To the Graduate Council:

I am submitting herewith a thesis written by Cheryl Lynn Beckett entitled "Physicochemical properties of modified antimicrobials and the effects on efficacy against foodborne pathogens." I have examined the final paper 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.

P. Michael Davidson, Major Professor

We have read this thesis
and recommend its acceptance:

Jochen Weiss

David Golden

Accepted for the Council:

Vice Provost and Dean of Graduate Studies

PHYSICOCHEMICAL PROPERTIES OF MODIFIED ANTIMICROBIALS AND THE EFFECTS ON EFFICACY AGAINST FOODBORNE PATHOGENS

A Thesis

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Abstract

The objective of this study was to examine the relationship between physicochemical properties and antimicrobial activity of certain chemical compounds. Antimicrobial activity against *Listeria monocytogenes* and adsorption kinetics data were examined to determine if a correlation exists. For potassium sorbate, a commonly used antimicrobial, a relationship appears to exist between the pseudo-critical micellar concentration and the minimum inhibitory concentration. Lysozyme, an naturally occurring antimicrobial, was treated with ultrasound at various power levels and treatment times to examine the effects of sonication on both physicochemical properties and antimicrobial activity.

Ultrasound-treated lysozyme was found to give different adsorption kinetic data than native lysozyme, and variation between treatments was observed. Initially it was believed that three states of lysozyme were observed, native, partially denatured, and fully denatured. The partially denatured lysozyme had increased antimicrobial activity against *L. monocytogenes*, and the fully denatured lysozyme retained some of its antimicrobial activity. This contrasted previous work that reported that fully denatured lysozyme lost antimicrobial and enzymatic activity.

Thermal analysis using differential scanning calorimetry was performed on native and ultrasound-treated lysozyme. Analysis showed that lysozyme that gave adsorption kinetic data indicating full denaturation was not actually completely unfolded. Thermal analysis of heat-treated lysozyme showed full denaturation, as did adsorption kinetics studies. It was concluded that ultrasound treatment of lysozyme resulted in different states of partial denaturation, rather than partial and full denaturation.

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1. Review of the Literature

1.1 Introduction

According to the Centers for Disease Control (CDC), foodborne diseases cause approximately 76 million illnesses each year in the United States. These illnesses result in 325,000 hospitalizations and 5,000 deaths. The symptoms of these foodborne illnesses may range from mild gastroenteritis to life-threatening neurological, hepatic, or renal syndromes. Of these illnesses, 38.6 million are caused by known pathogens, and among all illnesses caused by foodborne transmission, 30% are caused by bacteria (CDC, 2000). Seventy-five percent of all foodborne deaths are attributed to *Salmonella, Listeria*, or *Toxoplasma.* The total annual costs caused by foodborne illnesses are not known, but medical costs and lost wages due to foodborne salmonellosis alone have been estimated at over $1 billion per year (CDC, 2000).

1.2 *Listeria monocytogenes*

1.2.1 Microorganism Characteristics

Listeria monocytogenes is a microorganism of particular concern to the food industry. While the number of listeriosis incidents is relatively low when compared to incidents of other foodborne illnesses, the severity of the disease makes it a major concern. Ninety-five percent of patients infected with *Listeria* are hospitalized, and *Listeria* accounts for approximately half of all reported deaths from a foodborne illness (Donnelly, 2001). *Listeria* is a psychrotrophic gram-positive coccobacillus, as well as a facultative anaerobe. The genus *Listeria* is comprised of six subspecies, only one of which is a human pathogen, *Listeria monocytogenes*. One of the difficulties in

controlling the growth of *Listeria monocytogenes* is the highly adaptable nature of the bacteria. It can grow over a wide range of temperatures (–1.5°C to 50°C) and survive in media that have a pH ranging from 4.3 to 9.6 (Jay, 1996). *Listeria monocytogenes* survives freezing and drying and is salt tolerant (up to 25%) (Rocourt and Cossart, 1997).

Typical sources of *Listeria monocytogenes* are environmental reservoirs such as water, soil, silage, and decaying vegetation. The large variety of *Listeria*-contaminated environments is a challenge to food processors. Sources of contamination include, for example, worker's shoes, equipment, clothing, animal feces and raw plant tissue. *Listeria monocytogenes* grows well under nutrient-rich, high humidity conditions, and thus is often found in moist areas such as drains, floors, residues, or processing equipment (Cox et al., 1989). Due to its presence in food plants, *Listeria monocytogenes* can be found in a wide variety of food products, both raw and processed. Because it is psychrotrophic, *L. monocytogenes* can grow during refrigerated storage. The primary foods that harbor *L. monocytogenes* are dairy products, meats and meat products, and seafood. *Listeria monocytogenes* can also be found in low numbers on raw vegetables such as carrots, cabbage, radishes, and potatoes, but these are generally not good substrates for growth (Rocourt and Cossart, 1997).

1.2.2 Illnesses and Outbreaks

The first clinical descriptions of both animal and human disease caused by *L. monocytogenes* were published in the 1920s. *Listeria monocytogenes* was reported to be the cause of neonatal sepsis and meningitis in East Germany after World War II. Rhombencephalitis in domestic animals has for many years been associated with

ingestion of poor quality silage contaminated with *L. monocytogenes* (Low and Renton, 1985). This led to the speculation that foodborne infection could cause listeriosis in humans as well, which was proven by analysis of an outbreak of listeriosis in Canada in 1981 that was caused by contaminated coleslaw (Schlech et al., 1983).

Listeriosis is not defined by any one set of symptoms, since the course of the disease depends greatly on the state of the host. Healthy adults are generally resistant to infection by *L. monocytogenes* and there is little evidence that such individuals ever contract listeriosis. Pregnant women and the elderly are at a greater risk than the general population. Adults with conditions such as AIDS, alcoholism, diabetes, cardiovascular disease, renal transplant, neoplasm, and corticosteriod therapy are also more susceptible to the disease (Jay, 1996). The most commonly recognized symptoms in susceptible adults are sepsis and meningitis. Listeriosis in pregnant women can cause abortion, premature birth or stillbirth (Rocourt and Cossart, 1997). In addition, the infection may be passed on to her child, resulting in meningitis in the newborn child.

In the United States, there have been a number of outbreaks of listeriosis since 1979. In 1979, an outbreak involving 23 hospitalized patients occurred in Boston, MA and was linked to hospital food as the vehicle of infection. Occurrence of bacteremia or meningitis resulted in a 15% mortality rate (Donnelly, 2001). In 1983, 49 cases of listeriosis were reported in Massachusetts, with pasteurized whole and 2% milk being the vehicle. Forty-two of these patients suffered from underlying illnesses such as cancer or alcoholism. The reported fatality rate was 29% (Schlech, 2000). In June of 1985, 142 cases were reported in Southern California due to a Mexican-style cheese. Out of these 142 cases, 93 cases involved pregnant women or their offspring and 49 involved

immuno-compromised adults. The outbreak resulted in a total of 48 deaths (33.8% mortality rate) (Donnelly, 2001). In 1994, an outbreak was linked to chocolate milk served at a picnic during a Holstein cow show in Illinois. Forty-five people developed listeriosis with symptoms ranging from fever to gastroenteritis. None of the patients reported immune deficiency or chronic illness and a pregnant female who was amongst the infected patients in fact delivered a healthy baby (Donnelly, 2001). Over a seven-month period (August, 1998 to March, 1999), 101 cases of listeriosis were reported in 22 states. In the outbreak involved there were 21 fatalities with 15 adults and 6 miscarriages, and was linked to hot dogs (CDC, 1999). The CDC reported that from May to November, 2000 a 29-case-outbreak across 10 states resulted in 4 deaths and 3 miscarriages or stillbirths. This particular outbreak was linked to turkey deli meat. From October, 2000 to January, 2001 a 12 case outbreak in North Carolina was associated with homemade Mexican-style soft cheese. Ten of the cases involved pregnant women, and the outbreak resulted in 5 stillbirths, 3 premature births, and 2 infected infants (CDC, 2001).

Internationally there have been six major outbreaks of listeriosis between 1980 and 1999. An outbreak in Canada in 1981 was linked to contaminated coleslaw, which had a 34% mortality rate. In Switzerland between 1983-1984, 57 cases were caused by consumption of a soft cheese resulting in a 32% mortality rate. In both 1993 and 1997, outbreaks of febrile gastroenteritis were reported in Italy. The first outbreak involved 39 cases and was associated with rice salad, and the second outbreak numbered 1566 cases and was linked to corn salad. No deaths were reported in either outbreak. Finally, outbreaks were reported in France in both 1992 and 1999. The source of both outbreaks

was pork, rillettes in 1992 and tongue in 1999. The 1992 outbreak reported 38 cases with a 32% mortality rate, while the 1999 outbreak involved 32 cases with a 31% mortality rate (Schlech, 2000).

Although outbreaks of listeriosis do not occur as frequently as other foodborne illnesses, the 20-30% mortality rate is one of the highest in foodborne diseases. These high mortality rates are a concern for the food industry and regulators. Control of this pathogen is therefore of interest to many processors, and new methods of control are a focal point for current process development projects.

1.2.3 Control Methods

As previously mentioned, *Listeria* is widely present in the environment, and can easily be introduced into food plants. Due to its psychrotrophic nature, it grows well in refrigerated or cooled storage, and prevention of introduction of the organism into processed foods is thus difficult. However, a number of measures are available that can be used both in the processing plant and in the product itself to control growth of *Listeria*.

Methods of *Listeria* growth inhibition include modification and control of water activity, pH, salt concentration, temperature, irradiation, and addition of chemical preservatives. Farber et al. (1992) reported that *Listeria monocytogenes* was able to grow at water activities as low as 0.90. In this respect, the microorganism is second only to staphylococci in its ability to grow at water activities below 0.93. *Listeria* can also grow over a wide range of pHs with growth being prevented at pH < 4.0. In addition, *Listeria* is tolerant of salt levels of up to 25%. The combined inhibitory effect of low pH and addition of NaCl were found to be additive and not synergistic (Jay, 1996). Tienungood

et al. (2000) reported on the growth limits of *L. monocytogenes* as a function of temperature, water activity, and pH. They found that as the water activity increased *Listeria* was able to survive at lower pHs. In addition it was reported that as temperature increased the microorganism became less sensitive to lower pH. This type of data is useful in designing new hurdle technologies by determining which combinations of temperature, pH, and water activity are most effective in control of *L. monocytogenes*.

Listeria can grow in a wide range of temperatures and has been shown to be fairly heat resistant, however the microorganism can be destroyed by pasteurization (Jay, 1996). D values, or the time required to destroy 90% of the organism, vary for *L. monocytogenes* depending on strain and food composition. Strain Scott A heated at 71.5°C in milk had D values ranging from 0.9 to 2.0 seconds, while strain F5069 had slightly higher D values ranging from 3.1 to 5.0 seconds (Bradshaw et al., 1985). Mackey et al. (1990) reported that D values for beef and two poultry meats were approximately 6.6 to 8.4 seconds. Gaze et al. (1989) reported that heating meats to an internal temperature of 70°C for 2 min destroyed *L. monocytogenes*.

Neither vacuum packaging nor modified atmosphere packaging significantly affect the survival of *L. monocytogenes* (Rocourt and Cossart, 1997). However, vacuum packaging in combination with other control methods, specifically chemical control methods and refrigeration, has had some degree of success in controlling *Listeria* growth. Wederquist et al. (1994) reported that growth of *L. monocytogenes* was inhibited when sodium acetate, sodium lactate, or potassium sorbate was added to vacuum packaged turkey bologna stored under refrigerated conditions. This supported the finding of Unda et al. (1990) that sodium acetate extended the shelf life of vacuum packaged beefsteak.

Ionizing radiation can also be used as a preservative process. Ionizing radiation inhibits DNA synthesis, thus preventing cell reproduction. Irradiation used in the food industry is either based on the interaction of foods with electron beam or gamma ray radiation. These types of irradiation do not cause radioactivity in foods and are available at commercially feasible costs (Farkas, 1997). *Listeria monocytogenes* has been shown to be sensitive to low doses of irradiation (Rocourt and Cossart, 1997).

Some of the most effective methods to control *L. monocytogenes* are the addition of chemical antimicrobials or antimicrobials in combination with other process factors can be used to successfully establish "hurdles". There are approximately 30 different antimicrobials that have been approved for use in food products. The choice of a suitable antimicrobial depends on food product composition, preservation system used, cost of antimicrobial, and type, characteristics, and number of microorganism (Branen, 1993). Different antimicrobials have different modes of action that generally fall into one of three categories: (a) reaction with the cell membranes, (b) enzyme inactivation, and (c) destruction or functional inactivation of genetic material required for reproduction (Branen, 1993).

A class of chemical compounds that has been successfully used to control growth of *Listeria* is organic acids. Bal'a and Marshall (1998) reported that catfish fillets dipped in organic acids were contaminated with significantly lower populations of *L. monocytogenes* than untreated samples. A study by Palumbo and Williams (1994) showed that organic acid dips on the surface of frankfurters reduced the growth of *L. monocytogenes* under refrigerated vacuum packaged conditions. Polylactic acid, lactic

acid, and nisin were reported to have both bactericidal and inhibitory activity against *L. monocytogenes* in vacuum-packaged raw beef (Ariyapitipun et al., 2000).

Gill and Holley (2000a) illustrated that addition of lysozyme, nisin, and EDTA (a chelating agent) to ham and bologna sausages prior to cooking reduced the initial populations of both *Brochothrix thermosphacta* and *Leuconostoc mesenteroides*. Growth of *Listeria monocytogenes* and *Escherichia coli* O157:H7 was reduced for two and four weeks, respectively. Surface application of the antimicrobial gel on cooked ham and bologna was bactericidal to the Gram-positive organisms such as *L. monocytogenes* (Gill and Holley, 2000b). Oh and Marshall (1992) showed that monolaurin at pH levels of 5.0 and 5.5 was more effective against *L. monocytogenes* than at pH 6.0 or 7.0. In addition, monolaurin was more effective than tertiary butylhydroquinone, butylated hydroxyanisole, and propyl paraben.

In general, consumers can reduce the risk of listeriosis by (a) thoroughly cooking raw foods from animal sources such as beef, pork, or poultry, (b) thoroughly washing raw vegetables before consumption, (c) keeping all uncooked meats separate from other food products, and (d) avoiding the consumption of unpasteurized milk or milk products (Schlech, 2000). Consumers in high-risk groups should also avoid soft cheeses such as Brie, Camembert, feta, and Mexican-style cheese. Left-over and ready-to-eat foods, as well as cold cuts should be thoroughly reheated prior to consumption to ensure that foods are free of contaminants.

1.3 Chemical Control of Foodborne Pathogens

1.3.1 Sorbic Acid

1.3.1.1 Chemical and Physical Properties

Sorbic acid is a straight-chain trans-trans unsaturated fatty acid. The carboxyl group of sorbic acid is extremely reactive and can easily form salts and esters, such as potassium sorbate. The molecular formula for sorbic acid is CH_3-CH=CH-CH=CH-COOH. Potassium sorbate is formed if a potassium molecule replaces the hydrogen on the carboxyl group. The aqueous solubility of sorbic acid is relatively low (0.16g/100g water), compared to the solubility of potassium sorbate (138g/100g water) (Luck and Jager, 1997). Thus salts of sorbic acid are primarily used in the food industry.

1.3.1.2 Spectrum of Activity

Sorbic acid can effectively inhibit yeasts and molds at concentrations between 0.05 and 0.30% (Sofos and Busta, 1993). Sofos (1989) reported that sorbates inhibited yeasts in the genera *Brettanomyces, Candida, Cryptococcus, Debaryomyces, Endomycopsis, Hansenula, Kloeckera, Pichia, Rhodotorula, Saccharomyces, Sporobolomyces, Torulaspora, Torulopsis*, and *Zygosaccharomyces*. Sofos (1989) also reported the effectiveness of sorbates against molds species such as *Aspergillus, Penicillium*, and *Trichoderma*. Sorbates are capable of inhibiting both Gram-positive and Gram-negative bacteria, and studies have suggested that catalase-positive organisms are more inhibited than catalase-negative organisms (Luck and Jager, 1997). Sorbic acid is bacteriostatic not bactericidal.

The exact mechanism of action of sorbic acid in inhibiting microorganisms is the focus of ongoing investigations. The point of attack may differ depending on whether bacteria, yeasts, or molds are targeted. The general mechanism of sorbic acid is an elimination of the electrochemical membrane gradient by the passing of the undissociated lipophilic acid from the outside to the inside of the cell membrane and dissociating in the interior (Eklund, 1989). Several enzymes may in fact be inhibited by sorbic acid, including those found in the Krebs cycle (Eklund, 1989). Sorbic acid has been shown to react with sulfhydryl groups thus altering protein structures. In addition, reactions with phospholipid, glycolipid and protein cell wall components are likely (Luck and Jager, 1997). Contrary to these observations, Buazzi and Marth (1991) reported that the inhibitory mechanism of potassium sorbate against *L. monocytogenes* was not due to membrane damage, as no leakage of cellular contents was observed.

1.3.1.3 Factors Affecting Activity

Lower pH values increase antimicrobial activity of sorbate. This is because the highest inhibitory activity is related to the undissociated form of the acid (Sofos, 1989). Therefore, sorbic acid is not effective above pH 6.5. The use of sorbic acid is not recommended in products that will be pasteurized since the compound breaks down rapidly at higher temperatures (Giese, 1994). In order for sorbic acid to have activity against a microorganism it must penetrate the cell membrane. Only the undissociated form of the acid is able to pass through the membrane bilayer structure since it does not carry any electrical charge. Activity of the undissociated form is therefore more pronounced (Luck and Jager, 1997).

An optimal set of environmental conditions exists in which sorbic acid is highly active. For example, microbial growth inhibition by sorbates is greater at lower storage temperatures indicating that sorbates may be especially suited to preserve refrigerated foods (Robach, 1980, Tuncan and Martin, 1985). In addition, the activity of sorbates is generally enhanced under vacuum or modified atmosphere storage conditions (Wagner et al., 1982, McMeekin et al., 1984). Elliot and Gray (1981) showed that sorbate in combination with carbon dioxide can be an effective inhibitor of microbial growth. El-Shenawy and Marth (1991) reported that the addition of organic acids, specifically acetic, tartaric, lactic, and citric acid, enhanced the inhibitory aspects of potassium sorbate against *L. monocytogenes*. Organic acids were found to increase the lag phase of the growth curve.

1.3.1.4 Application to Foods

Sorbic acid can be directly added to foods. Other forms of application include dipping, spraying, and dusting of food and/or incorporation into or on packaging materials (Sofos and Busta, 1993). Sorbic acid and sorbate salts are among the most commonly used preservatives in the world, and are primarily used as yeast and mold inhibitors. Major food groups that may be preserved by sorbates include dairy products, bakery items, fruit and vegetable products, food emulsions, hard and frankfurter type sausages, dried and lightly salted oriental fish, and sugar and confectionery items (Sofos and Busta, 1993, Luck and Jager, 1997). Typical concentrations of sorbates in foods range from 0.02% to 0.3%.

1.3.2 Parabens

1.3.2.1 Chemical and Physical Properties

Parabens are phenolic compounds that are alkyl esters of *p*-hydroxybenzoic acid. Three of these esters (methyl, propyl, and heptyl) have been approved for use in foods in the United States (Davidson, 1993). The length of the side chain of the ester group may vary. Parabens are extremely stable compounds, that is, they are resistant to oxidation and elevated temperatures (Davidson, 1993).

1.3.2.2 Spectrum of Activity

Parabens are effective against both Gram-positive and Gram-negative bacteria. The inhibitory activity of parabens increases as the alkyl chain length increases. Gram-positive bacteria are generally more susceptible to parabens than Gram-negative bacteria, although parabens may be effective against both (Davidson, 1993). The mode of action of parabens may include the destruction of the cell membrane and/or protein denaturation inside the cell (Luck and Jager, 1997). The lipopolysaccharide of the outer cell membrane of Gram-negative bacteria may act as a barrier to parabens rendering them less susceptible to the inhibitory effects of the compounds (Eklund, 1989). Parabens also have activity against fungi. Thompson (1994) for example, reported that butyl and propyl paraben were effective against mycotoxigenic species of *Aspergillus, Fusarium*, and *Penicillium*. Thompson (1994) reported that when parabens were combined with ethanol to deactivate fungi a synergistic effect was observed.

1.3.2.3 Factors Affecting Activity

Parabens are effective over a wide range of pH (pH 3 - 8). Therefore they are of greater overall value than other pH-sensitive antimicrobials, especially in foods with pH

values near neutrality (Davidson, 1993). The reason for this is that parabens have a pK of 8.5 and thus their activity is relatively independent of food pH (Luck and Jager, 1997). The inhibitory activity of parabens depends on food composition. Dje et al. (1989) for example, reported little inhibition of *L. monocytogenes* in hot dogs that were treated with propyl paraben, while a 3 log reduction of *L. monocytogenes* was reported on chicken. It was hypothesized that this difference was due to the high fat content of hot dogs. Dje et al. (1990) also reported that propyl paraben by itself was not as effective as a methyl-propyl paraben combination in inhibiting growth of *L. monocytogenes* in salt brine and on hot dogs dipped in brine.

1.3.2.4 Application in Foods

The use of parabens in foods such as bakery products, soft drinks, beer, fish, flavor extracts, fruit products, jams and jellies, olives, pickles, salad dressings, syrups, and wines has been suggested. At present however their application has not been widely adopted in the food industry (Davidson, 1993). The exact reason for this is unknown but may be related to unfavorable sensory characteristics. Luck and Jager (1997) reported that a "definite" taste was associated with the presence of parabens in food at concentrations below 0.2%. Parabens may be dissolved in water, ethanol, propylene glycol, or the food product itself. It is also possible to mix the dry paraben with other components prior to addition to foods (Davidson, 1993).

1.3.3 Lysozyme

1.3.3.1 Chemical and Physical Properties

Lysozyme is an enzyme that exhibits antimicrobial properties. A common source for lysoyzme is egg white, cow milk, human colostrum, cauliflower, and cabbage

(Masschalck et al., 2001). Lysozyme consists of 129 amino acids cross-linked by four disulfide bridges. If all disulfide bonds are broken, the molecule loses its enzymatic activity. Activity is maintained if less than 3 bonds are broken (Jolles et al., 1963). The protein has a globular structure in water with polar groups exposed to water molecules and non-polar tails buried at the inside of the globular structure.

1.3.3.2 Spectrum of Activity

Lysozyme catalyzes the hydrolysis of the main component of bacterial cell walls, peptidoglycan (Proctor and Cunningham, 1988). Specifically, it catalyzes the hydrolysis of the β-(1-4) linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine residues (Board, 1968). As with parabens, the lipoprotein-lipopolysaccharide outer membrane of Gram-negative bacteria does not allow lysozyme to reach the peptidoglycan of the cell wall, making Gram-negative bacteria more resistant to the decomposition catalyzed by lysozyme.

Hughey and Johnson (1987) showed that lysozyme was able to lyse non-growing cells of *L. monocytogenes*, but had no lytic or inhibitory effect on growing cells. They also reported that several strains of *Clostridium botulinum* and *Clostridium sporogenes* were not inhibited if cells were in their growing stage. It was hypothesized that cell wall synthesis in rapidly growing cultures exceeds the rate of degradation by lysozyme

Gram-negative cells may be inhibited or lysed by lysozyme if the outer membrane of microorganisms is permeabilized by physical or chemical means or if the structure of lysozyme is changed. Warren et al. (1955) showed that *Pseudomonas aeruginosa* cells treated with either heat or acetone were lysed by lysozyme. In addition, several studies

have investigated the effects of a combination of lysozyme with ethylenediaminetetraacetic acid (EDTA) against Gram-negative organisms. Repaske (1956) reported that lysozyme in combination with EDTA was capable of lysing several Gram-negative bacteria, including *E. coli, Azotobacter* and *P. aeruginosa*. Wang and Shelef (1992) and Chander and Lewis (1980) showed that lysozyme in combination with EDTA totally inhibited growth of natural microflora in codfish and shrimp.

1.3.3.3 Factors Affecting Activity

Early work with lysozyme indicated that activity of lysozyme increases as the pH decreases. Davies et al. (1969) however reported that lysozyme activity depended not only on pH but also on salt concentration. A specific ionic strength was found at which the activity of lysozyme reached a peak. This optimal ionic strength was high at low pH values and decreased as the pH increased. Chang and Carr (1971) also reported that lysozyme was active over a pH range of 5-10 if salt concentrations were varied appropriately. This was explained in terms of the interaction of cations with the lysozyme molecule. At alkaline pH, cations may interact with negatively charged residues on the lysozyme molecule, which in turn may affect the conformation of the lysozyme and thus block or distort the active site.

Smith et al. (1991) found that the anti-listerial activity of lysozyme increased as growth temperature decreased indicating that lysozyme may be useful against *L. monocytogenes* in refrigerated foods. Johansen et al. (1994) reported that both temperature and pH had an effect on the anti-listerial activity of lysozyme. Activity of lysozyme was greater at 5°C than at 25 or 37°C. It was concluded that the antibacterial

effect of lysozyme on growing *L. monocytogenes* cells at low pH was due primarily to a change in sensitivity of cells rather than an increase in the activity of the enzyme. In milk, Kihm et al. (1994) showed that *L. monocytogenes* was resistant to lysozyme. However, when the milk was demineralized, the activity of lysozyme increased dramatically. Kihm et al. (1994) hypothesized that the presence of minerals negatively impacted lysozyme structure, which could also explain why EDTA (a chelating agent) enhances the activity of lysozyme. Liberti et al. (1996) showed that a polyphosphate-lysozyme combination did not enhance the effectiveness of lysozyme against non-growing *L. monocytogenes* cells. However, lysozyme combined with crude porcine pancreas lipase had greater activity than lysozyme alone. It was hypothesized that this was because lipase exposed microbial cell wall components thus allowing enhanced cell wall catalysis by lysozyme.

The conformation of the lysozyme also has an effect on the activity of the enzyme. Lysozyme in its native state has very little activity against Gram-negative organisms and denatured lysozyme has no enzymatic or antimicrobial activity at all (Masschalck et al., 2001). Partially denatured lysozyme, however, has been reported to have increased activity against Gram-negative bacteria. Ibrahim et al. (1996b) showed that partially heat denatured lysozyme was capable of causing membrane damage to *E. coli*. It was also reported that partially denatured lysozyme did not retain any of its enzymatic activity but still showed strong bactericidal activity, suggesting that antimicrobial and enzymatic activity are not related (Ibrahim et al., 1996a). Masschalck et al. (2001), however, reported an inability to replicate these results, and concluded that the effects seen were very strain dependent. This study also reported that partial heat denaturation can extend

the spectrum of activity under pressure. Partially heat denatured lysozyme was active against *Salmonella* Typhimurium under high hydrostatic pressure.

1.3.3.4 Application to Foods

The most common use of lysozyme is in cheeses such as Edam and Gouda to prevent a defect known as "late blowing." This defect creates holes in the cheese as well as off flavors and is caused by the growth of *Clostridium tyrobutyricum* (Vakil et al, 1969). Proctor and Cunningham (1988) reported that patents for lysozyme application to oysters, shrimp, other seafood, sushi, sake, kimchi, Chinese noodles, potato salad, and custard have been issued in Japan. Hughey et al. (1989) demonstrated that lysozyme was more effective against *L. monocytogenes* Scott A in vegetables than either sausage or Camembert cheese and that activity was increased with addition of EDTA.

1.3.3.5 Regulatory Aspects

Lysozyme did not receive GRAS status in the United States until the FDA issued a tentative final rule in 1998 allowing egg white lysozyme to be recognized as GRAS to prevent late blowing in cheese (FDA, 1998). Lysozyme however has been approved in other countries, such as Japan and the United Kingdom (Conner, 1993). Egg white lysozyme is considered safe because humans have been consuming eggs, a primary source of lysozyme, for a long time. Nevertheless allergic reactions in sensitive individuals may occur. It is not known if lysozyme added to cheese causes allergic reactions. Consequently, the FDA requires that lysozyme-containing cheese must carry a special label (FDA, 1998).

1.4 Application of Ultrasound

Ultrasound treatment, or sonication, is a process in which high frequency sound waves are applied to a food product to alter its physicochemical properties such as color or texture. Ultrasound waves are mechanical waves that cause compressional and shear stresses to propagate through the food material. In a relatively novel application, ultrasound may be used to alter the functional properties of proteins. Nakamura et al. (1988) reported that sonication improved for example the emulsification properties of egg yolk lecithin. The change in physicochemical properties may also affect catalytic functions of biopolymers. Taylor and Richardson (1980) showed that sonication of skim milk improved antioxidant activity of the product.

Another application of ultrasound is to control the growth of microorganisms. Harvey and Loomis (1929) observed that vegetative cells of microorganisms were inactivated with the use of high intensity ultrasound. The lethal effect was attributed to cavitation, which is the growth and collapse of microscopic bubbles in the wake of the propagating sinusoidal pressure wave. Cavitation can affect a biological system either through direct application of mechanical stress or through secondary localized temperature increases (Scherba et al, 1991). It has been theorized by Riesz and Kondo (1992) that the extremely high temperature and pressure fluctuation caused by cavitation can dissociate water molecules into H+ and OH- free radicals. Reaction of free radicals with cell components such as DNA or proteins may then cause the destruction of microorganisms. The precise mechanism for ultrasonic inactivation of microorganisms requires further investigation.

For inactivation or inhibition of microorganisms, ultrasound treatment may be combined with static pressure (manosonication (MS)) or combined with heat treatment and static pressure (manothermosonication (MTS)). In addition, ultrasound combined with reduced pH and/or antimicrobials can enhance antimicrobial activity. Lillard (1994) reported that ultrasound and chlorine were more effective in reducing numbers of *Salmonella* on poultry skin than either process alone. Manothermosonication was shown to be more effective than heat treatment alone in inactivating enzymes produced by *Pseudomonas fluorescens* (Vercet et al., 1997). It was further suggested that under appropriate operating conditions, MTS may replace ultra-high temperature processing of milk. Raso et al. (1998) reported that manosonication treatment of *Bacillus subtilis* spores sensitized them to lysozyme. However, direct sonication of lysozyme was shown to eliminate its enzymatic activity (Krishnamurthy et al., 2000). As was mentioned previously, however, loss of enzymatic activity does not necessarily equate to loss of antimicrobial activity.

1.5 Physicochemical Properties of Antimicrobials

1.5.1 Surface Tension

Surface tension can be used to determine such properties as critical micellar concentration and adsorption kinetics. For substances such as potassium sorbate that may self assemble, the surface tension measurements can be used to determine the critical micellar concentration (CMC). The critical micellar concentration is the point at which the substance begins to form micelles. This occurs when all interfaces are covered with the substance, but molecules are still present in the solution. These molecules have hydrophobic tails that do not want to be in contact with the water of the solute, so the

molecules self assemble with the tails oriented towards the inside of the micelle (Weiss, 2001). Surface tension also can be used to measure the adsorption kinetics of a substance at the air-water interface. Proteins like lysozyme adsorb slowly at interfaces. In the process the surface tension decreases gradually over time. The adsorption kinetics can be used to investigate structural changes such as degree of denaturation.

Xu and Damodaran (1992) reported that at freshly formed air-water interfaces, undenatured lysozyme tends to desorb from the interface due to its high electrochemical potential at the interface. At the subsurface the lysozyme actually undergoes a partial unfolding, which then allows for positive adsorption. It should be noted, however, that the lysozyme used in this experiment was labeled with ^{14}C through reductive methylation of the lysyl residues, and this may not be directly comparable with other studies. In addition, they worked with very long time periods (>1000 min) and on the assumption that adsorption at the interface was instantaneous. Green et al. (2000) examined competitive adsorption of lysozyme and pentaethylene glycol monododecyl ether ($C_{12}E_5$), a small molecular surfactant, at the air-water interface. It was reported that low surfactant concentration ($2x10^{-6}$) generated surface tension data indicative of co-adsorption at the interface, but that high surfactant concentration ($8x10^{-5}$) prevented adsorption of lysozyme at the interface.

1.5.2 Thermal Analysis

Differential scanning calorimetry is a technique in which the temperature difference between a sample cell and a reference cell is measured (Wunderlich, 2001). The reference and sample cell are placed in an oven and the temperature of the oven is then varied as a function of time. Simulatenous to the applied external thermal stress the

temperature (response) of the sample is measured and compared to the temperature of the reference. Heat capacity can thus be measured on a single run by assessing the heat flow into the sample and comparing it with the heat flow into the reference material (Wunderlich, 2001). DSC analysis can be used to measure heat capacity, identify material composition, and perform quantitative analysis of both glass transition and heat of fusion.

In a study by Zhang and Wunderlich (1997), the heat capacity of several proteins, including lysozyme, were examined. The object of the study was to calculate and determine quantitative information on enthalpy and entropies of transformation. This was done using by linking the macroscopic heat capacity of solids to the microscopic cause, vibrational motion. The authors were able to determine a new optimization procedure to calculate basic thermodynamic functions.

1.5.3 Free Sulfhydryl Groups

The presence or absence of sulfhydryl bonds in lysozyme influences its stability. As stated previously, native lysozyme has four disulfide bonds. If all four bonds are broken, lysozyme loses all of its enzymatic activity. If two of the bonds remain intact, the activity is preserved. Moriyama et al. (2000) examined the effects of selectively reducing disulfide bonds of lysozyme on the conformation of the molecule. It was reported that lysozyme with 2 or more disulfide bridges retained its native conformation at pH 7.0. However, when all four disulfide bridges were reduced, significant structural changes occurred at both neutral and acidic pH. It was also reported that native lysozyme in the presence of sodium dodecyl sulfate (SDS) behaves differently than lysozyme with all four disulfide bridges reduced.

1.6 Summary

Much work has been done on the control of *Listeria monocytogenes,* including using the antimicrobial lysozyme. Lysozyme in its native state is not very effective against *L. monocytogenes*, and has virtually no activity against Gram-negative bacteria. Combinations of lysozyme and EDTA have been shown to enhance activity, and modification of the lysozyme itself has also shown promise. Heat induced denaturation was reported to increase lysozyme activity against *E. coli*, and improve activity against *Salmonella* when used under high hydrostatic pressure. Ultrasound treatement has been used in direct application to foods to kill microorganisms, and in combination with antimicrobials to enhance their activity. In this study, ultrasound induced denaturation of lysozyme was examined to determine if the activity of the antimicrobial could be enhanced.

2 Materials and Methods

2.1 Test Antimicrobials

Potassium sorbate (2,4-Hexadienoic acid, Sigma Chemical Co., St. Louis, MO) was dissolved in deionized water and filter sterilized (Corning .22 μm cellulose acetate membrane). *N*-propyl *p*-hydroxybenzoate (propyl paraben, Sigma Chemical Co., St. Louis, MO) was dissolved in 95% ethanol. For minimum inhibitory concentration (MIC) studies, the propyl paraben was filter sterilized.

Lysozyme (3x crystallized, dialyzed, and lyophilized, 48,800 units/mg protein, Sigma Chemical Co., St. Louis, MO) was dissolved in sterile water. In initial inhibition studies the lysozyme was filter sterilized. Sterile water was used in all experiments where lysozyme was treated with ultrasound.

2.2 Test Microorganisms

Listeria monocytogenes Scott A, 101, 310, 108, and V7 were obtained from the University of Tennessee, Knoxville Department of Food Science and Technology culture collection. Cultures were maintained in tryptose phosphate broth (TPB) that was adjusted to pH 6 with 1N HCl. The cultures were incubated at 32°C, and transferred 24 hours prior to use.

2.3 Minimum Inhibitory Concentration Testing

A microplate assay was used to determine the minimum inhibitory concentrations of potassium sorbate, propyl paraben, and lysozyme against all five strains of *L. monocytogenes*. Stock solutions of antimicrobials were prepared using the appropriate

solvent (sterile water for lysozyme and potassium sorbate, 95% ethanol for propyl paraben). Concentrations of potassium sorbate solutions were 0, 1500, 1750, 2000, 2250, 2500, 2750, and 3000 μg/ml. Propyl paraben concentrations were 0, 200, 400, 600, 800, 1000, and 2000 μg/ml. Test solutions of lysozyme were 0, 100, 200, 250, 300, 350, 400, 450, 500, 550, 600, and 1000 μg/ml. For the preliminary sonication work, lysozyme concentrations of 100, 250, 500, 1000, and 2000 μg/ml were used to determine adsorptions kinetics and surface activity.

For each inhibition test, a total volume of 240 μl in microtiter plates with a well capacity of 300 μl were used. For potassium sorbate and lysozyme this consisted of 180 μl of inoculated TPB (~10^4 CFU/ml) and 60 μl of antimicrobial test solution. For propyl paraben, the wells contained 180 μl of inoculated TPB, 50μl of uninoculated TPB, and 10 μl of the propyl paraben solution. This was done to dilute the total volume of the 95% ethanol used to a concentration of 4% to not inhibit the test microorganisms. Each treatment was run in duplicates, and each experiment was replicated at least once. For positive controls, 180 μl of inoculated TPB and 60 μl sterile water (or 50 μl uninoculated TPB and 10 μl of 95% ethanol in the case of propyl paraben) were used.

Plates were incubated at 32°C for 24 hours. The optical density (OD) of each well was determined at 630 nm at 24 hours with an Elx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). Minimum inhibitory concentrations at 24 hours were defined as the lowest concentration at which growth was completely inhibited. Growth inhibition was defined as an OD of <0.05. Branen (1998) defined inhibition at optical density of <0.01, but for these experiments slightly higher levels were used due to the preliminary results and visual determination of inhibition. The average OD of

positive control wells ranged from 0.5 to 0.6, depending on the strain of *L. monocytogenes.*

For kinetic studies of growth inhibition, microtiter plates were prepared as previously described and incubated at 32°C. Initial OD readings were recorded prior to placing plates in the incubator. At intervals of 1, 2, 4, 6, 8, 12, and 24 hours plates were removed from the incubator and OD readings were taken.

2.4 Sonication Treatment

Lysozyme was treated with ultrasound in an attempt to completely denature or partially denature the protein. Two different Ultrasonic Homogenizers (Cole-Palmer Instrument Co., Vernon Hills, IL) and a Misonix 3000 (Misonix, Farmingdale, NY) were used to treat lysozyme samples. Samples were initially sonicated at power levels of 300 watts, 100 watts, and 45 watts for 15 minutes. Due to difficulties in maintaining constant power levels during the procedures, subsequent tests were conducted at constant input (amps) levels rather than output (watts) level. In addition, the application time was reduced. Power levels ranged from 40 amps to 10 amps and time intervals 1, 2, 5, 7.5, 10, 12.5, and 15 min. The Misonix 3000 was used at the lowest possible output, which gave a reading of 24 watts with a 0.75" horn tip, and a reading of 15 watts with a 0.5" horn tip. With the larger horn the sonication treatment lasted 30 sec, while the smaller horn treatment was 15 sec.

All lysozyme solutions were prepared at room temperature one hour prior to ultrasonication to allow the lysozyme to fully hydrate. Solutions were then rapidly cooled using an ice-water bath until the temperature of the lysozyme solution dropped

below 10°C. Solutions were kept in an ice-water bath and were constantly stirred. Temperature of lysozyme solutions was monitored throughout sonication treatments. The temperature of solutions never exceeded 50°C. (Note: the denaturation temperature of native lysozyme was experimentally determined to be 75°C) After sonication, solutions were allowed to return to room temperature.

2.5 Density Determination

The density of antimicrobial solutions was determined prior to surface tension measurements. Density of both native and sonicated solutions was measured. A modified Hubbard-Carmick specific gravity bottle was used (Fisher Scientific, Pittsburg, PA). The alpha value for the density equation was calculated as 26.5052. The temperature of antimicrobial solutions was measured prior to all mass determinations. The empty mass of the bottle was measured. The bottle was filled with test solution and the mass was determined. Final density was calculated as:

$$D = \frac{M_{Full} - M_{Empty}}{V_{Corrected}} \tag{2.1}$$

2.6 Drop Shape Analysis/ Surface Tension

Surface tension of potassium sorbate and lysozyme solutions was measured using a DSA 10 drop shape analyzer (Krüss, Hamburg, Germany). Surface tension for propyl paraben could not be determined due to the rapid evaporation of ethanol. A cuvette was filled with approximately 10 ml of test solution, and was placed on the test stand of the DSA 10. An inverted syringe was submerged into the test solution and an air bubble was formed at the tip of the needle. The image of the bubble was captured with a video

camera that was connected to a computer. The drop shape was then fit to the Young-Laplace equation to obtain the surface tension (Equation 2.2).

$$\Delta p = \sigma(\frac{1}{r_1} + \frac{1}{r_2}) \qquad (2.2)$$

Where Δp is the difference in pressure between the inside and outside of the drop, σ is the interfacial tension, and $r_{1,2}$ are the main radii of curvature of a sectional area of the drop surface. Surface tension was measured every 2 seconds to obtain the adsorption kinetics. Total measurement time was varied depending on length of ultrasonic treatment and lysozyme concentration. Data was exported into Microsoft Excel for further analysis. Surface tension data were evaluated for correctness using HPLC grade water (Aldrich Chemical Co., Milwaukee, WI) to obtain 72 mN/m. The cuvette and syringe used were cleaned using a dilute solution of chromic acid.

2.7 Thermal Analysis

Thermal analysis of lysozyme solution was performed using a TGA 2050 Thermogravimetric Analyzer (TA Instruments, New Castle, DE) and a DSC 2920 Modulated DSC (TA Instruments, New Castle, DE). For thermal analysis, a 20% lysozyme solution was prepared using the previously described procedure and sonicated for 1-15 minutes. An empty DSC pan was weighed using the TGA 2050, and a drop of sample was placed in the bottom of the pan. The pan was then sealed and reweighed. The weight of sample was recorded and entered into the DSC for each sample and run.

The sample pan was carefully placed into the DSC 2920 next to a reference pan. An initial holding temperature of 20°C was programmed and the oven temperature was then raised to 90°C with a scanning speed of 5°C/min. After an initial run the start

temperature was changed to 50°C, and the ramp changed to 1°C per minute. The parameter was adjusted to increase the resolution at the denaturation peak All DSC measurements were performed twice.

2.8 Statistical Analysis

Statistical analysis was performed using analysis of variance (ANOVA) and means separated using Student-Newman-Keuls mean separation test (SAS 2000). MIC values for all strains and successful treatments were averaged. Mean values were compared to determine if differences between the strains existed. Treatments which showed no inhibition of any strain were not included in the statistical analysis. Differences were found to be statistically significant at $P < 0.05$.

3. Results and Discussion

3.1 Propyl Paraben

The minimum inhibitory concentration (MIC) of propyl paraben was independent of type of strain for *Listeria monocytogenes* when tested in tryptose phosphate broth (TPB) at pH 6. The MIC was 600 μg/ml for all strains (Table 3.1). The optical density (OD) was measured for cultures containing 0, 200, 400, 600, 800, and 1000 μg/ml propyl paraben. A decrease in the OD from 0.341 (± 0.027) to 0.179 (± 0.006) was observed at 200 μg/ml propyl paraben, indicating that the propyl paraben reduced growth at concentrations below 600 μg/ml. The MICs were similar to those found by Payne et al. (1989) of 512 μg/ml.

3.2 Potassium Sorbate

As with propyl paraben, the MIC values for potassium sorbate was independent of type of strain of *L. monocytogenes* grown in TPB at pH 6. *Listeria monocytogenes* was inhibited by potassium sorbate concentrations above 1750 μg/ml (Table 3.2). Optical density values decreased from approximately 0.4 to 0.2 for cultures containing 1500 μg/ml propyl paraben. This indicates that potassium sorbate slightly reduces growth of *L. monocytogenes* below the inhibitory concentration of 1750 μg/ml. It may be noted that additional experiments at propyl paraben concentrations below 1500 μg/ml may be required to adequately assess this effect.

Table 3.1 Growth of 5 strains of *Listeria monocytogenes* as determined by OD_{630} in tryptose phosphate broth at pH 6 containing propyl paraben incubated at 32°C for 24 hr

Propyl Paraben Concentration [μg/ml]	*L. monocytogenes* Strain				
	Scott A	101	310	108	V7
	OD at 630 nm				
0	0.314	0.341	0.348	0.365	0.340
200	0.180	0.179	0.176	0.185	0.174
400	0.188	0.161	0.171	0.167	0.187
600	0.004*	0.006*	0.008*	0.009*	0.007*
800	0.004	0.007	0.012	0.006	0.007
1000	0.003	0.002	0.007	0.006	0.004

* indicates the minimum inhibitory concentration [MIC]

Table 3.2 Growth of 5 strains of *Listeria monocytogenes* as determined by OD_{630} in tryptose phosphate broth containing potassium sorbate at pH 6 incubated at 32°C for 24 hr.

Propyl paraben Concentration [μg/ml]	*L. monocytogenes* Strain				
	Scott A	101	310	108	V7
	OD at 630 nm				
0	0.497	0.378	0.449	0.312	0.299
1500	0.309	0.248	0.285	0.276	0.188
1750	0.009*	0.004*	0.060*	0.007*	0.013*
2000	0.015	0.005	0.052	0.020	0.052
2250	0.004	0.007	0.003	0.003	0.020
2500	0.009	0.007	0.021	0.014	0.009
2750	0.002	0.004	0.013	0.014	0.004
3000	0.002	0.004	0.200	0.006	0.010

* indicates the minimum inhibitory concentration [MIC]

The surface tension of aqueous potassium sorbate solutions was measured as a function of potassium sorbate concentration to determine the interfacial properties of the potassium sorbate (Figure 3.1). The surface tension decreases as more potassium sorbate is added to the system, possibly indicating that more material is adsorbed at the air-liquid interface. The surface tension becomes concentration independent at potassium sorbate concentrations above 2000 μg/ml. This could be explained in terms of the interface being saturated with potassium sorbate molecules, thus any additional potassium sorbate molecules would remain in the aqueous phase. This could be seen as a pseudo-critical micellar concentration (CMC) even though the potassium sorbate is not expected to form micelles. Interestingly, we observed a possible relationship between the pseudo-CMC and the MIC since both have the same value. This would indicated that for potassium sorbate to become fully active against strains of *L. monocytogenes*, as much potassium sorbate as possible must be present at the interface (cell membrane). This could indicate that a manipulation of the surface activity, that is, a reduction of the CMC could improve antimicrobial efficiency.

3.3 Lysozyme

A number of authors (Ibrahim et al., 1996a, 1996b, 1996c, Masschalck et al, 2001) have reported that modification of lysozyme through either heat or high hydrostatic pressure treatment can alter antimicrobial activity of lysozyme. These studies indicated that antimicrobial activity of the lysozyme against certain bacteria may be increased by applying one of these processing techniques. The mechanisms involved were suggested to be attributed to partial denaturation of lysozyme. Enzymatic activity of partially denatured lysozyme was reduced while antimicrobial activity increased, indicating no

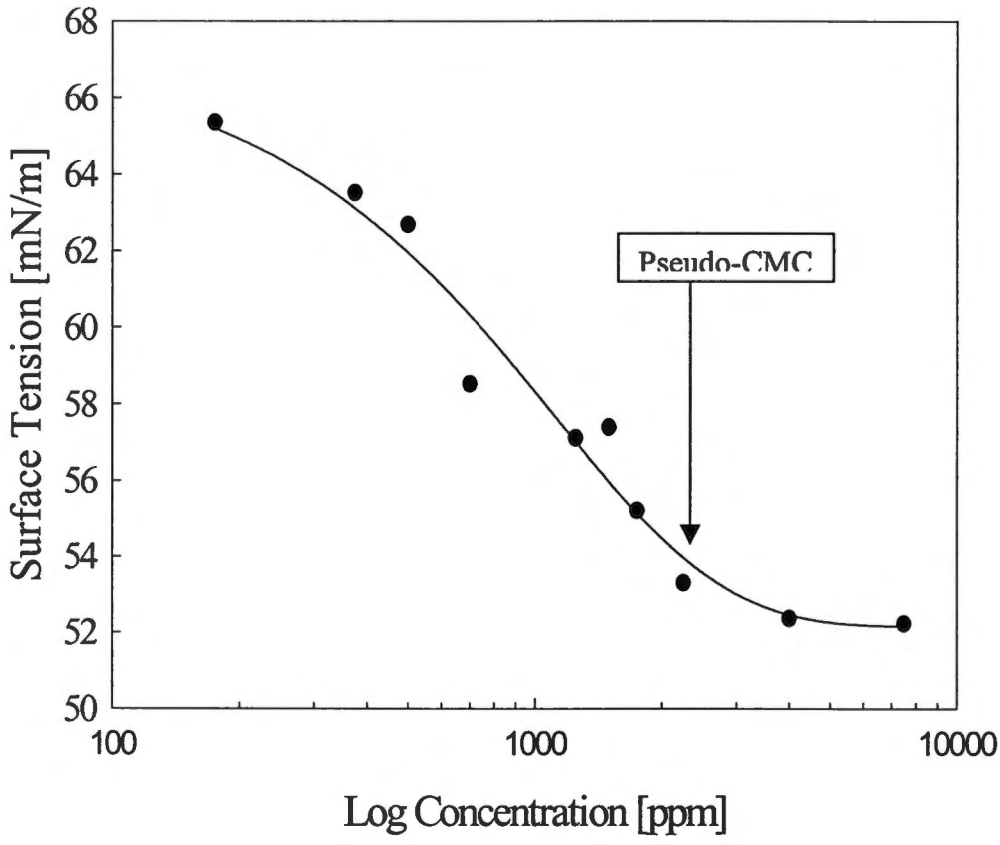

Figure 3.1 Surface tension of aqueous potassium sorbate solution as a function of potassium sorbate concentration. The critical micellar concentration (CMC) is the concentration above which the surface tension is independent of potassium sorbate concentration.

link between the two. This suggests that perhaps a second mechanism of action exists for the partially denatured lysozyme.

The objective of the present study was to investigate if application of ultrasound could be used to enhance the antimicrobial activity of lysozyme by modifying the degree of unfolding of the molecule. In preliminary studies, the activity of native lysozyme against the strains of *L. monocytogenes* was investigated. In subsequent studies, the effect of ultrasound treatment on antimicrobial activity and physicochemical properties of lysozyme was determined. The selection of ultrasound power levels was based on earlier studies conducted in our laboratory using bovine serum albumen (BSA). Results obtained in the experiments with BSA indicated that protein structure could be changed by varying power level of sonicators between 0 and 300 watts.

Unlike previously tested antimicrobials (propyl paraben and potassium sorbate), the MICs for lysozyme against strains of *L. monocytogenes* depended on thestrain used (Table 3.3). Scott A had the highest MIC of 350 μg/ml lysozyme. The four remaining strains, 101, 310, 108, and V7 were inhibited at lysozyme concentrations above 300 μg/ml. The OD readings did vary both between and within strains. The values given are means from all replications. Inhibition may have been observed for 3 of the 4 reps for a particular strain at a particular lysozyme concentration, but the OD value for the fourth rep may be responsible for the variation in the means. Various authors reported that pure lysozyme did not inhibit growth of *L. monocytogenes* at concentrations of 100-200 μg/ml (Hughey and Johnson, 1987, Payne et al., 1994, Masschalck et al., 2001). Hughey and Johnson (1987) for example reported that four strains of *L. monocytogenes* were not inhibited at lysozyme levels below 200 μg/ml, but that 100 μg/ml lysozyme combined

Table 3.3 Growth of 5 strains of *Listeria monocytogenes* as determined by OD_{630} in tryptose phosphate broth at pH 6 containing lysozyme incubated at 32°C for 24 hr

	L. monocytogenes Strain				
Lysozyme Concentration [μg/ml]	Scott A	101	310	108	V7
	OD at 630 nm				
0	0.452	0.393	0.360	0.367	0.465
300	0.095	0.006*	0.000*	0.006*	0.012*
350	0.004*	0.001	0.001	0.003	0.094
400	0.063	0.002	0.000	0.003	0.003
450	0.116	0.002	0.000	0.002	0.002
500	0.021	0.002	0.000	0.011	0.028
550	0.062	0.078	0.000	0.012	0.009
600	0.012	0.006	0.003	0.016	0.008

*indicates minimum inhibitory concentration (MIC)

with 0.37 mg/ml EDTA did inhibit microbial growth on brain heart infusion agar. Payne and coauthors (Payne et al., 1994) showed that lysozyme at concentrations of 100-200 μg/ml had no effect on growth of *L. monocytogenes*.

To determine the effect of ultrasound treatment on lysozyme, the adsorption kinetics of lysozyme were measured. Measurements of surface tension of native lysozyme as a function of time were obtained as a standard for comparison to later treatments (Figure 3.2). The adsorption kinetics were influenced by concentration, that is, the surface tension of 100 μg/ml lysozyme after 500 sec was 72 mN/m compared to 59 mN/m for 2000 μg/ml. This indicates that the lysozyme migration to the air-water interface depends on the concentration in the subsurface and is a diffusion controlled process. The more lysozyme in the subsurface, the faster the adsorption. This has been previously reported by Miller et al. (2001) and a detailed theoretical description of the process can be found there. The adsorption kinetics did not increase above 500 μg/ml, which is fairly close to the MICs of 300 and 350 μg/ml. This may indicate that lysozyme behaves like potassium sorbate in that when the interfaces are covered no further change in surface tension is detected. With lysozyme the rate of change becomes constant, and with potassium sorbate the surface tension becomes constant.

The adsorption kinetics of lysozyme after sonication for 15 minutes at 20 amps are shown in Figure 3.3. Unlike results obtained with native lysozyme, the adsorption of sonicated lysozyme at the air-water interface is nearly independent of lysozyme cocentration. That is, they show a similar time-dependent decrease in surface tension. The adsorption of native lysozyme at a concentration of 100 μg/ml requires a much longer time than the adsorption of native lysozyme at 1000 μg/ml. Sonicated

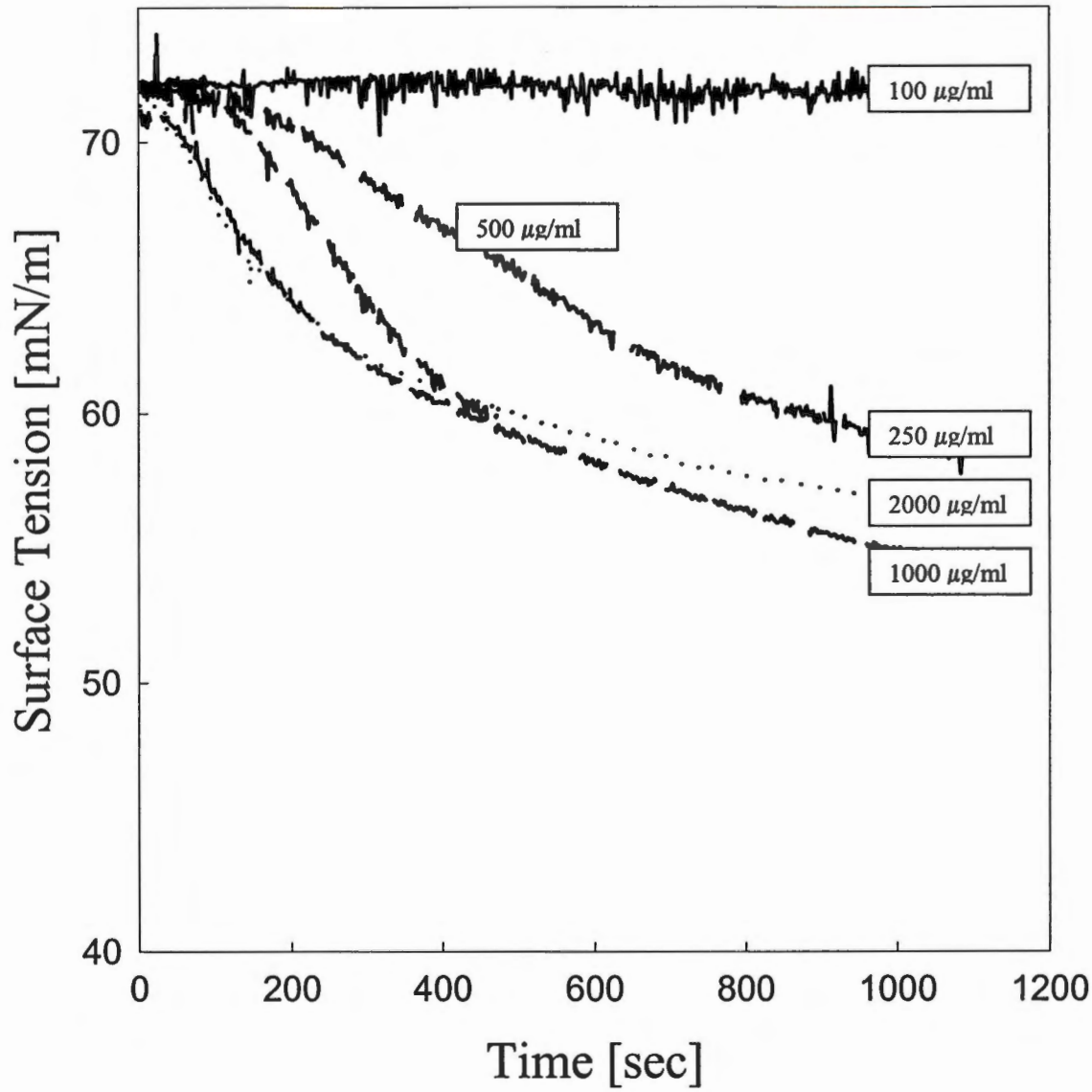

Figure 3.2 Surface tension of aqueous solutions of native lysozyme at concentrations of 100, 250, 500, 1000, and 2000 μg/ml as a function of time of adsorption at the air-water interface.

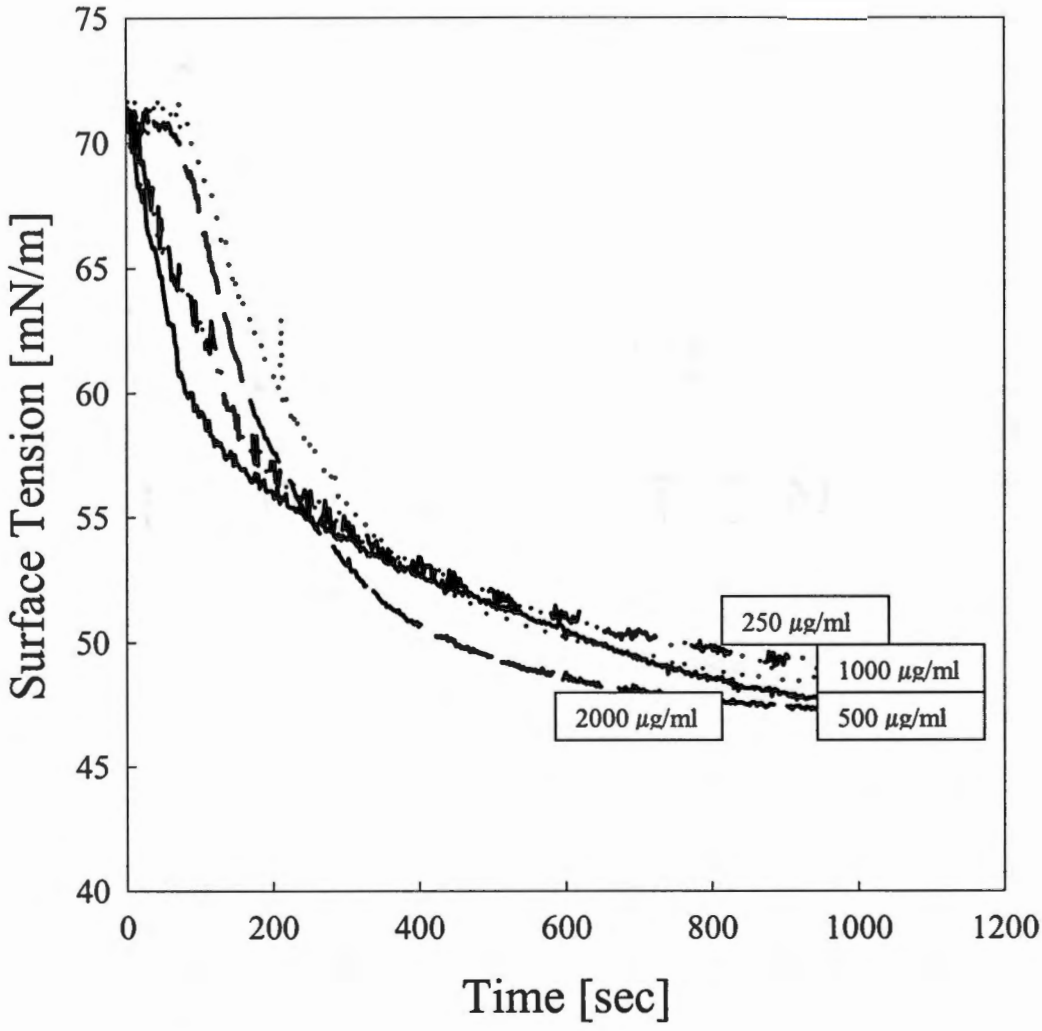

Figure 3.3 Surface tension aqueous lysozyme solution at 250, 500, 1000, and 2000 μg/ml sonicated at 20 amps for 15 minutes as a function of adsorption time.

lysozyme at 100 μg/ml adsorbs as rapidly as sonicated lysozyme at 1000 μg/ml. This would seem to indicate sonicated lysozyme reaches full surface coverage at lower concentrations. A direct comparison of native and 20 amp sonicated lysozyme surface tension curves at 100 μg/ml is shown in Figure 3.4. The difference observed in the curves could possibly be explained by denaturation or partial denaturation of lysozyme. The altered structure of lysozyme would have a marked effect on decrease of surface tension as a function of time. No effect was found with longer sonication times on the adsorption kinetics (data not shown). A possible explanation is that lysozyme obtained after 15 min sonication at 20 amps was fully denatured. If the protein were fully denatured, there would be an associated change in the tertiary structure of lysozyme. Hydrophobic groups that had previously been buried in the inside of the molecule could be exposed to the aqueous phase (DeMan, 1999). These exposed groups could increase the thermodynamic tendency of proteins to be removed from the interface and also alter unfolding processes that have to occur at the interface. This would result in a faster migration, causing the surface tension to decrease more rapidly with time than the surface tension of the native lysozyme.

The changes in lysozyme indicated by interfacial properties did not result in increased antimicrobial activity of the compound against *L. monocytogenes* (Table 3.4). Minimum inhibitory concentrations increased from 300-350 μg/ml for native lysozyme to 500-2000 μg/ml for sonicated lysozyme for strains Scott A and 101. Strains 310 and V7 were not inhibited by 2000 μg/ml ultrasound treated lysozyme. Strain 108 was inhibited at 250 μg/ml but not at 500 μg/ml or 1000 μg/ml. The results were therefore inconclusive for this strain. These results would seem to indicate that denaturation or partial

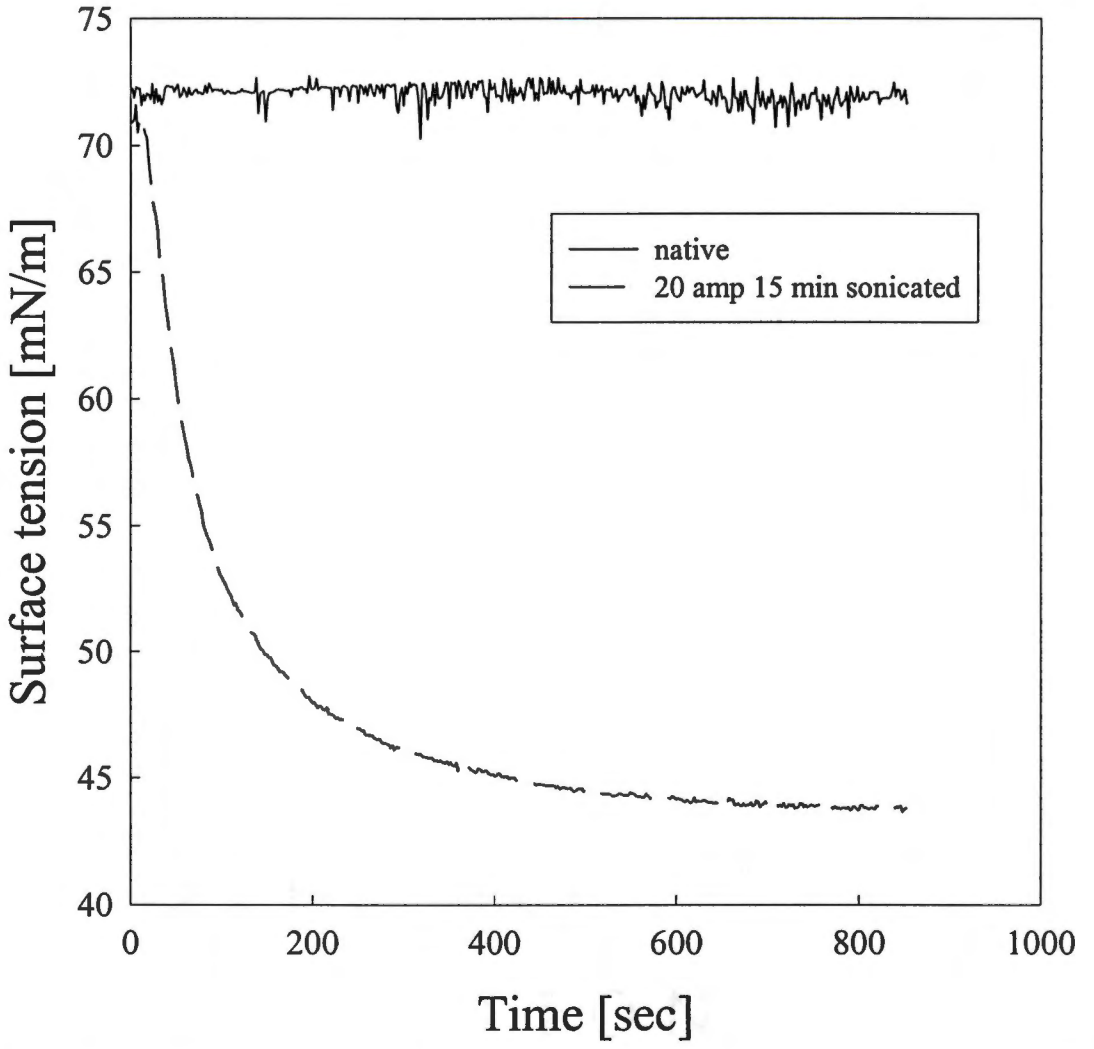

Figure 3.4 Surface tension of 100 μg/ml native lysozyme and lysozyme sonicated at 20 amps for 15 minutes as a function of time.

Table 3.4 Growth of 5 strains of *Listeria monocytogenes* as determined by OD_{630} in tryptose phosphate broth at pH 6 containing lysozyme sonicated at 20 amps for 15 minutes incubated at 32°C for 24 hr.

	L. monocytogenes Strain				
Lysozyme Concentration [μg/ml]	Scott A	101	310	108	V7
	OD at 630 nm				
0	0.277	0.229	0.333	0.240	0.269
100	0.208	0.243	0.255	0.112	0.327
250	0.203	0.188	0.242	0.028	0.318
500	0.169	0.011*	0.309	0.119	0.129
1000	0.217	0.208	0.312	0.285	0.230
2000	0.034*	0.023	0.373	0.004*	0.082

*indicates minimum inhibitory concentration [MIC]

denaturation of lysozyme using ultrasound treatment at 20 amps for 15 minutes has a negative effect on the antimicrobial activity. In contrast to native lysozyme, ultrasound treated lysozyme was much less consistent in its antimicrobial activity against *L. monocytogenes*.

To further examine the effects of ultrasound on interfacial properties and antimicrobial activity of lysozyme, power levels ranging from 0-300 watts or 0-40 amps were tested. Lysozyme sonicated at 300 watts did indeed show different adsorption kinetics than either native lysozyme, or 20 amp sonicated lysozyme (Figure 3.5). The lag time for 300 watt sonicated lysozyme is longer than that of 20 amp treated lysozyme, but shorter than that for native lysozyme. We hypothesize that this is because lysozyme was partially denatured. The antimicrobial activity of partially denatured lysozyme is shown in Table 3.5. Analogous to the 20 amp sonicated lysozyme, strain V7 was not inhibited. Strain Scott A was inhibited but the ultrasound MIC was higher than that of native lysozyme (500 μg/ml vs. 350 μg/ml). In contrast, strains 101, 310, and 108 were inhibited at lower concentrations of ultrasound-treated than native lysozyme (250 μg/ml vs. 300 μg/ml). This was particularly noticeable in strain 108 (100 μg/ml vs. 300 μg/ml). This is comparable to an inhibition with lysozyme in combination with synergists such as EDTA. Hughey and Johnson (1987) reported that lysozyme in combination with EDTA at below 100 μg/ml inhibited growth of *L. monocytogenes* compared to no inhibition at 200 μg/ml lysozyme alone. Branen (1998) reported that lysozyme acted synergistically with EDTA to inhibit *L. monocytogenes*.

An explanation for this enhanced inhibitory effect of sonicated lysozyme is that the exposure of hydrophobic groups that were previously located at the interior of the

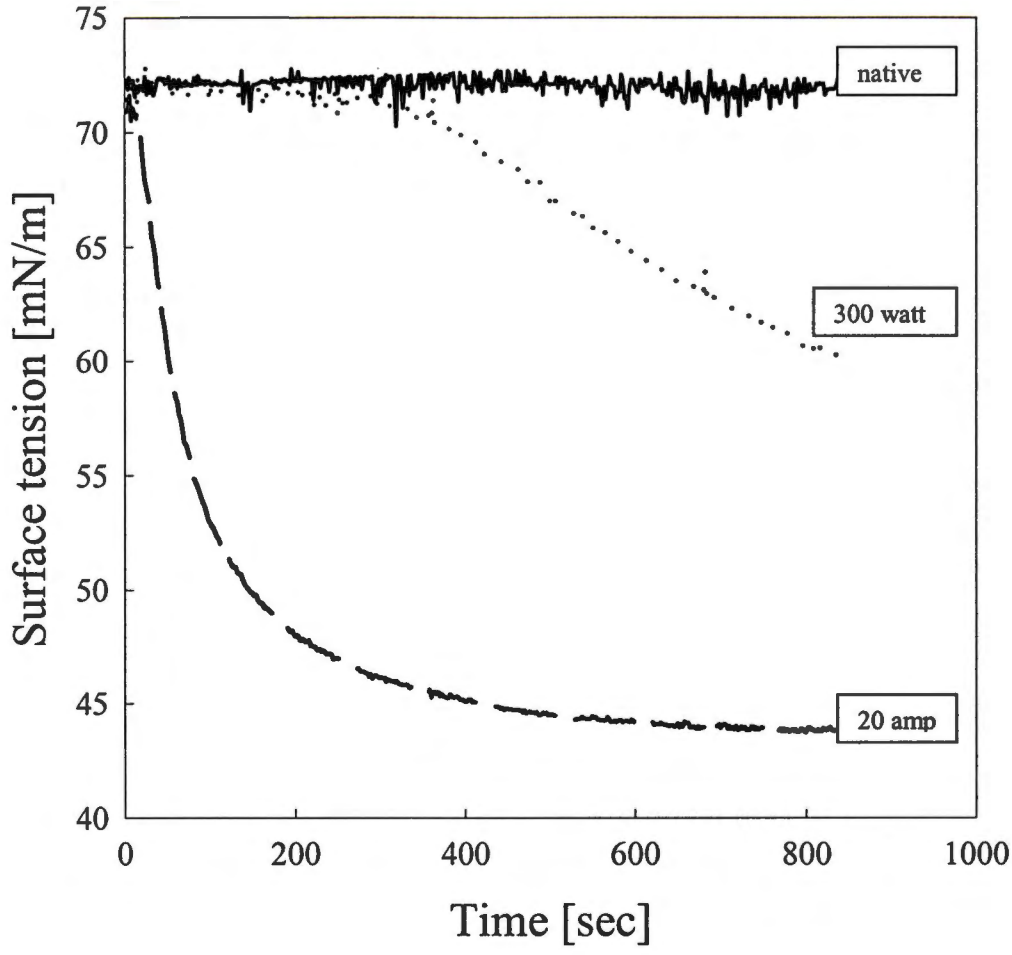

Figure 3.5 Adsorption kinetics curves for native, 300 watt/15 min sonciated, and 20 amp/15 min sonicated lysoyzme at a concentration of 100 μg/ml.

Table 3.5 Growth of 5 strains of *Listeria monocytogenes* as determined by OD_{630} in tryptose phosphate broth at pH 6 containing lysozyme sonicated at 300 watts for 15 minutes incubated at 32°C for 24 hr

Lysozyme Concentration [μg/ml]	*L. monocytogenes* Strain				
	Scott A	101	310	108	V7
	OD at 630 nm				
0	0.315	0.304	0.330	0.222	0.388
100	0.185	0.242	0.271	0.048*	0.328
250	0.173	0.028*	0.039*	0.035	0.318
500	0.028*	0.018	0.030	0.029	0.296
1000	0.015	0.004	0.029	0.022	0.275
2000	0.018	0.005	0.021	0.021	0.287

*indicates minimum inhibitory concentration [MIC]

molecule enables the lysozyme to attack the surface of the bacterial cells more efficiently. The increased surface activity of lysozyme due to the exposed hydrophobic groups results in acceleration of accumulation of antimicrobial at the bacterial cell membrane. Various researchers (Moriyama et al., 2000, Krishnamurthy et al., 2000, Massachalck et al., 2001) reported that disulfide free lysozyme no longer retained its structure or enzymatic activity. If the protein was only partially denatured it may retain some enzymatic activity in addition to enhanced binding properties. Moriyama and coauthors (2000) reported that, if all four disulfide bridges of lysozyme are reduced, the conformation of the lysozyme changed. The helicity of lysozyme with no disulfide bonds was lower than of lysozyme with 2, 3, or 4 disulfide bonds. If only two of the disulfide bonds were reduced, the protein conformation remained virtually unchanged. Krishnamurthy and coauthors (2000) reported that lysozyme sonicated at 50 watts for 15 sec was denatured and lost all enzymatic activity. This was determined by measuring changes in absorbance at 450 nm that occur when the bacterial membrane is degraded. Krishnamurthy observed no decrease in absorbance indicating that the enzyme was indeed inactive. A study by Masschalck et al. (2001) concluded that partially denatured lysozyme was not effective against Gram-negative bacteria at atmospheric pressure conditions, but that under high hydrostatic pressure, partially denatured lysozyme had a greater activity against *Salmonella* Typhimurium than native lysozyme.

In subsequent experiments, an attempt was made to replicate or improve partial denaturation of the lysozyme. Attempts were unsuccessful. It was found that the Cole Parmer ultrasonic homogenizer had not been tuned during this particular run as noted by the nonturbulent flow and the high-pitched sound of the sonicator. The Misonix 3000,

which is auto-tuning, could not reproduce these results. Different power levels and time combinations were tried with both machines and the adsorption kinetics results were generally very similar to those found with the 20 amp 15 minute treatment which showed apparent full denaturation. Substantially lower energy levels would be required to reproduce the partial denaturation. The effect of power level with a constant time (15 min) is shown in Figure 3.6. Sonication at 15 watts for even 5 seconds was found to give a similar adsorption kinetic result as the 20 amp 15 min curve (Figure 3.7). Next, 250 μg/ml lysozyme solutions were sonicated at 40 amps for increasing times (0, 1, 5, 7.5, 10, 12.5, 15 minutes) to investigate the effect of sonication time on antimicrobial activity (Table 3.6). The data illustrates that there was no inhibition of *L. monocytogenes* regardless of the sonication time. Strains 101 and 108 were affected by sonicated lysozyme, but it should be noted that those strains were also inhibited by the native lysozyme at similar concentrations. Therefore, it cannot be concluded that ultrasonication increased or decreased lysozyme efficiency.

From these experiments, we concluded that lysozyme is extremely sensitive to ultrasound treatment and even treatments for a few seconds are sufficient to irreversibly alter the protein configuration. This hypothesis is supported by the work of Krishnamurthy (2000) and Tabata (1993) both of whom reported that sonication for short time periods resulted in a loss of lysozyme activity. Using the auto-tuning Misonix 3000, we were not able to decrease the power level to a point where the lysozyme could be partially denatured.

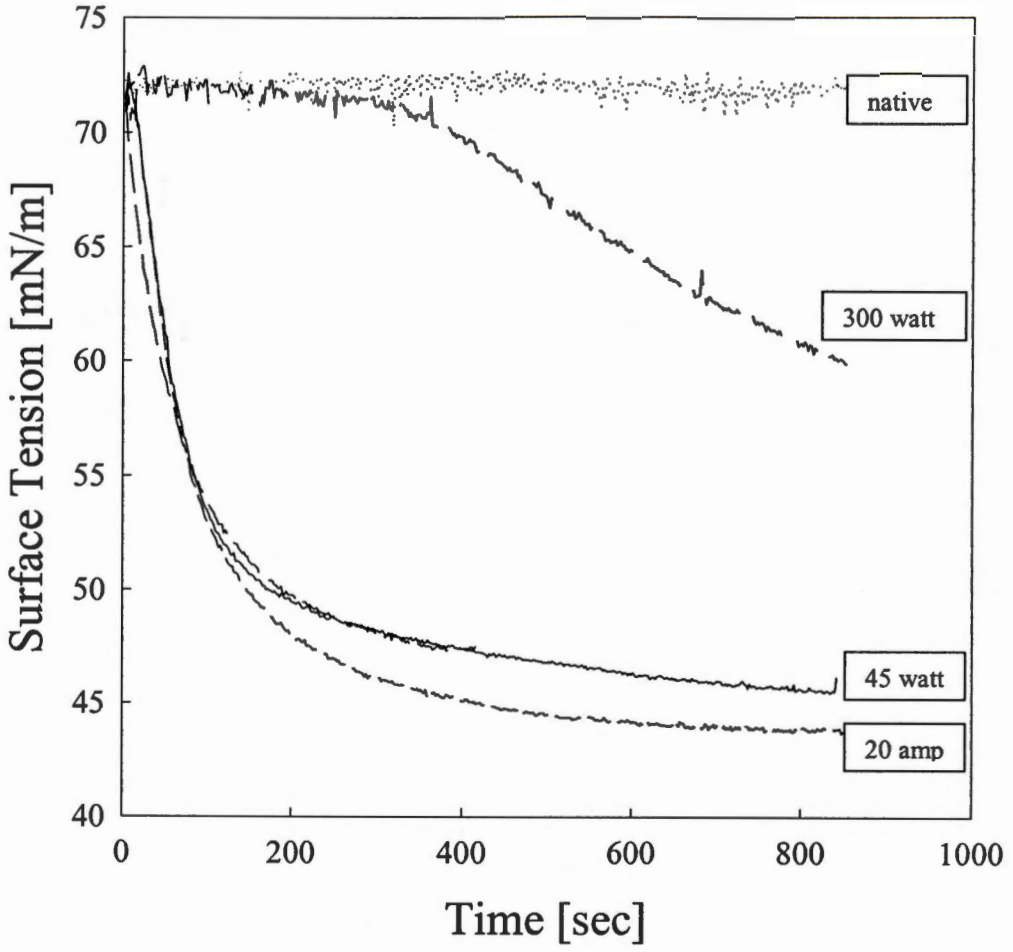

Figure 3.6 Adsorption kinetics of native lysozyme and lysozyme solutions sonicated at 300 watts for 15 minutes, 45 watts for 15 minutes, and 20 amps for 15 minutes. All solutions had a lysozyme concentration of 100 μg/ml.

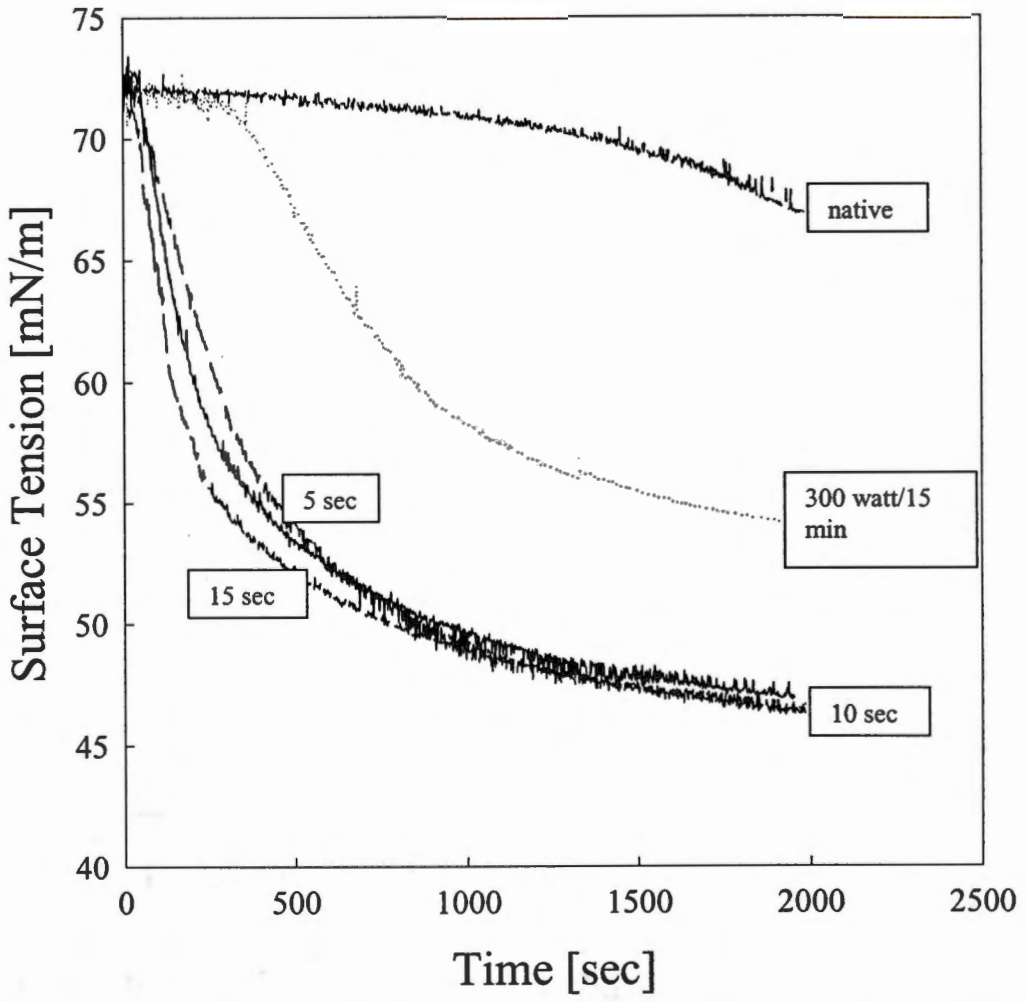

Figure 3.7 Adsorption kinetics of native lysozyme and lysozyme solutions sonicated at 15 watts for 5, 10, and 15 sec and sonicated out-of-tune at 300 watts for 15 minutes. All solutions were at concentration 250 μg/ml.

Table 3.6 Growth of 5 strains of *Listeria monocytogenes* as determined by OD_{630} in tryptose phosphate broth at pH 6 containing 250 μg/ml lysozyme sonicated at 40 amps for 1-15 minutes incubated at 32°C for 24 hr

	L. monocytogenes Strain				
Sonication Time [min]	Scott A	101	310	108	V7
	OD at 630 nm				
0	0.320	0.285	0.375	0.224	0.284
native	0.176	0.005*	0.237	0.006*	0.269
1	0.174	0.005	0.244	0.008	0.249
5	0.177	0.002	0.221	0.000	0.264
7.5	0.160	0.007	0.214	0.003	0.214
10	0.182	0.006	0.233	0.006	0.239
12.5	0.181	0.011	0.247	0.011	0.223
15	0.200	0.234	0.286	0.032	0.257

*indicates minimum inhibitory concentration (MIC)

To determine more precisely the degree of denaturation, thermal analysis experiments were conducted using differential scanning calorimetry (DSC). The heat flow into a 20% native and sonicated lysozyme solution was measured to detect possible phase transitions. The native state lysozyme was found to have a denaturation temperature of approximately 75°C, that is, an endothermic peak was found at this temperature. Lysozyme that was sonicated for less than fifteen minutes did not exhibit changes in the denaturation temperature. Lysozyme sonicated for fifteen minutes had a denaturation temperature of approximately 74°C, a difference of only about one degree (Figure 3.8). If the lysozyme had in fact been fully denatured, no phase transition would be observed, that is, the graph would be a straight line. The surface tension data does indicate that lysozyme sonicated at 40 amps for 15 minutes shows the decrease in the surface tension curves we associate with fully denatured lysozyme (Figure 3.9). However, the DSC data would indicate that the denaturation of lysozyme at this power level and time is not complete. Changes in adsorption kinetics that were observed for increasing sonication levels may actually be due to different levels of partial denaturation. In future studies circular dichroism should be used to determine in detail how the structure of the protein is altered by the application of ultrasound.

Several growth kinetic studies were conducted to determine the effect of ultrasound-treated lysozyme on *L. monocytogenes* strains. A preliminary study of *L. monocytogenes* growth without added lysozyme showed normal increases (Figure 3.10). The initial cell concentration was ~10^5 CFU/ml. The OD for all strains except 310 decreased after 12 hours likely indicating cell clumping.

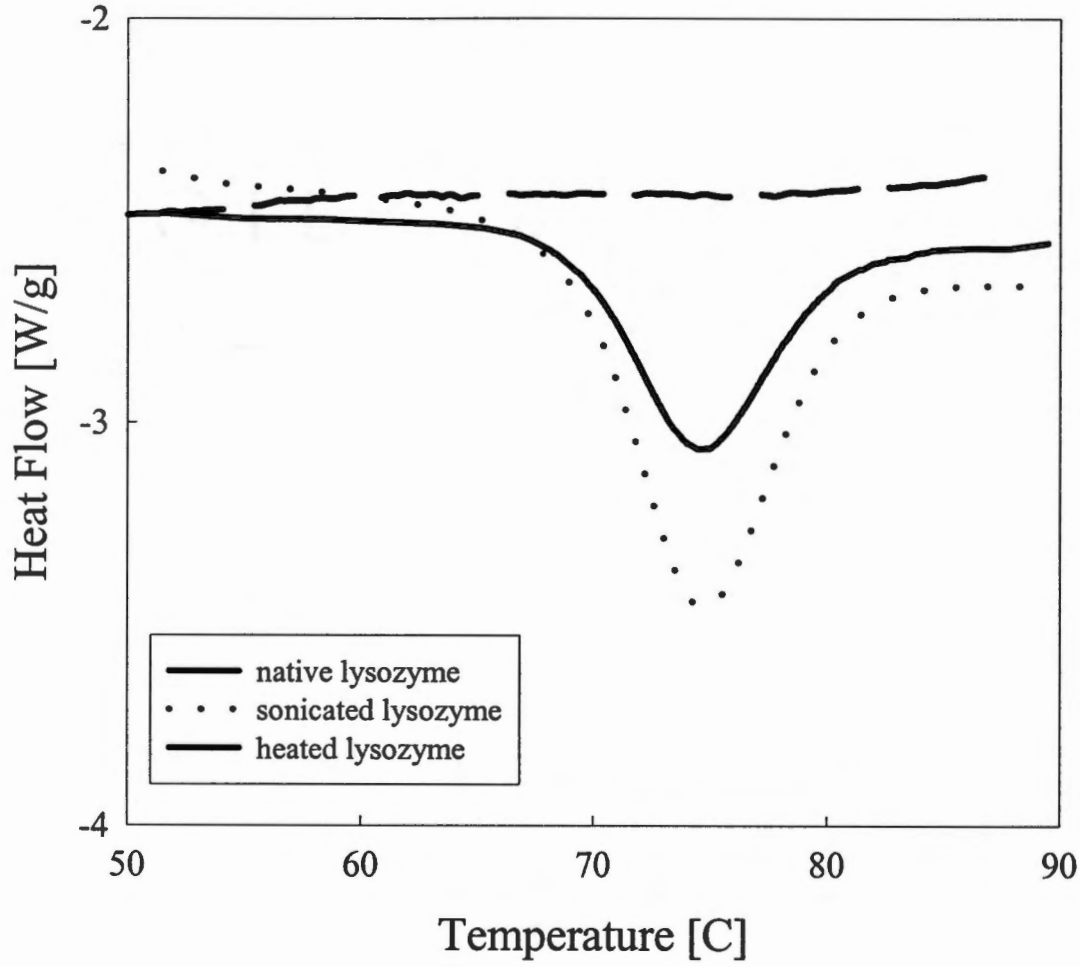

Figure 3.8 Denaturation temperature of native and 40 amp/15 min sonicated lysozyme as measured using differential scanning calorimetry (DSC)

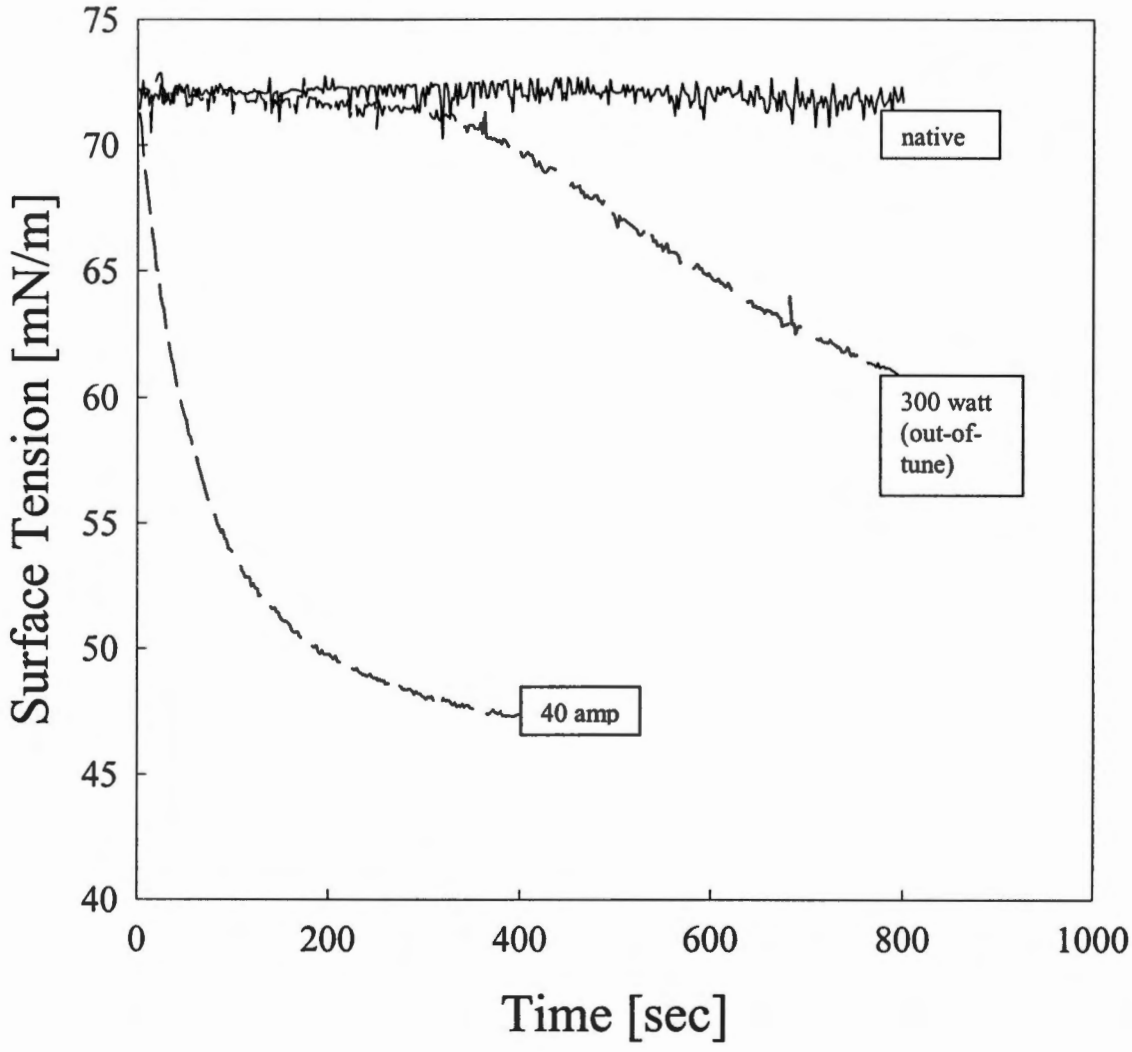

Figure 3.9 Adsorption kinetics of native lysozyme, out of tune 300 watt/15 minute sonicated lysozyme, and 40 amp/15 minute sonicated lysozyme at 100 μg/ml.

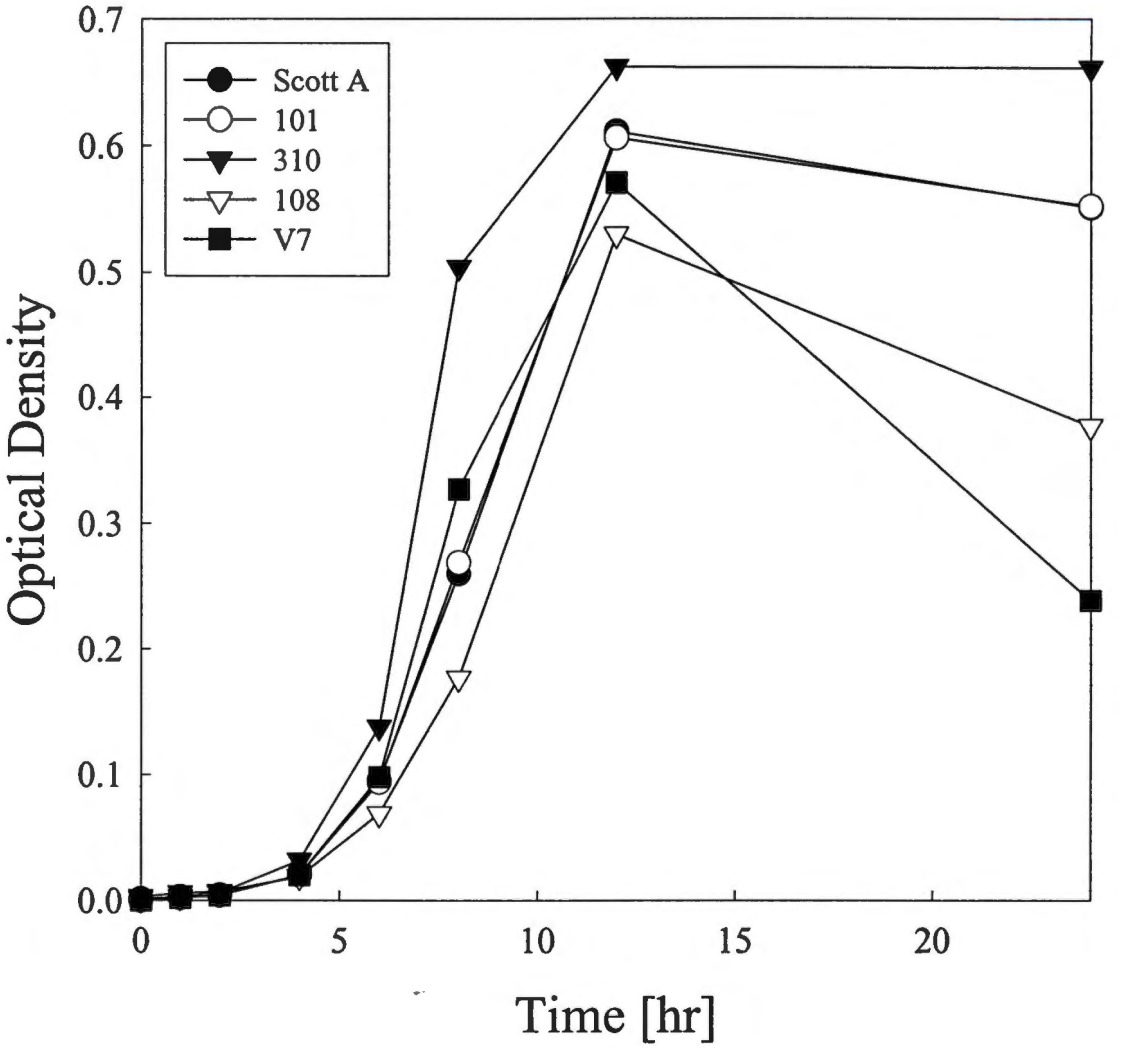

Figure 3.10 Optical density of 5 strains of *Listeria monocytogenes* grown in tryptose phosphate broth at pH 6 at 32°C as a function of incubation time.

The change in optical density readings as a function of incubation time for *L. monocytogenes* strain Scott A at pH 6 at 32°C containing various concentrations (0-600 μg/ml) of lysozyme sonicated at 40 amps for 15 minutes is shown in Figure 3.11. Similar results were obtained for strains 101, 310, and 108 as shown in Figures 3.12-14. The inhibition of *Listeria* with lysozyme varied for different strains. Strains Scott A and 101 showed significant inhibition at 300 μg/ml or greater. Strain 310 is slightly more resistant, with inhibition seen at 500 μg/ml or greater. And finally, strain 108 is the most susceptible, with inhibition seen at 200 μg/ml. All four strains of *L. monocytogenes* were inhibited with the native lysozyme at 500 μg/ml. These wide strain-dependent differences were in contrast to the consistency seen with native lysozyme. While lysozyme sonicated at 40 amps for 15 minutes was not more effective than untreated lysozyme, it was apparently as effective indicating that changes in protein structure did not eliminate activity. According to surface activity measurements, lysozyme treated at 40 amps for 15 minutes should be fully denatured and as such, should have no activity.

Two strains of *E. coli* O157:H7 were also exposed to ultrasound-treated lysozyme and the OD measured for up to 24 hr (Figures 3.15-16). Lysozyme was found to be ineffective against *E. coli*, whether treated with ultrasound or not. This contradicts a study by Ibrahim et al. (1996b), who reported that partially denatured lysozyme at 250 μg/ml was more effective against *E. coli* than native lysozyme, which did not exhibit any activity.

A second kinetic study was performed using the lowest possible power setting (15 watts) and a short treatment time (15 sec) to sonicate lysozyme. Again four strains of *L. monocytogenes* and two strains of *E. coli* O157:H7 were used. The results are shown in

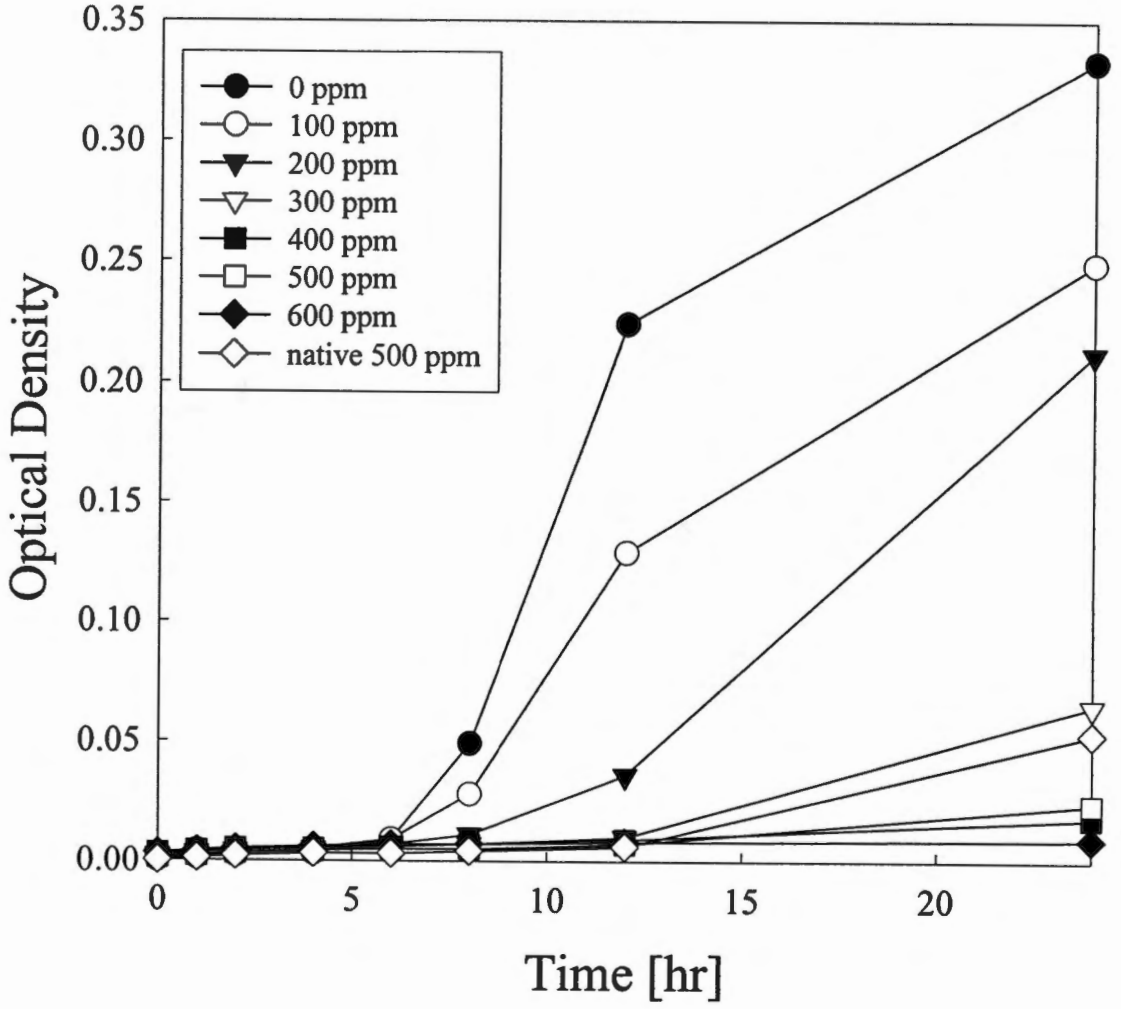

Figure 3.11 Optical density readings of *L. monocytogenes* Scott A treated with lysozyme sonicated at 40 amps for 15 minutes in concentration ranging from 0-600 μg/ml in tryptose phospate broth at pH 6 and 32°C as a function of incubation time.

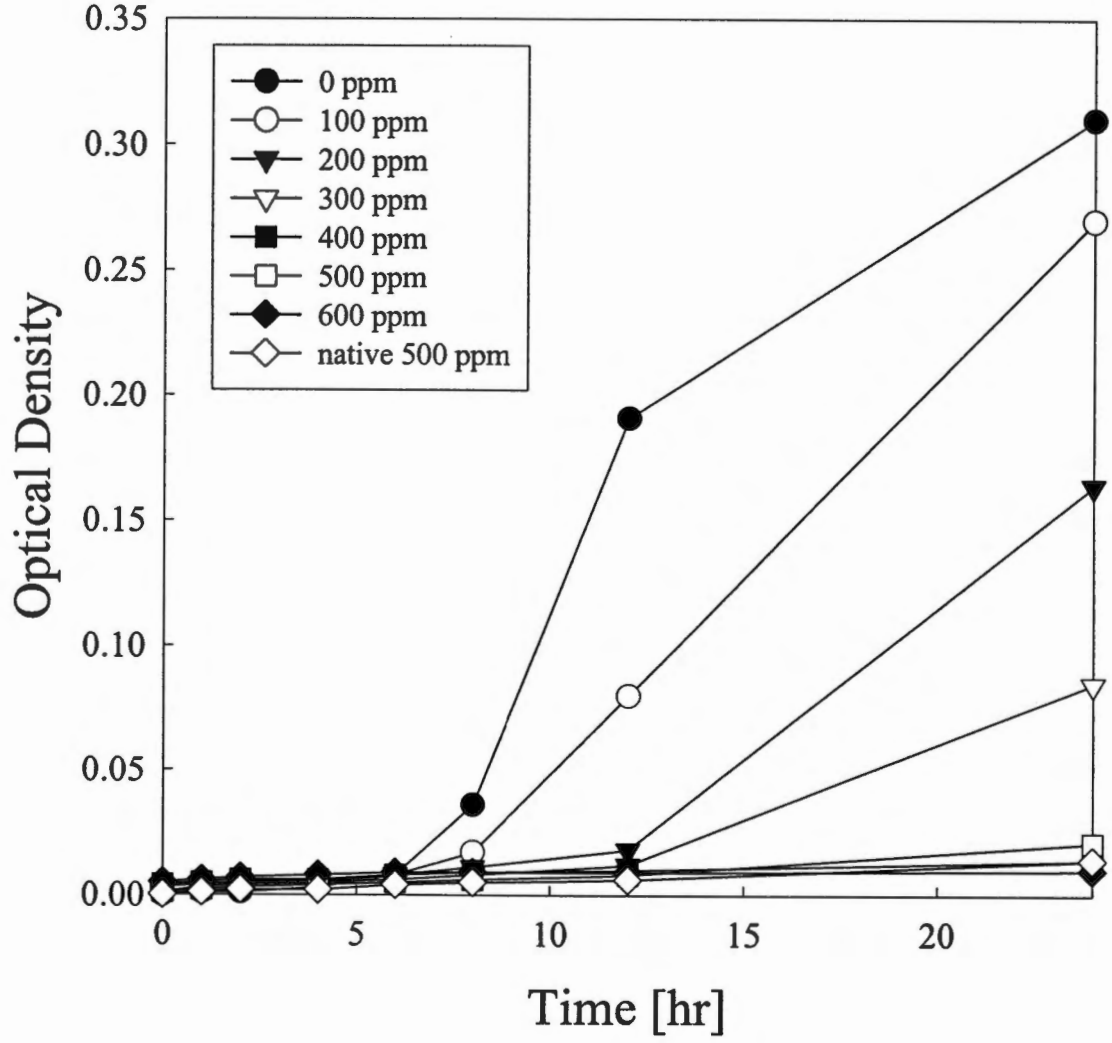

Figure 3.12 Optical density of *L. monocytogenes* 101 containing lysozyme sonicated at 40 amps for 15 minutes at concentrations ranging from 0-600 μg/ml in tryptose phospate broth at pH 6 and 32°C as a function of incubation time.

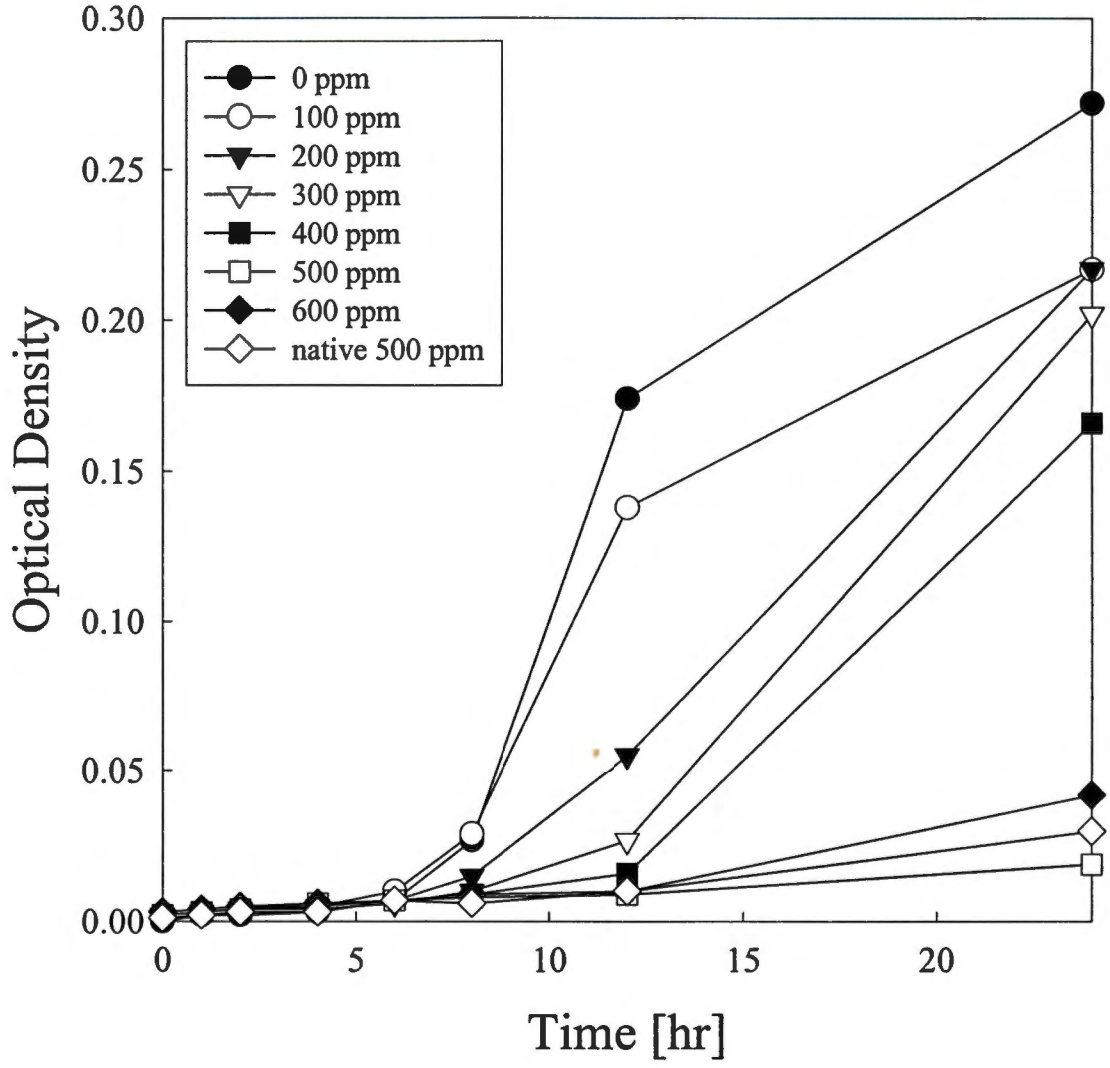

Figure 3.13 Optical density of *L. monocytogenes* 310 containing lysozyme sonicated at 40 amps for 15 minutes at concentrations of 0-600 μg/ml in tryptose phospate broth adjusted to pH 6 and incubated at 32°C over time

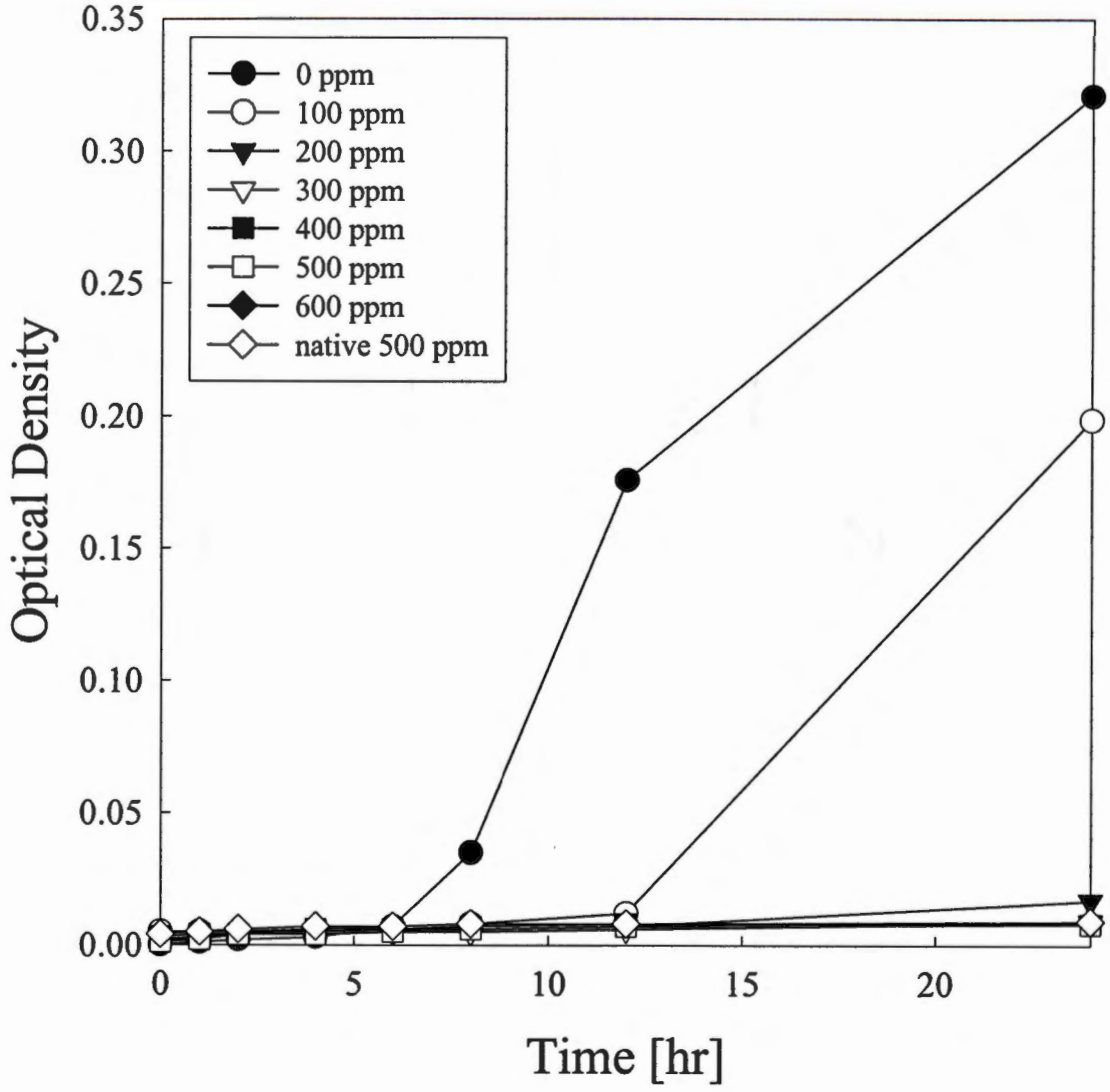

Figure 3.14 Optical density of *L. monocytogenes* 108 cultures containing lysozyme sonicated at 40 amps for 15 minutes at concentrations ranging from 0-600 μg/ml in tryptose phospate broth at pH 6 and 32°C as a function of time.

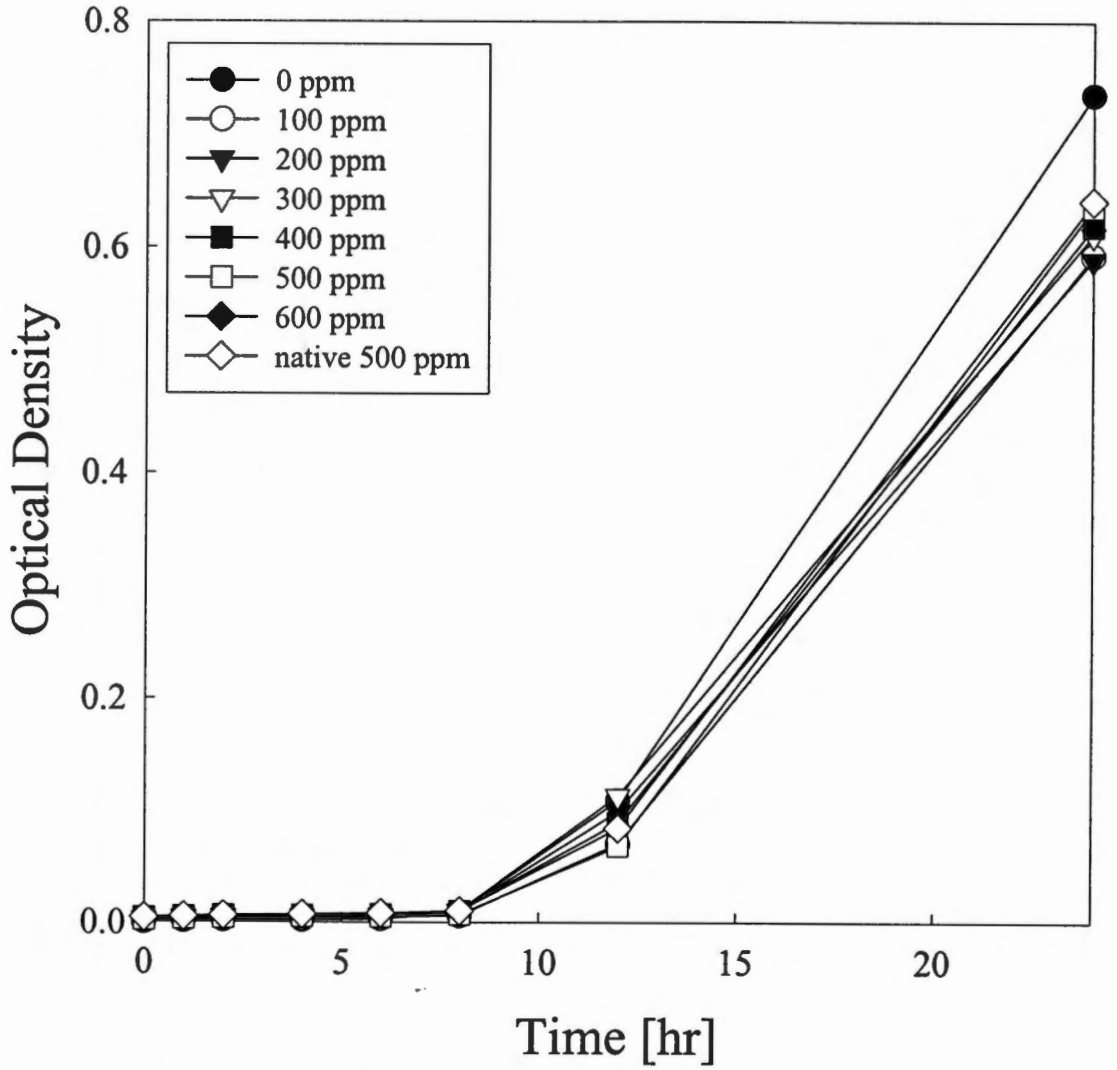

Figure 3.15 Optical density of *E. coli* O157:H7 E0019 cultures containing lysozyme sonicated at 40 amps for 15 minutes at concentrations ranging from 0-600 μg/ml in tryptose phospate broth at pH 6 and 32°C as a function of incubation time.

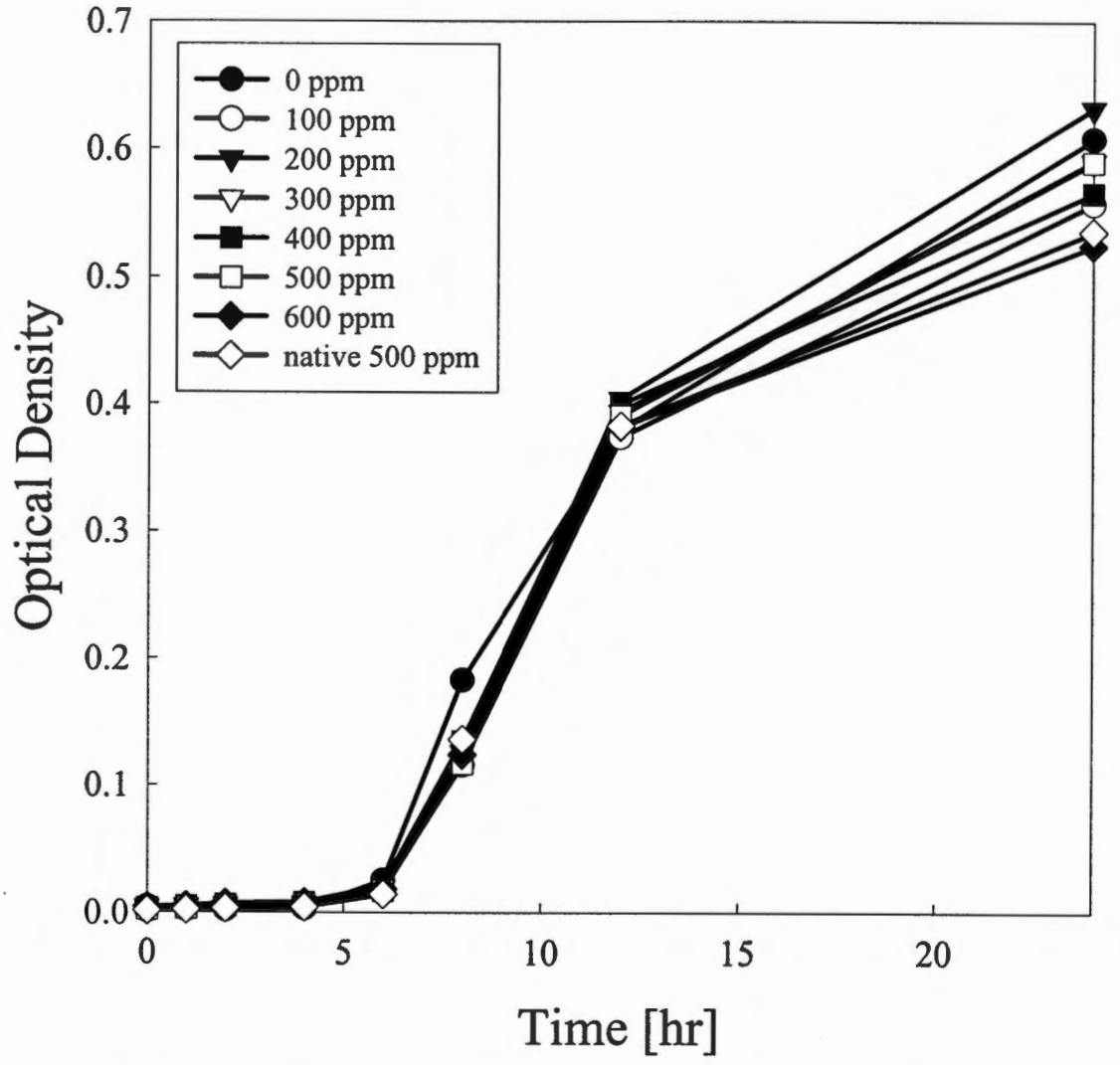

Figure 3.16 Optical density of *E. coli* O157:H7 932 cultures containing lysozyme sonicated at 40 amps for 15 minutes at concentrations ranging from 0-600 μg/ml in tryptose phospate broth at pH 6 and 32°C as a function of incubation time.

Figures 3.17 through 22 and are very similar to those obtained with the 40 amp sonicated lysozyme. Both strains Scott A and 101 were inhibited at 300 to 400 μg/ml, strain 310 was inhibited at 500 μg/ml, and strain 108 was the most susceptible with inhibition level of 200 μg/ml. Once again, *E. coli* was not inhibited by lysozyme in either the native or sonicated state. As in the case of the 40 amp sonicated lysozyme, it must be concluded that ultrasound treatment of lysozyme did not enhance the activity of the enzyme against Gram-negative bacteria and did not enhance nor eliminate the activity of lysozyme against *Listeria monocytogenes*. Again, one of four strains of *L. monocytogenes* tested in the kinetic study was inhibited at a lower lysozyme concentratoin than using native lysozyme.

Ibrahim et al. (1996b) reported increased activity of lysozyme that was partially heat denatured against *E. coli*. An experiment was conducted to replicate those results and prove whether partially denatured lysozyme could be more effective against *Listeria* than native lysozyme. A 250 μg/ml lysozyme solution was made and heated in a water bath for 20 minutes at 80°C. The solution was immediately placed in an ice-water bath for 10 minutes. The quantity of solution used in this experiment was slightly greater than the quantity used by Ibrahim, but this would not account for the differences observed. Iin contrast to the findings of Ibrahim et al. (1996b), we found that heat-treated lysozyme had no antimicrobial activity against either *E. coli* O157:H7 or *L. monocytogenes* (Figure 3.23). The adsorption kinetics of the heat-treated lysozyme was measured to obtain information about the state of the protein. Heated lysozyme had similar adsorption kinetics to that sonicated at 20 amps for 15 minutes (Figure 3.24). This and DSC measurements would indicate that heat-treated lysozyme was not partially denatured as

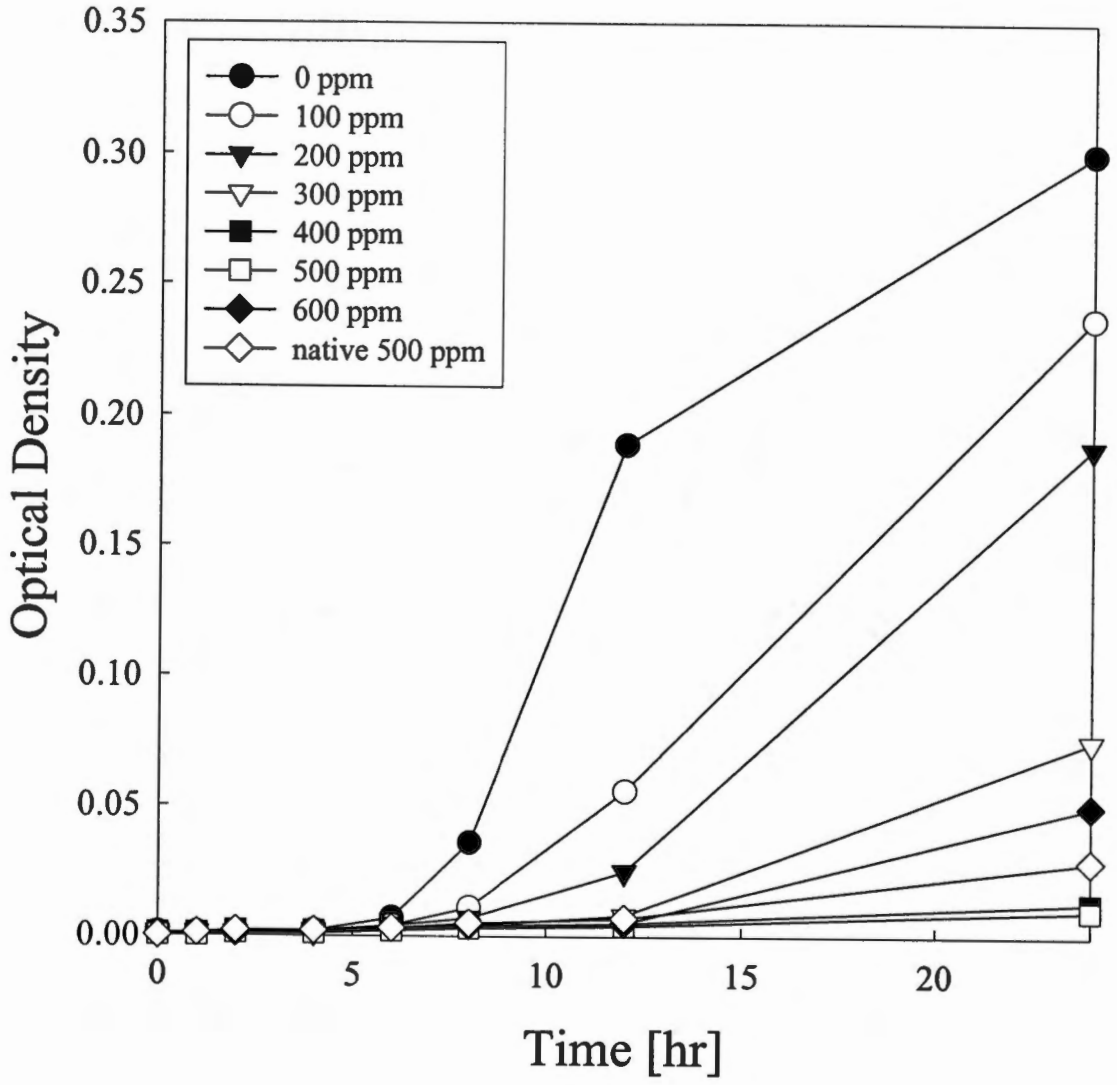

Figure 3.17 Optical density of *L. monocytogenes* Scott A cultures containing lysozyme sonicated at 15 watts for 15 seconds at concentrations ranging from 0-600 μg/ml in tryptose phospate broth at pH 6 and 32°C as a function of incubation time.

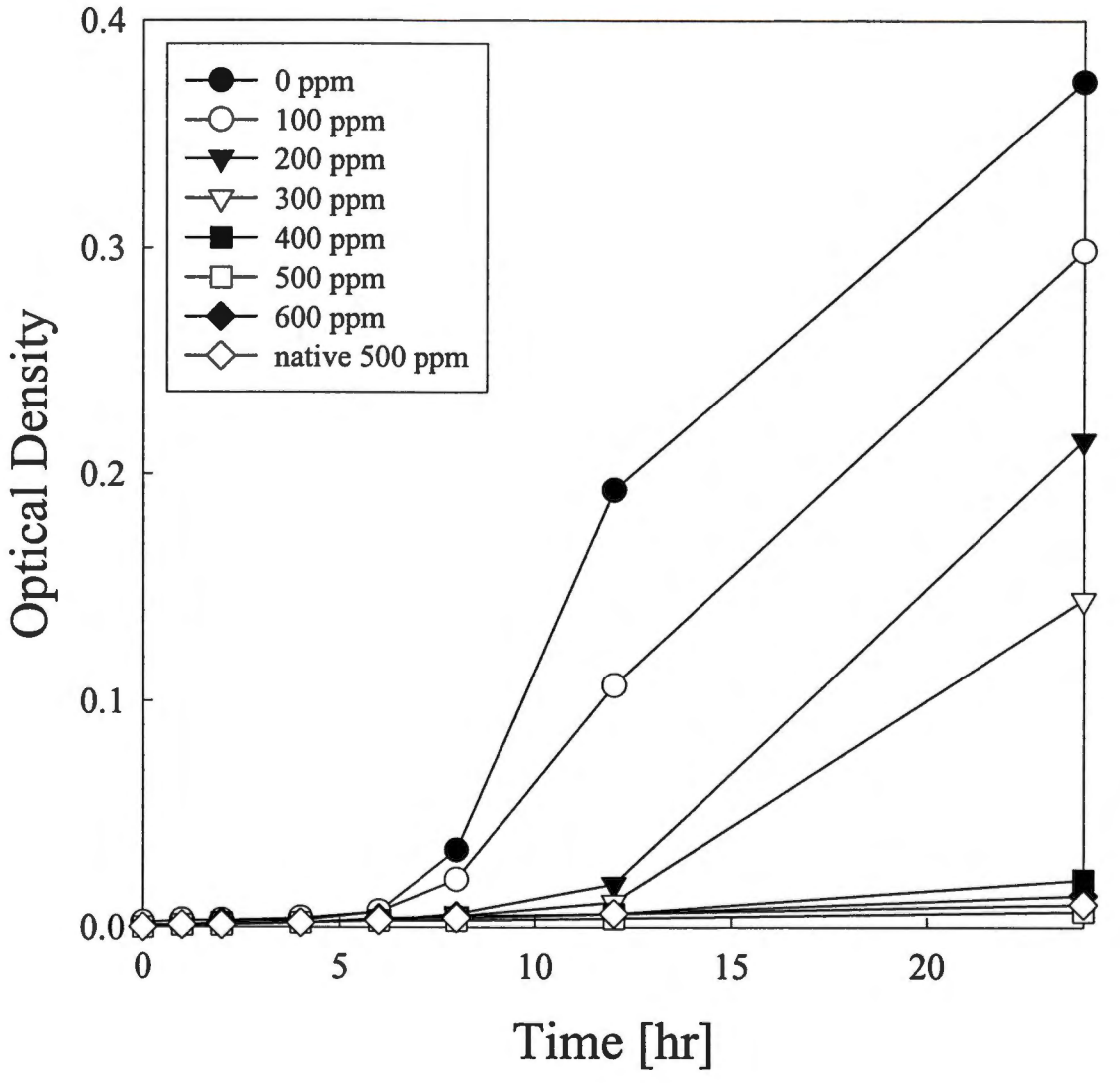

Figure 3.18 Optical density of *L. monocytogenes* 101 cultures containing lysozyme sonicated at 15 watts for 15 seconds at concentrations ranging from 0-600 μg/ml in tryptose phospate broth at pH 6 and 32°C as a function of incubation time.

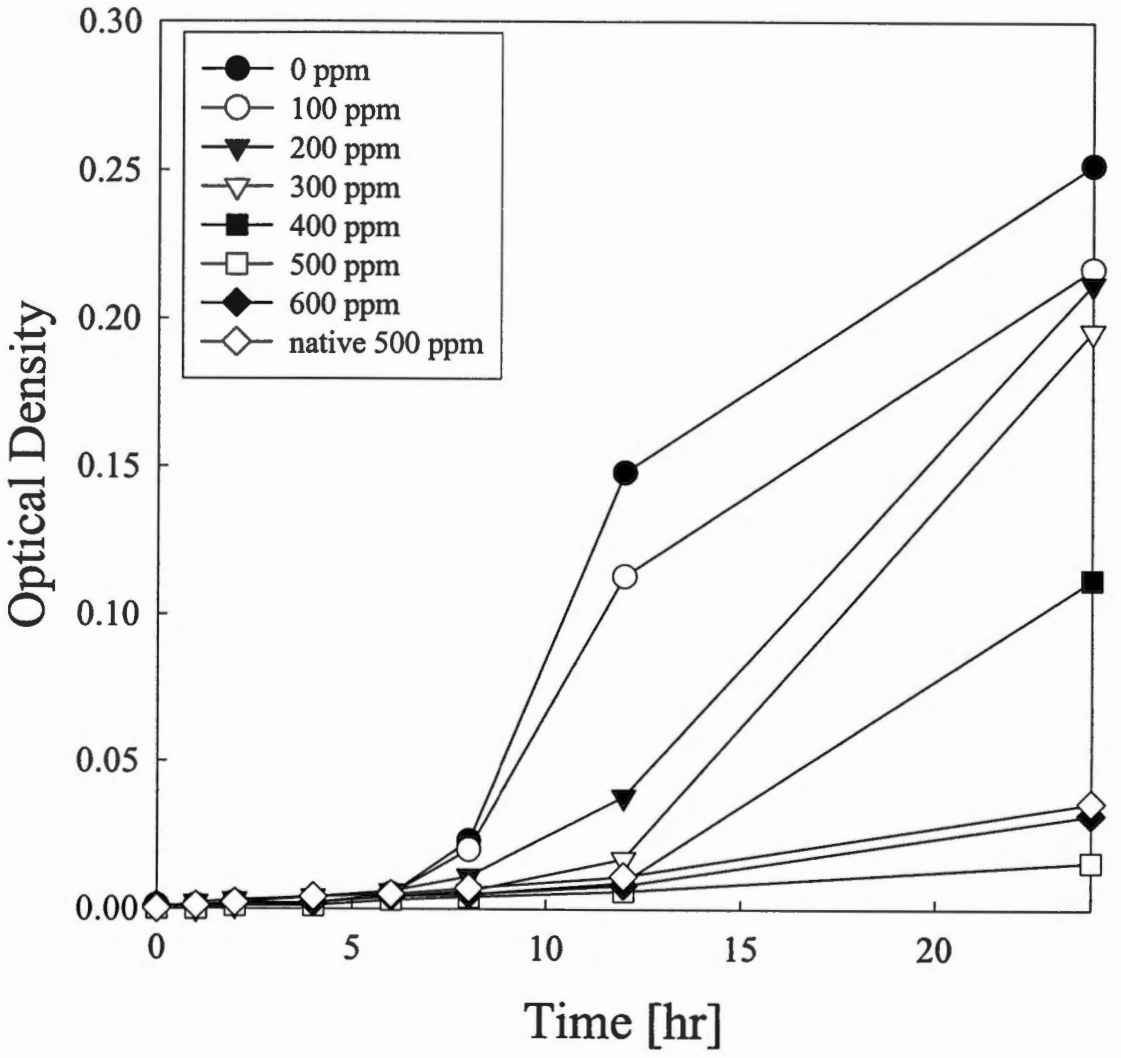

Figure 3.19 Optical density of *L. monocytogenes* 310 cultures containing lysozyme sonicated at 15 watts for 15 seconds at concentrations ranging from 0-600 μg/ml in tryptose phospate broth at pH 6 and 32°C as a function of incubation time.

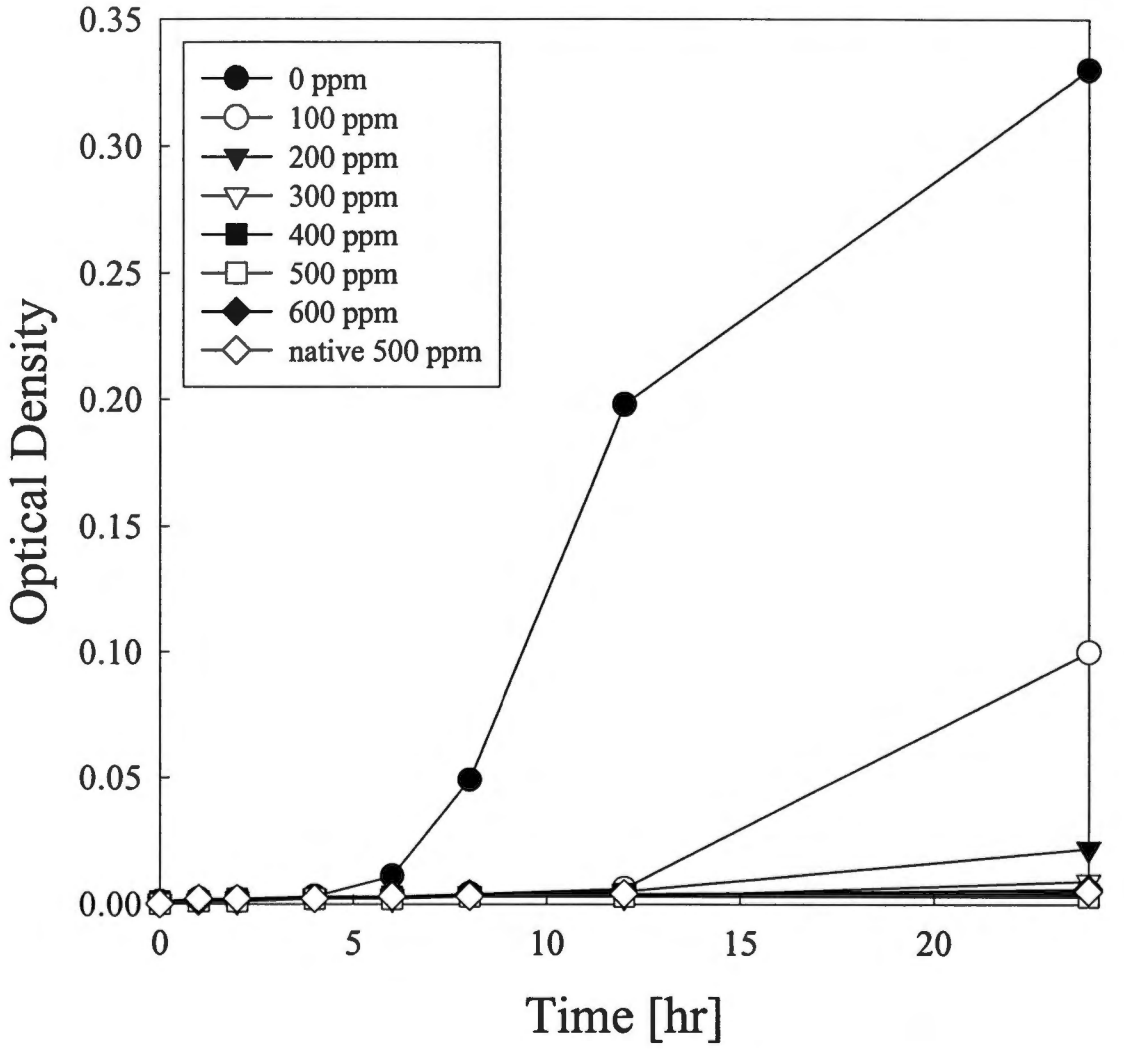

Figure 3.20 Optical density of *L. monocytogenes* 108 cultures containing lysozyme sonicated at 15 watts for 15 seconds at concentrations ranging from 0-600 μg/ml in tryptose phospate broth at pH 6 and 32°C as a function of incubation time.

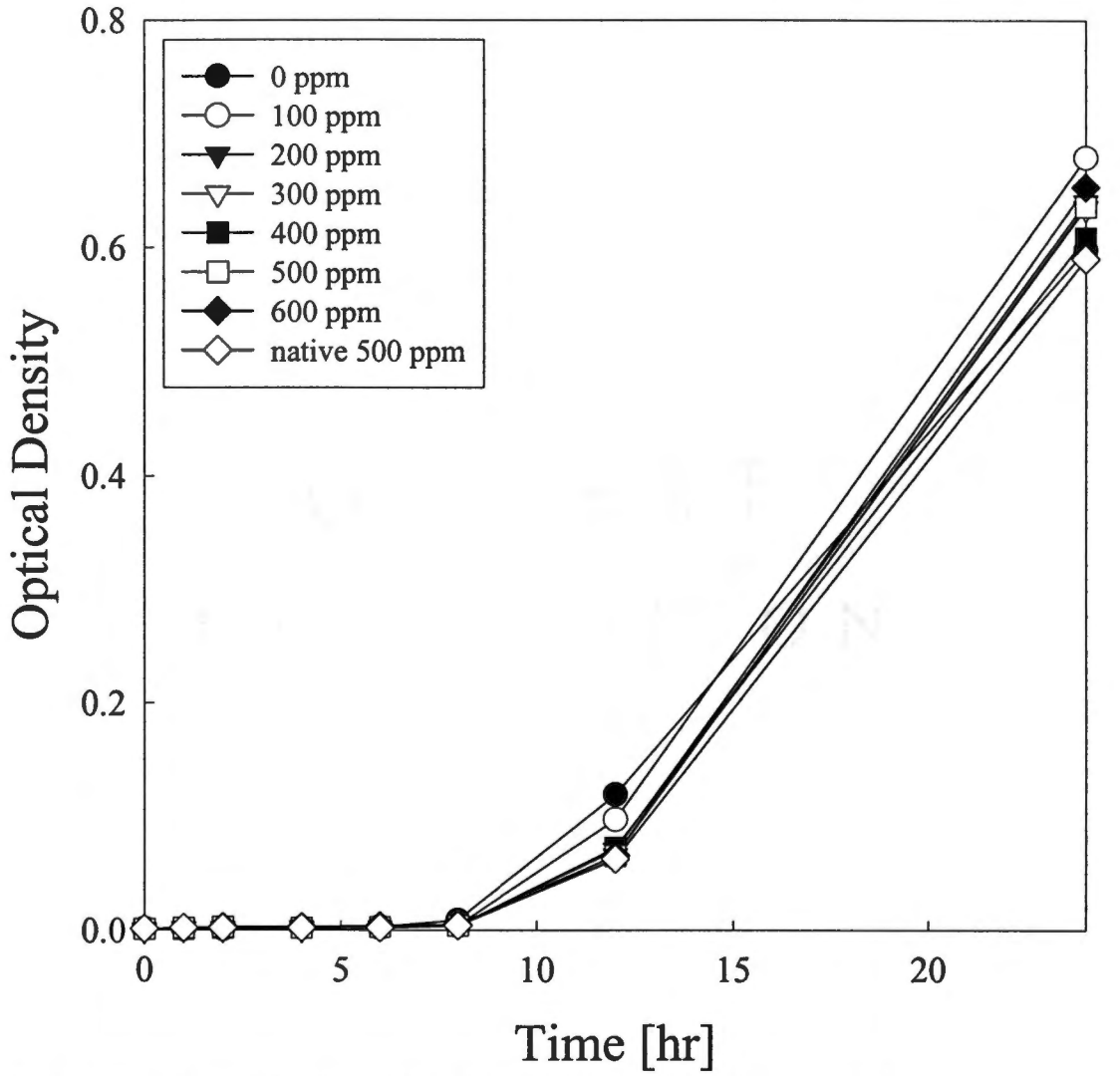

Figure 3.21 Optical density of *E. coli* O157:H7 E0019 cultures containing lysozyme sonicated at 15 watts for 15 seconds at concentrations ranging from 0-600 μg/ml in tryptose phospate broth at pH 6 and 32°C as a function of incubation time.

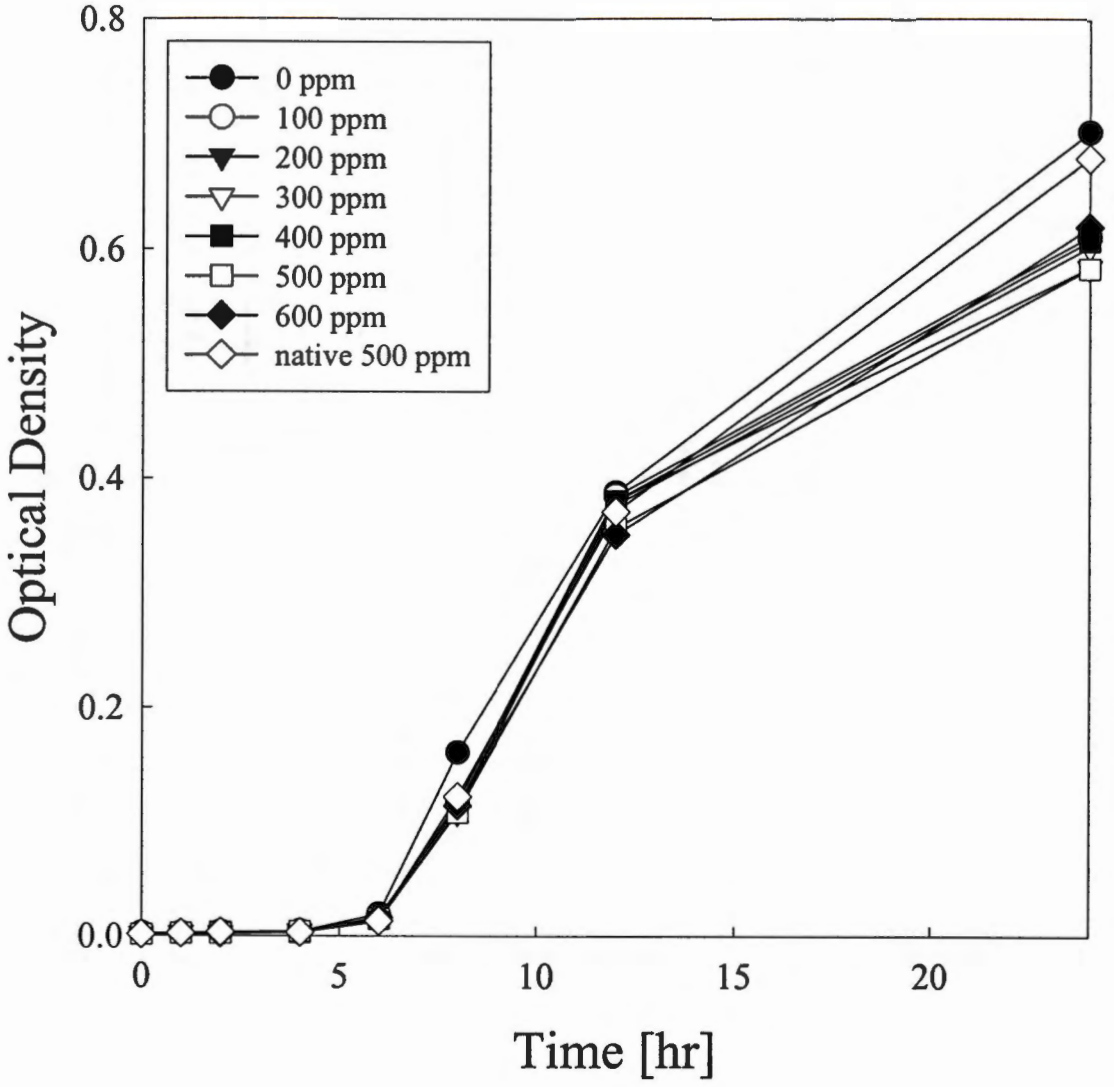

Figure 3.22 Optical density of *E. coli* O157:H7 932 cultures containing lysozyme sonicated at 15 watts for 15 seconds at concentrations ranging from 0-600 μg/ml in tryptose phospate broth at pH 6 and 32°C as a function of incubation time.

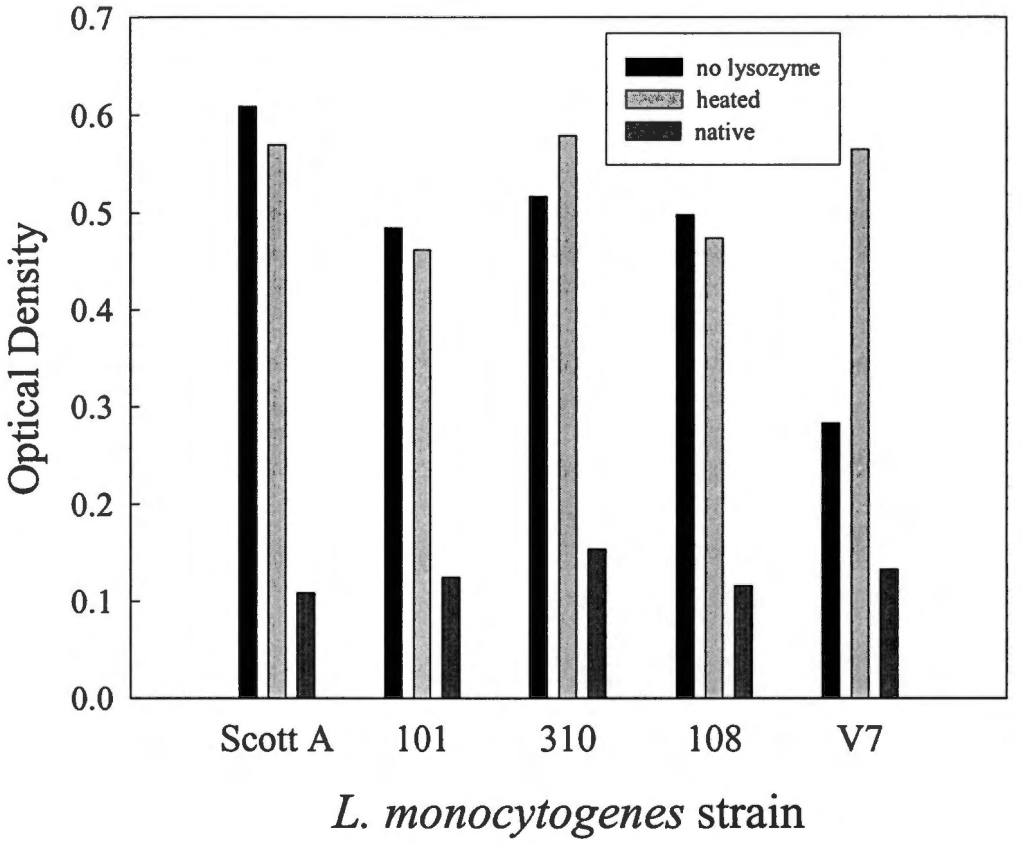

Figure 3.23 Effect of heat at 80°C for 20 minutes on the activity of 250 μg/ml lysozyme against 5 strains of *Listeria monocytogenes* incubated in tryptose phosphate broth at pH 6 for 24 hr at 32°C compared to native lysozyme and no antimicrobial

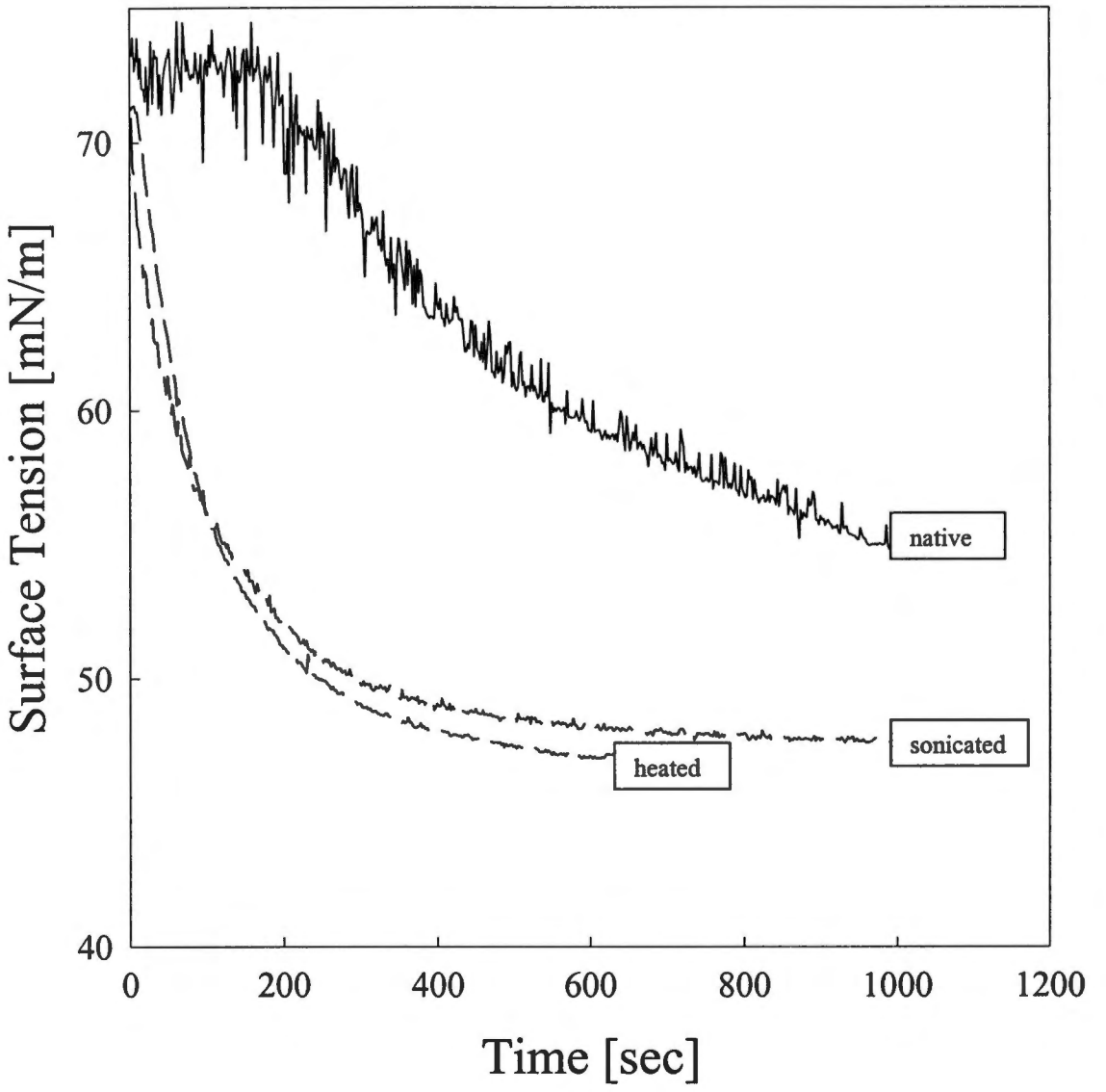

Figure 3.24 Adsorption kinetics of native lysozyme, lysozyme sonicated at 20 amps for 15 minutes, and lysozyme heated at 80°C for 20 minutes. All solutions were at a 250 μg/ml concentration.

reported by Ibrahim et al. (1996b), but instead was fully denatured, thus resulting in loss of enzymatic activity. It should be noted, however, that the strain of *E. coli* used by Ibrahim et al. (1996b) was not the same strain that was used in our experiments.

Statistical analysis (ANOVA) was performed to determine if the observed differences in resistance to ultrasound-treated lysozyme between strains of *L. monocytogenes* were significant. A mean separation test using the Student-Newman-Keuls test was performed on the mean MICs (Table 3.7). Strain 310 had significantly greater MICs than strains 101 and 108. Strain Scott A was significantly more resistant than strain 108. Overall, strain 310 was the most resistant to lysozyme and strain 108 was the most susceptible. Future studies may include research into what causes the differences in resistance between strains, and why one strain is more susceptible to a specific treatment than another.

Table 3.7 Mean minimum inhibitory concentrations of lysozyme treated with various ultrasound treatments against five strains of *Listeria monocytogenes*

Listeria monocytogenes Strain	Ultrasound Treatment										
	none	300w	100w	45w	40amp	20amp	10amp	15w30s	15w15s	Mean	Mean Separation[a]
	MIC [μg/ml]										
Scott A	350	500	1000	1000	1000	2000	2000	2000	2000	1316.7	AB
101	300	250	250	500	250	500	2000	1000	1000	672.2	BC
310	300	250	2000	2000	2000	2000	2000	2000	2000	1616.7	A
108	300	100	250	500	500	250	500	500	1000	433.3	C
V7	300	500	2000	2000	2000	500	2000	100	250	1072.2	ABC

[a]Student-Newman-Keuls analysis

4. Conclusions

This study has shown the antimicrobial activity and interfacial properties of potassium sorbate may be related. This could suggest that controlling physicochemical properties of antimicrobials could enhance their activity. Future work in this area should be focused on expanding the range of test antimicrobial compounds to determine whether this relationship is systematic or accidental.

The results also show that ultrasound treatement is not an effective method to enhance the activity of lysozyme but also does not necessarily eliminate activity. Lysozyme apparently denatures to some extent under the influence of ultrasound which results in a change of activity. Based on adsorption kinetics lysozyme appeared to have both a partially denatured and fully denatured form. This "partially" denatured molecule was observed to have enhanced activity against some strains of *L. monocytogenes*. The "fully" denatured molecule did not show enhanced activity, but did retain some antimicrobial activity. Differential scanning calorimetry analysis showed that lysozyme which appeared to be "fully" denatured with adsorption kinetics actually was not completely denatured. This indicates that there may be several different states of "partial" denaturation when lysozyme is treated with ultrasound. Some "partially" denatured states exhibit more activity than others, but even with high power and long treatment time lysozyme retained some antimicrobial activity. More research into the structure of the ultrasound-treated lysozyme would be beneficial in determining exactly what is occurring to the molecule. In addition, more research into the exact mechanism of action of "partially" denatured lysozyme is needed.

Surface tension analysis has been shown to be a rapid method indicating the degree of denaturation. Differences in adsorption kinetics are based on the partial unfolding of the protein and thus relate to protein structure. While this method may not be quantitatively accurate, a qualitative analysis of the degree of denaturation may be obtained. Adsorption kinetics can be measured over a short period of time and thus served as a diagnostic tool in these experiments to validate successful ultrasound treatment.

The results of Ibrahim et al. (1996a, 1996b, 1996c, 1997) were brought into question by the results of this study. Ibrahim reported that heat treatment of lysozyme at 80°C for 20 minutes resulted in a partially unfolded molecule that was more effective against Gram-negative bacteria. Replication of these conditions resulted in lysozyme that had an adsorption kinetic similar to that of denatured lysozyme. In this case, lysozyme did not exhibit any antimicrobial activity against either Gram-negative or Gram-positive organisms. Thermal analysis of lysozyme indicated that the denaturation temperature of lysozyme was around 75°C. It is therefore highly questionable that Ibrahim was able to partially denature lysozyme at a temperature above the denaturation point.

The differences observed in antimicrobial activity between various strains are remarkable. The precise reason for these differences remains unknown but one can hypothesize that composition and structure of membrane cell wall (i.e. peptidoglycan content or teichoic acids) of these bacteria may account for the results. Activity of lysozyme is based on interaction with peptidoglycan in the bacterial cell wall, thus the amount of peptidoglycan in the cell wall will have a substantial influence on their susceptibility. Future studies should focus on a closer examination of observed

differences in susceptibility. The results also confirm the necessity of using multiple strains in experimental work. This may also contribute to the fact that results obtained by Ibrahim et al. (1996b) were significantly different.

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Vita

Cheryl Lynn Gohr was born on April 8, 1978 in Elmhurst, Illinois. She attended public school in the Elmhurst Public School System, and graduated from York Community High School in 1996. She began study at the University of Tennessee, Knoxville in August of 1996, and received a Bachelor's Degree in Science with a major in Animal Science in December of 1999. In January, 2000 she joined the Department of Food Science and Technology at the University of Tennessee to pursue a Master's Degree. In July of 2000 she married her college sweetheart, Michael Beckett. She plans to work in the industry for several years before deciding whether or not to continue her education.