

PURIFICATION OF LYSOZYME FROM SHELL LIQUOR OF EASTERN OYSTERS  
(*CRASSOSTREA VIRGINICA*) AND ITS USE IN ANTIMICROBIAL FILMS TO  
PRESERVE SMOKED FISH

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## ABSTRACT

The objective of our study was to purify lysozyme from the fluid filling cavity (shell liquor) of the eastern oysters (*Crassostrea virginica*) and determine the antimicrobial activity of purified lysozyme against foodborne pathogens in smoked salmon samples.

Oyster shell liquor was collected over four seasons namely summer 2003, fall 2003, winter 2004, and spring 2004. Spring season showed the highest lysozyme concentration. Lysozyme was purified from 300 liters of oyster shell liquor by a two-step ion exchange chromatography using SP- and CM- Sepharose Fast Flow columns, respectively. Two hundred five mg of pure lysozyme was obtained which represented a recovery of 11.1%. The purity and molecular size of the isolated lysozyme showed a single band of about 18 KDa by SDS-PAGE.

MIC assays of the oyster lysozyme were carried out by serially diluting oyster and hen egg white lysozyme to get a concentration range of 160-2.5 $\mu$ g/ml. Twenty four hours bacterial suspension, Brain heart Infusion or APT broth (as required) was added to each well of a 96-well plate followed by incubating the microtiter plates and measuring absorbance at 640 nm with a microplate reader. MIC results showed that oyster lysozyme had antimicrobial activity against both gram-positive and gram-negative bacteria causing food spoilage and poisoning.

Smoked salmon samples were cut into 1 g pieces, inoculated with 24 h broth cultures of *L. monocytogenes* and *S. anatum*, dipped into zein propylene glycol, 0.75% agar gel, and calcium alginate coating. Calcium alginate coating was chosen as the best coating out of the three coatings. Various treatments of calcium alginate edible coatings

were incorporated with oyster lysozyme or hen egg white lysozyme on the surface of the smoked salmon, allowed to air dry for 20 min and refrigerated at 4°C. Bacterial counts were determined at 0, 7, 14, 21, 28, and 35 days at 37°C for 24 h and CFU/g were determined. Combination of 160 µg/ml of oyster lysozyme and 1000 IU/g of nisin treatment was found to be the most effective treatment and was shown to retain its antimicrobial activity inside the calcium alginate coating for 35 day period.

## CHAPTER 1 INTRODUCTION

In the United States, foodborne diseases affect almost 76 million people of which 325,000 hospitalizations and 5,000 deaths occur (Frenzen, 2004). The major bacterial pathogens responsible for most of the reported foodborne infections are *Escherichia coli* O157, *Campylobacter*, *Listeria monocytogenes*, and *Salmonella*.

Food preservation by inhibiting the growth of these undesirable microorganisms is achieved by the use of antimicrobial agents. These antimicrobial agents are either biologically derived substances which occur naturally in food systems or are safe synthetic compounds intentionally added foods (Sofos et al., 1998). Naturally occurring antimicrobials are used in food systems in which they are found or may be used commercially as additives in other foods which require preservation ( Sofos et al., 1998).

One such antimicrobial agent of natural origin is lysozyme. The biological function of lysozyme is mainly self defense from bacterial infection which it does by lysis of the bacterial cell wall (Salton, 1957).

The traditional source of lysozyme is hen egg white lysozyme. It is currently used commercially in a variety of food products as a preservative (Proctor and Cunningham, 1988; Johnson, 1994; Losso et al., 2000). Lysozyme is approved for food use in Asian, Japan, European, and Latin American countries. In the United States, lysozyme has been granted GRAS (Generally Recognized as Safe) status by Food & Drug Administration but is awaiting regulatory approval for use in foods. The effectiveness of lysozyme in food systems is based on its ability to control the growth of susceptible bacteria.

Lysozyme activity has also been detected in the body fluids and tissues of many bivalve molluscs where the enzyme is believed to be involved in the host defense mechanism and

in digestion (Mcdade and Tripp, 1967; McHenery and Birbeck, 1982; Chu and La Peyre, 1989). The biochemical properties of bivalve mollusc lysozymes are diverse and distinct from those of hen egg white lysozyme. Since bivalve molluscs are osmoconformers and poikilotherms, they are exposed to wide range of environmental conditions and thus lysozymes from bivalve molluscs have been evolved to be active under different environmental conditions. Since the specific activities range of mollusc lysozyme is outside the range of activity of chicken egg white lysozyme, mollusk lysozyme would be a good candidate for use in the food industry.

The consumption of refrigerated, ready-to-eat foods can cause foodborne illnesses if the food is undercooked or becomes cross-contaminated on the surface with major food borne pathogens. There have been reports of contamination in a wide variety of ready-to-eat foods due to food poisoning and spoilage bacteria. Lysozyme of bivalve molluscs are active at lower temperatures and have higher activities than the specific activities of egg white lysozyme at which the ready-to-eat foods are stored and thus makes it better suited to preserve refrigerated minimally processed food.

High lysozyme activity is detected in the fluid filling the shell cavity of oysters. This shell liquor which is a combination of fluid and plasma released when oysters are shucked is referred to as 'Shell liquor'. Shell liquor is currently discarded by the oyster industry when oysters are washed after shucking to remove the dirt and shell fragments before packing. Large volumes of the shell liquor, which goes up to millions of liters, could be collected and a new by-product of the oyster industry could be made. In order to evaluate the use of oyster shell liquor, our objectives were to purify oyster lysozyme from eastern oysters and determine the antimicrobial activity of purified lysozyme against

bacteria causing food spoilage and poisoning. In order to achieve this objective study was divided into three distinct phases:

- 1) To determine whether oyster lysozyme can be consistently purified from shell liquor throughout the year and to use scale up protocol to purify large volumes of shell liquor.
- 2) To determine the antimicrobial activities of oyster lysozyme against major foodborne pathogens.
- 3) To evaluate the feasibility of using oyster lysozyme in antimicrobial films to enhance the preservation of food such as smoked fish and to determine the effect of edible film containing lysozyme on the persistence of *Listeria monocytogenes* and *Salmonella* species inoculated on smoked fish held at refrigerated temperatures.

## **CHAPTER 2 LITERATURE REVIEW**

### **2.1 Properties of Lysozyme**

Lysozymes are peptidoglycan N-acetylmuramoylhydrolases and are referred to by the name muramidases (EC 3.2.1.17) (Chipman and Sharon, 1969). They are antimicrobial enzymes that cleave the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan, the components that make up the bacterial cell walls (Salton, 1957; Chipman and Sharon, 1969; Mine et al., 2004). Lysozyme was discovered and characterized in the early 1920s by Alexander Fleming, who called it ‘a remarkable ‘bacteriolytic element’. He demonstrated that lysozyme had antimicrobial activity against Gram-positive bacteria, present in many tissues and secretions, and was particularly abundant in the white of hen’s egg (Johnson, 1994). The possibility of obtaining lysozyme in large quantities, the easy purification and the fact that it is a small protein which can be readily crystallized has made lysozyme an ideal biomolecule to study (Pellegrini, 2003).

#### **2.1.2 Types of Lysozyme**

Lysozymes have been derived from microbial, viral, insect, plant, and animal sources and they are classified in distinct subfamilies based on their origin, amino acid sequence, and three dimensional structures (Jolles et al., 1996; Masschalack and Michiels, 2003). Egg white lysozyme is the richest source of lysozyme containing about 0.3-0.4 g of lysozyme in one egg. It is the classical representative of the lysozyme family and is called *c*-type (chicken- or conventional-type) lysozyme. Studies have shown that the cuticle and the shell of egg contain less lysozyme whereas the outer membrane and inner membranes are rich in lysozyme (Proctor and Cunningham, 1988; Losso et al., 2000). The presence of *g*-type

lysozyme has been shown in birds and *g*-type lysozyme stands for “goose type” lysozyme after Embden goose. It was also believed that both *c*- and *g*-type lysozyme have different molecular weights and behaviors, but belongs to a common ancestral protein (Proctor and Cunningham, 1988). Lysozyme is also found in animal tissues, sera, and organs. The spleens of cattle contains the largest amount of lysozyme (Chandan et al.,1964). Bovine milk has lysozyme which has approximately same molecular weight as that of egg white but has twice the specific activity (Chandan et al., 1964). A viral type or *v*-type lysozyme has been identified which is different from other lysozymes as it is a transglycosidase (Losso et al., 2000). Lysozyme from plants is classified as *h*- and *b*- type depending on the source from which it has been identified (Beintema and Van Scheltinga, 1996). Different types of lysozyme are listed in table 2.1. Lysozyme is also found to be present in a variety of fruits and vegetables at different concentrations (Table 2.2) (Chandan and Ereifej, 1981).

### **2.1.3 Evidence of the Presence of Lysozyme in Oysters**

In recent years, there has been growing interest in a new type of lysozyme, which is the invertebrate-type (*i*-type) (Mcdade and Tripp, 1967; Jolles and Jolles, 1975; Jolles et al., 1996; Mchenery and Birkbeck, 1986; Bachali et al., 2004; Xue et al., 2004). The first study with invertebrate species was carried out by McDade and Trip in 1967 when they showed the evidence for the existence of high lysozyme activity in the hemolymph of the oyster *Crassostrea virginica* (Mcdade and Tripp, 1967). Later Jolles and Jolles (1975) purified and partially sequenced a lysozyme from starfish *Asterias rubens*. On the basis of N-terminal sequence starfish lysozyme was classified as *i*-lysozyme (Jolles and Jolles,1975). Lysozyme has been detected in the digestive system of many bivalve species such as *Mytilus edulis*, *Modiolus modiolus*, *Chlamys opercularis*, *Tellina tenuis*, in the gill of *Mya*

*arenaria*, flat oyster *Ostrea edulis*, and mantle of *Crassostrea virginica* (Mcdade and Tripp, 1967; Mchenery et al, 1986; Cronin et al., 2001)

Table 2.1 Types of Lysozymes

Type		References
<i>c</i> -type( Chicken type)	Chicken egg white	Chipman and Sharon, 1969
<i>g</i> -type(Goose type)	Birds	Prager and Jolles, 1996
<i>h</i> type (hevamine type)	Plants	Beintema et al.,1996
<i>b</i> type (barley type)	Plants	Beintema et al.,1996
<i>i</i> - type (invertebrate type)	Molluscs, insects	Jolles and Jolles, 1975

Table 2.2 Concentration of Lysozymes from Different Sources

Type	Concentration	References
Body fluid lysozyme <ul style="list-style-type: none"> <li>• Tears</li> <li>• Saliva</li> <li>• Urine</li> <li>• Human milk</li> </ul>	2.6 mg/ml 0.13 mg/ml Trace 0.2-0.4mg/ml	Grossowicz and Ariel, 1983
Fruits and vegetables lysozyme <ul style="list-style-type: none"> <li>• Cauliflower</li> <li>• Papaya</li> <li>• Cabbage</li> <li>• Red radish</li> <li>• Turnip</li> <li>• Broccoli</li> </ul>	27.6 µg/ml 7.9 µg/ml 2.3 µg/ml 3.3 µg/ml 1.8 µg/ml 8.1 µg/ml	Ereifej and Markakis, 1980

. The biochemical properties of purified lysozymes from the bivalve species are diverse and distinct from the biochemical properties of chicken egg white lysozyme (Mchenery and Birkbeck, 1982; Ito et al., 1999; Xue et al., 2004). The enzymatic properties of lysozyme can be confirmed if an enzyme has the following three properties: 1) It can cause reduction in turbidity of isolated cell wall structures; 2) It can lyse intact bacterial cells; 3) It can liberate complex reducing groups and acetylamino sugar complex of glucosamine and acidic hexosamine and muramic acid (Salton,1957). All these properties were fulfilled by

the bacteriolytic substance in oyster hemolymph and it was concluded that the protein present was indeed lysozyme (McDade and Tripp, 1967).

#### 2.1.4 Structure of Lysozyme (Hen Egg White Lysozyme)

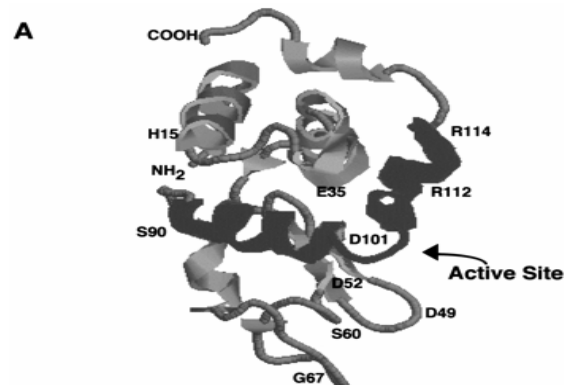


Figure 2.1: Structure of Hen Egg White Lysozyme.  
The active site is indicated by the arrow (Ibrahim et al., 2001)

Hen egg white lysozyme was the first enzyme whose three dimensional structure was determined and characterized (Blake et al., 1965). It is a single polypeptide chain of 129 amino acids having a molecular weight of 14,307 Da and isoelectric point of 10.7. The molecule is crosslinked by four disulfide bridges between the residues Cys6-Cys12, Cys30-Cys115, Cys64-Cys80 and Cys76-Cys94 (Blake et al., 1965). When the disulfide bonds are reduced, lysozyme loses its activity and in order to maintain the enzymatic activity two out of four disulfide bonds must be intact. Lysozyme molecule consists of two domains,  $\alpha$ - and  $\beta$ -domains, linked by a long  $\alpha$ -helix between which lies the active site of the enzyme. The hydrophilic and hydrophobic amino acids are scattered through out the length of the amino acid chain.

#### 2.1.5 Mechanism of Lysozyme Action

Since the discovery of lysozyme by Alexander Fleming, many studies have been made on the susceptibility of different micro-organisms to lysozyme. Several authors have also

proposed different methods for determining the activity of various preparations containing lysozyme ( Boasson, 1938; Smolelis and Hartsell, 1949; Salton, 1957). The lytic action of lysozyme on bacterial cells has been determined qualitatively, by noting if the clearing has been partial or complete, or quantitatively by measuring the change in turbidity of bacterial suspensions (Salton, 1957). There are many factors which affect the activity of lysozyme. pH, temperature, ionic environment, presence of electrolyte or ionic strength, accessibility of the linkages in the bacterial cell wall attacked by lysozyme are few of the factors which can affect the activity of lysozyme.

- **pH:** For hen egg white lysozyme, when experiments were done in neutral and in acidic solutions, the activity of lysozyme in acid solution was always found to be more than the activity of lysozyme in neutral solution (Boasson,1938). It was found that the inhibition starts at pH 5.7 the optimum pH for lysozyme activity is about 6.2 and the maximum lysis was found to occur between pH 6.0-7.0 (Boasson, 1938; Smolelis and Hartsell, 1949). For oyster lysozyme, optimal pH and ionic conditions were observed at pHs between 5.5 and 6.0 (Xue et al., 2004).
- **Temperature:** The lysis of bacterial cell increases as the temperature increases up to 60°C with hen egg white lysozyme (Smolelis and Hartsell, 1949). The lysozyme activity of purified oyster plasma lysozyme increased with increasing temperatures from 0° - 45°C and then decreased after 55°C (Xue et al., 2004).
- **Presence of electrolytes:** Maximum lytic activity was found in presence of potassium salts with ionic strength of about 0.1 when experiments were done with egg white lysozyme (Salton, 1957) and solutions of magnesium or calcium salts showed less lysis than that of potassium and sodium salts (Smolelis and Hartsell, 1949). In case of oyster

plasma lysozyme, ionic strengths were found to be in between  $I=0.180$  and  $0.200$  (Xue et al., 2004).

- **Cultural conditions:** Lysis of bacterial cell involves breakdown of surface structures of the cells. Thus, factors which affect the stability of the cell surface structure or any substance which blocks the interaction of lysozyme with its substrate affects the lytic activity of the enzyme (Salton, 1957).

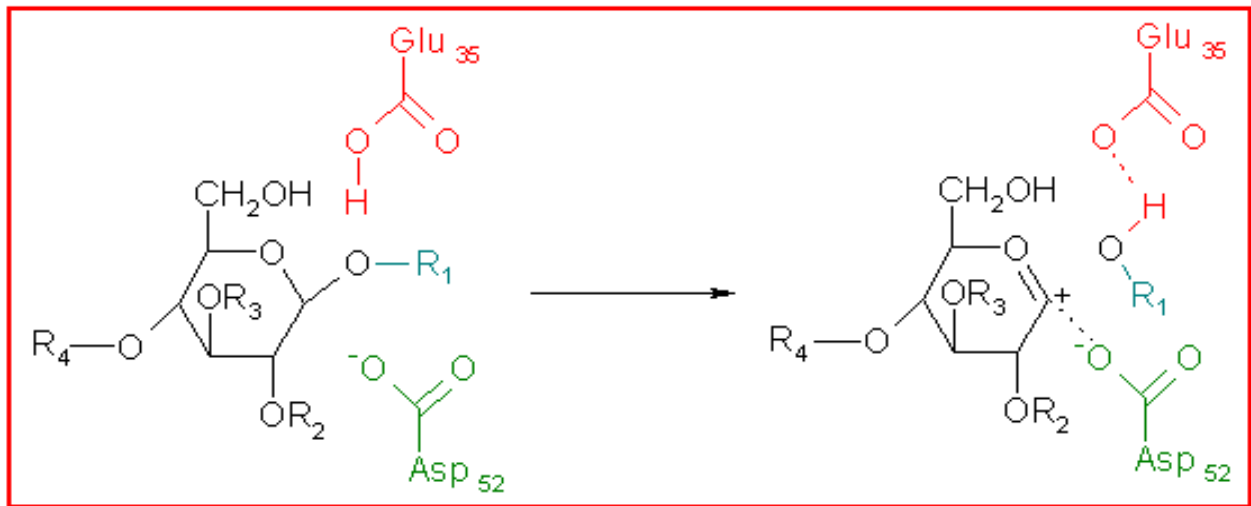


Figure 2.2: Mechanism of Lysozyme Enzymatic Action (Masschalack and Michiela, 2003)

### 2.1.6 Inactivation of Bacteria

Lysozyme has been reported to inactivate gram-positive and gram-negative bacteria by enzymatic and non enzymatic action respectively (Masschalack and Michiela, 2003; Pellegrini et al., 1992).

Enzymatic cleavage of peptidoglycan layer consists of breaking the bond between C1 of N-acetylmuramic acid (NAM) and C4 of N-acetylglucosamine (NAG) which makes up the bacterial cell wall. As seen from figure 2.2 , the two amino acids which take part in the catalysis are Asp<sub>52</sub> and Glu<sub>35</sub>. The sugar residues of the cell wall are positioned in between the catalytic groups Glu<sub>35</sub> and Asp<sub>52</sub>. Glu<sub>35</sub> which is protonated, lies in the hydrophobic

environment and acts as a proton donor in the catalysis reaction. Asp52, is a nucleophile which lies in the dissociated form and lies in the hydrophilic environment.

The reaction starts when Glu35 transfers a proton to the glycosidic oxygen which leads to cleaving of the bond between the oxygen and C1 of the D-sugar residue. This reaction creates a positively charged carbonium ion ( $C^+$ ). The distortion of the D-sugar from the chair to sofa form favors this process. The carbonium ion is stabilized by its interaction with negatively charged Asp52 until a hydroxyl ion diffuses into position from the surrounding water completing the reaction (Masschalack and Michiela, 2003).

Studies have shown that even when enzymatic activity of lysozyme was inhibited, lysozyme still exhibited antimicrobial activity (Ibrahim et al., 1996). It was suggested that the amphiphatic C-terminal domains of the lysozyme were responsible for the antimicrobial activities of the denatured lysozyme. Others have suggested that the nonenzymic bactericidal properties of lysozyme were due to its highly cationic nature (Masschalack and Michiela, 2003).

## **2.2 Antimicrobial Properties of Lysozyme (Hen Egg White Lysozyme)**

The natural substrate for lysozyme action is the peptidoglycan layer of the cell wall which forms a close network of the entire cell, giving the cell its shape and stability against cellular turgor pressure. Thus, the hydrolysis of the peptidoglycan layer leads to cell lysis. The chemical composition of the cell walls of gram negative bacteria differs from that of gram positive bacteria. The cell wall of Gram positive bacteria is composed of thick layer of peptidoglycan with embedded chains of teichoic acids and lipoteichoic acids. The cell wall of gram negative bacteria is composed of two layers, inner layer composed of a single layer of peptidoglycan without teichoic acids and an outer layer composed of thick layer of lipopolysaccharide. The outer layer of gram negative bacteria prevents the access of lysozyme

to the peptidoglycan layer thus making it less sensitive to lysozyme action (Salton, 1958). However, research in this area has shown that the killing mechanism of gram-positive bacteria is independent of enzymatic activity but attributed mainly to the cationic and hydrophobic properties of lysozyme (Pellegrini et al., 1992). These studies have shown that enzymatic and lytic activity are not linked with each other as denatured lysozyme lacking in enzymatic activity was still able to inhibit bacterial growth (Pellegrini et al., 1992; Ibrahim et al., 1996; Ibrahim et al., 2001).

### **2.2.1 Action against Gram-Positive Bacteria**

Hen egg white lysozyme is highly specific in action and is effective against relatively few species of bacteria associated with foods (Proctor and Cunningham, 1998). Lysozyme brings about the lysis of certain bacteria by altering the properties of the surface structures on the cell. Early observations of the microscopic sequence of changes affected by lysozyme from several different sources have shown that there is a marked swelling of the cells before cell lysis and this swelling is due to an alteration of the cell wall (Salton, 1957). The mechanism of action of lysozyme was investigated by Salton in 1957, who used three gram positive bacteria to show that lysozyme completely digested the bacterial cell wall. When *Micrococcus lysodeikticus*, *Sarcina lutea*, and *Bacillus megaterium* were digested with lysozyme, there was liberation of reducing and acetyl amino sugars (Salton, 1957). Lysozyme activity was determined by measuring the decrease in the turbidity of the cell-wall suspension and by estimating the amounts of liberated reducing substances. A complex mixture of fragments, differing in molecular size, electrophoretic properties, and chemical composition resulted when lysozyme acted on the cell walls of certain lysozyme sensitive bacteria. The main dialyzable substances liberated by lysozyme appeared to be disaccharides of acetlyglucosamine and acetyl muramic acid. The cell wall amino acid and

sugars were not detected as the free substances in the dialyzable fractions of the digests (Salton, 1958). This evidence suggested that lysozyme was splitting the glycosidic bonds of the cell wall amino sugars thus liberating disaccharides of acetylglucosamine and acetylmuramic acid (Salton, 1958).

### **2.2.2 Action against Gram-Negative Bacteria**

Early investigation with certain gram negative bacteria showed that when isolated cell walls of certain gram-negative bacteria were incubated with lysozyme, only small turbidity decrease was detected. The soluble, non-dialysable components which were released by lysozyme consisted of alanine, glutamic acid, diaminopimelic acid (DAP) and glucosamine as the predominant substances, smaller amounts of muramic acid, and few other amino acids (Salton, 1958). Recent studies showed that the action of lysozyme on gram negative bacteria did not depend on its enzymatic activity but depend on its structural phase transition (Ibrahim et al., 2001). There is an evidence of a specific bacterial domain, namely residue 98-112, which is known to be involved in the antimicrobial action of lysozyme against gram positive and gram negative bacteria (Ibrahim et al., 2001). Genetic evidence has also been provided which clearly demonstrated that the antimicrobial activity of lysozyme was independent of its muramidase activity (Ibrahim et al., 2001). Heat-denatured hen egg white lysozyme (HEWL) was investigated for its bactericidal activity and it was found that after denaturation, there was an enhancement in the bactericidal activity towards gram negative bacteria with a partially unfolded, enzymatically inactive and hydrophobic form of lysozyme (During et al., 1999). It was demonstrated that the bactericidal activity was due to the membrane insertion of the dimeric form of lysozyme which lead to membrane disruption. The bactericidal activity in this case could be uncoupled from the enzymatic activity (During et al., 1999; Ibrahim et al., 1996; Pellegrini et al., 1992).

### 2.3 Lysozyme Use in the Food Industry

Lysozyme is used in the food industry as a food preservative. Lysozyme has been extensively used in Japan and Japanese hold several patents for the use of lysozyme in food systems (Proctor and Cunningham, 1991). It is awaiting regulatory approval in the United States and has been granted GRAS (Generally Recognized as Safe) status by the FDA (Kahl, 1998). In 1968, Yajima found that lysozyme was a strong inhibitor of *hiochi* bacteria in sake. The enzymatic activity of lysozyme was not lost during pasteurization and it remained the same during one year at room temperature. There was no objectionable flavor in sake treated with lysozyme (Yajima et al., 1968). Lysozyme-glycine mixture at 1.5% has been used to extend the shelf life of potato salad at a temperature of 35-37° C from 23 to 43 h (Nakagawa and Maeshigi, 1980). Butyric acid bacteria *Clostridium trybutyricum* is a problem in the cheese industry as it causes late blowing of cheese. Egg white lysozyme is used to kill the resting vegetative cells of *Clostridium trybutyricum* and it was found that spores were resistant to lysozyme while proliferating vegetative cells were inhibited (Wasserfall et al., 1979). Lysozyme, like rennin, destabilizes casein micelles (Bakri and Wolfe, 1971). Several microorganisms were also shown to be susceptible to milk lysozyme, thus proving the significant role of antimicrobial activity of lysozyme (Vakil et al., 1969). Addition of lysozyme to Edam cheese did not affect the organoleptic quality of the cheese. (Wasserfall et al., 1976). Use of lysozyme in meat has been studied widely. Lysozyme has been used to preserve meat products either alone or in combination with other compounds such as NaCl or NaNO<sub>2</sub> (Akashi et al., 1969). The effects of lysozyme on different bacterial strains associated with food have been studied widely and are shown in Table 2.2. Different patents for the use of lysozyme in the food industry are shown in Table 2.3.

## **2.4 Antimicrobial Films and Fish Products Preservation**

### **2.4.1 Foodborne Pathogens Associated with Fish and Fish Products**

Fish and related products belong to high risk foodstuff group which have been explained in Codex Committee on Food Hygiene and also in European institution (Novotny et al., 2004). Outbreaks occur due to eating of insufficiently heated fish or its products contaminated after or during their processing. Temperature and pH are also limiting factors for the survival of foodborne pathogens in fish products.

### **2.4.2 Cold Smoked Salmon**

Cold smoked fish is a ready-to-eat (RTE) food product which is classified as a high risk product with respect to *listeriosis*. Several studies have shown the evidence of smoked mussels, ‘gravad’ trout, smoked trout and smoked salmon contamination by *Listeria monocytogenes* (Brett et al., 1998; Ericsson et al., 1997 and Dillon et al., 1994).

Cold-smoked salmon is a ready-to-eat (RTE) food product which is normally purchased vacuum-packed and has a shelf life of 3-5 weeks at refrigerated temperatures (Rovik, 2000).

The salt content, pH and water activity of the product allow the growth of *Listeria monocytogenes* (Rorvik, 2000). Vacuum-packed and sliced, cold salmon is a perishable product, due to addition of less preservative (Leroi et al., 2001). Traditionally, seafood was smoked for the purpose of preservation but nowadays seafood products are lightly salted and also lightly smoked for maintaining the flavor and texture of the product (Dillon et al., 1994). Two types of smoking process are used in the seafood industry: ‘Hot smoking’ and ‘Cold smoking’. Hot smoking consists of smoking the seafood under temperatures which ranges from 30 to 80°C over a period of several hours and cold smoking consists of smoking at temperature below 28°C (Dillon et al., 1994).

Table 2.3: Effects of Lysozyme on Different Bacterial Strains Associated with Various Food Products

Food	Organisms	Effects of lysozyme	References
Cooked sausage	<i>Pseudomonas</i> <i>Achromobacter</i> <i>Flavobacterium</i>	m+ m+ m+	Akashi, 1971
Cheese	<i>Streptococcus cremoris</i>	+	Akashi, 1972
Turkey drumstick and whole carasses	<i>Salmonella senftenberg</i>	+	Teotia and Miller, 1975
Vegetables	<i>L. monocytogenes</i> Scott A	m+	Hughey et al., 1989
Low acid canned food	<i>B. stearothermophilus</i> , <i>C. thermosaccharoliticum</i>	+ +	Hughey and Johnson, 1987
Cheese	<i>C. botulinum</i> <i>L. monocytogenes</i>	- m+	Hughey and Johnson, 1987
Canned vegetables	<i>C. thermosaccharolyticum</i>	+	Frazier and Westhoff, 1988
Sake	<i>L. heterohiochii</i>	m+	Yajima et al., 1968
Fresh cod	<i>L. monocytogenes</i>	m+	Wang and Shelef, 1991
Whole milk	<i>L. monocytogenes</i>	m+	Carminati and Carini, 1989
Cheese	<i>C. tyrobutyricum</i>	+	Johnson et al., 1994

m+, moderate inactivation; +, inactivation; -, resistance

Table 2.4: A Partial List of Patents on the Use of Lysozyme in the Food Industry

Patent number	Food product	Antimicrobial composition	Reference/Company
6,451,365 (U.S.)	Hot dogs, cooked ham	Nisin + Lysozyme + Beta hops acids	King et al., 2003
6,287,617 (U.S.)	Cut fruits,vegetables, seafood or meat	Trisodium orthophosphate + Nisin/lysozyme	Bender et al., 2001
5,019,411 (U.S.)	Vegetable foods	Lysozyme + ETDA+ Citric acid	Johnson et al., 1991
5,573,801 (U.S.)	Meat products	Nisin + Lysozyme + Chelating agent	Wilhoit, 1996
5,458,876 (U.S.)		Lysozyme /antibiotic formulations	Monticello, 1995
5,393,545 (U.S.)	Animal and/or vegetable origin	Lysozyme + Chelating agent	Johnson et al., 1995
4,810,508 (U.S.)	Dairy and meat products & by products	Lysozyme + Lysozyme salts	Dell'Acqua et al., 1989
5710 (Japanese)	Fish	Lysozyme + NaCl	Eisai Company,1972
19576 (Japanese)	Seafood	Lysozyme + Amino Acid+ NaCl	Eisai Company, 1971
5535105 (Japanese)	Sake	Lysozyme + <i>p</i> -hydroxybenzoic esters	Eisai Company, 1980
4810508 (Japanese)	Butter and Cheese	Lysozyme	Dell' Auua et al.,1989
46-336 (Japanese)	Tofu bean curd	Lysozyme + soya milk during processing	Taiyo Food company, 1972
4831-905 (Japanese)	Fresh vegetables, fish, meat , fruits	Coating lysozyme on surface	Kanebo Ltd, 1973
16-780 (Japanese)	Dried milk product	Lysozyme + Ovalbumin+ Ovomucin	Morinaga Milk Industry Co., 1970

### **2.4.3 Edible Film Coatings**

Physical, chemical, and microbiological changes take place during storage of ready-to-eat (RTE) food products which affect the quality and shelf life of these products when kept at refrigerated temperatures. These food products are frequently exposed to surface contamination which leads to reduction in the shelf life (Cha and Chinnan, 2004).

The main cause of food spoilage is due to the growth of microorganisms on the surface of the food products. An antimicrobial film acts as a protective barrier which retards the deterioration of food when the film is incorporated with antimicrobial agents, the later in turn extend the shelf life of the food product. The application of an antimicrobial agent in the film creates an environment inside the film that delays or prevents the growth of microorganism on the surface of the product (Cha and Chinnan, 2004). Antimicrobial agents are incorporated into the edible films and are released onto the surface of the food. These coatings also serve as a barrier to moisture and oxygen. These coatings are cost effective and protect the food material even when the package on the food material is opened (Debeaufort et al., 1998).

Antimicrobials could be bound either to the surface of the food packaging materials or could be coated onto the surface of the food. Enzymes are then released into the foods from the packaging materials thereby inhibiting the growth of the pathogenic microorganisms. Apart from the main function against pathogenic microorganisms, edible coatings serve to control the gas exchanges allowing reduction of oxidation in oxygen sensitive food. Edible coatings also retain the aroma of the product during storage and improve the mechanical properties of food making it easy to use during handling. Sensory

properties such as color and shininess are also improved when food products are coated with edible films (Debeaufort et al., 1998).

Edible film coatings could be polysaccharide-based films, protein-based films or lipid-based films. One of the widely used polysaccharide film is Alginate based films. Alginates extracted from the brown seaweeds belong to the *Phaeophyceae* class. Alginates are salts of alginic acid, which is a linear polymer of D-mannuronic and L-guluronic acid monomers. The interactions between alginate and di-valent or trivalent cations lead to alginate film formation. Calcium ions are widely used as effective gelling agents (Cha and Chinnan, 2004). Calcium alginate gels incorporation of antimicrobial agents have been used to preserve food products against spoilage by undesirable microorganisms (Wan et al., 1997; Cutter and Siragudsa, 1996; Williams et al., 1978; Lazarus et al., 1976).

#### **2.4.4 *Listeria monocytogenes* in Seafood**

*Listeria monocytogenes* is a gram positive, food borne pathogen. It is widely found in the environment and naturally occurs in many raw foods. *Listeria monocytogenes* is responsible for nearly one-fourth of all foodborne disease-related deaths in the United States each year (Mead et al, 1999). Due to public health significance of *L. monocytogenes*, US regulatory agencies have established a policy where Ready-to-eat (RTE) foods contaminated with food spoilage and food poisoning organisms which can be found at detectable level are said to be ‘adulterated’ products. Since 1980, the food industry has taken major efforts to eradicate *Listeria monocytogenes* from RTE products and processing environment (Chen et al., 2002; Eklund et al., 1994). *L. monocytogenes* has been proven to be a causative agent in the outbreak of many food-borne epidemics and the food associated with epidemics were milk, cheese and vegetables (Guyer and Jemmi, 1991). Recent studies have shown that *L.*

*monocytogenes* can be isolated from fish and seafood (Hoffman et al., 2002, Chen et al, 2002). It is psychotropic, halotolerant and can grow in the range of 1 to 45 ° C (34 to 113° F) and between 0 and 10% NaCl.

*Listeria monocytogenes* is frequently detected in smoked products which represent an important problem with respect to marketing of the products and human health if the pathogen reaches high level (Lakshmanan and Dalgaard, 2004). *L.monocytogenes* contamination is of concern in cold smoked salmon since the heat applied during smoking is not sufficient to inactivate potent *Listeria monocytogenes* and the smoked products are consumed without further cooking (Eklund et al., 1994). Studies were carried out where cold-smoked salmon processing plants were surveyed to determine the occurrence and sources of *Listeria monocytogenes* contamination (Eklund et al., 1994). The primary source of contamination proved to be the surface areas of frozen or raw fish coming into the plant. Even though hot smoking should have been bactericidal, studies have shown that the processes used by different processors vary and thus *Listeria* has been a problem in both hot and cold smoked products (Dillon et al., 1994). But cold smoked salmon products are at greater risk. Various studies have shown that after cold smoking there is around 15-40% increase in the percentage of samples containing *L. monocytogenes* (Heinitz and Johnson, 1998; Embarek, 1994; Dillon et al., 1994). The various parameters and steps of fish smoking influence the growth of *L.monocytogenes*. Salting of fish before smoking is a routine process but as *L. monocytogenes* is halotolerant, it is able to survive in salted fish as well (Eklund et al., 1995). Salt levels in smoked fish products range from 3.5-5% and this level has no inhibitory effect on the bacterium (Jorgensen at al., 2000). The Food and Drug Administrtaion (FDA) is currently requesting scientific data and information that would

help the agency to conduct risk assessment for *Listeria monocytogenes* in smoked finfish. The main aim for this risk assessment is to reduce or prevent the growth of *L. monocytogenes* during manufacturing or processing of hot and cold smoked finfish (Shuren et al., 2005)

#### **2.4.5 *Salmonella* in Seafood**

Smoked fish and shellfish are a source of microbial hazard by *Salmonella* species (Heinitz and Johnson, 1998). They serve as the passive carriers of salmonella, demonstrate no clinical symptoms in most cases and can be excreted without any trouble (Novotny et al., 2004). Contamination with *Salmonella* occurs from the terrestrial sources and this organism serves as a vector. Food products which are commonly involved with the transmission of *Salmonella* spp. are eggs, meat products, milk, and processed seafood products (Heinitz and Johnson, 1998). The Centers for Disease Control and prevention (CDC) estimate nontyphoidal *Salmonella* foodborne disease attributes to a total of 1,341,873 cases of which 15,608 hospitalizations and 553 deaths occurs in the United States annually (Mead et al., 1999). It has been shown that the incidence of *Salmonella* in fish or shellfish as the vehicle of transmission accounts for 8 of the 160 outbreaks (Heintz et al., 2000). Some of the *Salmonella* outbreaks associated with seafood are associated with smoked fish. *Salmonella paratyphi* B infections associated with consumption of smoked halibut in Germany (Francis et al., 1989), outbreak of *Salmonella blockey* infections following consumption of smoked eel (Fell et al., 2000), and outbreak involving improperly prepared chilled, boiled salmon caused by *Salmonella Montevideo* are a few (Cartwright et al., 1988) of the studies which dealt with smoked fishes contaminated by *Salmonella* spp. Seafood products that are highly at risk with contamination with

*Salmonella spp.* are the ready-to-eat seafood products such as cooked crab, dried salted seafood, smoked food and prepared items. Incidence of *Salmonella* in smoked fish is 3.9% and the isolated species from smoked fish have shown that *Salmonella newport* is the most prominent species found in smoked fish in the United States (Heintz et al., 2000).

**CHAPTER 3**  
**PURIFICATION OF LYSOZYME FROM SHELL LIQUOR OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA* AND ITS POTENTIAL USE AGAINST BACTERIA CAUSING FOOD POISONING AND FOOD SPOILAGE**

**3.1 Introduction**

Lysozymes are ubiquitous enzymes that are widely distributed in nature. Egg white is the richest source of lysozyme which makes up about 3.4% of the egg white proteins and is classified as *c*-type lysozyme (Blake et al., 1965). For almost three decades, there has been a growing interest in invertebrate-type or *i*-type of lysozyme (Mcdade and Tripp, 1967; Jolles and Jolles, 1975; Jolles et al., 1996; Mchenery and Birkbeck, 1986; Bachali et al., 2004; Xue et al., 2004). Jolles and Jolles(1975) purified and partially sequenced invertebrate lysozyme from a starfish *Asterias rubens*. Lysozyme activity has also been shown in the hemolymph of eastern oyster (*Crassostrea virginica*), digestive systems, gills, and mantel of several bivalves (Mcdade and Tripp, 1967). The N-terminal amino acid sequence analysis indicated that bivalve lysozyme belongs to the *i*- type (Jolles and Jolles., 1975; Ito et al., 1999).

The biochemical properties of lysozyme from bivalve species are diverse and distinct from those of chicken egg white lysozyme (Mchenery and Birkbeck, 1982; Ito et al., 1999; Xue et al., 2004). Bivalves use lysozyme as a digestive enzyme and for self defense against bacterial infection (Ito et al., 1999). Lysozyme from bivalve mollusks is less affected by temperature fluctuations as bivalves are exposed to a wide range of environmental conditions, thus making them active under different environmental conditions. They work well at lower pH , temperature and different ionic strengths than the hen egg white lysozyme.

The combination of the fluid and plasma released when oysters are shucked is referred to as 'Shell liquor'. Millions of liters of shell liquor are produced and discarded annually by the oyster industry. Xue et al. (2004) identified the presence of high lysozyme activity in the fluid filling the shell cavity of oysters. It is possible to collect millions of liters of oyster shell liquor for use as a source of value added product for the oyster industry. In particular, oyster shell liquor lysozyme may find application in the food and pharmaceutical industries.

Foodborne diseases affects millions of people each year and the major bacterial pathogens responsible for most of the reported foodborne infections are *Escherichia coli* O157, *Campylobacter*, *Listeria monocytogenes*, and *Salmonella*. Apart from these, there are several other foodborne pathogens which cause food spoilage and poisoning in a wide range of food products. Hen egg white lysozyme is used to control many of these bacteria in food products (Proctor and Cunningham, 1988; Johnson, 1994; Losso et al., 2000).

The objective of our study was to purify oyster lysozyme from eastern oysters and determine the antimicrobial activity of purified lysozyme against bacteria causing food spoilage and poisoning.

## **3.2 Materials and Methods**

### **3.2.1 Materials**

Sephadex G-25, SP-Sepharose Fast Flow, CM- Sepharose Fast Flow were purchased from Amersham Pharmacia Biotec (Piscataway, NJ). Micro BCA Protein Assay Kit was obtained from Pierce Biotechnology (Rockford, IL). Chemicals for sodium-dodecylsulfate-polyacrylamide gel, molecular weight markers (14.4-97.4 KDa) were purchased from Sigma-Aldrich (St Louis, MO).

## **3.2.2 Methods**

### **3.2.2.1 Seasonal Variation of Protein and Lysozyme Concentration in Oyster Shell Liquor**

#### **3.2.2.1.1 Collection of Oyster Shell Liquor**

Oyster shell liquor was obtained from P & J Oyster Co. in New Orleans, LA in the summer, fall, and winter of 2004, and spring of 2004. The volume of shell liquor per sack (about 40 kg) of oysters averaged 8 liters. The shell liquor was placed in 4 L bottles in coolers filled with ice and transported to the laboratory in the Department of Veterinary Science at Louisiana State University. The shell liquor was centrifuged at 4,000 x g for 30 min to remove any shell fragments and pieces of tissue cuts from oyster meat during shucking. The supernatants were collected and pooled. Protein concentration and lysozyme activity were measured as follows.

#### **3.2.2.1.2 Protein Concentration and Lysozyme Activity Determination**

Protein concentration was measured using the Micro BCA Protein Assay (Pierce Biotechnology, Rockford, IL). All measurements were carried out in triplicates. Lysozyme activity was measured spectrophotometrically as reported by Xue et al., (2004). Twenty  $\mu$ l of oyster shell liquor sample was mixed with 180  $\mu$ l of *Micrococcus lysodeikticus* suspended in 0.2 M acetate buffer at pH 5.8 in a 96-well microplate at room temperature. The absorbance of the mixture was immediately measured at 450 nm with a microtiter plate reader (Dynatec, Chantilly, VA.). Absorbance was measured 5 min after the initial reading and the decrease in absorbance at 450 nm per min was calculated. All measurements were done in triplicates. One unit of lysozyme was defined as that quantity which causes a decrease in absorbance of 0.001 per min of *Micrococcus Lysodeikticus* suspended in 0.2 M acetate buffer at pH 5.8. Lysozyme concentration in

shell liquor equivalent to purified oyster plasma lysozyme was calculated from the lysozyme activity taking purified plasma oyster lysozyme as a standard. The results of protein concentration and lysozyme concentration yield, percentage recovery and specific activity of purified oyster lysozyme for each season are shown in Table 3.1. and Table 3.2.

#### **3.2.2.1.3 Purification of Lysozyme from Oyster Shell Liquor**

The protocol of Xue et al., (2004) to purify lysozyme from oyster plasma was adapted to purify lysozyme from one liter oyster shell liquor collected during each season. The concentration of lysozyme in 1 liter of shell liquor was determined as described above in section 3.2.2.1.2.

#### **3.2.2.1.4 Molecular Weight and Purity of Oyster Shell Liquor Lysozyme**

The approximate molecular weight and purity of the purified oyster lysozyme was estimated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using a 12.5% running gel and 4% stacking gel. A low range molecular weight markers (14.4-97.4 KDa) was used as standards to calculate the molecular weight of lysozyme. Protein separation was carried in a vertical slab unit (Bio-Rad, Richmond, CA).

#### **3.2.2.1.5 Statistical Analysis**

Protein and lysozyme concentration in oyster shell liquor data for all the four seasons were  $\log_{10}$  transformed and the statistical comparisons of the four different seasons were analyzed by statistical comparisons using Students *t*-test following one –way analysis of the variance (ANOVA) (SAS insititute Inc., Cary, N.C., U.S.A.).

### **3.2.2.2 Large Scale Purification of Lysozyme from Oyster Shell Liquor**

#### **3.2.2.2.1 Sample Processing**

Oyster shell liquor was collected from P& J Oyster Co., (New Orleans, LA) during the spring of 2004. Three hundred liters of oyster shell liquor samples were brought from the processing company in batches consisting of 8 gallons and were lyophilized using a Genesis 35 X L lyophilizer (Virtis Co. NY, NY). The dried powder was suspended in distilled water to one tenth of the original volume, stirred overnight at 4° C, and centrifuged at 4000 x g for 30 min at 4° C. The supernatant was collected and designated as “concentrated shell liquor”. Protein concentration and lysozyme activity were determined as described above. The concentrated liquor was desalted in a Sephadex G-25 column (5 x 70 cm) equilibrated with 0.02 M sodium acetate buffer, pH 5.0. Two hundred ml of sample was loaded and the column was washed with 0.02 M sodium acetate buffer, pH 5.0 at an elution rate of 6ml /min. The elution was monitored for absorbance at 280 nm with an UV Monitor Bio-Rad, Richmond, CA). Fractions collected in the first peak were pooled and designated as “crude shell liquor” sample. Protein concentration and lysozyme activity were determined as described above.

#### **3.2.2.2.2 Strong Cation Exchange Chromatography**

Crude shell liquor was loaded at the rate of 6 ml/min onto a SP-Sepharose FF column (2.6 x 35 cm), equilibrated with 0.02 M sodium acetate buffer at pH 5.0. The column was successively washed with 0, 0.1, 0.3, and 0.6M of NaCl in 0.02 M sodium acetate buffer pH 5.0 at an elution rate of 6 ml/min. The elution was monitored by measuring the absorbance of fractions at 280 nm. Fractions from the 0.6 M NaCl eluted peak were collected and designated as “lysozyme enriched sample”. Lysozyme enriched sample

from seven runs were pooled and concentrated by freeze drying. Final volume, protein concentration and lysozyme activity of the lysozyme enriched sample were determined as described above.

#### **3.2.2.2.3 Weak Cation Exchange Chromatography**

The lysozyme enriched sample was loaded onto a CM- Sepharose Fast Flow column (1.6 x 35 cm) at the rate of 6 ml/minute. The column was washed with a linear gradient of NaCl using 0.3 M - 0.65 M NaCl in 0.02 M sodium acetate buffer, pH 5.0 at an elution rate of 6ml/min. The elution was monitored by measuring the absorbance of fractions at 280 nm. The lysozyme activity of each fraction was measured and an aliquot from each fraction containing high lysozyme activity was subjected to SDS-PAGE as described above. The fractions showing a single and similar size protein band between 14.4 and 21.5 KDa were pooled and desalted using a Sephadex G-25 column equilibrated with distilled water. The desalted preparations were designated as “oyster shell liquor lysozyme”. The sample was lyophilized, reconstituted in distilled water, adjusted to 1 mg/ml, and stored at -20°C as a stock solution until use.

#### **3.2.2.2.4 Molecular Weight and Purity of Oyster Lysozyme**

Oyster lysozyme was analyzed by SDS-PAGE as described in section 3.2.2.1.2 above using plasma lysozyme as a reference.

#### **3.2.2.3 Determination of Minimum Inhibitory Concentration (MIC) of Oyster lysozyme and Hen Egg White Lysozyme**

##### **3.2.2.3.1 Preparation of Bacterial Cultures**

A 10 µl of a culture mixture, containing 19 foodborne pathogens (Table 3.4) from the slant was taken and transferred to 10 ml Brain Heart Infusion broth or APT broth. The cultures were grown overnight and transferred to 10 ml BHI or APT broth the next day.

On the third day (after two transfers), 1 ml of culture was taken in an eppendorf tube and centrifuged at 4,000 x g for 2 min. The pellet was suspended with 1 ml phosphate buffer saline (PBS) and centrifuged twice at 4,000 x g for 2 min. To verify the bacterial concentration before carrying out the experiment, plate counts were carried out and colony forming units were calculated. The plate count was determined to be about  $10^7$  bacteria/ml and the cell suspension was serially diluted to  $10^3$  cells/ml.

#### **3.2.2.3.2 Preparation of Lysozyme Solutions**

Two mg per ml of Hen egg white lysozyme was prepared and used as stock solution. The stock solution was filter sterilized thorough a low protein binding filter (0.22 $\mu$ m). One ml aliquots were prepared and kept at -20°C until further use. Eight hundred microgram per ml of working lysozyme solution was made from the stock at the time of the experiment.

Similarly, eight hundred microgram per ml of working oyster shell liquor lysozyme solution was prepared from a 1 mg per ml of stock solution.

#### **3.2.2.3.3 MIC Assays**

Twenty microliters of bacterial suspension were added to 20 $\mu$ l of two fold serially diluted lysozyme (160 - 2.5  $\mu$ g/ml) in PBS or to 20 $\mu$ l of distilled water alone (control) in 96 well plates. 60 $\mu$ l of BHI or APT broth was added to each well. The plates were incubated at 37° C for the required incubation period (differs according to the bacteria) (Table3.3). The bacterial growth was measured at 640 nm with a microtiter plate reader at 12, 24, and 36 h as per bacterial requirements (Xue et al., 2004). The results were expressed as the minimum concentration of lysozyme which significantly inhibited the bacterial growth compared to the control (distilled water).

### 3.3 Results

#### 3.3.1 Seasonal Variation of Protein and Lysozyme Concentration in Oyster Shell Liquor

Protein concentration was found to be in the range of 0.26 to 0.76 g/L and lysozyme concentration was in the range of 0.319 to 1.15 mg/L. (Table 3.2). Shell liquor collected in winter season yielded 5.27 mg lysozyme per liter and was the highest amongst the four seasons. Lysozyme amount was expressed as equivalence of purified plasma lysozyme. Lysozyme yield was lowest in the summer season. The highest percentage recovery of 39% was obtained in the fall season with the lowest recovery of 9.4% in the spring season. The specific activity ranged from  $1.13 \times 10^4$  U/mg in the fall to  $2.52 \times 10^4$  U/mg in the spring.

Table 3.1 Protein and Lysozyme Concentration in Oyster Shell Liquor

Batch	Date collected	Protein concentration (g/L)	Lysozyme concentration*(mg/L)
Summer-1	5/15/03	2.97	2.30
Summer-2	5/29/03	1.63	2.05
Summer-3	6/16/03	1.31	1.95
Fall-1	10/16/03	1.71	3.24
Fall-2	11/6/03	6.41	11.10
Fall-3	11/11/03	5.05	8.22
Winter-1	1/24/04	5.02	15.88
Winter-2	2/18/04	5.71	11.43
Winter-3	2/27/04	3.27	10.90
Spring-1	4/6/04	6.68	13.96
Spring-2	4/28/04	6.11	23.14
Spring-3	5/12/04	5.04	9.24

\*Lysozyme concentration was expressed as equivalence of purified oyster plasma lysozyme with an activity of  $1.5 \times 10^5$  U/mg

Table 3.2 Yield, Percent Recovery, and Specific Activity of Purified Oyster Lysozyme

Batch	Lysozyme yield (mg)	% Recovery	Specific Activity (U/mg)
Summer	0.54	23.6	$2.11 \times 10^4$
Fall	4.33	39.0	$1.13 \times 10^4$
Winter	5.27	33.2	$1.19 \times 10^4$
Spring	2.18	9.4	$2.52 \times 10^4$

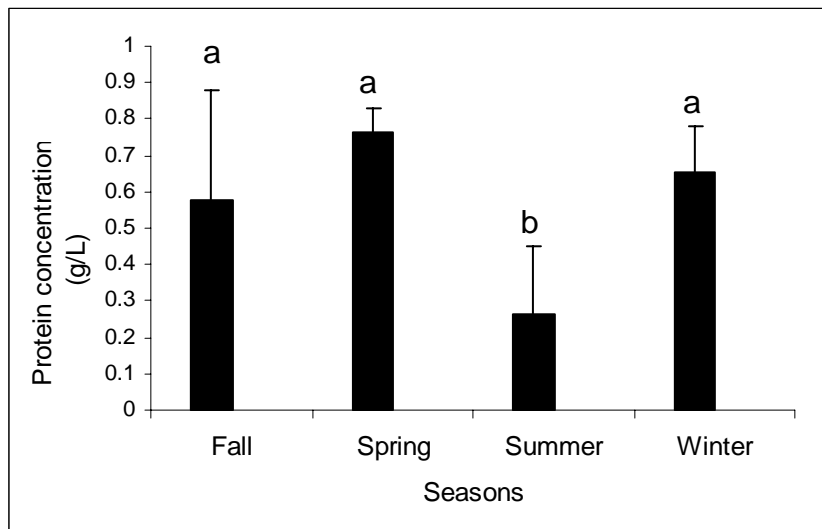


Figure 3.1 A: Protein Concentration of Purified Oyster Shell Liquor Lysozyme

All analysis are based on four separate experiments. Means followed by the same letter are not significantly different ( $P = 0.05$ ) from each other. Statistical comparisons of all pairs were analysed using Student's *t*-test following one-way analysis of the variance (ANOVA) (SAS Institute, Inc., Cary, NC).

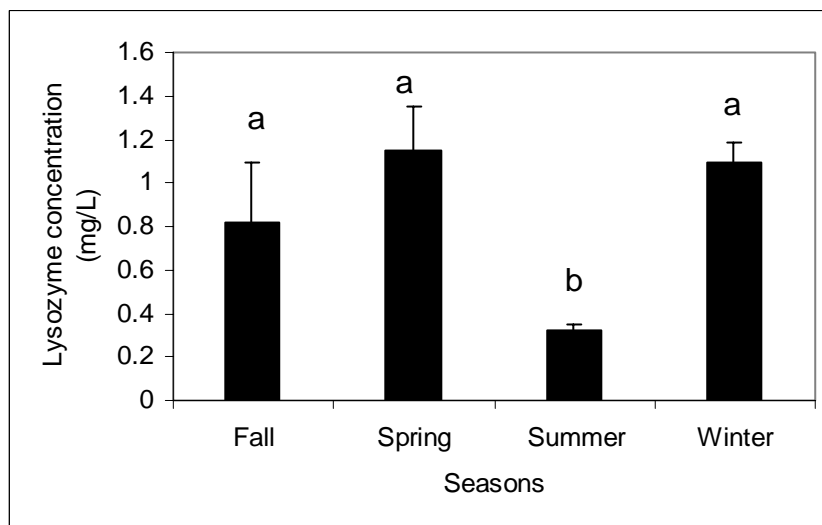


Figure 3.1 B: Lysozyme Concentration of Purified Oyster Shell Liquor Lysozyme

All analysis are based on four separate experiments. Means followed by the same letter are not significantly different ( $P = 0.05$ ) from each other. Statistical comparisons of all pairs were analysed using Student's *t*-test following one-way analysis of the variance (ANOVA) (SAS Institute, Inc., Cary, NC).

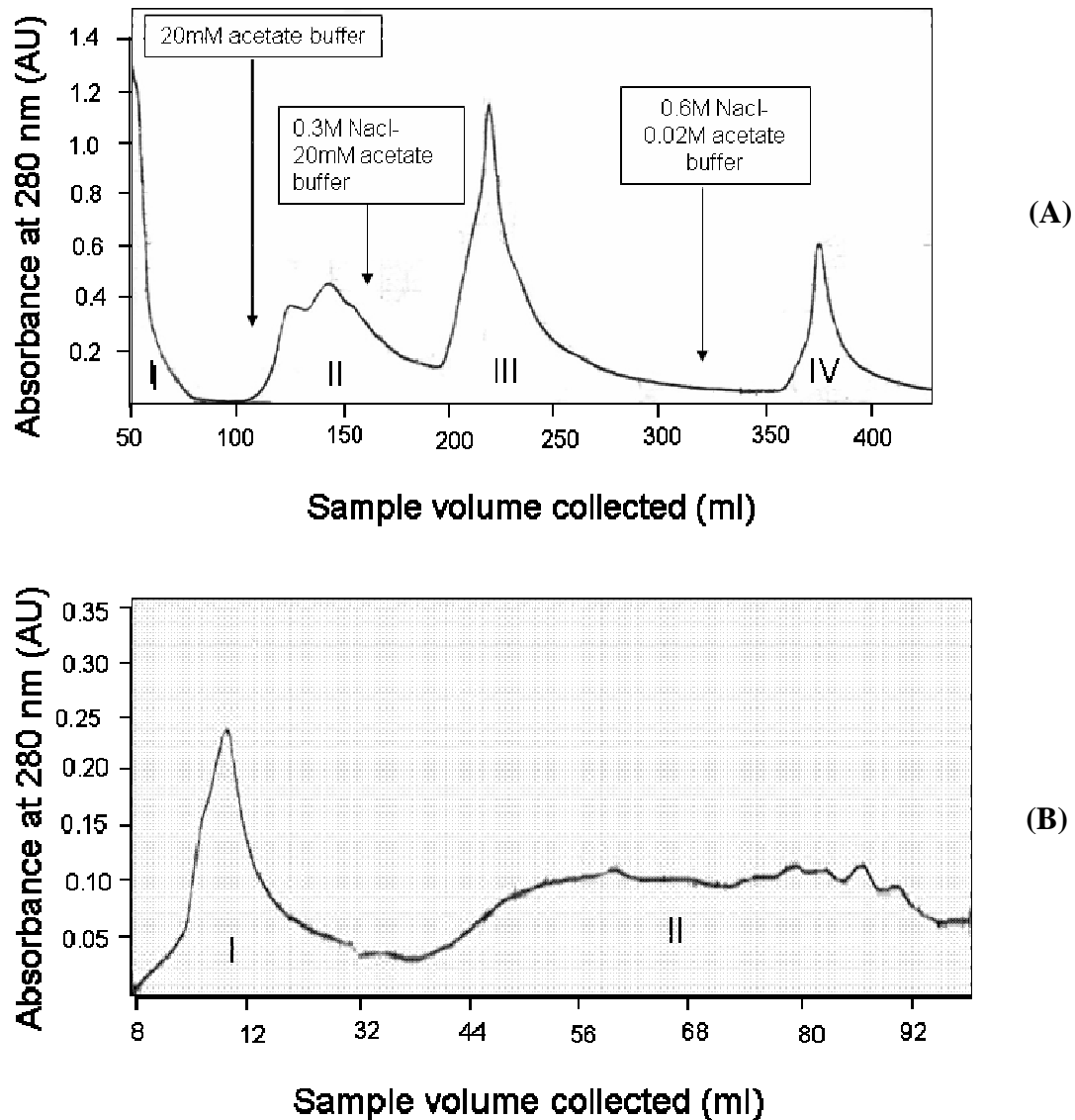
Table 3.3: List of Bacterial Cultures and their Sources, Growth Conditions, Incubation Period for MIC Assay

<b>Bacteria</b>	<b>Source</b>	<b>Broth</b>	<b>Growth Conditions</b>	<b>Incubation period</b>
<i>Staphylococcus aureus</i>	UW- Madison, Lindquist	BHI	Aerobic	12 h
<i>Clostridium perfringens</i>	UW- Madison, Lindquist	BHI	Anaerobic	48 h
<i>Listeria monocytogenes</i>	USDA	BHI	Aerobic	24 h
<i>Yersinia enterocolita</i>	ATCC 23715	BHI	Aerobic	12 h
<i>Bacillus cereus</i>	ATCC 11778	BHI	Aerobic	12 h
<i>Enterococcus faecium</i>	UW- Madison, Lindquist	BHI	Aerobic	12 h
<i>Enterococcus faecalis</i>	UW- Madison, Lindquist	BHI	Aerobic	12 h
<i>Lactobacillus viridescens</i>	UW- Madison, Lindquist	APT	Aerobic	24 h
<i>Lactobacillus plantarum</i>	UW- Madison, Lindquist	APT	Aerobic	24 h
<i>Pediococcus cerevisiae</i>	M.J. Johnson, Univ Of Arkansas	APT	Aerobic	24 h
<i>Aerococcus viridans</i>	M.J. Johnson, Univ Of Arkansas	BHI	Aerobic	24 h
<i>Pseudomonas fluorescens</i>	M.J. Johnson, Univ Of Arkansas	BHI	Aerobic	24 h
<i>Pseudomonas aeruginosa</i>	M.J. Johnson, Univ Of Arkansas	BHI	Aerobic	24 h
<i>Campylobacter jejuni</i>	ATCC 29428	BHI	Anaerobic	48 h
<i>Campylobacter coli</i>	ATCC 43480	BHI	Anaerobic	36 h
<i>E.coli O157:H7</i>	ATCC (Salvik) 43889	BHI	Aerobic	12 h
<i>Shigella spp</i>	M.J. Johnson, Univ Of Arkansas	BHI	Aerobic	12 h
<i>Salmonella anatum</i>	ATCC 27869	BHI	Aerobic	12 h
<i>Salmonella enteritidis</i>	UW- Madison, Lindquist	BHI	Aerobic	12 h

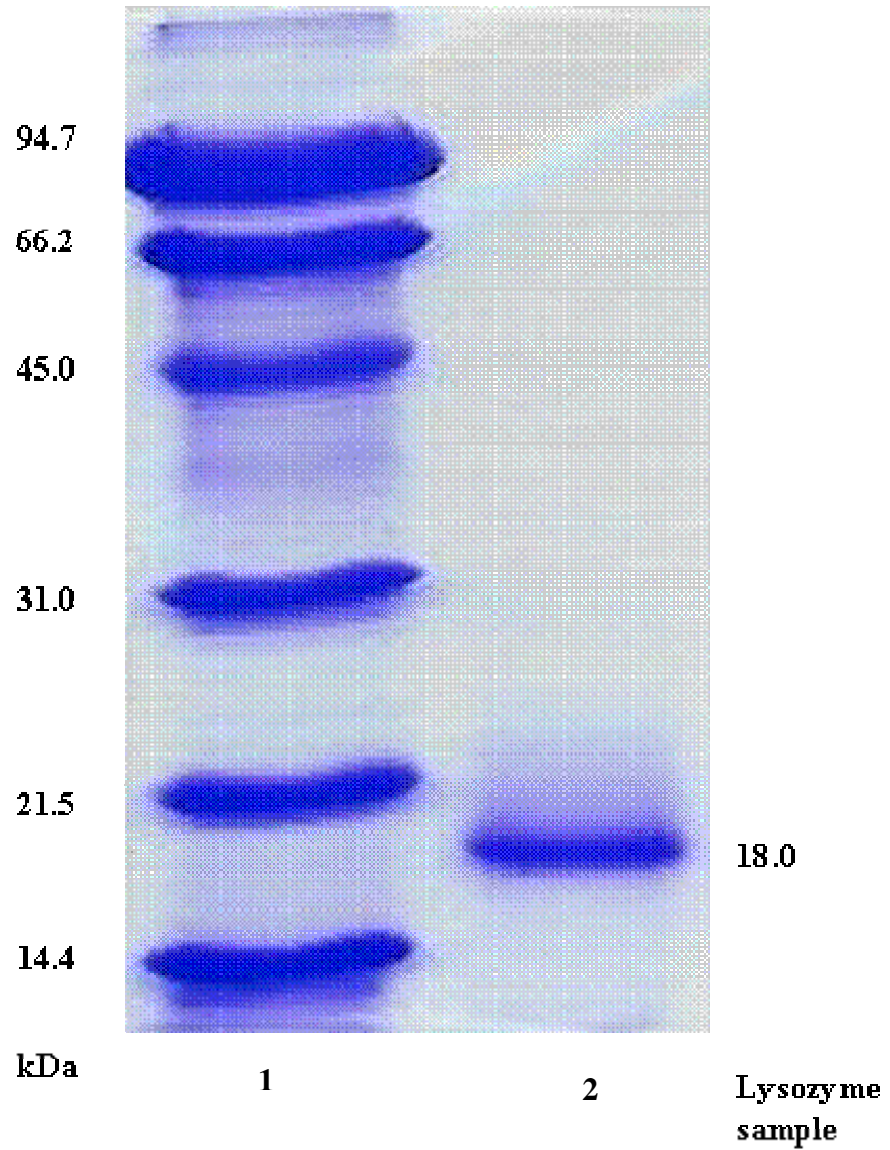
### 3.3.2 Large Scale Purification of Lysozyme from Oyster Shell Liquor

A protein with high lytic activity against *M. lysodeikticus* was purified from oyster shell liquor by a two step ion exchange chromatography. The first step ion-exchange chromatography using SP-sepharose resulted in elution of large quantity of non lysozyme protein which was eluted out during sample loading and column washing steps with 20mM sodium acetate buffer at pH 5.0 (Figure 3.2 A, peak I). No lysozyme activity was detected in fractions from peak II and peak III were checked for lysozyme activity. Fractions collected in peak IV showed lysozyme activity and hence were pooled together and designated as 'Lysozyme enriched sample'. Twenty eight percent of lysozyme was recovered after the initial ion exchange chromatography (Table 3.5). The specific activity of the crude shell liquor was  $2.06 \times 10^2$  U/mg. The specific activity of lysozyme enriched sample was  $1.47 \times 10^4$  U/mg, a 71 fold increase over the crude shell liquor sample. Sodium chloride linear gradient of 0.3-0.65 M in basic buffer was used for the elution of lysozyme enriched sample for the second step ion exchange chromatography using CM Sepharose. Samples from peak IV were distributed in a narrow (peak I) and broad peak (peak II) respectively (Figure 3.2 B). Peak I contained no lysozyme activity. Fractions collected in peak II showed a single protein band with high lysozyme activity using SDS-PAGE. All the fractions from peak II were pooled together and designated as 'purified lysozyme'. Oyster lysozyme purified from shell liquor gave a single band when stained with Coomassie brilliant blue. Molecular mass under reducing conditions was found to be about 18 KDa which was comparable to reference oyster plasma lysozyme (Figure 3.3). The specific activity of purified lysozyme was  $1.90 \times 10^5$  which was 922 times greater than the specific activity of the crude shell liquor sample with a recovery of 11.1%

Two hundred and five mg of purified lysozyme was purified from 300 liters of shell liquor.



**Figure 3.2** Purification of Plasma Lysozyme from Eastern Oysters (*C.virginica*) by Ion Exchange Chromatographies. A) Ion Exchange Chromatography of Crude Shell Liquor Lysozyme on SP-Sepharose FF column. A stepwise elution with NaCl. Fractions collected in peak IV were pooled as ‘lysozyme enriched sample’. B) Ion Exchange Chromatography of Lysozyme Enriched Sample on CM-Sepharose Fast Flow column. The column was eluted with a linear gradient of NaCl from 0.3 to 0.65 M in 0.02 M sodium acetate buffer, pH 5. The fractions in peak II were collected and designated as ‘purified lysozyme’.



**Figure 3.3** SDS-PAGE of Purified Oyster Lysozyme (*C.virginica*). Lane 1 represents the protein markers and Lane 2 represents purified oyster shell liquor lysozyme. SDS-PAGE was performed on a 12.5% gel under reduced conditions. Separated proteins were stained with Coomassie Brilliant Blue.

Table 3.4: Summary of the Oyster (*C.Virginica*) Lysozyme Purification from Shell Liquor using the Large Scale Protocol

Sample	Total Protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)
Crude shell liquor	$1.73 \times 10^6$	$3.5 \times 10^8$	$2.06 \times 10^2$	
Lysozyme-enriched IE	$6.70 \times 10^3$	$9.8 \times 10^7$	$1.47 \times 10^4$	28
Purified oyster lysozyme	$2.05 \times 10^2$	$3.9 \times 10^7$	$1.90 \times 10^5$	11.1

IE- Ion exchange

### 3.3.3 Determination of MIC of Oyster Lysozyme against Foodborne Pathogens

Antimicrobial activity of purified oyster lysozyme and Hen egg white lysozyme against 19 major foodborne pathogens was determined using Minimum inhibitory concentration assays (MIC). The results presented in Table 3.6 show that oyster lysozyme inhibited *Clostridium perfringens* at 2.5 µg/ml. *Lactobacillus plantarum*, *Pediococcus cerevisiae*, *Campylobacter jejuni* and *Campylobacter coli* were inhibited at 5 µg/ml lysozyme. *Enterococcus faecium* and *Enterococcus faecalis* were inhibited at a concentration of 20 µg/ml. *Listeria monocytogenes*, *Salmonella anatum* and *Lactobacillus viridescens* were inhibited at a higher concentration of 160 µg/ml. There was no growth inhibition of *Staphylococcus aureus*, *Bacillus cereus*, *Yersinia enterocolita*, *Aerococcus viridans*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *E.coli* 0157:H7, *Shigella spp* and *Salmonella enteriditis* at the highest concentration tested (160 µg/ml). Hen egg white lysozyme showed similar MIC for all the bacteria except for *Staphylococcus aureus* with a MIC of 40 µg/ml. Statistical analysis was carried out to see significant differences between oyster lysozyme and hen egg white lysozyme.

Table 3.5: Minimum Inhibitory Concentration (MIC) of Purified Oyster Lysozyme and Hen Egg White Lysozyme

<b>Bacteria</b>	<b>MIC (<math>\mu\text{g/ml}</math>)</b>	<b>MIC (<math>\mu\text{g/ml}</math>)</b>
<b>FOOD SPOILAGE</b>	<b>Oyster lysozyme</b>	<b>HEWL</b>
<i>Enterococcus faecalis</i>	20	20
<i>Enterococcus faecium</i>	20	20
<i>Lactobacillus viridescens</i>	160	160
<i>Lactobacillus plantarum</i>	5	5
<i>Pediococcus cerevisiae</i>	5	5
<i>Aerococcus viridans</i>	>160	>160
<i>Pseudomonas fluorescens</i>	>160	>160
<i>Pseudomonas aeruginosa</i>	>160	>160
	<b>MIC (<math>\mu\text{g/ml}</math>)</b>	
<b>FOOD POISONING</b>		
<i>Salmonella enteritidis</i>	>160	>160
<i>Staphylococcus aureus</i>	>160	40
<i>Clostridium perfringens</i>	2.5	2.5
<i>Listeria monocytogenes</i>	160	>160
<i>Yersinia enterocolita</i>	>160	>160
<i>Campylobacter jejuni</i>	5	5
<i>Bacillus cereus</i>	>160	>160
<i>E.coli</i> 0157:H7	>160	>160
<i>Campylobacter coli</i>	5	5
<i>Shigella spp</i>	>160	>160
<i>Salmonella anatum</i>	160	160

### 3.4 Discussion

Oyster shell liquor collected over a 1-year period in four seasons was checked for seasonal variations in protein concentration and lysozyme activity. Results show a wide variation in concentration and activity due to seasonal variations. Lysozyme concentration and yield were highest during the spring season and lowest in summer. Plasma lysozyme activity was shown to be higher in winter (Chu and La Peyre, 1989). Batches collected in the spring season contained more protein and lysozyme and we chose the spring season to collect shell liquor for large scale purification of lysozyme.

Shell liquor is an industrial waste of oyster processing which consists of hemolymph and plasma. Hemolymph, of oysters contains high levels of lysozyme activity (Mcdade and Tripp, 1967; Xue et al., 2004).

High lysozyme concentration was also found in the digestive system of many bivalves species such as *Mytilus edulis*, *Modiolus modiolus*, *Chlamys opercularis*, *Tellina tenuis*, the gill of *Mya arenaria*, flat oyster *Ostrea edulis*, and mantle of *Crassostrea virginica* (Mcdade and Tripp, 1967; Mchenery et al, 1986; Cronin et al., 2001).

The first purification of lysozyme from oyster species was done by Xue et al.,(2004) who purified and characterized lysozyme from the plasma of the eastern oyster (*Crassostrea virginica*). The same purification protocol was modified and scaled-up of the purification of lysozyme from 300 liters of oyster shell liquor. This is the first report where lysozyme was purified from the shell liquor of a bivalve mollusk on a laboratory and large scale levels. The modification of protocol consisted of omitting the gel filtration step and directly applying the sample to the first ion exchange chromatography (Xue et al., 2004). Large volumes of oyster shell liquor were purified using two-step ion exchange chromatography. The protein purified from the shell liquor showed high lytic activity against *M. lysodeikticus*. SDS-PAGE showed a single band at about 18 KDa which was of similar size to plasma lysozyme (Xue et al., 2004).

From the results of Minimum Inhibitory Concentration assays we found that purified oyster lysozyme from the shell liquor had antimicrobial activity against both gram-positive and gram negative bacteria. There have been few studies which dealt with the use of bivalve lysozyme for antimicrobial activities against Gram-positive and Gram-negative bacteria (Nilsen et al., 1999; Xue et al., 2004). Lysozymes are known to be active against Gram

positive bacteria due to thick peptidoglycan layer whereas Gram- negative bacteria have a single layer of peptidoglycan and a thick outer membrane which acts as a barrier preventing the access of lysozyme (Masschalck and Michiels, 2003). However, some investigators (During et al., 1999; Pellegrini et al., 1992; Ibrahim et al., 1996) have reported lysozyme to be active against gram negative bacteria. Our study also show that lysozyme from oyster shell liquor has activities against some Gram negative bacteria and Gram positive bacteria (Table 3.8).

Oyster lysozyme showed highest inhibition towards *Clostridium perfringens*. Adam (1974) showed that ultrahigh temperature treated *Clostridium perfringens* spores with EDTA sensitized the spores to lysozyme (Adams, 1974). However Hughey and Johnson (1987) reported *Clostridium perfringens* to be resistant to the action of hen egg white lysozyme. Hen egg white lysozyme is used in food industry against *C. botulinum* (Hughey et al., 1989; Johnson, 1994). It has been shown in studies that lysozyme may have different inhibitory activities against the same bacteria but different species (Johnson, 1994; Losso et al, 2000). Hen egg white lysozyme is known to be effective against certain gram negative bacteria when the cells are pre-treated with EDTA (Wooley et al., 1974). Pellegrini et al., 1992 showed that lysozyme could be effective against certain Gram negative bacteria even without the EDTA pretreatment. The bactericidal activity of lysozyme was attributed to the cationic and hydrophobic properties of lysozyme (Pellegrini et al., 1992). Our results also show that there is a marked decrease in absorbance of Gram negative *C. coli*, *C. jejuni* without any addition of EDTA. *C. jejuni* was shown to be weakly inhibited by lysozyme when the bacteria were inoculated into complex broth media containing 20-200 mg/liter of lysozyme (Hughey and Johnson, 1987).

Lysozyme is effective against *L. monocytogenes* and several studies have been carried out using lysozyme against *L. monocytogenes* (Carminati and Carini, 1989; Hughey et al., 1989; Wang and Shelef, 1991). Our study shows that at highest concentration tested i.e. 160 µg/ml, oyster lysozyme inhibited *L. monocytogenes*. The susceptibility of *L. monocytogenes* to lysozyme inactivation was found to be dependent on the physiological status of the bacterium and also the medium of the food in which lysozyme was suspended (Johnson, 1994).

*Staphylococcus aureus*, a gram positive bacterium is not inhibited by action of lysozyme (Salton and Pavlik, 1960; Wooley et al., 1974). Our study show that oyster lysozyme at 160 µg/ml did not inhibit the growth of *S. aureus*. Similarly, *Yersina enterocolitica* and *E.coli* O157:H7 were not inhibited by 160 µg/ml of oyster lysozyme. Hughey and Johnson (1987) reported *Yersina enterocolitica* and *E.coli* O157:H7 to be resistant to the action of hen egg white lysozyme.

Thus, from the minimum inhibitory concentration assays we conclude that oyster lysozyme and hen egg white lysozyme have antimicrobial activities against both Gram-positive and Gram-negative bacteria.

**CHAPTER 4**  
**CONTROL OF *LISTERIA MONOCYTOGENES* AND *SALMONELLA ANATUM***  
**ON THE SURFACE OF SMOKED SALMON COATED WITH EDIBLE**  
**COATINGS CONTAINING OYSTER LYSOZYME AND NISIN**

**4.1 Introduction**

Cold-smoked salmon is a ready-to-eat (RTE) food product which is normally purchased vacuum-packed and has a shelf life of 3 to 5 weeks at refrigerated temperatures (Rovik, 2000; Leroi et al., 2001). Regulatory agencies in the United States have adopted zero-tolerance policy towards contamination of RTE products with *Listeria monocytogenes* which can grow easily on the surface of these food products.

A major concern of the smoked fish industry is contamination of their products with *L. monocytogenes* and *Salmonella* species (Heintz and Johnson, 1998). The highest incidence of *L. monocytogenes* is associated with cold-smoked fish rather than hot smoked fish because this pathogen does not survive the hot smoke process (Eklund et al., 1994). Contamination of smoked fish with *L. monocytogenes* ranges from 17.9% to 22.3% (Heintz and Johnson, 1998). Studies have shown that cold-smoked salmon is a good substrate for the growth of *L. monocytogenes* even under vacuum conditions (Rorvik et al., 1991). *Salmonella* species have also been associated with smoked fish outbreaks (Heintz et al., 2000). The incidence of *Salmonella* in smoked fish is 3.9% with *Salmonella newport* or *Salmonella anatum* as the most prominent spp isolated from smoked fish in United States (Heintz et al., 2000).

Antimicrobial films act as a protective barrier which retards the food deterioration and when the film is incorporated with antimicrobial agents can extend the food shelf life (Cha and Chinnan, 2004). Antimicrobial agents incorporated into edible films are released slowly onto the surface of food products and these coatings also serve as a

barrier to moisture and oxygen which are cost effective and can protect the food material even when the package of the food material is opened (Cha and Chinnan, 2004).

Edible film coatings can be polysaccharide-based films, protein-based films or lipid-based films. One of the widely used polysaccharide film is alginate based films. Alginates are extracted from the brown seaweeds that belong to the *Phaeophyceae* class, They are salts of alginic acid, which is a linear polymer of D-mannuronic and L-guluronic acid monomers. Alginates have the ability to react with di-valent and tri-valent cations and this property is being utilized in alginate film formation. Calcium ions are widely used as effective gelling agents (Cha and Chinnan, 2004). Antimicrobial agents have been incorporated into calcium alginate gels and used to preserve a variety of food products (Wan et al., 1997; Cutter and Siragudsa, 1996; Williams et al., 1978; Lazarus et al., 1976).

The objective of this study was to determine the feasibility of using oyster lysozyme in antimicrobial films coatings to enhance the preservation of cold-smoked salmon.

## **4.2 Materials and Methods**

### **4.2.1 Culture Growth Conditions**

*Listeria monocytogenes* strain V7 (Serotype ½ a) and *Salmonella anatum* were obtained from the Centers for Disease Control, (Atlanta, Ga.). The bacterial cultures were grown for 24 h at 37°C in brain heart infusion (BHI) broth and decimally diluted with 0.1 M potassium phosphate buffer (pH 7.0) (PBS buffer). The pure cultures were stored at -70° C and subcultured twice in BHI broth at 37°C for 24 h before use.

#### **4.2.2 Preparation of Edible Coatings**

The agar gel coating was prepared by dissolving 0.75 g of Bacto agar (Difco) into 100 ml of sterile distilled water. Zein propylene glycol liquid solution was obtained from Freeman Industries (Tuckahoe, N.Y.). Calcium alginate coating was made by the combination of 1g of calcium carbonate (Sigma) and 1g of sodium alginate (ISP Alginates, Inc.) into 100 ml of sterile distilled water. All the solutions were autoclaved at 121 °C for 20 min and stored until use.

#### **4.2.3 Preparation of Antimicrobials and Fish Samples**

Purified oyster lysozyme was diluted to a concentration of 160 µg/ml because this is the minimum inhibitory concentration of oyster lysozyme needed against *L. monocytogenes* and *S. anatum* (Chapter 3). Hen egg white lysozyme crystals were obtained from Sigma. The concentration of hen egg white lysozyme used during this study was also 160 µg/ml for the same reasons as for oyster lysozyme. Nisaplin, a commercial source of nisin was obtained from Aplin & Barrett Ltd ( Trowbridge, Wilts, England). Nisaplin stock solution was made by dissolving 1g of Nisaplin into 1 ml of 0.02 N HCl to yield a final concentration of 1000 IU/g. The Nisaplin stock was filter sterilized through a 0.22µm low protein binding filter. The stock solution was autoclaved at 121 °C for 20 min and stored until used. Smoked salmon samples were purchased from a local supermarket. They were cut into 1g pieces and kept in sterile Whirl-Pack bags that were frozen at -20 °C until used.

#### **4.2.4 Determination of the Most Effective Film Coating for Enhancing the Antimicrobial Activity of Oyster Lysozyme (Appendix 1)**

Fish samples (1g) were immersed into 24 h broth cultures of *L. monocytogenes* V7 (Serotype ½ a) or *Salmonella anatum* for 1 min, allowed to drip free of excess inoculum

and dried for 20 min. The control samples were dipped into the broth cultures. Smoked salmon samples with and without *L. monocytogenes* or *S. anatum* were dipped into various treatments. The following six treatments were examined for bacterial counts during this study: 1) Control smoked salmon with no treatment; 2) Inoculated control smoked salmon; 3) inoculated smoked salmon dipped into 1000IU/g of nisin (N), Hen egg white lysozyme at 160 µg/ml (HEWL), or Oyster lysozyme at 160 µg/ml (LYO); 4) Inoculated smoked salmon dipped into edible coatings without antimicrobial treatments; 5) Inoculated smoked salmon dipped into edible coatings containing either N, HEWL or LYO; 6) Inoculated smoked salmon with N and HEWL or LYO incorporated inside edible coatings. The samples were allowed to air dry for 20 min before putting them into sterile bags and refrigerated at 4° C for 24 h. Bacterial counts were determined the next day by adding PBS to make a 1/10 dilution in each bag, stomaching for 2 min then plating serial dilutions onto XLD agar for *Salmonella anatum* and Oxford medium base with Oxford supplement for *L. monocytogenes*. The plates were then incubated at 37°C for 24 h and CFU/g was determined. The effect of three different edible coatings; calcium alginate, 0.75% agar edible coating and zein coatings containing antimicrobial agents against *Listeria monocytogenes* and *Salmonella anatum* on the surface of the smoked fish at 4° C after 24 h were determined.

#### **4.2.5 Effect of Oyster Lysozyme Incorporated into Calcium Alginate Coatings against *Listeria monocytogenes* and *Salmonella anatum* during 35 days Storage (Appendix 2)**

Smoked salmon samples (1g) were immersed into 24 h broth cultures of *L. monocytogenes* V7 (Serotype ½ a) or *S. anatum* for 1 min, allowed to drip free of excess inoculum and dried for 20 min. The following six treatments were examined for bacterial

counts during this study 1) Control smoked salmon with no treatment; 2) Inoculated control smoked salmon; 3) Inoculated smoked salmon dipped into 1000 IU/g of nisin (N), Hen egg white lysozyme at 160 µg/ml (HEWL) or Oyster lysozyme at 160 µg/ml (LYO); 4) Inoculated smoked salmon dipped into edible coatings without antimicrobial treatments; 5) Inoculated smoked salmon dipped into edible coatings containing either N, HEWL or LYO; 6) Inoculated smoked salmon with N and HEWL or LYO incorporated inside edible coating. The samples were allowed to air dry for 20 min before putting them into sterile bags and refrigerated at 4 °C. Bacterial counts were determined at 0, 7, 14, 21, 28, and 35 d by adding PBS to make a 1/10 dilution in each bag, stomaching for 2 min, and then plating serial dilutions onto XLD agar for *S. anatum* or Oxford medium base with Oxford supplement for *L. monocytogenes*. The plates were incubated at 37 °C for 24 h and CFU/g was determined.

#### **4.2.6 Statistical Analysis**

The inhibitory effects of the edible coatings with or without the antimicrobial agents against growth of *L. monocytogenes* or *S. anatum* on the ready-to-eat cold-smoked salmon samples at refrigerated temperatures were analyzed by statistical comparisons of all pairs using Student's *t*-test following one-way analysis of the variance (ANOVA) (SAS Institute Inc., Cary, N.C., U.S.A.). Statistical significance occurs for  $P > 0.05$ .

#### **4.3 Results**

After 24 h, *L. monocytogenes* counts were reduced by 0.52 log CFU/g when oyster lysozyme was incorporated into 1% calcium alginate coatings on the surface of the smoked salmon (Figure 4.1). When nisin was added along with oyster lysozyme into the coating, it caused further reduction of bacterial counts by 1.63 log CFU/g. There was no

significant difference between oyster lysozyme and hen egg white lysozyme in reducing the bacterial counts, either when used alone or when used in combination with nisin inside the calcium alginate coating.

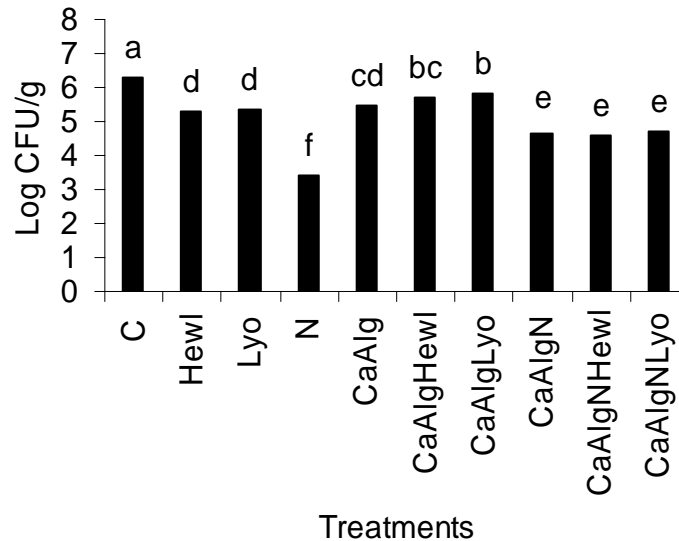


Figure 4.1: Effect of Calcium Alginate Edible Coating containing Antimicrobial Agents against *Listeria monocytogenes* on the Surface of Smoked Salmon at 4° C after 24 h.

<sup>a</sup> All analysis were based on two separate experiments. Means followed by the same letter are not significantly different ( $P = 0.05$ ) from each other. Statistical comparisons of all pairs were analysed using Student's *t*-test following one-way analysis of the variance (ANOVA) (SAS Institute, Inc., Cary, NC).

<sup>b</sup> Abbreviations of treatments are as follows:  
 C, Control; CaAlg, Calcium alginate coating; Hewl, Hen Egg White Lysozyme; N, Nisin; Lyo, Oyster lysozyme; CaAlgn, Calcium alginate coating with Nisin; CaAlgHewl, Calcium alginate coating with Hen Egg White Lysozyme; CaAlgLyo, Calcium alginate coating with Lysozyme; CaAlgnHewl, Calcium alginate coating with Nisin and Hen egg white lysozyme; CaAlgnLyo, Calcium alginate coating with nisin and Lysozyme

*L. monocytogenes* counts were reduced by 0.82 log CFU/g when oyster lysozyme was incorporated into 0.75% agar coatings on the surface of the smoked salmon after 24 h (Figure 4.2). Nisin along with oyster lysozyme inside the coating caused further reduction of bacterial counts by 1.82 log CFU/g. There was no significant

difference between oyster lysozyme and hen egg white lysozyme in reducing the bacterial counts, either when used alone or when used in combination with nisin inside the 0.75% agar coating.

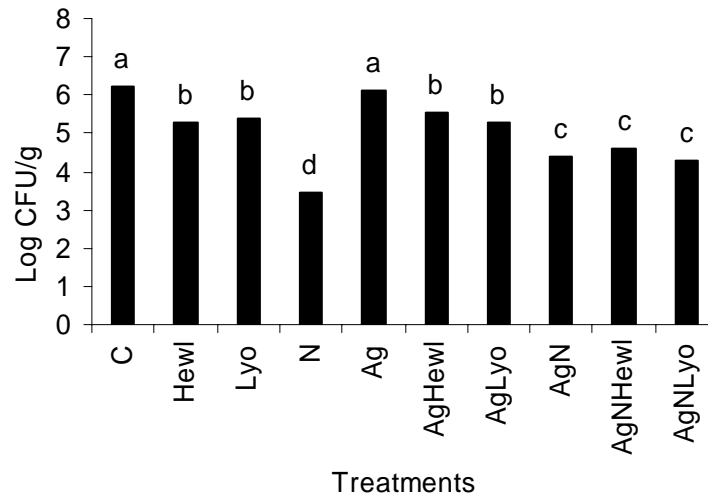


Figure 4.2: Effect of 0.75% Agar Edible Coating containing Antimicrobial Agents against *Listeria monocytogenes* on the Surface of Smoked Salmon at 4° C after 24 h.

<sup>a</sup> All analysis were based on two separate experiments. Means followed by the same letter are not significantly different ( $P = 0.05$ ) from each other. Statistical comparisons of all pairs were analysed using Student's *t*-test following one-way analysis of the variance (ANOVA) (SAS Institute, Inc., Cary, NC).

<sup>b</sup> Abbreviations of treatments are as follows:  
 C, Control; Ag, Agar coating; Hewl, Hen Egg White Lysozyme; N, Nisin; Lyo, Oyster lysozyme; AgN, Agar coating with Nisin; AgHewl, Agar coating with Hen Egg White Lysozyme; AgLyo, Agar coating with Lysozyme; AgNHewl, Agar coating with Nisin and Hen Egg White Lysozyme and; AgNlyo, Agar coating with Nisin and Lysozyme

After 24 h, *L. monocytogenes* counts were reduced by 0.61 log CFU/g when oyster lysozyme was incorporated into zein coatings on the surface of the smoked salmon (Figure 4.3). When nisin was added along with oyster lysozyme inside the coating it caused further reduction of bacterial counts by 1.79 log CFU/g. There was no significant difference between oyster lysozyme and hen egg white lysozyme in reducing the bacterial

counts, either when used alone or when used in combination with nisin inside the zein coating.

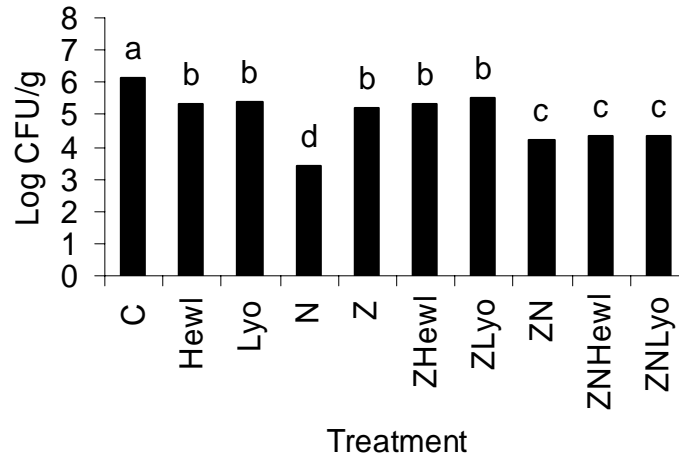


Figure 4.3: Effect of Zein Coating containing Antimicrobial Agents against *Listeria monocytogenes* on the Surface of Smoked Salmon at 4° C after 24 h

<sup>a</sup> All analysis were based on two separate experiments. Means followed by the same letter are not significantly different ( $P = 0.05$ ) from each other. Statistical comparisons of all pairs were analysed using Student's *t*-test following one-way analysis of the variance (ANOVA) (SAS Institute, Inc., Cary, NC).

<sup>b</sup> Abbreviations of treatments are as follows:  
 C, Control; Z, Zein coating; Hewl, Hen Egg White Lysozyme; N, Nisin; Lyo, Oyster lysozyme; ZN, Zein coating with Nisin; ZHewl, Zein coating with Hen Egg White Lysozyme; ZLyo, Zein coating with Lysozyme; ZNHewl, Zeincoating with Nisin and Hen Egg White Lysozyme ; ZNLyo, Zein coating with Nisin and Lysozyme

After 24 h, *S. anatum* counts were reduced by 1.82 log CFU/g when oyster lysozyme was incorporated into 1% calcium alginate coating on the surface of the smoked salmon Figure (4.4). Oyster lysozyme when incorporated into the calcium alginate coatings showed the most significant difference from all other treatment and was found to be the most effective treatment. Oyster lysozyme along with nisin when incorporated inside the calcium alginate coatings also showed significant reduction in bacterial counts.

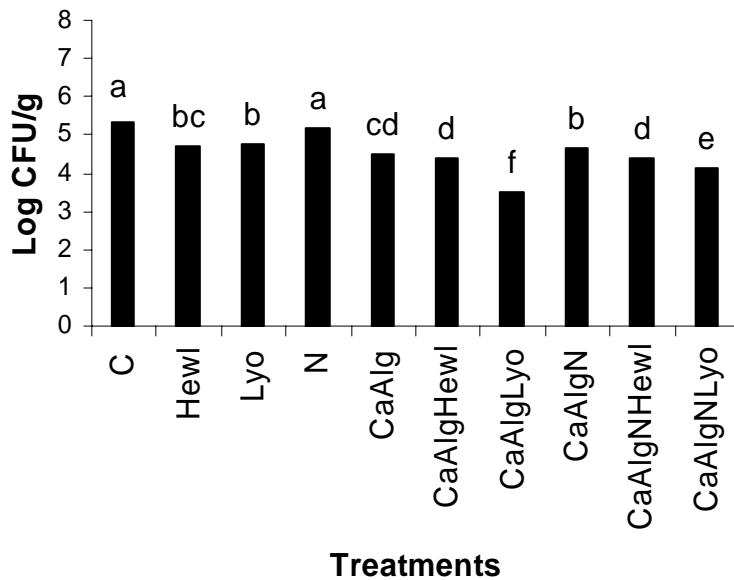


Figure 4.4: Effect of Calcium Alginate Coating containing Antimicrobial Agents against *Salmonella anatum* on the Surface of Smoked Salmon at 4° C after 24 h

<sup>a</sup>All analysis were based on two separate experiments. Means followed by the same letter are not significantly different ( $P = 0.05$ ) from each other. Statistical comparisons of all pairs were analysed using Student's *t*-test following one-way analysis of the variance (ANOVA) (SAS Institute, Inc., Cary, NC).

<sup>b</sup> Abbreviations of treatments are as follows:

C, Control; CaAlg, Calcium alginate coating; Hewl, Hen Egg White Lysozyme; N-Nisin; Lyo, Oyster lysozyme; CaAlgN, Calcium alginate coating with Nisin; CaAlgHewl, Calcium alginate coating with Hen Egg White Lysozyme; CaAlgLyo, Calcium alginate coating with Lysozyme; CaAlgNHewl, Calcium alginate coating with Nisin and Hen Egg White Lysozyme; CaAlgNLyo, Calcium alginate coating with nisin and Lysozyme

After 24 h, *S. anatum* counts were reduced by 0.4 log CFU/g when oyster lysozyme was incorporated into 0.75% agar coatings on the surface of the smoked salmon (Figure 4.5). When nisin was added along with oyster lysozyme inside the coating it caused further reduction of bacterial counts by 0.54 log CFU/g. The most

effective treatment was when nisin and oyster lysozyme were incorporated inside the calcium alginate film.

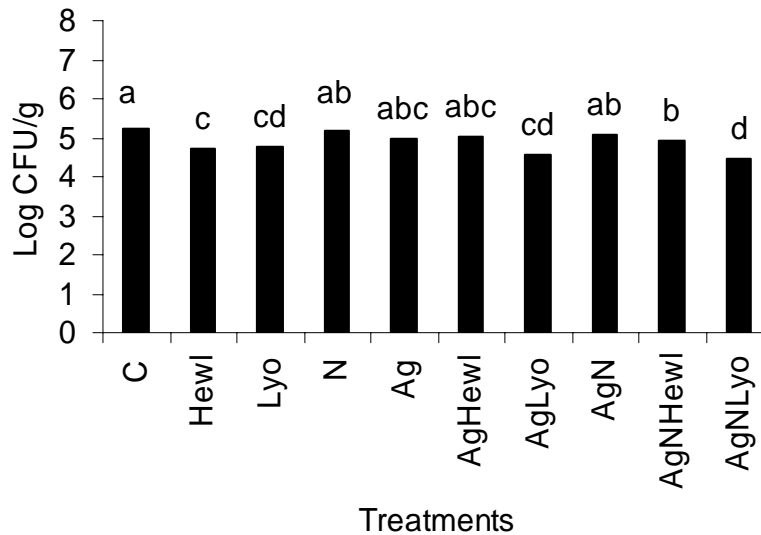


Figure 4.5: Effect of 0.75 % Agar Coating containing Antimicrobial Agents against *Salmonella anatum* on the Surface of Smoked Salmon at 4 °C after 24 h.

<sup>a</sup> All analysis are based on two separate experiment. Means followed by the same letter are not significantly different ( $P = 0.05$ ) from each other. Statistical comparisons of all pairs were analysed using Student's *t*-test following one-way analysis of the variance (ANOVA) (SAS Institute, Inc., Cary, NC).

<sup>b</sup> Abbreviations of treatments are as follows:  
 C, Control; Ag, Agar coating; Hewl, Hen Egg White Lysozyme; N, Nisin; Lyo, Oyster lysozyme; AgN, Agar coating with Nisin; AgHewl, Agar coating with Hen Egg White Lysozyme; AgLyo, Agar coating with Lysozyme; AgNHewl, Agar coating with Nisin and Hen Egg White Lysozyme ; AgNLYo, Agar coating with Nisin and Lysozyme

After 24 h, *S. anatum* counts were reduced by 0.97 log CFU/g when oyster lysozyme was incorporated into zein coatings on the surface of the smoked salmon (Figure 4.6) after 24 h. When nisin was added along with oyster lysozyme inside the coating it caused further reduction of bacterial counts by 1.14 log CFU/g. Hence, it was seen that for all the treatments, edible coating along with antimicrobials and nisin showed

the most reduction in bacterial counts compared to the control. The best film among the three films was then chosen for the shelf life study.

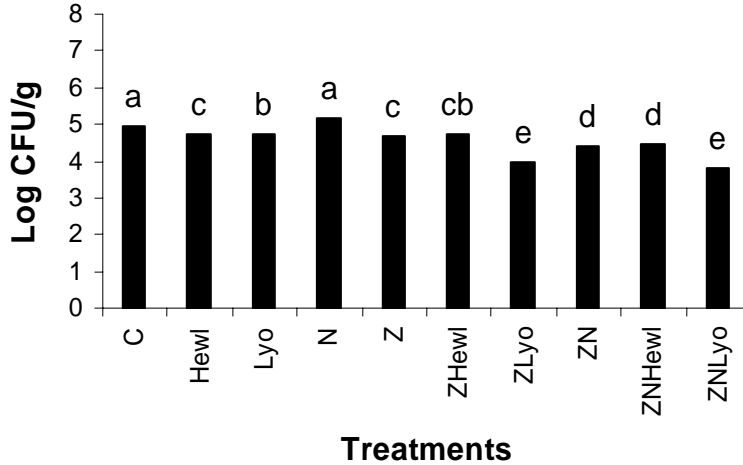


Figure 4.6: Effect of Zein Coating containing Antimicrobial Agents against *Salmonella anatum* on the Surface of Smoked Salmon at 4° C after 24 h.

<sup>a</sup> All analysis are based on two separate experiment. Means followed by the same letter are not significantly different ( $P = 0.05$ ) from each other. Statistical comparisons of all pairs were analysed using Student's *t*-test following one-way analysis of the variance (ANOVA) (SAS Institute, Inc., Cary, NC).

<sup>b</sup> Abbreviations of treatments are as follows:  
 C, Control; Z, Zein coating; Hewl, Hen Egg White Lysozyme; N, Nisin; Lyo, Oyster lysozyme; ZN, Zein coating with Nisin; ZHewl, Zein coating with Hen Egg White Lysozyme; ZLyo, Zein coating with Lysozyme; ZNHewl, Zeincoating with Nisin and Hen Egg White Lysozyme; ZNLyo, Zein coating with Nisin and Lysozyme

From the 35 days shelf life study, we observed that at 4° C an initial inoculation of *Listeria monocytogenes* increased from 6.57 log CFU/g to 9.86 log CFU/g on day 35 in the control samples (Table 4.1). Bacterial counts reached the control level when the surface of smoked salmon was treated with oyster lysozyme by the end of shelf life period. After 35 days of storage there was a 1.05 log CFU/g reduction of *L. monocytogenes* counts on the surface of smoked fish coated with calcium alginate coating containing 160µ/ml oyster lysozyme as compared to the control. With the

addition of nisin along with oyster lysozyme in the calcium alginate coatings, *L. monocytogenes* counts were further reduced by 2.75 log CFU/g on the surface of the smoked salmon by day 35. The most significant treatment was oyster lysozyme and hen egg white lysozyme when incorporated into the calcium alginate coating. There was no significant difference between oyster lysozyme and hen egg white lysozyme.

At 4° C, an initial inoculation of *Salmonella anatum* increased from 5.78 log CFU/g to 6.12 log CFU/g on day 35 in the control samples (Table 4.2). Bacterial counts were reduced by 0.73 log CFU/g when the surface of smoked salmon was treated with oyster lysozyme at the end of the shelf life period. After 35 days of storage there was a 1.52 log reduction of *S. anatum* counts on the surface of the smoked salmon coated with calcium alginate containing 160 µg/ml oyster lysozyme as compared to the control. With the addition of nisin along with oyster lysozyme in the calcium alginate coatings *S. anatum* counts were reduced by 2.25 log CFU/g on the surface of the smoked fish by day 35. The most effective treatment was obtained when oyster lysozyme and hen egg white lysozyme was incorporated into the calcium alginate coating.

#### **4.4 Discussion**

Smoked fish and shellfish products can be contaminated with foodborne pathogens like *Listeria monocytogenes* and *Salmonella species* (Heintz and Johnson, 1998). Studies carried out in cold-smoked salmon processing plant showed that the primary source of *L. monocytogenes* contamination was the surface of the frozen or raw fish coming into the plant (Eklund et al., 2004). *Listeria* can survive the cold-smoking process and has been isolated from cold smoked salmon (Guyer and Jemmi, 1991; Dillon et al., 1994; Jemmi,

1993). *Salmonella species* have also been associated with smoked seafood contamination with an incidence of 3.9% in smoked fish (Heinitz et al., 2000).

*Listeria monocytogenes* and *Salmonella anatum* counts were reduced by 0.80 CFU/g on the surface of smoked salmon when the samples were coated with calcium alginate or zein coatings compared to the control non-treated samples. These findings are in agreement with other studies that have shown that calcium alginate edible films coated on lamb or beef cuts reduced total bacterial counts by about 1 log cycle compared to the non-coated samples (Lazarus et al., 1976; Williams et al., 1978).

Our study has shown that adding oyster lysozyme with or without nisin into the edible coatings significantly reduced *L. monocytogenes* and *S. anatum* counts compared to the control nontreated samples on the surface of smoked salmon. Furthermore studies have shown that zein edible coatings are more effective with the addition of food grade antimicrobial agents against foodborne pathogens on the surface of ready-to-eat chicken (Janes et al., 2002). Zein films containing a high concentration of nisin reduced *L. monocytogenes* counts by 8 log cycles compared to zein films without nisin (Hoffman et al., 2001).

We chose calcium alginate edible coatings because it showed more promise for the control of *S. anatum* on the surface of smoked salmon with oyster lysozyme. The *S. anatum* counts were reduced to 1.82 log CFU/g when oyster lysozyme was incorporated into the calcium alginate film compared to 0.97 and 0.4 log CFU/g reduction in bacterial numbers when smoked salmon samples were coated with zein or agar coatings respectively.

Table 4.1 Effect of Different Antimicrobials with and without Calcium Alginate Coating on the Growth of *Listeria monocytogenes* at 4° C for 35 Days.

Treatment*	Log CFU/g*					
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
<b>C</b>	6.57± 0.03 <sup>a</sup>	6.81± 0.21 <sup>a</sup>	7.86 ± 0.25 <sup>a</sup>	8.88 ± 0.12 <sup>a</sup>	9.39 ± 0.14 <sup>a</sup>	9.86 ± 0.17 <sup>a</sup>
<b>CaAlg</b>	6.45 ± 0.01 <sup>a</sup>	6.19 ± 0.14 <sup>b</sup>	7.01 ± 0.12 <sup>b</sup>	8.24 ± 0.07 <sup>b</sup>	9.44 ± 0.13 <sup>a</sup>	9.93 ± 0.19 <sup>a</sup>
<b>N</b>	5.09 ± 0.16 <sup>d</sup>	4.58 ± 0.36 <sup>g</sup>	5.96 ± 0.23 <sup>c</sup>	7.68 ± 0.11 <sup>d</sup>	8.77 ± 0.43 <sup>b</sup>	9.39 ± 0.13 <sup>b</sup>
<b>LYO</b>	5.40 ± 0.07 <sup>cd</sup>	5.22 ± 0.12 <sup>de</sup>	6.95± 0.15 <sup>b</sup>	7.91 ± 0.02 <sup>cd</sup>	8.98 ± 0.18 <sup>abc</sup>	9.61 ± 0.07 <sup>a</sup>
<b>HEWL</b>	5.53 ± 0.09 <sup>b</sup>	5.17± 0.14 <sup>d</sup>	6.99 ± 0.02 <sup>b</sup>	7.9 ± 0.07 <sup>c</sup>	9.10 ± 0.19 <sup>ab</sup>	9.61 ± 0.14 <sup>ab</sup>
<b>CaAlgN</b>	5.22 ± 0.11 <sup>cd</sup>	4.78 ± 0.02 <sup>fg</sup>	5.83 ± 0.33 <sup>d</sup>	6.9 ± 0.15 <sup>e</sup>	7.99 ± 0.19 <sup>d</sup>	8.30± 0.10 <sup>d</sup>
<b>CaAlgLYO</b>	5.82 ± 0.27 <sup>b</sup>	5.64 ± 0.18 <sup>c</sup>	6.37± 0.77 <sup>c</sup>	7.16 ± 0.20 <sup>e</sup>	8.47 ± 0.26 <sup>cd</sup>	8.81 ± 0.27 <sup>c</sup>
<b>CaAlgHEWL</b>	5.61 ± 0.11 <sup>b</sup>	5.43 ± 0.09 <sup>cd</sup>	6.19 ± 0.12 <sup>cd</sup>	7.05 ± 0.10 <sup>e</sup>	8.57 ± 0.38 <sup>b</sup>	8.83 ± 0.21 <sup>c</sup>
<b>CaAlNHEWL</b>	5.71± 0.36 <sup>b</sup>	4.97 ± 0.12 <sup>ef</sup>	6.01 ± 0.23 <sup>c</sup>	6.11 ± 0.10 <sup>f</sup>	7.04 ± 0.16 <sup>e</sup>	7.15 ± 0.17 <sup>e</sup>
<b>CaAlgNLYO</b>	5.63 ± 0.19 <sup>b</sup>	4.90± 0.02 <sup>e</sup>	6.06 ± 0.21 <sup>c</sup>	6.35 ± 0.13 <sup>f</sup>	7.15 ± 0.15 <sup>e</sup>	7.11 ± 0.16 <sup>e</sup>

\* All analyses were based on two separate experiments with each mean ± standard deviation being average of three determinations. Means within each vertical column followed by the same letter are not significantly different (P = 0.05) from each other. Statistical comparisons of all pairs were analyzed using Student's *t*-test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

Table 4.2 Effect of Different Antimicrobials with and without Calcium Alginate Coating on the Growth of *Salmonella anatum* at 4° C for 35 Days.

Treatment*	Log CFU/g*					
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
<b>C</b>	5.78 ± 0.10 <sup>a</sup>	5.90 ± 0.34 <sup>a</sup>	5.91 ± 0.14 <sup>a</sup>	6.07 ± 0.11 <sup>a</sup>	6.05 ± 0.06 <sup>a</sup>	6.12 ± 0.01 <sup>a</sup>
<b>CaAlg</b>	5.70 ± 0.14 <sup>ab</sup>	5.68 ± 0.29 <sup>a</sup>	5.74 ± 0.24 <sup>a</sup>	5.89 ± 0.17 <sup>ab</sup>	5.78 ± 0.30 <sup>a</sup>	6.16 ± 0.15 <sup>a</sup>
<b>N</b>	5.59 ± 0.30 <sup>a</sup>	5.81 ± 0.07 <sup>a</sup>	5.98 ± 0.12 <sup>a</sup>	5.79 ± 0.02 <sup>a</sup>	6.06 ± 0.12 <sup>a</sup>	5.63 ± 0.72 <sup>a</sup>
<b>LYO</b>	5.21 ± 0.10 <sup>cd</sup>	5.58 ± 0.04 <sup>a</sup>	5.54 ± 0.07 <sup>b</sup>	5.81 ± 0.21 <sup>abc</sup>	5.60 ± 0.26 <sup>b</sup>	5.39 ± 0.08 <sup>b</sup>
<b>HEWL</b>	5.35 ± 0.07 <sup>abc</sup>	5.75 ± 0.36 <sup>a</sup>	5.61 ± 0.02 <sup>a</sup>	5.66 ± 0.14 <sup>b</sup>	5.58 ± 0.21 <sup>b</sup>	5.43 ± 0.13 <sup>b</sup>
<b>CaAlgN</b>	5.77 ± 0.16 <sup>a</sup>	5.70 ± 0.01 <sup>a</sup>	5.93 ± 0.20 <sup>a</sup>	5.52 ± 0.19 <sup>c</sup>	5.90 ± 0.10 <sup>ab</sup>	5.94 ± 0.02 <sup>ab</sup>
<b>CaAlgLYO</b>	4.88 ± 0.15 <sup>d</sup>	4.84 ± 0.31 <sup>b</sup>	4.79 ± 0.21 <sup>c</sup>	4.93 ± 0.02 <sup>d</sup>	4.83 ± 0.17 <sup>c</sup>	4.60 ± 0.36 <sup>c</sup>
<b>CaAlgHEWL</b>	4.88 ± 0.21 <sup>d</sup>	4.99 ± 0.22 <sup>b</sup>	5.03 ± 0.16 <sup>c</sup>	4.89 ± 0.14 <sup>d</sup>	4.93 ± 0.10 <sup>c</sup>	4.64 ± 0.20 <sup>c</sup>
<b>CaAlgNHEWL</b>	4.97 ± 0.09 <sup>d</sup>	4.88 ± 0.11 <sup>b</sup>	4.86 ± 0.18 <sup>c</sup>	4.71 ± 0.13 <sup>d</sup>	3.88 ± 0.12 <sup>d</sup>	3.91 ± 0.11 <sup>d</sup>
<b>CaAlgNLYO</b>	4.88 ± 0.07 <sup>d</sup>	4.64 ± 0.20 <sup>b</sup>	4.85 ± 0.22 <sup>c</sup>	4.64 ± 0.20 <sup>d</sup>	3.82 ± 0.16 <sup>d</sup>	3.87 ± 0.14 <sup>d</sup>

\* All analyses were based on two separate experiments with each mean ± standard deviation being average of three determinations. Means within each vertical column followed by the same letter are not significantly different (P = 0.05) from each other. Statistical comparisons of all pairs were analyzed using Student's t test following one-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC).

At 4°C, an initial inoculum of *Listeria monocytogenes* increased from 6.57 log CFU/g to 6.81 log CFU/g on day 7 in the control samples. Conversely, studies have shown that *L. monocytogenes* can grow rapidly on cold-smoked salmon increasing up to 5 log within 7 days at 4°C (Nilsson et al., 1997). The slow growth of *L. monocytogenes* in our case could be due to the presence of different microflora on the surface of the smoked salmon samples which can interfere with the growth of *Listeria* species on the surface of the smoked fish. Studies have shown that interaction between *Listeria* species and other microflora on the surface of raw food material is complex. Depending on the presence of the microflora, the growth of *Listeria* can either be inhibited or enhanced (Guyer and Jemmi, 1991). The low growth of *Listeria* could be due to the competition with the natural microflora on the surface of the fish (Ben Embarek, 1994). The bacterial flora of stored smoked fish consists of *Lactobacillus* spp and if it is predominantly present on the fish it could inhibit the growth of *Listeria* as a result of competition (Guyer and Jemmi, 1991; Ben Embarek, 1994; Nilsson et al., 1997).

On the other hand, at an initial inoculation level of 5.78 log CFU/g, *S. anatum* numbers on control samples remained almost constant throughout the experiment (Gill and Holley, 2000). The *Salmonella anatum* strain used in this study did not grow at refrigerated temperatures because this pathogen is a mesophilic organism and does not proliferate at refrigerated temperatures. However, *Salmonella* spp. can retain their biochemical and serological characters at low temperatures thus maintaining their pathogenic potential (Wilson et al., 1974).

Nisin is a food grade antimicrobial effective mainly against Gram-positive bacteria. In our study, Nisin significantly reduced *L. monocytogenes* counts on day 0

(Nattress et al., 2001; Cutter and Siragusa, 1997) but nisin did not reduce *S. anatum* counts. However, when *S. anatum* was treated with nisin and lysozyme a significant reduction in bacterial counts occurred. Nisin combined with a chelating agent was bactericidal against *Salmonella spp* (Steven et al., 1991). Our study has shown that nisin did not retain its antimicrobial activity throughout the 35 day period. It has been reported that nisin has significant antimicrobial activity at the starting of the storage during the refrigerated storage but its effectiveness decreases as the storage period increases (Rose et al., 1999; Janes et al., 2002)

Although lysozyme is known to be mainly effective against Gram-positive organism, our study shows that lysozyme was able to inhibit the growth of the Gram-negative *S. anatum*. Studies have shown that lysozyme acts on Gram-negative bacteria by a mechanism independent of its enzymatic action (Ibrahim et al., 2001; Pellegrini et al., 1992). Nattress et al.,(2001) reported that lysozyme in combination with nisin improves the efficiency of the mixture, show greater antimicrobial activity and extend the time during which nisin could be effective. Synergy between nisin and lysozyme against *Carnobacterium* was also observed and a combination of nisin and lysozyme was more effective against *Carnobacterium* than the individual component (Chung and Hancock, 2000).Electron microscopy results showed an increased surface disruption of the bacterial membrane and proposed that nisin could be inhibiting the energy dependent processes that repair lysozyme damage (Chung and Hancock, 2000). This synergy between the mixtures of nisin and lysozyme could be used to extend the shelf life of a variety of food products in the food industry when samples need to be stored for longer periods of time.

Calcium alginate edible coatings increased the effectiveness of nisin, hen egg white lysozyme, and oyster lysozyme when incorporated inside the coatings. Nisin and oyster lysozyme in the calcium alginate coatings reduced *L. monocytogenes* and *S. anatum* counts by 2.75 log CFU/g and 2.25 log CFU/g, respectively by day 35. Wan et al., (1997) showed that the effectiveness of nisin increased when incorporated in calcium alginate micro-particles. We found that there was no significant difference between Hen egg white lysozyme and oyster lysozyme on the bacterial growth when incorporated into the coating. Our results agree with Cutter and Siragusa (1996) who immobilized nisin in a calcium alginate film and found that bacterial reduction was greater when the bacteriocin was incorporated into the film rather than when applied alone.

Our results show that incorporation of antimicrobial agents into the calcium alginate edible film was able to retain the effectiveness of the antimicrobial agents throughout the 35 days storage making it more effective than when they were applied alone on the samples. These results are in agreement with studies using calcium alginate gel containing antimicrobial agents that effectively reduced foodborne pathogens on a wide variety of food products (Cutter and Siragudsa, 1996; Wan et al., 1997; Lazarus et al., 1976; Williams et al., 1978).

Our results indicated that the effectiveness of oyster lysozyme was enhanced when combined with nisin incorporated into the calcium alginate coating, which could be used to preserve ready-to-eat smoked salmon at refrigerated temperatures.

## CHAPTER 5 SUMMARY AND CONCLUSION

The present study aimed at purifying lysozyme from oyster shell liquor, and evaluating its use against major foodborne pathogens, and determining the feasibility of using oyster lysozyme in antimicrobial films to enhance the preservation of smoked salmon.

Shell liquor lysozyme was purified using a series of ion exchange chromatographies. There was a seasonal variation in lysozyme activity and the spring season showed the highest lysozyme activity. Three liters of shell liquor were collected during this season and scale up purification protocol was carried which yielded 205 mg of protein.

The purified shell liquor lysozyme was then tested for its antibacterial activities against major food borne pathogens. From the Minimum Inhibitory Concentration assay we found that purified oyster lysozyme from the shell liquor had antimicrobial activity against a number of both gram-positive and gram negative bacteria.

To control *Listeria monocytogenes* and *Salmonella anatum* on the surface of smoked salmon with edible film coatings, oyster lysozyme was incorporated into edible film coatings. Calcium alginate coating showed promise with the control of *S. anatum* and hence was chosen for 35 days shelf life study. Our results indicated that the effectiveness of oyster lysozyme was enhanced when combined with nisin incorporated in the calcium alginate coating, which could be used to preserve ready-to-eat smoked salmon at refrigerated temperatures.

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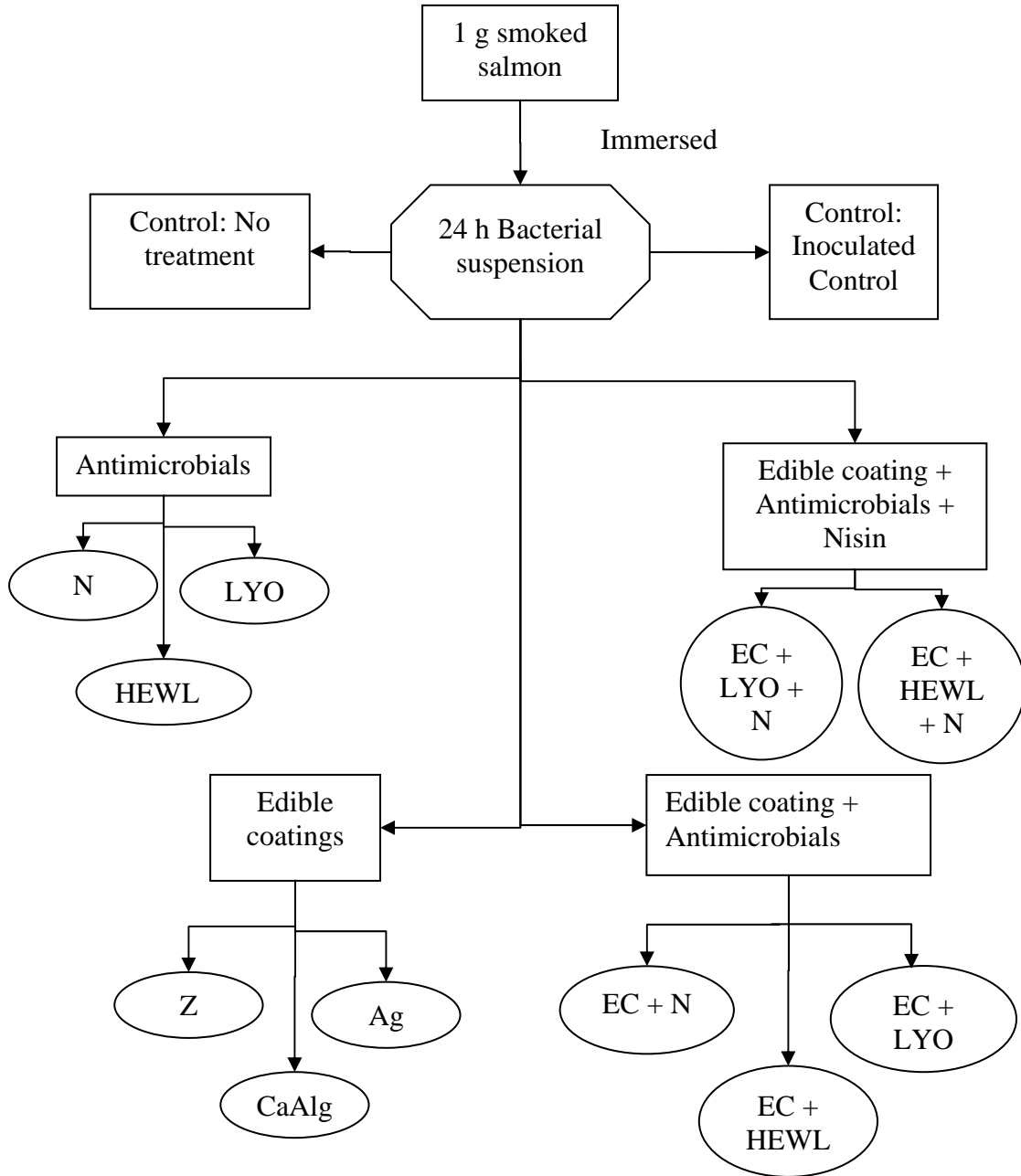
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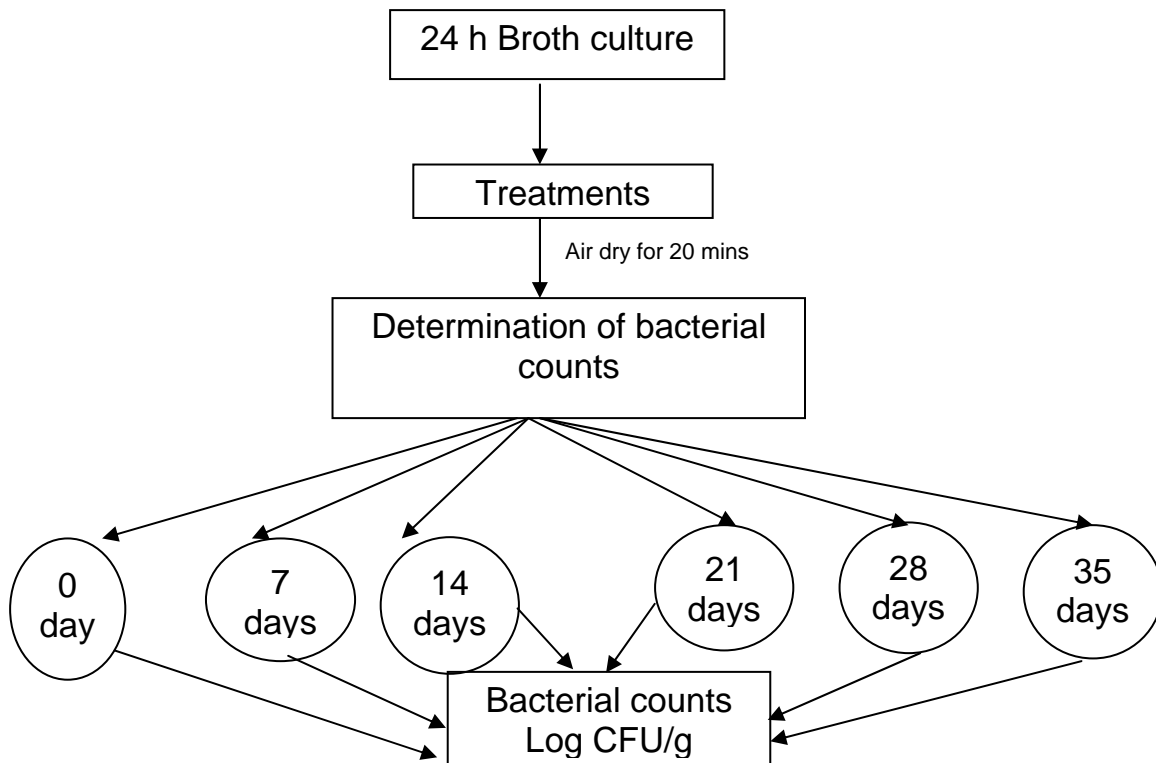
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**APPENDIX 1: DETERMINATION OF THE MOST EFFECTIVE FILM COATING FOR ENHANCING THE ANTIMICROBIAL ACTIVITY OF OYSTER LYSOZYME**



The following abbreviations stand for:  
 Z, Zein; CaAlg, Calcium alginate; Ag, Agar; EC, Edible coating; HEWL, Hen egg white lysozyme; LYO; Oyster lysozyme; N, Nisin

**APPENDIX 2: EFFECT OF OYSTER LYSOZYME INCORPORATED INTO CALCIUM ALGINATE COATINGS AGAINST *LISTERIA MONOCYTOGENES* AND *SALMONELLA ANATUM* DURING 35 DAYS STORAGE**



## **VITA**

Shreya Datta was born on February 4, 1978, in Bareilly, India. She earned her Bachelor of Science degree in microbiology in 1999 from Sardar Patel University, Gujarat, India. She then got her Master of Science degree in microbiology in 2001 from Department of Biosciences, Sardar Patel University, Gujarat, India. She worked as a lecturer in the Microbiology Department, V.P. Science College, Sardar Patel University, V.V.Nagar, India from 2001-2002. She joined as a master's student in the Department of Food Science at Louisiana State University, Baton Rouge, Louisiana, in 2003.