations were needed to inhibit *E. coli* O157:H7.

2.3.3 Micro-broth Dilution Assay

A checkerboard microassay was used to evaluate the antimicrobial activity of lysozyme-chelator combinations *(2)*. Sterile 96-well microtiter plates with a well capacity of 300 µl were used for the combination studies. For each test, a total volume of 240 µl was used consisting of 120 µl of inoculum in TSB (~4.0 log CFU/mL), 60 µl of lysozyme solution, and 60 µl of chelator solution. Microtiter plates were covered with a sterile lid and incubated 48 hr at 35°C.

The absorbance (630 nm) of each well was read at 0, 3, 6, 12, 24, 30, 36, and 48 hours with a microtiter plate spectrophotometer (El_x 800 Universal Microplate reader, BioTek Instruments, Winooski, VT). The micro-broth dilution assays were performed in triplicate and each experiment was replicated three times.

2.3.4 Determination of Chelator pKa Values

Solutions of EDTA, DSPP, and PSTPP were prepared at the maximum concentrations used in the antimicrobial tests. Volumetric titration with 0.01 M hydrochloric acid (HCl) (Fisher Scientific, Pittsburgh, PA) or 0.01 M sodium hydroxide (NaOH) (Fisher Scientific) was performed to determine the pKa values for each chelator. An Accumet Basic 15 pH meter (Fisher Scientific) was used to determine pH values during titration.

2.4 Results

2.4.1 Minimum Inhibitory Concentration of Chelators

The MIC (defined as no growth (OD ≤ 0.07) after 24 hr) for EDTA, DSPP, and PSTPP alone were determined against the four strains of *E. coli* O157:H7. At pH 6.0, EDTA was very effective with lysozyme in inhibiting all four strains of *E. coli* O157:H7 at 300 µg/mL (Table 2.1-See Appendix). DSPP inhibited strains F4546 and E0019 at 10,000 µg/mL. The DSPP MIC for strain 932 was 12,500 µg/mL DSPP, whereas strain H1730 was resistant to the highest concentration of 15,000 µg/mL DSPP. PSTPP did not inhibit any of the four strains at the highest concentration tested, 15,000 µg/mL, at pH 6.0.

In contrast, higher concentrations of EDTA were required to inhibit three of the four *E. coli* O157:H7 strains at pH 7.0. Strains F4546 and E0019 were inhibited by 1,000 µg/mL EDTA, while strain 932 was more resistant and required 1,250 µg/mL. Strain H1730 was inhibited by 300 µg/mL EDTA. At pH 7.0, DSPP at 15,000 µg/mL did not inhibit the four strains of *E. coli* O157:H7. PSTPP had an inhibitory effect at concentrations 12,500 µg/mL against strains 932, F4546, and E0019. Strain H1730 was inhibited by 15,000 µg/mL PSTPP.

At pH 8.0, inhibition by EDTA was similar to that at pH 6.0. The MIC for all four strains of *E. coli* O157:H7 was 300 µg/mL EDTA. In contrast, DSPP was not inhibitory to any of the strains at the maximum of 15,000 µg/mL. Strains F4546 and E0019 were inhibited by 5,000 µg/mL PSTPP and the remaining strains, 932 and H1730, were inhibited by ≥ 10,000 µg/mL PSTPP.

2.4.2 Lysozyme-Chelator Combinations

The optical density of the lysozyme-chelator combinations were measured as a function of time for two *E. coli* O157:H7 strains in the presence of three chelating agents. Strain 932 and H1730 are shown because they were the most resistant and susceptible to lysozyme-EDTA combinations, respectively. For strain 932, 200-600 µg/mL lysozyme and 300-1,500 µg/mL EDTA were required to inhibit the strain at pH 6.0 (Fig. 2.1). EDTA prevented growth of strain 932 at ≥ 500 µg/mL. At pH 6.0, DSPP also exhibited inhibitory properties when combined with lysozyme. 12,500-15,000 µg/mL DSPP and 200-600 µg/mL lysozyme inhibited strain 932. The combination of PSTPP and lysozyme did not completely inhibit strain 932 at pH 6.0.

Interaction of lysozyme and the chelating agents with strain H1730 at pH 6.0 is shown in Fig. 2.2 A-C. Inhibition of strain H1730 occurred with 200-600 µg/mL lysozyme and 300-1500 µg/mL EDTA. DSPP, with the addition of lysozyme, exhibited no inhibitory effect on strain H1730 at pH 6.0. However, inhibition did occur with 200-600 µg/mL lysozyme and 3,000-15,000 µg/mL PSTPP when used against strain H1730 at pH 6.0.

Lysozyme-chelator interactions for strain 932 at pH 7.0 are shown in Fig. 2.3 A-C. 400-600 µg/mL lysozyme and 1,250-1,500 µg/mL EDTA were needed to inhibit strain 932 at pH 7.0. < 400 µg/mL lysozyme and < 1,250 µg/mL EDTA produced absorbance readings > 0.20, indicating that only slight inhibition of the strain occurred at these concentrations. At pH 7.0, no combination of DSPP and lysozyme completely inhibited strain 932; however, higher concentrations are needed to reduce the extent of growth as measured by O.D. In contrast, PSTPP and lysozyme at pH 7.0 produced different results

than those recorded with DSPP against strain 932. 200-600 µg/mL lysozyme and 12,500-15,000 µg/mL PSTPP caused complete inhibition of strain 932.

Figure 2.4 shows the inhibition of strain H1730 with 200-600 µg/mL lysozyme and 300-1500 µg/mL EDTA at pH 7.0. DSPP in combination with lysozyme did not completely inhibit strain H1730 at pH 7.0. Higher concentrations of DSPP were needed to inhibit growth of the strain. PSTPP and lysozyme inhibited strain H1730 at 400-600 µg/mL lysozyme and 15,000 µg/mL PSTPP at pH 8.0.

The activity of lysozyme-chelator systems against *E. coli* O157:H7 strains 932 and H1730 are shown in Fig. 2.5 A-C. The combination of EDTA and lysozyme was effective in inhibiting strain 932 at pH 8.0 (Fig. 2.5). No growth of the strain occurred with 200-600 µg/mL lysozyme and 300-1,500 µg/mL EDTA. The DSPP and lysozyme combination was ineffective in completely inhibiting strain 932 at pH 8.0. In order for inhibition of the strain to occur, higher concentrations of DSPP were required. PSTPP and lysozyme resulted in inhibition at pH 8.0. Inhibition occurred with 200-600 µg/mL lysozyme and 5,000-15,000 µg/mL PSTPP.

Growth of strain H1730 was completely inhibited at pH 8.0 with 200-600 µg/mL lysozyme and 300-1500 µg/mL EDTA (Fig. 2.6). All combinations of DSPP and lysozyme were ineffective in completely inhibiting the strain at pH 8.0, however, OD readings at the highest concentration were less than the controls. Total inhibition occurred with 200-600 µg/mL lysozyme and 10,000-15,000 µg/mL PSTPP at pH 8.0.

The lysozyme-chelator combinations used for the micro-broth dilution assay demonstrate the vast difference in the resistance or susceptibility between the four *E. coli* O157:H7 strains. From the experiments, strain 932 was more resistant to lysozyme-

EDTA combinations than strain H1730. Although lower concentrations of EDTA were successful in inhibiting all strains at pH 6.0 and 8.0, strain 932 proved to be most resistant at pH 7 to the lysozyme-EDTA combination. 1,250 µg/mL EDTA was needed to inhibit strain 932, in comparison to the other strains which required lower inhibitory concentrations of EDTA. Strain H1730 proved to be more resistant to treatments of DSPP and PSTPP. At pH 6, 7 and 8, concentrations > 15,000 DSPP are required to inhibit strain H1730. Although the maximum concentration of DSPP was not inhibitory to strain 932 at pH 7.0 and 8.0, 12,500 µg/mL DSPP completely inhibited the strain at pH 6.0. At pH 6.0, complete inhibition of the strains was not present with PSTPP. However, at pH 7.0, a higher concentration of 15,000 PSTPP was needed to inhibit strain H1730, in contrast to inhibition of strain 932 with 12,500 PSTPP.

2.4.3 Chelator pKa Values

Titration tests were performed to determine the pKa values for the chelators. The pKa values for EDTA were 2.0, 2.7, 6.2, and 10.3. DSPP pKa values were recorded as 2.1, 6.0, and 9.1, in contrast to recorded pKa values of 8.1, 10.2, and 12.1 for PSTPP.

2.5 Discussion

Inhibition of four strains of *Escherichia coli* O157:H7 was achieved with each lysozyme-chelator combination. The results demonstrate that environmental conditions (pH, chelator concentration, and strain type) play an integral role in the inhibition of the strains.

The cations magnesium, calcium, iron, sodium and potassium are metal ions present in the LPS layer of Gram-negative bacteria *(4, 11, 23)*. Magnesium assists in cell division and many membrane-bound enzyme activities *(11)*. Magnesium also aids

calcium in cross-linking negatively charged groups to form salt bridges to bind polysaccharides on the surface of Gram-negative bacteria. Formation of metal complexes with magnesium and calcium can result in leakage of cell solutes and loss of viability *(7, 23)*. These strong interactions provide strength and stability to prevent the diffusion of lysozyme through the LPS layer. Iron is essential for growth, replication, respiration, and DNA synthesis of bacterial cells. Sodium and potassium are required in protein synthesis to balance RNA and other intracellular activities. Sodium additionally assists in the internal regulation of cytoplasmic pH within a cell. Chelation of essential metal ions from the LPS layer will disrupt the protective and influential effects of the metal complexes; thus allowing penetration of lysozyme to the cell interior *(4)*.

For chelation of metal ions to occur, two general principles must be fulfilled. The chelating agent must have the proper steric and electrochemical configuration in relation to the metal being sequestered. Secondly, environmental conditions (pH, ionic strength, and solubility) must favor the binding of metal ions (6,8). EDTA has six donor groups which are able to bind metal ions and form a metal-chelator complex (6). Metal ions generally have a coordination number of six, which EDTA is able to fully satisfy due to the presence of six donor atoms. The donor atoms allow for increased chelation and the removal of metal ions from the LPS layer, therefore explaining EDTA's superiority to the phosphate chelators used in the experiments *(6,8)*. We hypothesize that the availability of donor groups to accept and bind positively charged metal ions depends ultimately on the redox potential of these components. The ability to abstract hydrogen from negatively charged hydroxyl groups depends on solution pH and is governed by the pKa's of chelators.

The effects of pH on the chelating capacity of EDTA were evaluated by determining the pKa values. A pKa value of 6.2 would suggest that chelation is greater with EDTA at pH 6, resulting in the inhibition of strains with ≥ 300 µg/mL EDTA. Previous studies have indicated that the chelation of iron by EDTA from the LPS layer is greatest at pH 4-6 *(8)*. The MIC of the four strains of *E. coli* O157:H7 at pH 8 was ≥ 300 µg/mL EDTA. Since there was no pKa for EDTA in the vicinity of pH 8, there is no definite explanation for enhanced inhibition of the strains by EDTA. However chelators, such as EDTA, become increasingly dissociated at higher pH and the quantity of metal complexed by the compound increases *(6)*. Additional studies, as reported by Jeffrey et al. *(8)*, indicate that maximum chelation of magnesium and calcium by EDTA is greatest at pH 8-10. At relatively greater concentrations than at pH 6 and 8, EDTA inhibited three strains of *E. coli* O157:H7 at pH 7. The chelation of metal ions by EDTA at pH 7 was not as great as at pH 6, possibly because of the absence of a pKa value near pH 7. Additional factors for increased inhibition at pH 6 and 8 could be attributed to the presence of a slightly stressful environment for the strains.

The four strains of *E. coli* O157:H7 were inhibited by DSPP at pH 6. The four binding sites linked to the DSPP structure lowers its ability to effectively chelate metal ions from the LPS layer when compared to EDTA. A titration study indicated a pKa value of 6.0 for DSPP. The close proximity of a pKa value at pH 6 could have resulted in enhanced chelation of metal ions, increasing inhibition of the strains. Inhibition did not occur for the strains at pH 7 and 8 possibly due to the absence of an additional pKa value at this pH range. Three of the four strains of *E. coli* O157:H7 were inhibited with selected concentrations of DSPP. Strain 932 was designated as the most resistant strain to the

EDTA-lysozyme combination and was inhibited by ≥ 12,500 µg/mL DSPP. However, strain H1730, designated as the most sensitive strain to the EDTA-lysozyme combination, was the most resistant to DSPP. ≥ 15,000 µg/mL DSPP did not inhibit strain H1730. This particular strain may contain high amounts of one or more essential cations. Due to the lower number of binding sites present with DSPP, these cations may have remained bound in the LPS and prevented penetration of lysozyme to the cell interior.

Strains of *E. coli* O157:H7 were inhibited by PSTPP at pH 8. The dissociation of PSTPP in an aqueous media creates five potential binding sites for metal cations. Because of the lower number of binding sites, PSTPP possesses a lower chelating capacity in comparison to EDTA *(5)*. Titration studies determined that PSTPP has a pKa of 8.1. The close proximity of a pKa value at pH 8 allows PSTPP to chelate metal ions; therefore resulting in potentially greater inhibition of strains. Inhibitory concentrations of PSTPP were much lower than DSPP. When compared to DSPP, the additional binding site found within the PSTPP structure could allow smaller concentrations of PSTPP to be inhibitory. All lysozyme-chelator interactions exhibited inhibitory activity under different experimental conditions. Studies have shown that changes in pH with the addition of phosphates play an important role in the chelation of essential metal ions needed for bacterial metabolism and growth *(12)*. PSTPP was the only chelating agent to show potential as a replacement chelator for EDTA. However, the principal disadvantage of using PSTPP is the requirement for high concentrations to inhibit Gram-negative bacteria. These high concentrations may lead to altered food properties, formation of residues, and increased wear in food processing equipment *(1)*.

Although it is uncertain which metal ions are chelated by polyphosphates, it has been hypothesized that microbial inactivation of Gram-negative bacteria by polyphosphates occurs due to the chelation of magnesium *(11)*. Studies have also indicated that polyphosphates have the ability to chelate small amounts of iron and calcium *(11, 20)*. It is well known that calcium ions are sequestered by polyphosphates in controlling phage infection for natural cheese starter cultures *(22)*. Additional studies have reported that polyphosphates may affect the FtsZ protein in bacterial cells. The GTPase activity of FtsZ proteins relies on metal ions to initiate bacterial cell division *(25)*. In addition, studies have shown that the FtsZ protein exhibits a strict magnesium dependent behavior for GTPase activity. It is hypothesized that polyphosphate compounds, such as PSTPP, bind to bacteria cell walls, chelate essential metal ions, and create an acute metal ion deficit. This in return would block GTPase activity of the FtsZ protein and prevent replication of bacterial cells *(16)*.

Even though inhibition occurred with DSPP and PSTPP, neither polyphosphate compound achieved the maximum chelating capacity exhibited by EDTA. The binding capacity of chelating agents significantly depended on the pKa value for each compound and the number of binding sites present for each chelator. The results indicate that, on an equal concentration basis, inhibition by EDTA was approximately 10-times greater than that of DSPP or PSTPP. Although not as effective as EDTA, DSPP and PSTPP enhanced the antimicrobial spectrum of lysozyme at specific pH values and higher concentrations. This information could be beneficial in future applications of lysozyme and chelators to food products to control *E. coli* O157:H7 and possibly other Gram-negative bacteria.

References

1. Alexandra, M.S., D.M. Carneiro, C.A. Cassar, and R. J. Miles. 1998. Trisodium phosphate increases the sensitivity of Gram-negative bacteria to lysozyme and nisin. J. Food Prot. 61:839-844.

2. Barry, A.L. 1976. The Antimicrobic Susceptibility Test: Principle and Practices. Lea and Febiger, Philadelphia.

3. Cutter, C.N. and Siragusa, G.R.1995. Population Reductions of Gram-Negative Pathogens Following Treatments with Nisin and Chelators under Various Conditions. J. Food Prot. 58:977-983.

4. Elliason, D.J. and S.R. Tatini. 1999. Enhanced inactivation of *Salmonella typhimurium* and verotoxigenic *Escherichia coli* by nisin at 65°C. Food Microbiol. 16:257-267.

5. Ellinger, R.H. 1972. *Phosphates as Food Ingredients*. R.C. Weast (Ed). CRC Press, Cleveland, OH.

6. Furia, T.E. 1968. Sequestrants in Food. In: *Handbook of Food Additives*. The Chemical Rubber Company, Cleveland, OH.

7. Gray, G.W. and S.G. Wilkinson. 1965. The action of ethylenediaminetetraacetic acid on *Psuedomonas aeruginosa*. J. Appl. Microbiol. 28:153-164.

8. Jeffrey, G.H., J. Basset, J. Medham, and R.C. Denny (Eds.), 1989. Vogel's Textbook of Quantitative Chemical Analysis, 5th edition., Longman, London.

9. Jen, C.M.C. and L.A. Shelef. 1986. Factors affecting the sensitivity of *Staphylococcus aureus* 196E to polyphosphates. Appl. Environ. Microbiol. 52:842-846.

10. Klaassen, C.D. 1980. Absorption, distribution, and excretion of toxicants. In: *Toxicology, the Basic Science of Poisons*, 2nd edition. Macmillan, New York.

11. Knabel, S.J., H.W. Walker, and P.A. Hartman. 1991. Inhibition of *Aspergillus flavus* and selected Gram-positive bacteria by chelation of essential metal cations by polyphosphates. J. Food Prot. 54:360-365.

12. Kim, C.R., J.O. Hearnsberger, A.P. Vickery, C.H. White, and D.L. Marshall. 1995. Extending Shelf Life of Refrigerated Catfish Fillets Using Sodium Acetate and Monosodium Phosphate. J. Food Prot. 58:644-647.

13. Kim, C.R. and D.L. Marshall. 1999. Microbiological, colour, and sensory changes of refrigerated chicken legs treated with selected phosphates. Food Res. Intl. 32:209-215.

14. Lee, R.M., P.A. Hartman, H.M. Stahr, D.G. Olson, and F.D. Williams. 1994. Antibacterial mechanism of long-chained polyphosphates in *Staphylococcus aureus*. J Food Prot. 57:289-294.

15. Leive, L. 1968. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetic acid. J. Biol. Chem. 243:2373-2375.

16. Maier, S.K., S. Scherer, and M.J. Loessner. 1999. Long-Chain Polyphosphate Causes Cell Lysis and Inhibits *Bacillus cereus* Septum Formation, Which is Dependent on Divalent Cations. Appl. Environ. Microbiol. 65:3942-3949.

17. Masuda, T., Y. Ueno, and N. Kitabatake. 2001. Sweetness and enzymatic activity of lysozyme. J. Agric. Food Chem. 49:4937-4941.

18. Molins, R.A. 1991. *Phosphates in Foods*. CRC Press, Inc., Boca Raton, FL.

19. Moustafa, H.H. and E.B. Collins. 1969. Effects of selected food additives on growth of *Pseudomonas fragi*. J. Dairy Sci. 52:335-339.

20. Nelson, K.A., F.F. Busta, J.N. Sofos, and M.K. Wagner. 1983. Effect of Polyphosphates in Combination with Nitrite-Sorbate or Sorbate on *Clostridium botunlinum* Growth and Toxin Production in Chicken Frankfurter Emulsions. J. Food Prot. 46:846-850.

21. Stevens, K.A., B.W. Sheldon, N.A. Klapes, and T.R. Klaenhammer. 1991. Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. Appl. Environ. Microbiol. 57:3613-3615.

22. Tompkins, R.B. 1983. Indirect Antimicrobial Effects in Foods: Phosphates. J. Food Safety 6:13-27.

23. Vaara, M. 1992. Agents that increase the permeability of the outer membrane. Microbiol. Rev. 56:395-411.

24. Van Wazer, J.R. and C.F. Callis. 1958. Metal complexing by phosphates. Chem. Rev 58:1009-1017.

25. Zaika, L.L., O.J. Scullen, and J.S. Fanelli. 1997. Growth inhibition of *Listeria monocytogenes* by sodium polyphosphate as affected by polyvalent metal ions. J. Food Sci. 62:867-869.

Appendix

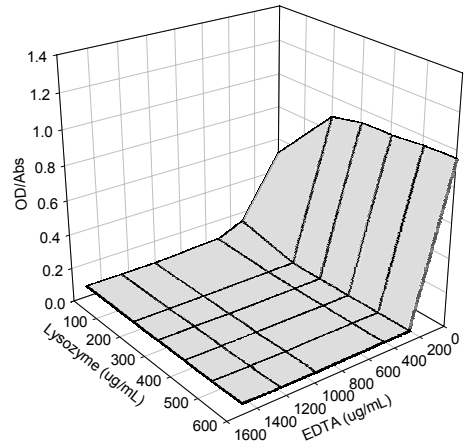
Table 2.1. Minimum inhibitory concentration of EDTA[1], DSPP[2], and PSTPP[3] against *Escherichia coli* O157:H7 at pH 6, 7, and 8 at 35˚C for 24 hr. in TSB.

pH	Strain	EDTA[1](µg/mL)	DSPP[2](µg/mL)	PSTPP[3](µg/mL)
6	932	300	12,500	>15,000
	F4546	300	10,000	>15,000
	E0019	300	10,000	>15,000
	H1730	300	>15,000	>15,000
7	932	1,250	>15,000	12,500
	F4546	1,000	>15,000	12,500
	E0019	1,000	>15,000	12,500
	H1730	300	>15,000	15,000
8	932	300	>15,000	10,000
	F4546	300	>15,000	5,000
	E0019	300	>15,000	5,000
	H1730	300	>15,000	10,000

[1]EDTA = Disodium ethylene diaminetetraacetic acid

[2]DSPP = Anhydrous disodium pyrophosphate

[3]PSTPP = Anhydrous pentasodium tripolyphosphate



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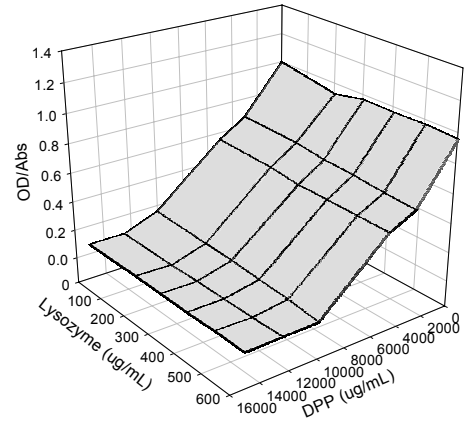

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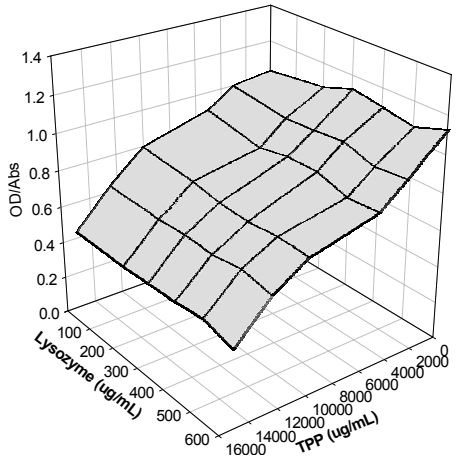
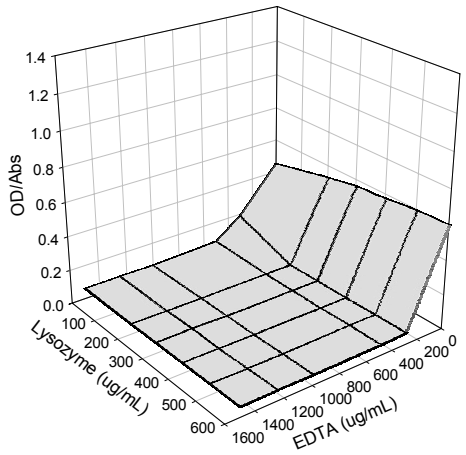


C.

Figure 2.1: Growth of *E. coli* O157:H7 strain 932 as measured by OD_{630} in the presence of combinations of lysozyme and chelator at pH 6.0 after 24 hr. (A) EDTA (B) DSPP (C) PSTPP



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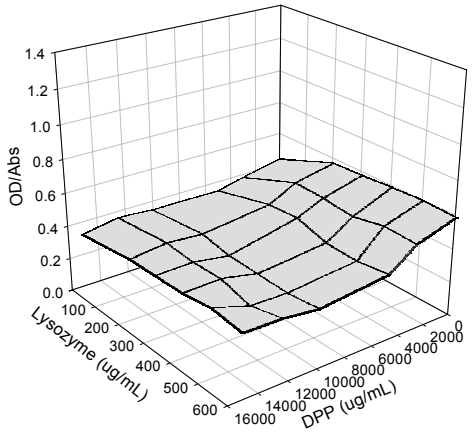

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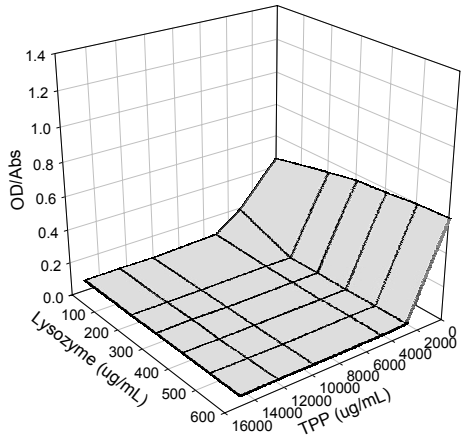
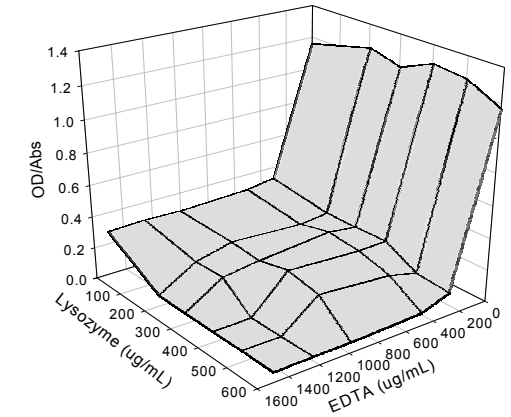


C.

Figure 2.2: Growth of *E. coli* O157:H7 strain H1730 as measured by OD_{630} in the presence of combinations of lysozyme and chelator at pH 6.0 at 24 hr. (A) EDTA (B) DSPP (C) PSTPP



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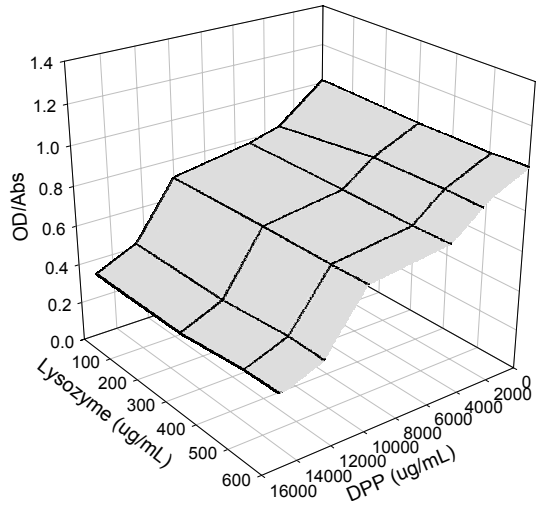

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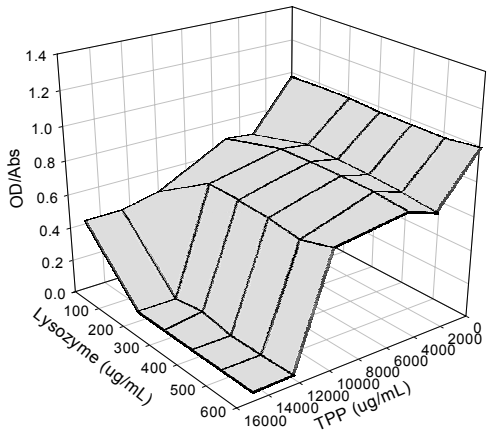
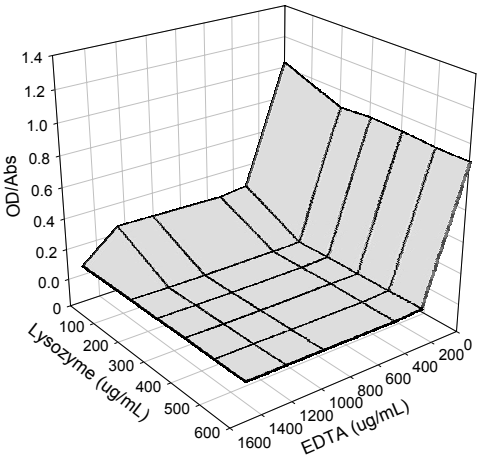


C.

Figure 2.3: Growth of *E. coli* O157:H7 strain 932 as measured by OD_{630} in the presence of combinations of lysozyme and chelator at pH 7.0 at 24 hr. (A) EDTA (B) DSPP (C) PSTPP



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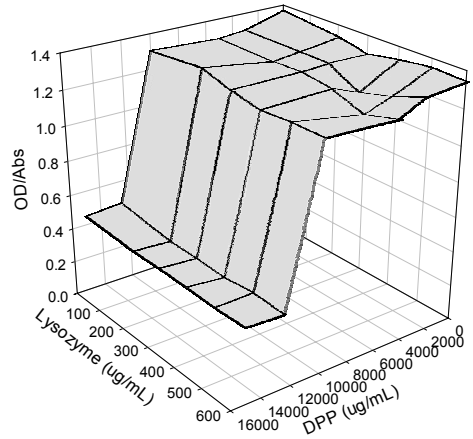

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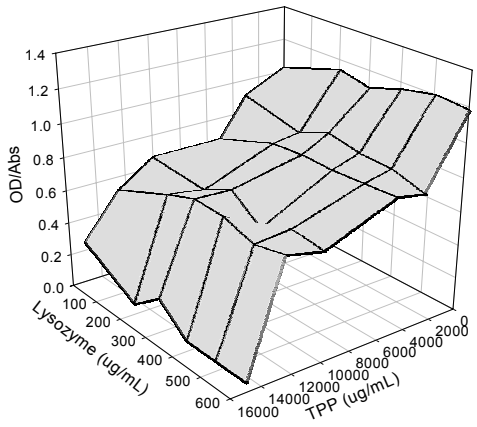

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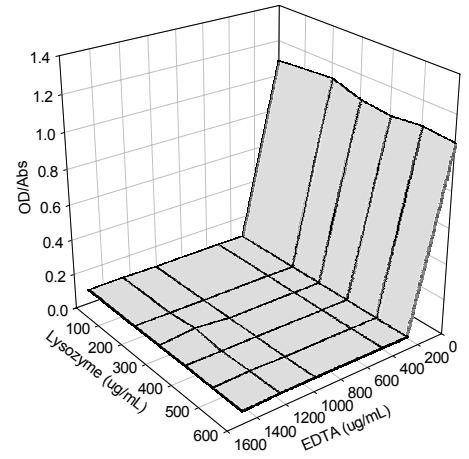
Figure 2.4: Growth of *E. coli* O157:H7 strain H1730 as measured by OD_{630} in the presence of combinations of lysozyme and chelator at pH 7.0 at 24 hr. (A) EDTA (B) DSPP (C) PSTPP



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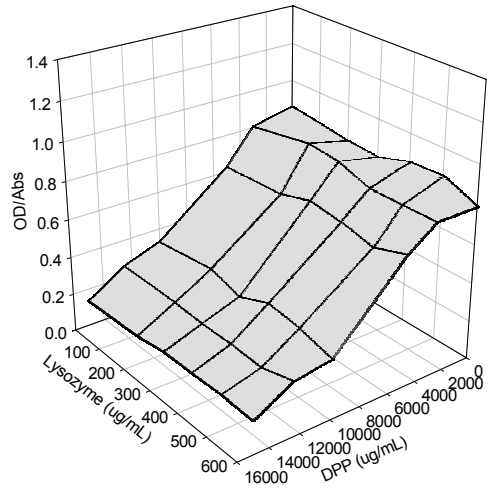

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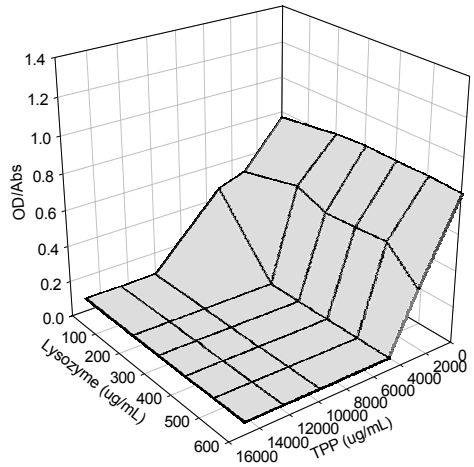
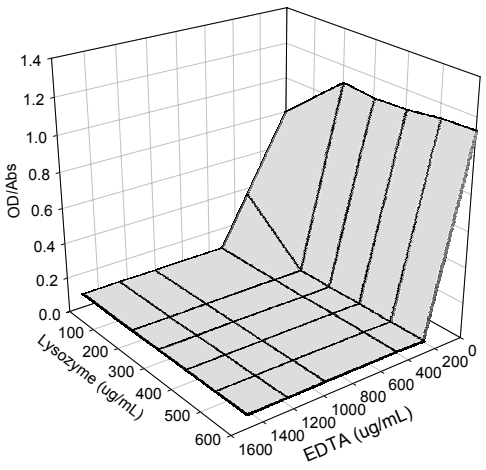


C.

Figure 2.5: Growth of *E. coli* O157:H7 strain 932 as measured by OD_{630} in the presence of combinations of lysozyme and chelator at pH 8.0 at 24 hr. (A) EDTA (B) DSPP (C) PSTPP



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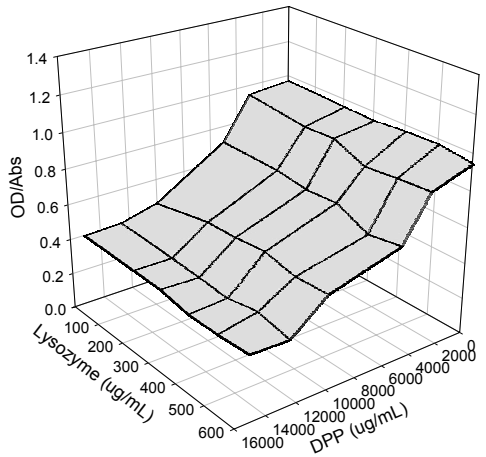

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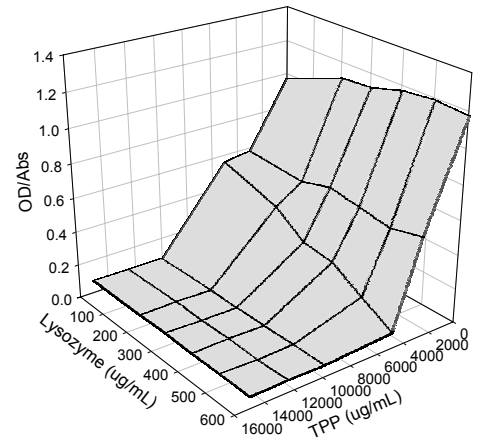


C.

Figure 2.6: Growth of *E. coli* O157:H7 strain H1730 as measured by OD_{630} in the presence of combinations of lysozyme and chelator at pH 8.0 at 24 hr. (A) EDTA (B) DSPP (C) PSTPP

Chapter 3:

Cations Reduce Antimicrobial Efficacy of Lysozyme-Chelator Combinations

3.1 Abstract

This study examined the reduction of antimicrobial efficacy of lysozyme-chelator combinations against two *E. coli* O157:H7 strains upon addition of mineral salts. Seven salts at concentrations ranging from 1.0 to 10.0 mM were added to aqueous solutions of lysozyme and disodium ethylenediamine tetraacetic acid (EDTA), disodium pyrophosphate (DSPP) or pentasodium tripolyphosphate (PSTPP) at pH 6.0, 7.0, or 8.0 and applied to cultures of *E. coli* O157:H7 strains 932 and H1730. Inhibitory activity of lysozyme chelator combinations against both strains was lost after addition of ≥ 1.0 mM Ca^{++} and Mg^{++} at pH 7.0 and 8.0. At pH 6.0, antimicrobial activity against strain 932 and H1730 was retained with EDTA-lysozyme despite the presence of calcium and magnesium cations. DSPP-lysozyme inhibited strain H1730 at pH 6.0 in the presence of Mg^{++}. Concentrations ≥ 4.0 mM Fe^{++} neutralized activity of all lysozyme-chelators combinations. Reverse inhibition of lysozyme-chelator complexes by Na^{+} and K^{+} depended on *E. coli* O157:H7 strain type. Addition of Na^{+} and K^{+} to lysozyme-chelator complexes indicated minimal to no reverse inhibition of strain 932. However, Na^{+} and K^{+} reversed lysozyme-chelator inhibition of strain H1730. The addition of ≥ 1.0 mM Fe^{+++} or Al^{+++} was effective in overcoming inhibition of lysozyme and EDTA at pH 6.0, 7.0 and 8.0. Isothermal titration calorimetry was used to explain results in terms of ion-specific competitive binding of free cations by the EDTA-lysozyme combinations. A mechanistic model for the antimicrobial functionality of chelator-lysozyme combinations was proposed.

3.2 Introduction

Chelating compounds are often added to food products to sequester metal ions that may act as pro-oxidants*(21)*. Additionally, chelators can function synergistically with food antimicrobials to inhibit bacteria *(4)*. For example, chelation of metal ions in microorganisms, specifically Gram-negative bacteria, may increase the antimicrobial spectrum of compounds, such as lysozyme *(12, 16, 24)*. Usually, lysozyme has little or no antimicrobial effect against Gram-negative bacteria because of the protection provided to the cells by the outer cell membrane. Sequestration of membrane-bound cations with suitable chelators apparently destabilizes the outer cell membrane or lipopolysaccharide layer allowing lysozyme to act on the Gram-negative cell wall *(11, 12)*. Ethylenediamine tetraacetic acid (EDTA), a generally recognized as safe (GRAS) (21 CFR 172) chelator, has a particularly high affinity for sequestering cations in the cell envelope of bacteria. To a lesser degree, polyphosphate chelators , e.g., disodium pyrophosphate (DSPP) and pentasodium tripolyphosphate (PSTPP) can increase the activity spectrum of food antimicrobials such as lysozyme, as well *(4, 22)*.

Metal cations are vital in stabilizing the molecular architecture of the LPS layer, which affords protection to the cell from the inhibitory activity of certain antimicrobials *(23)*. Studies have indicated that the predominant cations in the LPS layer of *E. coli* are calcium, magnesium, and sodium *(12, 23, 31)*, but lower concentrations of potassium, iron and aluminum have also been reported *(9, 14, 15, 25)*.

If sequestration of these metal ions is indeed the key to the synergistic activity of chelator-antimicrobial combinations, then addition of compounds reducing the capability of chelators to bind membrane-bound cations may alter the growth inhibition. For

example, the addition of salts used as functional ingredients in foods may result in competitive binding to chelators which may reduce the activity of antimicrobials. The objective of this study was therefore to determine the effect of type and concentration of mono-, di-, and trivalent mineral salts on the antimicrobial effectiveness of lysozyme and EDTA, DSPP or PSTPP combinations against two strains of *E. coli* O157:H7 at pH 6.0, 7.0, and 8.0.

3.3 Materials and Methods

3.3.1 Microorganisms

Escherichia coli O157:H7 strains 932 and H1730 were obtained from Dr. L.R. Beuchat of the University of Georgia, Griffin. All cultures were grown in tryptic soy broth (TSB; Difco, Sparks, MD) and transferred every two weeks to maintain viability. Working cultures were obtained by inoculating a loopful of culture into TSB and incubating for 24 hr at 35°C. After incubation, the cultures were diluted to ca. 4.0 log CFU/mL.

3.3.2 Antimicrobial Preparation

Lysozyme (58,100 units/mg protein; Sigma, St. Louis, MO.) was dissolved in distilled water and passed through a 0.45 µm sterile filter (Corning, Corning, NY). Lysozyme solutions were prepared to contain 600 µg/mL. Disodium ethylenediamine tetraacetic acid (EDTA; Sigma) was dissolved in distilled water and tested at 1500 µg/mL, while aqueous solutions of anhydrous disodium pyrophosphate (DSPP; Sigma) and anhydrous pentasodium tripolyphosphate (PSTPP; Sigma) were evaluated at 15,000 µg/mL. These concentrations were selected because they completely inhibited strains of *E. coli* O157:H7 for 48 hr in an earlier study *(2)*.

3.3.3 Cation Solutions

Solutions were prepared to contain 1.0, 4.0, 7.5 and 10.0 mM of $CaCl_2 \cdot 2\ H_2O$ (Ca^{++}), $MgCl_2 \cdot 2\ H_2O$ (Mg^{++}), NaCl (Na^{+}), KCl (K^{+}), $FeSO_4 \cdot 7\ H_2O$ (Fe^{++}), $FeCl_3 \cdot 6\ H_2O$ (Fe^{+++}) and $AlCl_3 \cdot 6\ H_2O$ (Al^{+++}) (all Sigma). All solutions were prepared and filter sterilized using a 0.45 µm sterile filter (Corning).

3.3.4 Micro-broth Dilution Assay

A checkerboard microassay was used to evaluate the antimicrobial activity of lysozyme-chelator combinations (*2*). Sterile 96-well microtiter plates with a well capacity of 300 µl were used for combination studies. For each test, a total volume of 240 µl was used consisting of 120 µl of inoculum in TSB (ca. 4.0 log CFU/mL) and 40 µl each of lysozyme, chelator and salt solution. Microtiter plates were covered with a sterile lid and incubated 48 hr at 35° C. The absorbance (630 nm) of each well was recorded at 0, 3, 6, 12, 24, 30, 36, and 48 hours with a microtiter plate spectrophotometer (El_x 800 Universal Microplate reader, BioTek Instruments, Winooski, VT). The micro-broth dilution assays were performed in duplicate and each experiment was replicated three times.

3.3.5 Spiral Plate Studies

Flocculation of Fe^{+++} and Al^{+++} in microtiter assays prevented accurate absorbance determinations. Therefore, TSB was prepared to contain the inoculum, lysozyme (600 µg/mL) and EDTA (1500 µg/mL). Fe^{+++} and Al^{+++} were added to 1.0, 4.0, 7.5, and 10.0 mM in the TSB. TSB was incubated at 35°C and samples taken at 0, 6, 12, 24, and 48 hr. *E. coli* O157:H7 was enumerated on tryptic soy agar (TSA; Difco) using a spiral plating system (Don Whitley Scientific Limited, West Yorkshire, UK). Plates were

incubated for 48 hr at 35°C and counts performed using a ProtoCol Plate Counter (Synbiosis; Cambridge, UK). Studies were performed in duplicate.

3.3.6 Isothermal Titration Calorimetry

Binding of salts to EDTA and lysozyme was determined using an Isothermal Titration Microcalorimeter (VP-ITC, Microcal, Northhampton, MA). After degassing, 1.5 ml of 4 mM EDTA or 4 mM EDTA and 0.4 mM lysozyme solutions were loaded into the calorimetric cell using a 2.5 ml syringe previously treated with EDTA to remove residual metal ions and washed with double distilled and demineralized water. Salt solutions at concentrations ranging from 3 mM to 3 M were titrated into the cell with sequential additions of 3 μl at a 150s-holding time between injections. Heat effects associated with dilution of salts was obtained by injection of salts into water and subtracted from the total heat effects. All measurements were conducted at 25°C.

3.4 Results

3.4.1 Inhibition Studies

E. coli O157:H7 strains 932 and H1730 were selected because they were the most resistant and most susceptible to lysozyme-EDTA combinations, respectively, in an earlier study *(2)*. Ca^{++} did not neutralize inhibition by lysozyme-EDTA at pH 6.0 (Fig. 3.1-See Appendix). However, at pH 7.0 and 8.0 the combination was neutralized by ≥ 4.0 mM Ca^{++} for both strains. At pH 6.0, inhibition of 932 by lysozyme-DSPP was eliminated with ≥ 1.0 mM Ca^{++}. For strain H1730, ≥ 4.0 mM Ca^{++} was required under the same conditions. For PSTPP-lysozyme, inhibition of 932 was neutralized by ≥ 4.0 mM Ca^{++} at pH 7.0 and 8.0 while for H1730 neutralization occurred at ≥ 1.0 mM Ca^{++} at pH 7.0 and ≥ 7.5 mM Ca^{++} at pH 8.0.

At pH 6.0, the lysozyme-EDTA combination remained inhibitory to both 932 and H1730 in the presence of all Mg^{++} concentrations tested (Fig. 3.2). At pH 7.0 and 8.0, ≥ 4.0 mM Mg^{++} neutralized inhibition of both strains. At pH 6.0 with DSPP, 1.0 mM Mg^{++} neutralized inhibition of 932 but 10.0 mM did not influence inhibition of H1730. For PSTPP-lysozyme, ≥ 1.0 mM Mg^{++} neutralized inhibition of strains 932 and H1730 at pH 7.0, while ≥ 7.5 mM Mg^{++} was required at pH 8.0.

Neither Na^{+} nor K^{+} had any influence on inhibition of strain 932 by EDTA, DSPP nor PSTPP, except Na^{+} at pH 8.0 (Fig. 3.3). However, Na^{+} and K^{+} neutralized the activity of lysozyme with EDTA, DSPP and PSTPP at 1.0 to 7.5 mM for strain H1730 (Fig. 3.4).

In contrast to the monovalent cations, Fe^{++} was more effective in neutralizing lysozyme-chelator combinations for strain 932 than H1730 (Fig. 3.5). For strain 932, 1.0 mM Fe^{++} partially neutralized EDTA, DSPP and PSTPP at all pH conditions. With H1730, 7.5 mM Fe^{++} was needed to neutralize EDTA and DSPP while only 1.0 mM Fe^{++} was required with PSTPP at pH 7.0 or 8.0.

Flocculation caused by addition of Fe^{+++} and Al^{+++} prevented use of the micro-broth assay because of interference with absorbance readings. Therefore, cells were enumerated using plate counts. Only EDTA was evaluated in this portion of the study. Lysozyme (600 μg/ml) and EDTA (1,500 μg/ml) were bacteriostatic to *E. coli* O157:H7 strains 932 and H1730 for the entire 48 hr incubation. Addition of 1.0 to 7.5 mM Fe^{+++} partially neutralized the inhibitory effect of lysozyme and EDTA at pH 6.0, 7.0 and 8.0, resulting in ca.1.5-2.0 log CFU increase in cell growth compared to the lysozyme-EDTA control (Fig. 3.6). However, addition of 1.0 mM Fe^{+++} to strain 932 at pH 6.0 resulted in inhibition similar to that of the lysozyme-EDTA control. In contrast, at pH 7.0 and 8.0,

1.0 mM Fe^{+++} effectively neutralized the lysozyme-EDTA. With *E. coli* O157:H7 strain H1730, ≥ 4.0-7.5 mM Fe^{+++} at pH 6.0, 7.0, and 8.0 resulted in ca. 1.5 - 2 log CFU increase compared to the lysozyme-EDTA control (Fig. 3.8). In the presence of Al^{+++}, neutralization of the inhibition by lysozyme-EDTA combinations of both strains of *E. coli* O157:H7 was nearly dose dependent (Fig. 3.7 and Fig. 3.9). Partial neutralization was demonstrated at all Al^{+++} concentrations and pHs, except strain 932 with 1.0 and 4.0 mM Al^{+++} at pH 8.0.

3.4.2 Isothermal Titration Calorimetry Studies

In order to further explain the pronounced changes observed in the inhibition of *E.coli* strains upon addition of cations with varying size and valency, isothermal titration calorimetry experiments were conducted using EDTA and lysozyme as the model binding agents. Because of problems with flocculation in the presence of trivalent salts and Fe^{++}, only Na^{+}, K^{+}, Mg^{++} and Ca^{++} were used in the calorimetric experiments. For calculation of molar ratios, EDTA was selected as he reference binding agent in all subsequent plots. Solution enthalpy effects due to dilution of salts in solvent were substracted from all results by titrating salts in water. It should be noted that both exothermic and endothermic heat dilution effects were observed in accordance with tabulated solution enthalpy data (Table 1 *(17)*). Figure 3.10 shows the calorimetric titration of a 3.0 M KCl solution in a 4.0 mM EDTA- 0.4 mM lysozyme complex which is equivalent to the concentration of 1500 μg/ml EDTA and 600 μg/ml lysozyme concentration found in microtiter plate wells. Each injection resulted in binding of K^{+} cations to EDTA-lysozyme combinations which were associated with an exothermic heat effect with enthalpy changes corresponding to the integrated area under the peaks. A plot

of the enthalpy change normalized by the molar concentration of the salt solution versus the molar ratio of K^+ to the EDTA-lysozyme complex illustrates that enthalpy of binding was very low (approx. 9 cal/mol KCl) and saturation occurred only at high molar ratios above 80.

The titration isotherms of K^+, Na^+, Ca^{++} and Mg^{++} in EDTA-lysozyme are shown in Figure 3.11. Isotherms of salt binding to EDTA-lysozyme combinations were fitted to an exponential binding model in the case of monovalent cations and a sigmoidal binding model indicative of the highly cooperativite nature of EDTA-divalent cation binding . Table 3.1 shows the binding enthalpy ΔH_{bin} and the molar ration $R_{m,sat,50\%}$ (i.e. the concentration at which 50% of all receptor molecules (EDTA and lysozyme) were saturated) calculated from the binding models *(17)*. Binding of Ca^{++} and Mg^{++} was associated with significantly higher enthalpy changes (30 kcal/mol $CaCl_2$ and 2.6 kcal/mol $MgCl_2$) and saturation was reached at lower molar ratios (8×10^{-3} and 4×10^{-2}). A slighly higher molar ratio of 50 in the case of K^+ titration in EDTA was required to achieve 50% saturation of pure EDTA solutions compared to the molar ration required to saturate EDTA-lysozyme combinations. Results suggest that a fraction of EDTA molecules may associate with functional groups on the lysozyme molecule to form a complex. A subsequent titration experiment of lysozyme in EDTA solution did confirm EDTA-lysozyme binding, at an $R_{m,sat,50\%}$ of 0.2 (Table 3.1).

3.5 Discussion

Studies performed by Boland and coauthors *(2)* indicated that EDTA, DSPP, and PSTPP were effective in increasing the antimicrobial activity of lysozyme against strains of *E. coli* O157:H7. The objective of the present study was to determine which metal

cations were able to reduce or eliminate the inhibitory activity of lysozyme-chelator complexes against strains of *E. coli* O157:H7. The purpose of the studies was threefold. Firstly, was to better understand which metal ions play an important role in the inhibition of *E. coli* O157:H7 by determining the extent of the reduction of antimicrobial activity upon addition of specific salts. Secondly, we hope to better predict in which type of foods lysozyme and chelators might best function. Lastly, investigation of the interaction of metal cations with chelator-lysozyme combinations may help to develop a model for the mode of action of chelator-lysozyme combinations.

Several factors may influence the ability of metal ions to reduce or eliminate activity of lysozyme-chelator complexes, including: the availability of metal binding sites per chelator, chelator pK_a values, metal-chelator stability constants, cation size and valency, *E. coli* O157:H7 strain type, and localization of cations within the *E. coli* LPS. Earlier experiments indicated that EDTA was ten times as effective in chelating metal ions as DSPP and PSTPP *(2)*. This can be attributed to the number of metal ion binding sites present within the chelator structure. Metal ions generally have a coordination number of six, which EDTA is able to fully satisfy due to the presence of six donor atoms *(18, 19)*. In comparison, DSPP and PSTPP have fewer binding sites, which reduces their ability to chelate metal ions. Accordingly, higher concentrations of cations were needed to overcome the inhibitory action of lysozyme and EDTA.

The extent of chelation of metal ions by EDTA, DSPP, and PSTPP depends on solution pH and is normally greatest when the pH is at or near the pK_a value of the chelator *(19)*. With pK_a values for EDTA, DSPP, and PSTPP being 6.2, 6.0 and 8.1, respectively, this may explain why higher cation concentrations were needed to neutralize

the inhibitory effect of lysozyme-chelator complexes at pH 6, 7 or 8, respectively. As pH values increasingly differed from chelators pK_a, lower concentrations of calcium and magnesium were able to neutralize inhibition by lysozyme and chelators indicating that complexes became more rapidly saturated with metal cations.

Chelators have variable affinities for metal ions. The stability constants of metal-chelator complexes can influence how much salt is needed to reverse inhibition of lysozyme-chelator complexes. The larger the stability constant for a metal-chelator complex, the more stable the complex. For example, sodium and potassium possess a low stability constant of 1.7 with EDTA and generally < 1.0 with phosphates at pH 7.0. Divalent cations, such as calcium and magnesium have higher stability constants and form stronger complexes with EDTA and most phosphates *(19, 20)*. For example, the stability constants at pH 7.0 for EDTA with calcium and magnesium are 10.7 and 8.7, respectively. Although phosphates such as DSPP and PSTPP have also been shown to effectively chelate calcium and magnesium, they possess much smaller stability constants for these divalent cations compared to EDTA *(21, 22)*. Stability constants for DSPP are 5.0 for calcium and 5.7 for magnesium *(19, 20)*. These lower stability constants would allow smaller concentrations of magnesium and calcium, as well as other metal ions, to be effective in saturating the binding capacity of the phosphates.

Generally, as the size of the cation increases, so does the stability constant between the metal and chelator. Studies have shown that EDTA forms stable complexes with Fe^{++} and Fe^{+++} and other trivalent cations such as Al^{+++}. Studies have also indicated that phosphates have high stability constants for the binding of iron *(8, 23)*. The stability constants for EDTA and Fe^{++}, Fe^{+++} and Al^{+++} complexes are 14.3, 25.1 and 16.3,

respectively *(17)*. However, experimental results indicated that smaller concentrations of Fe^{++}, Fe^{+++}, and Al^{+++} were needed to overcome the inhibitory effects of lysozyme and EDTA. Fe^{++} is sufficiently soluble and can easily be transported and acquired by *E. coli* O157:H7. In comparison to Fe^{+++} and Al^{+++}, the smaller binding constant of Fe^{++} to chelators requires greater concentrations to overcome lysozyme-chelator inhibition *(24, 25)*. EDTA chelates Fe^{+++} extremely well; but in the process it may be subject to physicochemical changes that result in the formation of Fe^{+++} hydroxide aggregates that precipitate at pH values between 6 and 10 *(7)*. Many trivalent cations, such as Al^{+++}, are unstable in aqueous solutions and hydroxide formation can result in the flocculation of microtiter assays *(11)*. It could be hypothesized that EDTA's high affinity for Fe^{+++} and Al^{+++} results in quick saturation of the EDTA complex, therefore allowing smaller concentrations of Fe^{+++} and Al^{+++} to overcome lysozyme-EDTA inhibition.

E. coli O157:H7 strain type had an apparent effect on the reduction or elimination of lysozyme-chelator inhibition, which could be partially attributed to the location of the cations in the LPS layer *(12)*. Results would indicate that calcium and magnesium are important components of the LPS layer of both strain 932 and H1730 while sodium and potassium appear to only be a factor in strain H1730 with little or no effect on inhibition of strain 932. The addition of Fe^{++} was more effective in reversing inhibition of strain 932 than strain H1730. However, Fe^{+++} and Al^{+++} were effective in overcoming inhibition of both strains by lysozyme and EDTA. Due to its structural complexity, the LPS layer can be expected to exhibit a certain degree of selectivity with regard to cation composition in different *E. coli* O157:H7 strains. This selectivity may explain why some

metal ions are able to overcome the chelator effect and re-establish their specific functions within the LPS layer *(11)*.

The extent of metal ion chelation from the LPS layer of many Gram-negative bacteria depends on the location of metal ions within the LPS layer. Calcium and magnesium have been suggested to be the principle metal ions extracted by chelators from the LPS layer of *E. coli (3, 8, 12, 14, 18)*. Studies have shown that calcium was the principle metal cation with magnesium playing a smaller role in the stabilization of the LPS layer. magnesium and calcium may complement each other in the cross-linking of negatively charged groups to form salt bridges to bind polysaccharides on the surface of Gram-negative bacteria *(3, 8, 12, 14, 26)*. Leive *(14)* proposed that calcium in *E. coli* was extracted to the greatest extent by EDTA due to it being readily accessibility in the LPS layer. Investigations have also concluded that calcium is important in the stabilization of the LPS into a lipid bilayer conformation *(10, 12, 29*. The chelation of calcium possibly increases the electrostatic repulsion between anionic groups, resulting in destabilization of the LPS layer. Magnesium binds very tightly to LPS-protein complexes therefore reducing its interactions with aqueous environments and accessibility to chelation *(12, 14)*.

Monovalent cations are generally confined to areas that cannot be reached by EDTA chelation, such as the phospholipid domain of the LPS layer *(12, 14, 30)*. Addition of sodium and potassium in the present study were effective in reversing inhibition of strain H1730, but not strain 932. It is possible that sodium and potassium do not play a significant role in strain 932. It has been hypothesized that sodium and potassium aid in the regulation of protein synthesis to balance RNA and other

intracellular activities or assist in the internal regulation of cytoplasmic pH within a cell *(3)*. Therefore it is possible that strain H1730 is dependent on sodium and potassium to stabilize internal cell functions.

EDTA's high affinity for Fe^{++}, Fe^{+++} and Al^{+++} may quickly saturate the complex, allowing remaining ions to interact with the LPS and re-establish their specific function within the LPS layer. Iron is essential for growth, replication, respiration, and DNA synthesis of bacterial cells. Iron is also believed to aid in virulence of some pathogenic Gram-negative bacteria, such as *E. coli* O157:H7 *(24, 31, 33)*.

Isothermal titration calorimetry measurements confirm the high cation specificity of lyzoyme-chelator combinations. Binding of divalent ions Mg^{++} and Ca^{++} is energetically much more favorable compared to binding of Na^{+} and K^{+} (Table 3.1). The sigmoidal concentration dependence of the binding enthalpy indicates a much tighter binding of divalent than monovalent ions. Significantly higher concentrations of monovalent ions are required to saturate the complex. Given a microbial cell surface that contains a variety of monovalent and divalent ions, this would suggest that addition of EDTA would results in selective association with primarily divalent ions. Thus, the functional efficiency of EDTA to improve the antimicrobial activity of lysozyme may be closely related to the ability to selectively remove ions that have shown to be the dominant components in stabilizing the LPS layer i.e. divalent ions. The thermodynamic analysis of lysozyme–EDTA interactions indicated a small degree of binding between the two compounds. Lysozyme consists of 129 amino acids with free amine side groups. At pH 6-8, these free amine groups are predominantly positively charged and could serve as a potential binding site for EDTA.

Based on our results we propose a mechanistic model for the action of lysozyme chelator combinations (Figure 3.12). In the absence of chelators, the LPS layer structure is stabilized by bound cations that prevent physical association of lysozyme with lipoproteins at the site of the catalytic action. Addition of chelators to the system causes association of chelators with bound cations disrupting the structure of the LPS layer. Lysozyme or lysozyme-EDTA combinations are now able to access the site of the catalytic action resulting in further breakdown of not only the membrane layer but the cell wall. At present, there is insufficient data to conclude whether the binding of EDTA to lysozyme is important for the synergistic functionality between EDTA and lysoyme. For example, binding of EDTA-lysozyme may alter electrostatic interactions which could alter transport of lysozyme to the catalytic site. On the other hand, the formation of an EDTA-lysozyme complex reduces the availability of free EDTA to bind cations contained in the LPS layer. Isothermal titration calorimetry of KCl with EDTA compared to titration of KCl to lysozyme/EDTA combination indicated that higher concentrations of ions were required to saturate pure EDTA suggesting that the formation of such a complex is unfavorable because it reduces the intrinsic activity of EDTA. A better understanding may come from investigating changes in surface charge of microorganisms upon addition of chelators in subsequent studies.

3.6 Summary

Calcium and magnesium are important metal ions for the reversal of lysozyme-chelator inhibition of *E. coli* O157:H7 strains 932 and H1730. These divalent cations probably play an integral role in the stability and structure of the LPS layer. Additionally, iron is effective in neutralizing lysozyme-chelator inhibition, probably because of its

wide range of functions and binding constants to chelators. However, the role of sodium and potassium is variable and strain dependent.

These experiments are beneficial in predicting to which food products lysozyme-chelator combinations may be successfully applied. For example, antimicrobial-chelator combinations against *E. coli* O157:H7 probably would not be effective in whole pasteurized milk due to the high concentration of calcium (118 g calcium/ 100 g whole milk) (*30*). However, for a product such as apple juice that contains little calcium (6.0 g calcium / 100 g apple juice) (*32*), the lysozyme-chelator combination would possibly be an effective tool for inhibiting Gram-negative pathogens, such as *E. coli* O157:H7. Therefore, for antimicrobial and chelator combinations, it is important to analyze the metal ion content of food products to which these combinations will be used. Metal ions will play a role in how effective these combinations are against Gram-negative pathogens.

References

1. Anderegg, G. 1975. Critical Survey of Stability Constants of EDTA Complexes, Part A: Stability Constants of Metal Complexes. Pergamon Press, Elmsford, New York.

2. Barry, A.L. 1976. The Antimicrobic Susceptibility Test: Principle and Practices. Lea and Febiger, Philadelphia.

3. Beveridge, T.J. and Koval, S.F. 1981. Binding of metals to cell envelopes of *Escherichia coli* K-12. Appl. Environ. Microbiol. 42: 325-335.

4. Boland, J.S., P.M. Davidson, and J. Weiss. 2003. Enhanced Inhibition of *Escherichia coli* O157:H7 with lysozyme and chelators. J. Food Prot. In Press.

5. Boziaris, I.S. and M.R. Adams. 1999. Effect of chelators and nisin produced in situ on inhibition and inactivation of Gram-negatives. Intl. J. of Food Microbiol. 53: 105-113.

6. Braun, V. and H. Killman. 1999. Bacterial solutions to the iron supply problem. Trends in Biochem. Sci. 24: 104-109.

7. Busheli-Witschel, M. and T. Egli. 2001. Environmental fate and microbial degradation of aminopolycarboxylic acids. FEMS Microbiol. Rev. 25: 69-106.

8. Bullen, J.J. and E. Griffiths. 1999. Iron and Infection. 2nd edition. John Wiley and Sons, New York.

9. Coughlin, R.T., S. Tonsager and E.J. McGroarty. 1983. Quantitation of metal cations bound to membranes and extracted lipopolysaccharide from *Escherichia coli*. Biochem. 22: 2002-2006.

10. Cowart, R.E. 2002. Reduction of iron by extracellular iron reductases: implications formicrobial iron acquisition. Arch. Biochem. Biophys. 400: 273-281.

11. Cutter, C.N. and G.R. Siragusa. 1995. Population reductions of Gram-negative pathogens following treatments with nisin and chelators under various conditions. J. Food Prot. 58: 977-983.

12. Elliason, D.J. and S.R. Tatini. 1999. Enhanced inactivation of *Salmonella typhymurium* and verotoxigenic *Escherichia coli* by nisin at 65° C. Food Microbiol. 16: 257-267.

13. Furia, T.E. 1968. Sequestrants in Food. In: Handbook of Food Additives. The Chemical Rubber Company, Cleveland, OH.

14. Ferris, F.G. 1989. Metallic Ion Interactions with the Outer Membrane of Gram-negative bacteria. In: Metal Ions and Bacteria. John Wiley and Sons, New York.

15. Ferris, F.G. and T.J. Beveridge. 1986. Physiocochemcial roles of soluble metal cations in the outer membrane of *Escherichia coli* K-12. Can. J. Microbiology. 32: 594-605.

16. Gray, G.W. and S.G. Wilkinson. 1965. The action of ethylenediaminetetracetic acid on *Pseudomonas aeruginosa*. J. Appl. Microbiol. 28: 153-164.

17. Griko, Y.V. 1999. Energetics of Ca^{2+}-EDTA interactions: calorimetric study. Biophys. Chem. 79: 117-127.

18. Hoyle B. and T.J. Beveridge. 1983. Binding of metallic ions to the outer membrane of *Escherichia coli*. Appl. Environ. Microbiol. 46: 749-760.

19. Jeffrey, G.H., J. Basset, J. Medham, and R.C. Denny (Eds.), 1989. Vogel's Textbook of Quantitative Chemical Analysis, 5th edition. Longman, London.

20. Jen, C.M.C. and L.A. Shelef. 1986. Factors affecting the sensitivity of *Staphylococcusaureus* 196E to polyphosphates. Appl. Environ. Microbiol. 52: 842-846.

21. Klaassen, C.D. 1980. Absorption, distribution, and excretion of toxicants. In: Toxicology: The Basic Science of Poisons, 2nd edition. Macmillian, New York.

22. Knabel, S.J., H.W. Walker, and P.A. Hartman. 1991. Inhibition of *Aspergillus flavus* and selected Gram-positive bacteria by chelation of essential metal cations by polyphosphates. J. Food Prot. 54: 360-365.

23. Lee, R.M., P.A. Hartman, H.M. Stahr, D.G. Olson and F.D. Williams. 1994. Antibacterial mechanism of long-chained polyphosphates in *Staphylococcus aureus*. J. Food Prot. 57: 289-294.

24. Leive, L. 1968. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetic acid. J. Biol. Chem. 243: 2373-2375.

25. Leive, L. 1974. The barrier function of the Gram-negative envelope. Ann. NY. Acad. Science. 235: 109-133.

26. Linde, D.R. 1999. CRC Handbook of Chemistry and Physics. Boca Raton, CRC Press.

27. Lugtenberg, D. and L. van Alphen. 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other Gram-negative bacteria. Biochim. Biophys. Acta. 737: 51-75.

28. Moustafa, H.H. and E.B. Collins. 1969. Effects of selected food additives on growth of *Pseudomonas fragi*. J. Dairy Sci. 52: 335-339.

29. Nelson, K.A., F.F. Busta, J.N. Sofos and M.K. Wagner. 1983. Effect of polyphosphates in combination with nitrite-sorbate or sorbate on *Clostridium botunilinum* growth and toxin production in chicken frankfurter emulsions. J. Food Prot. 46: 846-850.

30. Payne, K.D., P.M. Davidson, S.P. Oliver, and G.L. Christen. 1990. Influence of bovine lactoferrin on the growth of *Listeria monocytogenes*. J. Food Prot. 53: 468-472.

31. Vaara, M. 1992. Agents that increase the permeability of the outer membrane. Microbiol. Rev. 56: 395-411.

32. Watt, B.K. and A.L. Merrill. 1963. Composition of Foods. In: Agriculture Handbook Number Eight. United States Department of Agriculture, Washington, D.C.

33. Welch, K.D., T.Z. Davis and S.D. Aust. 2002. Iron Autoxidation and Free Radical Generation: Effects of Buffers, Ligands, and Chelators. Arch. Biochem. Biophys. 397: 360-369.

Appendix

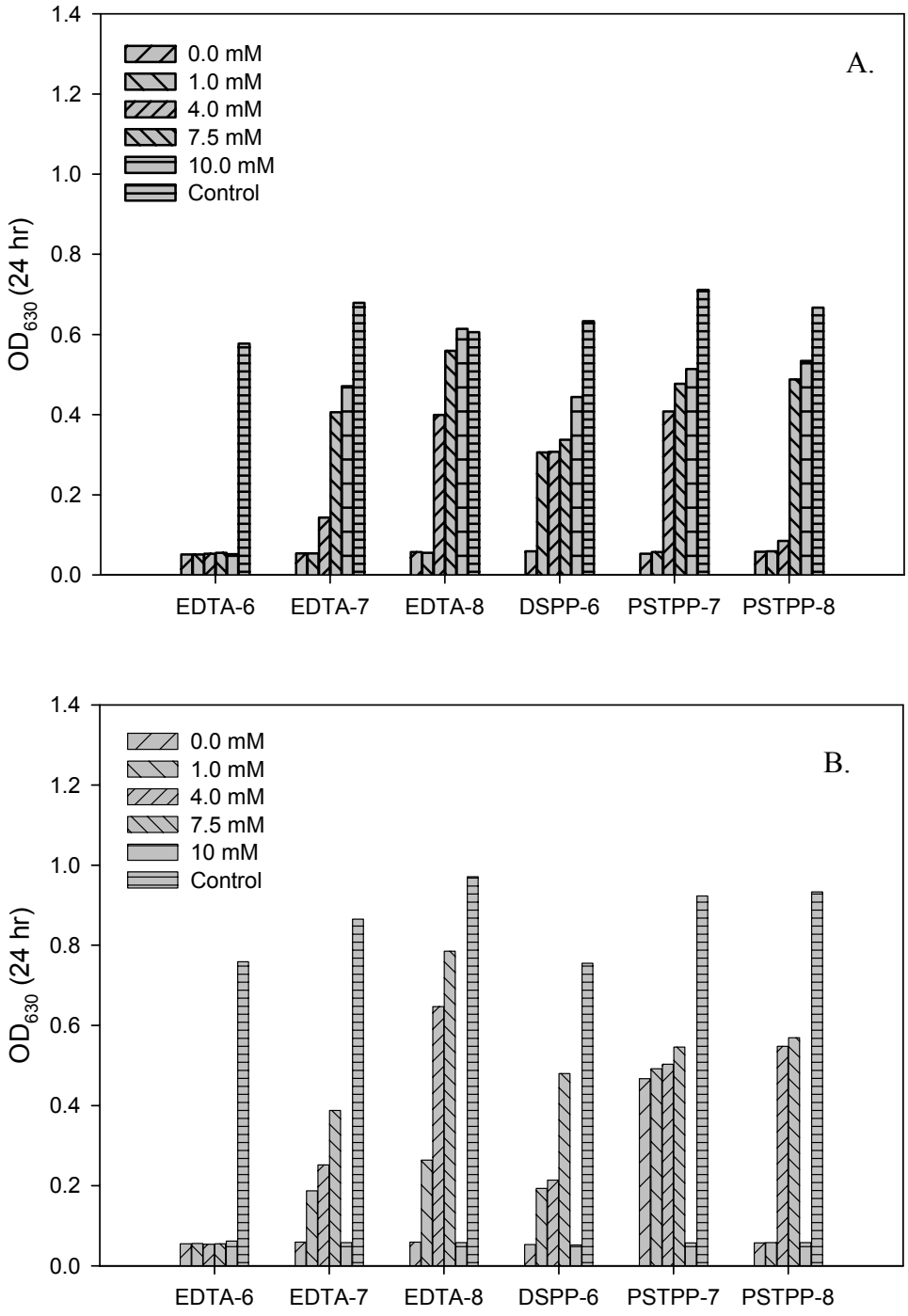


Figure 3.1: Reverse Inhibition of *E. coli* O157:H7 strains 932 and H1730 as measured by OD_{630} in the presence of combinations of lysozyme (600 µg/ml) and EDTA (1,500 µg/ml) or DSPP (15,000 µg/ml) or PSTPP (15,000 µg/ml) calcium chloride at pH 6.0, 7.0, and 8.0 after 24 hr. (A) Strain 932 (B) Strain H1730.

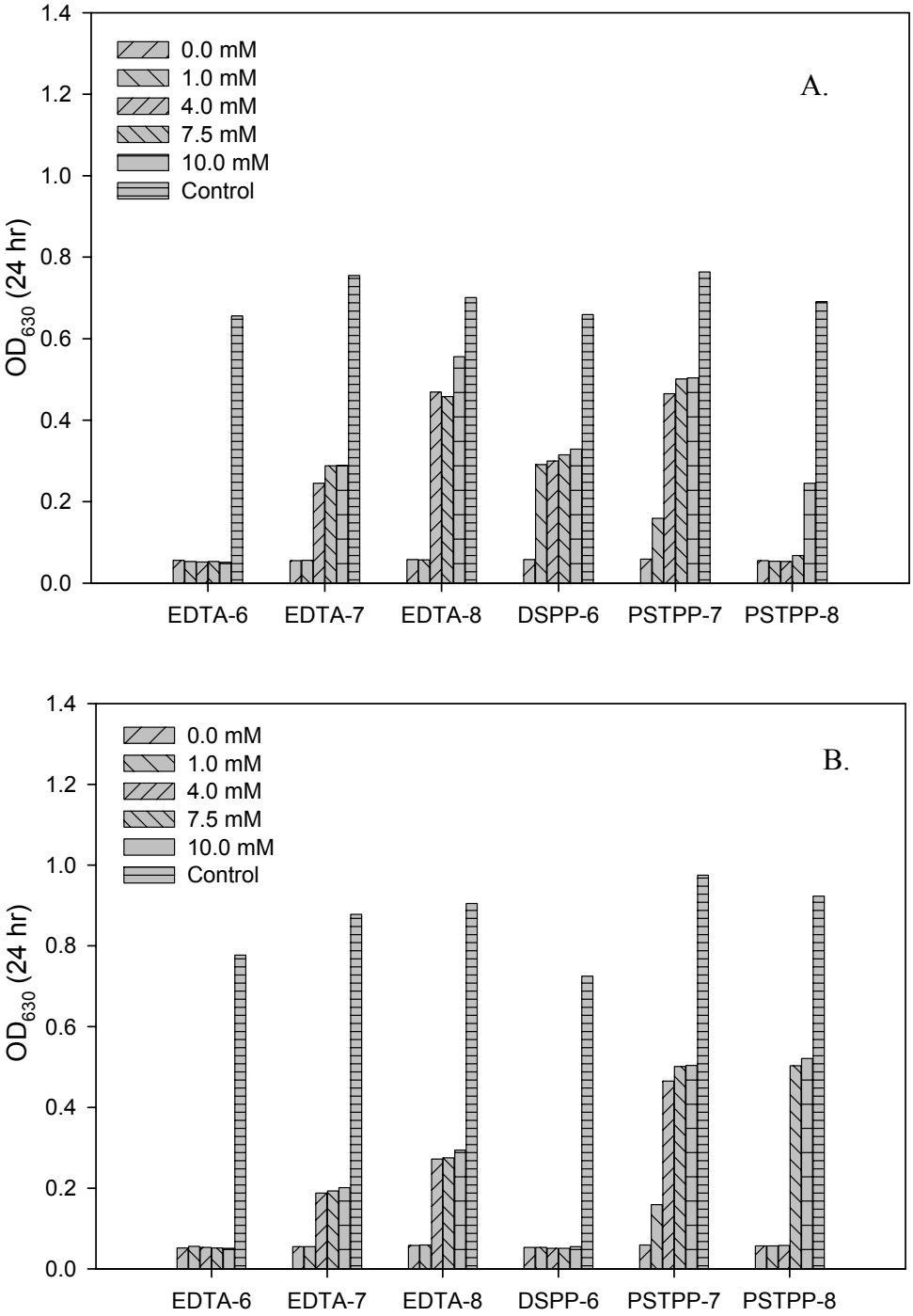


Figure 3.2: Reverse inhibition of *E. coli* O157:H7 strains 932 and H1730 as measured by OD_{630} in the presence of combinations of lysozyme (600 µg/ml) and EDTA (1,500 µg/ml) or DSPP (15,000 µg/ml) or PSTPP (15,000 µg/ml) and magnesium chloride at pH 6.0, 7.0, and 8.0 after 24 hr. (A) Strain 932 (B) Strain H1730.

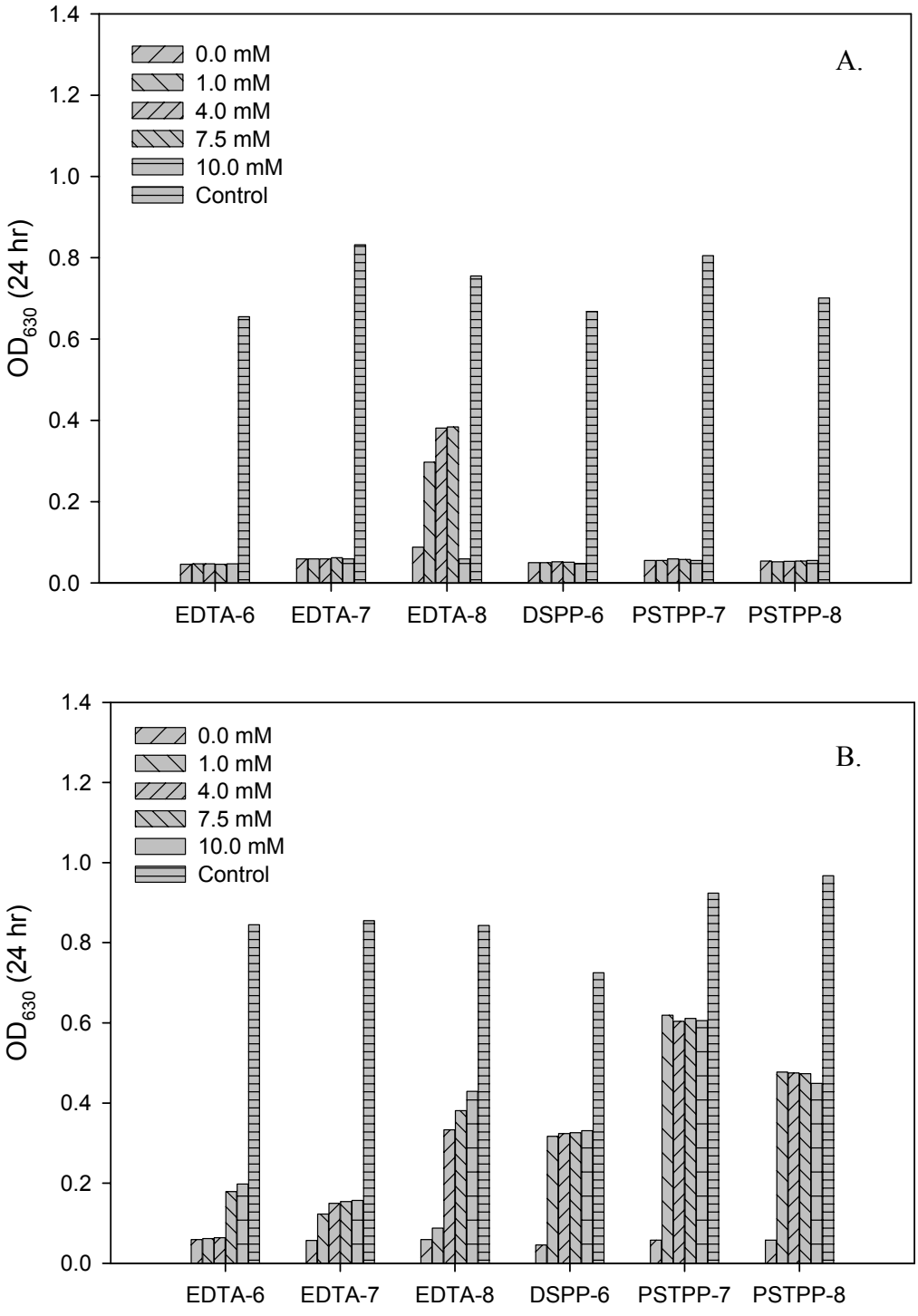


Figure 3.3: Reverse inhibition of *E. coli* O157:H7 strains 932 and H1730 as measured by OD_{630} in the presence of lysozyme (600 µg/ml) and EDTA (1,500 µg/ml) or DSPP (15,000 µg/ml) or PSTPP (15,000 µg/ml) and sodium chloride at pH 6.0, 7.0, and 8.0 after 24 hr. (A) Strain 932 (B) Strain H1730

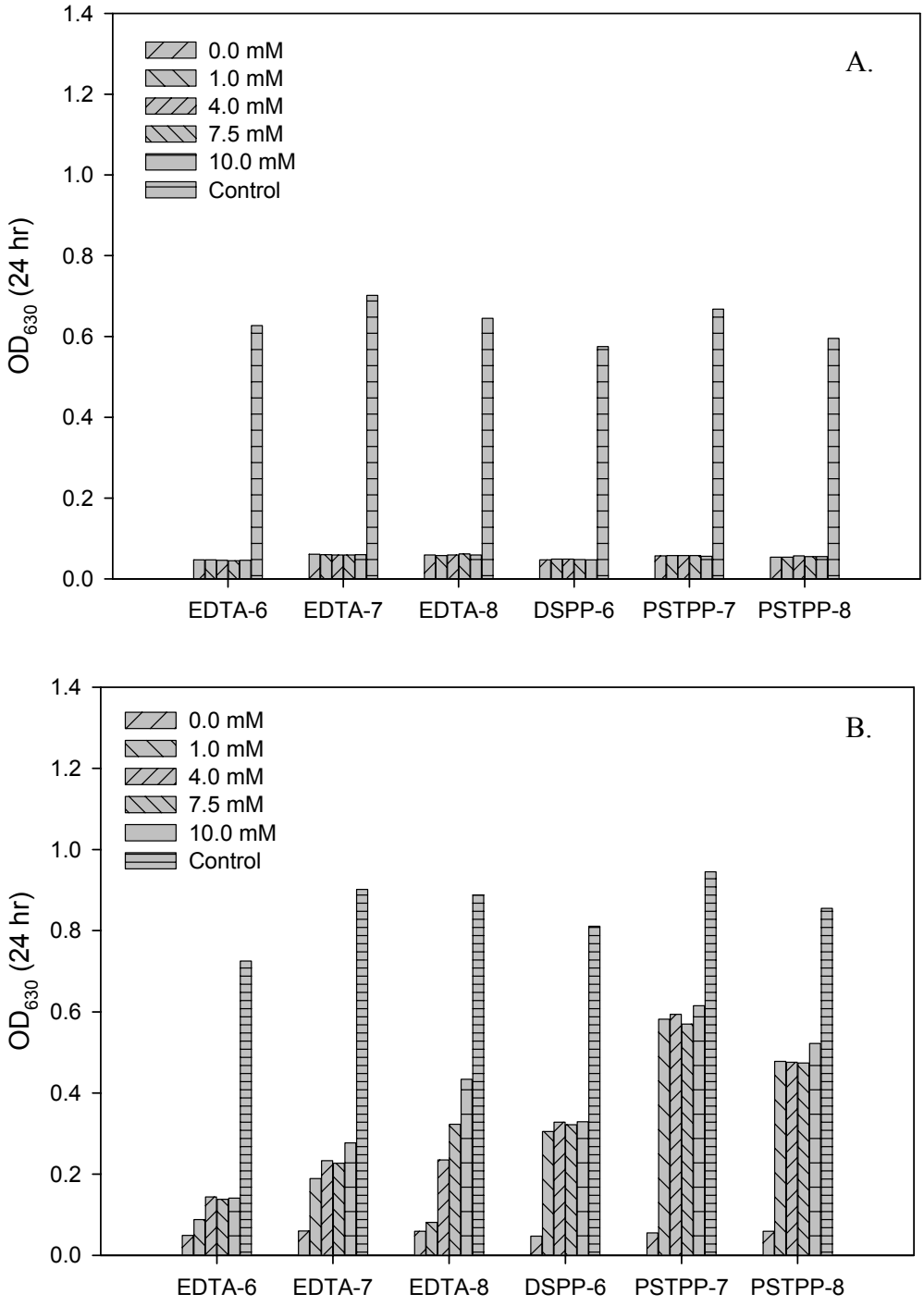


Figure 3.4: Reverse inhibition of *E. coli* O157:H7 strains 932 and H1730 as measured by OD_{630} in the presence of combinations of lysozyme (600 µg/ml) and EDTA (1,500 µg/ml) or DSPP (15,000 µg/ml) or PSTPP (15,000 µg/mL) and potassium chloride at pH 6.0, 7.0, and 8.0 after 24 hr. (A) Strain 932 (B) Strain H1730

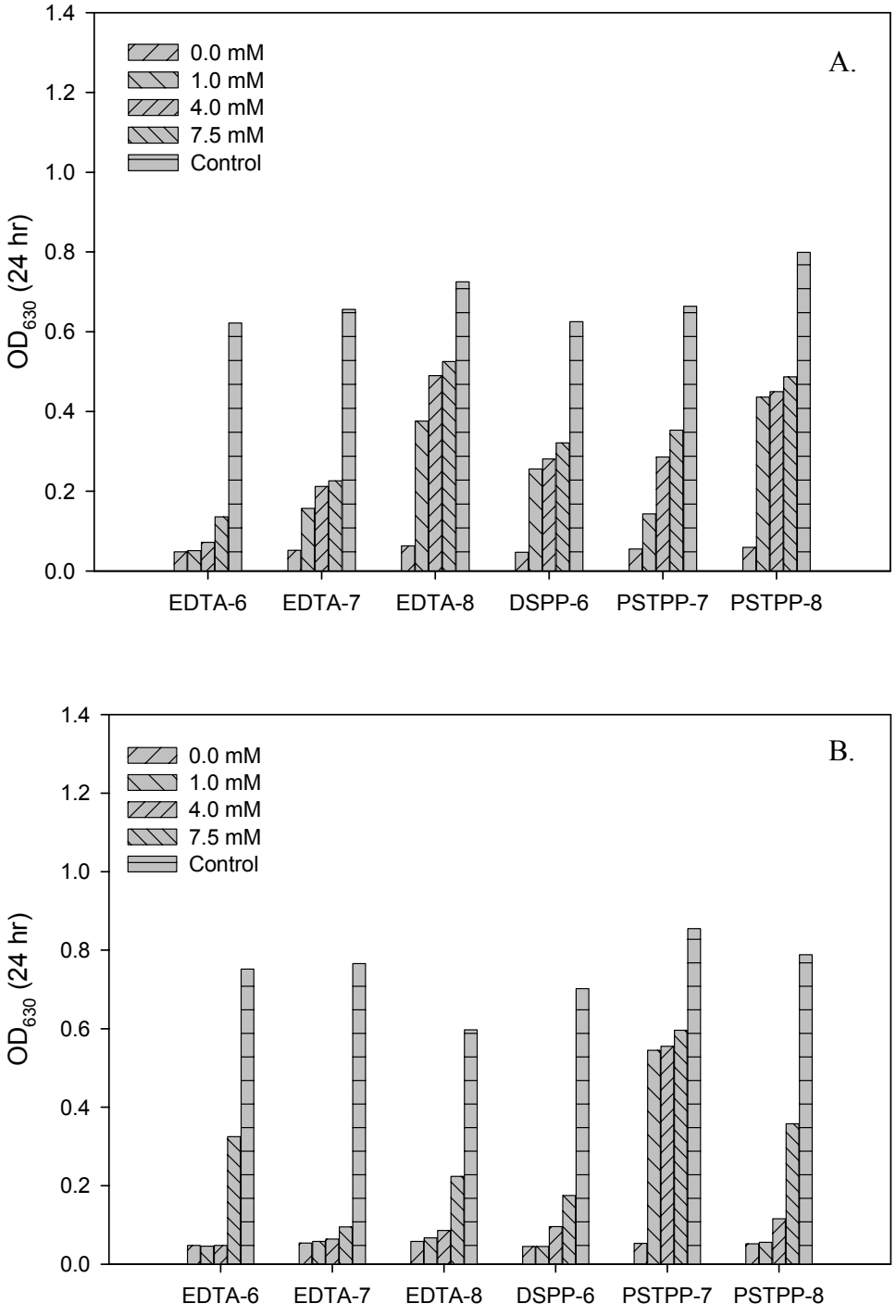
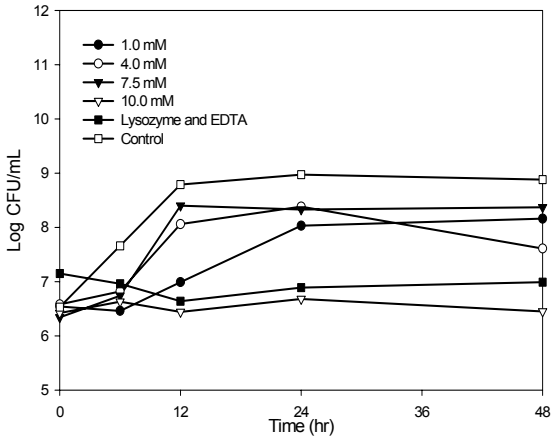


Figure 3.5: Reverse inhibition of *E. coli* O157:H7 strains 932 and H1730 as measured by OD_{630} in the presence of combinations of lysozyme (600 µg/ml) and EDTA (1,500 µg/ml) or DSPP (15,000 µg/ml) or PSTPP (15,000 µg/ml) and ferrous sulfate at pH 6.0, 7.0, and 8.0 after 24 hr. (A) Strain 932 (B) Strain H1730

A.



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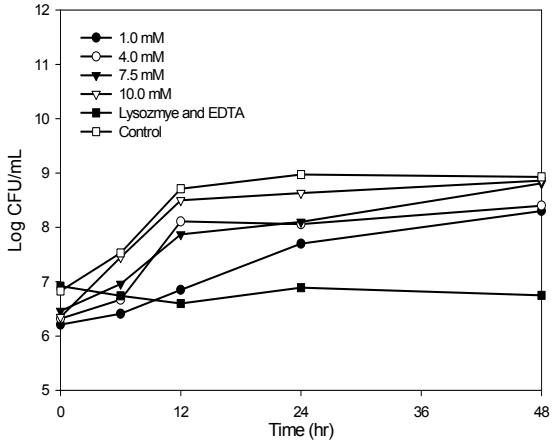

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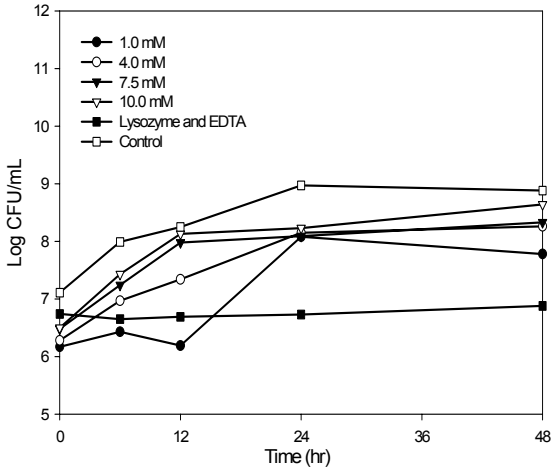
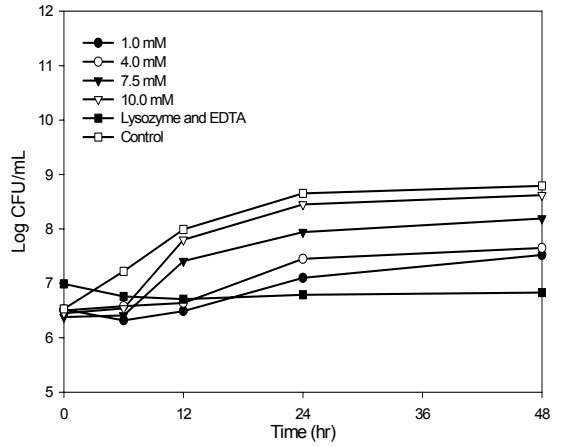


Figure 3.6: Reverse inhibition of *E. coli* O157:H7 strain 932 performed with standard plate counts in the presence of combinations of lysozyme (600 ug/ml), EDTA (1,500 µg/ml), and ferric chloride at pH 6.0, 7.0, and 8.0 with plate counts taken at 0, 6, 12, 24, and 48 hr. (A) ferric chloride, EDTA at pH 6.0 (B) ferric chloride, EDTA at pH 7.0 (C) ferric chloride, EDTA at pH 8.0.

A.



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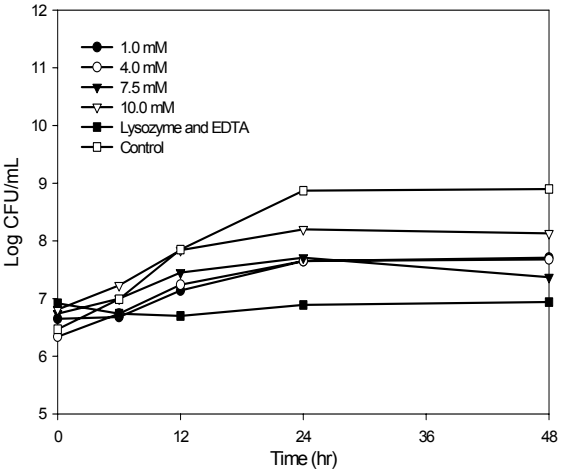


C.

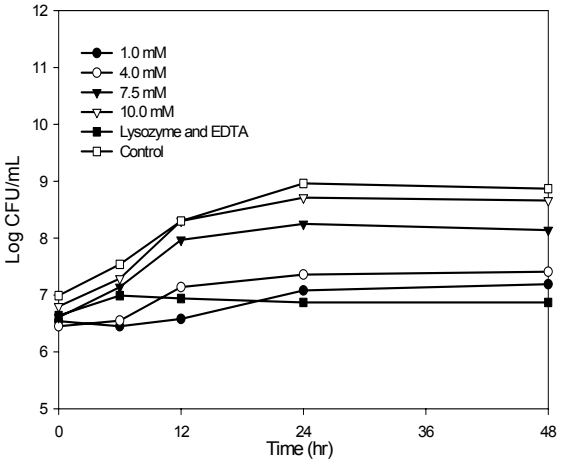
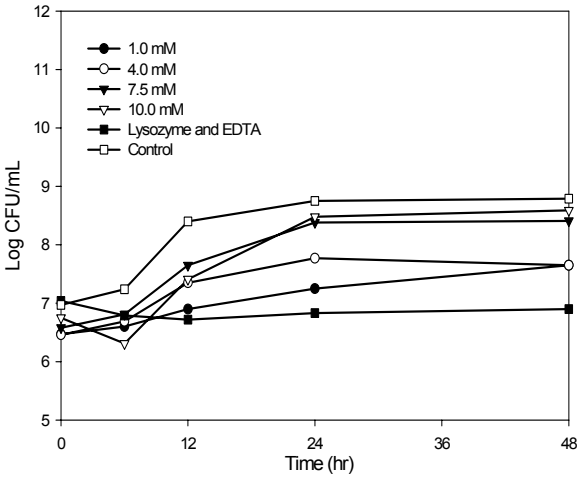


Figure 3.7: Reverse inhibition of *E. coli* O157:H7 strain 932 as performed with standard plate counts in the presence of combinations of lysozyme (600 µg/ml), EDTA (1,500 µg/ml), and aluminum chloride at pH 6.0, 7.0, and 8.0 with plate counts taken every 0, 6, 12, 24, and 48 hr. (A) aluminum chloride, EDTA at pH 6.0 (B) aluminum chloride, EDTA at pH 7.0 (C) aluminum chloride, EDTA at pH 8.0.

A.



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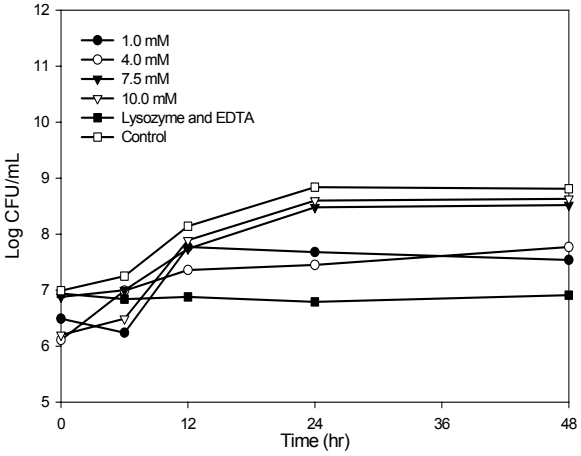

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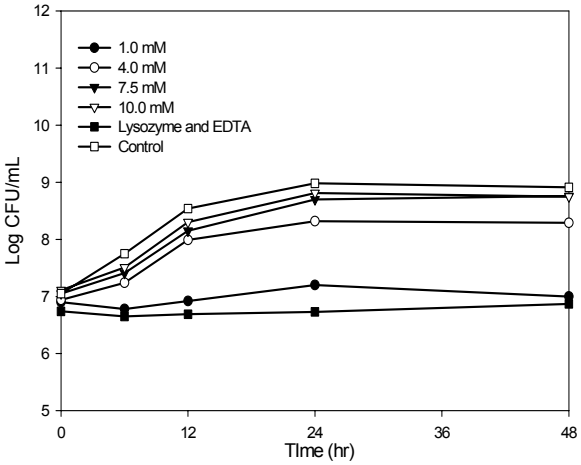
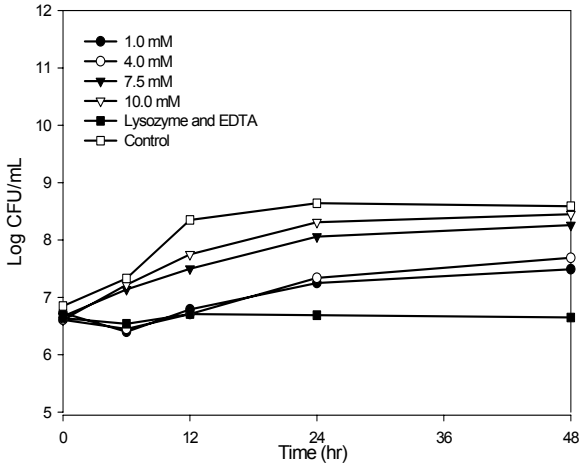


Figure 3.8: Reverse inhibition of *E. coli* O157:H7 strain H1730 as performed with standard plate counts in the presence of combinations of lysozyme (600 µg/ml) and EDTA (1,500 µg/ml), EDTA (1,500 µg/ml), and ferric chloride at pH 6.0, 7.0, and 8.0 with plate counts taken at 0, 6, 12, 24, 48 hr. (A) ferric chloride, EDTA at pH 6.0 (B) ferric chloride, EDTA at pH 7.0 (C) ferric chloride, EDTA at pH 8.0.

A.



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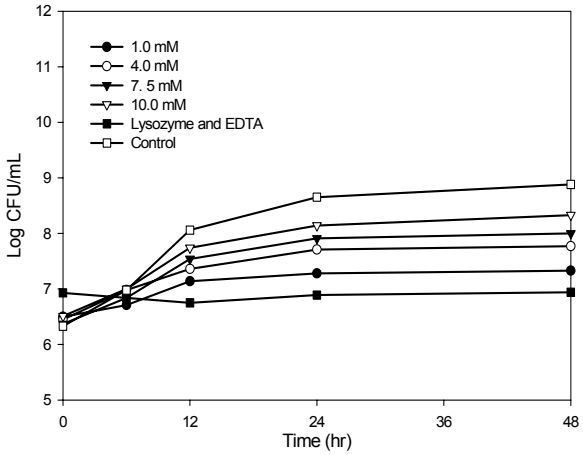

C.

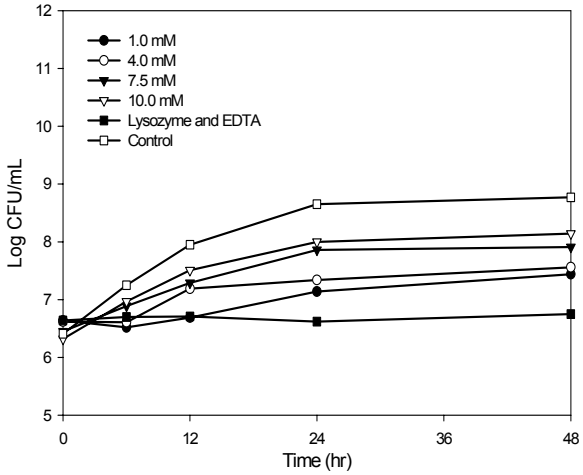

Figure 3.9: Reverse inhibition of *E. coli* O157:H7 strain H1730 as performed with standard plate counts in the presence of lysozyme (600 µg/ml), EDTA (1,500 µg/ml), and aluminum chloride at pH 6.0, 7.0, and 8.0 with plate counts taken at 0, 6, 12, 24, and 48 hr. (A) aluminum chloride, EDTA at pH 6.0 (B) aluminum chloride, EDTA at pH 7.0 (C) aluminum chloride at pH 8.0.

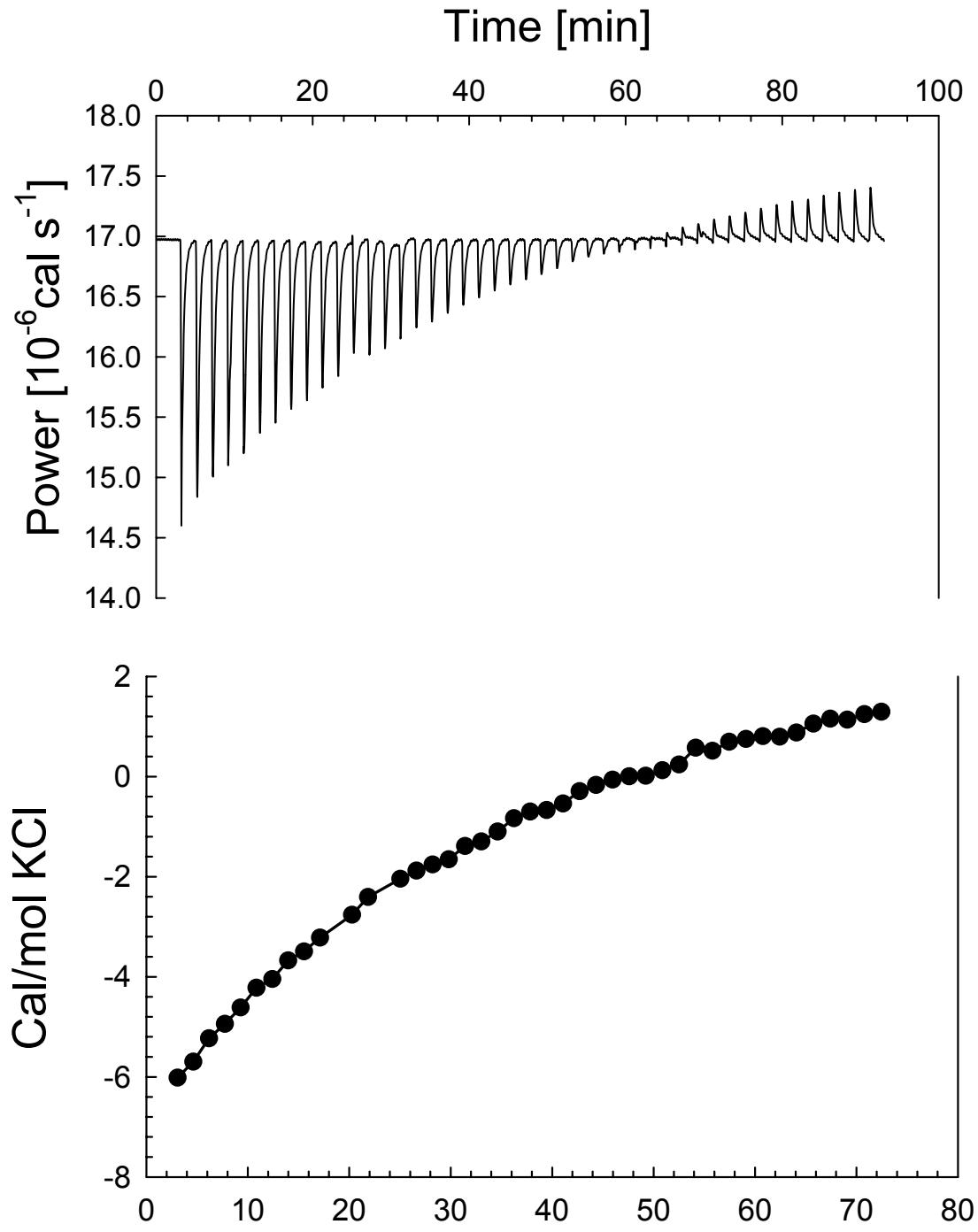

Figure 3.10: Calorimetric titration isotherm of the binding interaction between K^+ and lysozyme-EDTA combinations at 25°C. Forty 3-µl volume of 3 M KCl were injected in the calorimeter cell containing 0.4 mM lysozyme and 4mM EDTA (A) Calorimetric response (power in mcals) as a function of time (B) Integrated calorimetric response per injection normalized per one mole of KCl injected versus the molar ratio in the cell after injection using EDTA as a reference.

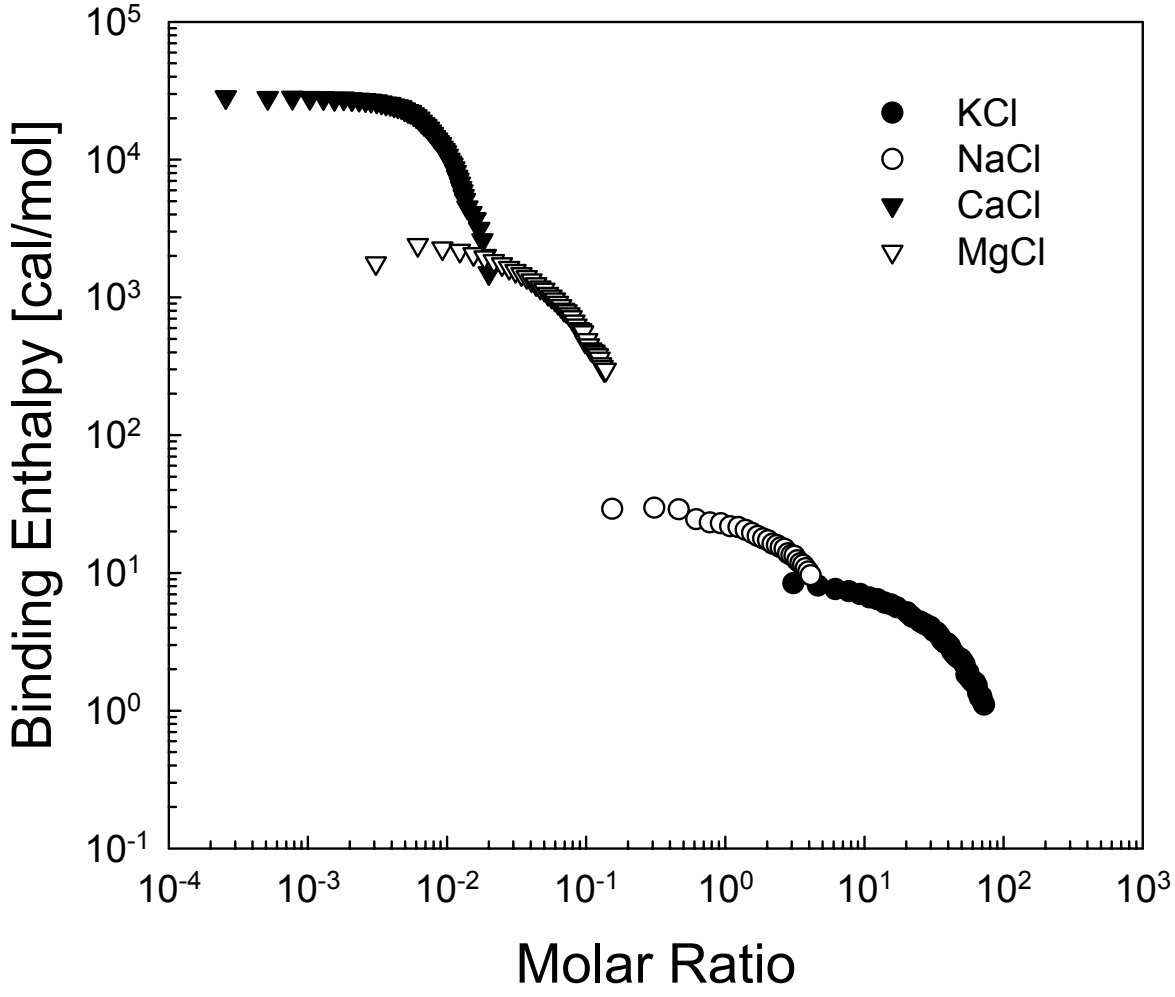

Figure 3.11: Calorimetric titration isotherm of the binding interaction between K^+, Na^+, Mg^{++}, Ca^{++} and lysozyme-EDTA combinations.

Table 3.1: Binding enthalphy ΔH, molar ratio of 50% complex saturation $R_{m,sat,50\%}$ of Na^{+}, K^{+}, Ca^{++}, Mg^{++} binding to EDTA, lysozyme and EDTA-lysozyme combination in water at 25°C.

Salt	ΔH_{bin} [kcal mol^{-1}]*	$R_{m,sat,50\%}$ [-]
K^{++} in comb.	9×10^{-3}	20
K^{++} in EDTA	12×10^{-3}	25
NaCl in comb.	4×10^{-2}	1.2
$MgCl_2$ in comb.	2.6	4×10^{-2}
$CaCl_2$ in comb.	30	8×10^{-3}
EDTA in lysozyme	4.1	0.2

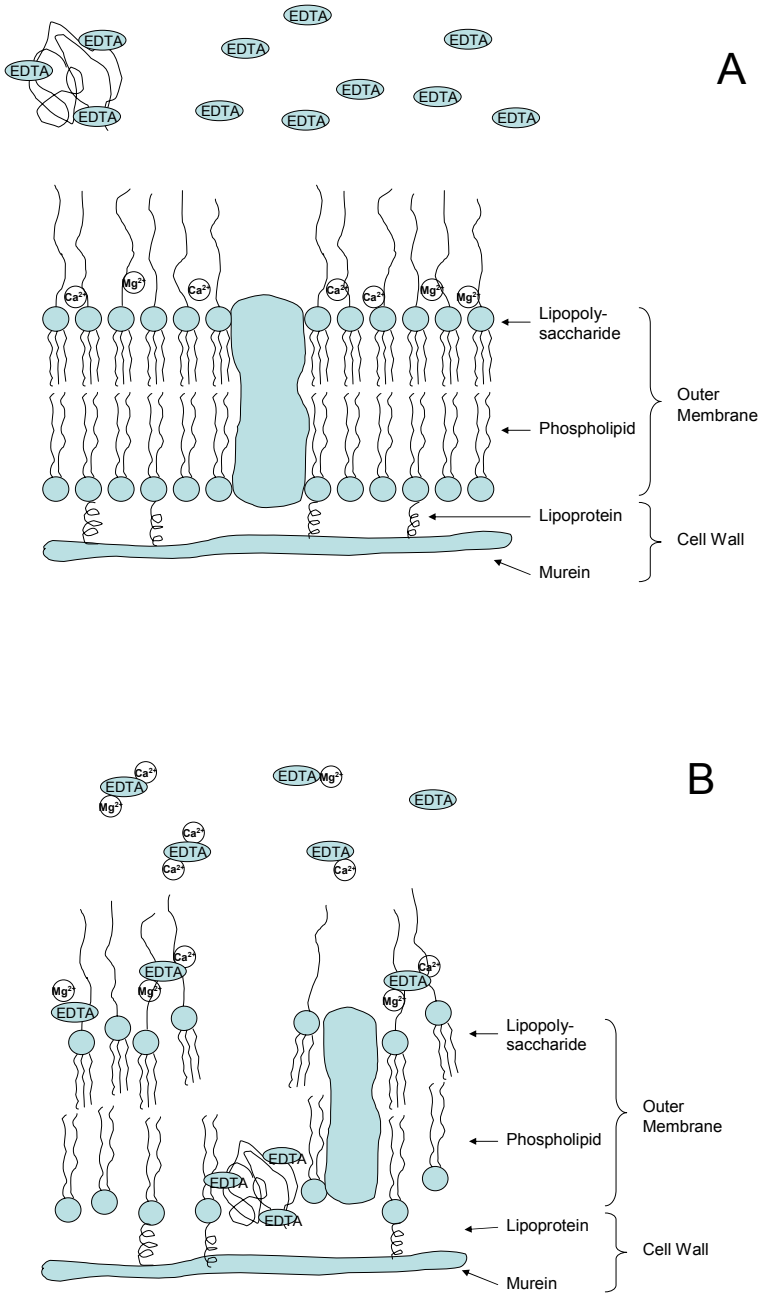

Figure 3.12: Schematic illustration of destabilization mechanism of chelator-lysozyme combinations (A) Influence of lysozyme-EDTA on cell membrane (B) Destabilization of cell membrane by lysozyme-EDTA

Chapter 4:

Impact of Lysozyme-EDTA Combinations on Surface Charge Properties of *Escherichia coli* O157:H7

4.1 Abstract

This study examined the influence of pH and lysozyme-EDTA treatments on the zeta potential of *Escherichia coli* O157:H7 strain 932. It has been accepted that inactivation of Gram-negative bacteria by antimicrobials-chelators combinations require sequesteration of cell membrane cations to allow the mass transport of lysozyme to the cell interior. However, little information is provided on the impact of lysozyme and EDTA on bacterial surface charge. Zeta potential was measured in emulsified systems for lysozyme. The change in *E. coli* O157:H7 surface charge upon addition of lysozyme-EDTA combinations were additionally measured. Over a pH range of 5-13, zeta potential for lysozyme emulsions ranged from 61.26 to – 42.14 mV. The addition of EDTA reduced the zeta potential of the lysozyme emulsion, with values ranging from 38.13 at pH 5.0 to – 33.45 mV at pH 13.0. Native *E. coli* O157:H7 strain 932 surfaces were negatively charged over a pH range of 5-13 with values from – 11.67 to – 36.95 mV. EDTA lowered the zeta potential of *E. coli* O157:H7. EDTA reduced zeta potential values from – 1.28 to – 27.47 mV for pH 5 and 13. Addition of lysozyme-EDTA combinations reduced zeta potential from – 3.76 to – 32.85 mV. Results were used to develop an antimicrobial-microorganism mechanistic model based on electrostatic interactions.

4.2 Introduction

The interfacial physiology of bacteria is an important characteristic with regard to the adaption and growth of bacteria in different environments. Parameters, such as electrostatic charge, influence the overall polarity of the bacterial cell wall and assist in the degree of hydrophobicity necessary for optimal cell functionality (*1*, *12*). Beveridge

and Graham (1991), emphasized the role of bacterial cell surfaces in envelope diffusion, cell growth and division and turgor support. Additionally, the cell wall provides protection against chemical and physical factors, including non-optimal environmental factors, fluctuations in temperatures, and antimicrobials (*12*).

Almost all solid and liquid particulate or macroscopic materials in contact with a liquid carry an electronic surface charge. The electrostatic charge of bacterial cells cannot be easily determined directly (*6*, *12*). Several methods have been used to measure the electrostatic properties of bacterial cell surfaces. Measurements of zeta potential is one method that can be used to indirectly assess net cell surface charge. Zeta potential is the electrostatic interaction potential of the interfacial region between the outer cell membrane and the aqueous environment at the plane of shear. It can be calculated from the cellular electrophoretic mobility in an electric field. (*7*, *8*, *12*).

At physiological pH conditions, Gram-negative bacteria possess a negative electrostatic surface charge. The negatively charge surface charge acts as a barrier to antimicrobials due to the electrostatic repulsion between the antimicrobial and the negatively charged bacterial surface (*4*, *12*). Negatively charged polypeptide antimicrobials, such as lysozyme, have little to no effect on Gram-negative bacteria because of the protection provide to the cells by the outer cell membrane (*2*). Chelating compounds can function synergistically with lysozyme to inhibit Gram-negative bacteria by a mechanism that likely involves the chelation of membrane cations. It has been hypothesized that chelating compounds, such as EDTA, reduce the electronegativity of the cell surface, therefore destabilizing the cell membrane and allowing penetration of

lysozyme to the cell interior (*2, 3*, *10*). However, no direct measurements have been reported to date.

The objective of this study was to determine the influence of pH and lysozyme-EDTA combinations on *E. coli* O157:H7 zeta potential. Results were used to develop a mechanistic model that may explain lysozyme-EDTA inactivation of *E. coli* O157:H7 strains.

4.3 Materials and Methods

4.3.1 Microorganism

Escherichia coli O157:H7 strain 932 was obtained from Dr. L.R. Beuchat of the University of Georgia, Griffin. The cultures were grown in tryptic soy broth (TSB; Difco, Sparks, MD) and transferred every two weeks to maintain viability. Working cultures were obtained by inoculating a loopful of culture into TSB and incubating for 24 hr at 35°C. After incubation, the cultures were diluted to ca. 4.0 log CFU/mL.

4.3.2 Lysozyme and EDTA Preparation

Lysozyme (58,100 units/mg protein; Sigma, St. Louis, MO.) was dissolved in 200 mL distilled water to create a four-percent weight-to-volume solution. Ten milliliters (mL) of n-hexadecane (Sigma) was added to 190 mL lysozyme solution. The obtained emulsion was sonicated at 75 Watts (Misonix Sonicator 3000, Misonix, Inc., Farmington, NY) for two minutes. Disodium EDTA (Sigma) was prepared and dissolved in distilled water to obtain 1500 μg/mL. After preparation of the lysozyme emulsion, EDTA solution was additionally added to the emulsion and stirred for 10 minutes.

4.3.3 Zeta Potential Analysis

Samples were analyzed using a ZetaPlus zeta potential analyzer (Brookhaven Instrument Co, Holtsville, NY). Lysozyme emulsion (0.1 g) and lysozyme-EDTA emulsions were dispersed into 25.0 g of distilled water and placed into a cuvette for analysis. The zeta potential of *E. coli* O157:H7 strain 932 was examined in an aqueous environment by diluting 100 µl of strain inoculum with distilled water to obtain particle concentrations suitable for electrophoretic measurements. The zeta potential of lysozyme and EDTA on strain 932 was measured by adding two milliliters of inoculum to 40 g distilled water containing 600 ppm lysozyme and 1500 ppm EDTA. The pH of bacterial cell suspensions was adjusted between pH 5.0 and 13.0. All zeta potential measurements were performed in triplicate and each experiment was replicated three times at 22°C.

4.4 Results

The zeta potential was recorded as a function of pH for a lysozyme emulsion (Fig 4.1-See Appendix). As pH increased from 5.0 to 13.0, the zeta potential of lysozyme decreased from 61.26 mV to – 42.14 mV and recorded a value of zero at or near pH 11.0. The addition of EDTA to the lysozyme emulsion is additionally shown in Fig. 4.1. In contrast, the addition of EDTA to the emulsion effectively lowered the zeta potential of lysozyme. Zeta potential values of 38.13 mV at pH 5.0 and – 33.45 mV at pH 13.0 were recorded.

The zeta potential of *E. coli* O157:H7 strain 932 was measured as a function of pH and in the presence of EDTA and lysozyme-EDTA combinations. A negative zeta potential was obtained between pH 5.0 and 13.0 with strain 932 (Fig. 4.2). A gradual decrease occurred with values ranging from – 11.67 mV at pH 5.0 to – 36.95 at pH 13.0.

Strain 932 was exposed to treatments of EDTA. The addition of EDTA considerably lowered the zeta potential of strain 932, ranging from – 1.24 mV at pH 5.0 to – 27.47 mV at pH 13.0. Treatments of lysozyme-EDTA combinations were added to strain 932. The lysozyme-EDTA combination reduced the overall zeta potential for strain 932. A value of – 3.76 mV was obtained at pH 5.0, before gradually decreasing to – 32.85 mV at pH 13.0. Although, lysozyme and EDTA reduced the overall zeta potential of strain 932, a greater zeta potential reduction was obtained with the addition of EDTA to the strain.

4.5 Discussion

Zeta potential measurements were performed with pure lysozyme emulsion. As the pH of a system increases, a decrease in zeta potential occurred with lysozyme (*9*). The isoelectric point of lysozyme is generally between pH 10.7-10.8 (*9*, *11*). As the pH approached pH 11.0, the decrease in lysozyme zeta potential slowed, before dropping off sharply after pH 11.0. Due to close vicinity of the isoelectric point, a neutral to slightly negative charge was present at pH 11.0.

The determination of bacterial surface charge properties is important to understand cell function and behavior in various environmental conditions. The negative charge for most bacteria can be attributed to the ionization of carboxylate, phosphate and amino groups found in the cell membrane and can be expected to change as environmental pH fluctuates (*5*, *12*). For *E. coli*, the presence of phosphoryl and 2-keto-3-deoxyoctonate carboxylate groups in the lipopolysaccharide layer are responsible for the negative surface charge, therefore possibly explaining why negative zeta potentials were obtained for strain 932 (*12*).

References

1. Beveridge, T.J. and L.L. Graham. 1991. Surface layers of bacteria. Microbiol. Rev. 55: 684-705.

2. Boland, J.S., P.M. Davidson, and J. Weiss. 2003. Enhanced inhibition of *Eshcerichia coli* O157:H7 with lysozyme and chelators. J. Food Prot. In Press.

3. Elliason, D.J. and S.R. Tatini. 1999. Enhanced inactivation of *Salmonella typhimurium* and verotoxigenic *Escherichia coli* by nisin at 65°C. Can. Food Microbiol. 16: 257-267.

4. Hart, M.E. and F.R. Champlin. Susceptibility to hydrophobic molecules and phospholipids composition in *Pasteurella multocida* and *Actinobacillus lignieresii*. Antimicrob. Agents Chemother. 32: 1354-1359.

5. Li, J. and L.A. McLandsborough. 1999. The effects of the surface charge and hydrophobicity of *Escherichia coli* on its adhesion to beef muscle. Inter. J. Food Microbiol. 53: 185-193.

6. Lytle, D.A., E.W. Rice, C.H. Johnson, and K.R. Fox. 1999. Electrophoretic mobilities of *Escherichia coli* O157:H7 and wild-type *Escherichia coli* strains. Appl. Environ. Microbiol. 65: 3222-3225.

7. Mozes, N. and P.G. Rouxhet. 1990. Microbial hydrophobicity and fermentation technology. In: R.J. Doyle and M. Rosenberg (Eds.), Microbial Cell Surface Hydrophobicity. American Society for Microbiology, Washington, D.C. pp. 75-105.

8. Saito, T., T. Takatsuk, T. Kato, K. Ishihara, and K. Okuda. 1997. Adherence of oral streptococci to an immobilized antimicrobial agent. Arch. Oral Biol. 42: 539-545.

9. Soltys, A., M. Lazarz, and E. Chibowski. 1997. Zeta potential and effective diameter of n-tetradecane emulsions in n-propanol solutions and in the presence of lysozyme. Colloids and Surfaces A: Physiochemical and Engineering Aspects. 127: 163-173.

10. Stevens, K.A. B.W. Sheldon, N.A. Klapes, and T.R. Klaenhammer. 1991. Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. Appl. Environ. Microbiol. 57: 3613-3615.

11. Wiacek, A. and E. Chibowski. 2000. Stability of oil/water (ethanol, lysozyme or lysine) emulsions. Colloids and Surfaces B: Biointerfaces. 17: 175-190.

12. Wilson, W.W., M.M. Wade, S.C. Holman, F.R. Champlin. 2001. Status of methods for assessing bacteria surface charge properties based on zeta potential measurements. J. Microbiol. Meths. 43: 153-164.

Appendix

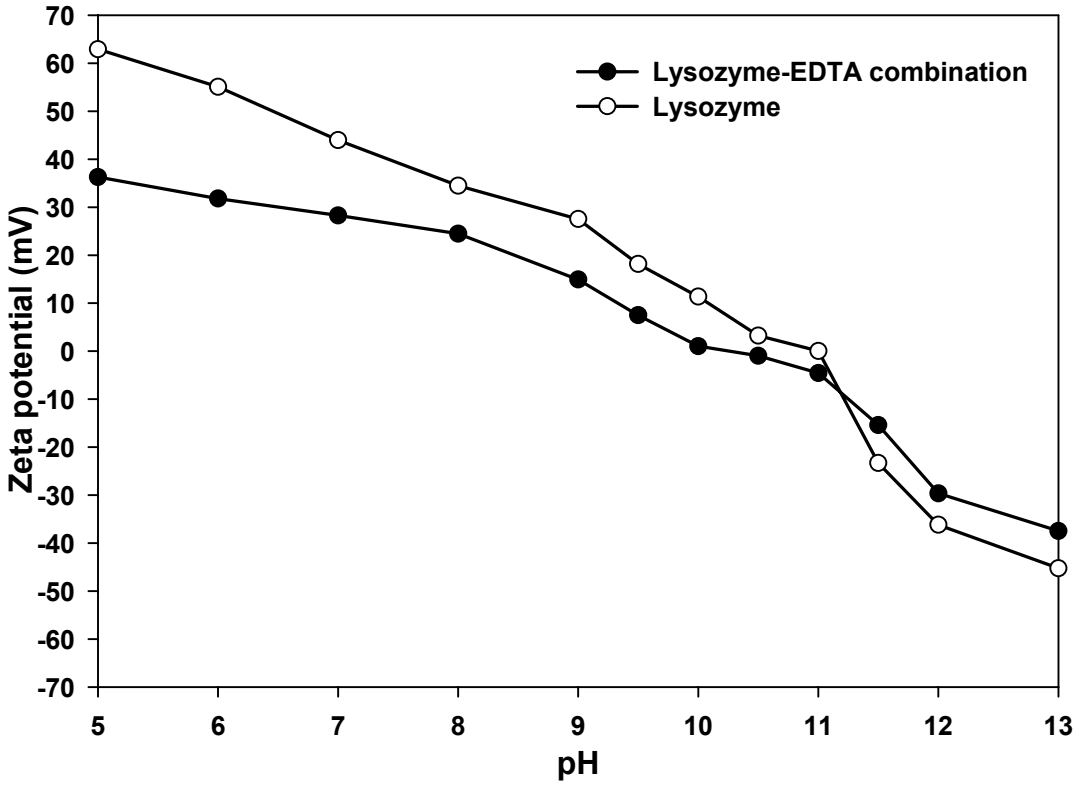

Figure 4.1: Zeta potential of emulsion containing lysozyme and lysozyme-EDTA combinations at pH 5-13.

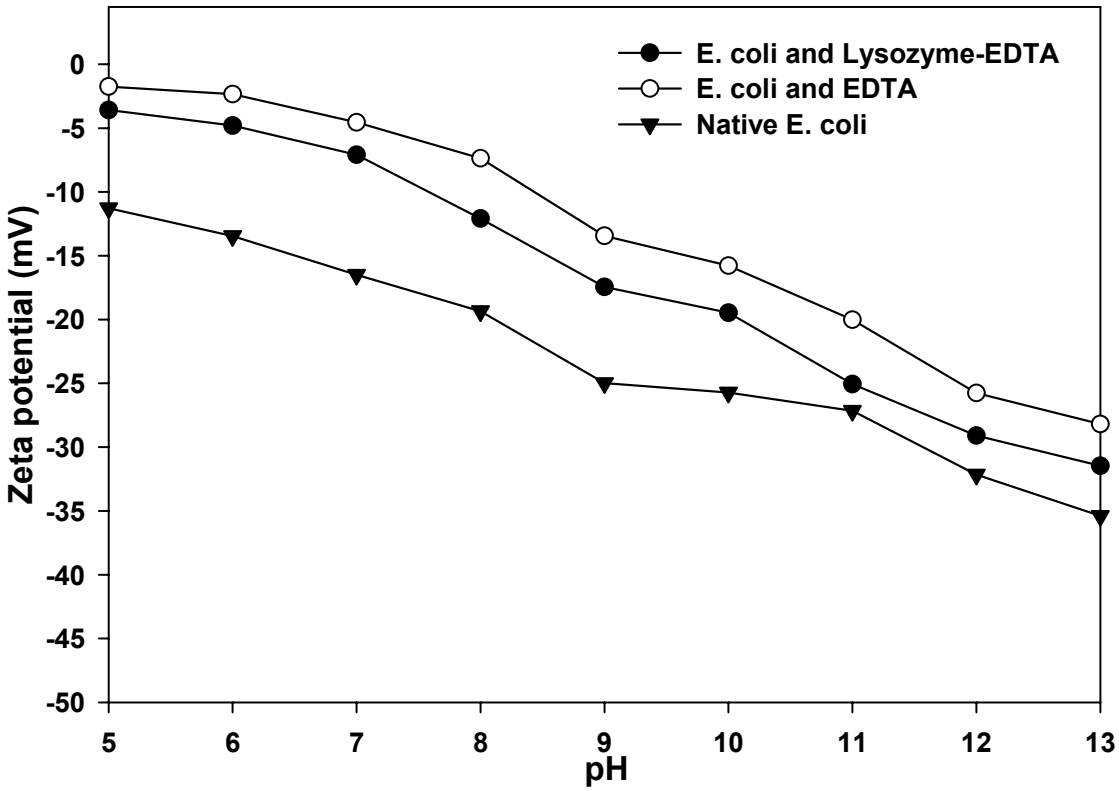

Figure 4.2: Zeta potential measurements of native *Escherichia coli* O157:H7 strain 932 in the presence of lysozyme-EDTA and EDTA alone.

Vita

Jeffrey Smith Boland was born on April 9, 1979 in Greer, South Carolina. He attended the Spartanburg County School District Five public school system and graduated from James F. Byrnes High School in 1997. Jeff attended Clemson University and graduated Cum Laude with a Bachelor's of Science Degree in Packaging Science in May 2001. In August 2001, he decided to continue his education by joining the Department of Food Science and Technology at the University of Tennessee to pursue a Master's Degree.

During his studies at the University of Tennessee, Jeff won second place at the 2002 IFT Food Microbiology graduate research competition with his oral presentation entitled "The Enhanced Inhibition of *Escherichia coli* O157:H7 by Lysozyme and Chelators." Futhermore, in 2003, he was awarded the Extraordinary Professional Promise award from the University of Tennessee for outstanding graduate research. After graduation, Jeff plans to pursue a career in the food industry.