DEVELOPMENT OF LYSOZYME INCORPORATED ANTIMICROBIAL ZEIN FILMS AND EVALUATION OF THEIR EFFECTS ON QUALITY OF COLD STORED BURGERS

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ABSTRACT

DEVELOPMENT OF LYSOZYME INCORPORATED ANTIMICROBIAL ZEIN FILMS AND EVALUATION OF THEIR EFFECTS ON QUALITY OF COLD STORED BURGERS

In this study antimicrobial edible food packaging films were obtained by incorporation of hydrophilic partially purified lysozyme into hydrophobic zein films. The antimicrobial enzyme was incorporated into films by homogenization or stirring methods to increase its distribution in the films and to modify the film structure. The soluble and bound lysozyme activities of different zein films, as well as antimicrobial activity of films on different bacteria including *Bacillus amyloliqufaciens*, *Listeria innocua*, *Eschericia coli, Pseudomanas fluorescens*, *Escherichia coli* O157:H7, *Salmonella typhimurium and Staphylococcus aureus* and on cold stored burgers were tested. The lysozyme was combined with disodium EDTA to increase sensitivity of G(-) bacteria to enzymatic action. The films obtained by incorporation of 175 to 700 μg/cm^2 lysozyme with stirring or homogenization methods showed good antimicrobial activity on most of the tested bacteria, except *S. aureus*. In general, due to their higher free soluble lysozyme content, the zein films incorporated with lysozyme by the stirring method gave higher antimicrobial activity on tested bacteria than films obtained by the homogenization method. However, the homogenization method caused better distribution of resulting antimicrobial activity in films than the stirring method. The films incorporated with 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA and prepared by stirring or homogenization method successfully suppressed the total viable counts and total coliform counts of cold stored turkey and beef burgers. The films also effectively reduced the oxidative changes in beef burgers during cold storage, but they showed no beneficial effects on beef color and sensory properties.

ÖZET

LİSOZİM İÇEREN ANTİMİKROBİYAL ZEİN FİLMLERİN ÜRETİMİ VE SOĞUKTA DEPOLANAN BURGERLERİN KALİTELERİ ÜZERİNDEKİ ETKİLERİNİN BELİRLENMESİ

Gerçekleştirilmiş olan bu çalışmada hidrofilik yapıdaki lisozim enzimi hidrofobik yapıdaki zein filmler içerisine ilave edilerek antimikrobiyal paketlemede kullanılabilecek yenilebilir filmler üretilmiştir. Lisozimin filmler içerisindeki dağılımını artırmak ve film yapısını değiştirmek amacıyla enzimin filmlere ilavesi homojenizasyon veya karıştırma teknikleriyle gerçekleştirilmiştir. Elde edilen filmlerin çözünür ve bağlı lisozim aktiviteleri belirlendiği gibi, antimikrobiyal aktiviteleri de test edilmiştir. Bu amaçla geliştirilen filmler öncelikle *Bacillus amyloliqufaciens*, *Listeria innocua*, *Eschericia coli, Pseudomanas fluorescens*, *Escherichia coli* O157:H7, *Salmonella typhimurium and Staphylococcus aureus* gibi bakteriler üzerinde test edilmiş ve daha sonra gerçek gıda uygulamasında burgerler üzerinde denemiştir. Özellikle G(-) bakteriler üzerinde yürütülen denemelerde bu bakterilerin lisozime olan hassasiyetini artırmak amacıyla disodyum EDTA kullanılmıştır. 175-700 $\mu g/cm^2$ arasında lisozim içeren filmler, *S. aureus* haricinde test edilmiş olan bakterilerin pek çoğu üzerinde etkili bulunmuştur. Karıştırma yöntemiyle elde edilen filmler genellikle daha yüksek oranda çözünür lisozim aktivitesi içerdiklerinden test edilen bakteriler üzerinde daha etkili bulunmuşlardır. Ancak, homojenizasyon yöntemiyle elde edilen disodyum EDTA ve lisozim içeren filmlerde antimikrobiyal aktivite film içerisinde daha homojen bir dağılım göstermektedir. Üretilmiş olan 700 $\mu g/cm^2$ lisozim ve 300 $\mu g/cm^2$ disodyum EDTA içeren filmler hindi ve dana etinden üretilmiş olan burgerlerin toplam canlı ve koliform sayısının artışını soğukta depolama sırasında başarıyla baskılamıştır. Aktif paketleme uygulaması depolama sırasında burgerlerdeki oksidatif değişimleri de minimize etmiştir, ancak gerçekleştirilen uygulamanın burgerlerin renk ve duyusal özellikleri üzerinde bir etkisi belirlenememiştir.

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CHAPTER 1

INTRODUCTION

Due to concerns over the risk of food-borne illnesses and marketing demands to extend food shelf life, there has been a great interest in antimicrobial packaging technologies (Dawson, et al. 2002). To reduce environmental pollution and high waste disposal costs of plastic packaging materials, use of edible and biodegradable films in antimicrobial packaging have also become very popular among food scientists (Fang, et al. 2002, Ghanbarzadeh, et al. 2006). Antimicrobial packaging is applied mainly to inhibit the growth of pathogenic bacteria and to suppress spoilage microorganisms at food surface, the most critical part of food for microbial development (Han 2000). The antimicrobial packaging is conducted by (1) the addition of antimicrobial containing sachets or pads into food packages; (2) the coating, immobilization or direct incorporation of antimicrobials into food packaging materials or (3) the use of packaging materials that are inherently antimicrobial (Appendini and Hotchkiss 2002). Different chemicals, such as organic or inorganic acids, metals, alcohols, ammonium compounds or amines, can be incorporated into biodegradable packaging materials. However, because of the health concerns of consumers related to chemical preservatives and environmental problems, there is a demand for the food industry to use natural biopreservatives with edible and/or biodegradable packaging materials (Labuza and Breene 1989, Suppakul, et al. 2003, Mecitoglu, et al. 2007). Thus, the natural antimicrobial additives such as bacteriocins, enzymes, proteins, plant extracts and essential oils are increasing used in edible packaging materials such as zein, cellulose derivatives, carrageenan, alginate, and whey proteins (Han 2000, Cha, et al. 2002, Quintavalla and Vicini 2002, Suppakul, et al. 2008).

Recently, Mecitoglu et al. (2006) have produced partially purified lysozyme from hen egg white by a simple method based on ethanol precipitation of undesirable egg white proteins and incorporated the lyophilized enzyme into zein films for antimicrobial packaging. The partially purified antimicrobial enzyme was very stable and lost almost no activity in lyophilized form or in cast edible zein films stored at -18 and 4 ^{o}C for up to 8 and 4 months, respectively. The only disadvantage of using partially purified lysozyme is the non-homogenous distribution of hydrophilic enzyme

preparation in hydrophobic zein films. Thus, the main objectives of this study are; (1) to develop zein films with homogenous distribution of partially purified lysozyme, (2) to test antimicrobial activity of the developed films on different pathogenic and spoilage bacteria and (3) to test the effects of developed lysozyme incorporated films on microbial load of cold stored turkey and beef burgers, and oxidative quality and sensory properties of cold stored beef burgers. This study has been conducted to prepare the basis of using partially purified lysozyme in industrial scale active packaging.

CHAPTER 2

PACKAGING

2.1. Food Packaging

Food packaging plays a significant role to protect food products during storage and transport in food supply chain from producer to consumer and it is an integral part both for the food processes and the whole food supply chain. The main function of food packaging, when regarded as a food preservation technology, is to protect food products from environmental and processing factors such as microbial and chemical contamination, oxygen, water vapor and light. Other functions are to provide consumers with ingredient and nutritional information by adequate labeling, and to ensure product traceability and a proper convenience to consumer (Ahvenainen 2003, Marsh and Bugusu 2007).

Developments of novel food packaging technology, which can play an active role in product processing, preservation and in retaining the safety and quality of foods, have a growing interest for a long time. These new developments have been generally regarded as active packaging (Lopez-Rubio, et al. 2004).

2.2. Active Packaging

Innovative food packaging concepts serve as a mode of continuous changes in current consumer demands and market trends (Quintavalla and Vicini 2002). Active packaging, which is one of the innovative food packaging concepts, provide some additional functions in comparison to traditional passive packaging materials which are limited to protection of packed food product against external effects (Devlieghere, et al. 2004). It has been defined as the interactions between food, the packaging material and the environment to prolong shelf life or to improve safety, nutritional and sensory quality of food products (Cha and Chinnan 2004).

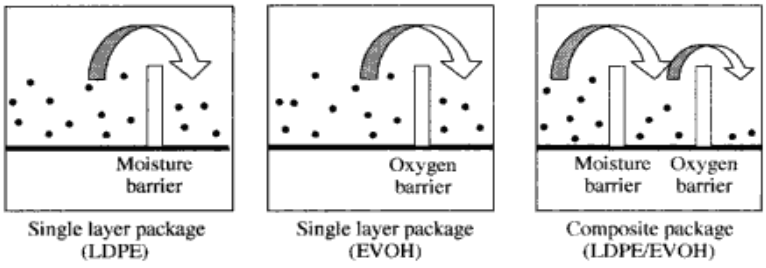
Principal active packaging systems include oxygen scavenger, carbon dioxide scavengers or emitters, humidity absorbers or controllers, ethylene scavengers, aroma

emitters/absorbers, enzymatically active systems, and antimicrobial systems (Lopez-Rubio, et al. 2004).

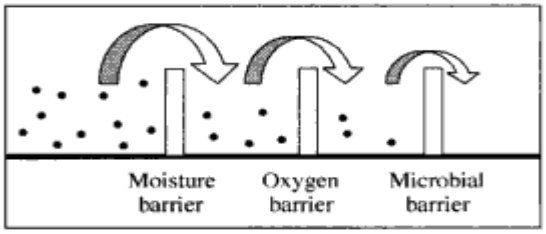
2.3. Antimicrobial Food Packaging

Antimicrobial packaging is a promising form of active packaging. It acts to extend the lag phase and reduce the growth rate of microorganisms to inhibit or retard the growth of microorganisms that may be present in the packed food or packaging material itself (Han 2000). The new antimicrobial function can be achieved by adding antimicrobial agents in the packaging system and/or using antimicrobial polymers that satisfy conventional packaging requirements.

Antimicrobial packaging is specifically designed to carry out safety assurance, quality assurance and shelf-life extension of food products, which is in reverse order to primary goals of conventional packaging systems (Figure 2.1). Since most foods are perishable and sensitive to microbial spoilage, the antimicrobial packaging system, which its primary goal is safety assurance, may apply to many food products (Ahvenainen 2003).



(a) Conventional packaging systems



(b) Antimicrobial packaging systems

Figure 2.1. Hurdle technology in antimicrobial packaging systems compared to the conventional packaging systems (Source: Ahvenainen 2003)

There are many antimicrobial agents that are suitable for incorporation into packaging materials. Selected antimicrobial packaging applications are given in Table 2.1.

Table 2.1. Selected natural components incorporated into packaging materials (Source: Han 2000, Quintavalla and Vicini 2002, Ahvenainen 2003)

Active component	Packaging Material	Application area
Organic acid		
Potassium sorbate	LDPE	Cheese
	LDPE	Culture media
	MC/palmitic acid	Culture media
	MC/HPMC/fatty acid	Culture media
	MC/chitosan	Culture media
	Starch/glycerol	Chicken breast
Calcium sorbate	CMC/paper	Bread
Propionic acid	Chitosan	Water
	Chitosan	Bologna, cooked ham, pastrami
Acetic acid	Chitosan	Water
	Chitosan	Bologna, cooked ham, pastrami
Benzoic acid	PE-co-MA	Culture media
Benzoic acid anhydride	PE	Fish fillet
	LDPE	Culture media
Sodium benzoate	MC/chitosan	Culture media
Sorbic acid anhydride	PE	Culture media
p-aminobenzoic acid	WPI	Culture media
Peptide/protein/enzyme		
Lysozyme, nisin, EDTA	SPI/zein	Culture media
Lysozyme, nisin, EDTA, propyl paraben	WPI	Culture media
Immobilized lysozyme	PVOH, nylon, cellulose acetate	Culture media
Glucose oxidase	Alginate	Fish

(Cont. on next page)

Table 2.1 (cont.). Selected natural components incorporated into packaging materials (Source: Han 2000, Quintavalla and Vicini 2002, Ahvenainen 2003)

Bacteriocins		
Nisin	PE	Beef, Phosphate buffer
	SPI, WPI, WG, EA	Phosphate buffer
	HPMC	Culture media
	Corn zein	Shredded cheese
	Silicon casing	Beef tissue
Nisin, lactisin	Polyamide/LDPE	Culture media
Nisin, lactisin, salts	Polyamide/LDPE	Culture media
Nisin, EDTA	PE, PE-co-PEO	Beef
Nisin, citrate, EDTA	PVC, nylon, LLDPE	Chicken
Nisin, organic acids mixture	Acrylics, PVA-co-PE	Water
Nisin, lauric acid	Zein	Stimulants
Nisin, pediocin	Cellulose casing	Turkey breast, ham, beef
	Starch/glycerol	Chicken breast
Nisin (peptide)	Silicon coating	Culture media
	SPI, corn zein	Culture media
Alcohol/Thiol		
Ethanol	Silica gel sachet	Culture media
	Silicon oxide sachet	Bakery
	Alginate	Fish
Hinokithiol	Cyclodextrin/plastic	Bakery
Fungicides		
Benomyl	Ionomer	Culture media
Imazalil	LDPE	Bell pepper
	PE	Cheese
Polymers		
Chitosan	Chitosan/pepper	Strawberry
Chitosan, herb extract	LDPE	Culture media
UV/excimer laser, irradiated nylon	Nylon	Culture media

(Cont. on next page)

Table 2.1 (cont.). Selected natural components incorporated into packaging materials (Source: Han 2000, Quintavalla and Vicini 2002, Ahvenainen 2003)

Oxygen absorber/antioxidant		
Reduced iron complex	Sachet	Bread
BHT	HDPE	Breakfast cereal
Gas		
CO_2	Calcium hydroxide sachet	Coffee
	-	Fruit/vegetable
SO_2	Sodium metabisulfite	Grape
ClO_2	Plastic film	-
Natural extracts		
Grapefruit seed extract	LDPE, nylon	Ground beef
	LDPE	Lettuce, soybean sprouts
Clove extract	LDPE	Culture media
Herb extract, Ag-Zirconium	LDPE	Lettuce, cucumber
	LDPE	Strawberry
Eugenol, cinnam aldehyde	Chitosan	Bologna, ham
Horseradish extract	Paper	Ground beef
Allyl isothiocyanate	PE film/pad	Chicken, meats, smoked salmon
Others		
UV irradiation	Nylons	Culture media
Silver zeolite, silver nitrate	LDPE	Culture media
Antibiotics	PE	Culture media
Hexamethylenete tramine	LDPE	Orange juice

Abbreviations: LDPE: low-density polyethylene; MC: methyl cellulose; HPMC: hydroxypropyl methyl cellulose; CMC: carboxyl methyl cellulose; PE: polyethylene; MA: met hacrylic acid; SPI: soy protein isolate; PVOH: polyvinyl alcohol; BHT: butylated hydroxytoluene; HDPE: high-density polyethylene; WPI: whey protein isolate; WG: wheat gluten; EA: egg albumen

2.3.1. Developing an Antimicrobial Food Packaging Systems

Most antimicrobial food packaging systems are classified into two types including a package / food system and a package / headspace / food systems if the void volume of solid food products is assumed as a kind of headspace (Han 2000).

Package/Food Systems: In package / food systems, packaging materials contact with the food surfaces or low viscosity or liquid food without headspace. Main migration phenomena in this system is diffusion between the packaging material and food and partitioning at the interface (Han 2000) (Figure 2.2). Firstly, active substance may be impregnated into the package and then migrate into food through diffusion and partitioning. Individually wrapped cheese and ready-to-eat meat products, aseptic brick are good examples (Quintavalla and Vicini 2002).

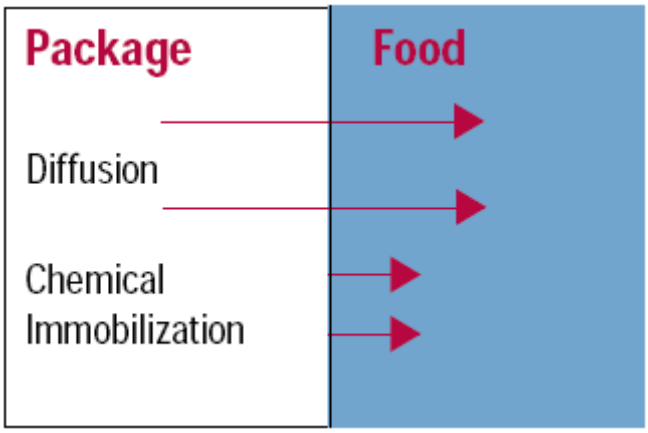


Figure 2.2. Package / food systems
(Source: Han 2000)

Package/Headspace/Food Systems: Evaporation or equilibrated distribution of a substance among the headspace, packaging material and/or food has to be considered as a part of main migration mechanisms to estimate the interfacial distribution of the substance (Figure 2.3). Examples of this system are flexible packages, bottles, cans, cups, and cartons. Active substance could be volatile in this system since nonvolatile substance can only migrate through the contact area between the package and the food, while volatile active substance can migrate through air gaps or headspace (Han 2000).

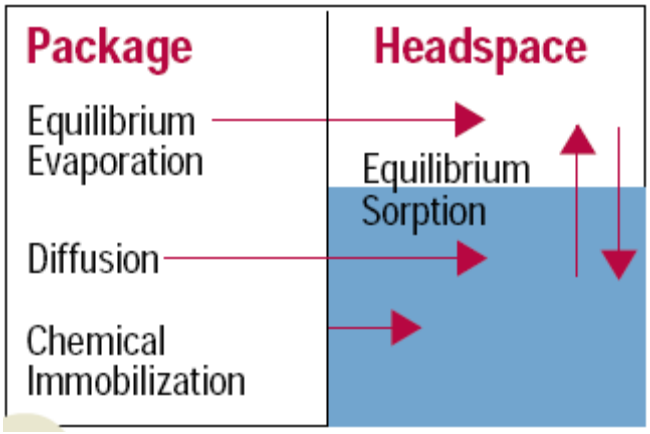


Figure 2.3. Package / headspace / food systems

(Source: Han 2000)

Besides diffusion and sorption systems, chemical immobilization utilizes the covalent binding of antibiotics or fungicides, or active moieties such as amine group into chemical structures of packaging material (Quintavalla and Vicini 2002). Covalently immobilized non-food-grade antimicrobial substance inhibits the growth of microorganisms on the contact surface of packed food without diffusional mass transfer (Han 2000, Suppakul, et al. 2003).

2.3.2. Types of Antimicrobial Food Packaging

2.3.2.1. Addition of Sachets/Pads Containing Volatile Antimicrobial Agents into Packages

Small sachet containing volatile antimicrobial agents that are inserted into the package or are bonded to inner wall of package is the most successful commercial application of antimicrobial packaging. Sachet systems have been used to control gas composition inside a package in order to inhibit the growth of microorganisms. The common forms are oxygen absorbers, moisture absorbers and ethanol vapor generators (Appendini and Hotckiss 2002).

2.3.2.2. Oxygen Scavenging Systems

The presence of oxygen in a packaged food is key factor that trigger many food deterioration reactions. Oxidation can cause changes in flavor, color, and odor as well as destroy nutrients and facilitate microbial growth. It also has an important effect on the respiration rate and ethylene production of respiring foodstuffs such as fruits and vegetables. Therefore, the removal of residual oxygen that remains after packaging is an effective way to minimize quality changes and prevent growth of aerobic bacteria and molds in O_2-sensitive food products especially in dairy, bakery and meat products. Oxygen concentration should be less than or equal to 1% (v/v) in the headspace to carry out this aim. In general, existing oxygen scavenging technologies use one or more of the following concepts: iron powder oxidation, ascorbic acid oxidation, unsaturated fatty acids, enzymatic oxidation, hydrocarbon oxidation, immobilization of yeast in solid holders, and photosensitive dye oxidation (Appendini and Hotchkiss 2002, Suppakul, et al. 2003, Lopez-Rubio, et al. 2004). Most of the commercially available oxygen scavengers incorporated into a sachet are based on the principle of iron oxidation (Coma 2008).

2.3.2.3. Carbon Dioxide Emitting/Absorbing Systems

Although carbon dioxide concentration inside the package have a microbial inhibitory effect on products such as meat, cheese, and baked goods, high CO_2 levels cause color and flavor changes and the development of undesirable anaerobic metabolism and pH reduction in some food products. CO_2 absorbers might therefore be useful. Adversely, in some cases, CO_2 emitters are mainly used to prolong the shelf life of foods such as fresh meat, poultry and fish because of their inhibitory activity, resulting in an increased lag phase and decrease the growth rate during logarithmic phase of growth of aerobic bacteria and fungi (Suppakul, et al. 2003). Moreover, in food products for which the volume of the package and its appearance are critical, sachets containing combinations of O_2 scavengers/CO_2 emitters could be commercially used to overcome package collapse or partial vacuum as a result of O_2 absorption. Basically, emitting/scavenging systems are based on a mixture ascorbic acid and sodium bicarbonate in the sachet (Lopez-Rubio, et al. 2004).

2.3.2.4. Ethanol Generating Systems

Ethanol generation is only used in intermediate moisture foods, cheese, and bakery products to prevent microbial spoilage, reduce the rate of staling and oxidative changes. Sachets including ethanol encapsulated or absorbed release its vapor into the package headspace thereby maintaining the preservative effect. Off-flavor development is the major drawback of ethanol generation systems (Appendini and Hotchkiss 2002, Suppakul, et al. 2003).

2.3.2.5. Moisture-Absorbing and Controlling Systems

Foods susceptible to moisture should be packaged with a high humidity barrier material. However, a certain amount of moisture can remain trapped during packaging process or develop during the storage and transportation. Unless this water is removed, it can be retained by the product, or it can form a condensate with attendant spoilage and/or low consumer appeal (Vermeiren, et al. 1999). Moisture absorbers have been devised in absorbent pads, desiccant films, or sachets to remove moisture from packaged foods (Cutter 2002). The primarily purpose of moisture control is to lower water activity, thereby reducing the growth of moulds, yeast and spoilage bacteria on foods with high water activity such as ready-to-eat meals, to remove melting water from frozen fish, meat or other frozen foods, and to prevent condensation when fresh horticultural produce respires (Lopez-Rubio, et al. 2004).

2.3.2.6. Incorporation of Antimicrobial Agents Directly into the Packaging Material

There are two methods to incorporate the antimicrobial agents directly into the packaging materials. These methods are addition of antimicrobials into polymers either in the melt or by solvent compounding. The choice of method depends on heat stability of incorporated antimicrobial substances. Classical thermal processing methods, extrusion and injection molding, may be applied to heat stable antimicrobials. For instance, since silver substituted zeolites used in antimicrobial packaging can withstand up to 800 ºC, they have been incorporated as a thin co-extruded layer with other

polymers. In contrast, solvent compounding is more suitable for incorporation of heat sensitive antimicrobial substances such as enzymes and volatile compounds into polymers. High pressure and temperature conditions in extruder during extrusion affect their chemical stability and therefore reduce antimicrobial activity of heat sensitive compounds. Lysozyme, for example, has been incorporated into cellulose ester films by solvent compounding in order to prevent heat denaturation of the enzyme. In solvent compounding method both antimicrobial substance and polymer should be dissolved in the same solvent (Appendini and Hotchkiss 2002, Han 2000).

The antimicrobial agents used in packaging may be volatile or non-volatile substances. If they are non-volatile, a contact between the food and the package is clearly necessary (Fig. 2.4). At this point, the release rate of the antimicrobial agent from the packaging material to food is specifically controlled to match the release rate with the growth kinetics of target microorganism because the concentration of the antimicrobial agent is maintained over the minimal inhibitory concentration on the food surface to provide sufficient antimicrobial activity (Appendini and Hotchkiss 2002, Quintavalla and Vicini 2002). On the other hand, volatile antimicrobials have some benefits compared to non-volatile antimicrobial agents. Volatile antimicrobials can penetrate and evaporate into the bulk matrix of the food that can not be reached by non-volatile antimicrobial. Examples of volatile substances used in packaging are chlorine dioxide, sulfur dioxide, carbon dioxide and ally isothiocyanate (Appendini and Hotchkiss 2002, Suppakul, et al. 2003).

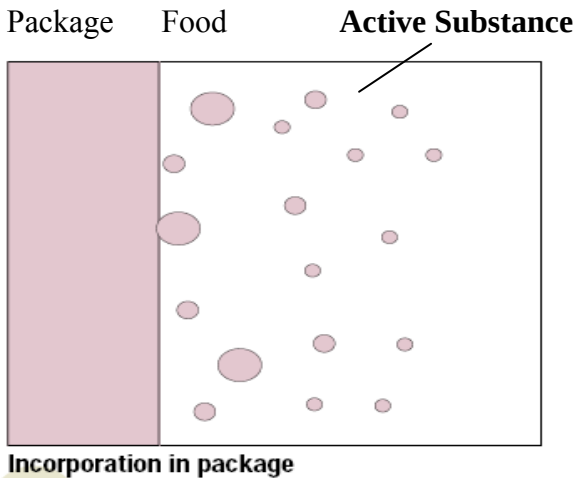


Figure 2.4. Diffusion of antimicrobial from package to food

(Source: Han 2000)

2.3.2.7. Coating or Adsorbing Antimicrobials to Polymer Surfaces

Heat sensitive antimicrobial substances can not be involved in thermal polymer processing such as extrusion and injection molding. For this reason, they are often coated onto the material after forming or are added to cast films. Cast edible films, for example, can be utilized as carriers for antimicrobials and applied as coating food contact surface or outer surface of packaging material in order to minimize the exposure of the product to contamination (Appendini and Hotchkiss 2002) and provide slowly migration from package onto the surface of food, thereby remaining at high concentrations for extended periods of storage time (Durango, et al. 2006) (Figure 2.5). Proteins have an increased capacity for adsorption due to their amphiphilic structure (Appendini and Hotchkiss 2002). Moreover, it is claimed that a polymer-based solution coating would be the most suitable method in terms of stability and adhesiveness of adsorption a bacteriocin to polymer surface. Nisin/methylcellulose coatings applied on polyethylene, and nisin coatings for poultry based on an adsorption of nisin on polyethylene, ethylene vinyl acetate, polypropylene, polyamide, polyester, acrylics and polyvinyl chloride are good examples (Suppakul, et al. 2003, Coma 2008).

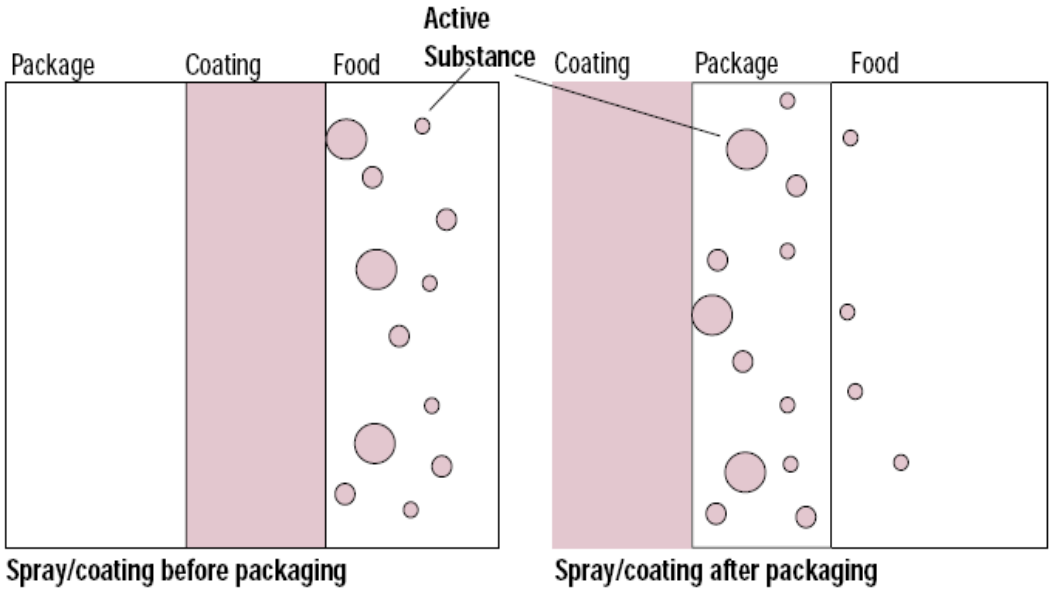


Figure 2.5. Effect of the coating location on the diffusion of antimicrobial agents (Source: Han 2000)

2.3.2.8. Immobilization of Antimicrobials to Polymers by Ion or Covalent Linkages

Immobilization system does not release antimicrobial agents but it suppresses the growth of microorganisms at the contact surface. Immobilization systems may be conducted more effective in the case of liquid foods compared to the solid foods due to less contact between the antimicrobial package and the whole food products for solid products (Ahvenainen 2003). Typically this is achieved through ionic and covalent attachment of the antimicrobial agent within the polymer matrix (Figure 2.6). This type of immobilization requires antimicrobials and polymers that have functional group and the use of spacer molecules that bound antimicrobial agent to polymer surface (Appendini and Hotchkiss 2002). For example, Scannel et al. (2000) investigated the immobilization of bacteriocins (nisin and lactisin 3147) to polyethylene / polyamide packaging materials. In a result of this study, immobilized nisin bound well and maintained its activity for 3 months under refrigerated storage and at room temperature as inhibitory action against lactic acid bacteria in ham was observed in this case. However, since lactisin 3147 did not appear to bind to the plastic film no zones were observed. Moreover, Wang et al. (1998) indicated that chitinase produced by *Pseudomonas aeuruginosa* K-187 was covalently immobilized onto the polymeric material and it inhibited the growth of fifteen target microorganisms except strain K-187 itself.

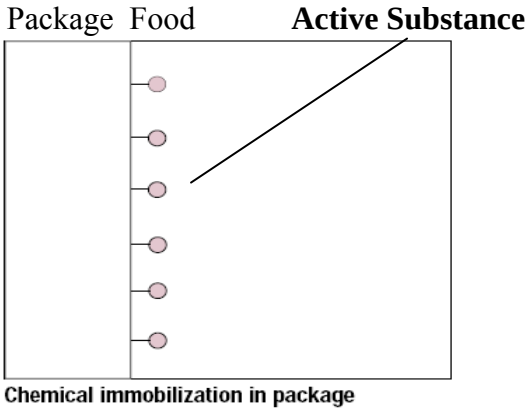


Figure 2.6. Immobilization of antimicrobial agents onto food packaging material (Source: Han 2002)

2.3.2.9. Use of Polymers that are Inherently Antimicrobial

Macromolecules such as cationic-amino polysaccharides used in film forming possess inhibitory antimicrobial effect naturally. The mechanism of antimicrobial action of these polymers may be based on interaction between positively charged polymer molecules and negatively charged microbial cell membranes, leading to leakage of their intracellular components (Appendini and Hotchkiss 2002). Recent researches have focused on natural antimicrobial chitosan and its derivatives due to problems caused by chemical preservatives (Prashanth and Tharanathan 2007).

CHAPTER 3

EDIBLE FILMS AND COATINGS

3.1. Definition and History of Edible Films and Coatings

Since environmental pollution caused by synthetic plastic films, which stick with the environment for many years after disposal (Aithani and Mohanty 2006), has recently become a major issue, research has been focused on developing edible packaging from renewable biopolymers (Sohail, et al. 2006). An edible film is defined as a thin, continuous layer of edible material formed, or placed, on or between, food components or on foods (Bravin, et al. 2006, Jongjareonrak, et al. 2006). Inherent biodegradability and edibility are the most beneficial characteristics of edible films and coatings not offered by plastic packaging materials (Han and Gennadios 2005). In addition to above properties, they have to be free of toxics and safe for health, cost-effective, have good sensory qualities, high barrier and mechanical efficiencies, enough biochemical, physico-chemical and microbial stability (Debeaufort, et al. 1998).

Although the use of edible films and coatings in food products may seem new, food products were first covered by edible films and coatings long time ago (Debeaufort, et al. 1998). The use of free standing edible films may have a shorter history than edible coatings. Wax, was the first edible coating, used on fruits to retard water loss, resulting in shelf-life extension in the early twelfth century in China (Tharantan 2003). However, waxes have been the only commercial protective coatings for apples and pears since 1930s. Hot-melt paraffin waxes have been commercially available to coat citrus fruits in the United States since 1930s, and carnauba wax and oil-in water emulsions have been used for coating fresh fruits and vegetables since 1950s. Lipid coatings on meats and cheeses have been used since the Middle Ages to prevent shrinkage. As an example of edible films, yuba, the first free-standing edible film obtained from skin of boiled soy-milk, was traditionally used in Asian countries to enhance appearance and preservation of some food products since the fifteenth century. Currently, edible films and coatings are used in various food applications, mostly fruits, vegetables, candies, and some nuts (Debeaufort, et al. 1998, Cagri, et al. 2004).

3.2. Functionality of Edible Films and Coatings

Edible films and coatings must have some functional and specific properties. Edible films and coating materials are potentially used to extend the shelf-life and improve the quality of almost any food system by serving as mass transfer barriers to moisture, oxygen, carbon dioxide, lipid, flavor and aroma between food components and the surrounding atmosphere (Cho and Rhee 2002) (Figure 3.1).

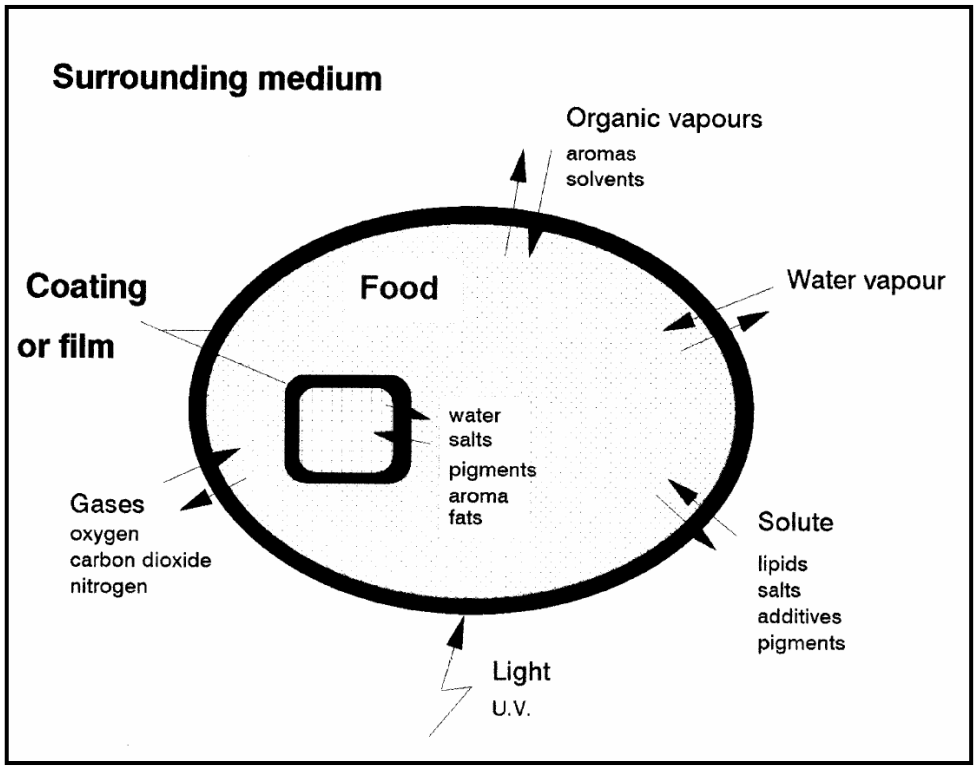


Figure 3.1. Selective functions of edible films and coatings
(Source: Debeaufort, et al. 1998)

Regarding the barrier properties of packaging materials, the critical compounds that can penetrate the packaging material and degrade food quality are the water vapor and oxygen of the surrounding atmosphere. The protection of food against oxygen is one of the most important requirements in packaging of food products (Hong and Krochta 2006). Edible films and coatings, having good gas barrier properties, can minimize the oxygen permeation into food (Han and Krochta 2007). As a consequence, they can achieve the control of respiration of fresh fruits and vegetables and prevent

lipid oxidation, which is one of the main factors limiting the quality and acceptability of food products, especially meat and meat products (Kilic and Richards 2003, Ghanbarzadeh, et al. 2007).

Moisture interchange between a food product and its environment is a critical factor affecting sensory quality and shelf life of food products. During storage of foods, many deteriorative chemical and enzymatic reactions (lipid oxidation, Maillard browning and enzymatic browning) as well as microbial growth occur at rates highly associated with water activity and water content of foods. In addition, the textural properties of certain foods such as dry and crispy cereal foods also largely depend on their water activity (Yang and Paulson 2000). Water activity of food is a paramount importance when selecting an edible film, since films of various compositions can develop different functional properties depending on relative humidity conditions (Olivas, et al. 2008).

Barrier properties of edible films can be affected by polymer structure, plasticizer concentration, solvent and other factors related to film dissolution, permeability, and diffusion properties. Also, the effect of thickness is a significant variable when barrier properties are taken into account (Yoshida, et al. 2002).

Moreover, edible films and coatings can carry food additives, such as antimicrobial agents, antioxidants, flavors and colors to enhance food quality and safety (Pranoto, et al. 2005b), and to ensure the safety of food surface through controlled release of these substances from the carrier film structure to food surface (Park, et al. 2004).

As it is mentioned above, edible films can apply to various foods in order to enhance food quality and safety. Some selected applications are given in Table 3.1.

Table 3.1. Application of edible films on various food
(Source: Cagri, et al. 2004)

Films	**Food**	**Benefits**
Casein	Peeled Carrots	Reduced dehydration
Casein acetylated monoglycerides	Celery sticks	Reduced dehydration

(Cont. on next page)

Table 3.1 (cont.) Application of Edible Films on Various Food
(Source: Cagri, et al. 2004)

Sodium caseinate	Green bell peppers	Reduced oxygen and carbon dioxide permeation
Collagen	Hot dogs, sausage	Lettuce, cucumber
Corn zein	Nuts	Delayed rancidity
	Tomatoes	Delayed color change, loss of firmness, and weight loss
Wheat gluten	Eggshells	Improved shell strength, reduced microbial contamination
Whey protein	Frozen King salmon	Reduced moisture loss and oxidation
Whey protein, acetylated monoglycerides	Nuts	Delayed rancidity
Whey protein	Eggshells	Improved shell strength, reduced microbial contamination
Soy protein	‘‘Fuji’’ and ‘‘Golden 95 delicious’’ apples	Retard changes in firmness, color, and acidity
	Eggshells	Improved shell strength, reduced microbial contamination
Alginate	Fresh meat, poultry, precooked ground pork patties	Reduce shrinkage, oxidative rancidity, moisture migration and oil absorption
Cellulose	Bell peppers	Reduced oxygen and carbon dioxide permeability
	Fried chicken	Reduced oil degradation, moisture loss

(Cont. on next page)

Table 3.1 (cont.) Application of Edible Films on Various Food
(Source: Cagri, et al. 2004)

Cellulose	Eggshells	Improved shell strength, reduced microbial contamination
Chitosan	Bell peppers and cucumbers	Reduced respiration, color loss, wilting, and fungal infection
	Strawberries	Delayed spoilage
	Tomatoes	Retard ripening and extended shelf life
Starch	Prunes	Extended shelf life
	Nuts	Delayed rancidity
Starch, alginate, stearic acid	Precooked beef patties	Controlled moisture loss
Starch, alginate, stearic acid, tocopherol	Precooked beef patties	Controlled lipid oxidation
Dextrin	Apples	Reduced oxidative browning
Xanthan gum	Carrots	Improved surface color
Wax or fatty acids	Fruits and vegetables	Delayed spoilage, reduced water loss
	Cheese	Prevent mold growth
Acetylated monoglyceride	Frozen King salmon and frozen Silver salmon	Reduced moisture loss and lipid oxidation

3.3. Film Composition

3.3.1. Film-Forming Materials

The main film-forming materials can be divided into three major categories: hydrocolloid, lipids, and composites. Hydrocolloids include proteins and

polysaccharides. Lipids include waxes, fats and oils. Composites contain both hydrocolloid components and lipids. These three categories are further outlined in Figure 3.2.

The choice of materials for film or coating is largely dependent on its desired function (Cha and Chinnan 2004). For example, when the purpose is to control the moisture balance within the heterogeneous food, hydrophobic materials are required to make a film with good water barrier properties (Talens and Krochta 2005).

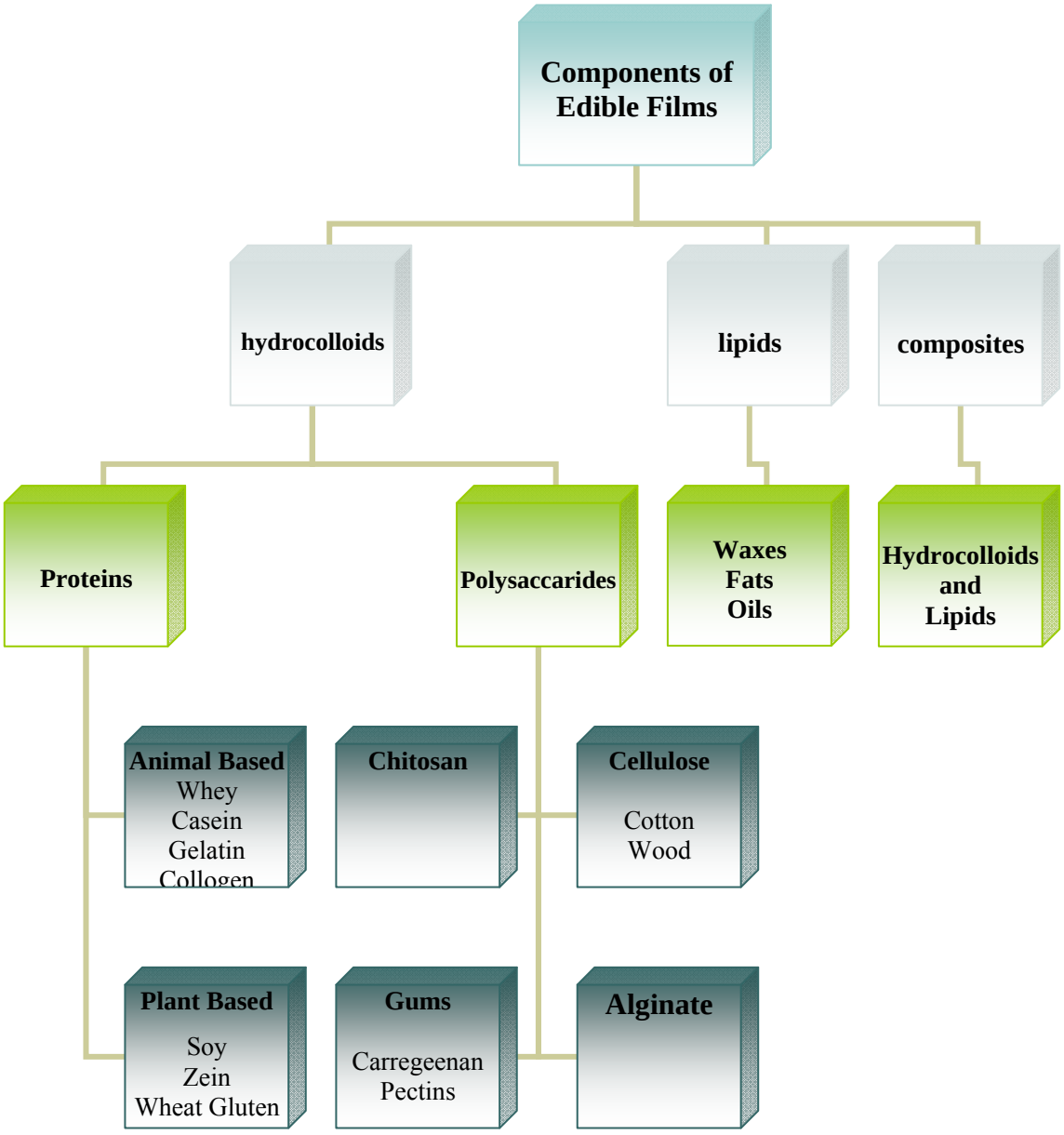


Figure 3.2. Schematic presentation of components of edible films

3.3.1.1. Hydrocolloid Films

Hydrocolloids used for films and coatings can be classified according to their composition, molecular charge, and water solubility. In terms of composition, hydrocolloids can be either polysaccharides or proteins (Danhowe and Fennema 1994).

Proteins and polysaccharides are good film formers exhibiting excellent oxygen, carbon dioxide, aroma, and lipid barrier properties, particularly at low relative humidity (RH), but due to their hydrophilic nature except corn zein and wheat gluten, corn zein and wheat gluten films and coatings are poor vapor barrier. Relatively low-molecular-weight hydrophilic plasticizers are often added to improve the mechanical properties of protein and polysaccharide films (Talens and Krochta 2005).

3.3.1.1.1. Protein-Based Edible Films

3.3.1.1.1.1. Edible Films from Animal Origin Proteins

Whey Protein Film: Whey protein, a by-product of the cheese industry, causes serious pollution and waste disposal problems (Ozdemir and Floros 2001). Whey protein fractions and whey protein isolate (>90%) have been studied for film formation because of their desirable functional properties (Gounga, et al. 2007). Edible film and coatings produced from whey protein can not only provide a novel usage of whey protein but also enhance safety and quality of food products (Ozdemir and Floros 2001). Particularly among biopolymers, whey protein produces transparent, bland, flexible films with excellent oxygen, aroma, and oil barrier at low relative humidity. On the other hand, whey protein films have high water vapor permeability (Perez-Gago, et al. 2003, Hong and Krochta 2006, Gounga, et al. 2007).

Whey protein films were successfully applied to food surfaces such as peanut and walnut surfaces and were shown to provide a good oxygen barrier and high gloss (Lee, et al. 2002). Cagri et al. (2002) reported that whey protein films containing sorbic acid and p-amino benzoic acid clearly inhibited the growth of *Listeria monocytogenes*, *E.coli* O157:H7, and *S. typhimurium* DT104 on both bologna and summer sausage slices. Moreover, percent elongation of the film increased as a result of contact with bologna and summer sausage while tensile strength sharply decreased.

Casein Film: Casein, a unique protein, is only synthesized in the mammary grand and is found nowhere else in nature. Casein is not homogeneous protein. Five different type of casein, namely α_{s1}, α_{s2}, β, κ and γ, are found in bovine milk. Their molecule weights range from 12 to 25 kDa. Casein structure is characterized as being mostly random coil, amphiphilic, and with the presence of phosphoseryl groups able to bind calcium. Peptide, amido, and carboxyl groups are the primary active groups in casein (Chick and Hernandez 2002).

Casein-based edible films can find broad application in food packaging because of their high nutritional quality, excellent sensory properties and potential to adequately protect food products from their surrounding environment. Caseins are quite soluble in water despite their high content of nonpolar amino acids (35–45% of total amino acids residues) and aqueous solutions with casein concentrations up to 20% can be prepared at elevated temperatures. Due to its random coil nature and ability to form extensive intermolecular hydrogen, electrostatic and hydrophobic bonds casein can be easily form films from aqueous solutions. The mechanical properties of casein and caseinate films, being neither too tough nor too fragile, also make them suitable for edible purposes. Though more permeable to water vapor than plastic films, they are good candidates for incorporation into edible films to control mass transfer in food system (Schou, et al. 2005).

Schou et al. (2005) used sodium caseinate film, containing a glycerol protein ratio of 0.16, as wrapping to prevent hardening of the bread and they found that films seemed to be the most suitable ones, having both good mechanical properties and reducing hardening of bread samples during storage.

Collagen Film: Collagen is a fibrous protein. The film forming ability of collagen traditionally has been utilized in the meat industry for edible sausage casings (Cha and Chinnan 2004). An edible collagen film, intended for use on netted roast, boneless hams, fish fillets, roast beef, and meat pastes, was commercialized in the U.S. in the late 1980s. Researchers have been investigated the potential of replacing plastic meat wrappings with collagen-based edible films. Beef cubes wrapped in collagen-based edible films and stored at -18 °C for 20 weeks were not significantly different than plastic-wrapped controls in terms of oxidation, color, microbial growth, and sensory attributes (Gennadios, et al. 1997).

Gelatin Film: Gelatin is prepared by hydrolyzing collagen, a naturally occurring fibrous protein found in animal such as bovine bones and pork skins. Gelatin is a derived using acid and alkali treatment followed by thermal degradation of collagen in the presence of water (Villegas, et al. 1999, Bower, et al. 2006). Additionally, since gelatin produced from porcin skin and bone can't be applied to some foods because of aesthetic and religious objections, and people have some concerns about usage of gelatin due to outbreaks of mad cow disease and the foot-and-mouth disease there has been growing interest for fish skin gelatin (Jongjareonrak, et al. 2006). Gelatin produces clear, flexible, strong, and oxygen-impermeable films when cast from aqueous solution in the presence of plasticizers. They have good gas and oil barrier properties but poor water barrier property due to their hydrophilic nature (Lacroix and Cooksey 2005).

Villages et al. (1999) dipped cooked ham and bacon in water and gelatin to test effectiveness of gelatin on oxidative and color stabilities. As a result of this study, gelatin coating improved oxidative and color stability of cooked ham and bacon during frozen storage (-18 °C). Antoniewski et al. (2007) investigated that the spray application of a bovine gelatin coat to beef tender-loins, pork loins, salmon fillets, and chicken breasts helped to extend the shelf life of the products stored at 4 °C in a modified atmosphere.

3.3.1.1.1.2. Edible Films and Coatings from Plant Origin Proteins

Zein Film: Zein is located in small round particles, 1-2 μm in diameter, called protein bodies in maize endosperm, and it constitutes 47% of the total protein in corn. Zein is produced commercially from corn gluten meal (CGM). CGM is a coproduct material that is obtained during starch production from corn by ethanolic extraction. CGM has low price and is used mainly for animal feeding. However, zein is an expensive product and thus extraction of zein and production of biodegradable film from it would produce important economic benefits (Ghanbarzadeh, et al. 2006). Interest in the industrial utilization of zein is due to mostly its excellent film-forming ability when cast from appropriate solvent systems (Lai and Padua 1997). It is insoluble in water or anhydrous alcohols because it has high amount of nonpolar amino acids such as leucine, proline and alanine. It dissolves only in organic solvents and has GRAS status for use in foods (Lungu, et al. 2005). Preparation of zein films generally involves

casting alcohol solutions on inert, flat surfaces. Formed films are peeled off after the solvent has evaporated (Torres-Giner, et al. 2008). Corn zein films are characterized by their ability to form though, glossy, hard, grease proof coatings (Lungu, et al. 2005). Corn zein forms films with high tensile strength and low water permeabilities compared to other protein based films, and has desirable heat seal property (Cho, et al. 2002). It is presently used to coat candy, dried fruit and nuts because this protein forms good barriers against oxygen penetration and moisture loss (Lungu, et al. 2005). Zein film is formed through the development of hydrophobic, hydrogen and limited disulfide bonds between zein chains. Zein without any plasticizer is resulted in a very brittle film. To increase film flexibility, plasticizers such as glycerol and sorbitol are needed to be incorporated into the film (Paramawati, et al. 2001).

Lungu et al. (2005) developed zein coating containing nisin and potassium sorbate and investigate its antimicrobial effect against *Listeria monocytogenes* on turkey frankfurters at 4 °C. Inoculated frankfurters treated with the solvents ethanol glycerol and propylene glycol used to dissolve zein had counts that were significantly lower than the control by 4 to 5 log CFU/g at day 28. Janes et al. (2002) showed with an inoculum level of 10^3 CFU/g, that zein coating with nisin and calcium propionate was able to prevent growth of *L. monocytogenes* inoculated on cooked chicken breast meat, but their combination was not able to prevent growth of 10^3 or 10^6 CFU/g inocula if the incubation temperature was 8 °C. Janes et al. (2002) also reported that nisin (1000 IU/mg) by itself without zein become inactive after 7 days. To reduce the 10^3 cell challenge to nondetectable levels in 24 d, the treatment combinations of zein, nisin, and calcium propionate with 4 °C. Carlin et al. (2001) showed that the population of *L. monocytogenes* was 10-fold lower on coated sweet corn with zein than on non-coated sweet corn indicating a barrier effect of zein coating after 8 days at 10 °C. However, sorbic acid introduced in zein coatings as a surface antimicrobial agent had no additional efficiency, probably because of high a_w of sweet corn, short time and low temperature of storage. In the other study, zein films, along with polyethylene films, were effective gas barriers that allowed the development of modified atmosphere inside the packaged broccoli florets stored at 5 °C. Broccoli florets maintained their original firmness and color after 6 days. Off-odors, present in all stored packages, except in those made with laminated-and-coated zein films, were attributed to anoxic conditions developed in the packages (Rakotonirainy, et al. 2001).

Soy Protein Film: Soy protein used in film formation is classified as soy protein concentrates and soy protein isolates. Commercially soy protein concentrate contains about 80% protein and is obtained by removing alcohol-soluble nonprotein compounds from defatted meal with 60-80% aqueous alcohol. Soy protein isolate contains more than 90% protein and is obtained by alkali extraction followed by acid precipitation (pH 4.5) (Cho, et al. 2007).

Soy protein films are typically prepared in two steps involving the heat denaturation of the proteins followed by surface dehydration. Denaturation of a protein involves its solubilization and unfolding. During drying intermolecular interactions such as disulfide cross-linking and hydrophobic bonds, formed between the unfolded proteins, provide network for film formation, In particular, intermolecular hydrogen-bonded β-sheet structure is essential for the network formation in soy protein film (Cho, et al. 2004, Cho, et al. 2007). Soy protein-based edible film is most commonly prepared from SPI rather than SPC because the nonprotein fraction in the latter adversely affects the film forming abilities. Soy protein films are more flexible, smooth, and clear films compared to other plant protein based edible films. Soy protein based edible films have received considerable attention for their excellent film forming abilities and barrier properties against oxygen (Cho, et al. 2007).

Theivendran et al. (2006) demonstrated that combination of nisin with grape seed extract or grape tea extract in soy protein based edible film suppressed the growth of *L. monocytogenes* on full fat turkey stored at 4 °C and 10 °C approximately by 2.8 and 2.3 log CFU/mL, respectively and thus provides additional safety and improve the quality of ready-to-eat meat.

Wheat Gluten Film: Wheat gluten is a biodegradable, inexpensive, abundant, and crop renewable raw material (Paz, et al. 2005). Film-forming capacity of wheat gluten is suitable for making films or coatings with remarkable functional properties due to the unique cohesive and elastic properties of the gluten proteins (Mastromatteo, et al. 2008). Wheat gluten edible films have transparent, mechanically strong and relatively water resistant. Wheat gluten films are good gas barrier (O_2 and CO_2) at low relative humidity (RH) (Paz, et al. 2005).

Tanada-Palmu et al. (2005) have shown that the wheat gluten coatings and films extended the shelf life of strawberries and retarded the senescence process compared with strawberries used as a control.

3.3.1.1.2. Polysaccharides-Based Edible Films

Chitosan Film: Chitosan is a cationic polysaccharide obtained by deacetylation of chitin, which is the major constituent of the exoskeleton of crustaceans such as crab and shrimp (No, et al. 2007, Beverlya, et al. 2008). When compared to chitin, chitosan is more soluble and has better antimicrobial activity due to the positive charge on the C-2 of the glucosamine monomer at pH 6 and below (Lacroix and Tien 2005). Chitosan is water-soluble and chitosan films are easily prepared by evaporating from dilute acid solutions of the polymer (Quattara, et al. 2000). Due to its high molecular weight and solubility in acidic aqueous solutions, chitosan can form film (Han, et al. 2005). Chitosan has been proved to be nontoxic, biodegradable, biofunctional, biocompatible, and it has widely been used in the food industry as a potential food preservative due to its antimicrobial characteristics (Li, et al. 2006). Long positively charged chitison molecules interact with negatively charged bacteria membranes causing disruption and death of cell. Antimicrobial and functional properties of chitosan depend on several factors including characteristics of chitosan molecule, other compounds in the system, and environmental conditions (Zivanovic, et al. 2005). The reported minimum inhibitory concentrations (MICs) vary widely from 0.01 to 1.0% and yeasts tend to be more sensitive than bacteria (Ryu, et al. 2002).

Some successful applications, especially against spoilage yeasts in juices and emulsified sauces, have been reported. In meat products, slight inhibition (1–2 log Cfu/g) of total microbial growth in refrigerated beef patties has been reported in the presence of 1.0% but not 0.5 or 0.2% chitosan (Roller, et al. 2002). Moreover, antimicrobial films prepared with chitosan showed inhibitory effects on surface spoilage bacteria. For example, Duan et al. (2008) indicated that all chitosan-lysozyme packaging applications resulted in 0.01 to 0.64 log reduction in yeast population in mozzarella cheese. However, acid soluble chitosan develop bitterness and astringency film, which has been one of the major sensory limitations (Han, et al. 2005).

Alginate Film: Alginate, which is extracted from brown seaweed, is a salt of alginic acid, a linear polymer of β-D-mannuronic acid and α-L-guluronic acid (Olivas, et al. 2008). Although alginate carries good film forming properties, they are quite brittle when dry. Addition of plasticizer agents like glycerol is required to make it flexible. Alginate films are good oil and oxygen barrier, but are poor water barrier because of its

hydrophilic nature (Lacroix and Tien 2005). However, the treatment of alginate film with calcium decreases water vapor permeability of alginate films, making them water-insoluble. Calcium has the ability to cross-link alginate by binding with guluronic acid therefore water vapor permeability depends on proportions of guluronic acid (Olivas, et al. 2008).

Rojas-Graü et al. (2007) indicated that alginate-apple puree edible coatings were successfully formulated with the addition of essential oils such as lemongrass, oregano oil and vanillin and resulted in a variety of beneficial effects on the shelf-life of fresh-cut 'Fuji' apple. Coatings with essential oils seemed to effectively inhibit the growth of *L. innocua* inoculated on apple pieces as well as psychrophilic aerobic bacteria, yeasts and molds.

Cellulose Film: Cellulose is the principal structural component of plants and the most abundant source of complex carbon hydrate in the world (Arvanitoyannis and Biliaderis 1999, Bifani, et al. 2007). It is highly crystalline, fibrous, and insoluble (Cha and Chinnan 2004). The usefulness of cellulose as a material for edible films can be extended by chemical modification to cellulosic derivatives (Brawin, et al. 2006). Several cellulose derivatives are widely produced commercially, most commonly carboxy methylcellulose (CMC), methylcellulose (MC), hydroxy propylcellulose (HPC), and hydroxypropyl methylcellulose (HPMC). They produce films with good film-forming properties by solubilizing in aqueous or aqueous-ethanol solution. Cellulose based edible films are generally transparent, flexible, odorless, tasteless, water soluble, and resistant to oil and fats (Lacroix and Tien 2005).

For instance in the study of Bifani et al. (2007), MC and HPMC can be used to reduce oil absorption in fried products such as potato and also reduce moisture loss during cooking of poultry and seafood. HPC can retard spoilage and moisture absorption in coated nuts and candies. CMC is an anionic cellulose ether and forms a complex in the presence of casein, increasing the coating formulation viscosity; it retains the firmness of fruits and vegetables, preserves important flavor components of some fresh commodities, reduces oxygen uptake without causing carbon dioxide increase in internal fruits and vegetables, and improves the puncture strength of films based on caseinate (Lacroix and Tien 2005).

Starch Film: Starch is a commonly used agricultural raw material for edible packaging because of its low cost, renewability, and biodegradability. Starch is normally a mixture of about 25% amylose and 75% amylopectin polymers (Mehyar and

Han 2005, Famá, et al. 2007). Amylose forms coherent, relatively strong and free-standing films in contrast to amylopectin films which are brittle and noncontinuos (Gennadios, et al. 1997). Starch based edible films have low mechanical resistance and high moisture sensitivity when compared to synthetic polymer films (Brawin, et al. 2006). Despite receiving much interest owing to their low cost, starch based edible films have failed to gain widespread use because they deteriorate very easily when subjected to high moisture environment. Therefore many researchers have tried to produce starch-based biodegradable materials subjected to chemical modifications such as acetylating and/or the addition of natural or chemical plasticizer in starch (Yoshido, et al. 2002).

Starch based edible coatings containing potassium sorbate have been applied to extend the storage life of strawberries. The incorporation with potassium sorbate reduced the initial microbial load, thus storage life of strawberries coated with starch extended from 14 to 28 days (Cha and Chinnan 2004).

3.3.1.2. Lipid-Based Edible Films

Materials such as fatty acids, lipids (triglycerides), and waxes are commonly used in edible coatings to reduce film water vapor permeability because these materials are nonpolar or hydrophobic and, thus are good barriers against moisture migration (Talens and Krochta 2005). Lipid based coatings are also used to prevent weight loss, to slow down aerobic respiration, and to enhance the visual appeal of fruits and vegetables providing gloss (Rhim and Shellhammer 2005). They exhibit poor mechanical properties because of their lack of cohesive structural integrity. Therefore, incorporating lipid or wax in protein-based or polysaccharide-based films may interfere with polymer chain-to-chain interactions and /or provide flexible domains within the films. The result can be a plasticizing effect, including reduction of film strength and increase of film flexibility (Talens and Krochta 2005). Carnauba wax, has GRAS status, is commonly added to edible coating formulation for fruits and vegetables (Weller, et al. 1998). Most natural waxes such as beeswax, carnauba wax, and candellila wax, also have emulsifying properties, as they are long-chain alcohols and esters. Limitations to their use include their poor mechanical properties and oily appearance in some products (Rhim and Shellhammer 2005).

3.3.1.3. Composite Films

Composite films with either polysaccharides/lipids or protein/lipids have been developed to combine the advantages of the individual film-forming materials. The primary function of a lipid compound in formulation can serve as a good barrier to water vapor, while the hydrocolloid component in the formulation can serve as a good barrier to oxygen and carbon dioxide and the necessary supporting matrix (Ryu, et al. 2002, Rhim and Shellhammer 2005). Therefore, it is desirable to develop the composite films, in laminated or emulsion forms, to protect from the moisture and oxygen surroundings (Weller, et al. 1998, Ryu, et al. 2002).

Both the laminate and emulsion films offer advantages. The laminate films are easier to apply with regard to the temperature, due to the distinct natures of the support matrix and lipid. During the casting of the lipid onto the protein or polysaccharide film, the temperatures of the film and lipid can easily be controlled separately. When producing the emulsion films, the temperature of the emulsion must be above the lipid-melt temperature but below the temperature for gelation and solvent volatilization of the structural network. However, since preparation of the laminated films requires four stages: two casting and two drying stages, laminated films are less popular in the food industry despite their good barrier against water vapor. The preparation of the emulsion films requires only one casting and one drying stage, but the finished films are still rather poor barriers against water vapor, since the water molecules still permeate through the non-lipid phase. Therefore, it would be interesting to form a bilayer film from the emulsion mixture where the lipid is concentrated at the surface during the drying process. Hence, a good barrier against water vapor would be achieved with a less time-consuming preparation method. This phase separation is, however, difficult to accomplish (Anker, et al. 2002).

In study of Bravin et al. (2006), the effectiveness of polysaccharides-lipid based edible coating in controlling moisture transfer in moisture-sensitive products was evaluated by coating crackers, a low water activity (a_w)-type cereal food. As a result of this study, they found that application of composite film to crackers, a low a_w food, confirmed the potential of edible packaging to become an integral part of food, and had longer shelf-life than uncoated samples, reducing the hydration kinetic in high a_w environment.

3.3.2. Plasticizers

In most cases, plasticizers, low molecular weight are added to edible films, which decreases the glass transition temperature of the films (Zhao and McDaniel 2005), especially polysaccharide and protein films, before drying in order to overcome the brittleness of films. These film structures are often brittle and stiff due to extensive interactions between polymer molecules. Plasticizers increase the film flexibility due to their ability to reduce internal hydrogen bonding between polymer chains while increasing molecular spacing (Cho and Rhee 2002). The addition of plasticizers affects not only the elastic modulus and other mechanical properties, but also the resistance edible films and coatings to permeation of vapors and gases (Han and Gennadios 2005). Moreover, studies have shown that the concentration, composition, size and shape of plasticizers affect the properties of film (Kim, et al. 2006).

The most common plasticizers used in edible films are glycerol, sorbitol, and polyethylene glycol (Zhao and McDaniel 2005). Glycerol, as a plasticizer, has been incorporated into most hydrocolloid films. It is a high boiling point plasticizer, water-soluble, polar, nonvolatile, and protein miscible. These properties make glycerol a suitable plasticizer for use with a compatible water-soluble polymer (Gounga, et al. 2007). Moreover, they could affect the flavor and taste of the films and coatings. Polyethylene glycol is tasteless. Glycerol and sorbitol taste sweet, but the sweetness of glycerol in protein films is negligible, while the sweetness of sorbitol noticeable (Zhao and McDaniel 2005). Water also plays an important role of plasticizer in edible films, and hydrophilic plasticizers generally attract additional water. Films commonly require plasticizers at 10% to 60% content (on a dry basis), depending on the rigidity of the polymer (Talens and Krochta 2005).

3.3.3. Food Additives

Edible films and coatings can carry various active agents, such as emulsifier, antioxidants, antimicrobials, flavors and colorants, thus enhancing food quality and safety. Emulsifiers are surface active agents of amphiplic nature able to reduce surface tension of the water-lipid interface and water-air surface. Emulsifiers are essential for formation of protein and polysaccharides films containing lipid emulsion particles. They

also modify surface energy to control adhesion and wettability of the film surface. In protein films, some film-forming proteins have sufficient emulsifying capacity due to their amphiplic structure. Incorporated flavors and colorants can improve the taste and visual perception of quality, respectively. Because of the various chemical characteristics of these additives, film composition should be modified to keep homogeneous film structure when heterogeneous additives are incorporated into film-forming materials (Han and Gennadios 2005). Antioxidant agents can be incorporated into edible films and coatings to retard oxidation if they inhibit the formation of free radicals in the initiation stage of the reaction or if they interrupt the propagation of free radical chain reaction (Han and Krochta 2007). Although synthetic antioxidants such as butoluene and butylhydroxyanisol (BHA) can inhibit lipid oxidation they have low water solubility and exhibit toxic properties. As a consequence, natural antioxidants have been suggested as a safe alternative to synthetic antioxidants to retard oxidative process and to improve the keeping quality of food products (Bekhit, et al. 2003).

3.3.3.1. Natural Antimicrobial Agents

Since food deterioration usually starts on food surfaces because of the presence and growth of spoilage or pathogenic microorganisms, antimicrobial sprays or dips have been done to overcome those contaminations. However, direct surface application of antimicrobial substances has some limitations because of active substances could be neutralized, evaporated or diffused in adequately into the bulk of food (Hotchkiss 1995, Pranoto, et al. 2005b, Han, et al. 2008). Moreover, surface microbial growth, the most common cause of food spoilage, can be controlled through the use of edible films carrying various active agents. Edible film and coatings with antimicrobials become active barriers to provide additional hurdles against microbial growth. In many cases, these compounds slowly release into the food surface and therefore remain at high concentration for extended periods (Pranoto, et al. 2005b). Different chemicals, such as organic or inorganic acids, metals, alcohols, ammonium compounds or amines, can be incorporated into biodegradable packaging materials. However, due to the health concerns of consumers related to chemical preservatives and environmental problems, there is a demand for the food industry to use natural biopreservatives with edible and biodegradable packaging materials (Mecitoglu, et al. 2007). Due to the perceived lower

risk to the consumer, the use of naturally derived antimicrobial additives such as bacteriocins, enzymes, proteins, plant extracts and essential oils is an increasing interest (Suppakul, et al. 2008) (Figure 3.3).

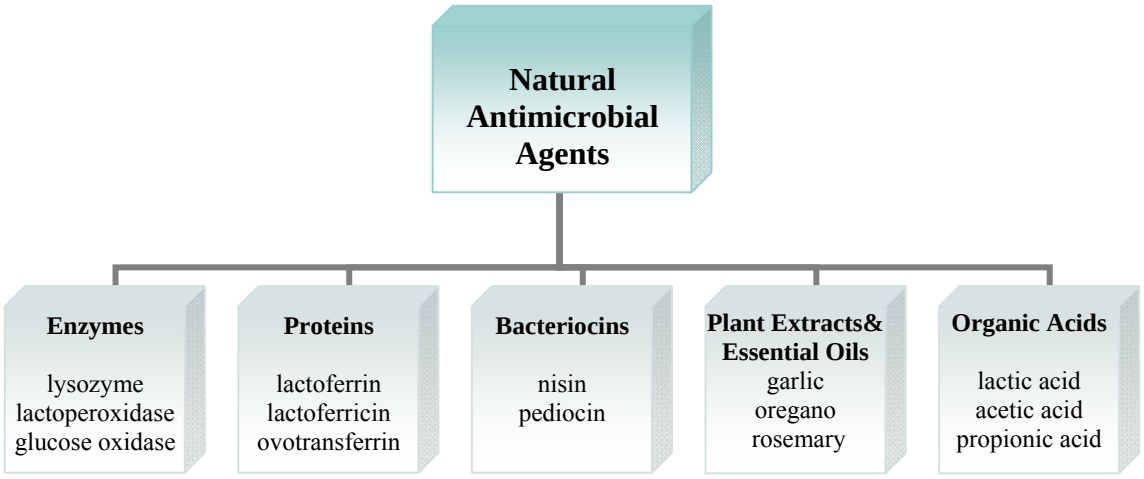


Figure 3.3. Schematic presentation of commonly used natural antimicrobial agents in edible films and coatings

3.3.3.1.1. Enzymes

Lysozyme: Lysozyme is a commercially important enzyme and is a currently used in food technology as a potent antibacterial agent and food additive, and is pharmacological technology as a drug for treatment of ulcers and infections. It is found in mammals, birds, and fishes. Lysozyme isolated from hen egg white is the most studied and the only one so far used commercially as a food preservative (Su and Chiang 2006). Lysozyme from hen egg white is a polypeptide of 129 amino acid residues with molecular weight of 14,400 Dalton (Figure 3.4). It is a basic protein (positively charged) with isoelectric point between 10.5 and 11.0 and is stable under acidic pH conditions, especially when thermally treatment (Jiang, et al. 2001). Matsuoka et al. (1966) found that lysozyme maintained 100% activity after thermal treatment at 100 °C for 3 min at pH 4.5, although an increase in pH decreased the lysozyme activity. Lysozyme content in the hen egg white has been determined 3.4 % of the total egg white protein (Jiang, et al. 2001). The molecule conforms to the principle of hydrophobic in, hydrophilic out of a protein. All its polar groups are on the

surface and the majority of nonpolar (hydrophobic) groups are buried in the interior (Kagoshima University 2008).

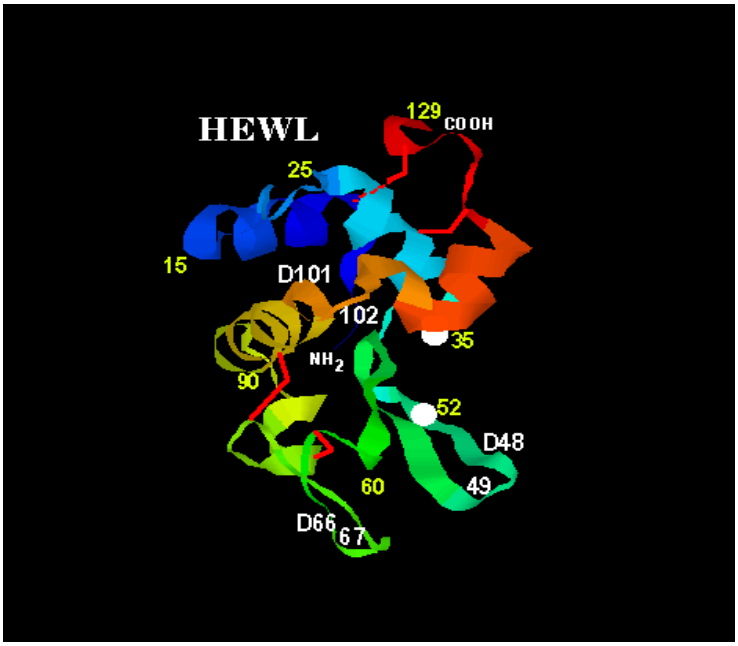
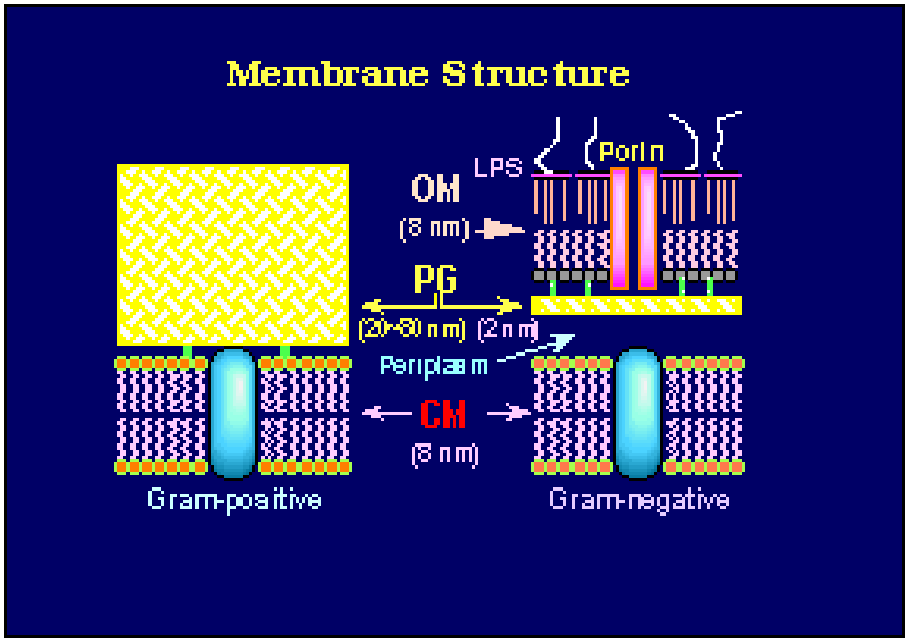


Figure 3.4. The three dimensional structure of lysozyme isolated from hen egg white (Source: Kagoshima University 2008)

Although many attempts have been made to use as a general antimicrobial agent because the lytic spectrum was certain Gram-positive bacteria and is less effective against Gram-negative bacteria, owing to the differences found in their membrane structure (Figure 3.5). Lysozyme attacks the *β*-1–4 glycosidic linkage between the *N*-acetylmuramic acid and *N*-acetylglucosamine groups found in the peptidoglycan layer in bacterial cell walls sensitive to the attack by lysozyme. However, Gram-negative bacteria are less susceptible, because its outer membrane mainly consisting of lipopolysaccharide prevents the access of lysozyme to the site of action on the peptidoglycan in cell walls (Nattres and Baker 2003).



OM= Outer Membrane; PG= PeptidoGlycan; CM= Cytoplasmic Membrane

Figure 3.5. Membrane structure of gram-positive and gram-negative bacteria (Source: Kagoshima University 2008)

The antimicrobial effect of lysozyme, particularly against Gram-negative bacteria can often be enhanced by the presence of specific chemicals or physical treatments. Sensitivity of Gram-negative species to lysozyme was increased by chelators, certain antibiotics, amino acids, alkaline pH, osmotic shock, drying, and freeze thawing and by hydrogen peroxide and ascorbic acid (Conner 1993). It has been recognized since 1960s that the susceptibility of Gram-negative bacteria to lysis by lysozyme can be increased by the use of membrane disrupting agents, such as detergents and chelators. Ethylene diamine tetraacetic acid (EDTA) is a chelating agent used in a wide variety of food products to prevent oxidation and other deteriorative reaction catalyzed by metal ions. The EDTA antimicrobial effect can be explained with its ability to limit the availability of cations (Cannarsi, et al. 2008). EDTA can destabilize the cell membranes of bacteria by complexing the divalent cations which act as salt bridges between membrane macromolecules such as lipopolysaccharides (Su and Chiang 2006). It has an antimicrobial property against common food spoilage and food-borne disease-causing bacteria, including *Bacillus cereus*, *Bacillus stearothermophilus*, *Campylobacter jejuni*, *Clostridium botulinum* types A,B, and E, *Clostridium butyricum*, *Clostridium perfringens*, *Clostridium sporogenes*, *Clostridium thermosaccharolyticum*,

Clostridium tyrobutyricum, *Escherichia coli* O157:H7, *Klebsiella pneumoniae*, *Listeria monocyotogenes*, *Salmonella Typhimurium*, *Staphylococcus aureus*, *Vibrio cholerae*, and *Yersinia enterocolitica* (Conner 1993).

Commercially, lysozyme has been used in the cheese manufacturing to prevent the growth of lactate fermenting, gas-forming *Clostridia* spp, especially *Clostridium tyrobutyricum*. Other potential applications include its use heat-sterilized products to reduce thermal requirements, its inclusion in immobilized enzyme columns to prevent contamination, and its use as a supplement to food such as poultry, shrimp, sausage, and sake as a preservative (Hugley and Johnson 1987). The potential use of lysozyme as a food preservative has invoked considerable interest, particularly in Japan (Nattress, et al. 2001). Another use of lysozyme in food technology involves the incorporation of the enzyme into plastic or edible films for the production of antimicrobial packaging material. In different studies, lysozyme has been used in the production of antimicrobial films for such plastic materials as polyvinlyalcohol (PVOH) and cellulose acetate, for edible and biodegradable materials, such as zein, whey proteins, alginate, carrageen, and chitosan (Appendini and Hotchkiss 1997).

Several methods have been used for isolating and purifying lysozyme. The classical crystallization method is widely used in the industry. Crystallization method requires a week until the enzyme is efficiently recovered (Jiang, et al. 2001). More advanced method, such as ion exchange and affinity chromatography, have been suggested by many researchers. Since these methods suffer from major drawbacks such as low capacity resins and the high cost of affinity supports these commercial applications may be limited (Chiang, et al. 1993). Even though commercial lysozyme have only 1-6% (w/w) protein impurities (Judge, et al. 1998), for the application of lysozyme in food industry, the use of cheaper partially purified lysozyme preparations obtained by some faster methods may be economically more feasible. For this reason, some rapid partial purification procedures have recently been developed based on combination of reductants and thermal treatment (Chang, et al. 2000) or selective precipitation of lysozyme using an anionic surfactant such as Aerosol-OT (Shin, et al. 2003).

Lactoperoxidase: Lactoperoxidase (LP) is the abundant enzyme, found in all cow's milk and in the most other mammalian milk. It constitutes about 1% of the whey proteins or 10-30 μg/ml milk. LP is a single chain protein and can survive at 70 °C for 15 min, is stable at pH 3-10.3. Studies demonstrated that LP is a "system" which

consists of three components: the lactoperoxidase, an oxidizable substrate (thiocyanite), and hydrogen peroxidase (H_2O_2) (Conner 1993). LP in lactoperoxidase system (LPOS) catalyzes the oxidation of thiocyanate ion (SCN^-) by hypothiocyanite ($OSCN^-$) and hypothiocyanous acid (HOSCN), which inhibit microorganisms by the oxidation of sulphydryl (SH) groups of microbial enzymes and other proteins (Min, et al. 2005b). The addition of LP and other components of this antimicrobial system to thermally processed skim milk, meat and vegetable products and prevention of the development of pathogenic bacteria have also been studied This system generally shows a bactericidal effect on Gram-negative bacteria and a bacteriostatic effect on Gram-positive bacteria and also inactivate viruses and fungi. The synergistic effect of LPS with nisin has also been demonstrated. Recently, the LPS was incorporated into edible whey protein films and tests of these films on different microorganisms and smoked salmon showed the good potential of this enzyme for use in antimicrobial packaging (Mecitoğlu and Yemenicioglu 2007).

3.3.3.1.2. Proteins

Lactoferrin (LF): LF is a single-chain glycoprotein with a molecular weight of about 80 kDa. The inhibition apparently results from the binding of essential iron needed for growth of microorganisms. LF is reported to be active against *Salmonella* and *E. coli*. LF was accepted as generally recognized as safe (GRAS) by U.S. Food and Drug Administration (FDA) in 2001.

Lactoferrin hydrolysate, derived from LF by pepsin digestion, contains an antimicrobial peptide termed lactoferricin, which has greater antimicrobial activity than lactoferrin. The mode of action of lactoferricin has not been clear, but it is believed to inhibit microorganisms by damaging the outer cell wall. It has fungistatic or fungicidal effects against *Candida albicans, Candida krusei,* and *Rhodotorula rubra* were reported (Min and Krochta 2005).

3.3.3.1.3. Bacteriocins

Bacteriocins are antimicrobial proteins or peptides produced by bacteria that kill or inhibit the growth of bacteria. Some bacteriocins produced by lactic acid bacteria

found in fermented and non-fermented foods are considered as GRAS, what means they are nontoxic and nonantigenic to humans (Santiago-Silva, et al. 2008). Generally, bacteriocins are low molecular weight, cationic, hydrophobic and amphiphilic peptides, with antibacterial activity against many Gram-positive bacteria (Scannell, et al. 2000, Marcos, et al. 2007). Although these biopreservatives have the potential to protect some food from spoilage, their application in raw or processed meat products limited because binding with meat particles and fat may cause loss of activity (Roller, et al. 2002).

Pediocin: Pediocin is a bacteriocin produced by some species of *Pediococcus* genera, is active against a broad spectrum of Gram-positive bacteria. This bacteriocin is used to overcome the post-processing contamination of meat products and it is notably effective against *L.monocytogenes*, pathogenic microorganism of great importance in food contamination. When bacteriocin is combined with sodium acetate, it has been shown an antilisterial effect in turkey slurries. Pediocin has also demonstrated antilisterial effect on sliced cooked sausages and frankfurter sausages (Santiago-Silva, et al. 2008).

Nisin: Nisin, produced by *Lactococcus lactis* subsp. *lactis*, is a 3500-Da hydrophobic peptide (Ko, et al. 2001, Lungu and Johnson 2005), heat-stable and acid-stable, and is the best known of the bacteriocins (Nattress, et al. 2001). Nisin was discovered over 50 years before most other bacteriocins and was the first compound of this type to be used in the food industry on a commercial scale (Cannarsi, et al. 2008). It has been approved as a GRAS compound by both the Food and Drug Administration (FDA) and World Health Organization (WHO) (Scannell, et al. 2000), but is approved only for use in processed cheese products in the United States (Lungu and Johnson 2005). Although the sensitivity of some Gram-negative bacteria to nisin has been reported, Gram-positive bacteria are more susceptible to nisin. However, to be effective against Gram-negative bacteria, it should be combined with a chelating agent such as EDTA (Hoffman, et al. 2001). The solubility and activity of nisin decrease as pH increases and this is one of the major challenges in the application of nisin by the food industry (Theivendran, et al. 2006). The use of nisin in fresh meat products is also not suggested since this agent forms inactive complex with glutathione in these products (Rose, et al. 1999). Nisin impregnated cast films are generally more bactericidal than heat-pressed films (Dawson, et al. 2003). It has been studied for its suitability to be incorporated into cellulose, whey protein isolate, soy protein isolate, egg albumen,

wheat gluten, hydroxypropyl methylcellulose, and zein films (Pranoto, et al. 2005a, Li, et al. 2006).

3.3.3.1.4. Plant Extracts and Essential Oils

Plant Extract: Plant extracts are used in a variety of food applications to preserve food quality and play role as an antioxidant. (Theivendran, et al. 2006). Plant extracts from garlic, oregano, rosemary, pimento, onion, cinnamon, cloves, thyme sage, green tea and grape seed are rich in phenolic compounds having antimicrobial activity. The antimicrobial compounds in plant materials are commonly present in the essential oil fraction. Thus, generally the antimicrobial effects of essential oils contain higher concentrations of phenolic compounds against various pathogenic and spoilage bacteria, molds, and yeasts than the plant extract (Pranoto, et al. 2005a). This effect is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and/or coagulation of cell contents (Seydim and Sarikus 2006).

Essential Oils: Antimicrobial activities of essential oils were recognized long ago, but their application as natural antimicrobials has recently received increased attention in the food industry. Some of the main chemical compounds of essential oil include alcohol, aldehydes, esters, ethers, ketones, phenols, and terpenes. Although each type of essential oil consists of more than 100 compounds, generally phenolics and terpenes are major contributors to antimicrobial effects of essential oil (Zivanovic, et al. 2005). Although the majority of essential oils are classified as Generally Recognized As Safe (GRAS), their use in foods as preservatives is often limited due to flavor considerations, since effective antimicrobial does may exceed organoleptically acceptable levels. Therefore, there is an increasing demand for accurate knowledge of the minimum inhibitory concentration (MIC) of essential oils to enable a balance between the sensory acceptability and antimicrobial efficacy. This can be achieved with *in vitro* and *in vivo* studies (Lambert, et al. 2001). Incorporating plant essential oil and oil compounds into edible films provide a novel way to enhance the safety and shelf-life in food systems (Rojas-Grau, et al. 2007). When edible films are enriched with essential oils, the drying temperatures usually employed to form the edible coating are high enough to volatilize a high percentage of the aromatic components (Ponce, et al. 2008).

Incorporation of essential oil in chitosan films may not only enhance the antimicrobial properties of films but also reduce water vapor permeability and slow lipid oxidation of the product on which the film is applied (Seydim and Sarikus 2006).

3.3.3.1.5. Organic Acids

Organic acids are either naturally present in fruits and vegetables or synthesized by microorganisms as a result of fermentation. Lactic, acetic, citric, succinic, malic, tartaric, benzoic, and sorbic acids are major organic acids that naturally occur in fruit and vegetables. The antimicrobial activity of organic acid is attributed to pH reduction, depression of internal pH of microbial cell by ionization of undissociated acid molecules, and disruption of substrate transport by altering cell membrane permeability or reduction of proton motive force (Eswaranandam, et al. 2004).

Lactic Acid: Lactic acid is manufactured by some *Lactobacillus* spp., which are able to produce L-lactate (or DL-lactate). The antibacterial effect is pronounced when utilized at 1 to 2 % levels (pH 5). At low pH 5, lactic acid can have a bactericidal activity particularly against Gram-negative bacteria (Ray 2004). It is capable of inhibiting the growth of various food spoilage bacteria, such as Gram-negative species of the families *Enterobacteriaceae* and *Pseudomonadaceae* (Alakomi, et al. 2000). However, it may not have an antimicrobial activity on fungi in the food environment (Ray 2004).

Acetic Acid: Acetic acid is commercially produced by *Acetobacter aceti.* In general, when the level of acetic acid is 0.2%, it shows bacteriostatic activity. However, when the level of acetic acid is above 0.3%, it shows bacteriocidal activity. It is more effective against Gram-negative bacteria. Actually, this effect depends on pH and the bactericidal effect is more definite at low pH (below pH 4.5). It is used as an antimicrobial agent to salad dressings, mayonnaise, and carcass wash (Ray 2004).

Propionic Acid: Propionic acid by *Propionibacterium* spp. has fungistic effect in food environment and also capable of inhibiting the growth of Gram-negative and Gram-positive bacteria. At pH 5 or below, Gram-negative bacteria are more sensitive than Gram-positive bacteria as used 0.1 - 0.2% level. It is applied to control molds in cheeses, butter, and bakery products and to hamper growth of bacteria and yeasts in syrup, apple sauce, and some fresh fruits (Ray 2004).

3.4. Film Forming Techniques

3.4.1. Solvent Removal

Hydrocolloid films are mainly formed by evaporation of solvent, usually water or ethanol, from appropriately prepared and cast solution. In this process, the film forming substances are able to form a continuous structure and stabilized by chemical and physical interactions between macromolecules. Several additives such as plasticizers, cross-linking agents and solutes are added to the film-forming solution dissolved in a suitable solvent. The film-forming solution is then cast in a thin layer, dried, and peeled from the surface (Cagri, et al. 2004).

Most film-forming proteins, except corn zein and wheat gluten, are soluble in water. Corn zein and wheat gluten films and coatings must be prepared in aqueous ethanol (Krochta 2002, Donhowe and Fennema 1994). Food-grade plasticizers, such as sorbitol, acetylated monoglyceride, glycerol, mannitol, sucrose, and polyethylene glycol, are often added to film forming solutions to enhance mechanical properties of the films and decrease brittleness and increase flexibility (Krochta 2002, Donhowe and Fennema 1994). The heat is applied to film-forming materials for protein gelation and coagulation, which involves denaturation, gelatification, or precipitation followed by rapid cooling. Intermolecular and intermolecular disulfide bonds in the protein complex are cleaved and reduced to sulfhydryl groups during protein denaturation (Cagri, et al. 2004). If a composite protein film or coating is based on an emulsion formation, a lipid material and a surfactant is also added, and the mixture is homogenized after it is heated above the melting point of the lipid. Degassing is also sometimes essential to eliminate bubble formation in the final film or coating. Finally, the protein film is formed by casting the prepared formulation on a suitable substrate and then drying (Donhowe and Fennema 1994). When the film-forming solution is cast, the film structure is produced by linking reformed disulfide bonds to the polypeptide chains together, with the aid of hydrogen and hydrophobic bonding (Cagri, et al. 2004).

3.4.2. Coacervation

Coacervation can be divided into two groups called as simple and complex coacervation. In simple coacervation, hydrocolloid dispersed in aqueous solution is precipited or gelified by removal of solvent, altering pH, adding non-electrolyte solute in which the polymer is not soluble and, an electrolyte substance having a salting out effect. In complex coacervation, at least two oppositely charged hydrocolloid solution are combined to induce interaction and an insoluble polymer mixture (Debeaufort, et al. 1998).

Coacervation may also be classified in aqueous or nonaqueous phase separation. While an aqueous separation system involves a hydrophilic coating deposited on a water-insoluble core particle, nonaqueous phase separation usually involve a hydrophobic coating deposited on either a water-soluble or a water-insoluble core particle (Donhowe and Fennema 1994).

3.4.3. Solidification of Melt

Solidification of melt is common method for producing lipid films. The rate of cooling plays an important role like the rate of solvent removal in the overall physical properties of resulting film. The rate of cooling affect the predominant polymorphic state, altering oxygen and water permeability of lipid films, as well as degree of recrystallization in the solidified film (Donhowe and Fennema 1994).

3.5. Film Application Techniques

A number of methods for application of edible films to foods have been employed, including but not limited to foaming, dipping, spraying, casting, brushing, wrapping, or rolling. Dipping, spraying and casting techniques are more common than other techniques. A thinner and a more uniform film required for certain surfaces could be best achieved by spraying. In fact, early coatings procedures involved sprays, with further distribution over food surfaces via roller or brushes, followed by tumbling to evenly spread the coating. This technique is also suitable to apply film to only one side

of food product or, to carry out dual applications used for cross-linking (Donhowe and Fennema 1994, Cutter and Sumner 2002).

Film thickness depends essentially on the application technique and on the solution viscosity. Indeed, since highly viscous solution can't be or very uneasily sprayed, and thus only falling coating or dipping techniques apply, giving high thickness to the coating (Debeaufort, et al. 1998). Dipping is the commonly used method for fruits, vegetables and meat products. In here food product is directly dipped into the composite coating formulation (in aqueous medium). After dipping, the excess coating usually drips off and the remaining material is allowed to set or solidify on food with air dry, whereby a thin film is formed over the food surface (Tharanathan 2003).

In casting process, film forming solution are poured into a confined area and subsequently dried. Casting produces free-standing films, which can be placed at food surfaces or between food layers, and allows film thickness to be controlled accurately on smooth and flat surfaces. Depending upon firmness and flexibility, cast films can then be used to wrap surfaces (Cutter 2006). Film can also be applied with brushes and/or with roller directly onto food surfaces and allowing them to set or dry in phase. The film casting is accomplished by use of applicators which can conduct spreading at the desired thickness and speed (Cutter and Sumner 2002).

CHAPTER 4

MATERIALS AND METHODS

4.1. Materials

The bacterial strains used in this study, *Listeria innocua* (NRRL B-33314), *Bacillus amyloliquefacines* (NRRL NRS-762), *Escherichia coli* (NRRL B-3008), *Pseudomonas fluorescens* (NRRL B-253), were supplied from the United States Department of Agriculture, Microbial Genomics and Bioprocessing Research Unit, Peoria, Illinois. In addition to these strains, *Escherichia coli* O157:H7 (ATCC 700728, Dr. Ali Aydın, Department of Food Hygiene and Technology, Faculty of Veterinary, İstanbul University, Turkey), *Salmonella typhimurium* (CCM 5445, Dr. A. Handan Baysal, Deparment of Food Engineering, İzmir Institute of Technology, Turkey) and *Staphylococcus aureus* (RSKK 95047, Dr. Gülsün Evrendilek, Department of Food Engineering, Abant Izzet Baysal University, Turkey) were used in this study. The frozen stock cultures were maintained in nutrient broth containing 15% glycerol at - 80 °C prior to analyses.

The turkey and beef burgers (containing 18 % fat and 1 % salt) used in this study were produced by Pınar Et A.Ş. (İzmir, Turkey) without using any antimicrobial or antioxidant agents. Fresh hen eggs, used in preparation of partially purified lysozyme, were obtained from a local supermarket in Izmir, Turkey. Zein, *Micrococcus lysodeikticus* and dialysis tubes (12000 MW, prepared as described in the product manual) were obtained from Sigma Chem. Co. (St. Louis, Mo., USA). Glycerol was purchased from Merck (Darmstadt, Germany). Disodium EDTA.2H_2O was purchased form Riedel-de haën (Sigma-Aldrich Laborchemikalien, Seelze, Germany).

4.2. Methods

4.2.1. Preparation of Partially Purified Lysozyme

Lysozyme was produced by slightly modifying the partial purification step given by Jiang et al. (2001). Briefly, the egg whites separated carefully without disturbing the egg yolks were first diluted with two volumes of 0.05 M NaCl solution. To precipitate the egg white proteins other than the lysozyme, the pH of this mixture was set to 4.0 by carefully adding several drops of 1 N acetic acid and it was diluted with equal volume of 60 % (v/v) ethanol. After 6 h incubation at room temperature in the presence of 30 % ethanol, the mixture was centrifuged at 15000 *g* for 15 min at 4 °C and the precipitate was discarded. The supernatant containing lysozyme was first dialyzed for 21 h at 4 °C by three changes of 2000 mL distilled water and then lyophilized by using a freeze drier (Labconco, FreeZone, 6 liter, Kansas City, MO, USA) working between -44 and -47 °C collector temperature and 50 x 10^{-3} and 100 x 10^{-3} mbar vacuum. The sample container volume was two to three times the sample volume. The lyophilized enzymes used in film making were stored at -18 °C and their activities were determined as U/mg prior to the preparation of each film.

4.2.2. Determination of Lyophilized Lysozyme Activity

The activity of lysozyme was determined spectrophotometrically at 660 nm by using a Shimadzu (Model 2450, Japan) spectrophotometer equipped with a constant temperature cell holder working at 30 °C. The reaction mixture was prepared by mixing 2.3 mL *Micrococcus lysodeikticus* cell suspension (at 30 °C for 5 min) prepared 0.26 mg/mL in 0.05 M Na-phosphate buffer at pH 7.0 and 0.2 mL enzyme solution (at 30 °C for 5 min). The reduction in absorbance was monitored for 120 s at 660 nm at 30 °C and enzyme activity was calculated from the slope of the initial linear portion of absorbance vs. time curve. The enzyme activity was expressed as unit. One unit was defined as 0.001 change in absorbance in 1 min. The average of three activity measurements was used in all tests.

4.2.3. Preparation of Zein Films

Zein films were prepared as described in Padgett et al. (1998). Briefly, 1.4 g zein was dissolved with 8.1 mL of ethanol (97 %) by mixing slowly with a magnetic stirrer for 25 min. Glycerol (0.39 mL) was then added to the medium and the temperature of the mixture was increased until it started to boil. The mixing was then ceased and the film solution was boiled for 5 min. After cooling to room temperature, hydrophilic partially purified lysozyme and/or $Na_2EDTA.2H_2O$ were incorporated into hydrophobic zein film forming solutions by stirring or homogenization methods. In stirring method, film forming solution containing lysozyme and/or $Na_2EDTA.2H_2O$ was further stirred for 25 min. In homogenization method, film forming solution homogenized at 8000 rpm for 2 min. The film forming solution (4.3 g) was then spread evenly onto a 8.5 x 8.5 cm glass plate cleaned previously with ethanol and the plates were dried at room temperature for 24 h. The films prepared by this method were peeled from the glass plates carefully and 6 x 6 cm pieces cut from the middle of films were used in all tests.

4.2.4. Photographs of Zein Films

The 3-dimensional surface and cross-sectional structures of the films were examined using a scanning electron microscope (SEM) (Philips XL 30S FEG, FEI Company, Eindhoven, Netherlands) in the Center for Materials Research of izmir Institute of Technology, İzmir, Turkey. Films were cut into 1 x 1 cm pieces using a sharp razor for the outer surface and cross-section observation. The surface of film samples was coated with a gold palladium for 2 min in a Magnetron Sputter Coating Instrument to eliminate charging effect, and then observed by SEM. The outer surface and cross-section micrographs of the films were viewed at a magnification of 1500x and 350x, respectively. In addition, average thickness of zein films was determined taking 10 measurements at different points of cross-sectional micrograph of each film.

4.2.5. Determination of Lysozyme Activity Released from the Zein Films

The release tests were conducted in a refrigerated incubator (Memmert Model ICP 500, Germany) at 4 ^{o}C. The films (6 x 6 cm) were placed in glass Petri dishes (10 mm in diameter) containing 50 mL distilled water (4 ^{o}C). The Petri dishes were then covered with parafilms and incubated for 1400 min with continuous stirring at 200 rpm with a magnetic stirrer (20 mm long teflon coated rod). The lysozyme activity released from films was monitored by taking 0.6 mL aliquots from the release test solution at different time intervals and conducting activity measurements for three times by using 0.2 mL of the taken aliquot in a single measurement. The activity of lysozyme was determined spectrophotometrically at 660 nm by using a Shimadzu (Model 2450, Japan) spectrophotometer equipped with a constant temperature cell holder working at 30 ^{o}C. The reaction mixture was prepared by mixing 2.3 mL *Micrococcus lysodeikticus* cell suspension (at 30 ^{o}C) prepared 0.26 mg/mL in 0.05 M, pH 7.0 Na-phosphate buffer and 0.2 mL enzyme solution (incubated at 30 ^{o}C for 5 min). The reduction in absorbance was monitored for 5 min and enzyme activity was calculated from the slope of the initial linear portion of absorbance vs. time curve. The enzyme activity was expressed as total units (0.001 change in absorbance in 1 min) released per cm^2 of the films (U/cm^2) for a given time period. All calculations were corrected by considering the total activity removed during sampling. The monitoring of enzyme release was continued until the activity increase in release test solutions ended and a slight reduction in activity initiated. The total soluble activity released from a film was determined by considering the peak points (maximum activity) in activity released vs. time curves. The percentage of activity recovered from a film in a release test was determined from the ratio of the total activity and amount of partially purified lysozyme added to film making solution.

4.2.6. Determination of Bound Lysozyme Activity Retained in Zein Films

In this study, no activity release was determined from the films at the end of release tests last for 1400 min. Thus, the enzyme retained in zein films after release test

was designated as bound enzyme. The films obtained from 1400 min release tests were cut into 3 x 3 cm pieces. For test of bound activity, the films were placed into glass Petri dishes containing 25 mL *Micrococcus lysodeikticus* solution (at 30 ^{o}C) prepared 0.26 mg/mL in 0.05 M, pH 7.0 Na-phosphate buffer. The Petri dishes were kept in an incubator at 30 ^{o}C and their contents' absorbance at 660 nm was monitored periodically under continuous magnetic stirring at 200 rpm. The lysozyme activity of the films were determined from the slopes of the initial linear portions of absorbance vs. time curve and given as U/cm^2.

4.2.7. *In Vitro* Antimicrobial Activity of Zein Films

The inhibitory effect of zein films against spoilage and pathogen bacteria was tested on agar and in broth media. Test of antimicrobial activity was conducted by using *Escherichia coli* (NRRL B-3008), *Pseudomonas fluorescens* (NRRL B-253), *Listeria innocua* (NRRL B-33314), *Bacillus amyloliquefacines* (NRRL NRS-762), *Escherichia coli* O157:H7 (ATCC 700728), *Salmonella typhimurium* (CCM 5445) and *Staphylococcus aureus* (RSKK 95047) as test microorganisms. The overnight cultures were prepared in nutrient broth and incubations were carried out for *L. innocua*, *E. coli* O157:H7, *E. coli, Salmonella typhimurium*, *S. auerus* at 37 °C, for *B. amyloliquefaciens* at 30 °C and *P. fluorescens* at 26 °C. For antimicrobial tests 16 discs (1.3 cm in diameter) were prepared from 6 x 6 cm films by a cork borer under aseptic conditions. The cutting was performed carefully to obtain samples from all film surface and 12 of the obtained discs were selected randomly and used in antimicrobial tests. During tests, 4 discs were placed into each Petri dish. The discs were placed carefully onto Petri dishes containing nutrient agar, which had been previously seeded with 0.1 mL of inoculum containing approximately 10^6-10^7 CFU/mL of tested bacteria. The Petri dishes were then incubated at optimum temperatures for 48 h and the area of the fully formed zones (ffz) observed was determined by measuring the zone diameter with a caliper. The zones with diameters ≤1.1 cm and zones formed on only one side of the discs were designated as partially formed zones (pfz) and their numbers were reported. The number of negative zone (nz) was also counted and reported.

4.2.8. Antimicrobial Activity of Zein Films on Turkey and Beef Burgers

Turkey and beef burgers, cut aseptically into 3 x 3 cm pieces weighing approximately 10 g, were coated with zein films (3 x 3 cm) produced by stirring and homogenization (Figure 4.1). Burger samples were then added to 0.1 % steril peptone water (90 ml), and homogenized in a stomacher (BagMixer® 400, Interscience, France) for 60 s at room temperature. Decimal dilutions were performed and microbial counts were determined. Plate count agar (PCA, Fluka, Spain) was used for total viable count and incubated at 30 °C for 48 h. Violet red bile agar (VRBA, Fluka, Spain) was used for total coliform count and incubated at 37 °C for 24 h. Experiments were performed in triplicate. The microbial counts were expressed as $\log_{10}$ colony forming units (CFU) per g of sample.

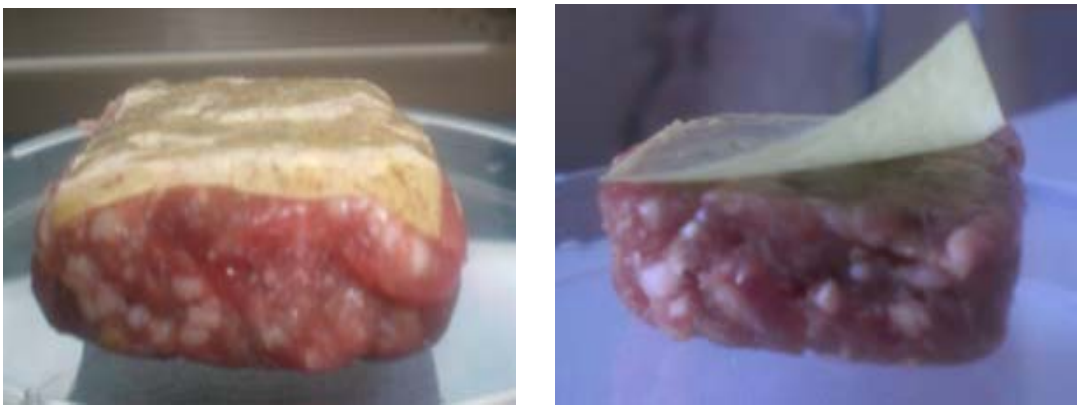


Figure 4.1. A photograph of beef burger with zein films placed on its surfaces

4.2.9. Determination of Oxidative Stability of Beef Burgers

For the determination of oxidative stability of cold stored beef burgers at days 0, 3 and 7, thiobarbituric acid-reactive substances (TBARS) assay was performed by slightly modifying as described by the Bekhit, Geesink, Ilian, Morton, and Bickerstaffe (2003). For analysis, 2.5 g sample was placed into a beaker containing 25 mL of 0.38 % TBA and 15 % TCA prepared in 0.25 N HCl solution. The sample was homogenized at 10000 rpm for 3 min and three 5 mL aliquots obtained from homogenate was heated for for 10 min in a boiling water bath to develop a pink color and then cooled in tap water.

The boiled samples were then clarified by centrifugation at 4500 *g* for 15 min and their absorbance was measured at 532 nm by using a Shimadzu (Model 2450, Japan) spectrophotometer. Average of three absorbance values was used to determine the oxidative stability of stored samples.

4.2.10. Color Analysis of Beef Burgers

The color of beef burgers was measured using a color machine vision system (ECS, Inc., USA). Fresh beef burgers were placed in a light box. An image of burgers was taken with a charged coupled device (CCD) video camera located inside the light box. The 24-bit color image was saved in the computer. The image was analyzed to generate a discrete spectrum of the colors present in the sample, and the average L*, a*, b* values of all the pixels representing the fresh beef sample. The L* value was reported as lightness value, whereas a*/b* value was reported as redness index. Images were taken at 0, 3 and 7 days of storage. The average of minimum two readings from burger surfaces was used for the calculation of L* and a*/b* values.

4.2.11. Sensory Analysis

Beef samples stored for 0, 3 and 7 days were analyzed by 10-trained panelists selected from graduate students and staffs at Department of Food Engineering, Izmir Institute of Technology. They were chosen among the ones who were experienced in judging fresh meat. They were trained at day 0 by presenting them reference samples of fresh beef burgers. The samples were coded with random 3-digit numbers and randomized before given to the panelists at each storage day. Duplicate samples with different codes (two coded samples) at room temperature from each treatment were arranged on different dinner plates and the plates were presented to all panelists in daylight laboratory conditions. They evaluated beef color (five-point scale: 1 = extremely discoloration; 5 = no discoloration), odor (five-point scale: 1 = extremely off-odor; 5 = extremely desirable), and overall acceptability (five-point scale: 1 = extremely unacceptable; 5 = extremely desirable) (Figure 4.2).

DIFFERENCE FROM CONTROL TEST Test No.1

Panelist No. ____ Name: _____ Age: _____ Date: _____
Type of sample: Beef Burger

Instructions

1. Receive the sample marked ‘‘Control’’ first.
2. Receive the test sample marked with the three digit code.
3. Assess the odor and color difference between the two samples using the scale below.
4. Compare the burger with the control.
5. Mark the scale to indicate the size of difference between them.

COLOR	**SAMPLE CODE**								
SCALE	Control	342	297	123	421	881	928	558	762
No discoloration (5)									
Slight discoloration (4)									
Moderate discoloration (3)									
Large discoloration (2)									
Extreme discoloration (1)									

ODOR	**SAMPLE CODE**								
SCALE	Control	342	297	123	421	881	928	558	762
Extremely desirable (5)									
Largely desirable (4)									
Moderately desirable (3)									
Slightly desirable (2)									
Extremely off-odor (1)									

OVERALL ACCEPTANCE	**SAMPLE CODE**								
SCALE	Control	342	297	123	421	881	928	558	762
Extremely desirable (5)									
Largely desirable (4)									
Moderately desirable (3)									
Slightly desirable (2)									
Extremely unacceptable (1)									

REMEMBER THAT A DUPLICATE CONTROL IS THE SAMPLE SOME OF THE TIME

COMMENTS:

Figure 4.2. Score sheet for difference from control test

4.2.12. Statistical Analysis

For the microbiological, oxidative stability, color and sensory studies, the effect of storage time and different packaging treatments were analyzed by ANOVA using PROC GLM procedure of Statistical Analysis System (SAS Institute, Cary, NC). Means with a significant difference ($P < 0.05$) were compared using the Duncan's multiple range test.

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1. Distribution of Ingredients in Zein Films

The partially purified lysozyme preparation used in this study was mainly hydrophilic. A major disadvantage of using partially purified lysozyme was the non-homogenous distribution of the hydrophilic enzyme preparation in hydrophobic zein films at high concentrations. Due to the problem, some hydrophilic, light yellow colored and semi transparent protein aggregates were formed in hydrophobic zein films (Figure 5.1). Thus, in this study hydrophilic partially purified lysozyme was incorporated into hydrophobic zein film forming solutions by homogenization or stirring method to obtain different degrees of distribution for lysozyme in the films and also to modify the film structure.

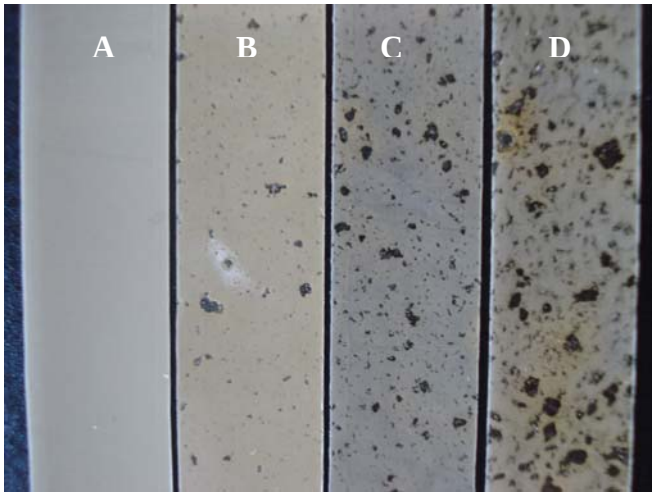


Figure 5.1. Photographs of different zein films produced by stirring method (Film contents: A, control film; B, 175 μg/cm^2 lysozyme; C, 350 μg/cm^2 lysozyme; D, 700 μg/cm^2 lysozyme. The background was black to make the light yellow semi-transparent protein aggregates visible)

As seen in Figure 5.2, homogenization method increased the number of protein aggregates in zein films significantly. However, most of the aggregates formed in these

films were small sized. As a result, zein films produced by homogenization method were more uniform compared to films produced by stirring method.

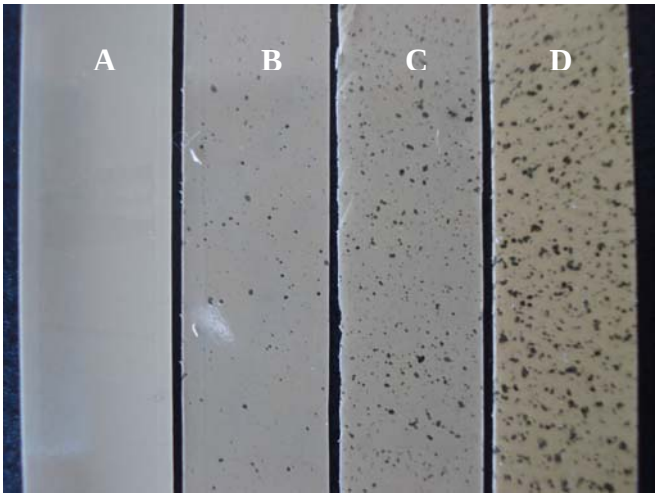


Figure 5.2. Photographs of different zein films produced by homogenization method (Film contents: A, control film; B, 175 μg/cm^2 lysozyme; C, 350 μg/cm^2 lysozyme; 700 μg/cm^2 lysozyme The background was black to make the light yellow semi-transparent protein aggregates visible.)

In zein films incorporated only with disodium EDTA, the chemical could not be solubilized completely, and it was observed as crystals distributed non-homogenously at different film locations. However, when disodium EDTA was incorporated in combination with lysozyme, these crystals were hardly observed since they were solubilized and distributed within the hydrophilic lysozyme aggregates formed in zein films. Additionally, as seen in Figures 5.3A and A^1, distribution of disodium EDTA in zein films produced by homogenization method was more homogenous than that in zein films produced by stirring method.

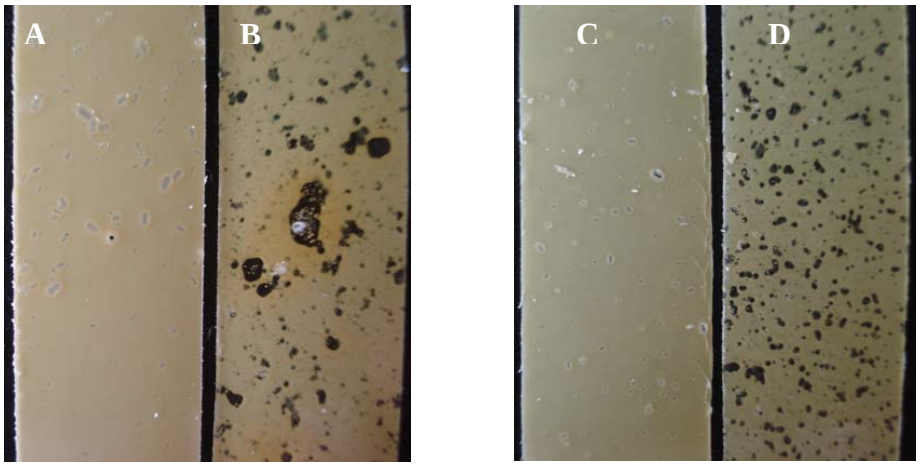


Figure 5.3. Photographs of different type of zein films (contents of films produced by stirring method: A; 300 μg/cm^2 disodium EDTA, B; 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA, contents of films produced by homogenization method: C; 300 μg/cm^2 disodium EDTA, D; 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA. The background was black to make the light yellow semi-transparent protein aggregates visible.)

Better distribution of lysozyme aggregates and disodium EDTA in zein films produced by homogenization method than the stirring method could also be observed from the surface photographs obtained by scanning electron microscope (SEM) (Figure 5.4).

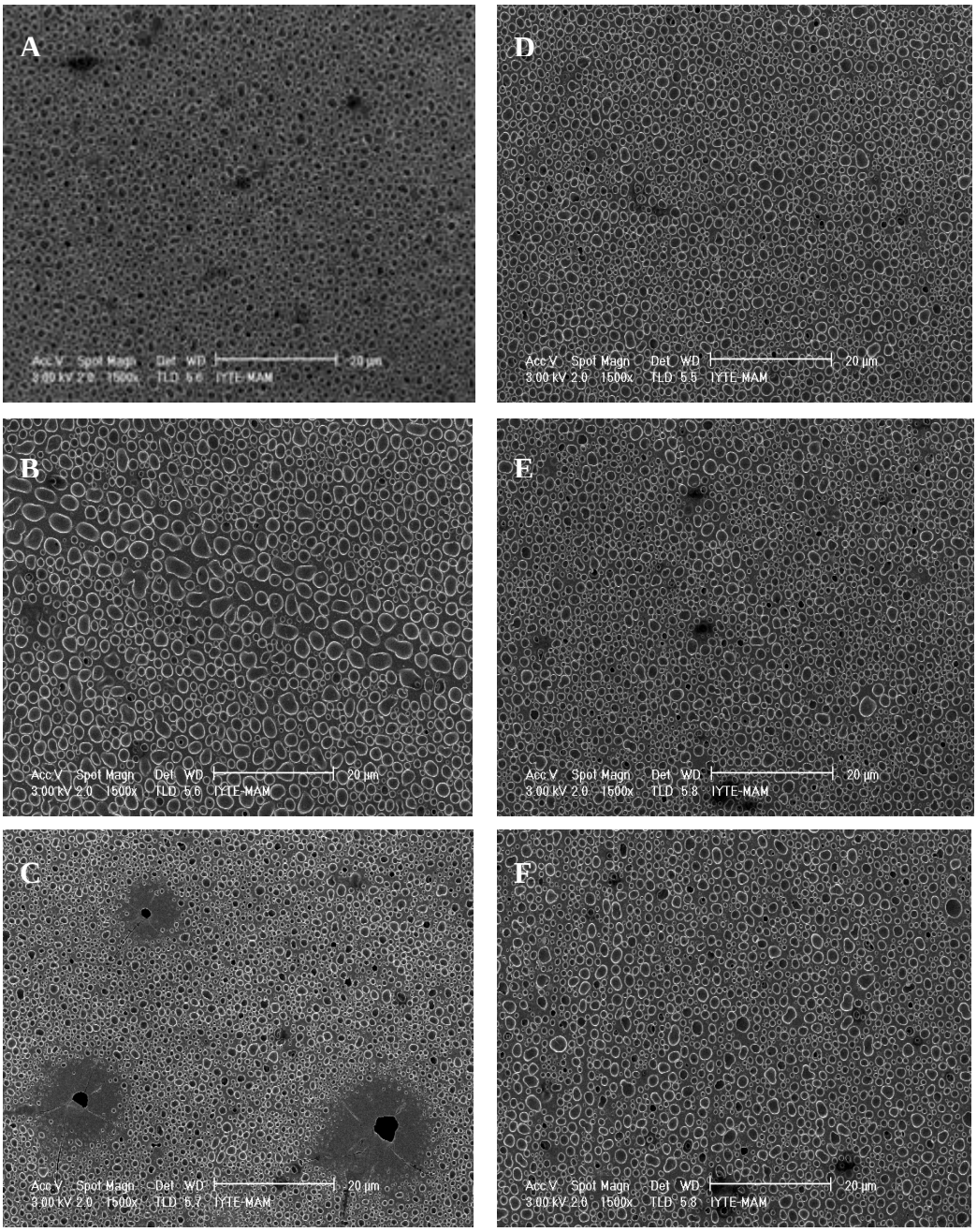


Figure 5.4. The surface photographs of different zein films obtained by scanning electron microscope (SEM) (bottom surface of films, magnification x1500; contents of films produced by stirring method: A; control film, B; 300 μg/cm^2 disodium EDTA, C; 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA, contents of films produced by homogenization method: D; control film, E; 300 μg/cm^2 disodium EDTA, F; 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

As seen in Figure 5.5, average thickness of zein films used in experiments changed between 150 and 200 µm. Incorporation of lysozyme and/or disodium EDTA and film making method did not significantly affect the thickness of zein films analyzed by measuring the thickness at 10 different points of the cross-sectional photographs.

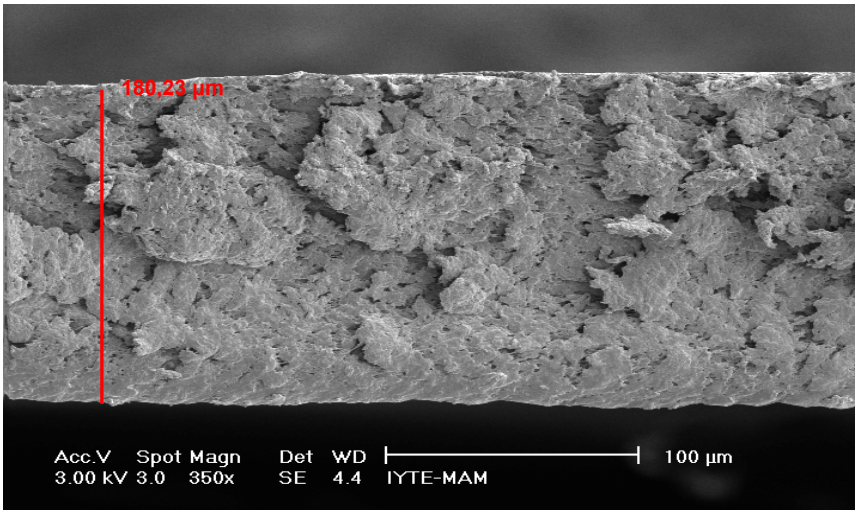


Figure 5.5. The cross-section photograph of zein film obtained by scanning electron microscope (SEM) (magnification x350; content of film produced by stirring method: 175 µg/cm^2 lysozyme)

5.2. Release of Lysozyme from Zein Films

The antimicrobial packaging is generally combined with refrigeration (Labuza and Breene 1989). Therefore, the release tests in this study were conducted at 4 °C (Figure 5.6). The rapid release of an antimicrobial agent from a film is undesirable since this allows regrowth of bacteria after initial reduction and lets the most risky food surface unprotected against microorganisms. The total lysozyme activity released from 8820 U/cm^2 lysozyme containing film produced by homogenization method was close to the activity of lysozyme incorporated into this film. On the other hand, in films, prepared by stirring method and incorporated with 2205 or 4410 U/cm^2 lysozyme, the total activities released were 59% and 33% greater than the incorporated activities in films, respectively. In films, prepared by homogenization method and incorporated with 2205 or 4410 U/cm^2 lysozyme, the total activities released were also 23% greater than the incorporated activities in films. This showed the activation of the lysozyme

incorporated into zein films. These results confirmed previous findings of Mecitoglu et al. (2006) that indicated the 32-215% activation of lysozyme during release tests from zein films produced by stirring method. It is thought that the lysozyme activation is related with conformational changes caused by ethanol used both in partial purification of enzyme and preparation of zein films. Release test periods to achieve total (maximum) lysoyzme activity were 60 or 300 min for films obtained by the stirring method, whereas films obtained by the homogenization method released maximum activity by 1400 min (Table 5.1). This result clearly showed the highest affinity of lysozyme to films obtained by the homogenization method.

Table 5.1. Some kinetic parameters related to lysozyme released from zein films at 4°C

Incorporated lysozyme activity (U/cm^2)	Film making method	Total lysozyme activity released (U/cm^2)	Recovery of lysozyme activity (%)[c]	Bound lysozyme activity at film surface (U/cm^2)[b]
2205 (175)[a]	Stirring	3498±77 (300)[b]	159	4.8
4410 (350)	Stirring	5848±362 (300)	133	6.0
8820 (700)	Stirring	8933±463 (60)	101	15.8
2205 (175)[a]	Homogenization	2702±78 (1400)	123	7.5
4410 (350)	Homogenization	5441±211 (1400)	123	6.5
8820 (700)	Homogenization	9546±305 (1400)	108	15.0

[a] Lysozyme incorporated into films as $\mu g/cm^2$
[b] Release test periods (min) to achieve total activity released
[c] (Total lysozyme activity released/incorporated lysozyme activity) x 100

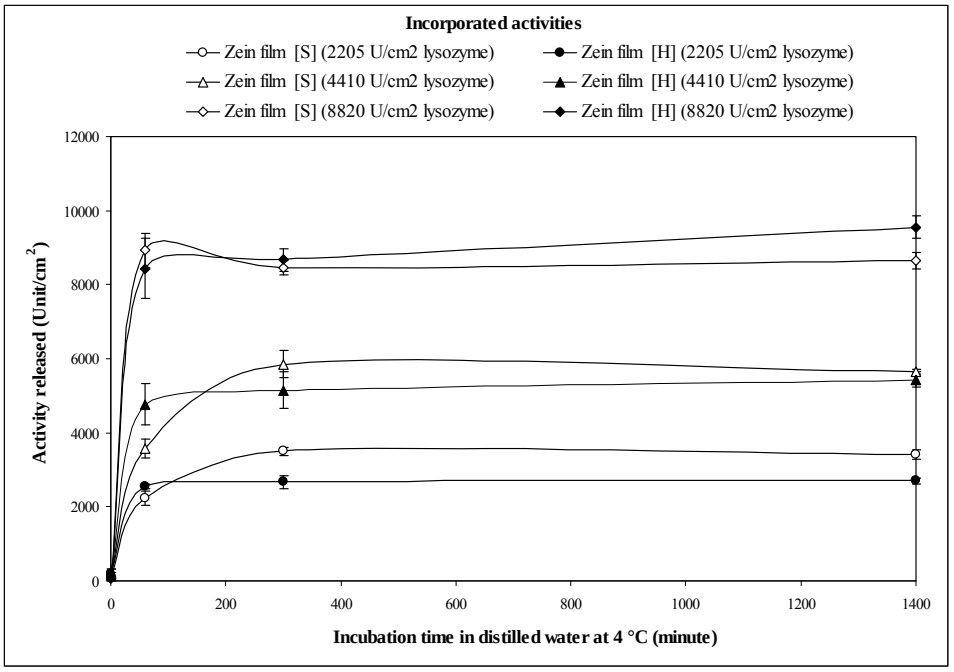


Figure 5.6. Release of lysozyme from different zein films, produced by stirring method [S] or homogenization method [H], used in distilled water at 4 °C

At the highest lysozyme concentration which also used in the food applications, the films also contained disodium EDTA. The release test results of these films were given in Figure 5.7. The total lysozyme activities released from these zein films incorporated with 11025 U/cm^2 lysozyme were almost the same for both stirring and homogenization methods (Table 5.2). Thus, no significant effects of film making method on recovery of lysozyme activity were determined at the highest lysozyme concentration. However, since large protein aggregates existed in films prepared by stirring method, greater interactions of the aggregates with water might caused a significantly decrease in release test periods to achieve maximum activity. Also, no enzyme activation was observed in films at this high enzyme concentration. Although the lysozyme is highly hydrophilic, this enzyme is known with its emulsifying activity. Thus, it seems that when used at high concentrations the enzyme was trapped in films due to its increased affinity to hydrophobic films.

Table 5.2. Some kinetic parameters related to lysozyme released from zein films at 4 °C

Incorporated activities or concentrations		Film making method	Total lysozyme activity released (U/cm^2)	Recovery of lysozyme activity (%)[c]	Bound lysozyme activity at film surface (U/cm^2)
Lysozyme (U/cm^2)	Na_2EDTA (μg/cm^2)				
11025 (700)[a]	300	Stirring	7723±293 (60)[b]	70	14.5
11025 (700)	300	Homogenization	7550±547 (1400)	68	20.3

[a] Lysozyme incorporated into films as μg/cm^2
[b] Release test periods (min) to achieve total activity released
[c] (Total lysozyme activity released/incorporated lysozyme activity) x 100

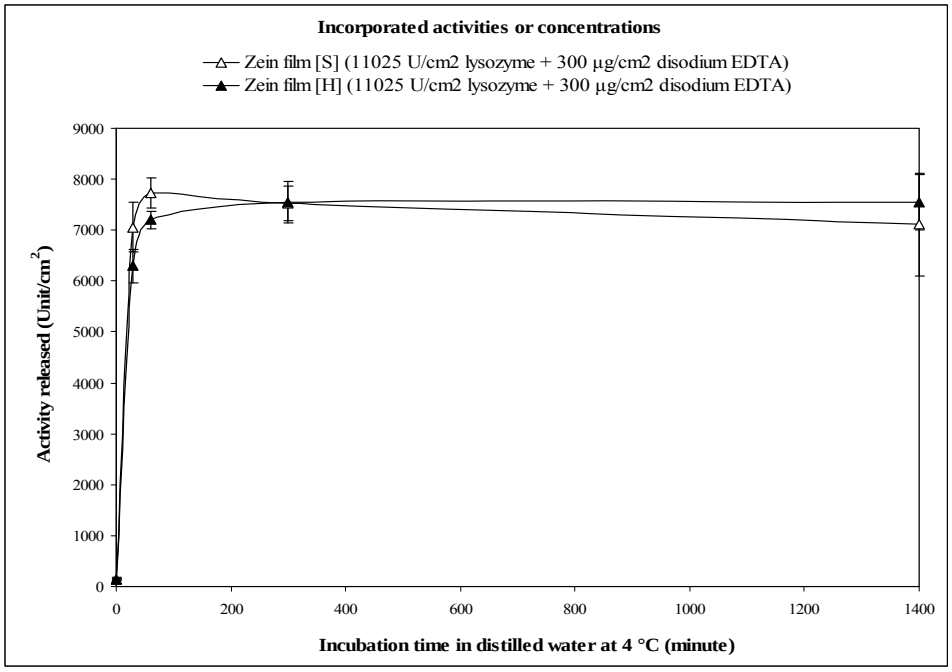


Figure 5.7. Release of lysozyme from different zein films, produced by stirring method [S] or homogenization method [H], used in distilled water at 4 °C

5.3. Bound Lysozyme Activity Retained at Film Surfaces

In antimicrobial packaging, the retention of some of the lysozyme activity in films may be beneficial to maintain the aseptic nature of films and control of microbial growth at food surfaces. Thus, the level of bound lysozyme activity retained at film surfaces after 1400 min release tests were also determined (Figure 5.8 and 5.9). As seen in Tables 5.1, the lysozyme activity retained at films produced by homogenization method incorporated with 2205 U/cm^2 was 58% higher than that retained at the zein films containing the same amount of lysozyme but produced by the stirring method. However, bound lysozyme activities at films produced by the homogenization method and incorporated with 4410 or 8820 U/cm^2 were similar with bound lysozyme activities retained at the zein films containing the same amounts of lysozyme but produced by the stirring method. Moreover, bound lysozyme activity retained at zein films containing 11025 U/cm^2 lysozyme increased significantly by application of homogenization method instead of stirring method. Thus, it seems that homogenization contribute more to the bound lysoyme activity of zein films at low and high lysozyme concentrations.

This may be explained by the fact that at these concentrations emulsifying property and resulting affinity of lysozyme increased due to appropriate enzyme/zein ratios.

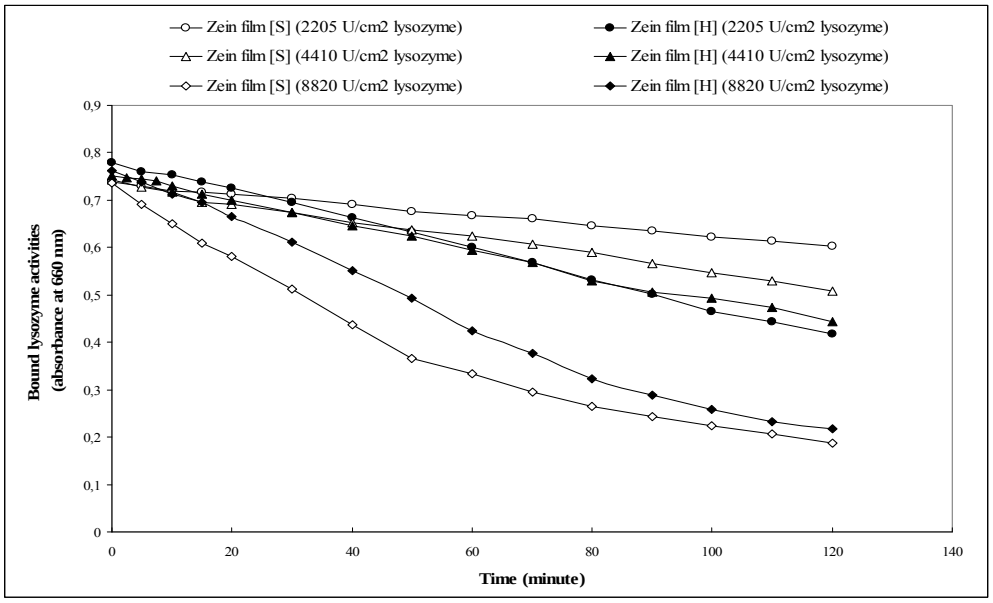


Figure 5.8. Bound lysozyme activities retained in zein film, produced by stirring method [S] or homogenization method [H], determined in *M. lysodeikticus* solutions after 1400 min release test conducted in distilled water at 4 °C

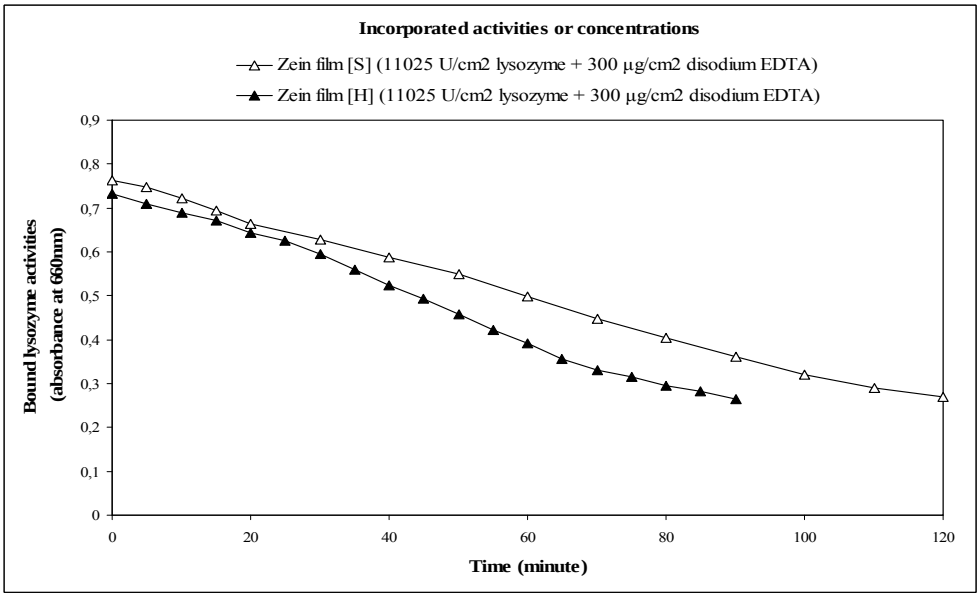


Figure 5.9. Bound lysozyme activities retained at zein film, produced by stirring method [S] or homogenization method [H], determined in *M. lysodeikticus* solutions after 1400 min release test conducted in distilled water at 4 °C

5.4. *In Vitro* Film Antimicrobial Activity

The results of the antimicrobial tests of partially purified lysozyme incorporated zein films produced by stirring or homogenization methods against different bacteria were given in Tables 5.3 and 5.4. The bacteria selected are commonly associated with meat products. For films incorporated only with the lysozyme, the greatest zone of inhibition was observed against *B. amyloliquefaciens* which seems very sensitive to lysozyme (Figure 5.10). The films incorporated only with lysoyme showed less antimicrobial effect on *L. innocua*. For *B. amyloliquefaciens* the antimicrobial effect of films increased slightly as the lysozyme concentration in the films increased. However, for *L. innocua* an interesting reduction was observed in antimicrobial activity at the highest concentration of lysozyme. For the other bacteria, including *E. coli* and *P. fluorescens* which were tested by films incorporated both with lysozyme and disodium EDTA, the zone areas observed were smaller that that observed on *B. amyloliquefaciens.* The increase of lysozyme concentration from 175 to 350 μg/cm^2 caused an increase of antimicrobial activity over these G(-) bacteria. However, as observed in results of *L. innocua,* the antimicrobial activity of films on *E. coli* and *P. fluorescens* reduced at the highest concentration of lysozyme (700 μg/cm^2). During release tests conducted in water we have observed increased soluble enzyme activity of films incorporated with 700 μg/cm^2 lysozyme. Thus, an increase in antimicrobial activity by the increased lysozyme concentration was expected. However, the antimicrobial tests were conducted on solid agar surfaces and it seems that too much enzyme and other egg yolk impurities (enzyme is partially purified) released from the films accumulated at the agar surface aggregated and this reduced lysozyme diffusion on agar plates.

Both types of films obtained by incorporation of 175 to 700 μg/cm^2 lysozyme with stirring or homogenization method showed good antimicrobial activity on *L. innocua* and *B. amyloliquefaciens*. The films also showed sufficient antimicrobial activity on *E. coli and P. fluorescens* when lysozyme was supported by the incorporation of 200 μg/cm^2 disodium EDTA. In general, the zein films incorporated with lysozyme by the stirring method gave larger zones than those films incorporated with lysozyme by the homogenization method. This occurred due to the higher solubility of lysozyme in zein films obtained by the stirring method than the

homogenization method. However, the lysozyme and disodium EDTA incorporated into zein films produced by the homogenization method caused better distribution of antimicrobial activity than the stirring method. In other words, there were less death points in zein films prepared with homogenization method due to more homogenous distribution of lysozyme in these films. As seen in Table 5.3, this was also clearly identified by the increased number of fully formed zones and reduced number of partially formed and no zone formation in films obtained by homogenization method

Table 5.3. Antimicrobial effects of partially purified lysozyme and/or disodium EDTA incorporated zein films produced by stirring or homogenization method against selected bacteria

Incorporated concentrations		**Number of fully formed zone (ffz), partially formed zone (pfz) or negative zone (nz)**		**Average area of fully formed zones (cm^2)**	
Lysozyme[a] (U/cm^2)	**Disodium EDTA ($\mu g/cm^2$)**	**Stirring[b]**	**Homogenization**	**Stirring**	**Homogenization**
Escherichia coli					
-	-	12nz	12nz	0	0
-	200	4ffz/8nz	6ffz/6nz	1.46±0.41	1.28±0.13
2205 (175)[c]	200	8ffz/4pfz	8ffz/3pfz/1nz	2.40±0.70	1.38±0.13
4410 (350)	200	8ffz/2pfz/2nz	9ffz/2pfz/1nz	2.81±1.13	1.75±0.94
8820 (700)	200	11ffz/1pfz	12ffz	2.66±1.83	1.57±0.40
Pseudomanas fluorescens					
-	-	12nz	12nz	0	0
-	200	7ffz/5nz	12ffz	2.89±1.27	2.43±1.19
2205 (175)[c]	200	8ffz/3pfz/1nz	12ffz	2.62±1.07	2.12±0.53
4410 (350)	200	9ffz/2pfz/1nz	12ffz	3.09±1.56	2.64±1.10
8820 (700)	200	11ffz	12ffz	2.25±1.88	2.36±1.68
Listeria innocua					
-	-	12nz	12nz	0	0
2205 (175)[c]	-	11ffz/1pfz	12ffz	2.26±0.72	1.49±0.21
4410 (350)	-	10ffz/1pfz/1nz	10ffz/2pfz	2.41±1.18	1.62±0.72
8820 (700)	-	10ffz/2pfz	11ffz/1pfz	1.93±0.87	1.25±0.69

(cont. on next page)

Table 5.3 (cont.) Antimicrobial effects of partially purified lysozyme and/or disodium EDTA incorporated zein films produced by stirring or homogenization method against selected bacteria

Bacillus amyloliquefaciens					
-		12nz	12nz	0	0
2205 (175)[c]	-	12ffz	12ffz	5.15±0.72	5.81±0.59
4410 (350)	-	12ffz	12ffz	6.84±1.85	6.55±0.84
8820 (700)	-	12ffz	12ffz	7.33±0.83	7.42±0.69

[a] Incorporated lysozyme activity was 12625 U/mg
[b] Film making methods
[c] Lysozyme incorporated into films as μg/cm^2

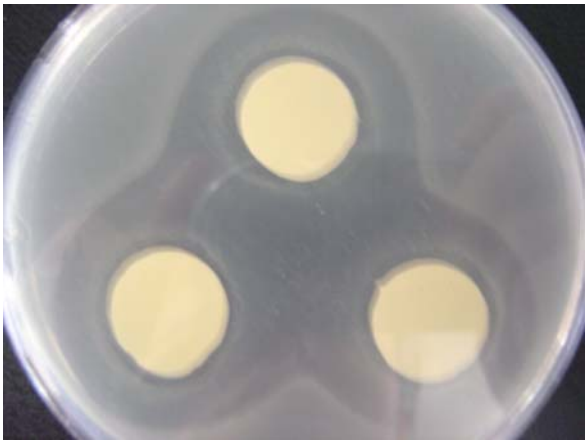


Figure 5.10. Antimicrobial effect of lysoyme incorporated zein films produced by homogenization method on *B. amyloliqufaciens* (film content: 175 μg/cm^2 lysozyme)

Antimicrobial effects of 700 μg/cm^2 lysozyme incorporated or 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA incorporated zein films produced by stirring or homogenization were also tested against selected pathogenic bacteria (Table 5.4). The zein films incorporating lysozyme by stirring method again gave larger zones on pathogenic bacteria including *Salmonella typhimurium* and *E. coli* O157:H7 than those films incorporating lysozyme by homogenization method. However, the lysozyme and disodium EDTA incorporated into zein films produced by homogenization method had better distribution of antimicrobial activity than stirring method (consider zone numbers and types given in Table 5.4. and Figures 5.11 and 5.12). For example, it is worth to

note that for *Salmonella typhimurium,* the tested films produced by the homogenization method gave two times greater number of fully formed zones than that of films produced by the stirring method. On the other hand, the lysozyme showed no antimicrobial activity on *S. aureus*. This result shows the resistance of this bacterium against lysozyme at the studies concentration range.

Table 5.4. Antimicrobial effects of partially purified lysozyme and/or disodium EDTA incorporated zein films produced by stirring or homogenization method

Incorporated concentrations		**Number of fully formed zone (ffz), partially formed zone (pfz) or negative zone (nz)**		**Average area of fully formed zones (cm^2)**	
Lysozyme[a] (U/cm^2)	**Disodium EDTA ($\mu g/cm^2$)**	**Stirring[b]**	**Homogenization**	**Stirring**	**Homogenization**
***Escherichia coli* O157:H7**					
-	-	12nz	12nz	0	0
-	300	9ffz/3nz	9ffz/2pfzz/1nz	3.04±0.83	2.28±1.30
11025 (700)[c]	300	9ffz/1pfz/2nz	10ffz/1pfz/1nz	3.61±1.49	3.04±1.16
Salmonella typhimurium					
-	-	12nz	12nz	0	0
-	300	4ffz/1pfz/7nz	8ffz/4nz	3.74±1.50	2.58±1.41
11025 (700)	300	6ffz/3pfz/3nz	12ffz	3.71±1.64	3.27±1.05
Staphylococcus aureus					
-	-	12nz	12nz	0	0
11025 (700)	-	12nz	12nz	0	0

[a] Incorporated lysozyme activity was 15750 U/mg
[b] Film making methods
[c] Lysozyme incorporated into films as $\mu g/cm^2$

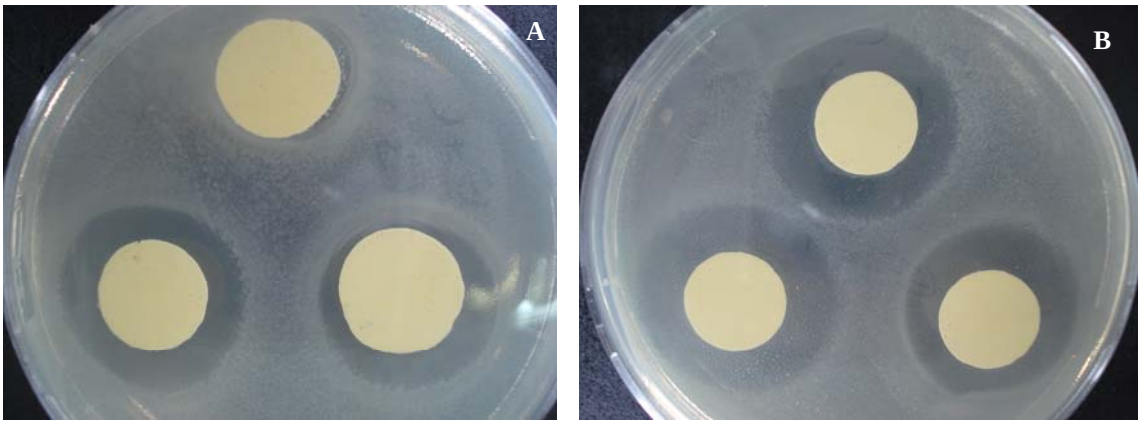


Figure 5.11. Antimicrobial effect of lysozyme incorporated zein films produced by stirring method (A) or homogenization method (B) on *E. coli* O157:H7 (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

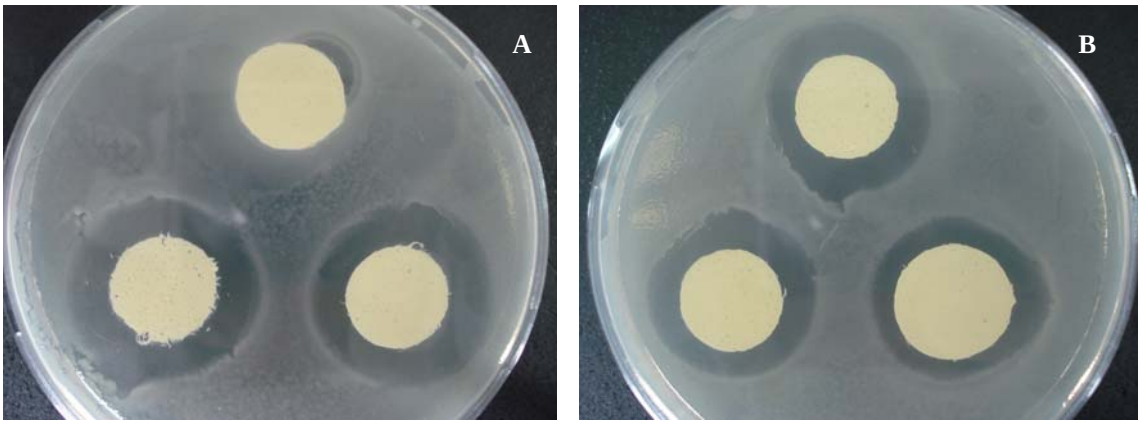


Figure 5.12. Antimicrobial effect of zein films produced by stirring method (A) or homogenization method (B) on *Salmonella typhimurium* (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

5.5. Effect of Zein Films on Total Viable Count (TVC) of Cold Stored Turkey Burgers

The effect of active packaging by zein films incorporated with 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA on TVCs of cold stored turkey burgers was investigated (Figures 5.14 and 5.15). The initial TVCs of turkey burgers packed without any zein film, with control zein films and with zein films incorporated with disodium

EDTA or lysozyme-disodium EDTA were almost the same (Table 5.6). At the end of 3 days cold storage, the only reduction in initial TVC (0.34 decimal) occurred in those burgers packed with lysozyme-disodium EDTA incorporated zein films produced by the stirring method. Between the 5th and 7th days of cold storage, a significant increase in TVCs (0.91 to 1.0 decimal) occurred in burgers packed with disodium EDTA and lysozyme-disodium EDTA incorporated zein films prepared by stirring, whereas there were no significant difference in burgers packed with disodium EDTA and lysozyme-disodium EDTA incorporated zein films prepared by homogenization. At the 5th day of cold storage, the TVC of burgers packed by using zein films produced by homogenization and incorporated with lysozyme-disodium EDTA reached to 6 $\log_{10}$ CFU/g, considered as a limit in the shelf-life determination studies. However, the TVCs of all other samples exceeded this limit. Thus, it seems that lysozyme released more rapidly from films produced by stirring method to burger surface and it became effective on initial TVC. On the other hand, it is likely that disodium EDTA and lysozyme-disodium EDTA released more slowly from films produced by the homogenization method and this maintained antimicrobial activity at burger surfaces for longer time periods. At the end of 7 days cold storage, all samples exceeded 6 $\log_{10}$ CFU/g. However, at the 3 th, 5 th and 7 th days of cold storage, turkey burgers packed by zein films incorporated with disodium EDTA and disodum EDTA-lysozyme had lower microbial load than those packed by without any zein film and the use of control film.

Table 5.6. Total viable counts of cold stored turkey burgers packed with different zein films (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

Type of film	**Total viable count ($\log_{10}$ CFU/g)**			
	Storage time at 4 ^{o}C (days)			
	0	**3**	**5**	**7**
No zein film	5.76±0.02 ^{a,D}	6.33±0.12 ^{a,C}	7.34±0.43 ^{a,B}	8.83±0.20 ^{a,A}
Zein film (Stirring)[a]	5.74±0.02 ^{ab,D}	6.33±0.03 ^{a,C}	7.44±0.24 ^{a,B}	8.72±0.24 ^{a,A}

(cont. on next page)

Table 5.6.(cont.) Total viable counts of cold stored turkey burgers packed with different zein films (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

Zein film incorporated with disodium EDTA (Stirring)				
	5.61±0.05 ^{d,C}	5.76±0.13 ^{b,C}	6.67±0.51 ^{b,B}	7.90±0.16 ^{b,A}
Zein film incorporated with lysozyme and disodium EDTA (Stirring)				
	5.66±0.03 ^{c,C}	5.32±0.08 ^{c,C}	6.32±0.25 ^{bc,B}	7.77±0.37 ^{b,A}
Zein film (Homogenization)				
	5.74±0.02 ^{ab,D}	6.33±0.09 ^{a,C}	7.66±0.10 ^{a,B}	8.68±0.22 ^{a,A}
Zein film incorporated with disodium EDTA (Homogenization)				
	5.71±0.02 ^{b,C}	6.25±0.13 ^{a,B}	6.30±0.49 ^{bc,B}	7.99±0.15 ^{b,A}
Zein film incorporated with lysozyme and disodium EDTA (Homogenization)				
	5.72±0.02 ^{ab,B}	5.79±0.46 ^{b,B}	6.01±0.09 ^{c,B}	7.71±0.54 ^{b,A}

^{a} : Film making method
$^{a-d}$: Means having different letters within each treatment denote significant difference at $p<0.05$.
$^{A-D}$: Means having different letters within each storage time denote significant difference at $p<0.05$.
Data are mean values ± S.D. (n=3)

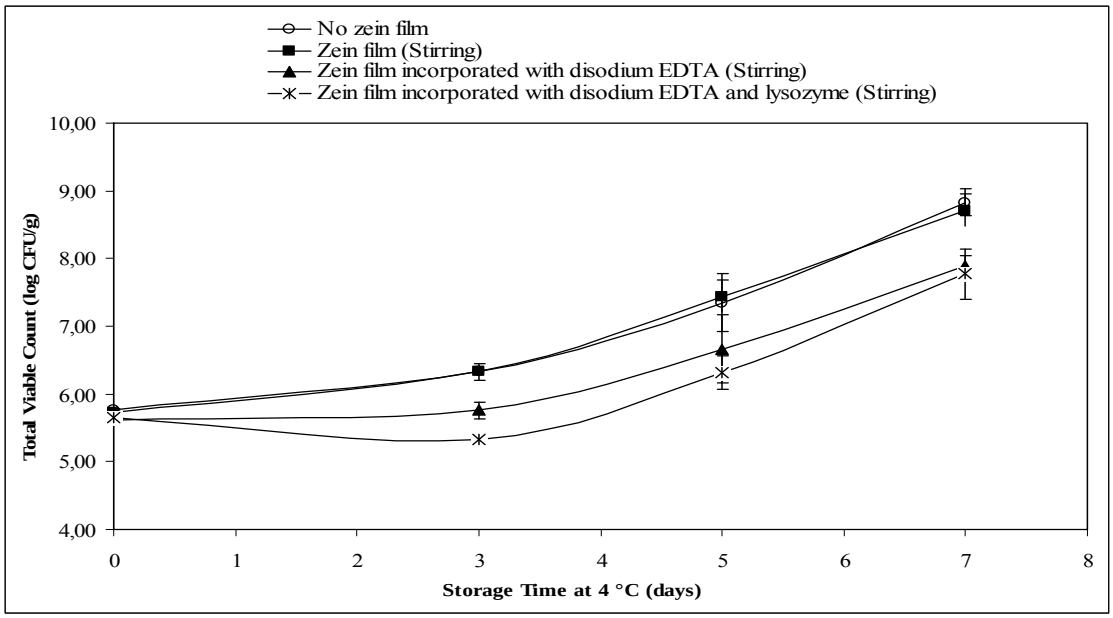


Figure 5.14. Total viable counts during cold storage of turkey burgers packed with different zein films produced by stirring method (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

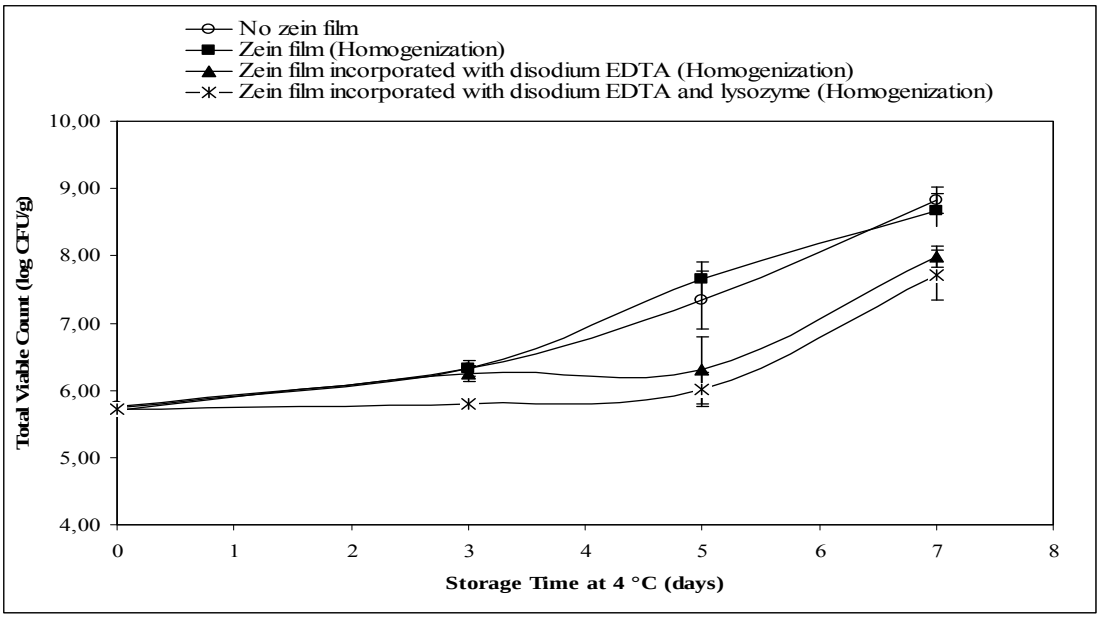


Figure 5.15. Total viable counts during cold storage of turkey burgers packed with different zein films produced by homogenization method (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA

5.6. Effect of Zein Films on Total Coliform Count (TCC) of Cold Stored Turkey Burgers

The effect of active packaging by zein films incorporated with 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA on TCCs of cold stored turkey burgers was also investigated (Figure 5.16 and 5.17). The initial TCC of turkey burgers packed without using any zein film, with standard zein films and with zein films incorporated with disodium EDTA or lysozyme-disodium EDTA were almost same (Table 5.7). At the end of 3 days of cold storage, only a slight increase in initial TCC was observed (0.24 decimal) for those burgers packed by lysozyme-disodium EDTA incorporated zein films produced by the stirring method. The TCC of burgers packed by zein films incorporated with lysozyme and disodium EDTA and produced by the homogenization method increased more significantly at the end of 3 days cold storage. However, between the 3th and 7th days of cold storage, only 0.29 decimal increases in TCCs occurred in burgers packed with lysozyme-disodium EDTA incorporated zein films produced by the homogenization method. In the same storage period, a more significant increase in TCCs (1.12 decimal) occurred in burgers packed with lysozyme-disodium

EDTA incorporated zein films prepared with the stirring method. At the 7 th day of cold storage, turkey burgers packed by disodum EDTA-lysozyme incorporated zein films produced by homogenization had the lowest microbial load. These results showed that the use of lysozyme-disodium EDTA combination in zein films produced by homogenization method is more effective than use of the indicated antimicrobial agents in zein films produced by the stirring method.

Table 5.7. Total coliform counts of cold stored burgers packed with different zein films (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA

Type of film	**Total coliform count (log_{10} CFU/g)**			
	Storage time at 4 ^{o}C (days)			
	0	**3**	**5**	**7**
No zein film	3.97±0.03 ^{ab,D}	4.37±0.08 ^{ab,C}	5.36±0.04 ^{a,B}	5.85±0.13 ^{a,A}
Zein film (Stirring)	3.97±0.03 ^{ab,B}	4.27±0.36 ^{ab,B}	5.18±0.09 ^{abc,A}	5.54±0.19 ^{abc,A}
Zein film incorporated with disodium EDTA (Stirring)	4.07±0.09 ^{a,C}	4.21±0.02 ^{ab,C}	4.92±0.16 ^{c,B}	5.45±0.37 ^{abc,A}
Zein film incorporated with lysozyme and disodium EDTA (Stirring)	3.90±0.22 ^{ab,B}	4.14±0.36 ^{b,B}	4.32±0.29 ^{d,B}	5.26±0.09 ^{c,A}
Zein film (Homogenization)	4.03±0.07 ^{ab,D}	4.62±0.14 ^{a,C}	5.23±0.02 ^{ab,B}	5.80±0.21 ^{ab,A}
Zein film incorporated with disodium EDTA (Homogenization)	3.89±0.02 ^{ab,D}	4.43±0.31 ^{ab,C}	5.05±0.08 ^{bc,B}	5.40±0.17 ^{bc,A}
Zein film incorporated with lysozyme and disodium EDTA (Homogenization)	3.82±0.14 ^{b,B}	4.46±0.10 ^{ab,A}	4.49±0.23 ^{d,A}	4.75±0.25 ^{d,A}

^{a} : Film making method
$^{a-d}$: Means having different letters within each treatment denote significant difference at $p<0.05$.
$^{A-D}$:Means having different letters within each storage time denote significant difference at $p<0.05$.
Data are mean values ± S.D. (n=3)

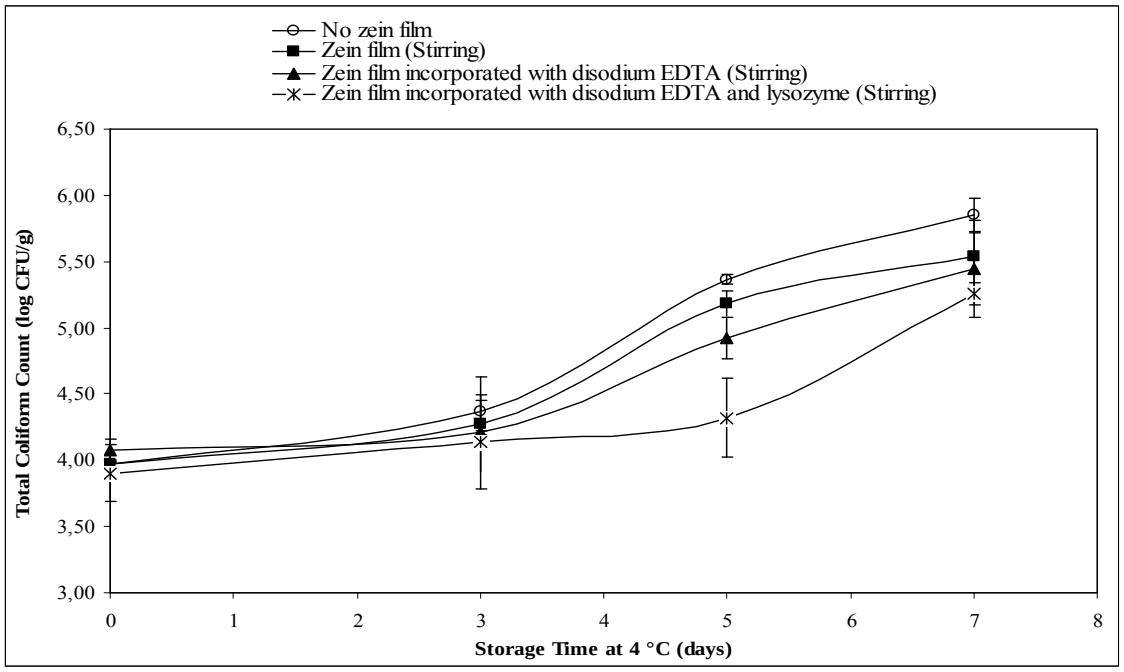


Figure 5.16. Total coliform counts during cold storage of turkey burgers packed with different zein films produced by stirring method (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

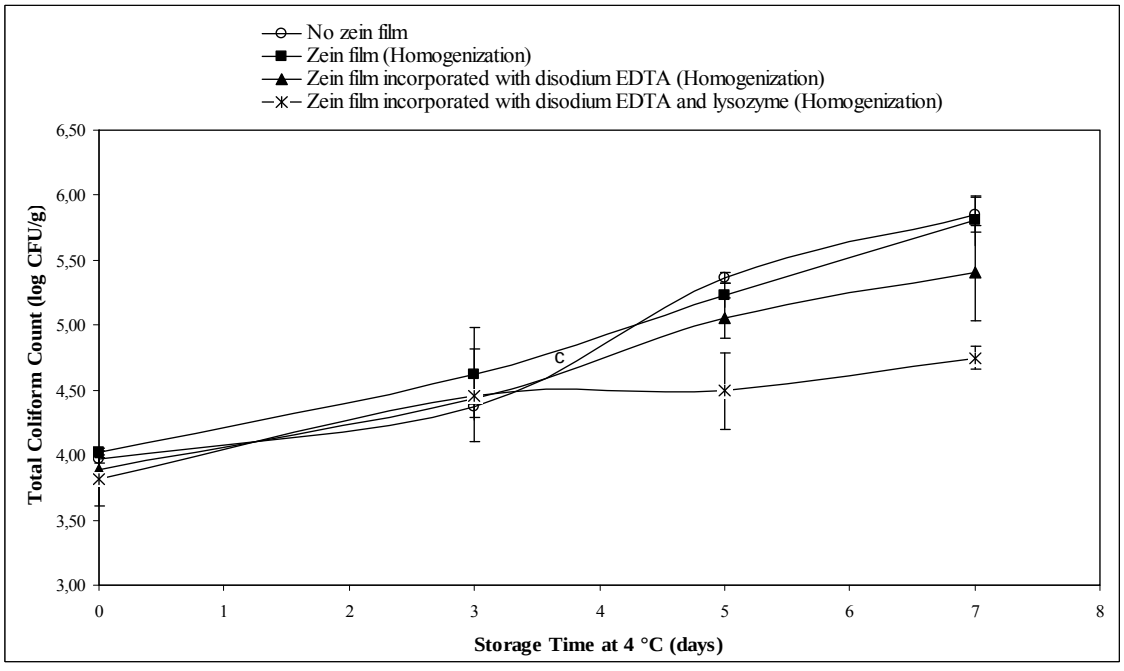


Figure 5.17. Total coliform counts during cold storage of turkey burgers packed with different zein films produced by homogenization method (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

5.7. Effect of Zein Films on Total Viable Count (TVC) of Cold Stored Beef Burgers

The effect of active packaging by zein films incorporated with disodium EDTA and lysozyme on TVCs of cold stored beef burgers was also investigated (Figure 5.18 and 5.19). The beef sample used in this study had a high initial TVC (Table 5.8). The initial TVCs of beef burgers packed without zein films, with control films and with zein films incorporated with disodium EDTA or lysozyme-disodium EDTA are almost same. At the end of 3 days cold storage, a reduction in initial TVC (0.34 decimal) was observed only for burgers packed by lysozyme-disodium EDTA incorporated zein films produced by the stirring method. At the 5^{th} day of cold storage, the TVCs of burgers packed by using zein films incorporated with lysozyme-disodium EDTA and produced by homogenization or stirring methods was almost 6 $\log_{10}$ CFU/g, considered as a limit in the shelf-life determination studies. However, the TVCs of all other samples exceeded this limit considerably. Between 5^{th} and 7^{th} days of cold storage, a significant increase in TVCs (1.29 and 1.09 decimal) occurred in burgers packed with disodium EDTA, and lysozyme-disodium EDTA incorporated zein films prepared with the stirring method. But, there were only slight increases in TVCs (0.71 and 0.76) of burgers packed with disodium EDTA, and lysozyme-disodium EDTA incorporated zein films prepared with the homogenization method. Since the initial TVCs were quite high, the difference between stirring and homogenization methods was not clearly understood. However, at the 7^{th} days of cold storage, beef burgers packed by with zein films incorporated with disodium EDTA-lysozyme had lower microbial load than all of the other packed burgers.

Table 5.8. Total viable counts of cold stored beef burgers packed with different zein films (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA

Type of film	Total viable count (log_{10} CFU/g)			
	Storage time at 4 ^oC (days)			
	0	**3**	**5**	**7**
No zein film	5.20±0.01 ^{ab,D}	5.38±0.02 ^{bcd,C}	7.84±0.04 ^{a,A}	7.71±0.08 ^{b,B}
Zein film (Stirring)[a]	5.46±0.43 ^{a,B}	5.63±0.21 ^{ab,B}	7.99±0.19 ^{a,A}	7.96±0.26 ^{ab,A}
Zein film incorporated with disodium EDTA (Stirring)	5.12±0.08 ^{ab,C}	5.25±0.05 ^{cd,C}	7.02±0.39 ^{b,B}	8.31±0.13 ^{a,A}
Zein film incorporated with lysozyme and disodium EDTA (Stirring)	5.28±0.35 ^{ab,C}	4.94±0.22 ^{e,C}	6.01±0.09 ^{c,B}	7.10±0.10 ^{c,A}
Zein film (Homogenization)	5.39±0.17 ^{ab,C}	5.82±0.24 ^{a,B}	8.02±0.30 ^{a,A}	8.15±0.03 ^{a,A}
Zein film incorporated with disodium EDTA (Homogenization)	4.99±0.09 ^{b,C}	5.44±0.08 ^{bc,C}	6.94±0.49 ^{b,B}	7.65±0.42 ^{b,A}
Zein film incorporated with lysozyme and disodium EDTA (Homogenization)	5.09±0.02 ^{ab,C}	5.10±0.16 ^{ed,C}	6.14±0.14 ^{c,B}	6.90±0.34 ^{c,A}

[a] : Film making method
[a-e] : Means having different letters within each treatment denote significant difference at $p<0.05$
[A-D]: Means having different letters within each storage time denote significant difference at $p<0.05$
Data are mean values ± S.D (n=3)

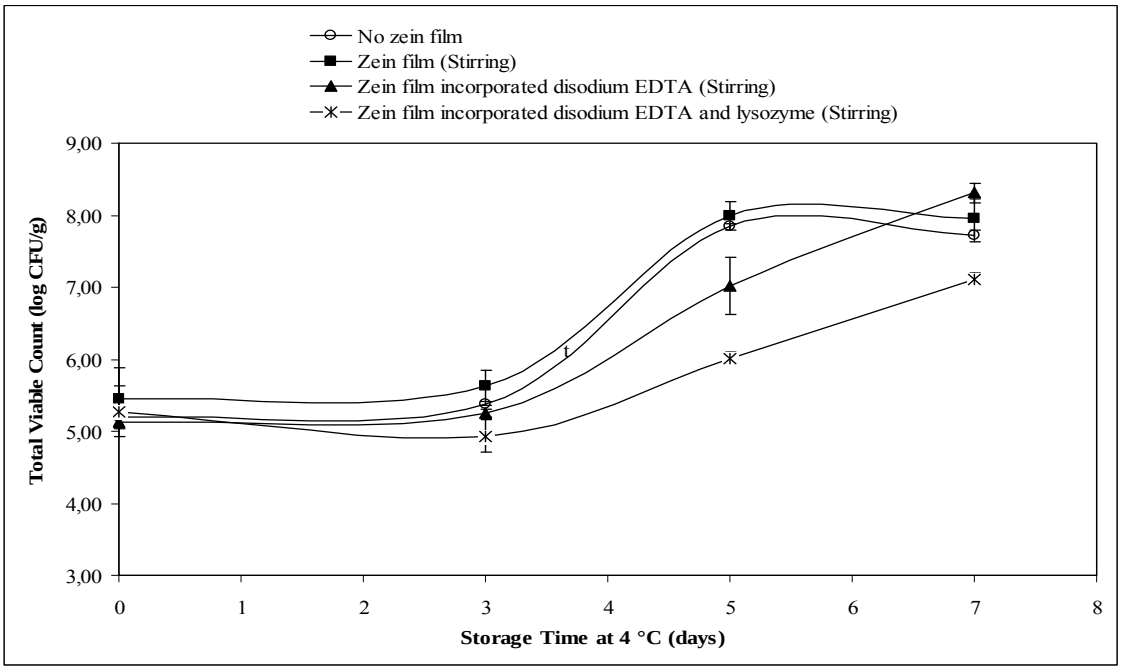


Figure 5.18. Total viable counts during cold storage of beef burgers packed with different zein films produced by stirring method (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

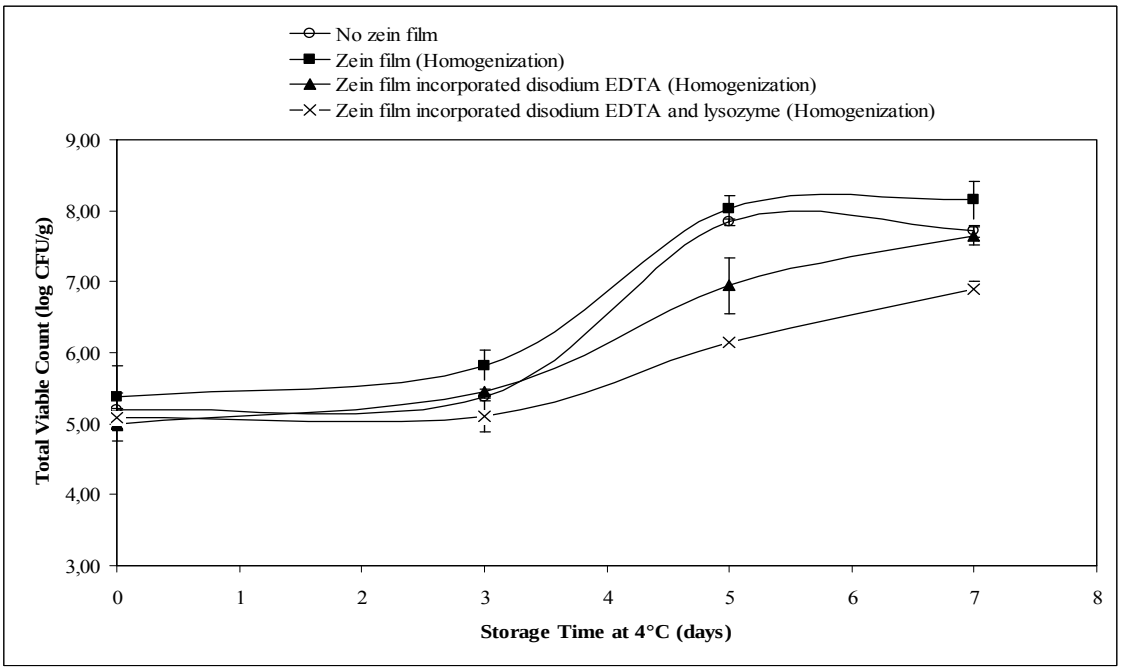


Figure 5.19. Total viable counts during cold storage of beef burgers packed with different zein films produced by homogenization method (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

5.8. Effect of Zein Films on Total Coliform Count (TCC) of Cold Stored Beef Burgers

The effect of active packaging by zein films on TCC of cold stored beef burgers was also investigated (Figure 5.20 and 5.21). The incorporation of disodium EDTA and lysozyme did not change the initial microbial load of beef samples considerably (Table 5.9). All burger samples packed without zein film or with different zein films had the same initial TCCs. However, the beneficial effects of lysozyme incorporated into zein film prepared with stirring or homogenization methods on microbial load were observed at the end of 3rd day of cold storage when compared to other treatments ($p<0.05$). At the end of 5 days cold storage, the burgers packed by using lysozyme-disodium EDTA incorporated zein films formed by stirring method had the lowest TCC. Between the 5th and 7th days of cold storage, 1.11 decimal increase in TCCs occurred in burgers packed with lysozyme-disodium EDTA incorporated zein films produced by stirring, whereas in the same time period, TCCs of burgers packed by using lysozyme-disodium EDTA incorporated zein film produced by homogenization method did not change significantly. These results showed that the use of lysozyme-disodium EDTA combination in zein films produced by homogenization method is more effective to suppress TCCs in packed burgers than beef burgers packed with other type of zein films.

Table 5.9. Total coliform counts of cold stored beef burgers packed with different zein films (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

Type of film	Total coliform count (log_{10} CFU/g)			
	Storage time at 4 ^oC (days)			
	0	3	5	7
No zein film	2.60±0.02 ^{a,D}	3.81±0.14 ^{c,C}	5.52±0.04 ^{a,A}	5.06±0.12 ^{bc,B}
Zein film (Stirring)[a]	2.68±0.03 ^{a,C}	4.46±0.21 ^{a,B}	5.70±0.17 ^{a,A}	5.35±0.30 ^{b,A}
Zein film incorporated with disodium EDTA (Stirring)	2.62±0.08 ^{a,D}	4.06±0.19 ^{bc,C}	4.89±0.33 ^{b,B}	6.21±0.22 ^{a,A}
Zein film incorporated with lysozyme and disodium EDTA (Stirring)	2.66±0.33 ^{a,D}	3.26±0.12 ^{d,C}	3.90±0.29 ^{d,B}	5.01±0.24 ^{bc,A}
Zein film (Homogenization)	2.90±0.34 ^{a,C}	4.35±0.09 ^{ab,B}	5.62±0.10 ^{a,A}	5.48±0.07 ^{b,A}
Zein film incorporated with disodium EDTA (Homogenization)	2.68±0.04 ^{a,C}	3.96±0.41 ^{c,B}	5.02±0.12 ^{b,A}	5.32±0.36 ^{b,A}
Zein film incorporated with lysozyme and disodium EDTA (Homogenization)	2.60±0.10 ^{a,C}	3.25±0.06 ^{d,B}	4.34±0.02 ^{c,A}	4.76±0.48 ^{c,A}

[a] : Film making method
[a-d] : Means having different letters within each treatment denote significant difference at $p<0.05$
[A-D]: Means having different letters within each storage time denote significant difference at $p<0.05$
Data are mean values ± S.D (n=3)

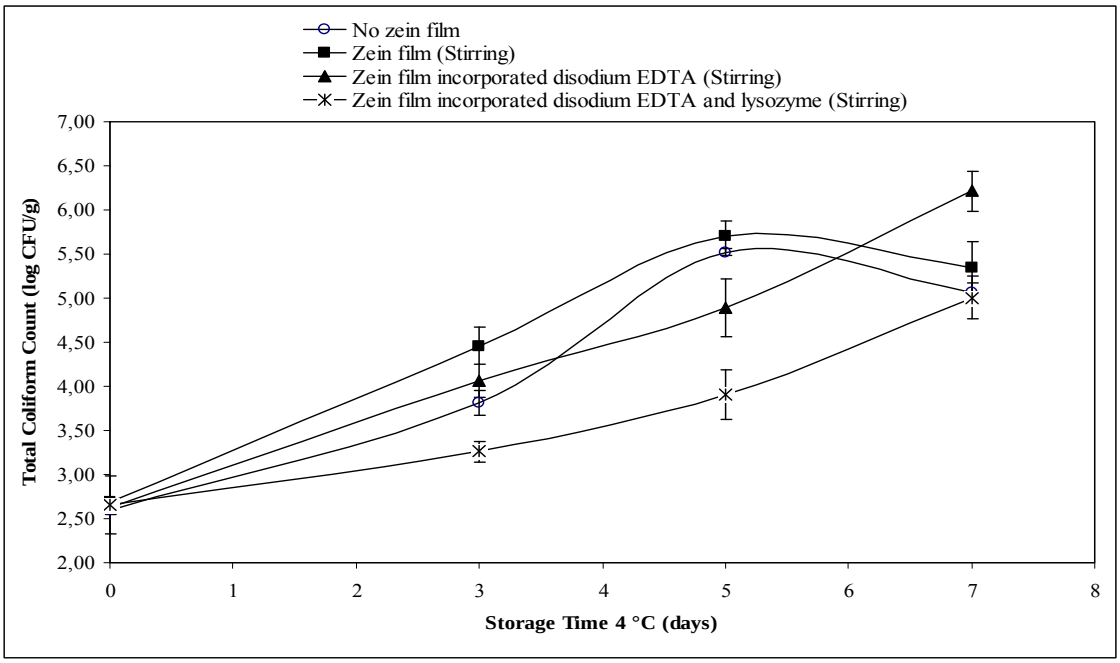


Figure 5.20. Total coliform counts during cold storage of beef samples packed with different zein films produced by stirring method (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

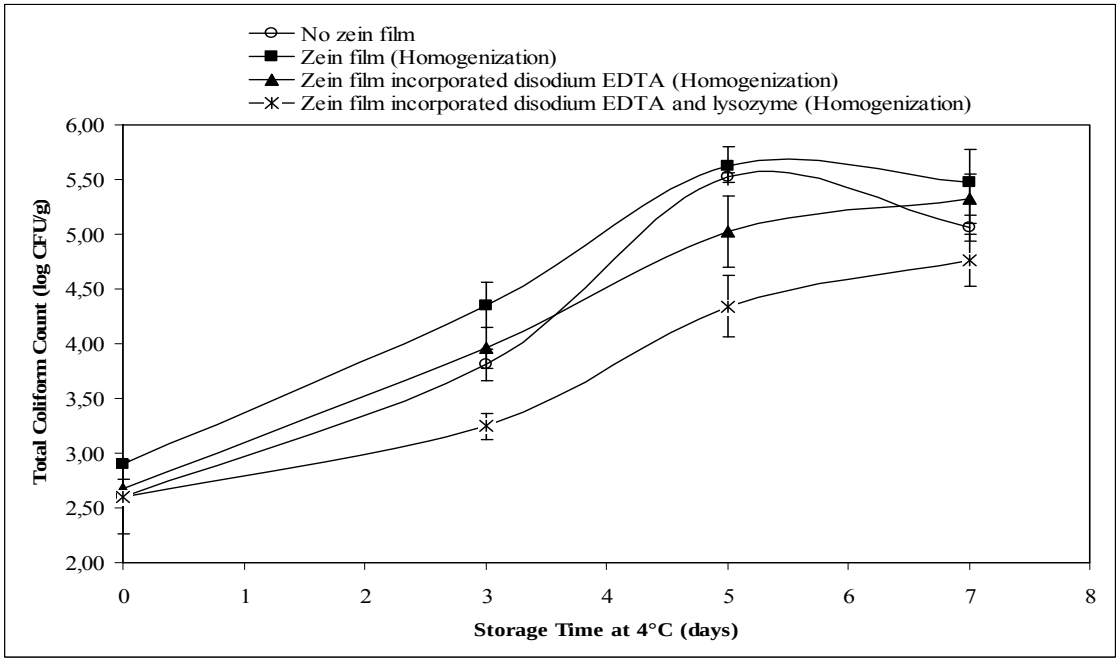


Figure 5.21. Total coliform counts during cold storage of beef burgers packed with different zein films produced by homogenization method (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

5.9. Effect of Zein Films on Oxidative Stability of Cold Stored Beef Burgers

One of the most undesirable changes occurs during food storage is lipid oxidation. This generally involves the degradation of poly-unsaturated fatty acids and the production of secondary decomposition products including aldehydes products leads to development off-flavors and off-odors (Sun et, al. 2001). The oxidation states of cold stored burgers were evaluated by considering their absorbance values at 532 nm determined by using the thiobarbituric acid-reactive substances (TBARS) assay (Table 5.10). Considerable increases occurred in the absorbance values of samples packed without any zein films (Figure 5.22). However, at the 7^{th} day of cold storage, the burgers packed by zein films incorporated with disodium EDTA or lysozyme-disodium EDTA had significantly low value in their absorbance at 532 nm. It appears that the increased oxidative stability of cold stored burgers is obtained mainly by the iron chelating capacity of disodium EDTA incorporated into films. These results clearly showed that zein films incorporated with lysozyme and disodium EDTA are bifunctional having both antioxidant and antimicrobial effect. The oxidation of burgers packed with control zein films were also slower than that of burgers packed without using zein films. In the literature the zein was reported as an antioxidant protein (Chiue, et al. 1997). However, due to water insolubility of zein the antioxidant activity of control zein films seems to be related with limitation of oxygen.

Table 5.10. Oxidation states of cold stored beef burgers packed with different zein films (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

Type of film	Absorbance values at 532 nm		
	Storage time at 4 ^{o}C (days)		
	0	3	7
No zein film	0.33±0,01 ^{a,C}	0.68±0.06 ^{a,B}	1.72±0.08 ^{a,A}
Zein film (Stirring)[a]	0.28±0.03 ^{b,C}	0.58±0.08 ^{b,B}	1.18±0.04 ^{b,A}
Zein film incorporated with disodium EDTA (Stirring)	0.23±0.01 ^{c,C}	0.28±0.02 ^{e,B}	0.63±0.00 ^{d,A}
Zein film incorporated with lysozyme and disodium EDTA (Stirring)	0.25±0.01 ^{bc,C}	0.27±0.01 ^{e,B}	0.40±0.01 ^{e,A}
Zein film (Homogenization)	0.32±0.03 ^{a,C}	0.50±0.03 ^{c,B}	0.99±0.04 ^{c,A}
Zein film incorporated with disodium EDTA (Homogenization)	0.22±0.05 ^{c,B}	0.35±0.06 ^{d,A}	0.38±0.03 ^{e,A}
Zein film incorporated with lysozyme and disodium EDTA (Homogenization)	0.23±0.00 ^{c,B}	0.33±0.00 ^{d,A}	0.35±0.02 ^{e,A}

[a] :Film making method
[a-e] : Means having different letters within each treatment denote significant difference at $p<0.05$
[A-C]: Means having different letters within each storage time denote significant difference at $p<0.05$
Data are mean values ± S.D. (n=3)

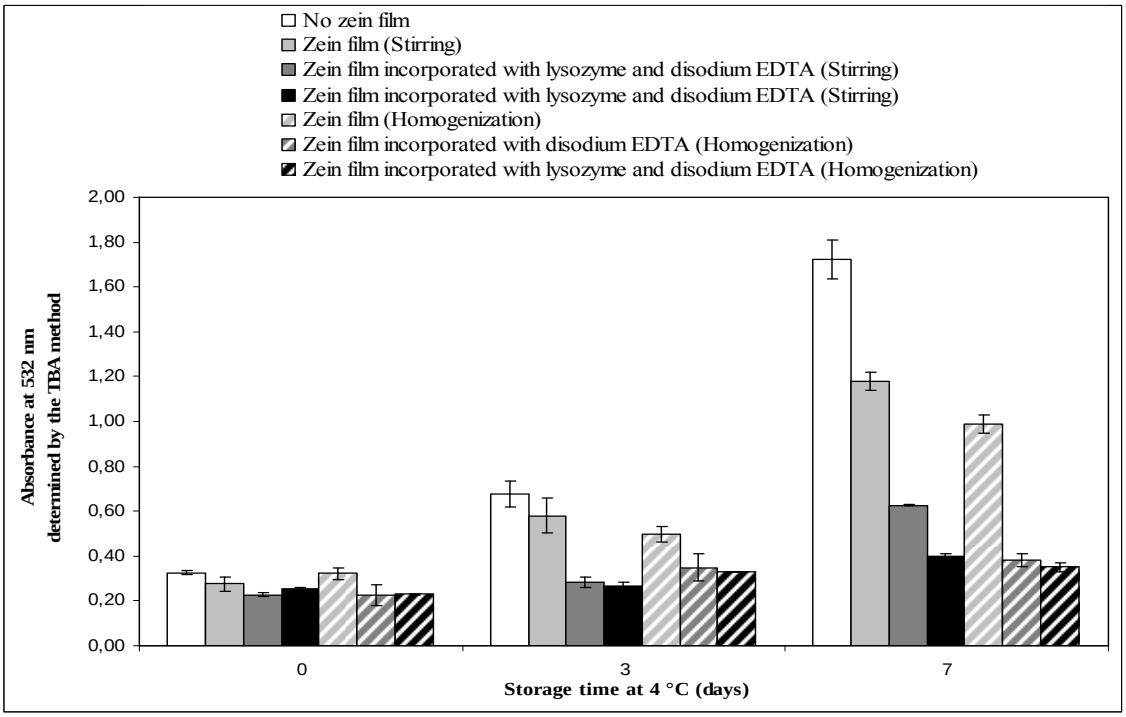


Figure 5.22. Oxidation states of cold stored beef burgers packed with different zein films (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

5.10. Effect of Zein Films on Color of Cold Stored Beef Burgers

The results of color measurements are given in Table 5.11. The redness indexes of packed burgers cold stored for 3 days changed between 1.48 and 2.09. The initial redness index of burgers was between 2.10 and 2.32 (Figure 5.24). Thus, it is clear that the packaging and cold storage caused a statistically significant reduction in the redness index of burgers, except uncoated burgers with zein film. Moreover, at the end of 7 days cold storage the redness indexes of packed burgers continued to decrease significantly and ranged between 0.85 and 1.58. The changes in meat color are very dynamic and related to the relative proportions of the three myoglobin forms deoxymyoglobin, oxymyoglobin (bright red) and metmyoglobin (brownish red) (O'Sullivan, et al. 2003). Thus, the color changes of burgers should have occurred by the interconversions of these pigments during storage.

On the other hand, the results showed that there was no beneficial effect of using disodium EDTA and lysozyme in zein films on maintenance of burger redness. The

comparison of the initial L* (lightness) value of burgers showed that there was no significant darkening or browning in burger color due to metmyoglobin formation among all beef samples in terms of both treatments and storage time ($p<0.05$) (Figure 5.23).

Table 5.11. Effect of different zein films on color of cold stored beef burgers (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

Type of film	Lightness (L*) value			Redness (a*/b*) index		
	Storage time at 4 °C (days)					
	0	3	7	0	3	7
No zein film	61.5±3.56	58.5±1.20	56.2±1.49	2.12±0.10 ^{ab,A}	2.09±0.05 ^{a,A}	1.58±0.19 ^{a,B}
Zein film (Stirring)[a]	59.72±1.02	57.5±2.32	57.9±0.01	2.18±0.02 ^{a,A}	1.77±0.09 ^{bc,B}	1.36±0.04 ^{b,C}
Zein film incorporated with disodium EDTA (Stirring)	60.0±0.22	58.5±0.51	58.1±0.94	2.30±0.04 ^{b,A}	1.82±0.03 ^{c,B}	0.93±0.00 ^{c,C}
Zein film incorporated with lysozyme and disodium EDTA (Stirring)	59.5±2.08	59.6±0.69	59.1±1.38	2.24±0.10 ^{ab,A}	1.57±0.05 ^{bc,B}	0.85±0.03 ^{c,C}
Zein film (Homogenization)	61.3±1.07	58.9±1.01	58.3±3.80	2.32±0.01 ^{ab,A}	1.73±0.01 ^{bc,B}	1.58±0.16 ^{ab,C}
Zein film incorporated with disodium EDTA (Homogenization)	58.2±2.05	60.9±0.09	59.6±0.47	2.10±0.04 ^{ab,A}	1.48±0.30 ^{ab,B}	0.85±0.04 ^{c,C}
Zein film incorporated with lysozyme and disodium EDTA (Homogenization)	61.8±2.34	58.1±1.98	57.9±1.03	2.18±0.09 ^{ab,A}	1.60±0.09 ^{bc,B}	1.06±0.08 ^{c,C}

[a] : Film making method
[a-c] : Means having different letters within each treatment denote significant difference at $p<0.05$
[A-C]: Means having different letters within each storage time denote significant difference at $p<0.05$

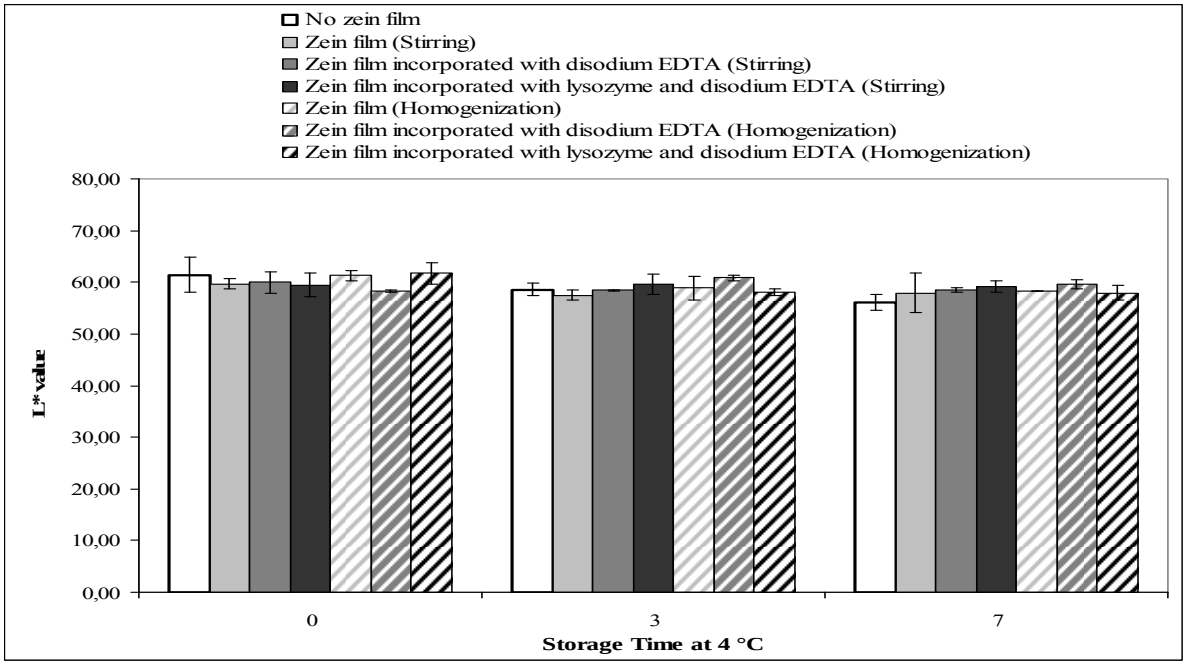


Figure 5.23. L* values of cold stored beef burgers packed with different zein films (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

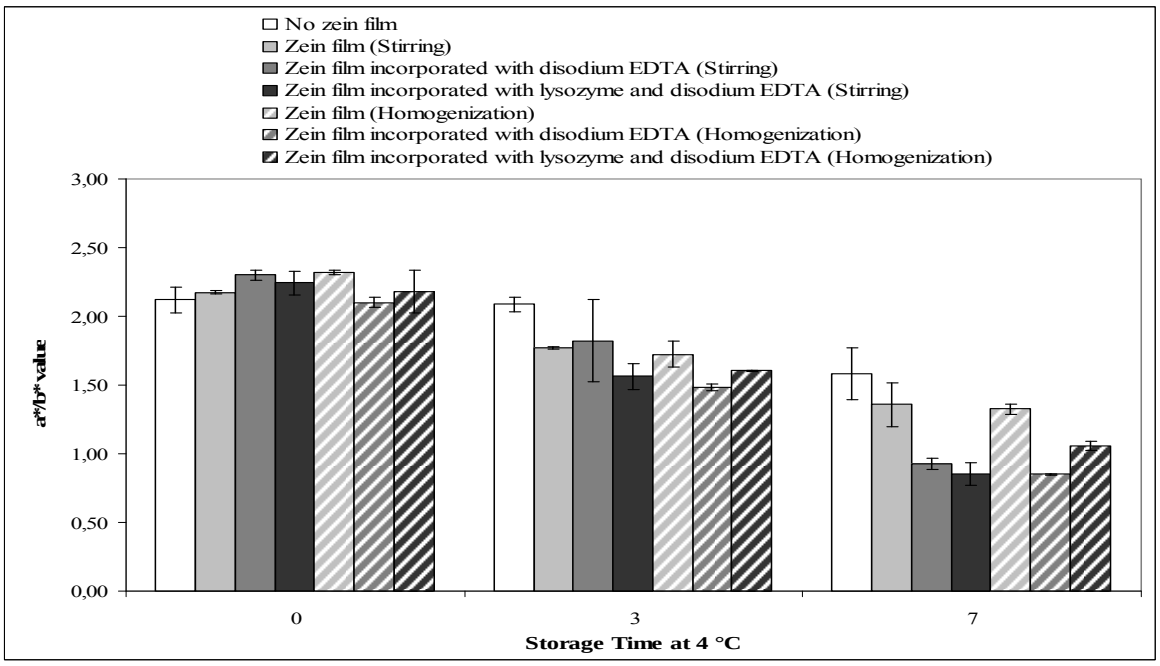


Figure 5.24. a*/b* values of cold stored beef burgers packed with different zein films (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

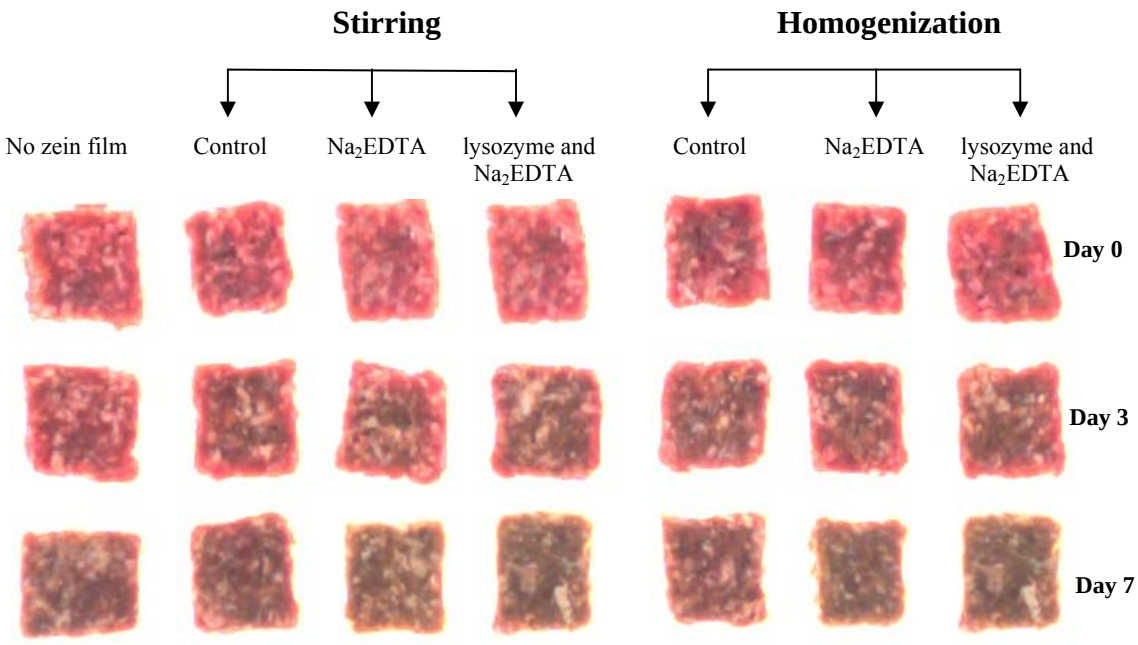


Figure 5.25. Photographs of beef burgers packed with different zein films during cold storage (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

5.11. Sensory Evaluation of Beef Burgers

Sensory evaluation by trained panelists gives a good estimate of the overall quality of foods. Sensory data are not objective for regulatory purpose. This may be only alternative way of assessing quality of foods. Figures 5.26, 5.27 and 5.28 show the judges' assessments regarding color, odor and overall acceptability, respectively, during 7 days of cold storage of beef burgers packed with different type of zein films or without films. On day 0, sensory panel could not detect significant differences among beef samples in all sensory attributes evaluated. However, it can be observed that, after the 3rd day and 7th day of storage, uncoated beef burgers showed the highest color and overall acceptability scores (Table 5.12 and 5.14), while there was no significant differences between samples packed with or without zein films in terms of odor on day 3 and 7 (Table 5.13). Beef burgers packed with zein films had lower scores than burgers packed without zein films in terms of color on day 3 and 7. Since zein film is a oxygen barrier, it prevents the formation of oxymyoglobin which gives bright red color to meat. It should be pointed out that overall acceptability scores of judges indicate similarity to color scores on day 0, 3 and 7. This result may be caused by the fact that while judges

were evaluated overall acceptance of beef burger they gave priority to color over other sensory attributes. All sensory attributes' scores decreased with storage time as related to microbial spoilage and physicochemical changes of burgers. The decrease in scores from day 0 to day 7 can be described significant or insignificant. As a result, there was no significant sensory difference between burgers coated with zein film produced by stirring and homogenization methods. Incorporation of lysozyme into zein film had no beneficial effect with storage time on sensory attributes of coated beef burgers.

Table 5.12. Color scores of beef burgers during cold storage (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

Type of film	**Color**		
	Storage time at 4 °C (days)		
	0	**3**	**7**
No zein film	4,75±0,21 [a,A]	4.45±0.07 [a,A]	2.95±0.35 [a,B]
Zein film (Stirring)[a]	4.75±0.07 [a,A]	3.95±0.07 [b,B]	2.18±0.11 [b,C]
Zein film incorporated with disodium EDTA (Stirring)	4.60±0.42 [a,A]	3.79±0.12 [b,B]	2.15±0.07 [b,C]
Zein film incorporated with lysozyme and disodium EDTA (Stirring)	4.40±0.42 [a,A]	3.65±0.09 [b,B]	2.40±0.00 [b,C]
Zein film (Homogenization)	4.70±0.00 [a,A]	3.70±0.14 [b,B]	2.47±0.19 [b,C]
Zein film incorporated with disodium EDTA (Homogenization)	4.65±0.07 [a,A]	3.84±0.21 [b,B]	2.20±0.28 [b,C]
Zein film incorporated with lysozyme and disodium EDTA (Homogenization)	4.65±0.21 [a,A]	3.90±0.14 [b,B]	2.10±0.14 [b,C]

[a] : Film making method
[a-b] : Means having different letters within each treatment denote significant difference at $p<0.05$
[A-C]: Means having different letters within each storage time denote significant difference at $p<0.05$

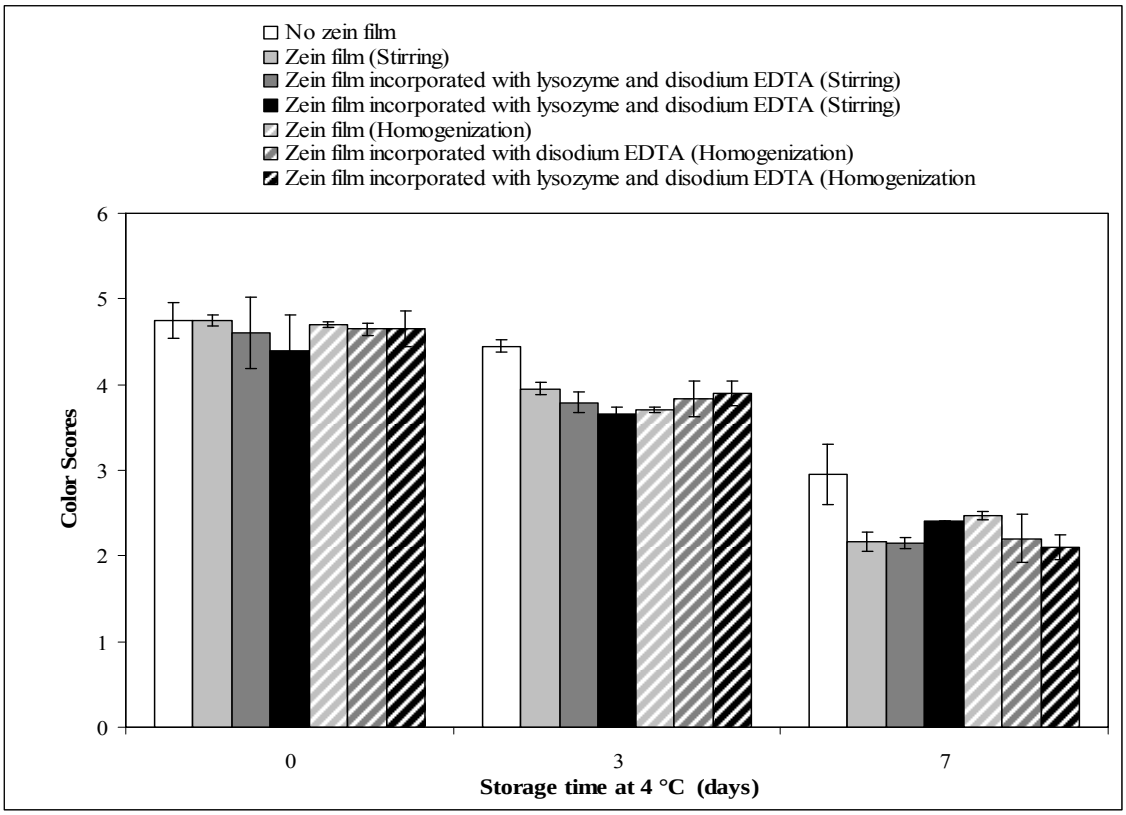


Figure 5.26. Color scores of beef samples during cold storage (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

Table 5.13. Odor scores of beef samples during cold storage (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

Type of film	**Odor**		
	Storage time at 4 °C (days)		
	0	**3**	**7**
No zein film	4,50±0,21 ^{A}	4.10±0.07 ^{A}	2.06±0.35 ^{B}
Zein film (Stirring) [a]	4.00±0.07 ^{A}	3.65±0.07 ^{A}	2.11±0.11 ^{B}

(cont. on next page)

Table 5.13.(cont.) Odor scores of beef samples during cold storage (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

Zein film incorporated with disodium EDTA (Stirring)			
	4.00±0.42 ^{A}	3.78±0.12 ^{A}	2.20±0.07 ^{B}
Zein film incorporated with lysozyme and disodium EDTA (Stirring)			
	3.94±0.42 ^{A}	3.59±0.09 ^{A}	2.21±0.00 ^{B}
Zein film (Homogenization)			
	4.29±0.00 ^{A}	3.65±0.14 ^{B}	2.20±0.19 ^{C}
Zein film incorporated with disodium EDTA (Homogenization)			
	4.24±0.07 ^{A}	3.67±0.21 ^{B}	2.00±0.28 ^{C}
Zein film incorporated with lysozyme and disodium EDTA (Homogenization)			
	4.12±0.21 ^{A}	3.47±0.14 ^{B}	2.00±0.14 ^{C}

[a] : Film making method
[A-C]: Means having different letters within each storage time denote significant difference at $p<0.05$.

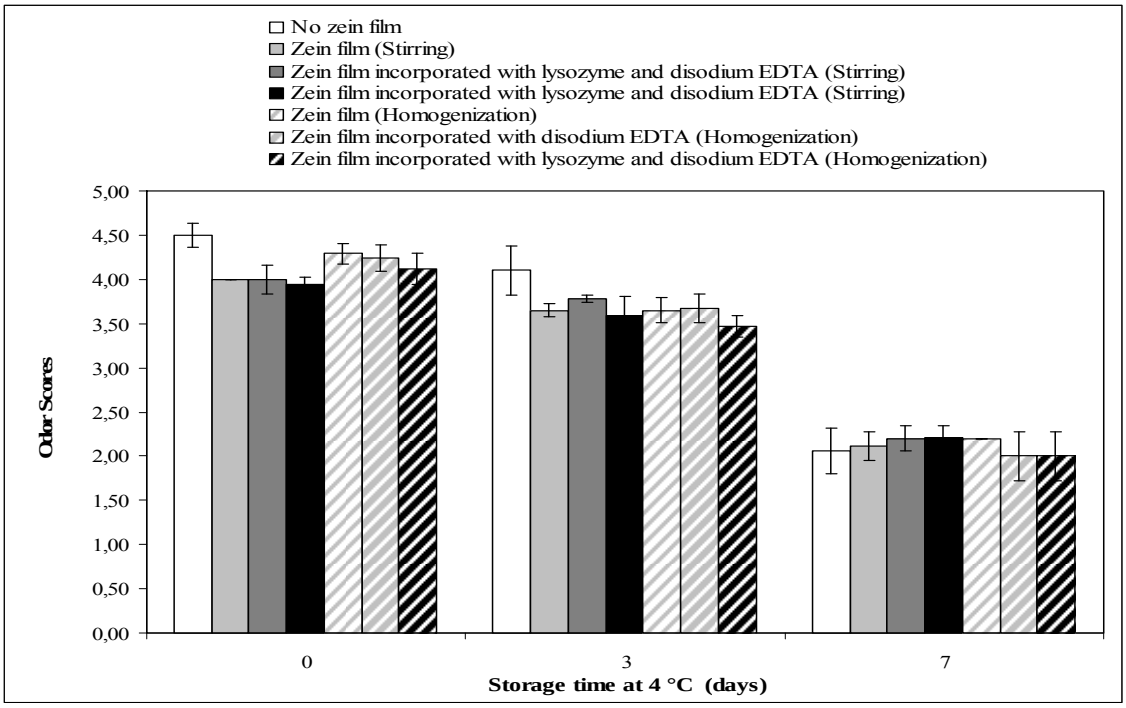


Figure 5.27.Odor scores of beef burgers during cold storage (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

Table 5.14. Overall acceptability scores of beef burgers during cold storage (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

Type of film	Overall Acceptability		
	Storage time at 4 °C (days)		
	0	3	7
No zein film	4,50±0,14 [a,A]	4.25±0.07 [a,A]	2.58±0.55 [a,B]
Zein film (Stirring)	4.25±0.25 [a,A]	3.47±0.04 [b,B]	2.05±0.15 [b,C]
Zein film incorporated with disodium EDTA (Stirring)	4.47±0.04 [a,A]	3.10±0.42 [b,B]	2.05±0.21 [b,C]
Zein film incorporated with lysozyme and disodium EDTA (Stirring)	3.95±0.37 [a,A]	3.10±0.28 [b,B]	2.15±0.07 [ab,C]
Zein film (Homogenization)	4.50±0.08 [a,A]	3.40±0.14 [b,B]	2.20±0.14 [ab,C]
Zein film incorporated with disodium EDTA (Homogenization)	4.44±0.31 [a,A]	3.50±0.00 [b,B]	2.00±0.28 [b,C]
Zein film incorporated with lysozyme and disodium EDTA (Homogenization)	4.39±0.24 [a,A]	3.25±0.07 [b,B]	1.90±0.28 [b,C]

[a] : Film making method
[a-b] : Means having different letters within each treatment denote significant difference at $p<0.05$.
[A-C]: Means having different letters within each storage time denote significant difference at $p<0.05$.

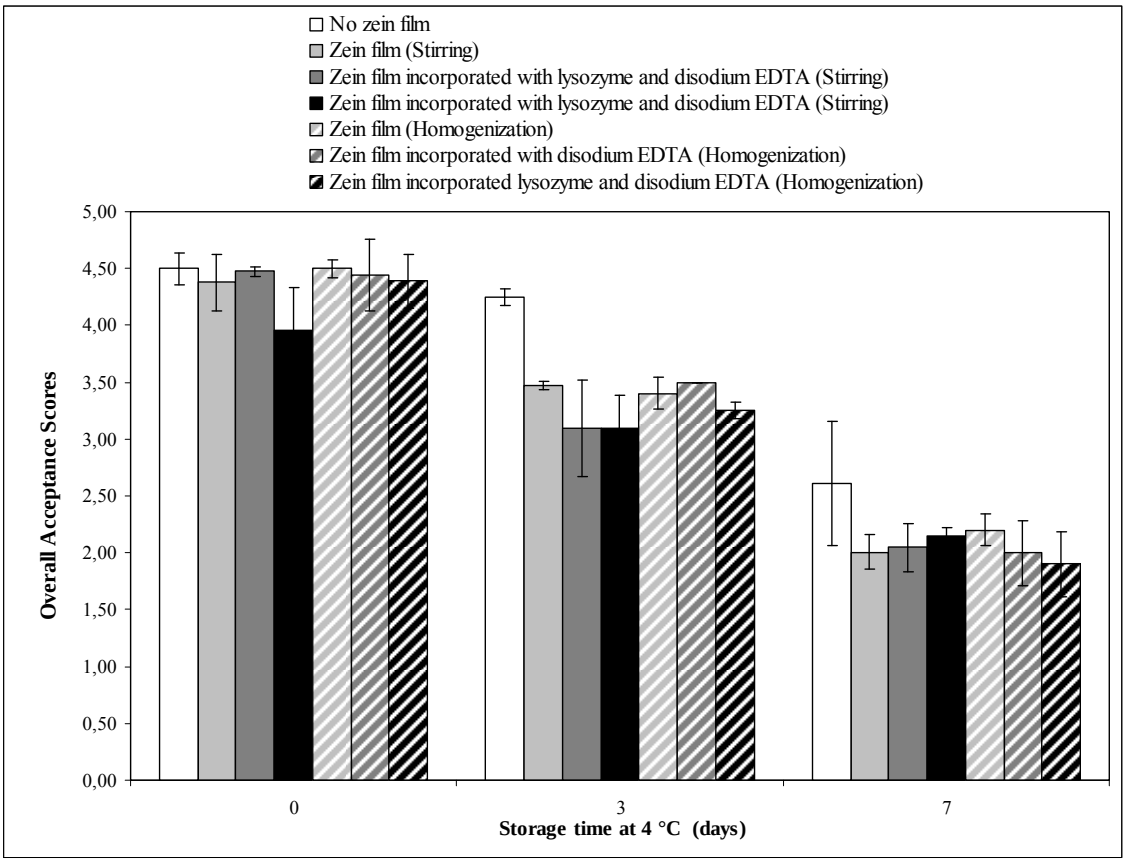


Figure 5.28. Overall acceptability scores of beef burgers during cold storage (film contents: 700 μg/cm^2 lysozyme and 300 μg/cm^2 disodium EDTA)

CHAPTER 6

CONCLUSION

The use of partially purified lysozyme in antimicrobial packaging is a critical process which needs use of appropriate films and homogenous distribution of the crude enzyme within the films. Otherwise, the enzyme and other protein residues may aggregate within the films and this cause heterogeneous distribution of enzyme and its resulting antimicrobial activity in the films. Thus, in this study, hydrophilic partially purified lysozyme was incorporated into hydrophobic zein films by homogenization or stirring of film forming solutions to obtain different degrees of distribution for lysozyme in the films and modify the film structure. Homogenization method provides more uniform antimicrobial zein films by minimizing protein aggregates formed due to low solubility of hydrophilic lysozyme in ethanol used to prepare zein films. Both types of films obtained by incorporation of lysozyme with stirring or homogenization showed good antimicrobial activity on *B. amyloliquefaciens* and *L. innocua.* The films also showed sufficient antimicrobial activity on *E. coli, P. fluorescens*, *E. coli* O157:H7 and *S. typhimurium* when lysozyme was supported by the incorporation of disodium EDTA. Zein films incorporated with lysozyme by the stirring method gave larger zones than those films incorporated with lysozyme by the homogenization method. This occurred due to higher soluble lysozyme activity of most zein films obtained by stirring method than those films obtained by the homogenization method. However, the lysozyme and disodium EDTA incorporated into zein films produced by the homogenization method caused better distribution of antimicrobials and their resulting antimicrobial activity in the films than the stirring method. The developed films incorporated with lysozyme and disodium EDTA by stirring or homogenization method successfully suppressed the microbial counts of turkey and beef burgers during cold storage. Due to the metal chelating dependent antioxidant effect of disodium EDTA, the developed films also effectively prevented oxidative changes in packed burgers. However, no beneficial effects of films on beef color and sensory properties were determined. This research clearly demonstrated that the partially purified lysozyme can be incorporated into zein films by using the homogenization method. This data is important to prove suitability of using partially purified lysozyme in industrial scale active packaging applications.

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