DEVELOPMENT OF pH-CONTROLLED TRIGGERING MECHANISMS FOR CONTROLLED RELEASE OF LYSOZYME

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ABSTRACT

DEVELOPMENT OF pH-CONTROLLED TRIGGERING MECHANISMS FOR CONTROLLED RELEASE OF LYSOZYME

In this study, zein and whey protein (WP) based films and their blends and composites have been prepared to obtain pH-controlled triggering mechanisms for controlled release of lysozyme. The total amount of lysozyme released from standard zein films was not considerably affected from changes in pH between 7.3 and 5.3 since this hydrophobic biopolymer lacked charged ionisable groups to bind lysozyme ionically. In contrast, incorporation of lentil protein isolate (LPI) into zein created a composite structure and caused binding of positively charged lysozyme (pI>9.0) on negatively charged groups of LPI (pI: 4.5-6) within the film matrix in release medium with pH between 5.3 and 7.3. The amount of bound lysozyme in zein-LPI composites increased linearly as LPI concentration increased between 1,5 and 4,5 mg/cm^2 at pH 7.3. The release of bound enzyme could be triggered by reducing pH of release medium from 7.3 to 4.3, down below pI of LPI. On the other hand, films of WP (pI≈5.2) bind considerable amount of lysozyme due to their inherent net negative charges close to neutrality. The release of bind lysozyme could be trigged as pH of release medium reduced from 6.0 to 3.0, down below the pI of WP. The preparation of WP-oleic acid blend and WP-bees wax composites increased the film porosity and amounts of released lysozyme from films at pH 4.5 and 5.0, by 2-4 and 1.2-1.5 folds, respectively. The zein and WP based films containing 0.7 to 1.4 mg/cm^2 lysozyme showed good antimicrobial activity against *Listeria innocua.* This work showed the potential of creating pH-controlled release systems during antimicrobial packaging of food.

ÖZET

LİSOZİMİN KONTROLLÜ SALIMI İÇİN pH TARAFINDAN KONTROL EDİLEN TETİKLEME MEKANİZMALARI GELİŞTİRİLMESİ

Bu tezde lisozimin pH'ya bağlı kontrollu salımını gerçekleştirebilecek tetikleme mekanizmaları oluşturulması amacıyla zein ve peyniraltı suyu (WP) proteini filmler ve bunların karışım ve kompozitleri hazırlanmıştır. Hidrofobik yapıları ve iyonik gruplarının olmaması nedeniyle zeinden elde edilen filmler lisozimi iyonik olarak bağlayamamakta ve filmlerden salınan toplam lisozim miktarları pH 5.3 ve 7.3 arasındaki değişimlerden oldukça az etkilenmektedir. Bunun aksine filmler içerisine mercimek protein izolatı (LPI) ilave edilmesi kompozit bir yapı oluşturmakta ve pH 5.3-7.3 aralığında pozitif yüklü lisozimin (pI>9) film matrisi içerisine dağılmış negatif yükler içeren LPI'ya (pI: 4.5-6.0) bağlanmasına neden olmaktadır. Zein-LPI kompozitlerine bağlanan lisozim miktarı filmler içerisindeki LPI miktarının 1,5 ile 4,5 mg/cm^2 arasında artırılması durumunda linear olarak artış göstermektedir. Bağlı olan lisozimin filmlerden salınması ortam pH değerinin aşamalı olarak 7.3'den 4.3'e yani LPI'nın pI değerinin altına düşürülmesiyle başarıyla tetiklenebilmektedir. Diğer yandan WP filmler doğal olarak yaklaşık 5.2 olan pI değerleri nedeniyle nötrale yakın pH değerlerinde net bir negatif yük taşıdıklarından yüksek oranda lisozim bağlayabilmektedirler. WP filmlere bağlanmış lisozimin salımı ortam pH değerinin aşamalı olarak 6.0'dan WP'nin pI değerinin altındaki pH 3.0'e düşürülmesiyle tetiklenebilmektedir. WP-oleik asit karışımları ve WP-mum kompozitlerinin hazırlanması filmlerin gözenekliliğini artırdığından özellikle pH 4.5 ve 5.0'de filmlerden salınan bağlı lisozim miktarı artırılabilmektedir. 0.7-1.4 mg/cm^2 düzeyinde lisozim içeren zein ve WP temelli filmler *Listeria innocua* bakterisi üzerinde kayda değer düzeyde antimikrobiyel aktivite göstermişlerdir. Elde edilen bu veriler antimikrobiyel paketleme uygulamasında pH'ya bağlı olarak tetiklenebilen kontroll salım sistemleri kullanılabileceğini göstermiştir.

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CHAPTER 1

INTRODUCTION

Today, the food safety mentality requires developing new and intelligent technologies in the food packaging area. For this reason, active packaging technology has become very popular and safe during the last 15 years. There is an increasing demand for developing natural, antimicrobial and intelligent active packaging systems. Contrary to synthetic chemicals, using natural antimicrobials and antioxidants incorporated in edible films is accepted as safe and environmentally-friendly by the consumers. Thus, developing biodegradable materials and edible films for these purposes has become inevitable for the food scientists.

Antimicrobial packaging, which is a type of active packaging application, is very effective on controlling microbial spoilage and growth in food systems and it has become very popular due to the food-borne microbial outbreaks in fresh or minimally processed food products. Microorganisms, which cause food spoilage and food-borne outbreaks, mostly grow in the food surface (Brody et al., 2001; Han, 2005). Antimicrobial agents have microbiocidal or microbiostatic effects against pathogen or spoilage microorganisms on the foods. To control and inhibit the growth of microorganisms, antimicrobial agents should be applied on the food surface. However, direct application of antimicrobials on food has some disadvantages such as the diffusion of antimicrobial agent to inner part of the food or antimicrobials' potential of form complex structures with food components. All of these situations cause neutralization of antimicrobial agents, a decrease in the inhibition effect and a risk for the food safety. Therefore, it is imperative to maintain the stable concentration of antimicrobials during the whole shelf life of packaged foods (Han, 2005). This necessity makes the release techniques of antimicrobials critical. To obstruct the loss of activity of antimicrobial agents on the surface, because of high concentration levels, controlled release mechanism is very effective. Controlled release packaging system is a new technology-application of active packaging. Although active packaging system has many functions enhancing the food safety by interacting with it, controlled release packaging applications have an extra function which can release active agents in a

controlled manner. Controlled release of antimicrobials can be triggered in several ways. Using multilayer composite films, development of temperature dependent release mechanisms, affect the release of antimicrobials by incorporating different components such as fibers are examples of controlled release studies. One of the triggering mechanisms of release is changing pH of the food media. pH is a dynamic parameter in foods such as meat and cheese. After 24h of slaughtering, meat pH drops from 7.3 to 6.3-5.3. Meat and meat products tend to spoil because of both spoilage microorganisms and enzymatic deterioration (Emiroğlu et al., 2010), as a result of these reactions pH changes dramatically in meat and meat products.

In this study, it is aimed to control the release of antimicrobial agent by pH-triggered mechanism and show its preservative activity. One of the most commonly used antimicrobial agents is lysozyme. It is an antimicrobial enzyme that is easy-produced and considered as natural additive by customers. Generally, meat becomes an easy target for microorganisms during maturation process, especially on the distribution and marketing stages. With the help of natural pH change in meat, it is possible to increase the release rate of lysozyme from the films on the food surface and maintain the inhibition of microorganisms. For this purpose; to obtain a high released rate of lysozyme, pH of the food can be dropped manually by a separate acidification step and its antimicrobial effect can be increased whenever it is necessary.

As a packaging material, protein films are widely used as an antimicrobial agent carrier due to their functional properties that can be used in controlling the release rate of agents onto food surface.

The aim of this study is to incorporate the partially purified or pure lysozyme into different edible films and increase or decrease its release rate dependent on pH. On the base of isoelectric point (pI) values of film-making materials, lysozyme and additive components, the release rate has been increased or decreased dependent on pH. Finally it can be said that the release rate of lysozyme can be controlled by developing different edible films incorporated with appropriate additives according to pH change of the food systems.

CHAPTER 2

FOOD PACKAGING

Packaging is a system that contains a confined product, a packaging material and an in-packaging atmosphere (Han, 2001). The main functions of the food packaging are to prevent microbial contamination and unwanted chemical reactions as well as to provide physical protection. However, protective role of packaging should be improved to increase the food safety because of tampering, unsafe treatments (Han, 2005), biological and chemical changes in food composition during storage and marketing (Jongjareonrak et al., 2008). Modern quality systems are important and effective in eliminating the microbiological or chemical disintegration problems in food, hence the use of new technologies is necessary (Han, 2005).

Traditional food packaging protects foods by providing an inert barrier to the environmental factors. Recently, there has been an increasing interest in active packaging. Instead of providing an inert barrier, active packaging has more active role in preservation of foods (Jongjareonrak et al., 2008). Also, active packaging applications are the most pertinent technology for consumer contentment (Mastreomatteo et al., 2010).

2.1. Problems in Packaging Industry

Today, plastic polymers are widely used in food packaging industry because of their transparency, softness, heat seal ability, good strength to weight ratio, low cost, efficient mechanical properties (tear and tensile strength) and good O_2 barrier. However, the mostly used plastic packaging materials are based on petrochemical products and have adverse effect on environment since they are not totally biodegradable and they do not meet the consumer demand due to health concerns. Basically, the negative effects of plastic packaging materials are;

- consumption of fossil fuels
- environmental pollution (white pollution)
- landfill depletion

- high energy use in production process
- migration of polymers into food materials (Mahalik and Nambiar, 2010)

Plastic materials can be recycled but this process is impractical and generally undesirable (Gross and Kalra, 2002) because of collection cost, difficulties in separation and cleaning steps, and requirement of energy. Also, recycled plastics are not used as food-contacting packaging materials since the prolyzing of all organic contaminants is not sufficient during the recycling process (Bugusu and Marsh, 2007).

Due to these serious issues and disadvantages in recycling of plastic there has been an increasing tendency to use biopolymers instead of plastic packaging materials (Mahalik & Nambiar, 2010; Emiroğlu et al., 2010). The worldwide consumption of biodegradable polymers has increased 600% from 2000 to 2008 (Anonymous).

2.2. Active Packaging

Food quality is closely related to the food safety and it is an indispensible requirement of food industry. For this reason, packaging has a substantial role in ensuring the safety and protection of food. However, classical packaging systems protect the quality in passive ones that are no longer sufficient. The inclination in developing new and advanced technologies in food packaging system results from the increasing demand for active packaging which is more dynamic and functional (Lim, 2011). For this purpose, active packaging is the most satisfying innovative technology in the food packaging (Mastromatteo et al., 2009). The definition of active packaging accepted by European FAIR-project is that "Active packaging is an innovative concept that can be defined as a type of packaging that changes the condition of the packaging to extend the shelf-life or improve safety or sensory properties while maintaining the quality of the food" (Ahvenainen and Vermeiren, 1999). It provides better barrier properties than conventional packaging system does and contains active ingredients (Ahvenainen, 2003). Generally the active packaging systems are;

- Oxygen and ethylene scavenging systems
- CO_2 scavengers and emitters
- Moisture regulators

- Antimicrobial packaging concepts
- Release or adsorption systems of flavours and odours

O_2-scavenging technology is used against oxidation or growth of aerobic bacteria and moulds. The modified atmosphere packaging or vacuum packaging cannot totally remove the O_2 in the package, which can result in changes in flavour and odour of food or an increase in respiration and ethylene production rate of vegetable and fruits. The basic O_2-scavenging techniques are iron powder oxidation, ascorbic acid oxidation, photosensitive dye oxidation, enzymatic oxidation, unsaturated fatty acid oxidation and immobilized yeast on a solid material. O_2 scavenging tools can be applied with sachets or they can be directly incorporated into packaging material (Vermeiren, 1999).

Ethylene (C_2H_4) is a plant hormone and it affects the physiology of fresh vegetables and fruits by accelerating the respiration rate. Scenescence, ripening, softening and yellowing are the some examples of deterioration caused by ethylene accumulation. To prevent the accumulation of C_2H_4, some adsorbing substances are used such as potassium permanganate ($KMnO_4$), activated carbon, zeolites and clays. The most encountered problem in ethylene-scavenger system is the insufficient ethylene adsorbing capacity.

CO_2 is produced by respiration and it can cause food deterioration and packaging destruction. In CO_2-scavenger systems, $Ca(OH)_2$ and CO_2-adsorbent sachets containing CaO are used. These compounds react with CO_2 and produce $CaCO_3$. On the other hand, CO_2 has an inhibiting effect on microorganism on food surfaces such as meat and poultry products. If O_2 is totally removed from the in-packaging atmosphere, the packaging will be collapsed, so replacing O_2 with CO_2 or maintaining the release of CO_2 when O_2 is absorbed by sachets will be beneficial for packaging and food protection.

Some foods need a protective barrier against moisture, or else microbiological spoilage can develop inside of the packaging or quality will be damaged i.e. softening of low-moisture foods. In order to tune the inside-moisture, water vapour absorbing or releasing films, pads and sachets are used. In order to control moisture, liquid water absorbing sheets are used to lower the a_w such as polyacrylate salts and graft copolymers of starch. Other application is hindering the moisture in the vapour phase. In order to do this, a humectants material is placed between two plastic film layers whose water vapour permeability is high. Glycol and carbohydrates placed between

polyvinylalcohol (PVOH) layers can be an example of this application and it is commercially produced as the trademark Pichit^{TM} (Showa Denko, Japan).

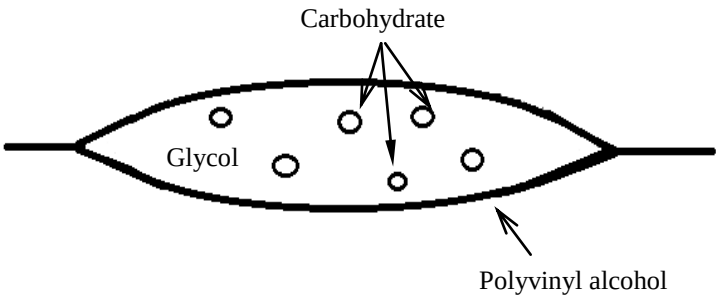


Figure 2.1. Pichit bilayer sheet for absorbing water
(Source: packaging-technology.org 2011)

The systems maintaining the release or absorption of flavours and odours have a working pirinciple based on sorption of undesirable flavour and odours in foods. One of the approaches in this system is eliminating bitterness in citrus juices i.e. caused by narginin in grapefruit juice. Cellulose acetate film loaded by enzymes (narginase) is placed inside the package hydrolyses the bitterness compounds. Other application is used to remove the undesirable odours sourced by aldehydes and amines. Acidic compounds incorporated into polymers are good solutions to eliminate the amines, also ferrous salt and organic acid containing polymers such as The Anico^{TM} (Japan) oxidize the off-odour compounds (Vermeiren et al., 1999).

Table 2.1. Active packaging systems of releasers with material types, working principles and applications (Source: Lee et al., 2008).

Active task type	**Material**	**Working principle**	**Desired effect and applications**	**Issues must be considered**
CO_2 containers	$FeCO_3$; $NaHCO_3$; Na_2CO_3; ascorbic acid	Generation of CO_2	Inhibition of bacteria and moulds, prevention of package collapse	$FeCO_3$ is very unstable not to be used without any halide metal catalysts
Ethanol emitters	Ethanol sachets	Generation of ethanol vapour	Antifungal and antibacterial activity	Insufficienct prevention to growth of yeast, volatilization, off-flavour

(Cont. on next page)

Table 2.1. (cont.)

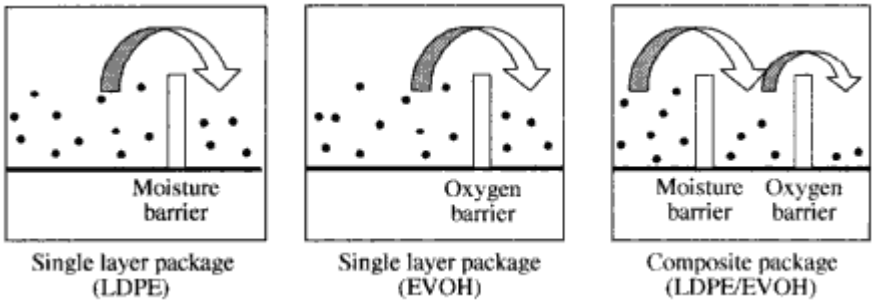
O_2 scavengers	Iron powder, ascorbic acid, unsaturated hydrocarbons, yeast, catechol, oxidative enzymes, ligands, polyamides	Removing O_2 by oxidation	Reducing O_2 concentration and preventing its oxidative effect	Enzymes are sensitive to temperature and pH.
Antimicrobial packaging	Antimicrobials incorporated or encapsulated in packaging materials	Release of antimicrobial agent onto the food surface or contacted inhibition of microorganisms without migration	Inhibition of microbial growth	Direct contact is necessary except volatile antimicrobials; Vapours such as ethanol, allyl, isothiocyanate and ClO_2 may cause undesired odour
Antioxidant packaging	BHT, α-tocopherol, ascorbic acid, TBHQ	Release of antioxidants or consumption of O_2	Reducing oxidation,	Antioxidants used for polymer protection can protect foods by absorbing O_2
Other releasers	Flavours incorporated in packaging matrix	Release of flavour components	Development of food flavours	Release of desirable flavour should be used not to disguise of microbial off-flavours.

Among these systems, antimicrobial packaging is a promising active packaging type that can be frequently used to protect foods (Emiroğlu *et al*., 2010).

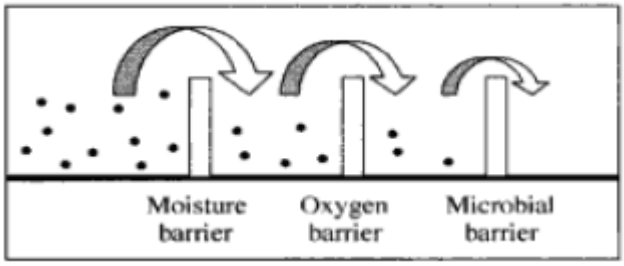
2.3. Antimicrobial Packaging

Antimicrobial packaging is one of the applications of active packaging system and defined as a system that can kill or inhibit the spoilage and pathogenic microorganisms contaminated to foods (Han, 2005). Antimicrobial packaging systems can protect food more actively by inhibiting the growth of pathogenic or spoilage organisms before any evidence of deterioration and reducing the surface contamination (Han, 2005; Yener et al., 2009). These systems are beneficial since they can reduce the

amount of preservatives added into the food (Ahvenainen, 2003). Antimicrobial systems generally include antimicrobial packaging materials, antimicrobial inserts or antimicrobial edible food ingredients (Han, 2005). The major function of antimicrobial packaging systems is controlling the microbial growth rather than to meet the other roles of conventional food packaging, so the primary roles of antimicrobial packaging system are; safety assurance, quality maintenance and shelf-life extension. Basically, providing combined barriers against moisture, oxygen and microorganisms is the principle of antimicrobial packaging system.



(a) Conventional packaging system



(b) Antimicrobial packaging system

Figure 2.2. Protective barrier systems of antimicrobial packaging system compared to conventional packaging system (Source: Ahvenainen 2003)

Antimicrobial activity can be shown by packaging material, headspace of food system or in packaging atmosphere but the important point is that it should be shown mainly on food surface. There are different application methods of antimicrobial agents; absorbation of antimicrobial agents before final extrusion, dissolving into film forming solvents, incorporating in edible packaging materials, coating onto surface of film material, adding a sachet containing antimicrobial agents and adding into headspace or packaging atmosphere if the agent is gaseous (Han, 2005; Emiroğlu et al., 2010).

2.3.1. Development of Antimicrobial Packaging System

Antimicrobial agents may be incorporated into non-food parts of the system such as packaging material, headspace and packaging atmosphere or into films, coatings, sheets, trays, containers in form of packaging materials; inserts, pads and sachets placed in package space (Han, 2005). Basically, antimicrobial packaging types are divided into two groups; packaging-food and packaging-headspace-food systems. The package-food system includes a solid or liquid-like food and a packaging material in contact with the food. Antimicrobial agents transfer into the food by diffusion and partitioning at the interface (Quintavalla, 2002). In the packaging-headspace-food system, main transfer mechanism is evaporation or equilibrated distribution of agents through the headspace. Due to this migration, volatile antimicrobial substances should be used in packaging systems containing headspace or air gaps between packaging material and food.

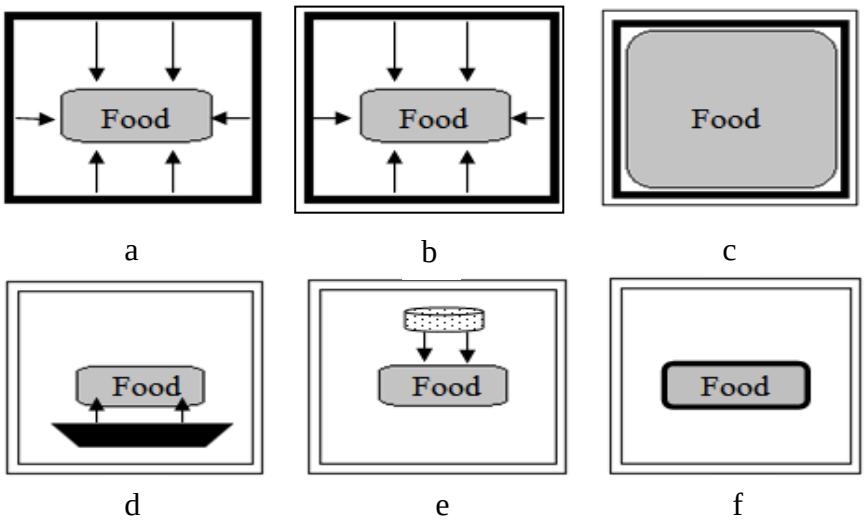


Figure 2.3. Different antimicrobial film packaging systems; (a) antimicrobial packaging, (b) antimicrobial coating on conventional packaging material, (c) immobilization of antimicrobial agents in packaging material, (d) antimicrobial tray or pad, (e) antimicrobial sachets, (f) antimicrobial edible coating on food (Source: Han, 2005)

Antimicrobial agents reach on food system by release, absorption or immobilization. In release method, antimicrobials migrate onto food surface or into headspace of package. In absorption model, antimicrobials eliminate the factors that are

essential for the microorganism growth. In immobilization system, antimicrobial agents do not migrate from packaging material, only inhibit the growth on the food surface by having a contact with the packaging material.

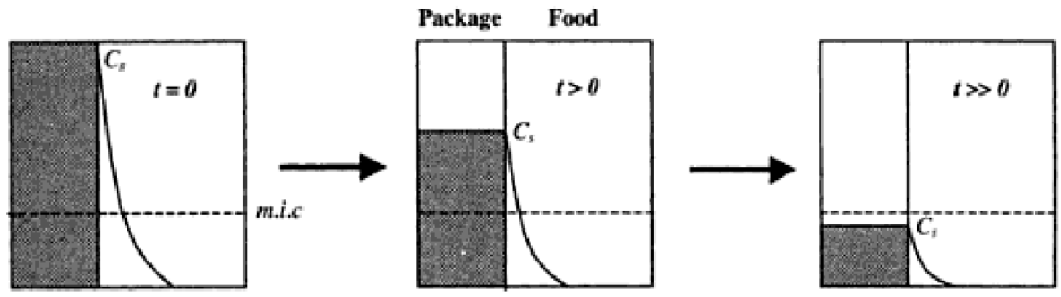
2.3.2. Factors Affecting Antimicrobial Films

Antimicrobial systems applied on real food systems, may be affected by many factors that should be considered.

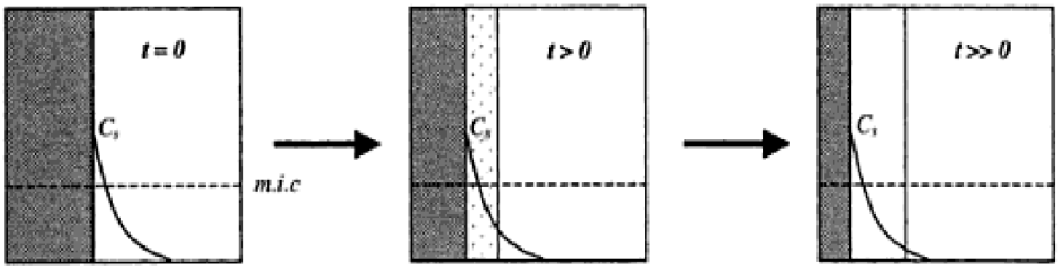
Formation methods are effective on antimicrobial activity of active packaging systems. The casting methods are; extrusion and solvent casting (wet casting). In extrusion method, extrusion temperature and specific energy input affect the antimicrobial activity. High temperature conditions cause degradation of antimicrobial agents and energy input refers to intention of film making conditions. Nam *et al*. (2002) indicated that high extrusion temperature causes serious loss of lysozyme activity.

In wet casting method, solubility of antimicrobial agents and polymers and their reactivity are effective. Solubility affects the homogeneous distribution of antimicrobials through the solvent and reactivity is related to the activity loss of antimicrobials.

The chemical properties of antimicrobial agents and their relations with polymeric film material affect the antimicrobial efficiency of system. Hydrophilicity or hydrophobicity of materials and active agents may cause solubility problems including crevice holes, the loss of physical integrity, powder-blooming and loss of transparency (Han, 2005). The compatibility of antimicrobial agent and film material and adequate amount of active agent can be incorporated into packaging material without any activity loss. Also, solubility of antimicrobial is related to its concentration on the food. If the agent is highly soluble in the food, it shows free diffusion profile and its concentration on the food surface will be very high. However, if the antimicrobial is not very soluble in the food, this creates a monolithic system in which the release is slow. Thus the concentration of antimicrobials stays above the m.i.c. for longer period of time.



(A) Release of soluble antimicrobial agents through free diffusion



(B) Release of antimicrobial from monolithic system

Figure 2.4. Changes in antimicrobial concentration in two different diffusion system

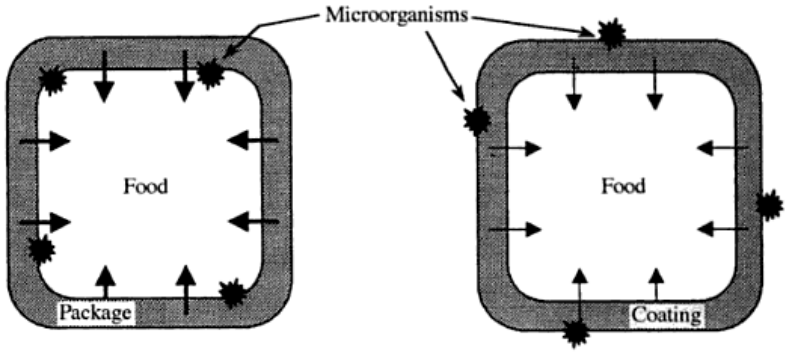
The activity of chemical and natural antimicrobials mostly changes with pH, especially, with the pH of the packaged food (Han, 2003). It is known that the activity of lysozyme is highly affected by pH. Other antimicrobial agent affected by pH is nisin; it is reported that nisin is more active at acidic pH and its activity is maximum at pH 5.5. In a study investigating the nisin activity on *L.innocua* showed that nisin caused more reduction in microbial cells at pH 5.5 rather than 6.5 and 7.2 (Sanjurjo et al., 2006).

pH is effective on strength and solubility of edible films, i.e. gelatin films (Bower, 2006) and muscle protein films (Hamaguchi et al., 2007). Solubility of proteins is affected by pH and in very high or very low pH conditions, solubility increases. When the solubility of film-forming components increases, tensile strength of film also increases (Hamaguchi et al., 2007).

The most important factors affecting the antimicrobial activity during the distribution and storage, are temperature and time. Related to temperature and time microbial stability, chemical reaction kinetics and optimum activity profile of antimicrobials show variability. To maximize the effectiveness of antimicrobial agents,

it is beneficial to choose the proper temperature which maintains the minimum microbial growth.

Release kinetic of antimicrobials is related to whether the application system is packaging or coating. Microorganisms become a threat for the food surface inside when a packaging system is used, but on a coated-food system, microorganisms contaminate the outer surface of coating. In an antimicrobial packaging system, antimicrobial agents migrate from the packaging material to food surface, keep the concentration above the m.i.c. and inhibit the microorganisms. However, in the coating system, antimicrobials diffuse into food, so the concentration inside the coating layer dilutes. This makes the coating layer ineffective for microbial protection in the further period of storage and distribution.



(a) packaging system (b) coating system

Figure 2.5. Antimicrobial packaging and antimicrobial coating systems

2.4. Antimicrobial Agents

In antimicrobial packaging systems, various types of antimicrobial agents are used. These agents may generally be categorized as chemical antimicrobials, antioxidants, biotechnology products, antimicrobial polymers, natural antimicrobials and gases (Han, 2005). Organic acids (benzoic acids, parabens, sorbates, sorbic acids, acetic acid, lactic acid, and medium-size fatty acids), fungicides (benomyl, imazalil), alcohols (ethanol) and antibiotics are examples of chemical antimicrobials. Antioxidants are effective on inhibiting the growth of aerobic bacteria and moulds by creating the anaerobic medium inside the package. Biotechnology products used as antimicrobial

agent are bacteriocins (nisin, lacticins, pediocin, diolococcin and propionicins) and fermentation products (reuterin). There are also many natural antimicrobials such as natural polymers (chitosan), natural plant extracts (cinnamon oil, horseradish oil, eugenol, thymol etc.) and natural enzymes (lysozyme, lactoperoxidase etc.) (Han, 2003; Mecitoğlu et al., 2006).

Table 2.2. Active agents used in different polymer films against different microorganisms

Antimicrobial	Packaging materials	Microorganisms	References
Organic acids			
Sorbates	Whey protein isolate	S. cerevisiae, Asp. niger	Ozdemir, 1999
Enzymes			
Lysozyme	Fish-skin gel and gelatine film	*Bacillus subtilis, Streptococcus cremoris*	Bower *et al.*, 2006
	Zein	*L. plantarum, B. subtilis*	Mecitoğlu *et al.*,2006
Lysozyme, EDTA	Pullulan film	*E. coli*	Kandemir *et al.*, 2005
Lactoperoxidase	Alginate	*L. innocua, P. fluorescens,*	Yener *et al.*, 2009
	Whey protein isolate	*Salmonella enterica* *E. coli* O157:H7, *L. monocytogenes*	Min, Harris and Krochta, 2005
Bacteriocins			
Nisin	Calcium alginate film	*Salmonella typhimirium*	Natrajan and Sheldon, 2000
	Corn-zein film	*Listeria monocytogenes*	Hoffman *et al.*, 2001;
		Brochotrix thermosphacta	Ku and Song, 2007
	Whey protein isolate		Rossi-Marquez *et al.*, 2009
Natural extracts			
Thyme, oregano	Soy protein film	*E. coli, S. aureus, E. coli* O157:H7, *P. aeruginosa, L. plantarum*	Emiroğlu *et al.*, 2010
Thymol	Zein film	-	Mastromatteo *et al., 2009*
Clove essential oil	Fish gelatin, chitosan	*L. acidophilus, Pseudomonas fluorescens, L. innocua, E. coli*	Gómez-Estaca *et al.*, 2009
Others			
Ovotransferrin	κ-carrageenan	*S. aureus, E. coli, S. typhimurium,, C. albicans*	Seol *et al.*, 2009

Antimicrobial agents inhibit microorganisms in two ways; (a) microbiocidal effect and (b) microbiostatic effect. In microbiocidal inhibition way, microorganisms

are killed or eliminated from food system. In microbiostatic inhibition way, antimicrobial agents keep the growth of microorganisms below a certain critical concentration. When designing an antimicrobial packaging system, it is important to select an appropriate antimicrobial agent which is effective on target microorganisms. For instance, sorbic acid and sorbates are antifungal agents but they do not have antibacterial activity. Also, herbs and spices are used as natural antimicrobial agents but their chemical stability is low and their acting kinetic is unknown (they may affect taste negatively) (Han, 2005). The way of inhibiting the microorganism may be different among the antimicrobial agents, i.e. some agents block the essential metabolic pathways like EDTA and lactoferrin, and some of them directly destroy the cell wall of microorganisms such as lysozyme. Selecting proper antimicrobial agent is closely related to characteristics of microorganisms such as their oxygen requirement (aerobes and anaerobes), cell wall composition (Gram positive or Gram negative), growth stage (spores or vegetative cells), optimum growth temperature (thermophilic, mesophilic or psychrotropic) and acid / osmosis resistance (Han, 2003).

2.4.1. Biopreservatives

Because of health and environmental concerns of chemical compounds, there is an increasing demand on using natural, disposable, recycling biopreservatives in active packaging (Mecitoğlu et al., 2006; Mastromatteo et al., 2009). There are various biopreservatives used in antimicrobial packaging including plant extracts, bacteriocins such as pediocin, lacticin, nisin and antimicrobial enzymes such as lysozyme, lactoperoxidase, chitinase, glucose oxidase (Labuza & Breene 1989, Suppakul et al. 2003 – Yemenicioğlu, 2005). Since polymer film technology requires severe thermal process conditions, incorporation of biopreservatives in polymer films is not suitable. Biodegredable films are more appropriate for using natural sensitive biopreservatives instead of plastic polymers because of their mild production conditions (Mecitoğlu et al., 2006).

Essential oils are effective since their phenolic compound content (such as flavanoids and phenolic acids) and they show antibacterial and antioxidant effect. Essential oils can directly be added into foods but if they are added at high concentrations, the organoleptic characteristics of food can be changed in an undesired

way. To prevent this problem, there are many edible film applications loaded with essential oils. However, essential oils have the risk of oxidation. To overcome this problem, microencapsulation of essential oils into carbohydrates (i.e. hydrolyzing and emulsifying starches and gums) can be a good solution (Mastromatteo et al., 2010). Natural plant extracts and essential oils have good antimicrobial activity such as thyme, oregano, rosemary, garlic oil (Emiroğlu et al.,2010; Pranoto et al., 2005; Seydim and Sarıkus; 2006)

Organic acids such as lactic acid, propionic acid, asetic acid, citric acid, sorbic acid and tartaric acid are effective against moulds, yeast and many bacteria. Organic acids are accepted as GRAS additives used in many edible film applications.

Natural antioxidants are the other widely used active agent. They retard the oxidation of lipids in high fat-containing foods and extend their shelf-life. Among the antioxidant compounds, tocopherols, known as vitamin E, have become very popular due to its non-toxic characteristic and it is approved by the regulations.

Bacteriocins are widely used in antimicrobial packaging applications and nisin is the one most commonly incorporated into edible films. It is produced by *Lactococcus lactis* subs. *lactis* bacterium. Nisin is effective mainly on Gram-positive bacteria (Marquez et al., 2009).

2.4.2. Lysozyme

Lysozyme is one of the most frequently used natural antimicrobial agent in antimicrobial packaging (Han, 2000; Mecitoğlu et al., 2006; Bower 2006). This hydrolyse-type enzyme split the bonds between N-acetylmuramic acid and N-acetyl glucosamine of the peptidoglycan layer on the cell wall of Gram-positive bacteria and inhibit them (Benkerroum, 2008). Since there is a protective lipopolysaccharide layer, Gram-negative bacteria are resistant to lysozyme (Mecitoğlu et al., 2006; Bower, 2006). All mammals, birds and fishes are the source of lysozyme; generally hen-egg white is used to produce lysozyme (Bower, 2006). Using lysozyme as a direct food additive is approved by regulations and widespread in foods such as cheese, vegetables and salads (Gemili et al., 2007). Hen egg-white is the richest source of lysozyme, 3400 and 5840 mg/L lysozyme can be produced from it.

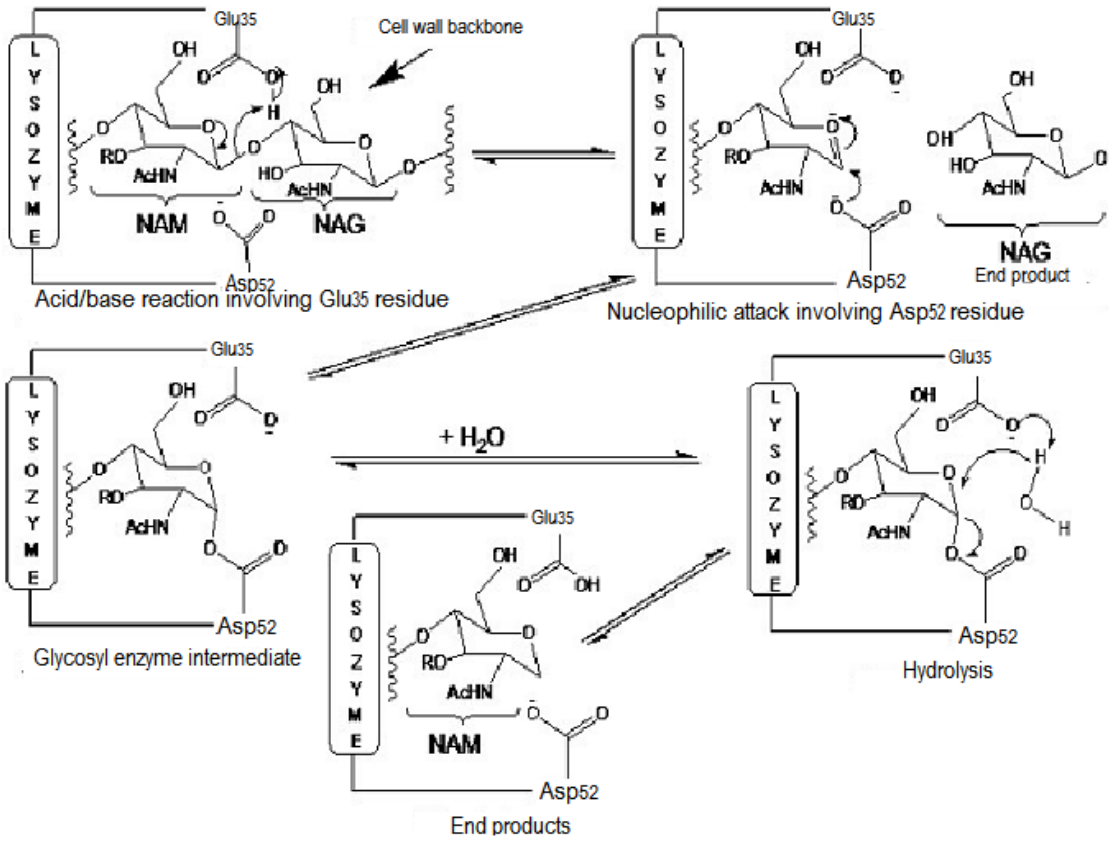


Figure 2.6. Mechanism of action of lysozyme on β1-4 linkages between N-acetylmuramic acid and N-acetylglucosamine on the bacterial cell-wall backbone

In many studies related to lysozyme incorporated antimicrobial films, commercial lysozyme was used. Although commercial lysozyme is almost pure [1-6% (w/w) protein impurities reported (Judge et al., 1998)] and show very high enzyme activity (20000 – 100000 U/mg), it is expensive and takes long time to be produced by salt crystallization method. If the use of lysozyme in food packaging industry is considered, partially purified lysozyme has some advantages such as its economic feasibility and faster producing method (Kandemir et al., 2005).

Kandemir *et al*. (2005) produced partially purified lysozyme by slight modified method of Jiang *et al*. (2001) and showed that production conditions affect the activity lysozyme, i.e. ethanol treatment. They also showed that incorporating the partially purified lysozyme into films with other components did not affect the initial activity of lysozyme but storage at 4°C may cause activity changes due to the enzyme conformation modifications or reactions between enzyme and other molecules, even so it has showed adequate stability.

CHAPTER 3

EDIBLE FILMS

Edible films are used for many years to protect food products by retarding the water vapour, oxygen, oil and solute migration. They prevent oxidation and lipid migration in high-fat containing foods and stabilize the water activity (Krochta, 1994).

Among the biodegradable materials, edible films are mostly used materials for fresh and minimally processed foods because of their edibility and biodegradability (Han, 2005; Krotcha and De Mulder-Johnston, 1997). Edible films and coatings containing antimicrobial agents protect the foods from microbial growth (Han, 2005).

Edible film components can be divided into three groups; hydrocolloids, lipids and composites. Hydrocolloids used in making edible films are proteins and carbohydrate such as cellulose derivatives, alginates, pectins, starches and other polysaccharides. Lipids proper to make film are waxes, acylglycerols and fatty acids. Composite films include lipid and hydrocolloid materials together (Krochta, 1994).

Biocomposites are widely used to develop the barrier and strength properties of edible films. Edible films have limited water resistance and strength when they are entirely made of natural compounds. Because of this drawback, edible films are modified by adding other natural fillers and stiffening materials. The natural fillers used may be fibers from various sources such as microfibrils from potato pulp, flax, ramine and wood (Mastromatteo et al., 2009)

3.1. Biodegradable Film Materials and Films

The interest in biodegradable film materials has increased, because they are environmentally-friendly and have potential to be used alternatively instead of plastic polymers. However today, it is not possible to totally replace plastic polymers with biodegradable films, in the future they have high potential for being used in antimicrobial packaging applications (Hamaguchi et al., 2007). Most of the biodegradable films are edible and they are formed under temperate conditions (Mecitoğlu et al., 2006).

3.1.1. Hydrocolloids

Hydrocolloid films are not successful to provide a good moisture barrier since they have hydrophilic nature, but they are good barriers to oxygen, lipid and carbon dioxide. Hydrocolloids are separated into groups as carbohydrates and proteins.

3.1.1.1. Protein Based Film Materials

Proteins have been predominantly used to make edible films because of their film-forming ability, relative abundance and nutritional quality (Hamaguchi et al., 2007). These materials are gelatin, casein, wheat gluten, soy protein, whey protein and zein.

Plant proteins are classified into four groups according to their solubility; albumins (water-soluble), globulins (saline-soluble), glutelins (alkali and acid-soluble) and prolamines (aqueous alcohol-soluble). Zein is the prolamin fraction of corn protein. Zein is also divided into two groups; α-zein which is soluble into 95% ethanol and β-zein which is not soluble into 95% ethanol solution. It is not soluble in water due to its highly hydrophobic amino acid composition.

Zein films are hydrophobic and consist of principally H-bonds in the film matrix. Films are tough, glossy and grease-resistant.

In related studies, different biopreservatives have been incorporated into zein films such as nisin, lysozyme (Mecitoğlu et al., 2006) and antioxidants like BHA (Mecitoğlu et al., 2006; Mastromatteo et al., 2009).

Zein protein has good ability to make pre-cast films which can be used as food wrapping or applicable as food coating (Mecitoğlu et al., 2006; Mastromatteo et al., 2009). Also there are examples of using zein films combined with modified atmosphere packaging (Mastromatteo et al., 2009).

Wheat endosperm consists of 70% wheat protein. It has two fractions; gliadin and glutenin refers to prolamin and glutelin fractions of plant proteins respectively. Gliadin fraction is soluble in 70% ethanol, so to make wheat gluten films aqueous ethanol solvent is used. Also alkaline and acidic conditions should be provided, in addition mechanical mixing and heating help the wheat gluten dispersion. When gluten disperses in alkaline solution, disulfide bonds are cleaved and reduced to sulfhydryl

groups, after casting film solution disulfide bonds are formed again and produce film structure.

Most of the soy proteins in the soybean are in globulin form. Soy protein isolate that is used in making soy protein-films contain mostly protein but also lipids and carbohydrates.

Gelatin is a protein which is obtained by partial hydrolysis or thermal degradation of collagen. Mostly used raw materials for gelatine production are fish and pork skin, as well as bovine skin and bones. Since fish-skin gelatine has less hydrogen-bonding capacity, it has not strong gelation ability and its gelation temperature is lower than cattle or pork originated gelatines (Bower, 2006).

Caseins constitute 80% of total milk proteins. It is found as rennet caseins, dry casein or caseinates commercially. When making casein films, aqueous solutions are used due to the high amount of H-bonds of casein. Casein films appear transparent, flavourless and flexible.

Total milk proteins are composed of 20% whey proteins which differentiate by the solubility characteristic at pH 4.6. Liquid whey is a by-product of cheese production and constitutes a serious waste problem, using it in edible film making may disburden this problem. Whey protein includes 5 different protein types; α-lactalbumin, β-lactoglobulin, bovine serum albumin, immunoglobulins and proteosepeptones. It also contains lactopreoxidase and lactoferrin. Whey protein isolate is the second most commonly used biodegradable film material in active packaging applications. It forms transparent, brittle films and coatings and provides good O_2, CO_2, lipid and aroma barrier properties (Marquez et al., 2009).

3.1.1.2. Carbohydrate Based Film Materials

Water-soluble polysaccharide based edible films are used to extend the shelf-life of food products by providing the oxygen and carbon dioxide barrier properties. Polysaccharides are widely used in edible film making because of their availability, low cost and non-toxic properties.

Cellulose is a polysaccharide in composition of linear (1-4)-β-D-glucopranozyl chain and exists in plant cell walls. It is not soluble in water due to the high amount of intramolecular H-bonds. Cellulose, naturally found in crystalline state, is isolated from

the cell walls in microfibrils by chemical extraction (Chandra and Rustgi, 1998). Cellulose films are tough, transparent and flexible.

Starch is a cheap and renewable material produced from cereals (i.e. corn, maize, wheat and rice) and tubers (i.e. potato, tapioca and arrowroot). It contains amylose (20-30 %) and amylopectin (70-80 %) at different ratios dependent on the source. If the viscosity, stability and thickening power properties of starch films are wanted to be good, amylopectin is used. In case of gel making and film forming properties are primarily desired, amylose should be preferred. Generally starch films are odourless, tasteless, colourless and nontoxic. However, it has some difficulties in film making methods such as problems in dispersing in water and retrogradation or making gel very rapidly.

Chitin which constitutes the skeletal construction of invertebrate animals and cell walls of fungi and green algae links up with alkali and form nontoxic material "chitosan". Chitosan films can inhibit the growth of fungi and phyto-pathogens.

CHAPTER 4

ACTIVE AGENT RELEASE MECHANISMS

Antimicrobial effect is obtained by adding antimicrobial agents into film materials and it is shown by the release of agent from film, absorption of agent by film or immobilization into film material.

4.1. Release of Antimicrobials

Diffusivity of an antimicrobial agent incorporated in an edible film is dependent on the mobility of agent in the system and the time necessary to reach steady state. Generally antimicrobial agents release kinetic is described by Fick's second law. "Fick's second law of diffusion describes species concentration change as a function of time and position." For the diffusion through a film having a thickness at one direction, the equation can be written as follows (Teerakarn et al., 2002);

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \qquad (4.1)$$

In this formula; C is the concentration of diffusing agent, D refers to diffusivity and x is the coordinate dimension in the direction of diffusion.

The antimicrobial films may contain volatile or non-volatile antimicrobial agents. Due to the physical distinctness of antimicrobial agents, their transfer occurs differently. The release of active agents can occur by direct contact of packaging material to food surface or through gas phase diffusion from packaging material to food.

Non-volatile agents should exist in packaging materials and directly contact to the food system. If the agent is exerted onto food surface, its initial concentration will be very high and it will start to form complex structures with the food components or it will be diffuse towards to the centre of the food. However, the diffusion coefficient and solubility play a significant role here, mass transfer kinetics should be proper to suppress the microbial growth (Han, 2005).

The antimicrobial packaging systems containing volatile antimicrobial agents, the direct contact is not necessary, but the concentration of the agent on the headspace should be equilibrium with the food surface and packaging materials (Han, 2005).

4.2. Types of Release Mechanisms

The release of low molecular compound from a swelling homogeneous polymeric matrix occurs by the phenomena of; (1) water penetration from the outer solution into the polymeric matrix, (2) macromolecular matrix relaxation and swelling, (3) reverse diffusion of the active agent from the swollen polymeric matrix into the outer solution (Mastromatteo et al., 2009). Diffusion of active agent from polymeric network to aqueous solution continues until a thermodynamic equilibrium between solution and film. According to Mastromatteo *et al*. (2009) there are three basic active agent release mechanisms; diffusion limited release, swelling induced release and biodegradation induced release.

4.2.1 Diffusion Limited Release (Reservoir System)

This system contains active agent and a barrier controlling the release of agent. The most frequently used barrier types that control the release are micro-porous, macro-porous and non-porous barriers. The release rate of active agent depends on the thickness of barrier, the release area and the permeability of barrier material. Release kinetics fits to zero-order kinetics which means release rate is constant. In the reservoir system, first, active agent diffuses within the reservoir and this is resulted as dissolving or fractionalizing between the reservoir carrier fluid and the barrier. Then, active agent diffuses out through the barrier layer and cause a partition between the barrier and surrounding solution (food medium) and finally it is transferred away from the barrier's outer surface to food.

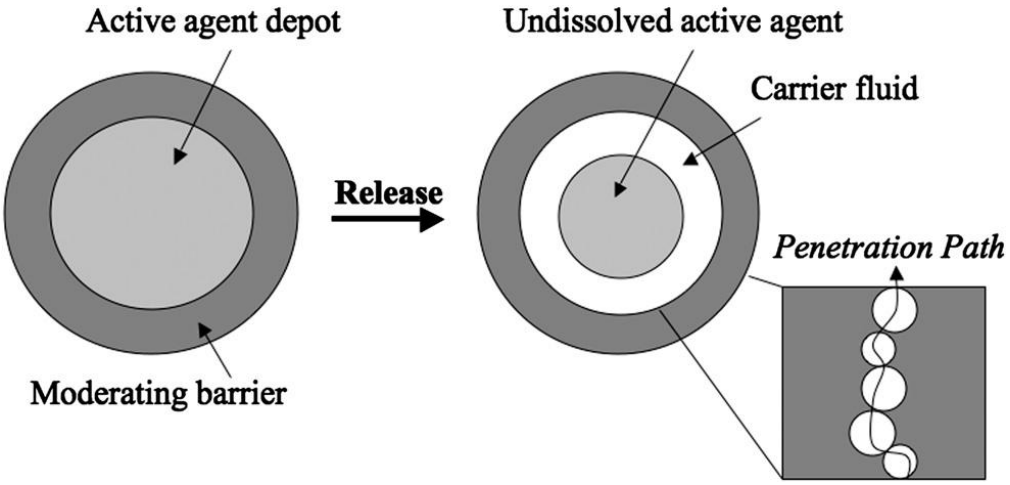


Figure 4.1. Reservoir system
(Source: Mastromatteo 2010)

4.2.2. Swelling Induced Release

In the swelling induced release mechanism, there is an active agent with a low diffusion coefficient dispersed or dissolved into a polymeric matrix and it is unavailable to diffuse. When the polymeric matrix has met with thermodynamically proper media such as aqueous solution, the matrix starts to absorb fluid and swells. This results in an increase in diffusion coefficient of active agent ant it diffused out of the polymer. The mechanism that provides to active agent to diffuse out easily is the transition of polymeric structure from glassy state to rubbery state by the help of the penetrating fluid. It is known that the polymer structure that has undergone rubbery state is more mobile than glassy state and this eases the diffusion of incorporated active agent.

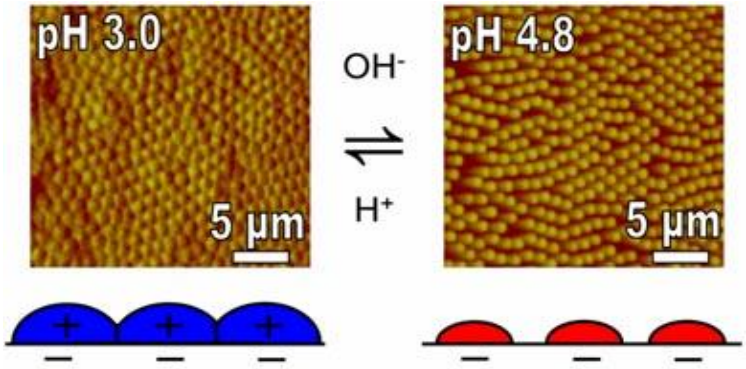


Figure 4.2. Structure change in pH-induced swelling of microgel film
(Source: newcastle.edu.au, 2011)

4.2.3. Biodegradation Induced Release

In this release system there are two different mechanisms; (1) surface erosion (heterogeneous erosion) and (2) bulk erosion (homogeneous erosion).

In surface erosion mechanism, degradation of polymeric matrix occurs before the diffusion of water into matrix. This action causes the degradation of the outer surface layer of polymer and erosion occurs heterogeneously. Mostly, the polymers have reactive functional groups, such as polyanhydrides, degrade heterogeneously.

In bulk erosion mechanism; first the hydration of polymer occurs and then biodegradation takes place through the whole polymeric matrix system homogeneously. This mechanism is mostly seen on polymers having less reactive functional groups such as poly(lactide) and poly(lactide-co-glycolide).

Release rate of active agent in biodegradable system is related with surface area.

Table 4.1. Overview of studies on release mechanism for food packaging applications

Matrix	**Active Agents**	**Release Mechanism**	**References**
Milk protein	Oregano and pimento essential oils	Swelling induced release	Ousallah *et al*., 2004
Protein microcapsules	Antimicrobial compounds	Biodegradation induced release	Kromidas *et al.,* 2006
Chitosan	Garlic oil	Swelling induced release	Pranoto *et al.,* 2005
Hydrogel	Vitamin B_{12}	Swelling induced release	Dengre *et al*., 2000
Corn zein	Thymol	Swelling induced release	Masromatteo *et al.,* 2008
Polyvinylalcohol	Lemongrass essential oil	Reservoir system	Leimann *et al*. 2009
Cellulose acetate	Theophylline	Reservoir system	Altinkaya and Yenal, 2006
Cellulose acetate	L-ascorbic acid	Swelling induced release	Gemili *et al*., 2010
Zein	Lysozyme	Swelling induced release	Güçbilmez *et al.*,2007

4.3. Controlled Release

Antimicrobial packaging system is a good way to prevent food spoilage on the surface but efficiency of antimicrobials is limited when the release is uncontrolled. Concentration becomes unbalanced with microbial growth because of the interaction with food components and diffusion into bulk of the food system (Quintavalla and Vicini, 2002; Han, 2005). The release rate should be controlled depending on the growth rate of target microorganism otherwise there some problems will occur. When the release rate of antimicrobial agent is faster than the microbial growth in the food system, concentration will be lower than the effective critical concentration during the shelf life of product. As a result of this, microorganisms will grow due to the loss of antimicrobial effect. In contrast, if antimicrobials release slowly and cannot reach the minimum inhibitory concentration before the microbial growth, food will be deteriorated (Han, 2005). To overcome all of these problems, various comprehensive researches of controlled release systems for active agents have been conducted.

The packaging system that can maintain the controlled release mechanisms is known as controlled release packaging; the basic devices in this system is packaging material act as a delivery tool and active agents such as antimicrobials, antioxidants and flavours. Controlled release packaging is the most innovative and ever-developing active packaging technology. Controlled release in drug delivery systems is used for many years but its applications on food system are new and also essential. There are various types of controlled release packaging systems being established (LaCoste et al., 2005).

Controlled release systems are able to retain the antimicrobial agents in network and transported from polymeric matrix to food surface and replenish the consumed antimicrobial components in the manner that maintaining predetermined concentration of agents on the surface during a determined period of time (Mastromatteo et al., 2009-2010). Controlled release systems can limit the consumption of antimicrobial concentration on the surface. When the antimicrobials decreases on the surface, even if it is not necessary, higher amount of antimicrobials should be added to maintain the stable concentration and this is unwanted when sensorial and consumer demands are considered.

In the figure below, the relation between solubility and the release rate of antimicrobials is shown. Figure A represents the highly soluble antimicrobial in the food. Because of the rapid dissolving of antimicrobial on the food surface, release type fits to free diffusion model and its concentration reaches very high level in a very short time. However, after a short period, the concentration decreases below the minimum inhibitory concentration. In figure B, slow-release system is given. The antimicrobial agent is less soluble and incorporated into a packaging layer. Diffusion is slower than free diffusion model, its concentration lasts longer above the m.i.c. than figure A but after a while it diffuses into bulk food. In figure C, antimicrobial agent release is controlled by a membrane and its concentration keeps above the m.i.c. during the whole storage time. Here the release kinetic fits to zero-order and release flux is constant. It is obvious that efficiency of antimicrobials lasts longer on the food surface when the release is controlled by a mechanism.

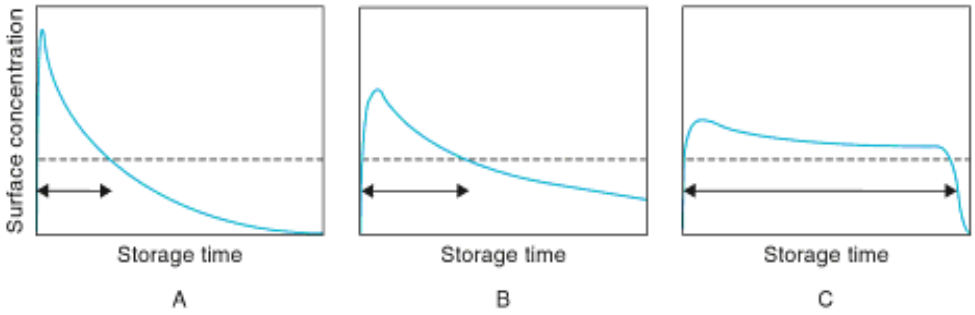


Figure 4.3. Release profiles of antimicrobials; (A) free diffusion from packaging material, (B) slow-diffusion from monolithic packaging material, (C) controlled release from permeable membrane

As much as the controlled release of antimicrobials is important on the food surface, release of antioxidants, essential oils and organic acids is also critical. The release of antioxidants should be controlled to maintain the effective concentration since the oxidative reactions initiates on the food surface (Benjakul, 2008). If high amount of antioxidant added directly into food, rapid depletion of antioxidant would have occurred (LaCoste, 2005).

To sum up, controlled release packaging has two advantages when compared to the active packaging applications;

- Preventing the diffusion of high concentration of antimicrobial into food bulk and keep the protective effect stable

- Providing a selective antimicrobial action on the food surface and prevent overuse of antimicrobials.

4.4. Application of Controlled Release Systems on Antimicrobial Films

Han (1996) and Floros (2000) showed that using multilayer film structure is an effective way to control the diffusion of active agent. The release of lysozyme from polyvinyl alcohol has been controlled by 3-layered film system. Also natural fibers were used to adjust the mass transport properties of edible films (Mastromatteo et al., 2009)

Mastromatteo *et al*. (2009) have developed multilayer zein films loaded with spelt bran to control the release of thymol. Here, natural fibers are used to adjust the release rate of active agent. According to the results it is shown that, as the film thickness and number of layers increases release rate of active agent decreases. However, adding fibres into film matrix has formed micro-channels that promoted release of thymol. As the fibre concentration increases, release rate has slowed down since there occurred micro-voids acts as a trap that prevent the release.

Han, Castell-Perez and Moreira evaluated the effect of electron beam irradiation on the stability of *trans*-cinnamaldehyde and the effect of pH and storage temperature on release kinetic from LDPE/polyamide film. In the results, crosslinking of *trans*-cinnamaldehyde in LDPE/polyamide and slow release of this compound had been obtained.

Yemenicioğlu *et al.* (2009) developed zein films incorporated with lysozyme, EDTA and legume protein extract and investigate the films effect on beef burgers. Addition of legume proteins into zein films had enhanced the distribution of lysozyme within the film matrix, increased the bind-lysozyme activity and reduced the soluble lysozyme activity. In this way, the released lysozyme concentration on the food surface had been decreased and antimicrobial effect of films had been sustained for longer storage period.

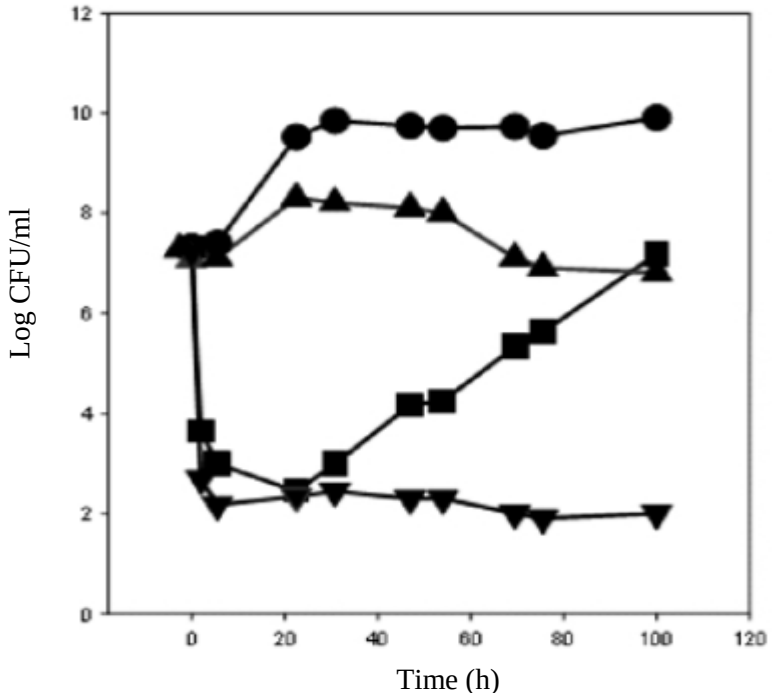


Figure 4.4. Sensitivity of *L.monocytogenes* under three delivery mechanisms of nisin; (●) is control,(■) is direct addition, (▲) is slow release of nisin and (▼) is combination of direct addition and slow release (Source: LaCoste et al., 2005)

According to the study of LaCoste *et al.* (2005) antimicrobial activity of nisin delivered by direct addition and slow release was challenged with *L. monocytogenes.* In the first model, nisin was instantly added into broth medium which is simulating the direct addition of antimicrobials into food systems. This model caused an immediate inhibition of microorganisms (5log) but approximately after 20 hours, survival organisms and adapted cells had kept on growing and microbial count increased again and reached the initial concentration. In the second model, same amount of nisin was released slowly into broth medium and only a slight reduction in the viable cell count. In the combination of both models, direct addition caused sharp inhibition and than continuous release of nisin into medium kept the living cell concentration at low levels. That combined model is parallel with the controlled release system, it is showed that controlled release packaging is the most effective way to inhibit and suppress the microbial growth on the surface.

In the study of Ouattara *et al.*, release of propionic and acetic acid was controlled by limitation of water diffuses into chitosan film matrix.

In the controlled release techniques, pH is a commonly used factor to control the diffusivity of active agent from the polymer film matrix.

Marquez *et al.* (2009) investigated the effect of pH on the diffusivity of nisin from whey protein isolate films. The release of nisin had been conducted at pH 7, pH 4 and pH 5. It was found that; the highest release had occurred at pH 4. At pH 7; very low release of nisin had observed. The reason of very small release at pH 7 is electrostatic interaction between proteins and nisin. At that pH, which is below the pI of nisin (~10), nisin has positive charge and whey proteins have negative charge because the pH is higher than whey proteins isoelectric point (4.2- 5.3). At pH 4 both nisin and whey proteins have net positive charge and due to the electrostatic repulsion release rate of nisin had reached its maximum level. At pH 5 electrostatic interaction between whey protein fractions (pI of β-lactoglobulin : 5.3 and pI of α-lactalbumin : 4.3) is bigger than ph 4, this causes a reduction in nisin release amount. The release rate of nisin is shown in Figure 4.3.

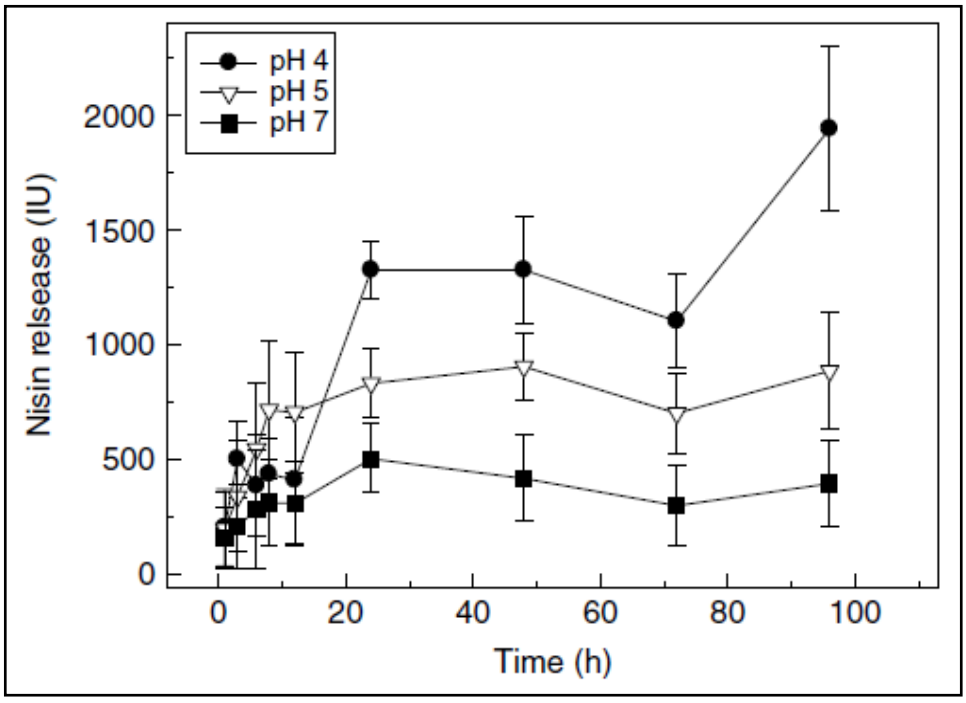


Figure 4.5. Release kinetic of nisin at pH 4, pH 5 and pH 7

(Source: Marquez et al., 2009)

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

Zein, commercial lysozyme, *Micrococcus lysodeicticus*, dialysis tubes (12000 MW, prepared as described in the product manual) and beeswax were obtained from Sigma Chem. Co. (St. Louis, Mo., USA). Whey protein isolate (BiPRO®, 97.8% protein) was obtained from Davisco Foods International, Inc. (MN, USA). Oleic acid (90%) was obtained from Sigma- Aldrich, USA. Fresh hen eggs, used in preparation of partially purified lysozyme, were purchased from a local supermarket in İzmir, Turkey. Glycerol was obtained from Merck (Darmstadt, Germany). The Ali dayı cultivar of Lentil, used to produce lentil protein extraction, was provided by the General Directorate of Agricultural Research in Ankara, Turkey. Bacterial strain of *Listeria innocua* (NRRL B-33314) used in antimicrobial zone tests was provided by United States Department of Agriculture, Microbial Genomics and Bioprocessing Research Unit, Peoria, Illinois.

5.2. Methods

In this study zein and WPI films are produced. To determine the lysozyme activity released from the films, released tests and antimicrobial zone tests are conducted.

5.2.1. Preparation of Partially Purified Lysozyme

Lysozyme was partially purified from hen egg white by given by Jiang et al. (2001). In substance, the egg white and egg yolk were separated and a certain volume of egg white was diluted 3-fold with 0.05 M NaCl solution. pH of the solution was set to 4.0 by adding certain volume of 1 N acetic acid to precipitate egg white proteins except

lysozyme and final solution was diluted 2-fold with 60 % (v/v) ethanol. After 6 h incubation at room temperature, the mixture was centrifuged at 15000 *g* for 15 min at 4 °C and the precipitate was discarded. The supernatant containing lysozyme was dialyzed by using 12000 MW dialysis tubes for 21 h at 4 °C with 3 changes of 2 L deionized water. The lysozyme solution remained after dialysis was lyophilized by using a freeze drier (Labconco, FreeZone, 6 litre, 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 before preparation of each film.

5.2.2. Preparation of Acetone Powders Used for Protein Extraction

To remove phenolic compounds and lipids, legumes were processed to acetone powder according to the method given by Arcan and Yemenicioğlu (2006). To prepare aceton powder, 50 g of dry lentil samples were dehydrated by soaking 150 ml deionized water for 16-18h. Dehydrated lentil samples were homogenized in a crusher (Waring 7011HS) for 2 min with 200 ml of cold acetone stored at -18 °C . The slurry obtained after crushing filtered using Whatman No:1 filter paper through the instrument of Buchner funnel under vacuum and the solid residue was collected. Same treatment was repeated twice more to solid residue by using fresh cold acetone. Last solid residue remained on filter paper was dried under room conditions and stored at -18 °C.

5.2.3. Preparation of Lentil Globulin Extract

The lyophilized lentil globulin extract was obtained from lentil acetone powder by alkaline extraction method given in Kaur (2006). Acetone powder dispersion was prepared at room temperature for 30 min under continuous magnetic stirring and its pH was adjusted to 9 by using 1 N NaOH. To remove the non-protein fractions (fiber and starch) alkali dispersion was centrifuged at 15000 g for 30 min (temperature of 4 °C) than the residue was discarded. The supernatant, containing solubilized proteins, was taken and its pH was set to 4,5 by using 1 N acetic acid solution. Isoelectrically precipitated globulin fraction of lentil protein was separated by centrifugation at 15000

g/30 min at 4 °C temperature. The precipitate was mashed with 150 ml distilled water (pH 7.0) and the solution was lyophilized with Labconco FreeZone freeze dryer.

5.2.4. Determination of Lyophilized Lysozyme Activity

Lysozyme activity was measured spectrophotometrically at 660 nm by using Shimadzu Model (2450) spectrophotometer equipped with a constant temperature cell holder set to 30°C. Reaction mixture was prepared by mixing 0.1 ml sample (incubated at 30 °C for 1 min) and 2.4 ml *Micrococcus lysodeicticus* suspension prepared in 7.0 pH Na-phosphate buffer. After fairly vortexing the reaction mixture, the decrease in absorbance of *Micrococcus lysodeicticus* was monitored for 120 sec. Enzyme activity was calculated from the slope of initial portion of absorbance vs. time curve and expressed as Unit per cm^2 of film area. To calculate the average activity of enzyme, the activity of three samples were taken. One Unit was defined as 0.001 change in absorbance within 1 min time interval.

5.2.5. Preparation of Zein Films

Zein films were prepared by following the method of Padgett et al. (1998). In this method, 1.4 g of corn zein was dissolved in 8.2 ml ethanol (96%) by mixing slowly with magnetic stirrer for 30 min. At the 25^{th} minute of stirring, 0.4 ml glycerol was added into solution. Film solution was heated until boiling point and boiled for 5 min for denaturation of zein proteins. After cooling to room temperature, commercial or partially purified lysozyme was added into film solution to maintain the final lysozyme concentration as 0.7 – 1.4 mg/cm^2. Lysozyme was homogeneously dispersed by homogenization at 10000 rpm for 4 min and the 4.3 g of film solution was spread onto 8.5x8.5 cm glass plates, previously cleaned ethanol. Casted films were dried under 50% relative humidity and 25 °C using controlled test cabinet. After drying, 4x4 cm film was smoothly peeled off and used release tests.

5.2.6. Preparation of Whey Films

Whey protein isolate (WPI) films are prepared by following the method of McHugh and Krochta (1994). Briefly, 10% (w/w) of WPI solution prepared in distilled water using magnetic stirrer. 0,6g of glycerol was added and solution was kept mixing 5 min. To obtain the film and denaturate the whey proteins, the solution was kept in water bath at 90 °C for 30 min. Once cooled in ice bath for 5 min, oleic acid (9%) or wax (30%) and lysozyme were added into the solution, than homogenization was performed at 10000 rpm for 4 min. 5 g of Active agent incorporated WPI film-forming solution was cast onto plastic Petri dishes (9-cm inner diameter) and dried under controlled test cabin conditions (50% relative humidity and 25 °C) for 24h.

5.2.7. Photographs and Thickness of Films

The 3-dimentional cross-sectional structure of films were scanned using a scanning electron microscope (SEM) (Philips XL 30S FEG, FEI Company, Eindhoven, Netherlands) in the Center for Materials Research of İzmir Institute of Technology, İzmir, Turkey. 0.8 mm film strips were broken in liquid nitrogen to view the surface of cross-section area clearly and films were covered by gold palladium for 1 min in a Magnetron Sputter Coating Instrument to eliminate charging effect. Cross-sectional micrographs of covered films were examined by SEM at a magnification of 500x, 1500x, 5000x and 10000x respectively. The average thicknesses of films were measured from the 500x magnified micrographs.

5.2.8. Release Test

Release tests of lysozyme incorporated films were conducted in an incubator (SANYO, MIR-154, JAPAN) at 4 °C. 4x4 cm zein films and 14.2 cm^2 WPI films were put in glass Petri dishes (10 cm inner diameter) containing 50 ml buffer solutions at determined pH values. During the release tests; pH 7.3 – 6.3 – 5.3 – 4.3, 0.05 M Na-phosphate buffers were used for zein films and pH 7.0 – 6.0 – 5.0 – 4.5 – 4.0 – 3.0, 0.05 M Na-phosphate and Na-acetate buffers were used for WPI films as release media. The

films placed into buffer solutions were incubated with continuous shaking at 80 rpm until the release of lysozyme reached a stable level.

Lysozyme activity released from films was monitored by taking 0.3 ml aliquots from the buffer media at different time intervals. Then, activity was measured for 3 times using 0.1 ml of the taken aliquot in a single measurement. The activity was determined as described in 5.2.4 section using Shimadzu Model (2450) spectrophotometer. Calculations were corrected by considering the total activity removed from the buffer solution during sampling. Monitoring was ended when the activity of lysozyme became stable and started to show slight reduction.

Two types of release ways was arranged; monitoring the release of same piece of film with changing pH media in every 24h and monitoring the release of more than one piece of films in the different pH buffers at the same time. At the first mechanism; only one piece of film (4x4 cm) was put into the buffer solution at determined pH. After 24h release in the first buffer has stopped, the film was taken and put into second buffer with different pH value. This arrangement was applied on the film started from the higher-pH buffer to lower one, which was simulating the pH drop on foods. In the second release schedule; more than one film pieces (4x4 cm) were put into buffer solutions with different pH values at the same time and release was started. Samples were taken from each buffer solution for 5-12 days related to the progression of lysozyme activity increase.

5.2.9. Mechanical Properties

Tensile strength, elongation at break and elastic modulus of films were determined using TA XT texture analyzer. 0.8 cm in width film strips were exposed to constant rate of stress at 5 mm/min until shear off. Test was conducted in accordance with ASTM D882-02 standard. Tensile properties were calculated from the plot of stress versus strain and film thicknesses obtained from SEM photographs (500x) were used in calculations.

5.2.10. Antimicrobial Activity of Films

Antimicrobial activity of zein and WPI films were determined with the zone inhibition assay by using *Listeria innocua* (NRRL B-33314) on agar media. The overnight cultures were incubated in nutrient agar medium at 37 °C. After incubation MacFarland was set to 1.0 with 1% peptone water and inoculated on nutrient agar medium. 15 disks were cut from 8.5x8.5 films by a cork-borer under aseptic conditions and randomly selected 3 discs were placed onto inoculated agar. Petri dishes were incubated at 37 °C for 48h and area of zones around the discs was calculated by measuring the diameter of zones with a micrometer.

5.2.11. Statistical Analysis

Statistical analysis was performed by using MINITAB® release 14 (Minitab Inc., State College, Pa., U.S.A.). LYS activities, antimicrobial inhibition zones and mechanical properties were analyzed by using one-way analysis of variance (ANOVA). Significance was accepted at $p < 0.05$.

CHAPTER 6

RESULTS AND DISCUSSION

6.1. Development of pH-controlled Triggering Mechanisms for Controlled Release of Lysozyme from Zein Films

6.1.1. Scientific Basis of Planned pH-controlled Triggering Mechanism in Zein Films

The zein was a highly hydrophobic protein which has little amounts of charged groups. Thus, in lysozyme incorporated zein films almost no charge-charge interactions are formed between hydrophobic film matrix and hydrophilic enzyme which has pI greater than 10.0 and bears a net positive charge in almost all food systems. To create a pH-controlled release system for lysozyme a composite film was obtained by incorporating different amounts of lentil protein isolate (LPI) into zein films. The LPI has pI between 4.5 and 6.0 and it is capable to form negative charges above pH 4.5 to bind positively charged lysozyme. It was proposed that the bind positively charged lysozyme by the negatively charged LPI within composite film matrix could be released from the films when release medium pH was reduced below 4.5 and LPI turned its charge form negative to positive.

6.1.2. Effects of pH and LPI on Lysozyme Activity

Before studying the lysozyme release profiles of zein and zein-LPI composites at different pH values, stability of lysozyme was determined at different pH values and in presence of LPI. As seen in Figure 6.1, the enzyme showed a high stability between pH 4.3 and 7.3. The LPI incorporated into zein films also did not contain any inhibitors or activators causing considerable changes in lysozyme activity (Figure 6.2). Thus, it was accepted that the changes in lysozyme activity in release test medium were related to release of enzyme from the films or absorption of enzyme by the films.

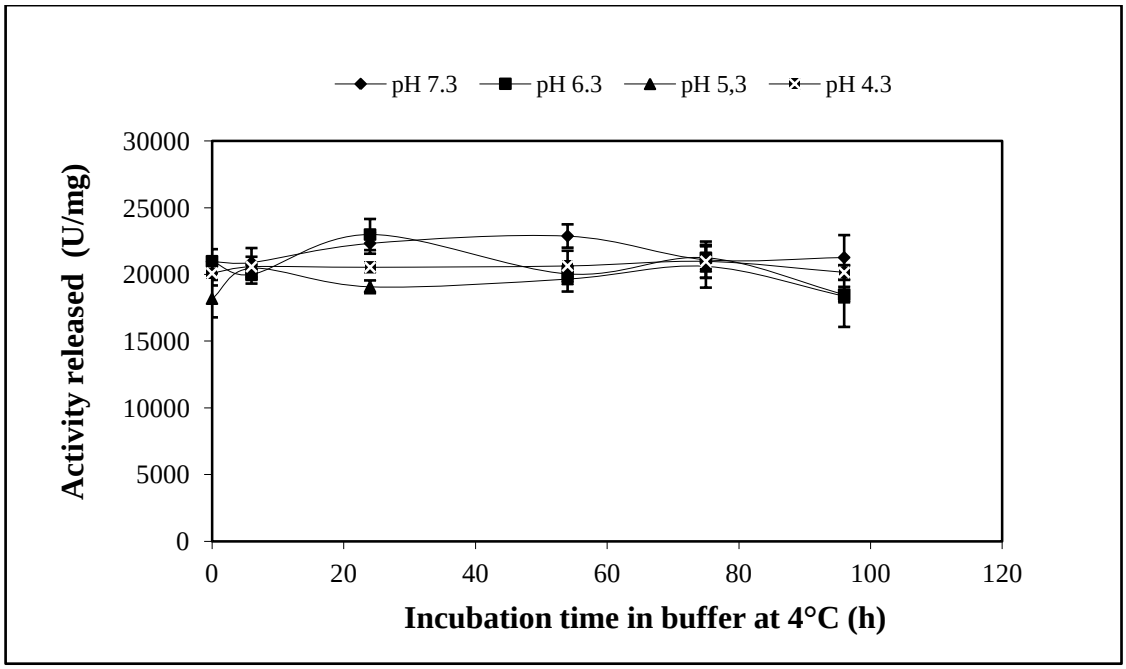


Figure 6.1. Effect of pH on lysozyme activity at 4°C (tests were conducted in different 0.05 M Na-phosphate buffers)

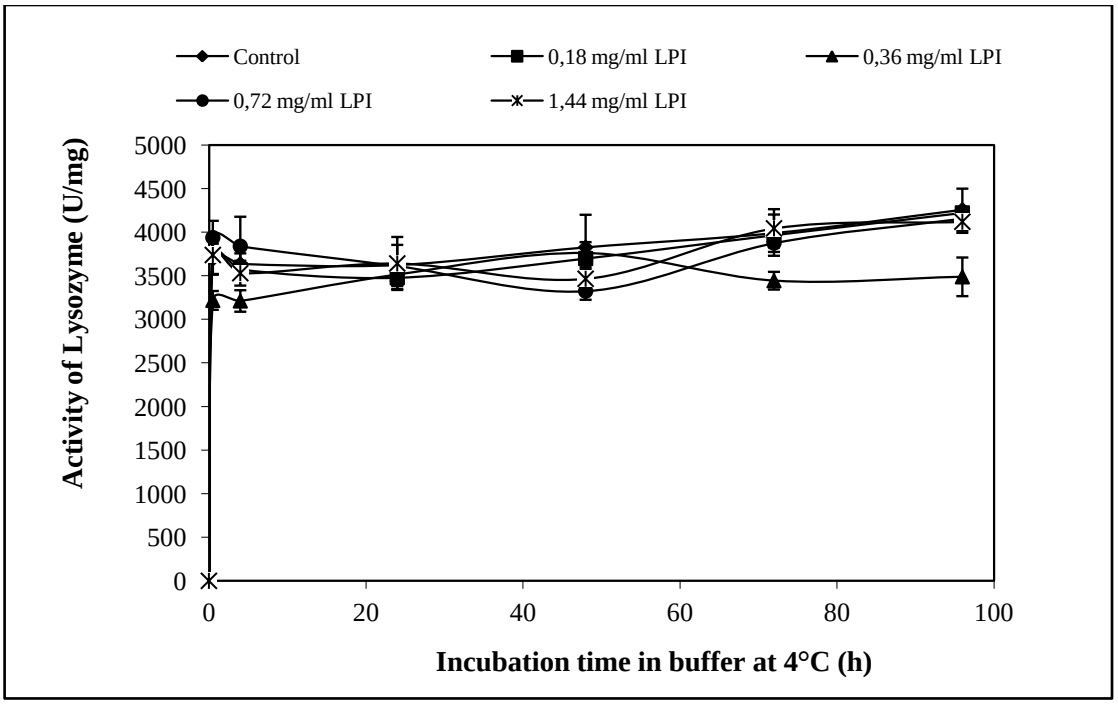


Figure 6.2. Effect of LPE on lysozyme activity 4°C (tests were conducted in distilled water containing different amounts of LPI)

6.1.3. pH-dependent Release Profiles of Partially Purified Lysozyme from Zein and Zein-LPI Composite Films

To determine their pH-dependent release profiles each film was kept in a series of different Na-phosphate buffers, first at pH 7.3 and then at pH 6.3, 5.3 and 4.3. The films were kept for 24h at different pH values with the exception of pH 4.3 buffer which was the final release medium for all films. At pH 7.3, the highest amount of lysozyme was released from standard zein films (Figure 6.3). However, the total amount of lysozyme released from the films at pH 7.3 reduced as LPI concentration in the films increased from 1.5 to 4.5 mg/cm2. This result clearly showed the binding of positively charged lysozyme by the LPI which bear a net negative charge at pH 7.3. The films did not release the bind lysozyme when they moved from pH 7.3 buffer after 24h and transferred into pH 6.3 buffer for the next 24h incubation. No enzyme release was also observed from the films when they were transferred to pH 5.3 buffer and incubated for additional 24h. In contrast, transfer of films into pH 4.3 buffer initiated the release of bind lysozyme from LPI containing films. It was clear that the reduction in pH of buffer to 4.3 caused the protonation of negatively charged groups of LPI (pI: 4.5-6.0) and triggered release of bound positively charged lysozyme from the films. The amount of released lysozyme at pH 4.3 increased as LPI concentration in the films was increased.

To determine the lysozyme binding capacity of zein-LPI composite films and increase the fraction of enzyme released by the triggering mechanism at pH 4.3, the amount of partially purified lysozyme within the films was increased from 0.7 to 1.4 mg/cm^2. As seen in Figure 6.4A, the increase of lysozyme concentration within the films increased the total amount of lysozyme released at both pH 7.3 and 4.3. The amount of bind lysozyme by the films released at pH 4.3 increased 1.5 to 2.5 folds by increase of lysozyme concentration from 0.7 to 1.4 mg/cm^2. Thus, it appeared that the increased lysozyme concentration within the films increases the interaction and charge-charge binding between lysozyme and LPI.

pI of lysozyme is 10.5 (Alderton and Fevold, 1946). It was expected that as the pH decreased, the released lysozyme activity would have increased due to the repulsing effect of same charges gained because of the isoelectric points of lentil globulins and lysozyme. As the pH of medium gets close to the isoelectric point of lentil globulins, positive charge of globulins increases. Also lysozyme is positively charged below pH 10.5 so as the pH decreases towards 4.5, both molecules would have positive charge and

repel each other. This mechanism induces the increase of released lysozyme concentration as the pH decreases.

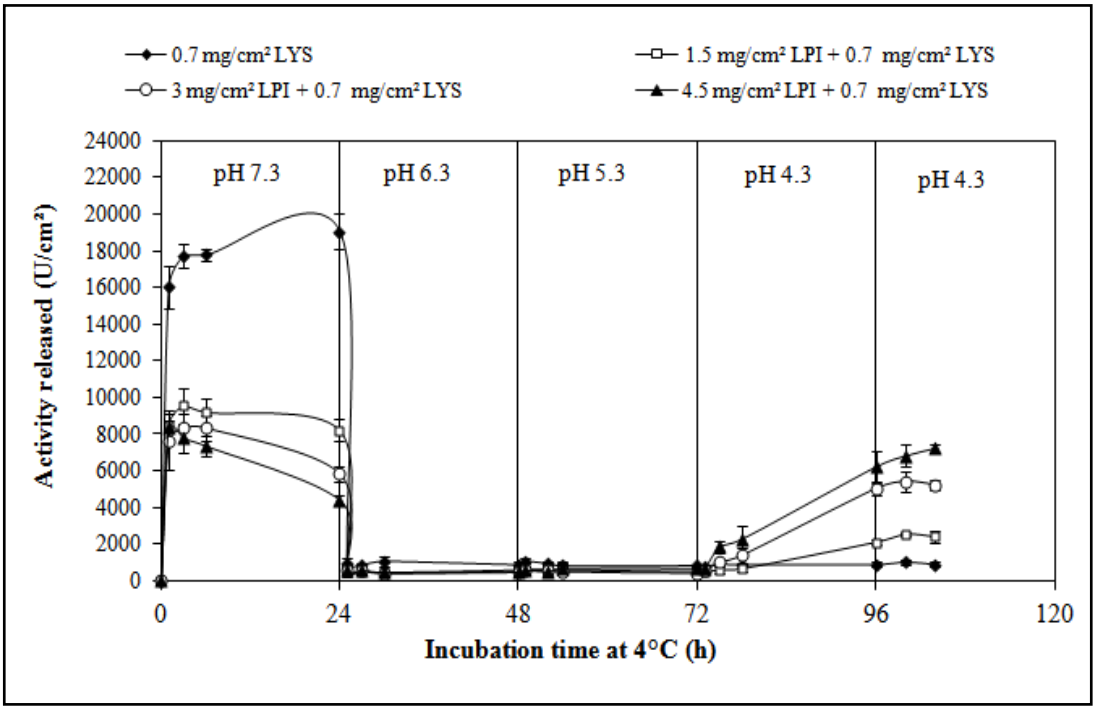


Figure 6.3. Relase profiles of partially purified lysozyme from zein and zein-LPI composite films kept in a series of buffers at different pH values (films contained 0.7 mg/cm^2 lysozyme)

To determine the storage stability of lyoszyme in zein and zein-LPI composites some of the films were cold stored at 4^{o}C for 1 month and they were once more tested for their release profiles. The release profiles of cold stored films were quite similar with those of controls which release tests were conducted immediately after film drying (Figure 6.4A and B). The total amounts of lysozyme released from cold stored films were also only slightly lower than those of respective controls (See Table 6.1). Thus, it is clear that the pre-cast zein-LPI composite films could be cold stored without loss of enzyme activity and pH-controlled release properties.

Table 6.1. Total amounts of lysozyme released from different zein and zein-LPI composite films at different pH buffers

Incorporated concentrations (mg/cm^2)		**Released lysozyme activity in 24h (U/cm^2)**				**Total activity (U/cm^2)**
LYS	**LG**	**pH 7.3**	**pH 6.3**	**pH 5.3**	**pH 4.3**	
Films containing partially purified lysozyme						
1.4	-	26060±1339 (21641±1661)[a]	1195±185 (1153,5±115)	1213±121 (1032±246)	1346±228 (1047±151)	29815 (24875)
1.4	1.5	17942±647 (16600±1627)	1228±84 (1213±102)	986±69 (1287±149)	6418±937 (5824±154)	26575 (24989)
1.4	3	14892±920 (11314±540)	1203±74 (874±90)	1911±446 (1469±184)	8855±739 (9217±59)	26863 (22875)
1.4	4.5	7333±774 (6354 ±789)	1244±189 (700±83)	1799±684 (1403±322)	11762±155 (11577±774)	22139 (20035)
0.7	-	19045±955	900±133	834±51.8	899±112	21680.3
0.7	1.5	8194±592	597±95	502±4.14	2411±150	11706
0.7	3	5838±424	515±80.7	434±51	5257±381	12046
0.7	4.5	4397±271	474±147	693±15.2	7236±381	12801
Films containing pure lysozyme						
0.7	-	69910±4991	3033±117	2938±66	2535±17	78416
0.7	1.5	71533±245	5733±370	5488±311	9834±761	92588
0.7	3	49429±3630	6436±586	7067±294	17469±613	80401
0.7	4.5	36865±2397	6785±419	8923±246	22815±433	75388

[a]the values within the parenthesis were activity of films cold stored for 1 month at 4°C.

The release profiles of lysozyme were determined also by using pure commercial lysozyme in place of partially purified lysozyme. This was done to better see charge-charge interactions between LPI and lysozyme without the presence of egg white protein impurities. The pure lysozyme had almost 4-6 fold higher activity than

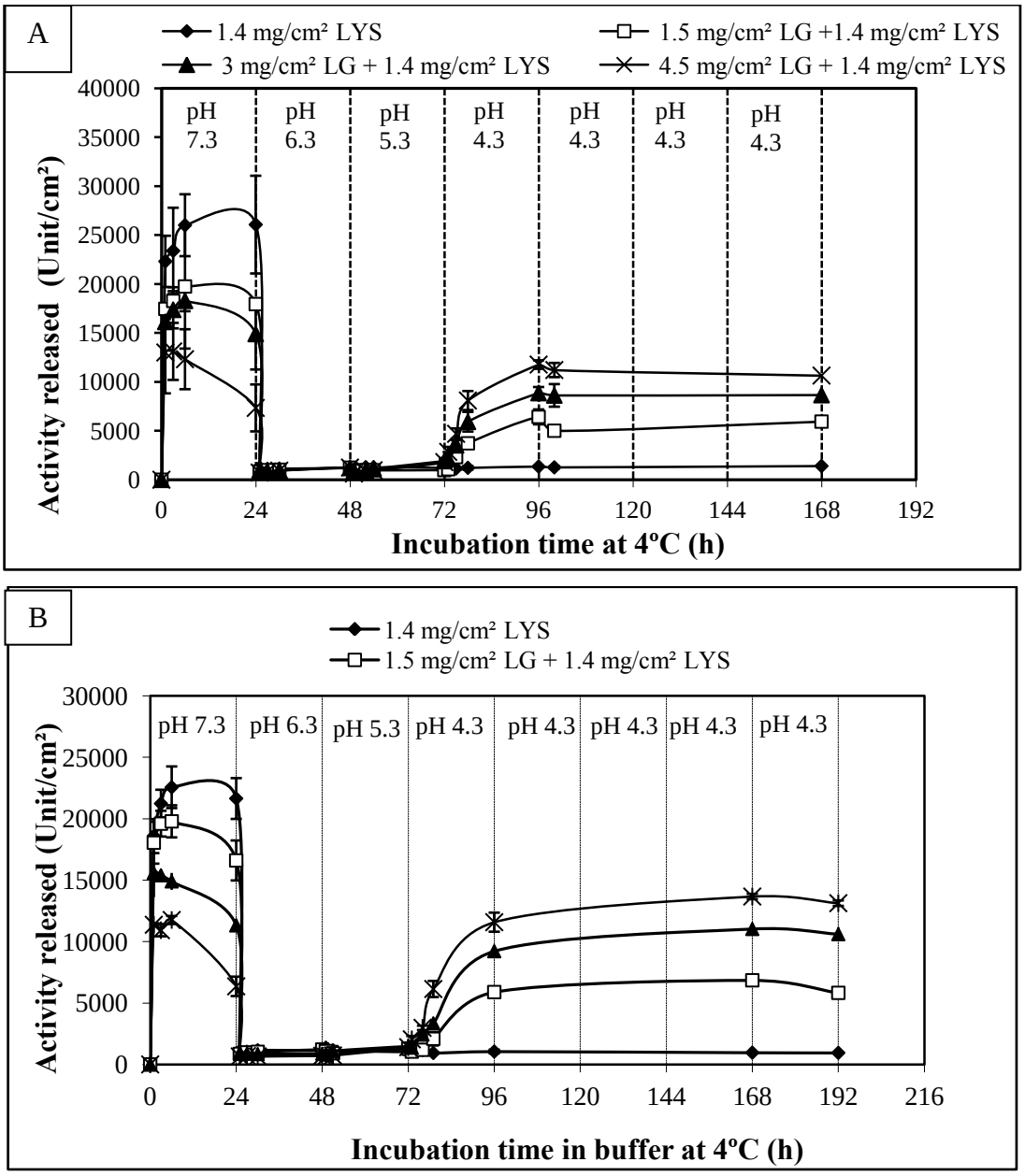


Figure 6.4. Release profiles of partially purified lysozyme from zein and zein-LPI composite films kept in a series of buffers at different pH values (Films were used in release tests immediately after drying (A) and after 1 month cold storage at 4°C (B); films contained 1.4 mg/cm^2 lysozyme)

partially purified lysozyme. Thus, use of pure enzyme was expected to increase the amount and activity of bind lysozyme and released lysozyme when pH was reduced below pI of LPI. The release profiles of zein and zein-LPI composites containing lysozyme showed some differences at pH 7.3. The incorporation of 1.5 mg/cm^2 LPI with 0.7 mg/cm^2 pure lysozyme did not cause a reduction in enzyme activity as observed for partially purified lysozyme. It seemed that the pure enzyme was not bound by LPI when it was used at 1.5 mg/cm^2 in zein-LPI composite. However, the lysozyme

activity reduced considerably as LPI concentration was increased to 3.0 and 4.5 mg/cm^2. Similar to release curves of partially purified lysozyme, small amounts of pure lysozyme released at pH 6.3 and 5.3. This showed the effectiveness of charge-charge interactions formed between pure lysozyme and LPI. As expected, the release of lysozyme triggered as films were transferred from pH 5.3 buffer to pH 4.3 buffer. Thus, this result once more confirmed the working of triggering mechanism based on pI of LPI. The higher the LPI within the films resulted with higher activity release at pH 4.3. The total amounts of pure lysozyme activity released from LPI containing films at pH 7.3 and 4.3 was 8-9 and 3-3.5 fold higher than those determined for partially purified lysozyme (comparison for 0.7 mg/cm2 lysozyme containing films).

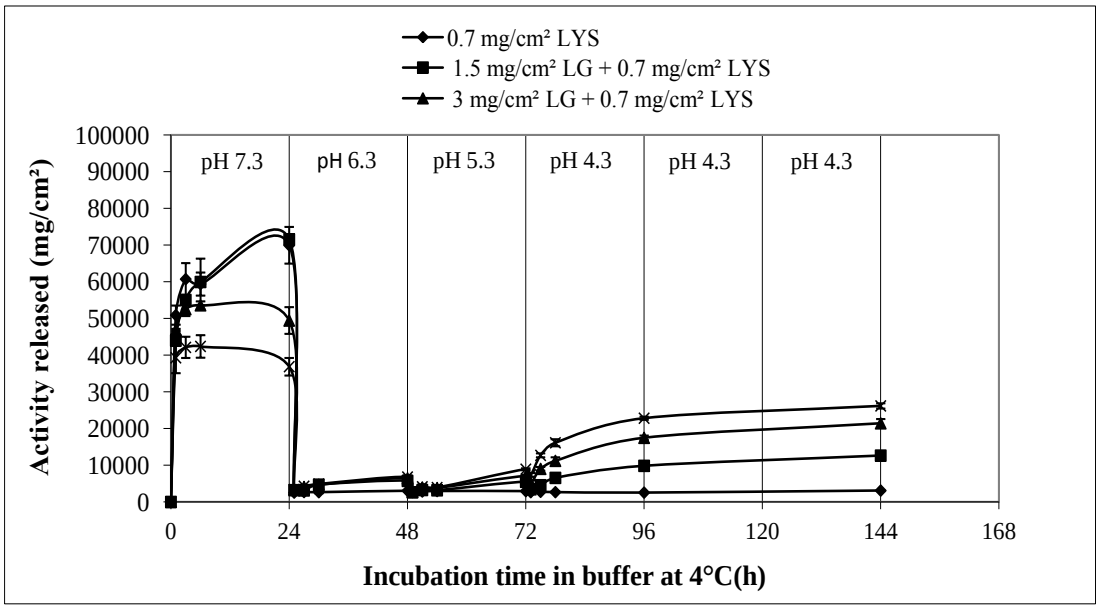


Figure 6.5. Relase profiles of pure commerical lysozyme from zein and zein-LPI composite films kept in a series of buffers at different pH values (films contained 0.7 mg/cm^2 lysozyme)

6.1.4. Antimicrobial Activity of Zein and Zein-LPI Composite Films

The antimicrobial activity of developed zein and zein-LPI films were tested against non-pathogenic *Listeria innocua*, which is used as an indicator about potential activity of films against the critical pathogenic bacteria *L. monocytogenes*. The classical zone inhibition tests clearly showed the antimicrobial activity of zein and zein-LPI composites containing pure of partially purified lysozyme. The films containing 1.4

mg/cm^2 partially purified lysozyme gave similar zone diameters with 0.7 mg/cm^2 pure lysozyme containing films. No negative effects of LPI on antimicrobial activity were determined. Thus, it seemed that the films still contained sufficient amounts of soluble enzyme to release and show antimicrobial effect on test microorganism.

Table 6.2. Antimicrobial activivty of pure lysozyme containing zein and zein-LPI composites on *L. innocua*

Incorporated concentrations (mg/cm^2)		**Average area of zones (cm^2)**
Pure lysozyme	Lentil globulin	
0.7	-	0.74 ± 0.27^a
0.7	1.5	0.68 ± 0.22^a
0.7	3	0.78 ± 0.26^a
0.7	4.5	0.78 ± 0.19^a

[a]different letter at each row indicate statistially significant changes at $p<0.05$

Table 6.3. Antimicrobial activivty of partially purified lysozyme containing zein and zein-LPI composites on *L. innocua*

Incorporated concentrations (mg/cm^2)		**Average area of zones (cm^2)**
Partially purified lysozyme	Lentil globulin	
0.7	-	0.63 ± 0.14^a
1.4	-	0.61 ± 0.15^a
1.4	1.5	0.64 ± 0.11^a
1.4	3	0.68 ± 0.15^a
1.4	4.5	0.75 ± 0.13^b

[a]different letter at each row indicate statistially significant changes at $p<0.05$

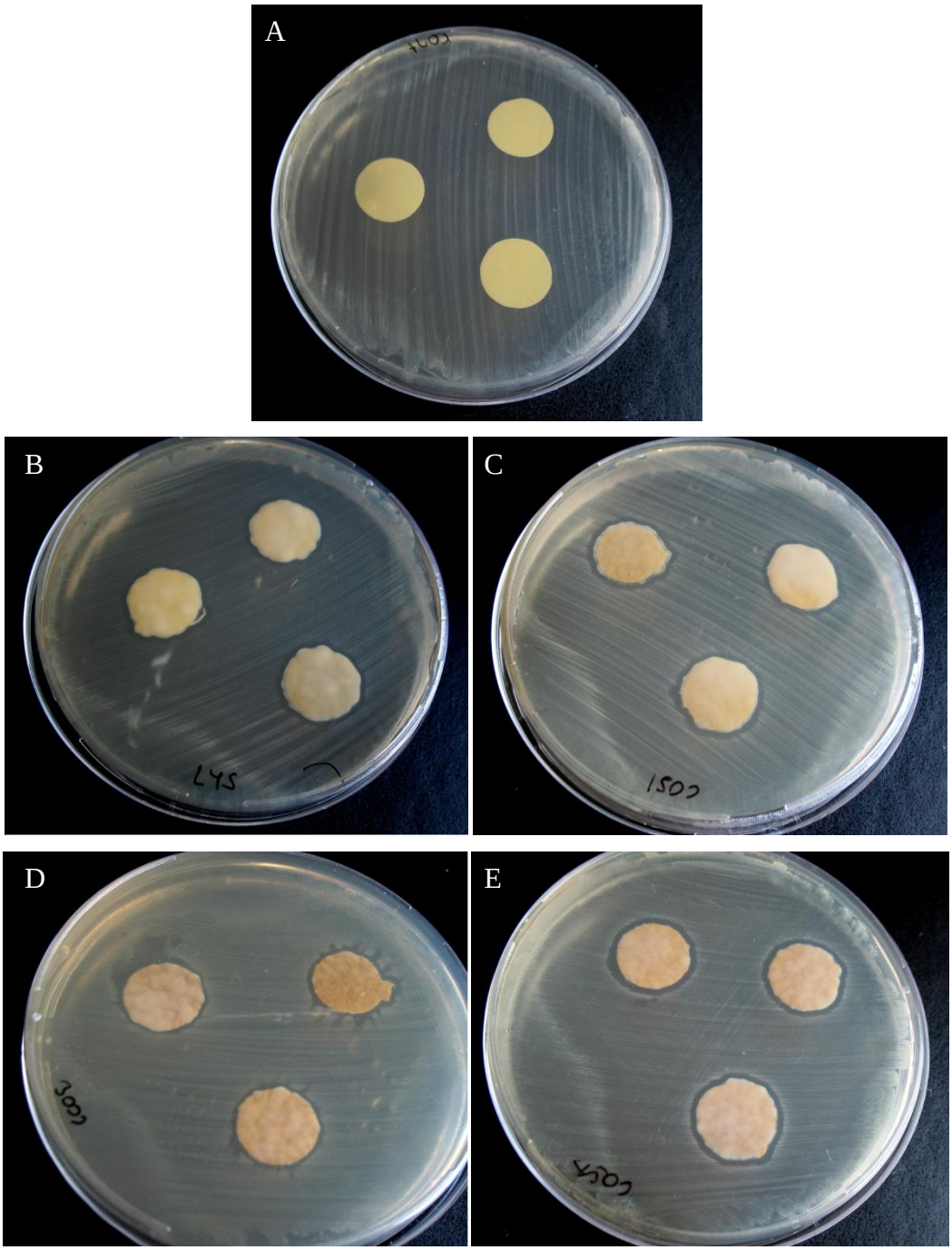


Figure 6.6. Zones formed by pure lysozyme containing zein and zein-LPI composites against *L. innocua* (A: Controls; B: 0.7 mg/cm^2 lysozyme; C: 0.7 mg/cm^2 lysozyme and 1.5 mg/cm^2 LPI; D: 0.7 mg/cm^2 lysozyme and 3 mg/cm^2 LPI; E: 0.7 mg/cm^2 lysozyme and 4.5 mg/cm^2 LPI)

6.1.5. Mechanical Properties of Zein and Zein-LPI Composite Films

Mechanical properties of the films were evaluated by determining their tensile strength, % elongation and Young's modulus values. The incorporation of lysozyme into zein films alone did not cause a statistically significant change in tensile strength and elongation of zein films. The LPI at 1.5 mg/cm^2 with 0.7 mg/cm^2 pure lysozyme did not affect the elongation of zein films, but some increases occurred in film elongation in presence of 3.0 and 4.5 mg/cm^2 LPI. The films containing pure lysozyme and LPI also showed insignificant changes in their tensile strengths with the exception of 1.5 mg/cm^2 LPI and 0.7 mg/cm^2 pure lysozyme containing films which showed a slight reduction in this parameter. In contrast, considerable reductions were observed in Young's modulus of the films by addition of 1.5, 3.0 or 4.5 mg/cm^2 LPI and/or 0.7 mg/cm^2 pure lysozyme. On the other hand, the addition of 1.4 mg/cm^2 partially purified lysozyme did not change the tensile strength and elongation of films. The addition of 1.5 or 3.0 mg/cm^2 LPI did not affect the tensile strength of films, but a slight increase occurred in tensile strength of films by use of 4.5 mg/cm^2 LPI. The elongation of the films also increased slightly by addition of partially purified lysozyme and increase of LPI concentration within the films. The addition of pure or partially purified lysozyme alone or in combination with LPI reduced the Young's modulus of the films. The LPI at different concentrations caused similar reductions in Young's modulus of the films. These results clearly showed the minimal effects of pure and partially purified lysozyme in mechanical properties of films. However, a limited plasticizing effect was observed in the films by addition of LPI.

6.1.6. Scanning Electron Microscopy (SEM) of Zein and Zein-LPI Composite Films

The SEM of zein and zein-LPI composites were given in Figure 6.7A to I. The additon of partially purified lysozyme in films caused the formation of some small aggregates and rarely distributed large sized pores within the films, but no drammatic changes were observed in film morphology by addition of partially purified enzyme. In contrast, the addition of LPI caused loss of film porosity. Similar changes were

observed also in pure lysozyme and/or LPI containing films. However, it was hard to detect the composite structure of films from cross-section photos.

Table 6.4. Mechanical properties of zein films incorporated with pure and partially purified lysozyme and lentil globulins

Incorporated concentration (mg/cm^2)		**Tensile Strength (MPa)[a]**	**Elongation (%)**	**Young's modulus (MPa)**
Commercial lysozyme	Lentil globulin			
-	-	6.40 ± 0.89^{a}	1.36 ± 0.13^{a}	470.29 ± 38.64^{a}
0.7	-	5.61 ± 0.32^{a}	1.52 ± 0.12^{a}	370.14 ± 30.45^{b}
0.7	1.5	4.39 ± 0.82^{b}	2.44 ± 0.38^{a}	180.59 ± 26.68^{c}
0.7	3	5.62 ± 0.57^{a}	3.45 ± 0.73^{b}	168.31 ± 31.70^{c}
0.7	4.5	5.00 ± 1.29^{ab}	3.46 ± 1.24^{b}	138.75 ± 23.10^{c}
Partially purified lysozyme				
-	-	6.40 ± 0.89^{a}	1.36 ± 0.13^{a}	470.29 ± 38.64^{a}
1.4	-	5.88 ± 1.08^{a}	1.56 ± 0.22^{a}	376.77 ± 56.28^{b}
1.4	1.5	7.11 ± 0.68^{a}	2.91 ± 0.54^{b}	248.29 ± 27.48^{c}
1.4	3	7.31 ± 0.15^{a}	3.64 ± 0.26^{c}	201.71 ± 12.51^{c}
1.4	4.5	8.37 ± 0.46^{b}	3.98 ± 0.73^{c}	216.27 ± 41.03^{c}

[a]different letter at each column indicate statistically significant changes at $p<0.05$

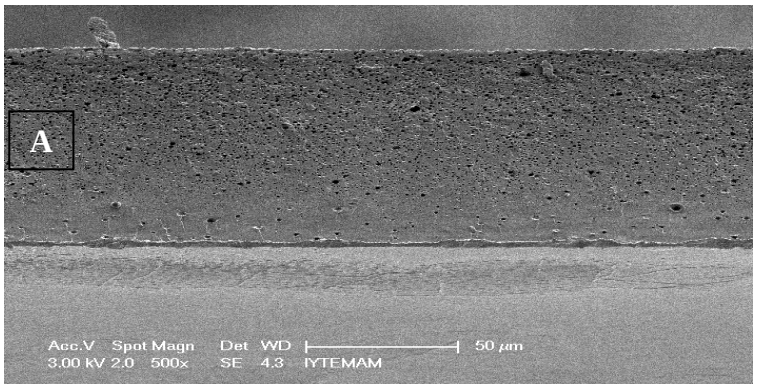


Figure 6.7. SEM photographs of different zein and zein-LPI films (Magnification x500; A: Control; B: 1.4 mg/cm^2 partially purified lysozyme; C: 1.5 mg/cm^2 LPI and 1.4 mg/cm^2 partially purified lysozyme D: 3 mg/cm^2 LPI and 1.4 mg/cm^2 partially purified lysozyme; E: 4.5 mg/cm^2 LPI and 1.4 mg/cm^2 partially purified lysozyme; F: 0.7 mg/cm^2 pure lysozyme; G: 1.5 mg/cm^2 LPI and 0.7 mg/cm^2 pure lysozyme; H: 3 mg/cm^2 LPI and 0.7 mg/cm^2 pure lysozyme; I: 4.5 mg/cm^2 LPI and 0.7 mg/cm^2 pure lysozyme

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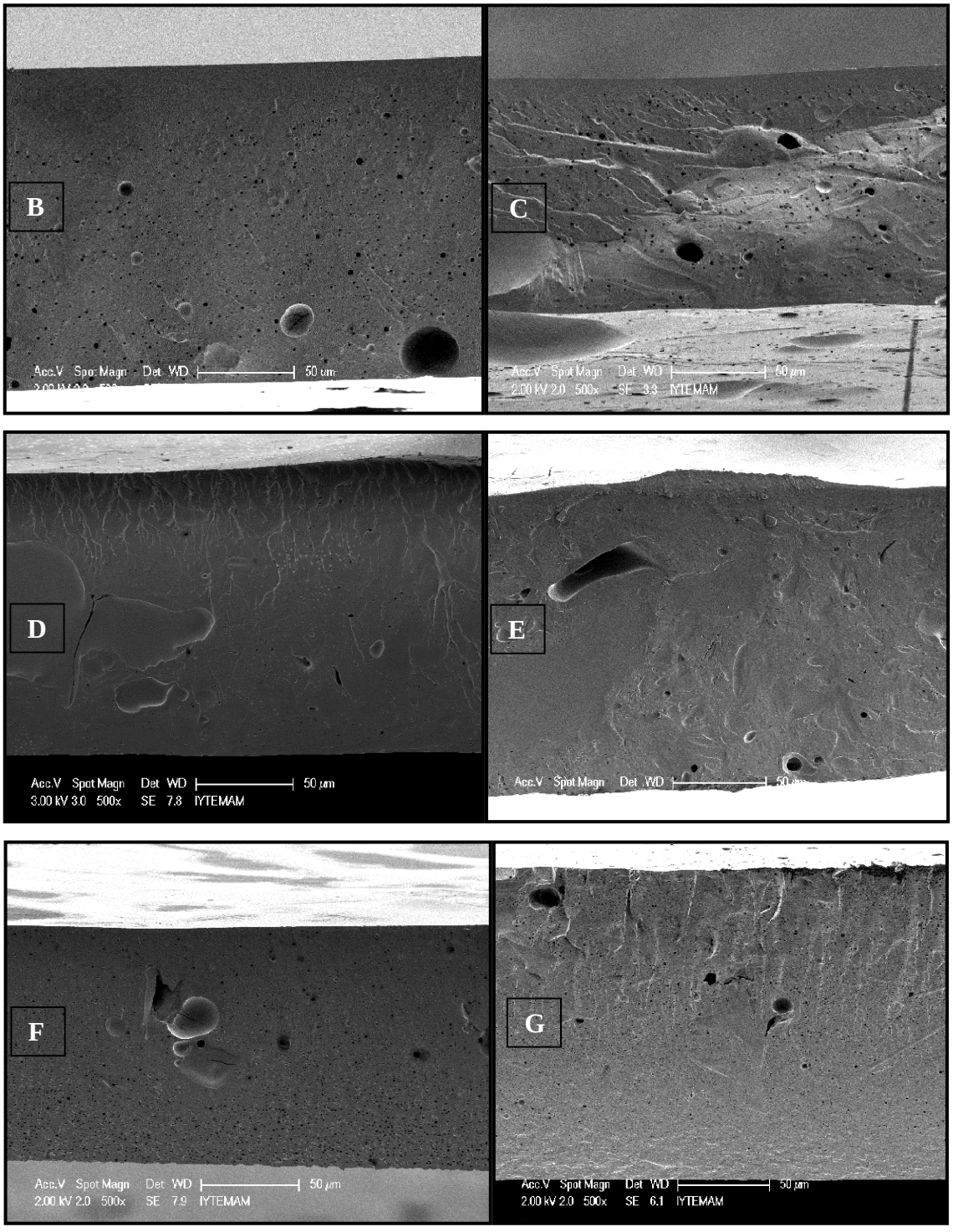

Figure 6.7. (cont.)

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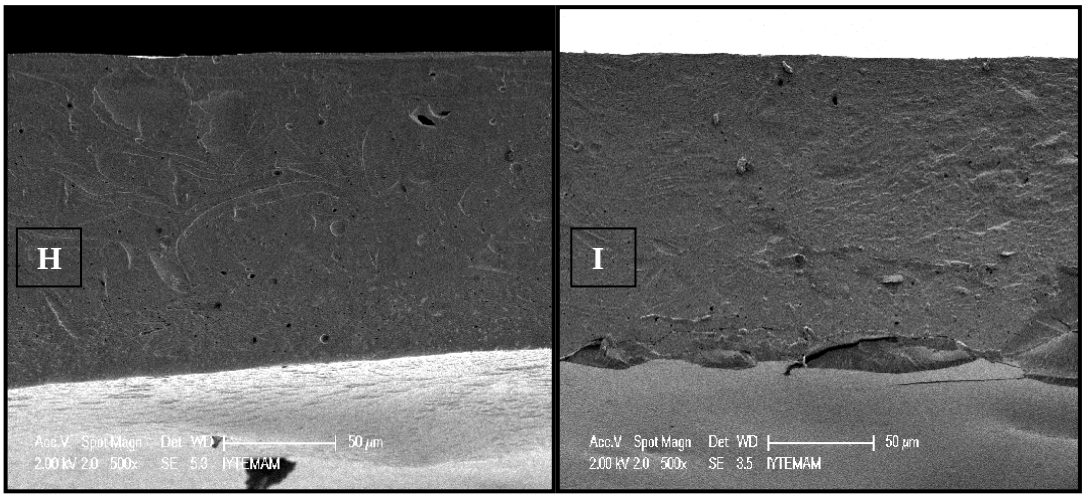

Figure 6.7. (cont.)

6.2. Development of pH-controlled Triggering Mechanisms for Controlled Release of Lysozyme from Whey Protein Films

6.2.1. Scientific Basis of Planned pH-controlled Triggering Mechanism in Whey Films

Unlike to zein, whey proteins (WP) contain considerable number of ionizable groups which showed pI around 5.2 (Kilara, 1998). Thus, it is not essential to incorporate some charged proteins like LPI within these films to create a charge-charge interraction between WP and lysozyme. It was expected that the negative charges of WP films will bind positively charged lysozyme (pI>10.0) above pH 5.2. In contrast, the reduction of the release test medium pH below 5.2 should trigger the release of lysozyme.

6.2.2. pH-dependent Release Profiles of Pure Commercial Lysozyme from Whey Protein Films

The release profiles of pure lyozyme containing WP films at different pH values were given at Figure 6.8. As expected no lysozyme release was expected at pH 6.0 which is above the pI of WP. Also, a very limited lysozyme release occurred when films were incubated at pH 5.0. However, reduction of pH below pI of WP initiated the

release of lysozyme from the films. The release rate and total amount of lysozyme released from the films increased as pH was reduced (Table 6.5).

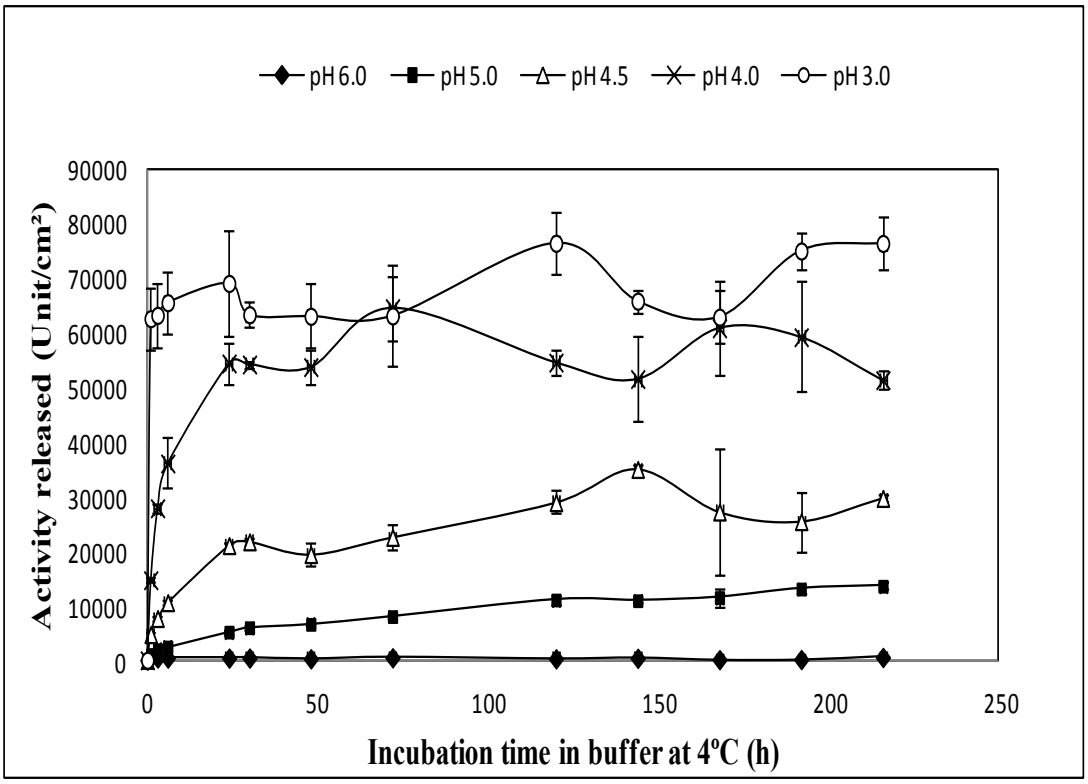

Figure 6.8. Release profiles of pure lysozyme containing whey protein films at different pH values (films contained 0.7 mg/cm^2 pure lysozyme

Table 6.5 Total released pure lysozyme activites from WP films at different pH values (films contianed 0.7 mg/cm^2 pure lysozyme)

Release medium pH	**Total released activity (U/cm^2)**
6.0	510.4±129.2
5.0	13587±669.5
4.5	29708±677.3
4.0	51395±1733
3.0	76481±4930

The release profiles of WP films incubated at different subsequent pH values for minimum 24h more clearly showed the triggering effect of pH on lysozyme release.

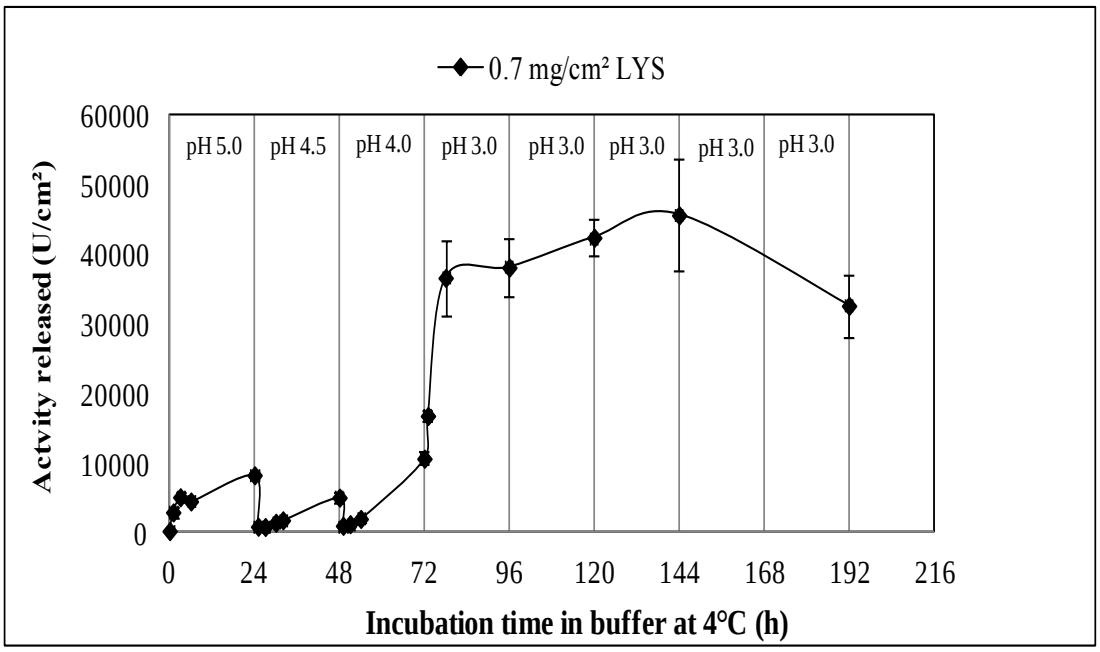

Figure 6.9. Relase profiles of pure lysozyme from WP films kept in a series of buffers at different pH values (films contained 0.7 mg/cm2 pure lysozyme)

6.2.3. pH-dependent Release Profiles of Pure Commercial Lysozyme from Whey Protein-Oleic Acid Blend and Whey Protein-Beeswax Composite Films

The WP-oleic acid blend and WP-beeswax composite films were prepared to see effects of these fatty acid and wax on lysozyme release rates, respectively. It is generally expected that the addition of hydrophobic fatty acid or wax reduces the release rates of antimicrobial agents form films. A reduction in release rate is generally a result of reduced film swelling caused by increased hydrophobicity by the addiiton of lipids. However, in some cases, particularly when fatty acid and lipids could not be effectively solubilized, emulsified and distributed within the film matrix, some lipid aggregates and pores might be formed within the films and this might cause an increase in release rate of the antimicrobials (Alkan et al., 2011). The results of release tests for zein-oleic acid blend

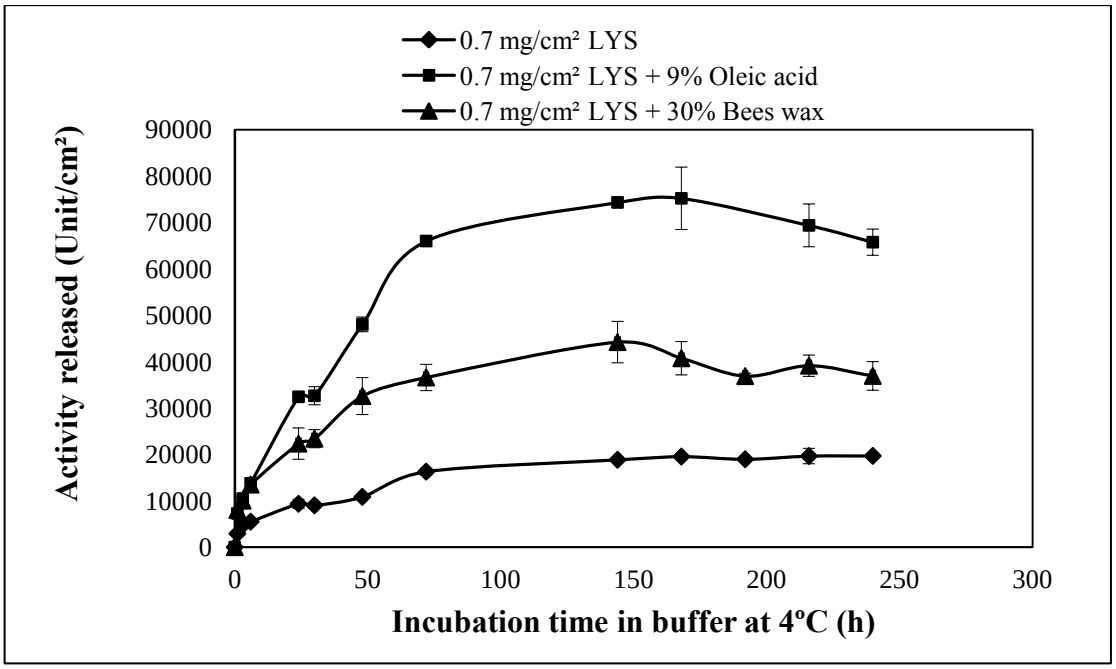

Figure 6.10. Release profiles of pure lysozyme containing WP, WP-oleic acid blend and WP-beeswax composite films at pH 4.5 (films contained 0.7 mg/cm^2 pure lysozyme)

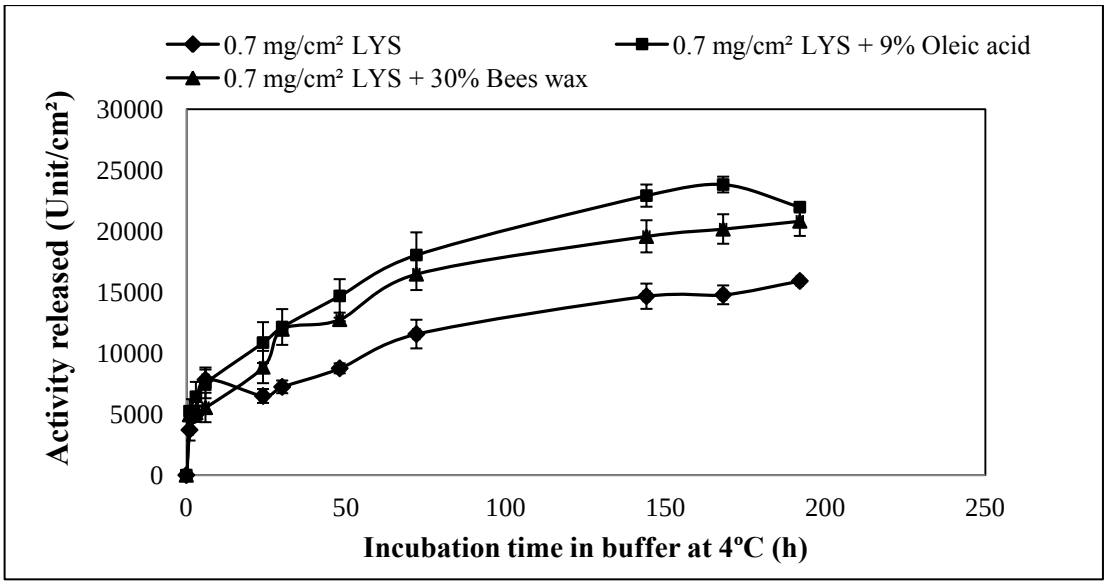

Figure 6.11. Release profiles of pure lysozyme containing WP, WP-oleic acid blend and WP-beeswax composite films at pH 5.0 (films contained 0.7 mg/cm^2 pure lysozyme)

and zein-beeswax composite films were given in Figures 6.10, 6.11 and 12. The release profiles at pH 4.5 and 5.0 clearly showed the increased release rate and released lysozyme content by incorporation of oleic acid and beeswax into films. The oleic acid was much more effective than the beeswax to increase the release rate and reelased

lysozyme content than the beeswax. These results suggested significant modifications in WP film morphologies by addition of waxes. It appeared that the addition of fatty acid and lipid increased the film porosity. Moreower, it is also clear that the amount of fatty acid and wax incorporated into films was insuffieint to increase hydrophobicity of highly hydrophilic WP films which show rapid sweilling in water. On the other hand, no considerable lysozyme release was observed in films when release tests were conducted at pH 5.5. It was clear that the strong charge-charge attractions immobilized and prevented lysozyme release even in more porose films.

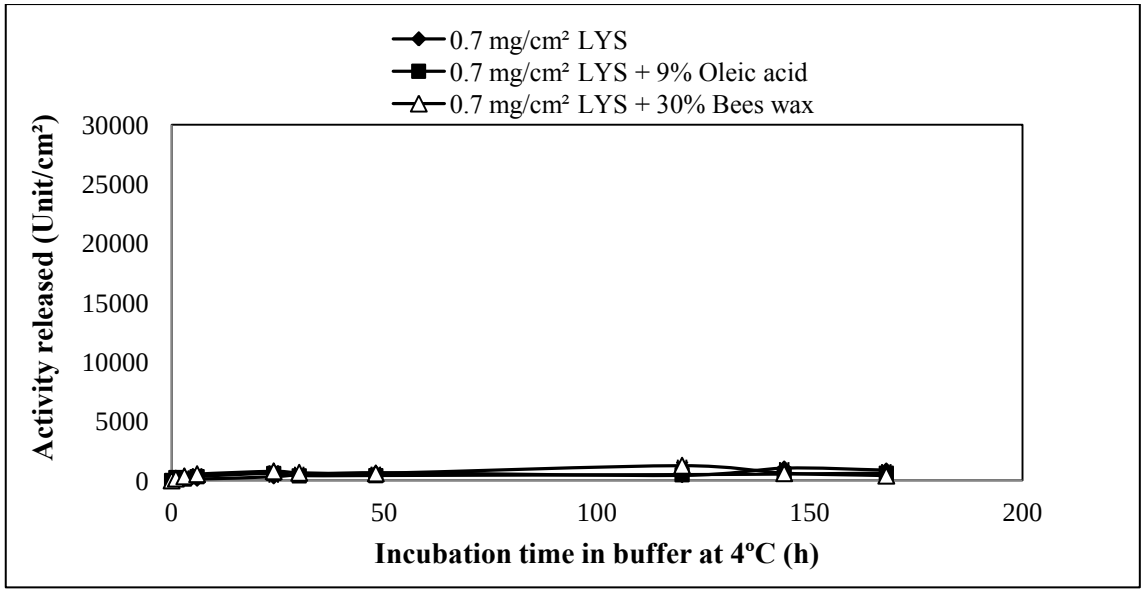

Figure 6.12. Release profiles of pure lysozyme containing WP, WP-oleic acid blend and WP-beeswax composite films at pH 5.5 (films contained 0.7 mg/cm^2 pure lysozyme)

6.2.4. Antimicrobial Activity of Whey Protein Films, Whey Protein-Oleic Acid Blend Films and Whey Protein-Beeswax Composite Films

Antimicrobial activity of WP based films were also determined by the classical zone inhibition method by using *L. innocua* as test microorganism. The results of antimicrobial tests were given in Table 6.6. The results clearly indicated the inherent antimicrobial activity of control WP films. It seemed that the WP contained some antimicrobial milk components like lactoferrin or peptides. This result confirmed the recent findings of Uysal *et al* (2011) who also determined inherent antilisterial activity of WP films. Based on microbiological test results for 24h incubation period, the

incorporation of lysozyme caused a statistically significant increase in antimicrobial activity of films compared to control. The increase of incubation period from 24 to 48h increased the antimicrobial activity of films with the exception of WP-beeswax films which showed a slight reduction in antimicrobial activity after 48h. At both short and long incubation periods, the highest antimicrobial activity was observed for WP-oleic acid blend films which were capable to release greater amounts of lysozyme than the other films (see release tests at Figure 6.9 and 6.10).

In the literature, there are no studies specifically reporting the pH controlled release properties of lysozyme from WP films. However, the antimicrobial activity of lysozyme containing WP films was reported previously for different bacteria including *L. monocytogenes* (Min et al., 2005).

Table 6.6 Antimicrobial activivty of pure lysozyme containing whey protein films and its blend and composite on *L. innocua*

Incorporated lysozyme concentration (mg/cm^2)	**Ingredient**	**Average area of zones (cm^2)**	
		24 h	**48h**
-	-	0.61±0.4[a]	0.70±0.5[a]
0.7	-	0.83±0.2[b]	0.95±0.2[a]
0.7	9 % oleic acid	1.24±0.6[c]	1.45±0.14[b]
0.7	30 % beeswax	0.89±0.1[b]	0.73±0.13[a]

[a]different letter at each column indicate statistially significant changes at $p<0.05$

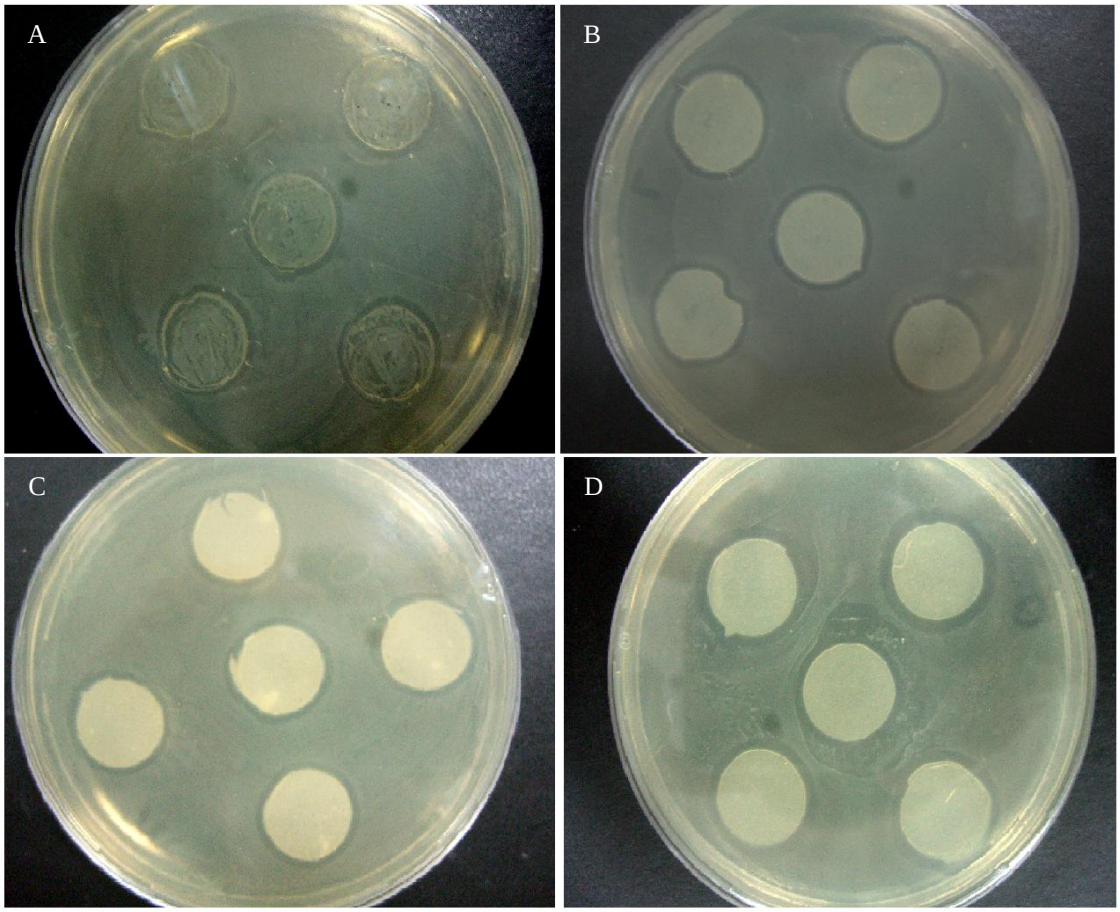

Figure 6.13 Antimicrobial inhibition zones of control WPI film (A) and WPI films containing 0.7 mg/cm^2 lysozyme (B), 30% beeswax (C) and 9% oleic acid (D) on *L. innocua*

6.2.5. Mechanical Properties of Whey Protein Films, Whey Protein-Oleic Acid Blend Films and Whey Protein-Beeswax Composite Films

The parameters showing mechanical properties of different WP films were given in Table 6.7. The results clearly showed the limited effects of blending WP with oleic acid and forming a composite with beeswax at the studies conditions. The only statistically significant change was the slight reduction in tensile strength of WP-beeswax film.

Table 6.7. Mechanical properties of different whey protein films incorporated with pure lysozyme, oleic acid and beeswax

Incorporated lysozyme concentration (mg/cm^2)	Additive components	Tensile Strength (MPa)	Elongation Percentage (%)	Young's Modulus (MPa)
-	-	2.79 ± 0.23^a	15.43 ± 1.63^a	18.26 ± 2.26^a
0.7	-	2.80 ± 0.44^a	33.23 ± 14.79^a	10.14 ± 5.79^a
0.7	9% Oleic acid	2.77 ± 0.26^a	30.12 ± 11.81^a	10.72 ± 5.06^a
0.7	30% Beeswax	2.13 ± 0.14^b	14.13 ± 3.54^b	15.66 ± 3.31^a

[a]different letter at each column indicate statistially significant changes at $p<0.05$

6.2.6. Scanning Electron Microscopy (SEM) of Whey Protein Films, Whey Protein-Oleic Acid Blend Films and Whey Protein-Beeswax Composite Films

The SEM photos given in Figure 6.14 clearly proved our hypothesis about possible dramatic effects of oleic acid and beeswax on WP film morphology. The incorporation of oleic acid caused extensive pore formation at the surfaces of WP films, but very little pores were observed at the depth of films. In contrast, the beeswax formed some rough structures at film surface, and tiny insoluble wax particles distributed within the films increased pores within the films. These photos explained the increase in released lysozyme content of WP films during release tests by addition of oleic acid and beeswax. It is clear that the increased film porosity by addition of fatty acid and wax minimized trapping of loosely bound lysozyme within the films.

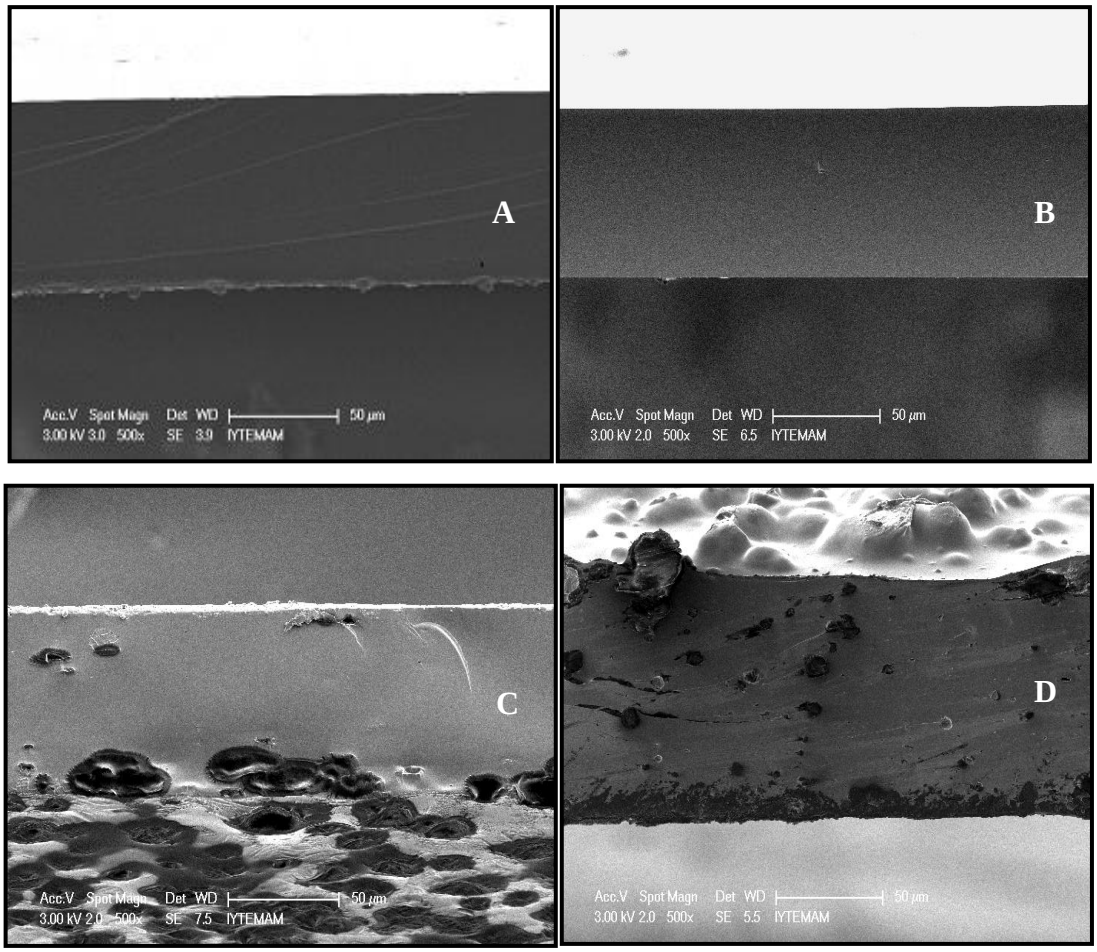

Figure 6.14 SEM photographs of different whey protein films (Magnification x500; A: Whey protein film; B: whey protein-oleic acid blend film; C: whey protein-beeswax composite film; all films contained 0.7 mg/cm^2 lysozyme)

CHAPTER 7

CONCLUSION

Due to their hydrophobic nature and poor charged amino acid content, standard zein films did not bind lysozyme by charge-charge interactions. Thus, the lysozyme release properties of these films could not be controlled by change of release medium pH. The incorporation of surface active lentil protein isolate into zein films caused a considerable increase in number of ionizable groups within the zein films and enabled forming a pH triggering mechanism which release lysozyme below pH 5.0, but bind it above this pH (release controlled at pH≈5.0 (fast))**.** The whey protein films contained sufficient ionizable groups and formed an inherent pH triggering mechanism which controlled both release rate and releasing and binding of lysozyme. The enzyme was released fast at pH 3.0, but slow at pH 4.0. The binding occurred above pH 4.0. (release rate controlled at pH 3.0 (fast) and 5.0 (slow))**.** The very slow release rate of whey protein films at pH 4.5 and 5.0 could be accelerated by preparing its more porous blends and composites with beeswax and oleic acid. The zein composites and whey blends and composites did not show considerably different mechanical properties than standard zein and whey protein films used frequently by the food industry. The developed zein and whey protein films showed sufficient antilisterial activity.

The potential applications of developed films: the developed films could be employed for coating of diary and meat products. The triggering system to release lysozyme could be activated by acidification of coated products by dipping into weak acid solutions when antimicrobial activity is needed (during transportation, during or after defrosting or during displaying for sell at the supermarket).

Future studies: food applications with different dairy and meat products inoculated with specific pathogenic bacteria

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