PREPARATION AND CHARACTERIZATION OF ANTIMICROBIAL POLYMERIC FILMS FOR FOOD PACKAGING APPLICATIONS

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by
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TO

MY MOTHER

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ABSTRACT

PREPARATION AND CHARACTERIZATION OF ANTIMICROBIAL POLYMERIC FILMS FOR FOOD PACKAGING APPLICATIONS

In this study, cellulose acetate (CA) based antimicrobial packaging materials containing lysozyme as an antimicrobial additive were developed. In order to achieve appropriate controlled release of antimicrobial agent, the structure of the films were changed from highly asymmetric and porous to dense ones by modulating the composition of the initial casting solution. The effectiveness of the films were then tested through measurement of soluble and immobilized lysozyme activity, release kinetics and antimicrobial activity on selected microorganisms. The highest release rate, soluble lysozyme activity and resulting antimicrobial activity (on *E.coli*) was obtained with the film prepared from 5 % CA solution including 1.5 % lysozyme. Increasing CA content in the casting solution decreased the porosity of the films, hence, reduced the release rate, maximum released activities and the antimicrobial activities of the films. On the other hand, immobilized activities and the tensile strength of the films increased. The mechanical properties of the antimicrobial films cast with 5 % and 10 % CA were similar to those of lysozyme free CA films. However, significant reductions in tensile strength and elongation values were observed for the antimicrobial films prepared with 15 % CA. Differences in the release rates, soluble, immobilized and antimicrobial activities at porous and dense surfaces of the films suggest that different surfaces of CA films can be employed for antimicrobial packaging according to the targeted shelf-life of the food products. When the films made with 5 % CA were stored at 4 ℃ for a maximal period of 105 days, an increase in soluble lysozyme and antimicrobial activities of the films were observed. The results demonstrate that CA films prepared in this study show promising potential to achieve controlled release in antimicrobial packaging.

ÖZET

GIDA PAKETLEME UYGULAMALARI İÇİN ANTİMİKROBİYEL POLİMERİK FİLMLERİN HAZIRLANMASI VE KARAKTERİZE EDİLMESİ

Bu çalışmada, antimikrobiyel madde olarak lisozim içeren selüloz asetat (SA) antimikrobiyel paketleme malzemeleri geliştirilmiştir. Ajanların uygun kontrollü salımlarına ulaşabilmek için filmlerin başlangıç ıslak çözelti komposizyonları değiştirilerek farklı asimetrik ve gözenekli yapıda filmler elde edilmiştir. Filmler daha sonra çözünür ve tutuklu lisozim aktiviteleri, salım kinetikleri ve seçilen mikroorganizmalar üzerine aktiviteleri için test edilmiştir. En yüksek salım hızı, çözünür lisozim aktivitesi ve sonuçlanan antimikrobiyel aktivite (*E.coli* üzerine) % 5 SA ile hazırlanmış %1,5 lisozim içeren filmlerden elde edilmiştir. Filmlerin başlangıç ıslak çözeltilerinde CA miktarındaki artış filmlerin gözenekliliğini dolayısıyla salım hızını, maksimum salınan aktiviteyi ve antimikrobiyel aktiviteyi azaltmıştır. Diğer taraftan, filmlerin tutuklu aktiviteleri ve gerilme dayanıklılıkları artmıştır. %15 SA ile hazırlanmış filmlerin m fekanik dayanıklılıklarında ve uzama değerlerinde lisozim içermeyenlere göre önemli bir azalma görülmesine rağmen %5 ve %10 SA ile hazırlanmış antimikrobiyel filmlerin mekanik özelliklerinin lisozim içermeyenlerle aynı olduğu bulunmuştur. Filmlerin gözenekli ve gözenekli olmayan yoğun yüzeylerinden elde edilen farklı sonuçlar, bu yüzeylerin gıda ürünlerinin hedeflenen raf ömürlerine göre antimikrobiyel paketleme için uygulanabilir olduğunu göstermektedir. %5 SA ile hazırlanmış filmler 4°C'de 105 gün depolandığında, zamanla çözünür lisozim ve antimikrobiyel aktivitelerinde artış gözlenmiştir. Bu sonuçlar, hazırlanan SA filmlerin antimikrobiyel ambalajlamada kontrollü salım elde etmek için kullanılabileceğini göstermektedir.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

LIST OF SYMBOLS

A_f	Area of the film (cm^2)
C_o	Initial concentration of active agent in the film (g/cm^3)
C_f	Concentration of active agent in the film at any time t (g/cm^3)
C_s	Concentration of active agent in the solution (g/cm^3)
D	Effective diffusion coefficient of active agent in the film (cm^2/sec)
K	Partition coefficient (cm^3 solution/cm^3 film)
L	Thickness of the film (cm)
M_t	Total amount of active agent desorbed from the film at any time t (g)
M_∞	Total amount of active agent desorbed from the film at equilibrium (g)
t	Time (sec)
x	Position in the film (cm)

CHAPTER 1

INTRODUCTION

In order to control microbial growth in foods and improve their shelf-life and safety, different antimicrobial agents are mixed with the initial food formulations or they are applied to food surface by dusting, dipping or spraying (Min and Krochta 2005). Incorporation of antimicrobial agents directly into food is appropriate when there is a risk of microbial growth at both surface and internal parts of final food product. However, when the main cause of spoilage of food is microbial growth at the food surface, the use of this method causes addition of excessive amounts of chemical additives into food materials. Thus, this traditional strategy does not fit to the current trend of food technology to develop healthier processed foods by using minimum amounts of chemical additives. The application of antimicrobial agents on food surface by different methods also has limited beneficial effects since this causes reduction of surface concentration of the antimicrobial due to its diffusion from food surface to interior parts (Min and Krochta 2005). Another disadvantage of direct application of antimicrobials (chemical or natural) to food materials is neutralization of the added agent due to its possible complex interactions with the food components (Rose et al 1999, Rose et al. 2002, Appendini and Hotchkiss 2002). Antimicrobial packaging is an alternative method to overcome these limitations by using small amounts of antimicrobial agent. The strategy of antimicrobial packaging depends on release of antimicrobial agent incorporated into a packaging material onto food surface. The release of antimicrobial agent should occur at such a suitable rate to maintain its inhibitory concentration against pathogenic or spoilage microorganisms during the targeted storage period (Quintavalla and Vicini 2002, Ozdemir and Floros 2003, Buonocore et al. 2004). The antimicrobial packaging can also be used to extend the shelf-life of a food or to reduce the risk of contamination occurred during food packaging.

In recent years there has been a great interest in antimicrobial food packaging technologies due to increased food-borne microbial outbreaks caused by minimally processed fresh products and refrigerated products (De Roever 1998, Devlieghere et al. 2004). Traditionally, antimicrobial additives are mixed into initial food formulations to

control microbial growth and extend shelf-life. However, this strategy is not always effective since the protective ability of the antimicrobial agent ceases once it is neutralized in reactions and/or intractions in the complex food system and the quality of food degrades at an increased rate. In addition, the antimicrobial compound directly added into the food cannot selectively target the food surface where spoilage reactions occur more intensively. Antimicrobial packaging is an alternative method to overcome these limitations since the agent slowly release from the film onto the food surface during the storage, hence, maintains its critical concentration necessary for inhibiting the microbial growth.

The release rate of antimicrobial agent is critical to maintain food quality and safety. A rapid release of an antimicrobial agent from film to food surface may reduce the success of packaging application considerably since this causes subsequent diffusion of the agent from food surface to internal parts which are less critical than the food surface for microbial growth and contamination (Appendini and Hotchkiss 2002). On the other hand, if the release rate of the antimicrobial agent is very slow, its inhibitory concentration cannot be reached, consequently, spoilage reactions on the surface may start and food quality and safety can no longer be maintained. Controlled release of the active agent is highly desirable since this helps maintaining inhibitory concentrations of the agent at the critical food surface during storage period. Controlled release systems have been mainly developed for pharmaceutical applications (Langer and Peppas 1981, Leong and Langer 1988, Brayden 2003). Different strategies for achieving controlled release of drugs are well established and studies on both preparation and modeling of drug delivery systems have been reported (Langer and Peppas 1981, Siepmann et al. 1999, Arifin et al. 2006, Mallapragada and Peppas 1997, Richard 1998, Siepmann and Peppas 2001). However, studies on the development of food packaging films with controlled release properties are really limited. The concept of controlled release for food packaging applications was first applied by Han and Floros. They suggested to control the release kinetics by using a multilayer structure which includes an outer barrier layer, a matrix layer containing the active agent and a control layer (Han and Floros 1998). Similar approach was also proposed by Lopez-Rubio et al. (Amparo et al. 2006). In these structures, the inner control layer governs the rate of diffusion of active agent or exert a barrier function to protect it from direct food or moisture contact while the outer layer prevents migration of the agent toward the outside of the package. On the basis of Han and Floros's work, Buonocore et al. developed two multilayer films

which consist of two external control layers and an inner layer containing the active agent (Han and Floros 1998, Buonocore et al. 2005). The same research group has also tried to regulate the release kinetics of active compounds by changing the degree of cross-link of the polymer matrix (Buonocore et al. 2003, Buonocore et al. 2004). Micro and nanoencapsulation of food ingredients have been proposed as another alternative method for achieving controlled release of active agents in food applications (Amparo et al. 2006, Guzey and McClements 2006). Guzey and McClements have discussed the potential application of microencapsulation for sensitive food components such as enzymes and bioactive compounds by means of polyelectrolyte multilayer self assembly. They have suggested that release rate of the active agent from small capsules directly added into the food can be controlled by changes in pH, temperature or ionic strength of the medium (Guzey and McClements 2006). La Coste et al. have proposed to use smart blending for developing novel controlled release packaging materials. They claim that this approach offers the ability to deliberately manipulate the film morphologies, hence, to provide a wide range of release properties (La Coste et al. 2005). Among all these strategies, only the uses of multilayer and crosslinked films have been tested as food packaging materials in order to control the release of the active compound from the films into foodstuff. The feasibility of other proposed methods in developing controlled release packaging materials for foods needs to be tested with in vitro and in vivo experiments.

To prepare antimicrobial films, different synthetic antimicrobial chemicals have been incorporated into packaging materials including organic or inorganic acids, metals, alcohols, ammonium compounds or amines (Appendini and Hotchkiss 2002, Suppakul et al. 2003). However, the increasing consumer health concern and growing demand for healthy foods have stimulated the use of natural biopreservatives such as antimicrobial enzymes and bacteriocins (Suppakul et al. 2003, Labuza and Breene 1989). Lysozyme is one of the most studied biopreservative for antimicrobial packaging applications (Han 2000 Quintavalla and Vicini 2002). This enzyme shows antimicrobial activity mainly on G (+) bacteria by splitting the bonds between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan in their cell walls. However, the enzyme does not show antibacterial activity against G (-) bacteria due to their protective outer membrane surrounding the peptidoglycan layer. To increase its antimicrobial spectrum and to destabilize the outer membranes of G (-) bacteria, lysozyme is generally combined with EDTA (Padgett et al. 1998, Branen and Davidson 2004). The lysozyme

has been incorporated into different edible or biodegredable packaging materials made from zein (Mecitoğlu at al. 2006, Güçbilmez et al. 2007), whey proteins (Suppakul et al. 2003), chitosan (Park et al. 2004), alginate and carrageenan (Cha et al. 2002) and tested on different bacteria. There are also several studies in the literature that used lysozyme with plastic films. Appendini and Hotchkiss have immobilized lysozyme on polyvinylalcohol (PVOH) beads, nylon 6,6 pellets and cellulose triacetate (CTA) films (Appendini and Hotchkiss 1997). Buonocore et al. and Conte et al. studied the incorporation of lysozyme into PVOH films (Buonocore et al. 2003, 2004, 2005, Conte et al. 2007).

The objective of this study is to develop asymmetric and porous cellulose acetate (CA) films for food packaging applications and investigate their feasibility in controlling the release rate of active agent. To achieve this goal, natural antimicrobial agent lysozyme obtained from hen egg white has been incorporated into cellulose acetate films with different morphological characteristics and the release rates from these films have been determined. The relationship among the initial composition of the film forming solution, the resulting morphology and release behavior of the active agent was demonstrated. The antimicrobial activity of the films has also been tested on selected G(+) and G(-) bacteria. Recently, different research groups showed the advantage of using asymmetric-membrane capsules and asymmetric coatings on drug tablets to control the release rate of drugs (Herbig et al. 1995, Cardinal et al. 1997, Wang et al. 1998, Thombre et al. 1999a, Thombre et al. 1999b Thombre et al. 1999c, Lin and Ho 2003, Prabakaran et al. 2004, Altınkaya and Yenal 2006). However, the potential use of asymmetric and porous structures in preparing controlled release food packaging materials has not been investigated. To our knowledge, this is the first study illustrating the development of asymmetric porous films for food packaging applications.

This thesis consists of six sections. After giving a brief introduction in the first part, in the second part, active packaging systems, especially antimicrobial food packaging systems are discussed. Models used for determining antimicrobial agent transport in food packaging systems are reviewed in the third section, while materials and methods used in this study were given in the fourth section. In the fifth section, the results are shown and discussed and finally in the sixth section conclusions are given.

CHAPTER 2

PACKAGING

Packaging is the science, art, and technology of enclosing or protecting products for distribution, storage, sale, and use. Packaging also refers to the process of design, evaluation, and production of packages (WEB_1 2007).

Many cooking and preservation processes still largely depend on effective packaging such as canning, aseptic and baking processes. Oxygen, light, water vapor, bacterial and other contaminants can affect the product without protective packaging after processes such as drying and freezing (Ahvenainen 2003).

2.1. Active Packaging

Active packaging is an innovative concept that can be defined as the interaction of the package, the product, and the environment to prolong shelf life or enhance safety or sensory properties of the product. (Suppakul et al. 2003).

There are three categories for active packaging techniques in order to preserve and improve quality and safety of food. These are absorbers (i.e. scavengers), releasing systems and other systems. Undesired compounds such as oxygen, carbon dioxide, ethylene, excessive water etc. are removed using absorbing (scavenging) systems. Packaging materials including preservatives add or emit active compounds to the packed food or into the head-space or the package using releasing systems. Self heating and self cooling packaging materials can be listed as other active packaging systems (Ahvenainen 2003).

2.2. Antimicrobial Food Packaging

Antimicrobial packaging is one of the many applications of active packaging. It is the packaging system that is able to kill or inhibit spoilage and pathogenic microorganisms that are contaminating foods (Han 2000).

Antimicrobial packaging is specifically designed to control growth of microorganisms unlike conventional food packaging systems which are used for shelf-

life extention, quality maintenance, and safety assurance which could be achived by various methods. Table 1. shows application area, antimicrobial agents and packaging materials used in antimicrobial food packaging.

Table 1. Application area, antimicrobial agents and packaging materials used in antimicrobial food packaging (Source: Han 2000, Appendini and Hotchkiss 2002).

Antimicrobial Agent	**Packaging Material**	**Application area**
Organic acid		
Patassium sorbate	LDPE	Cheese
	LDPE	Culture media
	MC/palmitic acid	Culture media
	MC/HPMC/fatty acid	Culture media
	MC/chitosan	Culture media
	Starch/glycerol	Chicken brest
Calcium sorbate	CMC/paper	Bread
Propionic acid	Chitosan	Water
Acetic acid	Chitosan	Water
Benzoic acid	PE-co-MA	Culture media
Sodium benzoate	MC/chitosan	Culture media
Sorbic acid anhydride	PE	Culture media
Benzoic acid anhydride	PE	Fish fillet
Imazalil		
Nisin (peptide)		
Peptide/protein/enzyme		
Lysozyme	LDPE	Bell pepper
	LDPE	Cheese
Glucose oxidase	Silicon coating	Culture media
	SPI, corn zein films	Culture media

(cont. on next page)

Table 1. (cont.)

Alcohol/thiol	PVOH, nylon, Cellulose acetate, corn zein films	Culture media Culture media
Ethanol	Alginate	Fish
Oxygen absorber/antioxidant		
Reduced iron complex	Slica gel sachet Silicon oxidase sachet	Culture media Bakery
BHT		
Chelating agents	Sachet	Bread
EDTA	HDPE	Breakfast cereal
Spicies		
Cinnamic Caffeic p-coumaic acids	Edible films	Culture media
Essential oils (plant extracts)		
Grapefruit seed extracts Hinokitiol Bamboo powder Rheum palmatum Coptis chinesis extracts	Nylon/PE, cellulose	Culture media
Gas		
CO_2 SO_2	LDPE, cellulose	Culture media
	Calcium hydroxide sachet Sodium metabisulfite	Coffee Grape

LDPE: low-density polyethylene; MC: methyl cellulose; HPMC: hydroxypropyl MC; CMC: carboxyl MC; PE: polyethylene; MA: met hacrylic acid; SPI: soy protein isolate; PVOH: polyvinyl alcohol; BHT: buthylated hydroxy toluene; HDPE: high-density PE

2.2.1. Types of Antimicrobial Food Packaging

Antimicrobial food packaging can be produced in various forms (Appendini and Hotchkiss 2002). These are:

- Addition of sachets / pads containing volatile antimicrobial agents into packages.
- Incorporation of volatile and non-volatile antimicrobial agents directly into polymers.
- Coating or adsorbing antimicrobials onto polymer surfaces.
- Immobilization of antimicrobials to polymers by ion or covalent linkages.
- Use of polymers that are inherently antimicrobial.

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

Addition of sachets / pads containing volatile agents into packages is the most successful commercial application of antimicrobial packaging. Oxygen absorbers, moisture absorbers and ethanol vapor generators are the main types of sachets used commercially. Oxygen and moisture absorbers are used especially in bakery, pasta and meat packaging to prevent oxidation and water condensation. Although oxygen absorbers are not an antimicrobial agent, a reduction of headspace oxygen in the package inhibits the growth of aerobes, particularly molds. Moisture absorbers reduce water activity and also indirectly affect microbial growth on the food (Appendini and Hotchkiss 2002).

2.2.1.2. Incorporation of Antimicrobial Agents into Packaging Materials

Incorporation of antimicrobial agents into polymers has been commercially applied in drug and pesticide delivery, household goods, textiles, surgical implants and other biomedical devices. Few food-related applications have been commercialized but

the research about the incorporation of antimicrobials into packaging for food applications has increased recently (Appendini and Hotchkiss 2002).

Addition of antimicrobials into the melt form of polymer and addition of antimicrobial into the wet polymer solution are two ways to incorporate the antimicrobial agents into the packaging materials.

If the incorporated antimicrobial agents are non-volatile, packaging materials must contact the surface of the food so that the antimicrobial agents can diffuse to the surface. The diffusion of antimicrobials from packaging material has been the subject of several research papers (Ouattara et al. 2000, Buonocore et al. 2003, Choi et al. 2005, Mecitoğlu et al. 2006). If the incorporated antimicrobial agents are volatile (e.g. chlorine dioxide, sulfur dioxide, carbon dioxide and allyl isothiocyanate), packaging materials do not need to contact the surface of the food (Appendini and Hotchkiss 2002, Suppakul et al. 2003).

2.2.1.3. Coating or Adsorbing Antimicrobials to Polymer Surfaces

Antimicrobials which are sensitive to high temperature can not be used in polymer processing. Because of that, they are often coated onto the material after forming or are added to cast films. Nisin / methylcellulose coatings applied on polyethylene films (Appendini and Hotchkiss 2002) are examples. Proteins have an increased capacity for adsorption due to their amphiphilic structure.

Figure 1 shows the effect of the coating location on the diffusion of antimicrobial agents.

2.2.1.4. Immobilization of Antimicrobials by Ionic or Covalent Linkages to Polymers

Immobilization of the antimicrobial agents to polymers by ionic or covalent bonding occurs when both antimicrobial agent and the polymer have functional groups. Examples of antimicrobials with functional groups are peptides, enzymes, polyamines and organic acids. Examples of polymers used for food packaging applications that have functional groups are ethylene vinyl acetate (EVA), ethylene methyl acrylate (EMA),

ionomer, nylon and polystyrene (PS). Table 2. shows the antimicrobial agents and their functional supports.

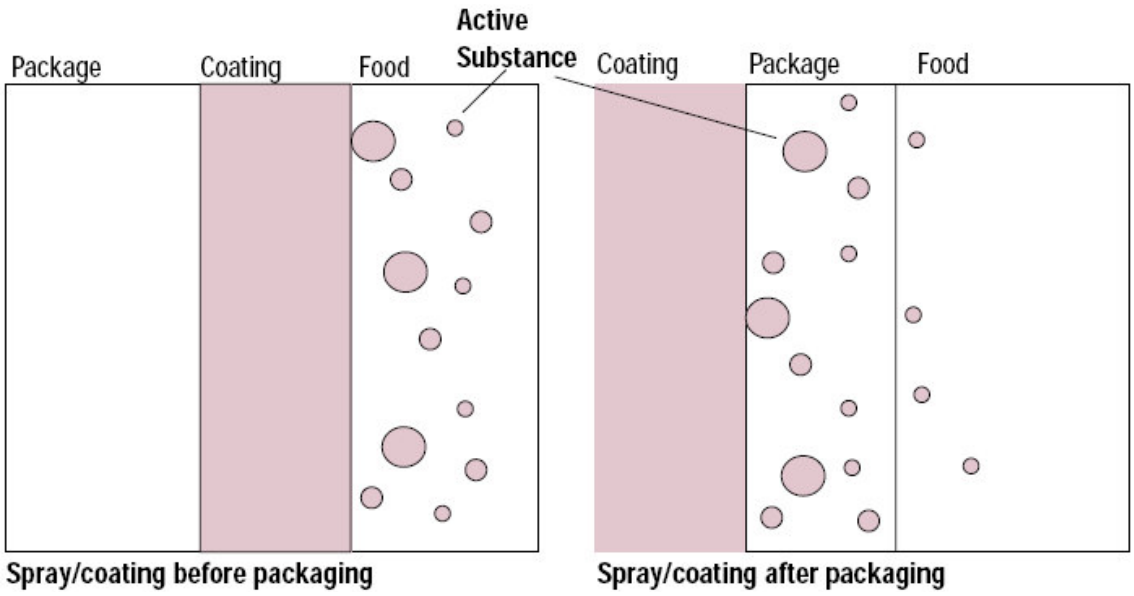


Figure 1. Effect of the coating location on the diffusion of antimicrobial agents (Source: Han 2000)

Table 2. Antimicrobials covalently/ionically immobilized in polymer supports (Source: Scannel et al. 2000, Appendini and Hotchkiss 2002)

Functional Support	**Antimicrobials**
Ionomeric films	Benomyl Benzoyl chloride Bacteriocin
Polystyrene	Lysozyme Synthetic antimicrobial peptides
Polyvinyl alcohol	Lysozyme
Nylon 6,6 resins	Lysozyme
Cellulose triacetate	Lysozyme
Polyethylene/polyamide films	Nisin

2.2.1.5. Use of Polymers that Are Inherently Antimicrobial

Cationic polymers such as chitosan and poly-L-lysine are inherently antimicrobial and have been used in films and coatings. These polymers interact with negative charges on the cell membrane and cause the leakage of their intracellular components (Appendini and Hotchkiss 2002). Chitosan has been approved as a food ingredient from FDA recently; because of that, the use of chitosan for new product development is popular as a natural antimicrobial agent (Ahvenainen 2003).

2.2.2. Antimicrobial Packaging Systems

Antimicrobial food packaging systems can be divided into two parts; package / food systems and package / headspace / food systems. There are some differences between two systems and they are discussed below.

2.2.2.1. Package / Food Systems

In these systems, packaging materials contact with the food surfaces or low viscosity or liquid food without headspace (Han 2000). Main migration phenomena in this system is the diffusion between the packaging material and food and partitioning at the interface (Figure 2.). Individually wrapped cheese, ready-to-eat meat products are examples of this system (Quintavalla and Vicini 2002).

2.2.2.2. Package / Headspace / Food Systems

Examples of package / headspace / food system are flexible packages, bottles, cans, cups, and cartons. In these systems, antimicrobial agents have to be volatile because of the migration through the headspace. Unlike nonvolatile substances, volatile substances can migrate through the headspace and air gaps between the package and the food (Figure 3.).

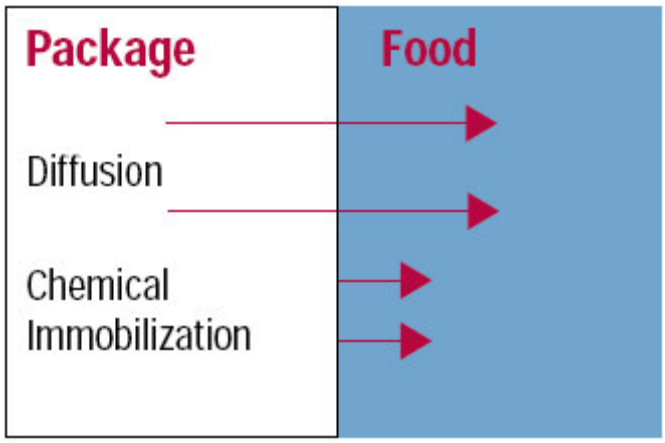


Figure 2. Package / food systems

(Source: Han 2000).

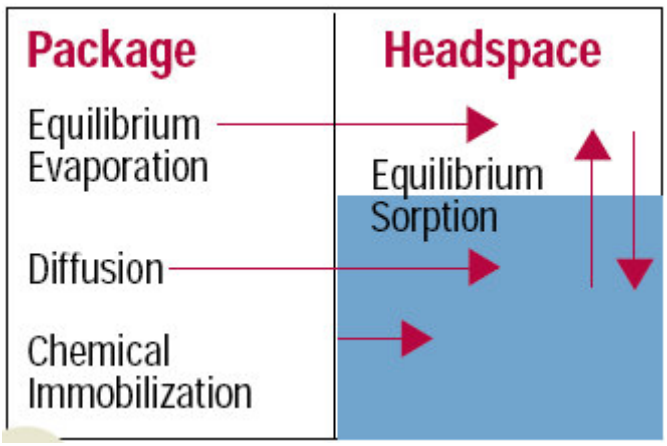


Figure 3. Package / headspace / food systems

(Source: Han 2000)

2.3. Types of Polymers

Polymeric films can be divided into two categories as synthetic and natural polymers.

2.3.1. Synthetic Polymers

Synthetic polymers are often referred to as "plastics", such as the well-known polyethylene and nylon. Synthetic polymers are made by reacting monomers or their derivatives under controlled conditions (Campbell 1994). Synthetic polymeric films usually have high tensile strength and good barrier properties. Some information for different synthetic polymers is given below.

2.3.1.1. Polyethylene (PE)

PE is a polymerized ethylene resin, used especially for containers, kitchenware, and tubing, or in the form of films and sheets for packaging. PE is classified into several different categories based mostly on its density and branching. The major classifications of PE are high-density polyethylene (HDPE), low-density polyethylene (LDPE), and linear low density polyethylene (LLDPE) (Baner 2000). The mechanical properties of PE depend significantly on variables such as the extent and type of branching, the crystal structure, and the molecular weight (Yam 1999). The repeating unit of the PE can be seen from figure 4

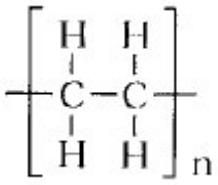


Figure 4. The repeating unit of poly(ethylene)
(Source: Yam 1999)

Han and Floros extruded an antimicrobial film using LDPE resins and potassium sorbate powder. Its tensile properties, transparency and antimicrobial activity were measured to examine the performance as a packaging material. Incorporation of potassium sorbate in the film did not affect the tensile properties significantly. However; transparency of the film decreased as the concentration of potassium sorbate increased (Han and Floros 1997).

2.3.1.2. Polypropylene (PP)

PP is a linear, crystalline polymer that has the lowest density (0.9) among all major plastics. Compared to PE, PP has higher tensile strength, stiffness, and hardness. It resists moisture, oils, and solvents. Since its melting point is 165°C, it is used in the manufacture of objects that are sterilized in the course of their use. PP is also used to make textiles, ropes that float, packaging material, and luggage. Food containers made from PP do not melt in the dishwasher, and during industrial hot filling processes. For this reason, most plastic tubs for dairy products are polypropylene sealed with aluminium foil. (Baner 2000). The structure of the PP can be seen from figure 5

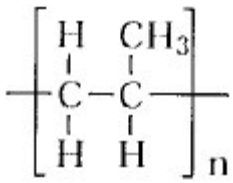


Figure 5. The structure of the polypropylene
(Source: Yam 1999)

2.3.1.3. Polyvinyl Chloride (PVC)

Polyvinyl chloride is a clear, amorphous polymer used mostly for films and containers. Most often, plasticizers (organic liquids of low volatility) are added to the polymer to yield widely varying properties, depending on the type and amount of plasticizers used. Plasticized PVC films are limp, tacky, and stretchable, and the films

are commonly used for packaging fresh meat and fresh produce. Unplasticized PVC sheets are rigid, and the sheets are often thermoformed to produce inserts for snacks such as chocolate and biscuits.

PVC bottles have good clarity, oil resistance, and barrier properties. However; the use of PVC bottles in food packaging is relatively small due to poor thermal processing stability and environmental concerns with chlorine-containing plastics (Yam 1999).

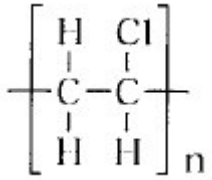


Figure 6. The structure of the PVC
(Source: Yam 1999)

2.3.2. Natural Polymers

Natural polymeric materials, such as wood and horn, have been used by humans since prehistoric times (Campbell 1994). Many important natural materials are organic polymers, including cellulose (from sugar monomers), lignin, rubber, proteins (from amino acids), and nucleic acids (from nucleotides) (WEB_3 2007).

2.3.2.1. Starch

Starches used in industrial applications are usually extracted from cereal seeds (corn, wheat, and rice), tubers (potato), and roots (tapioca). Starches from various sources are chemically similar, but their granules are heterogeneous with respect to their size, shape, and molecular constituents. Proportion of the polysaccharides amylose and amylopectin is the most critical descriptor. The chemical structures of amylose (Figure 7.) and amylopectin (Figure 8.) are shown below.

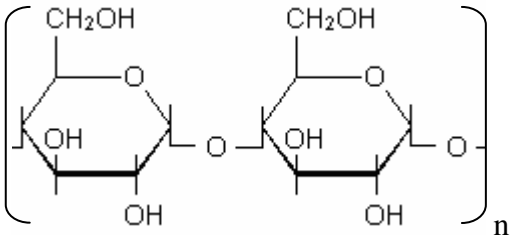


Figure 7. Structure of Amylose
(Source: WEB_4 2007).

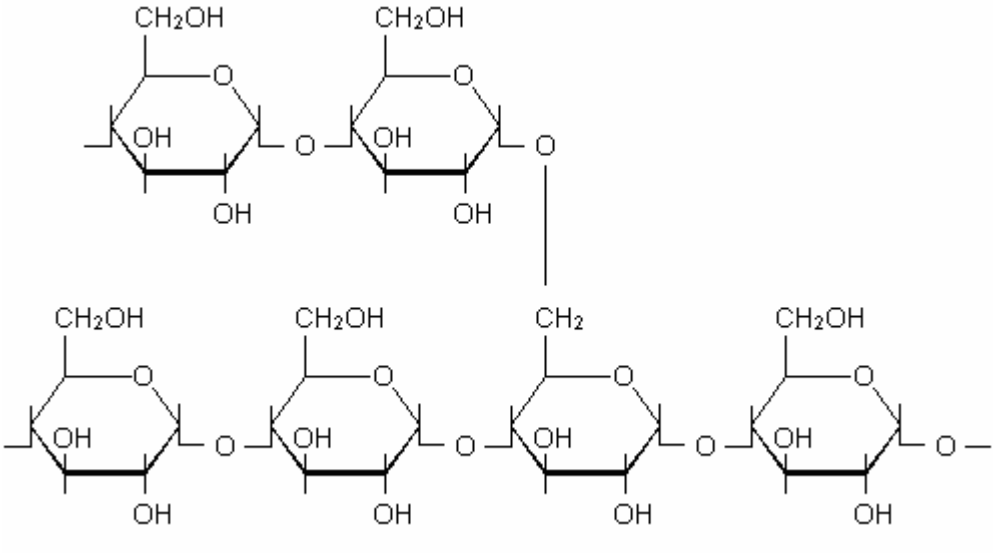


Figure 8. Structure of amylopectin
(Source: WEB_4 2007).

Starch has been widely used as a raw material in film production because of increasing prices and decreasing availability of conventional film-forming resins. Starch is also useful for making agricultural mulch films because it degrades into harmless products when placed in contact with soil microorganisms.(Chandra and Rustgi 1998, Mohanty et al. 2005).

Research on starch includes investigation of its water adsorptive capacity, the chemical modification of the molecule, its behaviour under agitation and high temperature, and its resistance to thermomechanical shear. Although starch is a polymer, its stability under stress is not high. At temperatures higher than 150°C, the glucoside links start to break, and above 250°C the starch grain endothermally

collapses. At low temperatures, a phenomenon known as retrogradation is observed. This is a reorganization of the hydrogen bonds and an aligning of the molecular chains during cooling. In extreme cases under 10°C, precipitation is observed (Chandra and Rustgi 1998).

Petersen et al. investigated the physical and mechanical properties of biobased materials (Polylactate (PLA), polyhydroxybutyrate (PHB), wheat and corn starch). They concluded that most of their properties were similar with synthetic materials but the major difference being the water vapor permeability, needs improvement if used for primary food packaging purposes (Petersen et al. 2001).

2.3.2.2. Protein

Proteins are heteropolymers containing up to 20 different types of amino acids of the general structure.

Molecular weight of proteins can be as low as 6000 and as high as one million. The number of possible conformations of proteins can be more than 10^{100} for a protein of only 100 amino acid residues (Fried 2003).

Proteins are very convenient sources to obtain packaging films because of their different molecular properties and chemical functions. Although the protein films are highly sensitive to moisture and show poor water vapor barrier properties, they have good gas barrier properties and suitable mechanical and optical properties. (Güçbilmez 2005).

2.3.2.3. Cellulose

Cellulose is the structural polysaccharide of plants and it exists in wood, cotton, cereal straws etc. The structure of the cellulose can be seen in figure 9.

Naturally, cellulose occurs in a crystalline state. From the cell walls, cellulose is isolated in microfibrils by chemical extraction. In all forms, cellulose is a very highly crystalline, high molecular weight polymer, which is infusible and insoluble in all but the most aggressive, hydrogen bond-breaking solvents such as *N*-methylmorpholine-*N*-oxide. Because of its infusibility and insolubility, cellulose is usually converted into derivatives to make it more processable. Cellulose acetate is one of the derivatives of

cellulose. It is currently used in high volume applications ranging from fibers, to films, to injection moulding thermoplastics (Chandra and Rustgi 1998).

Noishiki et al. investigated the mechanical properties of silk fibroin-microcrystalline cellulose composite films. The tensile strengths of the films increased with increasing cellulose contents (wt %) in their study (Noishiki et al. 2002).

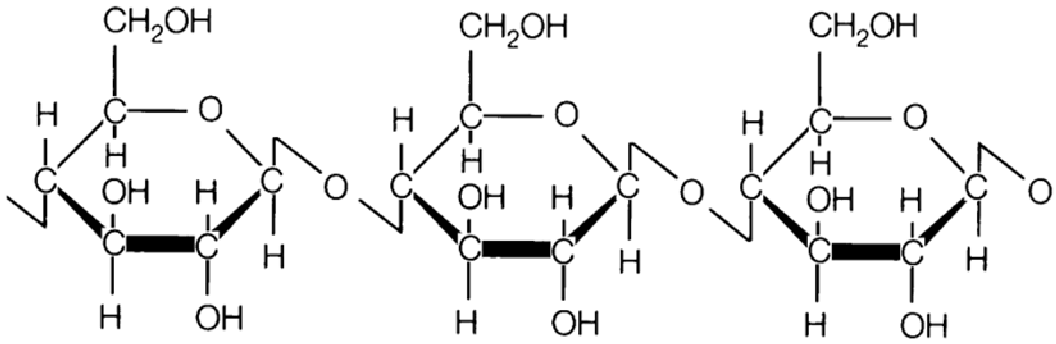


Figure 9. Structure of cellulose
(Source: WEB_5 2007).

2.3.2.3.1. Cellulose Acetate

Cellulose, a natural polymer produced by plants, is a polysaccharide composed of long chains of glucose molecules. This polymer can be chemically modified to produce cellulose acetate (CA), the properties of which are directly influenced by the degree of substitution by acetate groups. It is produced by replacing approximately 75% of hydroxyl groups on the backbone of the polymer with acetate group. CA is a semicrystalline polymer having excellent mechanical properties (Calil et al. 2007).

2.4. Antimicrobial Agents

2.4.1. Definition of Antimicrobial Agents

Antimicrobial agent is a chemical or natural compound that either destroys or inhibits the growth of microscopic and submicroscopic organisms. On the basis of their primary activity, they are more specifically called antibacterial, antifungal,

antiprotozoal, antiparasitic, or antiviral agents. A much larger number are used in almost every phase of human activity: in agriculture, food preservation, and water, skin, and air disinfection (WEB_6 2007). A compilation of some common uses for antimicrobials is shown in the Table 3.

Table 3. Common antimicrobial agents and their uses
(Source: WEB_6 2007).

Use Field	**Agents**
Antibacterials	Sulfonamides, isoniazid, p-aminosalicylic acid, penicillin, streptomycin, tetracyclines, chloramphenicol, erythromycin, novobiocin, neomycin, bacitracin, polymyxin
Antiparasitics (humans)	Emetine, quinine
Antiparasitics (animal)	Hygromycin, phenothiazine, piperazine
Antifungals	Griseofulvin, nystatin
Chemotherapeutics (plants)	Captan (*N*-trichlorothio-tetrahydrophthalimide), maneb (manganese ethylene bisdithiocarbamate), thiram (tetramethylthiuram disulfide)
Skin disinfectants	Alcohols, iodine, mercurials, silver compounds, quaternary ammonium compounds, neomycin
Water disinfectants	Chlorine, sodium hypochlorite
Air disinfectants	Propylene glycol, lactic acid, glycolic acid, levulinic acid
Gaseous disinfectants	Ethylene oxide, β-propiolactone, formaldehyde
Clothing disinfectants	Neomycin
Animal-growth stimulants	Penicillin, streptomycin, bacitracin, tetracyclines, hygromycin
Food preservatives	Sodium benzoate, tetracycline

2.4.2. Types of Antimicrobial Agents

Antimicrobial agents can be divided into two categories according to their sources. These are chemical antimicrobial agents and natural antimicrobial agents.

2.4.2.1. Chemical Antimicrobial Agents

Any chemical that when added to food tends to prevent or delay deterioration, but does not include common salt, sugars, vinegars, spices, or oils extracted from spices, or chemicals applied for their respective insecticidal or herbicidal properties are defined as chemical preservatives (Davidson and Branen 1993).

2.4.2.1.1. Sodium Benzoate and Benzoic Acid

Benzoic acid is one of the oldest chemical preservatives used in the cosmetic, drug, and food industries. Sodium benzoate was the first chemical preservative approved for use in foods by the U.S. Food and Drug Administration.

The structural formulas of benzoic acid (C_6H_5COOH) and sodium benzoate (C_6H_5COONa) shown in Figure 10. Benzoic acid (molecular weight 122.1) occurs in pure form as colorless or white needles or leaflets. It is soluble in water. Sodium benzoate (molecular wieght 144.1) is a white granular or crystalline powder. It is much more soluble in water than benzoic acid (Davidson and Branen 1993).

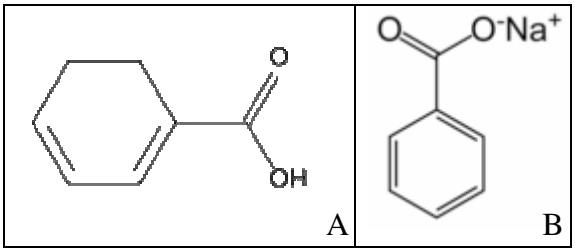


Figure 10. Chemical structures of benzoic acid (A) and sodium benzoate (B) (Source: WEB_7 2007).

2.4.2.1.2. Sorbic Acid and Sorbates

In recent years, sorbic acid (Figure 11.) and its more water soluble salts, especially potassium sorbate known as sorbates are used widely throught the world as preservatives for various foods, animal feeds, pharmaceuticals, cosmetics and in other applications.

They are very good preservatives because they inhibit or delay the growth of many microorganisms, including yeasts, molds, and bacteria. Under certain conditions, sorbates can not inhibit some microbial strains. In general, however, they are considered effective food preservatives when used under sanitary conditions and in products processed using good manufacturing practices (Davidson and Branen 1993).

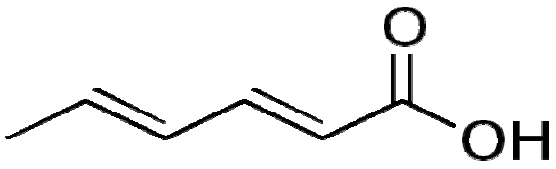


Figure 11. Chemical structure of sorbic acid
(Source: WEB_8 2007).

2.4.2.2. Natural Antimicrobial Agents

Recently, due to health concerns and increased awareness, people preferred not to consume the food including chemical preservatives. Because of this condition, natural antimicrobial agents have gained a major importance. The most popular antimicrobial agents are enzymes such as lysozyme, lactoperoxidase, etc.

2.4.2.2.1. Nisin

Nisin is an antibacterial polypeptide (molecular weight 3,510) produced by *Lactococcus lactis* subspecies *lactis* that broadly inhibits gram-positive bacteria and sporeformers (Padgett et al. 1998) but when combined with a chelator, nisin also can inhibit growth of some gram-negative bacteria (Dawson et al. 2005).

Generally all proteins have absorbance at 280 nm but nisin has no absorbance at this wavelength, since it contains no aromatic acids. The solubility and stability of nisin depend on the pH of the solution. In dilute HCl solutions at pH 2.5 its solubility is 12%. The solubility decreases to 4% at pH 5.0. Nisin is insoluble and irreversible inactivation occurs even at room temperature while pH are neutral and alkaline values (Davidson and Branen 1993).

2.4.2.2.2. Lysozyme

Lysozyme (EC3.2.1.17), found in different sources including plants, animals and microorganisms, is a single peptide enzyme (Ibrahim et al. 1996). Its molecular weight is 14400 and it contains 129 amino acid residues (Takahashi et al. 2000, Ibrahim et al. 1991). Its lytic activity on bacteria is occurred by hydrolyzing glycosidic β-linkages between N-acetylhexosamines of pepdidolycan (PG) in their cell walls. Because of that reason, many researchers have studied lysozyme for biopreservation of foods. Most of the studies related with lysozyme have been focused on hen egg white lysozyme because the enzyme is commercially purified from hen egg white (Chang et al. 2000). The antimicrobial activity of the enzyme is mainly against gram-positive bacteria but because of the PG layer surrounded by a protective lipopolysaccharide (LPS) membrane, it is ineffective against gram-negative bacteria (Nakamura et al. 1991, Ibrahim et al. 1991).

Many researchers have interested in increasing the antimicrobial spectrum of lysozyme. For instance, combination of lysozyme with EDTA makes lysozyme highly effective on gram-negative bacteria (Mecitoglu et al. 2006), and conjugates of lysozyme with dextran, galactomannan or xyloglucan have good antimicrobial activity against both gram-positive and gram-negative bacteria when applied in combination with mild heating at 50 ℃ (Nakamura et al. 1992).

In several studies, lysozyme was incorporated as a preservative in many packaging materials to extend the shelf life of the foods (Appendini and Hotchkiss 1997, Buonocore et al. 2004, Mecitoğlu et al. 2006).

Appendini and Hotchkiss investigated the feasibilty of incorporating lysozyme as an antimicrobial enzyme into polymers (PVOH, nylon 6.6, cellulose triacetate (CTA)) which are sutible for food contact (Appendini and Hotchkiss 1997).

Highest activity per gram polymer was obtained when lysozyme was immobilized on CTA films (Table 4.). Also activity retention after repeated use was highest in the CTA films. Lowest activity was obtained with nylon 6.6. pellets. This polymer not only showed low activity but also 40 and 60 % of this activity was lost when it was reused. PVOH had higher activity than nylon 6.6, however, almost all activity was lost when reused a second time. CTA was chosen for further studies because of that reasons.

Buonocore et al (2004) investigated the release kinetics of lysozyme, nisin, and sodium benzoate in the PVOH which is highly swellable polymer. They controlled the the release kinetics by controlling the degree of crosslinking using glyoxal as a crosslinking agent. Their results indicate that the release kinetics of both lysozyme and nisin can be modulated through the degree of crosslink of the polymer matrix, whereas multilayer structures need to be used to control the release kinetics of sodium benzoate (Buonocore et al., 2004).

Table 4. Efficiency of lysozyme immobilization on different polymers (Source: Appendini and Hotchkiss 1997).

Activity retained after	**added[a] (mg/g polymer)**	**(unigs[b]/g polymer) (units[b]/g polymer)**	**run[c] (%)**
PVOH	100	170	10
Nylon 6,6	10	43	60
(methylated Nylon 6,6)			
(GA-PEI)	48	30	40
CTA[d]	100	605	90

[a] Initial amount of enzyme added per gram polymer

[b] One unit is equal to 0.001 unit decrease in absorbance at 450 nm of a 0.015% *M.lysodeicticus* suspension in 66.6 mM phosphate buffer, pH 6.24.

[c] Activity of a sample was tested repeatedly. The data indicate the activity of a sample that was used twice as a percentage of the activity of the same sample when used once.

[d] Films formed by addition of 100 mg lysozyme/g CTA dissolved in 2 ml 66.6 mM phosphate buffer, pH 6.24, containing 30% glycerol to methylene chloride emulsion. The emulsion was stirred for 60 min.

Mecitoğlu et al. (2006) incorporated lysozyme into zein films and investigated its release profile from the films into water used as a liquid food. They have observed an increase in maximum released activities with increased activities of lysozyme incorporated into the films. In their study, because of ethanol treatment during partial purification and in film preparation, activation of the enzyme was observed. Some kinetic parameters of lysozyme release from zein films are listed in Table 5.

Table 5. Some kinetic parameters related to lysozyme release from zein films at 4 ^{o}C (Source: Mecitoğlu et al. 2006).

No	Incorporated lysozyme activity in films (U /cm^2)	Release Rate (U/ cm^2/min)	Maximum activity released (U/cm^2)	Activity recovered from films (%)[d]	Immobilized activity retained in films (U/cm^2)
1	187 (63)[a]	9 (0-10)[b]	246 ±60 (1380)[c]	132	1.1
2	374 (126)	8 (0-30)	323 ±25 (360)	86	4.6
3	541 (148)	7 (0-60)	731 ±85 (1380)	135	11
4	708 (244)	27 (0-60)	2229 ±71 (360)	315	5
5	1318 (455)	29 (0-120)	4034 ±37 (360)	306	5

[a] Lysozyme incorporated into films as µg/cm^2;

[b] Time periods of data used in best fit to calculate initial rates;

[c] Release test periods (min) to achieve maximum activity released;

[d] (maximum activity released / incorporated activity) x 100

CHAPTER 3

MODELING OF ANTIMICROBIAL AGENT TRANSPORT IN FOOD PACKAGING SYSTEMS

3.1. Previous Studies

Modeling of transport of active agent from the packaging film into food is very useful. Because, such a model allows to determine diffusion coefficient of active agent in the film, hence to estimate concentration profile throughout the film and to predict the time period in which the antimicrobial concentration will be maintained above the critical inhibitory concentration in the packaged food.

Jung H. Han selected two-layer system (package / food) as a model system in which solid foods and liquid or viscous foods are in direct contact with the packaging material (Han 2000). To explain mass transfer through this model system, he has proposed two approaches. In the first approach, it is assumed that diffusivity of active agent in the packaging film is much smaller than that in the film and volume of the food layer is very large compared to the volume of the packaging film. Thus, diffusion of active agent from the film into the semiinfinite volume was considered, and solution of Fick's second law given by Crank was utilized. In the second approach, transport of active agent both in the packaging film and food was considered. This approach was also extended for three layer structure containing outer antimicrobial barrier layer, matrix layer and food (Crank 1975).

Choi et al. used the first approach proposed by Jung H. Han to find the diffusion coefficient of potassium sorbate in κ-carrageenan based antimicrobial film at different pH and temperature (Choi et al. 2005, Han 2000).

According to this approach, fractional release of active agent with time is given by the following equation (Crank, 1975).

$$\frac{Mt}{M\infty} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} \exp\left[\frac{-(2n+1)^2 \pi^2 Dt}{l^2}\right] \qquad (1)$$

At short times, t→0, Eqn (1) reduced to following form:

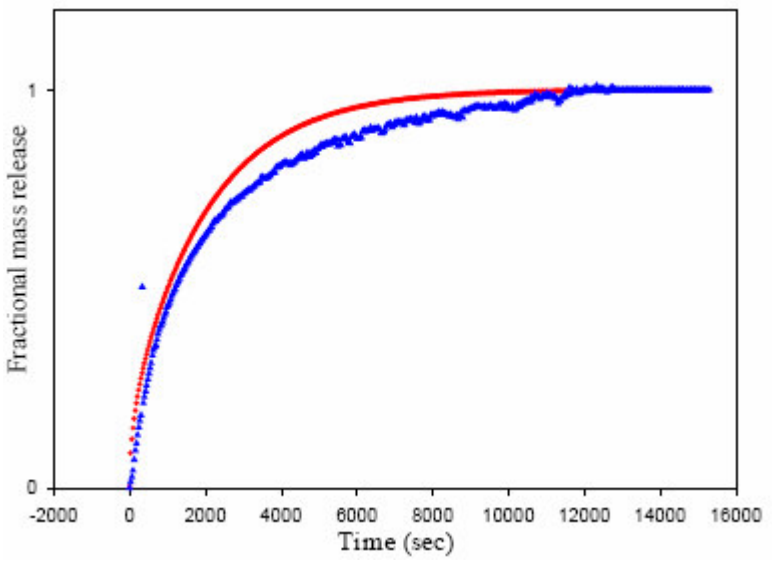
$$\frac{M_t}{M_\infty} = \frac{2}{\sqrt{\eta}}\left(\frac{D \cdot t}{L^2}\right)^{1/2} \tag{2}$$

At short times, fractional uptake curve becomes linear, thus, they have calculated the diffusion coefficient from the slope of the initial linear region of M_t / M_∞ vs $\sqrt{t}$ curve as follows (Choi et al. 2005):

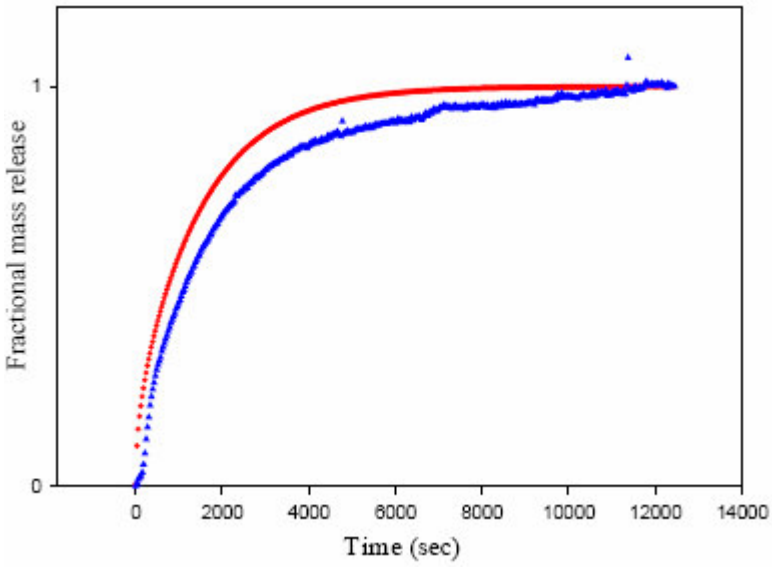
$$D = \left(\frac{kl}{4}\right)^2 \pi \tag{3}$$

where k corresponds to initial slope.

Figure 12a through 12c show experimental and theoretical fractional mass release curves measured at 25℃ and various pH values. Table 6. lists the diffusivities calculated and correlation coefficients determined for each curve. The diffusion coefficient of potassium sorbate in K-carrageenan was found to increase with temperature and pH of the medium. Figure 12a through 12c illustrate that agreement between experimental and theoretical uptake curves is not good. This result clearly indicate that the mathematical model, shown by Eqn (1) and based on neglecting mass transfer in the food environment is not sufficient to correlate the experimental data.



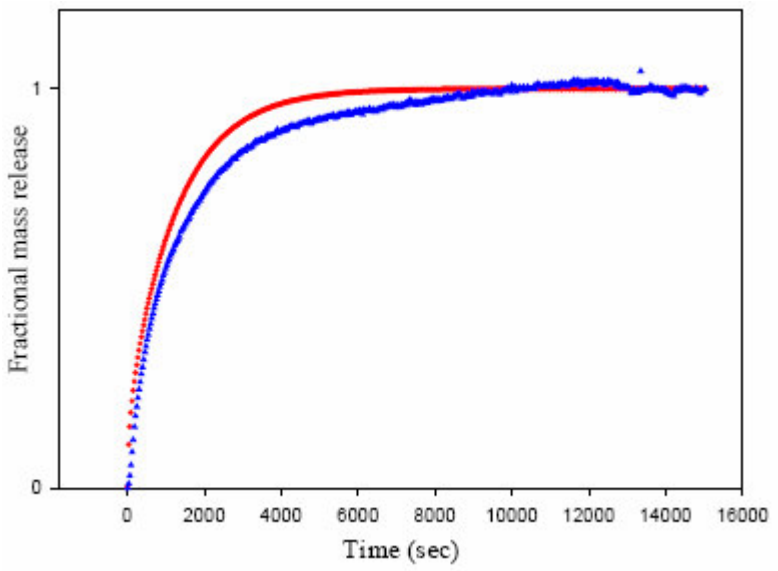
(a)



(b)

Figure 12.Experimental and theoretical fractional mass release of potassium sorbate at 25℃ (●, theoretical data; ▲, experimental data; (A) pH 3.8, (B) pH 5.2, (C) pH 7.0) (Source: Choi et al. 2005).

(cont. on next page)



(c)

Figure 12. Experimental and theoretical fractional mass release of potassium sorbate at 25℃ (●, theoretical data; ▲, experimental data; (A) pH 3.8, (B) pH 5.2, (C) pH 7.0) (Source: Choi et al. 2005). **(cont.)**

Table 6. Diffusion coefficient of potassium sorbate in k-carrageenan film at different pH and temperatures (Source: Choi et al. 2005).

pH	**Temp. (°C)**	**D ($\times 10^{-13}$ m^2/s)**	**r^2**
3.8	40	3.53±0.14	0.990
	25	2.95±0.18	0.994
	5	1.17±0.60	0.994
5.2	40	4.24±0.54	0.993
	25	2.98±0.81	0.983
	5	1.29±0.15	0.993
7.0	40	6.42±3.4	0.992
	25	2.60±0.76	0.994
	5	1.05±0.50	0.992

In another study, Buonocore et al. derived a mathematical model to describe the release kinetics of antimicrobial agents (lysozyme, nisin, and sodium benzoate) from crosslinked polyvinylalcohol (PVOH) into water (Buonocore et al. 2003).

The model takes into account the diffusion of water into polymeric film, the counter-diffusion of antimicrobial agent from the film into water and swelling and relaxation of the polymer matrix.

The model parameters were determined by first conducting a swelling experiment in which water penetrates into PVOH films containing no antimicrobial agents, then, measuring release kinetics of antimicrobial agnets from the films as shown in Figure 13.

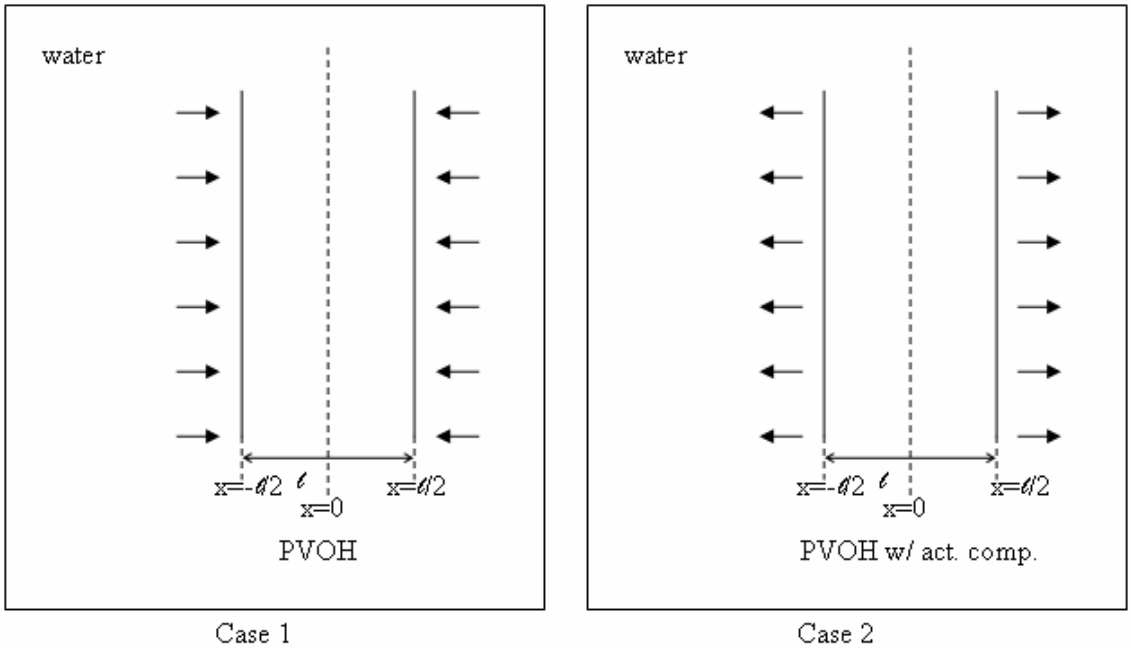


Figure 13. The model systems of the diffusion of the water (Case 1) and the active compound (Case 2).

The model was found to be successful in fitting the water sorption kinetics (Figure 14-15) as well as the release kinetics of antimicrobial agents, lysozyme, nisin and sodium benzoate. The comparison between experimental sorption kinetics and theoretical ones for the antimicrobial agent, lysozyme are shown in Figure 16. through 17.

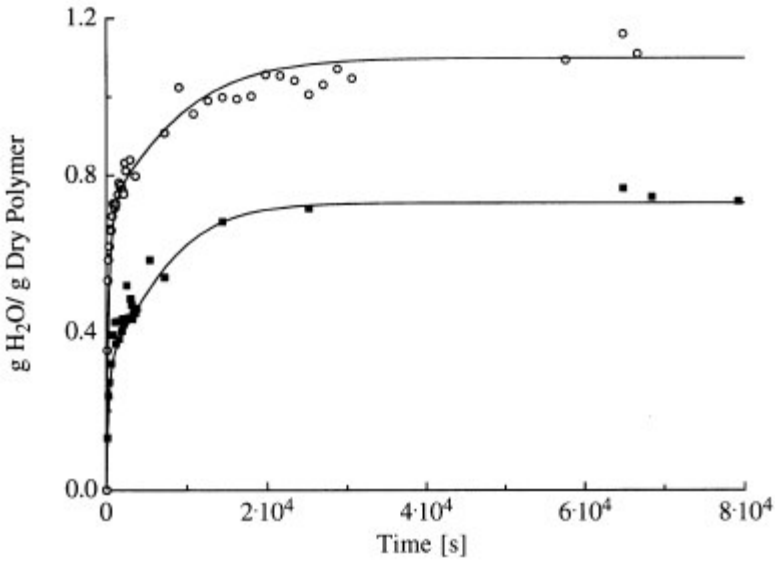


Figure 14. Swelling ratio plotted as a function of time. (■) Film A, (○) Film B, (—) best fit of the proposed model to the experimental data (Source: Buonocore et al. 2003).

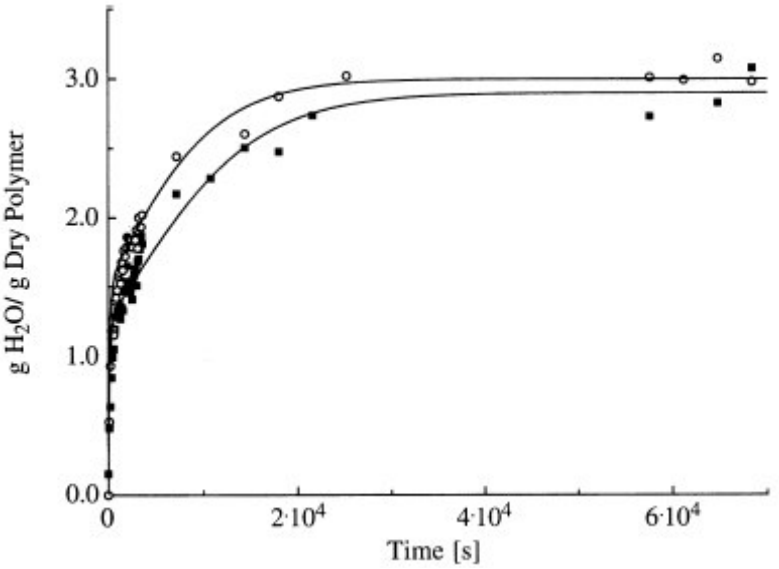


Figure 15. Swelling ratio plotted as a function of time. (■) Film C, (○) Film D, (—) best fit of the proposed model to the experimental data (Source: Buonocore et al. 2003).

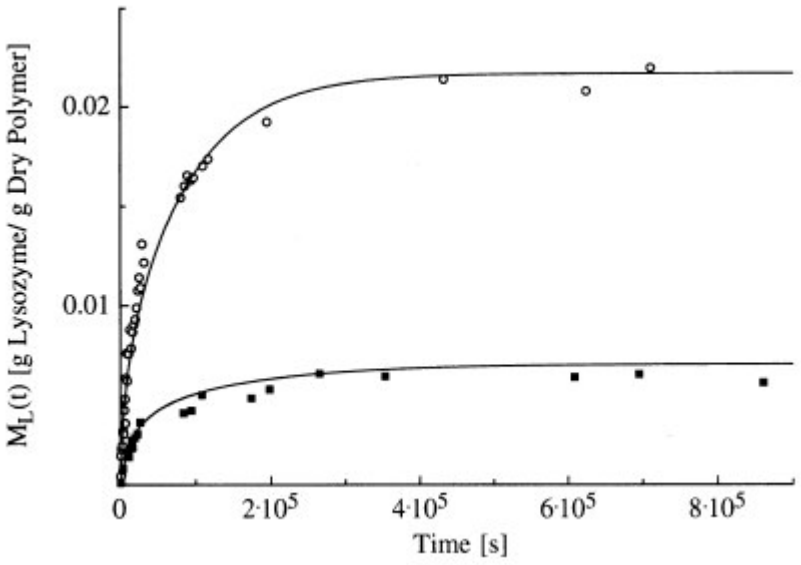


Figure 16. Amount of lysozyme released plotted as a function of time. (■) Film A, (○) Film B, (—)best fit of the proposed model to the experimental data (Source: Buonocore et al. 2003).

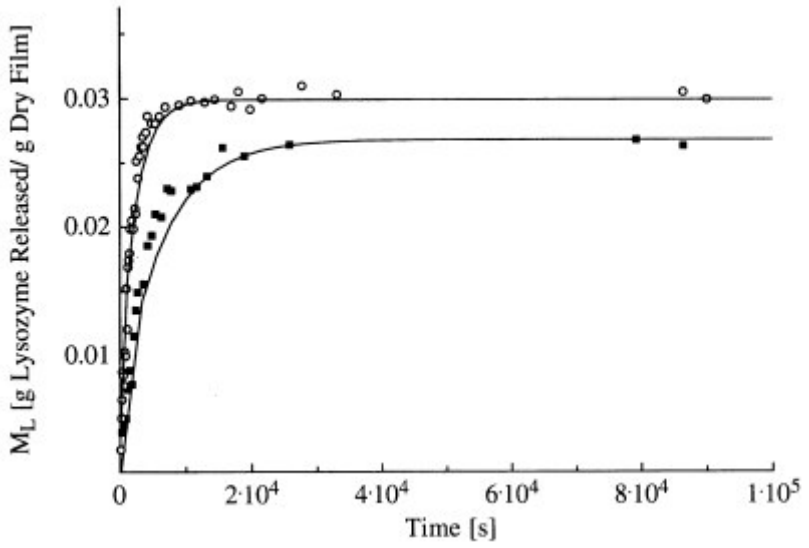


Figure 17. Amount of lysozyme released plotted as a function of time. (■) Film C, (○) Film D, (—)best fit of the proposed model to the experimental data (Source: Buonocore et al. 2003).

They have performed the experiments with four different types of films prepared by using different amount of crosslinking agent, glyoxal.

The diffusion and partition coefficients of antimicrobial agents through these films determined by fitting the experimental data with the model results are all listed in Table 7. The results indicate that as the degree of crosslinking increased, the diffusion coefficient of active compounds decreased while their partition coefficient increased.

Table 7. Values of the diffusion coefficient and partition coefficient of the active agents (Source: Buonocore et al. 2003).

Sample	**%(w) of crosslinking agent**	**Lysozyme (L)**		**Nisin (N)**		**Sodium benzoate (SB)**	
		$D_F^L (cm^2/s)$	K_L	$D_F^N (cm^2/s)$	K_N	$D_F^{SB} (cm^2/s)$	K_{SB}
Film A	7.700	3.83×10^{-11}	431.37	3.01×10^{-10}	152.80	1.25×10^{-8}	55.82
Film B	2.000	2.45×10^{-10}	45.62	3.16×10^{-9}	67.58	4.20×10^{-8}	60.01
Film C	0.770	2.10×10^{-9}	15.06	6.24×10^{-9}	34.57	2.55×10^{-8}	25.96
Film D	0.077	9.98×10^{-9}	6.33	8.61×10^{-9}	26.73	2.54×10^{-8}	21.39

3.2. Determination of Diffusion Coefficient of Active Agent in the Packaging Material

In this study, the diffusion coefficient of lysozyme in the films was determined by combining the release kinetics measurements with the mathematical model presented below. During a typical desorption experiment, a polymer film with a thickness of L is placed in a well stirred limited volume of solution. If it is assumed that there is no chemical reaction between the active compound and the film, mass transfer in the film takes place only by diffusion and diffusion coefficient of active compound in the film, D, is constant, then Fick's second law is used to describe the change in the concentration of active compound in the film with respect to time and position:

$$\frac{\partial C_f}{\partial t} = D\frac{\partial^2 C_f}{\partial x^2} \tag{4}$$

The solution of Eq. (4) requires one initial and two boundary conditions. As shown in Figure 18., one side of the film is made impermeable for the desorption of active compound into the solution,

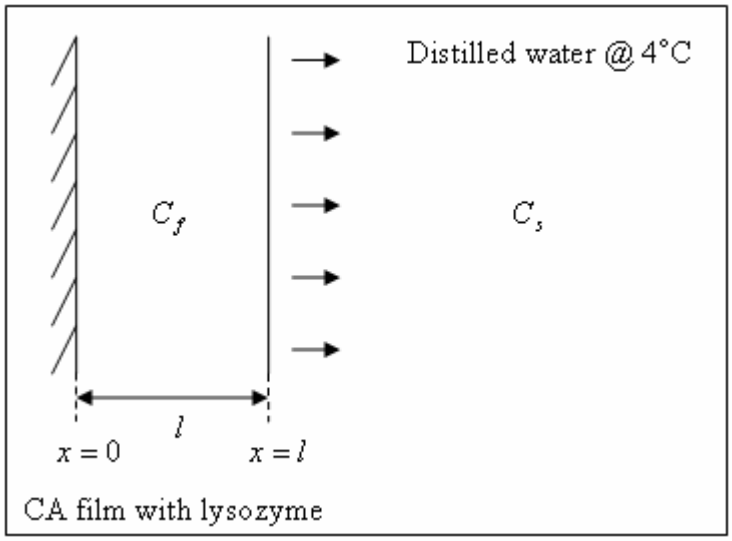


Figure 18. Schematic representation of the desorption system.

Thus:

$$x = 0 \qquad \frac{\partial C_f}{\partial x} = 0 \tag{5}$$

The rate at which active compound leaves the film from the upper surface is always equal to that at which it enters the solution. This condition is

$$x = L \qquad V_{sol}\frac{\partial C_s}{\partial t} = -DA_m\frac{\partial C_f}{\partial x} \tag{6}$$

Eq. (6) indicates the fact that the concentration of active compound in the solution depends only on time since the solution is well stirred, thus, its total amount in the solution and in the film remains constant as diffusion proceeds. If the concentration within the surface of the film is related to that in the solution through a partition coefficient, K,

$$x = L \qquad C_f = KC_s \tag{7}$$

then, Eq. (6) is rewritten as follows:

$$x = L \qquad a\frac{\partial C_f}{\partial t} = -D\frac{\partial C_f}{\partial x} \tag{8}$$

where a is given by

$$a = \frac{V_{sol}}{KA_m} \tag{9}$$

Initially, the antimicrobial agent is entrapped into the polymer film at a uniform concentration of C_o. Then, the initial condition is

$$t = 0 \qquad C_f = C_o \tag{10}$$

A solution of these model equations from Eq. (4) through Eq. (10) has been obtained by Carslaw and Jaeger and it is presented in a classical book of Crank (Carslaw and Jaeger 1951, Crank 1975). In a form expressing the ratio of total amount of active compound desorbed from the film at any time t, M_t, to the amount desorbed at equilibrium, M_∞, the solution is

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=1}^{\infty} \frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2 q_n^{\,2}} \exp\left(-Dq_n^{\,2}t/L^2\right) \tag{11}$$

where the q_ns are the non-zero positive roots of

$$\tan q_n = - \alpha q_n \tag{12}$$

and $\alpha = \frac{a}{KL}$. Using this analytical solution, the diffusivitiy of active compound in the film was determined through nonlinear least square analysis by minimizing the difference between Equation (11) and experimental uptake curves.

CHAPTER 4

MATERIALS AND METHODS

4.1. Materials

Micrococcus lysodeikticus (Sigma M3770), dialysis tubes (12000 MW, prepared as described in the product manual) were obtained from Sigma Chem. Co. (St. Louis, MO, USA). Disodium EDTA.$2H_2O$ (ethylenediaminetetraacetate dihydrate) was purchased from Riedel-de haën (Sigma-Aldrich Laborchemikalien, Seelze, Germany). Fresh hen eggs used in production of lysozyme were obtained from a supermarket in İzmir, Turkey. Cellulose acetate with a molecular weight of 50,000 and acetyl content of 39.8% was obtained from Eastman (Kingsport, TN, USA). Acetone (99 %) was obtained from Merck (Darmstadt, Germany). *Escherichia coli* (NRRL B-3008) and *Bacillus amyloliquefaciens* (NRRL NRS-762) were kindly provided by USDA Microbial Genomics and Bioprocessing Research Unit (Peoria, Illinois).

4.2. Methods

4.2.1. Production of Lysozyme

Lysozyme was produced by slightly modifying the partial purification step given by Jiang et al. (2001). Briefly, the egg whites separated carefully without disturbing the egg yolks were first diluted 3-fold with 0.05 M NaCl solution. To precipitate the egg white proteins other than the lysozyme, the pH of this mixture was set to 4.0 by carefully adding several drops of 1 N acetic acid and it was diluted with equal volume of 60 % (v/v) ethanol. After 6 hours incubation at room temperature in presence of 30 % ethanol, the mixture was centrifuged at 15 000 x g for 15 min at 4 ^{o}C and the precipitate was discarded. The supernatant containing lysozyme was first dialyzed for 21 h at 4 ^{o}C by three changes of 2000 mL distilled water and then lyophilized by using a freeze drier (Labconco, FreeZone, 6 liter, Kansas City, MO, USA) The lyophilized

enzyme was stored at – 18 ^{o}C and its activity was determined as U/mg before each film preparation.

4.2.2. Preparation of Films

For preparation of films, cellulose acetate (CA) and lysozyme dissolved in acetone and distilled water, respectively were mixed under continuous magnetic stirring for 30 minutes at 920 rpm in a tightly closed and sealed bottle to prevent evaporation. Lysozyme concentration in the film forming solution was kept constant at 1.5% (w/w) while CA , acetone and water concentrations were changed. The mixed solution was then cast on a polypropylene substrate with the aid of an automatic film applicator (Sheen, Automatic film applicator-1133N, Kingston, England) at a speed of 100 mm/sec. The thickness of the film was adjusted by a four-sided applicator with the gap size of 300 micron. The cast film was then immediately placed into an environmental chamber (Siemens, Simatic OP7, Massa Martana, Italy) and dried for 30 min at 25°C and 40 % relative humidity.

4.2.3. Release Tests

The release tests were conducted for both dense and porous sides of the films separately by using a hand made glass apparatus shown in Figure 19. In this apparatus, film samples 5 cm in diameter were squeezed between two glass plates one of which contains a circular hole with a diameter of 4 cm at the center. To prevent contact of water with the protected side of the film, two silicon o-rings (full diameter: 6 cm, inside diameter: 4 cm) were placed on top of the samples. The apparatus was then fixed with two rubber attachments from both sides and placed into a glass Petri dish (10 cm in diameter) containing 50 mL distilled water at 4^{o}C and stirred magnetically at 240 rpm with a 2cm long Teflon coated rod. The Petri dishes were covered tightly with parafilms and with their glass lids to prevent evaporation during long release period. The lysozyme activity in release medium was monitored by taking 0.6 mL samples at different time periods and assaying activity three times by using the method given in section 4.2.4. 1 mL samples were also taken periodically to determine lysozyme release kinetics from increased protein content in the solution. Protein concentration was

measured spectrophotometrically by reading absorbance of the samples at 280 nm. The sample used in determination of protein content was poured back into release test solution.

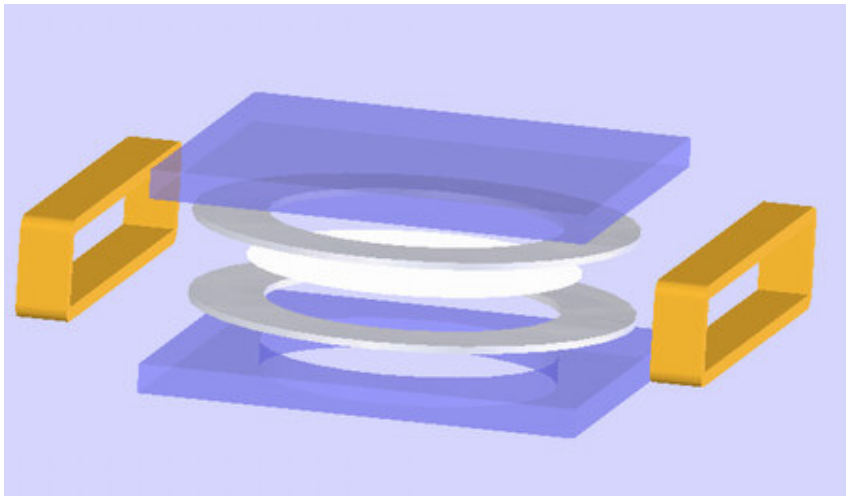


Figure 19. The illustration of the experimental set-up used in release tests of porous or dense surfaces of cellulose acetate films.

4.2.4. Determination of Soluble Lysozyme Activity

The activity of lysozyme was determined by measuring the decrease in absorbance of *Micrococcus lysodeikticus* suspension prepared in 0.05M Na-phosphate buffer at pH 7.0 with a concentration of 0.26 mg/ml. 2.3 ml of this suspension at 30 ℃ was mixed with 0.2 ml of lysozyme solution incubated at 30 ℃ for 5 minutes. The decrease in absorbance was monitored for 120 sec at 660 nm and 30 ℃ by using a UV/VIS spectrophotometer (PerkinElmer, Lambda 45, USA) equipped with a constant temperature cell holder. Activity was calculated from the slope of the initial linear portion of absorbance vs. time curve and expressed as Unit or percent initial activity. One Unit was defined as 0.001 change in absorbance within 1minute time interval. The average of three activity measurements was used in all calculations.

4.2.5. Determination of the Immobilized Lysozyme Activity Retained in Films

The films used in release studies were cut into 2 pieces, placed into glass Petri dishes and brought into contact with 25 ml *M. Lysodeikticus* solution (at 30 °C) prepared in 0.05M, pH 7.0 Na-phosphate buffer with a concentration of 0.26 mg/ml. The petri dishes were kept in an incubator (Nüve, FN 500) at 30 °C and the decrease in absorbance of *M. Lysodeikticus* suspension at 660 nm was monitored periodically. The lysozyme activity of the films were determined from the slopes of the initial linear portions of absorbance vs. time curves and these values were divided by the area of the films to obtain activity per cm^2 of the film (U/cm^2).

4.2.6. Determination of Protein Content

Protein content was determined according to Lowry procedure by using bovine serum albumin as a standard (Harris 1987). The average of five measurements was used to determine the protein content.

4.2.7. Test of Antimicrobial Activity of the Films

The antimicrobial activity of (1) control films and (2) lysozyme, (3) disodium EDTA or (4) lysozyme and disodium EDTA containing films was determined with the zone inhibition assay by using *Escherichia coli* (NRRL B-3008) and *Bacillus amyloliquefaciens* (NRRL-NRS 762) as test microorganisms. The concentrations of lysozyme and disodium EDTA in the film forming solution were adjusted as 1.5 % and/or 1.2 % by weight, respectively, unless other values are indicated in the text. The overnight cultures of *E.coli* and *B. amyloliquefaciens* were prepared in nutrient broth at 37 and 30 °C, respectively. For antimicrobial tests, 24 discs (1.3 cm in diameter) were prepared from each film by a cork borer under aseptic conditions. Half of these discs were tested for the antimicrobial activity of their porous sides, whereas the remaining films were tested for their dense sides. The disodium EDTA containing films were used only for *E. coli* to make it susceptible to lysozyme action. For both microorganisms, the discs were placed onto PCA plates containing 0.1 mL culture applied with spread plate

method. The initial cell concentrations of *E.coli* and *B. amyloliquefaciens* cultures were almost 7.1×10^9 CFU/mL and 1.4×10^8 CFU/mL, respectively. All Petri plates were incubated for 2 days at 37 °C and the diameter of the fully formed zones (ffz) were measured by a digital compass. The total area of ffz (as cm2) was used to evaluate the antimicrobial potential of a film. The number of partially formed zones (pfz) only at one side of the discs and the number of no zone (nfz) formation were also separately reported to better compare the nonhomogeneity of antimicrobial activity of the films.

4.2.8. Mechanical Properties of Films

The tensile strength of the films was measured with Testometric M500-100kN (Lancashire, England) testing machine. The film samples were stressed at a constant rate of 0.25 mm/min until failure. The test method and sample preparation was in accordance with ASTM D 882-02 standard. The samples 5 mm in width and 50 mm in gauge length were used for the tests. At least, five specimens of each type of film were used for the tests and tensile properties were calculated from the plot of stress versus strain.

4.2.9. Morphological Characterization of Films

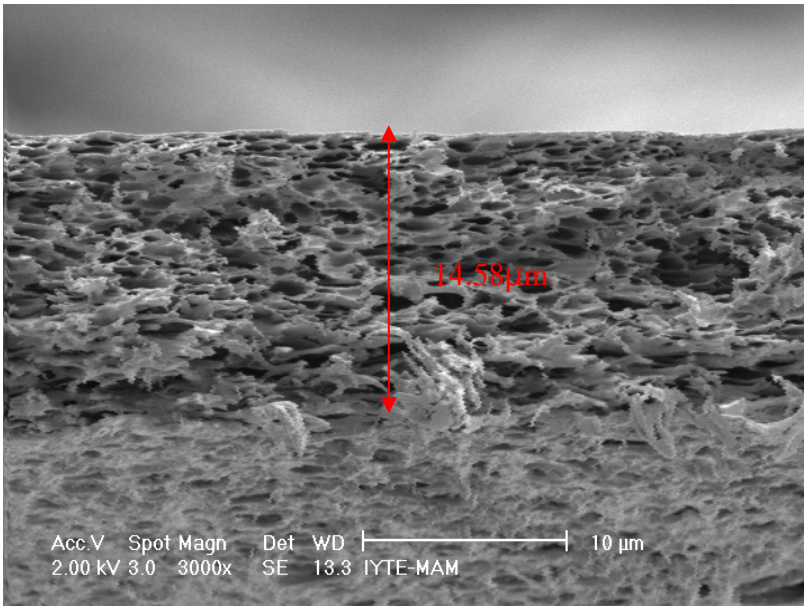
Morphology of the films was examined by scanning electron microscopy (SEM) on a Philips XL-30SFG model. Samples were coated with gold palladium using a Magnetron Sputter Coating Instrument.

CHAPTER 5

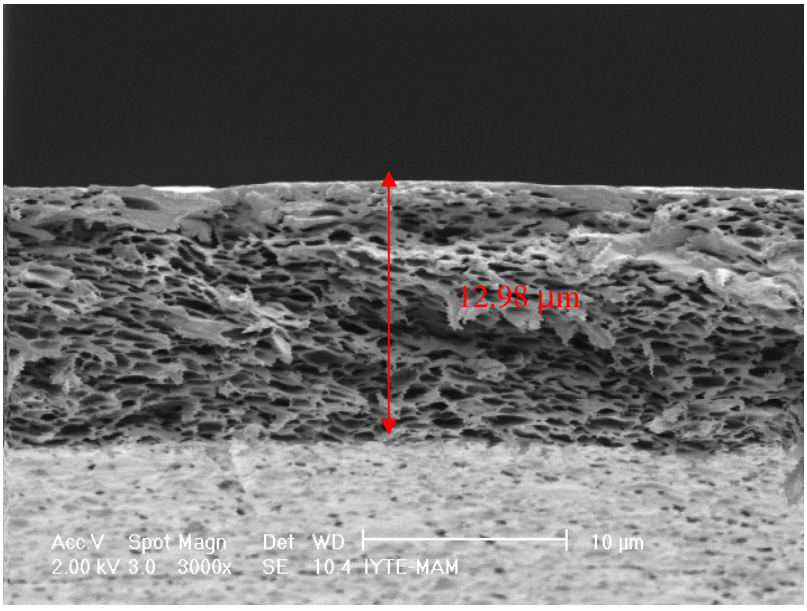
RESULTS AND DISCUSSIONS

5.1. The Morphology of the Films

The films used in this study were prepared by a dry phase inversion technique. In this technique, the polymer dissolved in a mixture of a volatile solvent (acetone) and a less volatile nonsolvent (water) is cast on a support and exposed to an air stream. During drying of the solution, fast solvent evaporation leads to a decrease in solubility of the polymer, then phase seperation into polymer lean and polymer rich phases takes place. The polymer rich phase forms the matrix of the film while the polymer lean phase fills the pores. Film structures ranging from dense to highly asymmetric ones can be obtained depending on the drying conditions, wet casting thickness as well as the composition of the casting solution. Figures 20a through 20c illustrate the influence of the initial casting composition on the structure of the films through SEM pictures. Morphological characterization results obtained from the analysis of SEM pictures are also listed in Table 8. As expected, addition of low concentration of polymer (5 %) to the casting solution created a highly porous structure with a very thin dense skin layer at the top surface as shown in Fig. 2a. On the other hand, relatively thicker skin layers and less porous substructures formed with increased CA/acetone ratio in the casting solution. In addition, average pore size of the films decreased. The film cast with 5 % CA was found to be thicker than that prepared with 10 % CA solution due to presence of macrovoids in that film.



(a)



(b)

Figure 20. SEM of the cross-sections of different cellulose acetate films prepared with CA/acetone/water/lysozyme weight percentages of a) 5/80/13.5/1.5 b)10/80/8.5/1.5 c) 15/80/3.5/1.5. Magnification, 3000x

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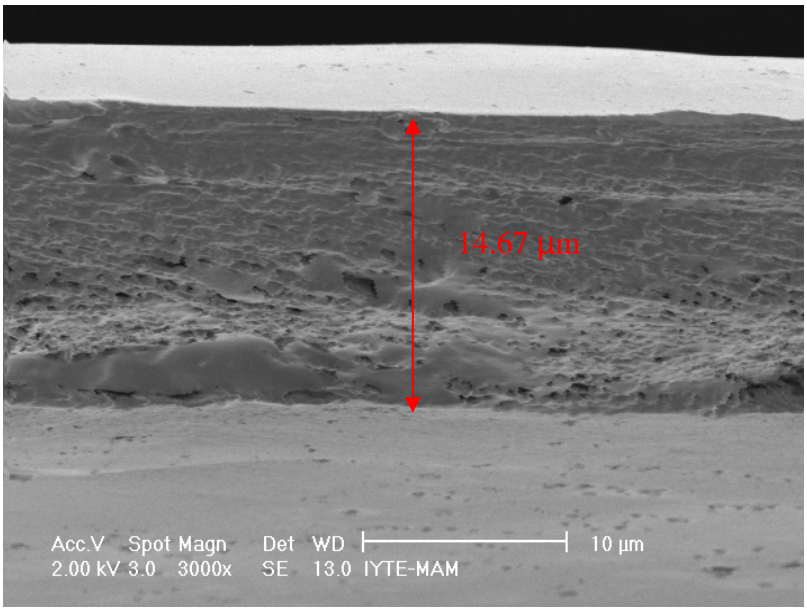


(c)

Figure 20. SEM of the cross-sections of different cellulose acetate films prepared with CA/acetone/water/lysozyme weight percentages of a) 5/80/13.5/1.5 b)10/80/8.5/1.5 c) 15/80/3.5/1.5. Magnification, 3000x **(cont.)**

Table 8. Morphological characterization results of the films.

Compositions[a] (wt %)				Total thickness (µm)	Skin layer thickness (µm)	Pore size (µm)	Porosity (%)
CA	Acetone	H_2O	Lysozyme				
5	80	13.5	1.5	14.58	-	0.995	30.53
10	80	8.5	1.5	12.98	1.27	0.716	29.06
15	80	3.5	1.5	14.67	2.63	0.403	1.37

[a]: Weight percentages of CA/acetone/water/lysozyme in film forming solutions.

5.2. Effect of Film Composition on Released Lysozyme Activity

Due to asymmetric structure of the films, release studies were conducted separately for both sides of the films in order to see the difference on released activity. Antimicrobial packaging is mostly applied to refrigerated foods, thus, the release tests were conducted at 4^{o}C. In fact, the low temperature is also beneficial to minimize loss of lysozyme activity. As seen in Table 9, the highest amount of activity release occurred from the films prepared with 5 % CA. At the dense and porous surfaces of these films, the maximum activities released reached to 530 and 701U/cm^2 which corresponded to 23 and 30 % of the total activities incorporated into the films, respectively. Thus, it seems that a significant portion of activity was maintained in the films in immobilized form or it was lost during film formation in presence of acetone. Then, the enzyme activity released from the porous surface decreased sharply within the first several hours of the release test. Part of the lost activity was recovered, however, the fluctuations in activity continued until the end of the release test. The initial release rate of enzyme was found to be slower from the dense surface of these films. In addition, fluctuations in released enzyme activity from dense surfaces were also less pronounced than those from porous surfaces and enzyme retained more activity between 1400-2000 min of release test. Similar trend was also observed in activity profiles released from the films cast with 10 % CA. On the other hand, significantly lower lysozyme activity was detected during release tests and the maximum activity released from the porous surface hardly reached to 11 % of total activity incorporated into this film. The slower release of lysozyme from dense surfaces also helped recovery of the reductions in enzyme activity during initial periods of the release tests. A slightly lower amount of lysozyme release occurred from porous surface of the film prepared with 15 % CA compared to the amount released from its dense surface. This can be explained by limited amount of lysozyme present in the thin porous area of these films. The results shown in Figure 21a and b. simply indicate that stability of lysozyme in distilled water has changed depending on the type of the surface. This may be due to different changes in conformation of enzyme exposed to different acetone concentrations for various times during the formation of the films.

Table 9. Maximum released and immobilized activities of lysozyme in different CA films prepared by changing initial composition of the film forming solution.

Compositions (wt %)	Film surface	Maximum released activities[b] (U/cm^2)	Recovery [d] (%)	Immobilized activity[e] (U/cm^2)
5/80/13.5/1.5[a]	Porous	701 ± 0 (6)[c]	30%	3.2
	Dense	530 ± 61 (1448)	23%	3.5
10/80/8.5/1.5	Porous	275 ± 65 (36)	11%	7.2
	Dense	109 ± 6 (126)	4.2%	7.4
15/80/3.5/1.5	Porous	63 ± 6 (6)	2.5%	2.7
	Dense	109 ± 6 (246)	4.3%	13.5

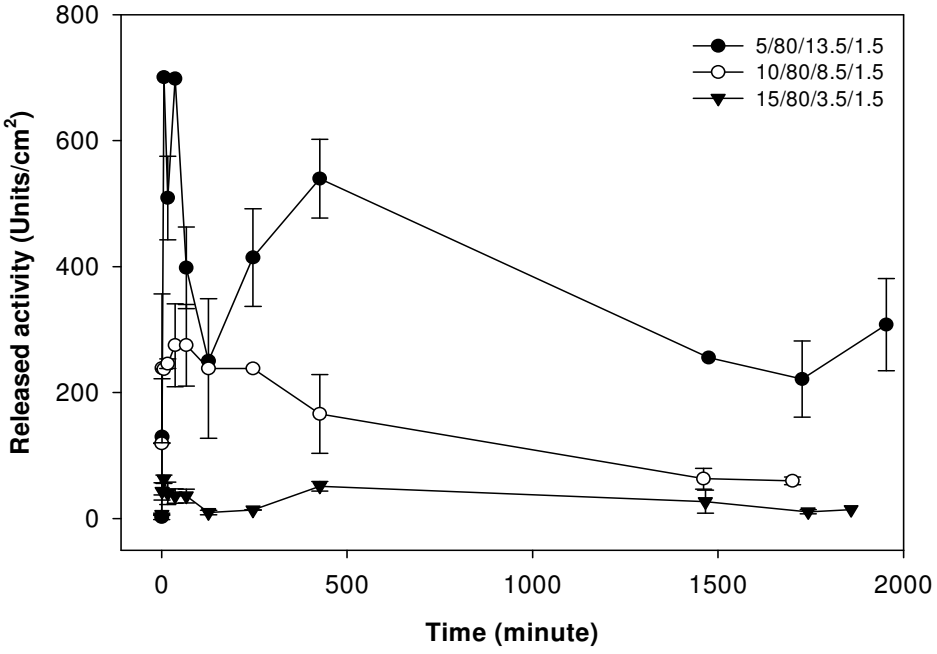
[a] Weight percentages of CA/acetone/water/lysozyme in film forming solutions

[b] The lysozyme used in the film forming solution contained 7666 U/mg activity

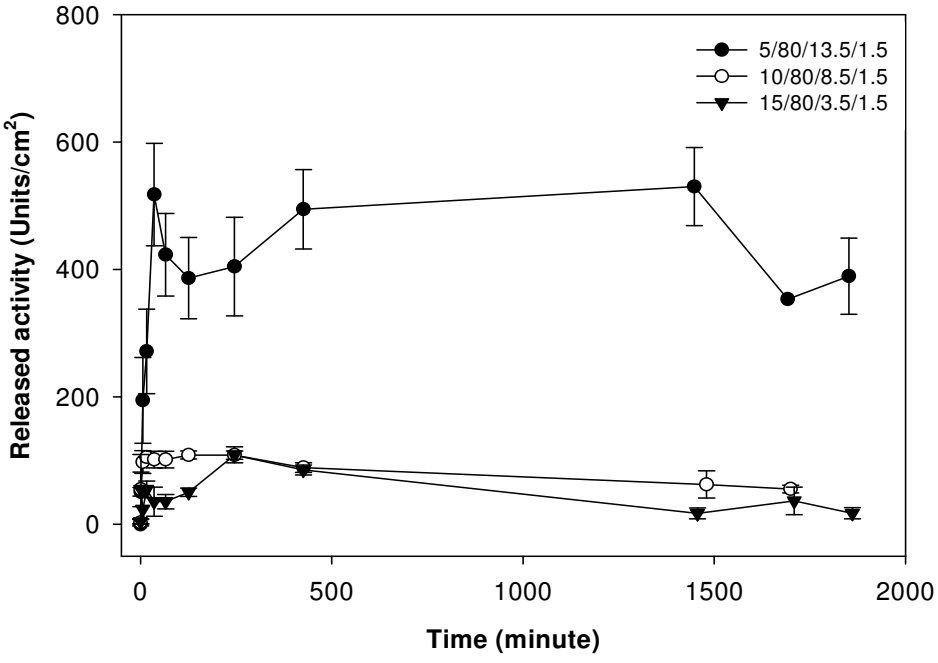
[c] Time (min) to reach maximum activity during release test

[d] (Maximum released activity/Incorporated activity in the films) × 100

[e] Immobilized activities left in films following 1700 min release test in distilled water at 4^{o}C.



(a)



(b)

Figure 21. Change of lysozyme activity during release tests of different cellulose acetate films incubated at 4°C, (a): porous surfaces, (b): dense surfaces of the films.

5.3. Effect of Film Composition on Immobilized Lysozyme Activity

In this study, the films used in release tests were also assayed for their remaining immobilized enzyme activity. In antimicrobial packaging, it is generally intended to maintain maximum amount of soluble antimicrobial activity in the films. However, some immobilized antimicrobial activity may also be beneficial to maintain film sterility during storage of foods. As seen in Table 9, the immobilized activities for the films prepared with 5 % and 10 % CA solutions were found similar when their dense or porous surfaces were used for the measurements. Increasing CA content from 5 % to 10 % in the films caused 2 fold higher immobilized lysozyme activity as the film structure changed from highly porous to dense one. It can be noticed that different immobilized activities were measured using the porous and dense surfaces of the film including 15 % CA. This can be explained by the fact that at the end of 1700 minutes, equilibrium was not achieved between the film and surrounding medium and the release of lysozyme into *M. lysodeikticus* solution still continues especially from the dense side of the film. Thus, lysozyme mostly concentrated throughout the dense region of the film produced much higher immobilized activity. The results in Table 9. clearly indicate that immobilized lysozyme activity depends on the structure of the films. Simply, the change from porous to dense film structures caused an increase in the immobilized lysozyme activities.

5.4. Effect of Film Composition on Antimicrobial Activity

For antimicrobial activity measurements, CA films without lysozyme, with lysozyme and lysozyme combined with EDTA were used. Lysozyme was mixed with EDTA to increase its antimicrobial activity against *E. coli* which is a G (-) bacteria. Part of the antimicrobial test results are reported in Table 10. in terms of number of fully formed zones (ffz), partially formed zones (pfz) and number of no zone formation (nzf).

Table 10. Number of different types of zones formed around different CA films placed onto PCA plates containing *E.coli* as a test microorganism.

Composition[a]	Number of fully formed zones (ffz), partially formed zones (pfz) or no zone formation (nzf)	
	Porous surface	Dense surface
5/80/15/0/0	12 nzf	12 nzf
5/80/13.8/0/1.2	4 ffz /1 pfz / 7 nzf	12 ffz
5/80/12.3/1.5/1.2	12 ffz	10 ffz / 2 nzf
10/80/10/0/0	12 nzf	12 nzf
10/80/8.8/0/1.2	12 ffz	2 ffz / 10 nzf
10/80/7.3/1.5/1.2	11 ffz / 1 pfz	4 ffz / 2 pfz / 6 nzf
15/80/5/0/0	12 nzf	12 nzf
15/80/3.8/0/1.2	2 ffz / 10 nzf	12 nzf
15/80/2.3/1.5/1.2	3 ffz/9 nzf	12 nzf

[a] Weight percentages of CA/acetone/water/lysozyme/EDTA in film forming solutions

In tests conducted for *E. coli*, no zone formation was observed around lysozyme and EDTA free control films as expected. On the other hand, clear zones surrounding most of the films containing a combination of EDTA and lysozyme were measured as illustrated in Figure 22. This result simply indicated diffusion of antimicrobials from the films and subsequent growth inhibition of *E.coli* in the medium. Figure 23. shows that total area of the fully formed zones, hence, the antimicrobial activities of the films decreased with increased CA content in the film forming solution. In addition, when the dense surfaces instead of porous surfaces were brought into contact with the agar medium, the antimicrobial activities reduced by 37 %, 70 % and 100 % for the films prepared with 5 % CA, 10 % CA and 15 % CA solution , respectively. In fact, this is an expected result since the porous surfaces of most of the films gave higher soluble

lysozyme activity during release tests. The dense surfaces of the films cast with 15 % CA solution have shown no clear zone formation. On the other hand, limited diffusion of antimicrobial agents from the porous surfaces of these films into the medium caused the formation of a few clear zones. In order to investigate the contribution of lysozyme on the antimicrobial activity of EDTA against *E.coli* , the tests were also conducted with the films including EDTA alone as an antimicrobial agent. The results shown in Figure 24. illustrates that when the porous surfaces of the films were in contact with bacteria, the presence of lysozyme in addition to EDTA increased the antimicrobial activities by 390 % and 62 % for the films prepared with 5 % and 15 % CA solution, respectively. However, the number of fully formed zones and total area of the fully formed zones around the porous surface of the films cast with 1.2 % EDTA alone in 10 % CA solution were found greater than those obtained with the porous surfaces of the films containing the combination of EDTA and lysozyme as antimicrobial agents. This may be due to protein aggregates formed by lysozyme which may block the pores, hence, decrease the diffusion of EDTA from the film into the agar medium. The antimicrobial release from the dense surfaces of the films prepared with 1.2 % EDTA alone in 5 % CA solution were also found to be higher than those from the dense surfaces of the films containing both EDTA and lysozyme. This observation may be attributed to nonhomogenoeus distribution and higher concentration of EDTA at the dense surfaces of these films in the absence of hydrophilic enzyme lysozyme. This result is confirmed by the number and types of zones reported in Table 10. Simply, all 12 discs used in the test produced clear zones around the dense surfaces of lysozyme free films while only 4 fully formed clear zones formed when their porous surfaces were brought into contact with the bacteria. For the dense surfaces of the films cast with 10 % CA solution, the antimicrobial activity enhanced by 125 % in the presence of lysozyme. The results shown in Figures 23 and 24 clearly suggest that the antimicrobial activity of lysozyme and EDTA against G(-) bacteria , *E.coli*, can be controlled by changing the composition of the casting solution.

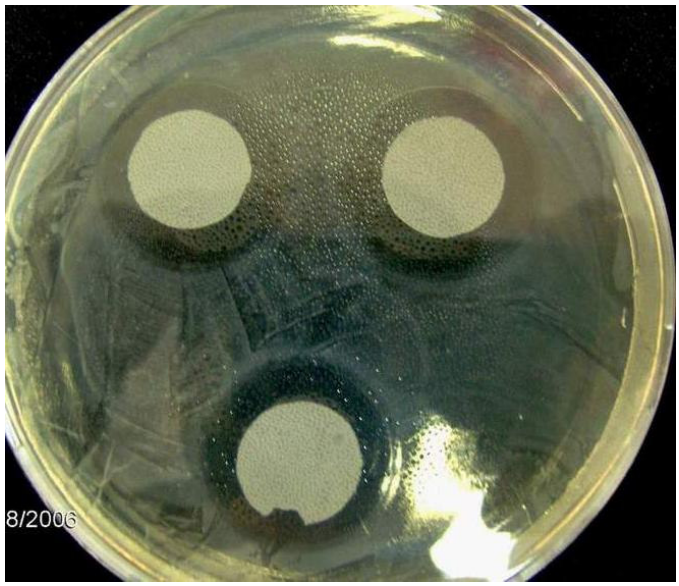


Figure 22. Antimicrobial activity of porous surfaces of cellulose acetate films on *E. coli*. The films were prepared with CA/acetone/water/lysozyme/EDTA weight percentages of 5/80/12.3/1.5/1.2.

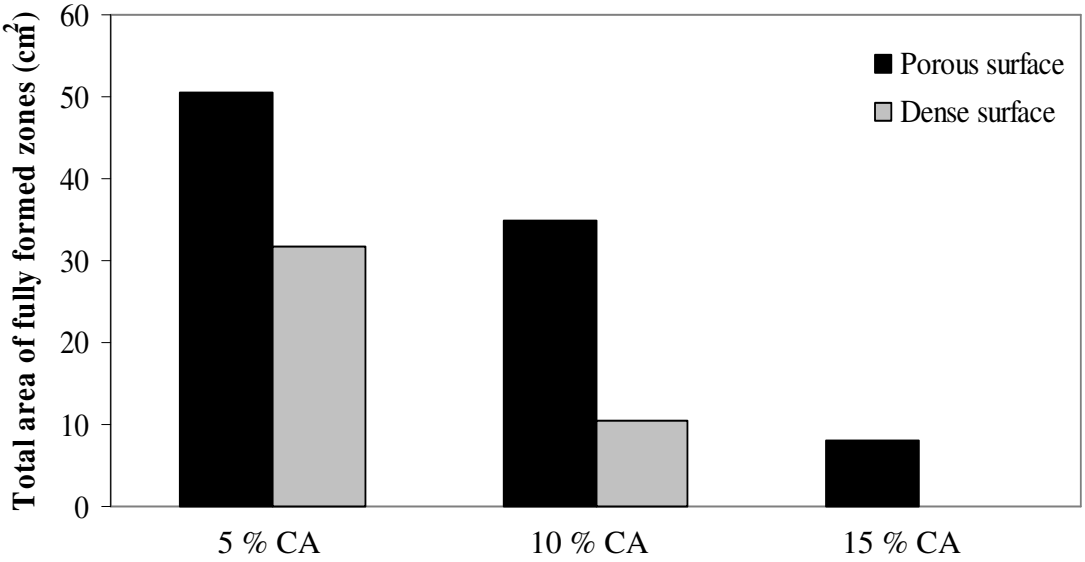


Figure 23. The influence of CA content in the casting solution on the antimicrobial activity of the films against *E. coli*. The weight percentages of acetone, lysozyme and EDTA in each film forming solution were kept at 80 %, 1.5 % and 1.2 %, respectively.

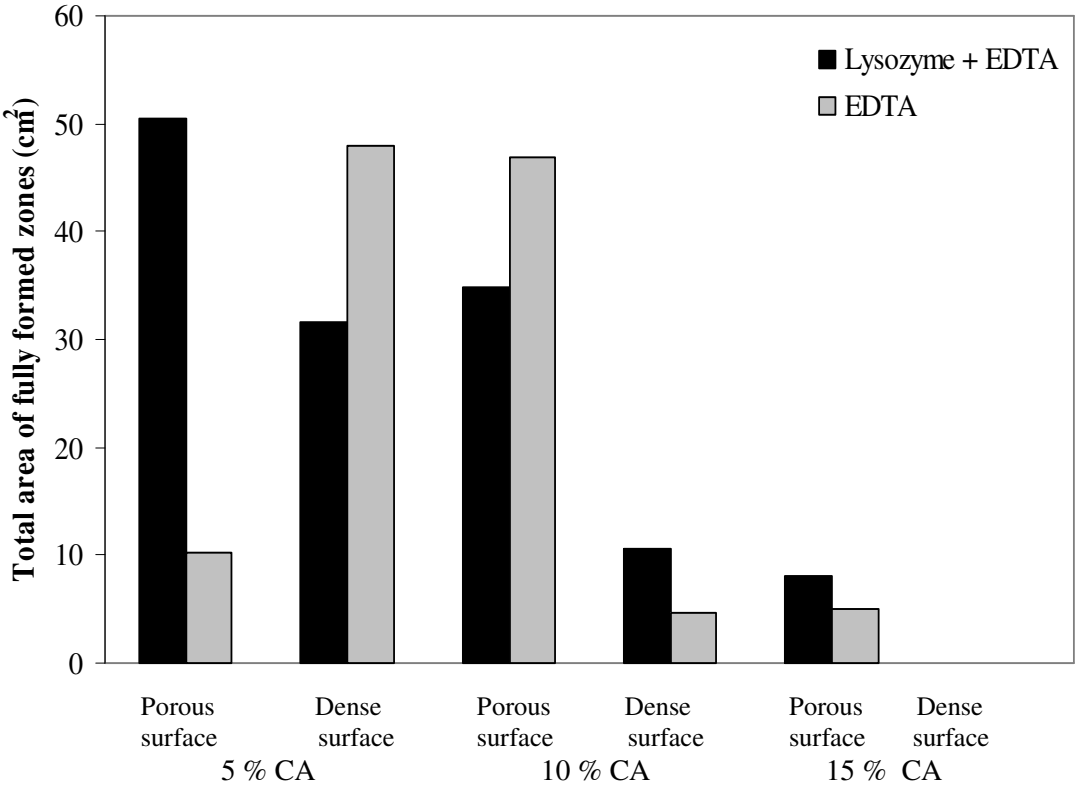


Figure 24. The contribution of lysozyme on antimicrobial activity of cellulose acetate films. The weight percentages of acetone and EDTA in each film forming solution were kept at 80 % and 1.2 %, respectively, while 1.5 % lysozyme was added to this solution to prepare films containing lysozyme and EDTA as antimicrobial agents.

In tests conducted with G(+) bacteria, *B. amyloliquefaciens* , no zone formation was observed around the discs of films prepared with 1.5 % lysozyme in different concentrations of CA solution. However, when film discs were removed carefully from the agar surface, a clear area was observed at all of the disc locations as shown in Figure 25. In lysozyme free control films, extensive growth was observed below the discs. This result clearly showed the presence of antimicrobial activity of lysozyme against *B. amyloliquefaciens*. On the other hand, it is clear that this effect was limited only to film contact surfaces at which released lysozyme concentration was maximal.

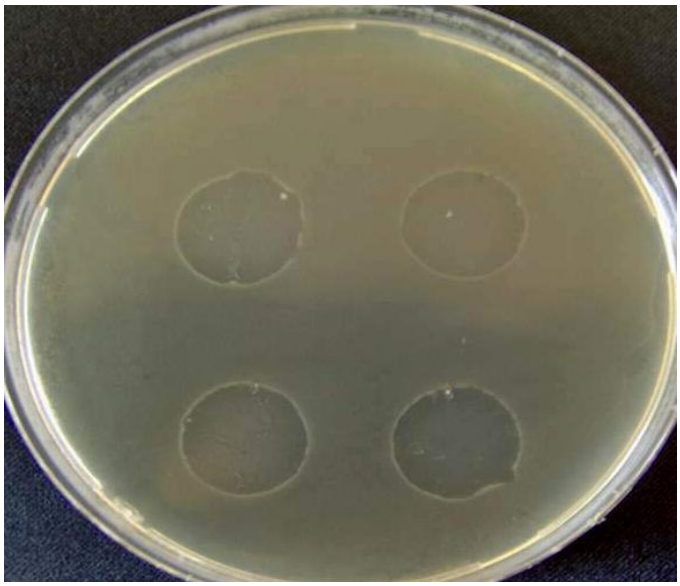


Figure 25. Antimicrobial activity of porous surfaces of cellulose acetate films on *B. amyloliquefaciens*. The films were prepared with CA/acetone/water/lysozyme weight percentages of 5/80/13.5/1.5.

5.5. Stability of Lysozyme in Cold Stored Films

In standard tests, the cast and dried films were used after they were cold stored overnight at +4^{o}C in order to compare lysozyme activities released from different asymmetric films. To evaluate the storage stability of lysozyme in CA films, the release tests were conducted for the films stored at +4^{o}C for a maximal period of 105 days. In these tests, the porous surfaces of the films prepared with 1.5 % lysozyme in 5 % CA solution were used since these films released maximum amount of enzyme activity. As seen in Figure 26, the lysozyme released from the film cold stored for one day lost most of its activity between 1400th and 1800th min of release test. In contrast, in all films cold stored for more than 15 days the released activity did not drop considerably. Maximum lysozyme activities released from the films increased continuously from 467 U/cm^2 to 703 U/cm^2 by the cold storage of the films as shown in Table 11. Moreover, the immobilized lysozyme activity remained in the films increased 1.8-2.4 fold for films cold stored ≥15 days. The increased stability of soluble and immobilized activities of lysozyme by cold storage suggests that the film production process caused some reversible changes in enzyme confirmation and resulted in temporary instability. It seems that the conformational changes in enzyme were caused by the solvent acetone

used to dissolve CA. In fact, it is well known that the reduction in dielectric constant of the medium in the presence of acetone favors the protein-protein interactions which may cause changes in quaternary structures of proteins. It is also reported that the interaction of acetone with proteins above 0^oC causes diffusion of acetone into interior parts of proteins and this may affect the hydrophobic interactions at these locations (Harris, 1995).

Table 11. Effect of cold storage of cellulose acetate films on the stability of soluble and immobilized activities of incorporated lysozyme.

Storage time at 4^oC (days)	Maximum released activity (Units/cm^2)	Immobilized activity (Units/cm^2)
1	467 ± 113 (36)[a]	2.51
15	541 ± 62 (426)	6.08
21	618 ± 64 (126)	5.79
42	699 ± 0 (66)	4.54
105	703 ± 0 (6)	5.19

[a] Time (min) to reach to maximum released activity

The increase of lysozyme activity by cold storage was also confirmed by test of antimicrobial activity of stored films on *E. coli*. For this purpose, the films prepared by 5% CA solution containing 1.5 % lysozyme and 0.5 % EDTA were tested on the target bacteria by the zone inhibition assay after 1 and 45 days of cold storage at 4^oC. The EDTA concentration used in these antimicrobial tests was reduced from 1.2 to 0.5 % to better evaluate the antimicrobial activity formed by lysozyme. The results have shown that the total area of the ffz has increased from 39 to 100.5 cm^2 at the porous surfaces when the films were cold stored for 45 days. Similarly, 45 days cold storage increased the total area of the ffz around dense surfaces of the films from 23.2 to 104.3 cm^2. These results confirmed our previous findings which showed increased activity and stability of lysozyme in CA films by cold storage. The stability of lysozyme in the films during cold storage is particularly important since this is essential for commercializing pre-cast films.

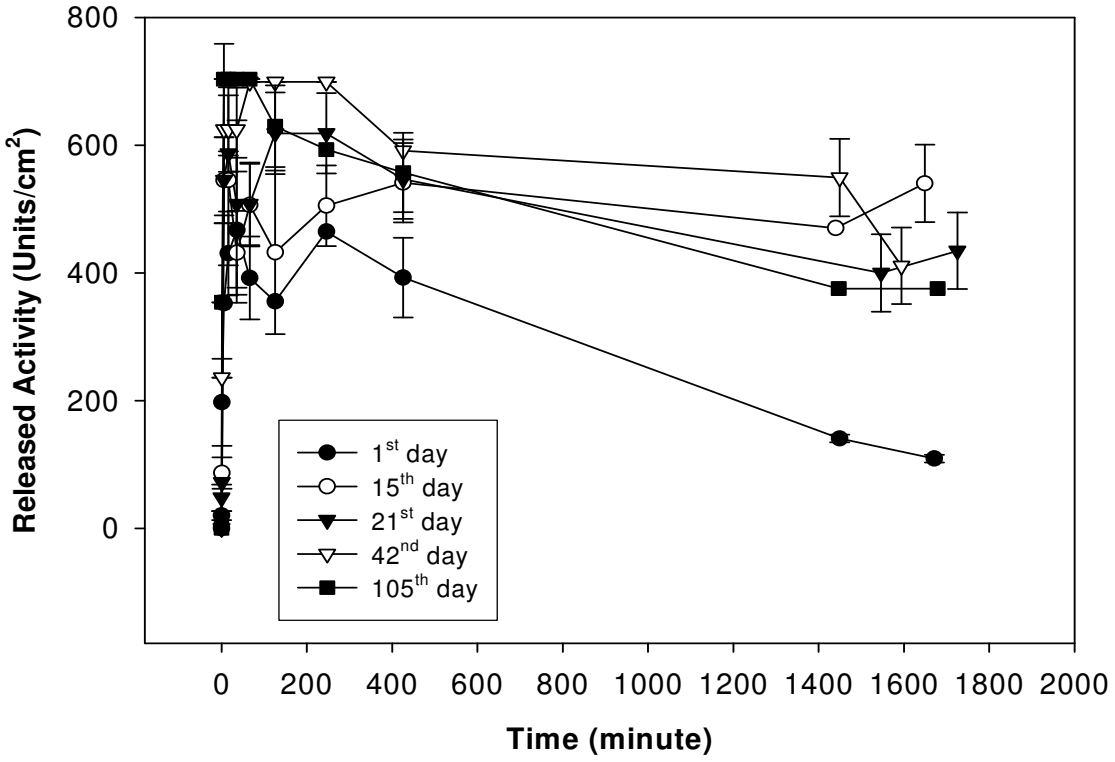
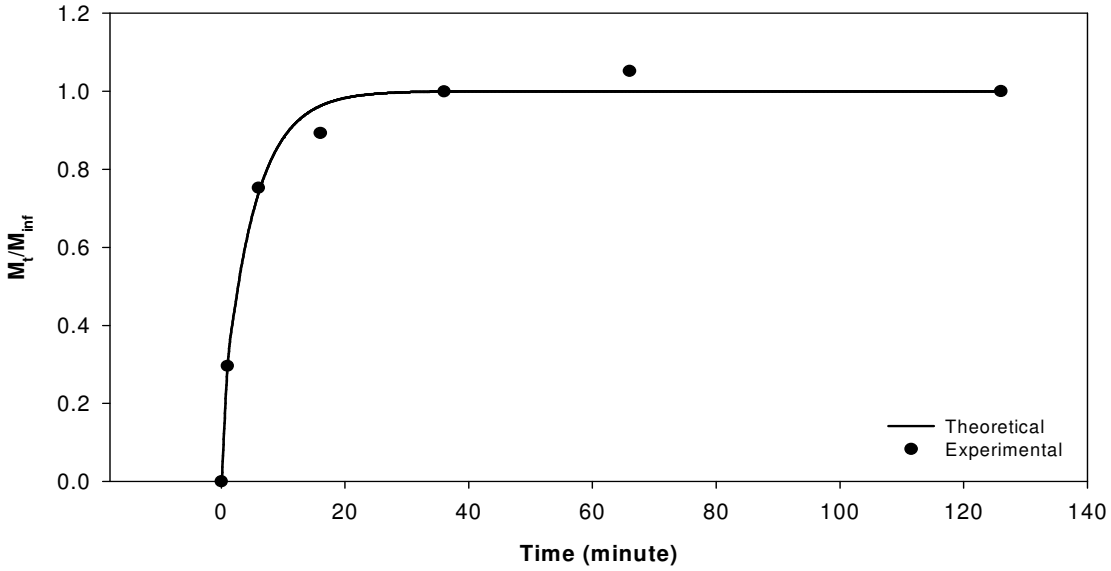


Figure 26. Change of lysozyme activity during release tests of cold stored cellulose acetate films at 4°C. The films were prepared with CA/acetone/water/lysozyme weight percentages of 5/80/13.5/1.5.

5.6. Effect of Film Composition on Diffusion of Lysozyme

The normalized release of lysozyme from CA films into water at 4 °C is shown in Figures 27a through 27c. These curves were obtained by plotting the ratio of the amount of lysozyme released at time t, M_t, to the total amount released when equilibrium is reached, M_∞. Table12. lists the effective diffusion coefficient of lysozyme and its partition coefficients calculated from the normalized release curves using Eq. (8) and the equilibrium condition, respectively. As expected, diffusion coefficient of lysozyme in all films was higher when the porous side of the films was in contact with water. The largest difference in diffusivities through dense and porous side was obtained for the film prepared with 5 % CA solution since large pores are present in that film. By increasing CA/acetone ratio from 5/80 to 15 /80 in the casting solution, the diffusivity of lysozyme decreased almost one order of magnitude while its partition

coefficient increased. The partition coefficients determined using both sides of the films were found close to each other within the experimental error limits. The results shown in Table 12. clearly suggest that release kinetics of lysozyme can be controlled by changing the composition of the film forming solution.



(a)

Figure 27. Experimental and theoretical fractional mass release of lysozyme from the films (a: the porous side of the film cast with 5% CA solution, b: the dense side of the film cast with 5% CA solution, c: the dense side of the film cast with 10% CA).

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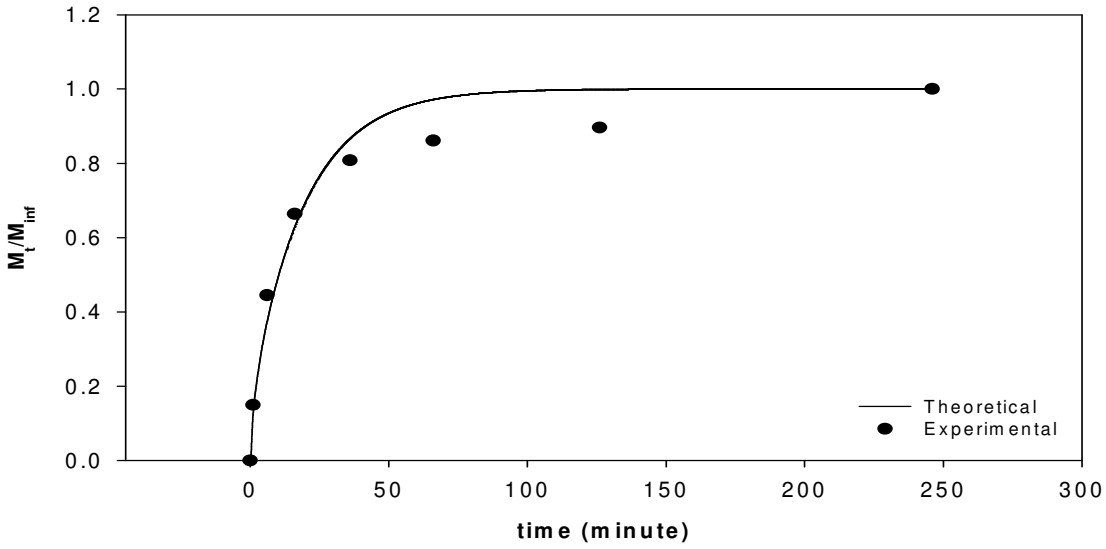
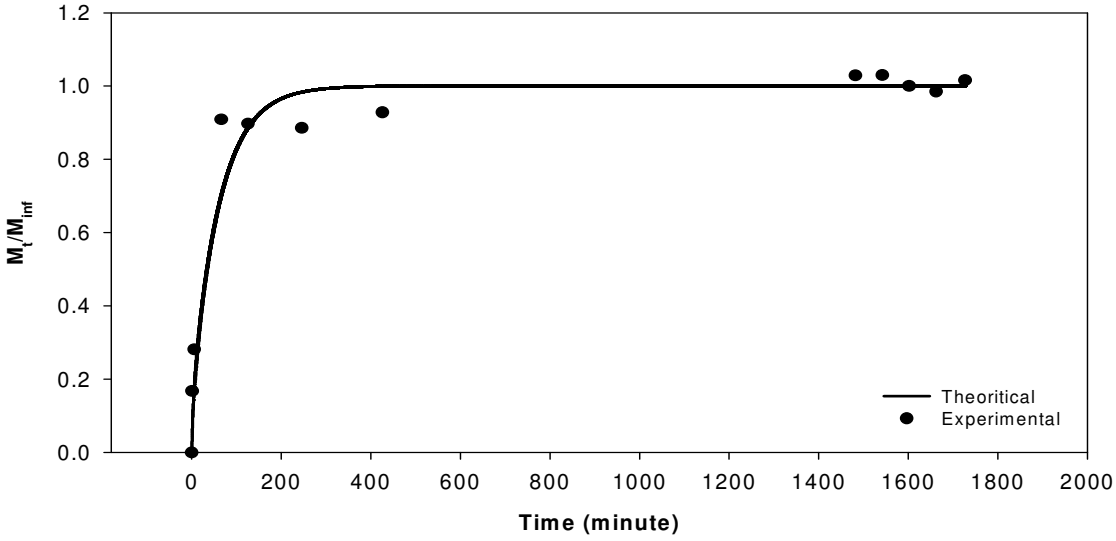


(b)



(c)

Figure 27. Experimental and theoretical fractional mass release of lysozyme from the films (a: the porous side of the film cast with 5% CA solution, b: the dense side of the film cast with 5% CA solution, c: the dense side of the film cast with 10% CA). **(cont.)**

Table 12. Effective diffusion coefficient of lysozyme in different CA films and its partition coefficients.

Composition (wt %)	Film surface	Partition coefficient	D (cm^2/s)
5/80/13.5/1.5[a]	porous	234.34 (126)[b]	2.33×10^{-9}
	dense	269.26 (246)	6.17×10^{-10}
10/80/8.5/1.5	porous	318.73 (1701)	2.33×10^{-10}
	dense	296.29 (1727)	1.83×10^{-10}
15/80/3.5/1.5	porous	814.56 (1466)	4.17×10^{-10}
	dense	825.80 (2968)	1.50×10^{-10}

[a] Weight percentages of CA/acetone/water/lysozyme in film forming solutions

[b] Equilibrium time

5.7. Mechanical Properties of Films

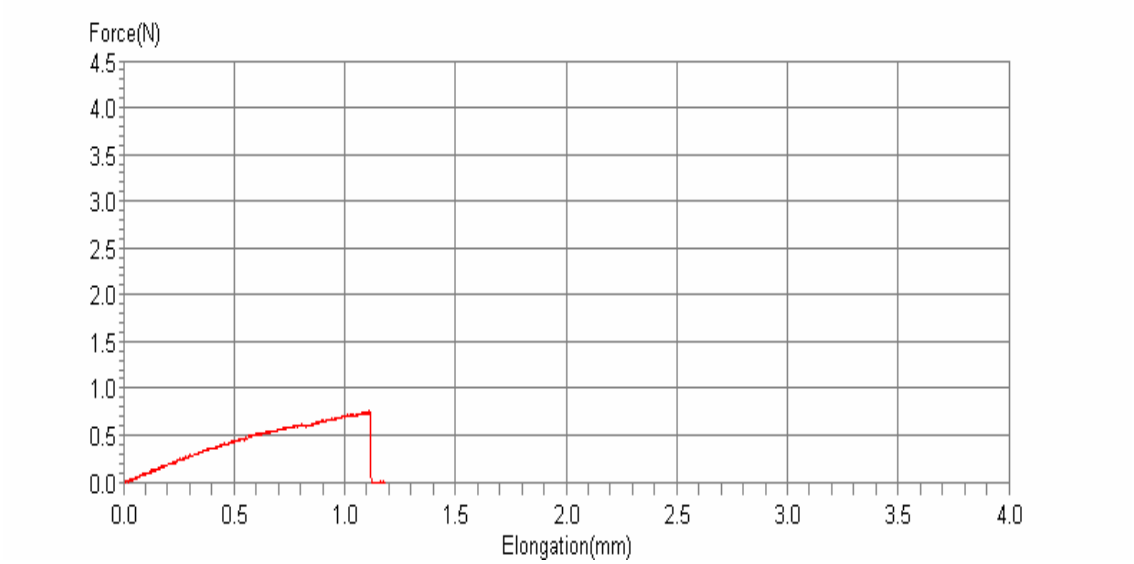
Mechanical properties of the films were evaluated in terms of tensile strength, % elongation and Young's modulus values which are related to structural characteristics of the films. The results are listed in Table 13 and also the force versus elongation graphs of the films with lysozyme can be seen from Figures 28a to 28c. As expected, the tensile strength and elongation of the films increased with increasing CA concentration in the casting solution due to reduced pore sizes and porosity of the films. It can also be seen that Young's modulus increased with CA content. This is quite expected, as dense structures become stiffer and have higher modulus values than the porous structures. Tensile strength and elongation values of the films prepared with 1.5 % lysozyme in 5 % and 10 % CA solutions were found to be similar to those obtained with control films containing no lysozyme. On the other hand, incorporation of lysozyme into the films prepared with 15 % CA solution caused a significant reduction in tensile strength and stretching ability (elongation) of the films prior to breakage. This can be attributed to incomplete dissolution of lysozyme in concentrated polymer solution including reduced amount of water, thus, nonhomogeneous distribution of

suspended lysozyme aggregates within the final film structure. In the presence of lysozyme, all films have shown slight increase in stiffness values.

Table 13. Mechanical properties of the films.

Composition (wt %)	Tensile Strength (MPa)	Elongation (%)	Young's Modulus (MPa)
5/80/15/0[a]	10.37 ± 1.61	1.76 ± 0.41	257.52 ± 46.84
10/80/10/0	37.05 ± 1.70	4.02 ± 1.19	1214.81 ± 63.24
15/80/5/0	86.31 ± 3.75	12.00 ± 1.58	2068.33 ± 94.73
5/80/13.5/1.5	10.35 ± 2.48	1.4 ± 0.55	387.22 ± 50.34
10/80/8.5/1.5	40.49 ± 3.40	3.46 ± 0.60	1240.74 ± 68.69
15/80/3.5/1.5	47.75 ± 6.63	4.27 ± 1.79	2057.09 ± 714.43

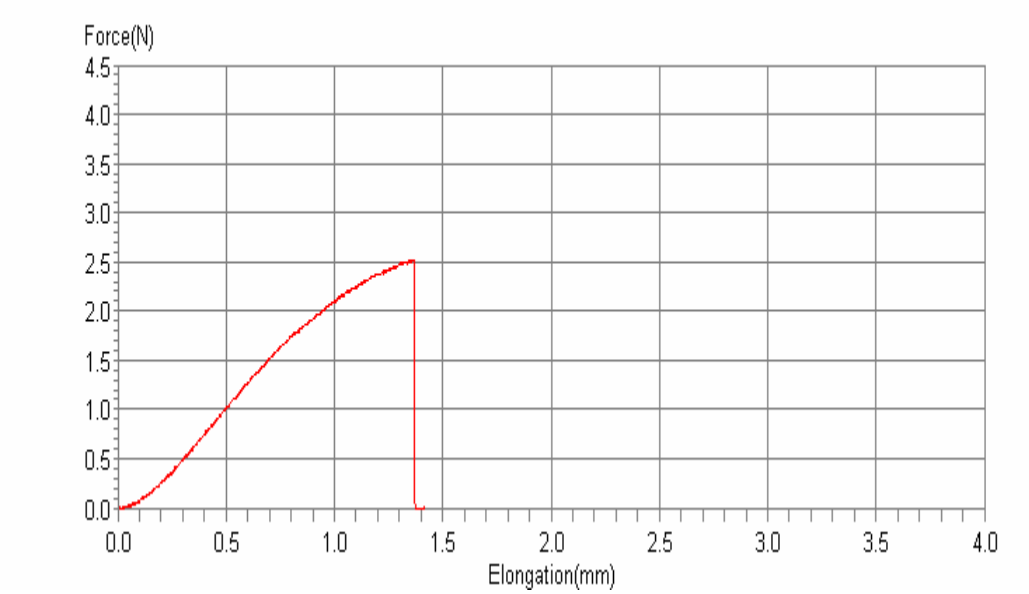
[a] Weight percentages of CA/ acetone / water / lysozyme in film forming solutions



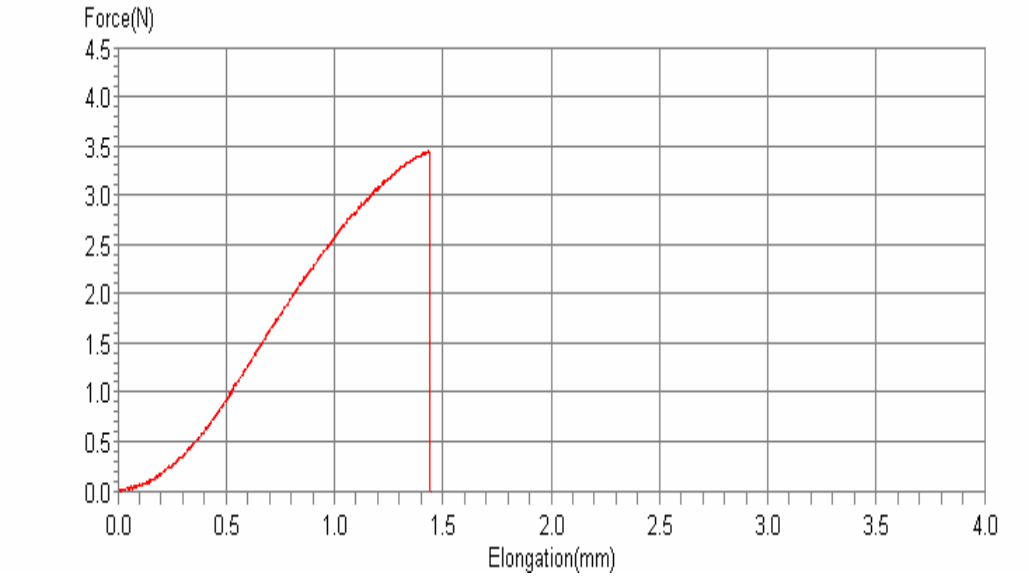
(a)

Figure 28. Force versus elongation graphs of the films with lysozyme (a): the film containing 5% CA, (b): the film containing 10% CA, (c): the film containing 15% CA.

(cont. on next page)



(b)



(c)

Figure 28. Force versus elongation graphs of the films with lysozyme (a): the film containing 5% CA, (b): the film containing 10% CA, (c): the film containing 15% CA. **(cont.)**

CHAPTER 6

CONCLUSION

In this study, lysozyme containing cellulose acetate based antimicrobial films were prepared using dry phase inversion technique. The technique allowed us to develop novel food packaging materials for the controlled release of the antimicrobial agent. The results have clearly shown that the release rate of lysozyme from the films could be tailored by changing the composition of the initial casting solution. Release rates, maximum activity of lysozyme released and antimicrobial activites decreased while immobilized activities and tensile strength of the films increased with increasing CA content in the solution. The incorporation of lysozyme into the films prepared with 5 % and 10 % CA solution did not cause major changes in the mechanical properties of these films. On the other hand, significant reductions in tensile strength and elongation values of the antimicrobial films prepared with 15 % CA solution were found compared to those of the lysozyme free films. Experimental studies conducted with different surfaces of the films have shown that the lysozyme release rate, hence, antimicrobial activities could also be varied significantly by contacting either dense or porous surface of the films with the food surface. Finally, it was found that maximum activity of lysozyme released into the medium and antimicrobial activity of the films cast with 5 % CA solution increased with time during storage at 4 ^{o}C.

Results obtained in this work suggest that asymmetric porous films containing antimicrobial agents can be used as novel food packaging materials with controlled release properties. However, further studies are needed to test the effectiveness of these films on selected food systems.

REFERENCES

Ahvenainen, Raija. 2003. *Novel food packaging techniques*, (CRC Press, Woodhead Pub. Ltd.), pp. 50-65.

Altınkaya, S.A., Yenal, H. 2006. "In Vitro Drug Release Rates From Asymmetric-Membrane Tablet Coatings: Prediction of Phase Inversion Dynamics",*Biomedical Engineering Journal*, Vol. 28, pp. 131.

Amparo, L.R., Rafael, G., Lagaron, J.M. 2006. "Bioactive Packaging: Turning Foods into Healtier Foods Through Biomaterials", *Trends in Food Science and Technology*, Vol. 17, pp. 567.

Appendini, P., Hotchkiss, J.H. 1997. "Immobilization of Lysozyme on Food Contact Polymers as Potential Antimicrobial Films", *Packaging Technology and Science*, Vol. 10, pp. 271.

Appendini, P., Hotchkiss, J.H. 2002. "Review of Antimicrobial Food Packaging", *Innovative Food Science & Emerging Technologies*. Vol. 3, pp. 113.

Arifin, D.Y., Lee, L.Y., Wang, C-H. 2006. "Mathematical Modeling and Simulation of Drug Release From Microspheres: Implications to Drug Delivery Systems", *Advanced Drug Delivery Reviews*, Vol. 58, pp. 1274.

Baner, A.L. 2000. *Plastic packaging materials for food:barrier function mass transport* , (Wiley-VCH Weinheim, New York), pp. 21.

Branen, J.K. Davidson, P.M. 2004. "Enhancement of Nisin, Lysozyme, and Monolaurin Antimicrobial Activities by Ethylenediamineetetraacetic Acid and Lactoferrin", *International Journal of Food Microbiology.* Vol. 90, pp. 63.

Brayden, D.J. 2003. "Controlled Release Technologies for Drug Delivery", *Drug Discovery Today*. Vol. 8, pp. 976.

Buonocore, G.G., Conte, A., Corbo, M.R., Sinigaglia, M., Nobile, M.A. 2005. "Mono- and Multilayer Active Films Containing Lysozyme as Antimcirobial Agent", *Innovative Food Science and Emerging Technologies*, Vol. 6, pp.459.

Buonocore, G.G., Del Nobile, M.A., Panizza, A., Bove, S., Nicolas, L. 2003a. "Modeling the Lysozyme Release Kinetics From Antimicrobial Films Intended for Food Packaging Applications", *Food Engineering and Physical Properties*, Vol. 68, pp. 1365.

Buonocore, G.G., Del Nobile, M.A., Panizza, A., Corbo, M.R., Nicolais, L. 2003b. "A General Approach to Describe The Antimicrobial Agent Release from Highly Swellable Films Intended for Food Packaging Applications", *Journal of Controlled Release.* Vol. 90, pp. 97.

Buonocore, G.G., Sinigaglia, M., Corbo, M.R., Bevilacqua, A., La Notte, E., Del Nobile, M.A. 2004. "Controlled Release of Antimcirobial Compounds From Highly Swellable Polymers", *Journal of food Products*, Vol. 67, pp. 1190.

Campbell, Ian M. 1994. *Introduction to synthetic polymers*, (Oxford University Press, New York)

Cardinal, J.R., Herbig, S.M., Korsmeyer, R.W., Lo, J., Smith, K.L., Avinash, G. 1997. "Use of Asymmetric-Membranes in Delivery Devices", U.S. Patent No. 5,612,059.

Cha, D.S., Choi, J.H., Chinnan, M.S., Park, H.J. 2002. "Antimicrobial Films Based on Na-alginate and K-carragenan", *Lebensm.-Wiss. U.-Technol.*, Vol. 35, pp. 715.

Chandra, R., Rustgi, R. 1998. "Biodegradable Polymers", *Prog. Polym. Sci.*, Vol. 23, pp. 1273.

Choi, J.H., Choi, W.Y., Cha, D.S., Chinnan, M.J., Park, H.J., Lee, D.S., Park, J.M. (2005). Diffusivity of potassium sorbate in K-carrageenan based antimicrobial film. *LWT*, 38, 417.

Conte, A., Buonocore, G.G., Sinigaglia, M., Del Nobile, M.A. 2007. "Development of Immobilized Lysozyme Based Active Film", *Journal of Food Engineering*, Vol. 78, pp. 741.

Crank, J. 1975. *The Mathematics of Diffusion*, Second Edition, (Oxford University Press, New York), pp. 41-50.

Craslaw, H.S., Jaeger, H.S. 1959. *Conduction of Heat in Solids*, (Clarendon Press, Oxford)

Davidson, P.M., Branen, A.L. 1993. *Antimicrobials in Foods*, (Marcel Dekker, INC., New York), pp. 11-91.

Dawson, P.L., Harmon, L., Sotthibandhu, A., Han, I.Y. "Antimicrobial Activity of Nisin-Adsorbed Silica and Corn Starch Powders", *Food Microbiology*. Vol. 22, pp. 93.

De Roever, C. 1998. "Microbiological Safety Evaluations and Recommendations on Fresh Produce", *Food Control*. Vol. 9, pp. 321.

Devlieghere, F., Vermeiren, L., Debevere, J.2004. "New Preservation Technologies: Possibilities and Limitations", *Internatıonal Dairy Journal*. Vol. 14, pp. 273.

Fried, J.R. 2003. *Polymer Science & Technology*. Second Edition. (Prentice Hall Professional Technical Reference, NJ), pp. 325-353.

Güçbilmez, Ç.M. 2005. "Production of Functional Packaging Materials by Use of Biopreservatives", Master of Science Thesis. İzmir Institute of Technology. İzmir

Güçbilmez, Ç.M., Yemenicioğlu, A., Arslanoğlu, A. 2007. "Antimicrobial and Antioxidant Activity of Edible Zein Films Incorporated wit Lysozyme, Albumin Proteins and Disodium EDTA", *Food Research International*, Vol. 40, pp. 80.

Guzey, D., McClements, D.J. 2006. "Formation, stability and properties of multilayer emulsions for application in the food industry", *Advances In Colloid And Interface Science*, Vol. 128-130, pp. 48.

Han, J.H. 2000. "Antimicrobial Food Packaging", *Food Technology.* Vol. 54 (3), pp. 56.

Han, J.H., Floros, J.D. 1997. "Casting Antimicrobial Packaging Films and Measuring Their Physical Properties and Antimicrobial Activity", *Journal of Plastic Film and Sheeting*. Vol. 13, pp. 287.

Han, J.H., Floros, J.D. 1998. "Simulating Diffusion Model and Determining Diffusivity of Potassium Sorbate Through Plastic to Develop Antimicrobial Packaging Films", *Journal of Food Processing and Preservation*, Vol. 22, pp. 107.

Harris, D.A. 1987. *Spectrophotometry and Spectrofluorometry*, (Oxford: I.R.L. Press, New York)

Harris, E.L.V. 1995. *Protein Prufication Tecniques*, (Oxford University Press, New York)

Herbig, S.M., Cardinal, J.R., Korsmeyer, R.W., Smith, K.L. 1995. "Asymmetric Membrane Tablet Coatings for Osmotic Drug Delivery", *Journal of Controlled Release*, Vol. 35, pp. 127.

Ibrahim, H.R., Kato, A., Kobayashi, K. 1991. "Antimicrobial Effects of Lysozyme Against Gram-Negative Bacteria due to Covalent Binding of Palmitic Acid", *Journal of Agric. Food Chem.* Vol. 39, pp. 2077.

Ibrahim, H.R., Higashiguchi, S., Jugena, L.R., Kim, M., Yamamoto, T. 1996a. "A Structural Phase of Heat-Denaturated Lysozyme with Novel Antimicrobial Action", *Journal of Agrici Food Chem.* Vol. 44, pp. 1416.

Labuza, T.P., Breene, W.M. 1989. "Applications of Active Packaging for Improvement of Shelf-Life and Nutritional Quality of Fresh and Extended Shelf-Life Foods", *Journal of Food Processing and Preservation*. Vol. 13, pp. 1.

LaCoste, A., Schaich, K.M., Zumbrunnen, D., Yam, K.L. 2005. "Advancing Controlled Release Packaging Through Smart Blending", *Packaging Technology and Science*, Vol. 18, pp. 77.

Langer, R.S., Peppas, N.A. 1981. "Present and Future Applications of Biomaterials in Controlled Drug Delivery Systems", *Biomaterials*. Vol. 2, pp. 201.

Leong, K.W., Langer, R. 1988. "Polymeric Controlled Drug Delivery", *Advanced Drug Delivery Reviews.* Vol. 1, pp. 199.

Lin, Y.K., Ho, H.O. 2003. "Investigation on The Drug Releasing Mechanisim From An Asymmetric-Membrane-Coated Capsule with An In Situ Formed Delivery Orifice", *Journal of Controlled Release*, Vol. 89, pp. 57.

Mallapragada, S.K., Peppas, N.A. 1997. "Crystal Dissolution-Controlled Release Systems: I. Physical Characteristics and Modeling Analysis", *Journal of Controlled Release*, Vol. 45, pp. 87.

Mecitoğlu Ç., Yemenicioğlu, A., Arslanoğlu, A., Elmacı, Z.S., Korel, F., Çetin, A.E. 2006. "Incorporation of Partially Purified Hen Egg White Lysozyme into Zein Films for Antimicrobial Food Packaging", *Food Research International.* Vol. 39, pp. 12.

Mohanty, A.K., Misra, M., Drzal, L.T., 2005. *Natural fibers, biopolymers, and biocomposites* (Taylor & Francis, Boca Raton, FL)

Nakamura, S., Kato, A., Kobayashi, K. 1991. "New Antimicrobial Characteristics of lysozyme-Dextran Conjugate", *Journal of Agric. Food Chem.* Vol. 39, pp. 647.

Nakamura, S., Kato, A., Kobayashi, K. 1992. "Bifunctional Lysozyme-Galactomannan Conjugate Having Excellent Emulsifying Properties and Bacterial Effect", Journal of Agric. Food Chem. Vol. 40, pp. 735.

Min, S. And Krochta, J.M. 2005. "Inhibition of *Penicillium Commune* by Edible Whey Protein Films Incorporating Lactoferrin, Lactoferrin Hydrosylate, and Lactoperoxidase Systems", *Journal of Food Science.* Vol. 70, No.2, pp. 87.

Ouattara, B., Simard, R.E., Piette, G., Begin, A., & Holley, R.A. 2000. "Diffusion of Acetic and Propionic Acids from Chitosan-Based Antimicrobial Packging Films", *Food Chemistry and Toxicology.* Vol. 65 (5), pp. 768.

Ozdemir, M, Floros, J.D. 2003. "Film Composition Effects on Diffusion of Potassium Sorbate Through Whey Protein Films", *Food Engineering and Physical Properties*, Vol.68, pp. 511.

Padgett, T., Han, I.Y., Dawson, P.L. 1998. "Incorporation of Food-Grade Antimicrobial Compounds into Biodegradable Packaging Films", *Journal of Food Protection*. Vol. 61 (10), pp. 1330.

Park, S.I., Daeschel, M.A., Zhao, Y. 2004. "Functional Properties of Antimicrobial Lysozyme-Chitosan Composite Films", *Food Microbiology and Safety*, Vol. 69, pp. 215.

Petersen, K., Nielsen, P.V., Olsen, M.B. 2001. "Physical and Mechanical Properties of Biobased Materials - Starch, Polylactate and Polyhydroxybutyrate", *Starch*. Vol. 53, pp. 356.

Prabakaran, D., Singh, P., Jaganathan, K.S., Vyas, S.P. 2004. "Osmotically Regulated Asymmetric Capsular Systems for Simultaneous Sustained Delivery of Anti-tubercular drugs", *Journal of Controlled Relaese*, Vol. 95, pp. 239.

Quintavalla, S. and Vicini, L. 2002. "Antimicrobial Food Packaging in Meat Industry", *Meat Science*. Vol. 61, pp. 373.

Richard, C. 1998. "Mathematical Modeling of Controlled Release from Implanted Drug Impregnated monoliths", *Pharmaceutical Science and Technology Today*, Vol. 1 pp. 269.

Rose, N.L., Palcic, M.M., Sporns, P., Mcmullen, L.M. 2002. "Nisin: A Novel Sustrane for Glutathione S-Transferase Isolated from Fresh Beef", *Journal of Food Science*. Vol. 67, No.6, pp. 2288.

Rose, N.L., Sporns, P., Stiles, M.E., McMullen, L.M. 1999. "Inactivation of Nisin by Glutathione in Fresh Meat", *Journal of Food Science*. Vol. 64, No.5, pp. 759.

Scannel, A.G.M., Hill, C., Ross, R.P., Marxs, S. Hartmeier, W., Arendt, E.K. 2000. "Development of Bioactive Food Packaging Materials Using Immobilized Bacteriocins Lacticin 3147 and Nisalpin", *International Journal of Food microbiology*. Vol. 60, pp. 241.

Siepmann, J., Lecomte, F., Bodmeier, R. 1999. "Diffusion-Controlled Release Systems:Calculation of The Required Composition to Achive Desired Release Profiles ", *Journal of Controlled Release*. Vol. 60, pp. 379.

Siepmann, J., Peppas, N.A. 2001. "Modeling of Drug Release From Delivery Systems Based on Hydroxypropyl Methylcellulose (HPMC)", *Advance Drug Delivery Reviews*, Vol. 48, pp. 139.

Suppakul, P., Miltz, J., Sonneveld, K., Bigger, S.W. 2003. "Active Packaging Technologies with an Emphasis on Antimicrobial Packaging and its Applications", *Concise Reviews and Hypotheses in Food Science.* Vol. 68 (2) pp. 408.

Takahashi, K., Lou, X.F., Ishii, Y., Hattori, M. "Lysozyme-Glucose Stearic Acid Monoester Conjugate Formed through the Maillard Reaction as an Antibacterial Emulsifier", *Journal of Agric. Food Chem.* Vol. 48, pp. 2044.

Thombre, A.G., Cardinal, J.R., DeNoto, A.R., Gibbes, D.C. 1999b. "Asymmetric-Membrane Capsules for Osmotic Drug Delivery. Part II: In Vitro and In Vivo Drug Reelase Performance", *Journla of Controlled Relaese*, Vol. 57, pp. 65.

Thombre, A.G., Cardinal, J.R., DeNoto, A.R., Herbig, S.M., Smith, K.L. 1999a. "Asymmetric-Membrane Capsules for Osmotic Drug Delivery. Part I: Development of A Manufacturing Process", *Journal of Controlled Release*, Vol. 57, pp. 55.

Thombre, A.G., DeNoto, A.R., Gibbes, D.C. 1999c. "Delivery of Glipizide From Asymmetric-Membrane Capsules Using Encapsulated Excipients", *Journal of Controlled Release*, Vol. 60, pp. 333

Wang, D.M., Lin, F.C., Chen, L.Y., Lai, J.Y. 1998. "Application of Asymmetric TPX Membranes to Transdermal Delivery of Nitroglycerin", *Journal of Controlled Release*, Vol. 50, pp. 187.

WEB_1, 2007. Wikipedia-the free encyclopedia, 19/06/2007. http://en.wikipedia. org/wiki/Packaging

WEB_2, 2007. Answers.com, 19/06/2007. http://www.answers.com/polymer

WEB_3, 2007. Answers.com, 19/06/2007. http://www.answers.com/organic%20 polymer

WEB_4, 2007. Polysaccharide, 19/06/2007. http://www.biorama.ch/biblio/b20gfach/ b35bchem/b10kh/kh040.htm

WEB_5, 2007. Life chemistry, 19/06/2007. http://www.greenspirit.org.uk/Resources/-LifeChemistry.htm

WEB_6, 2007. Answers.com, 19/06/2007. http://www.answers.com/topic/antimi-crobial-agent

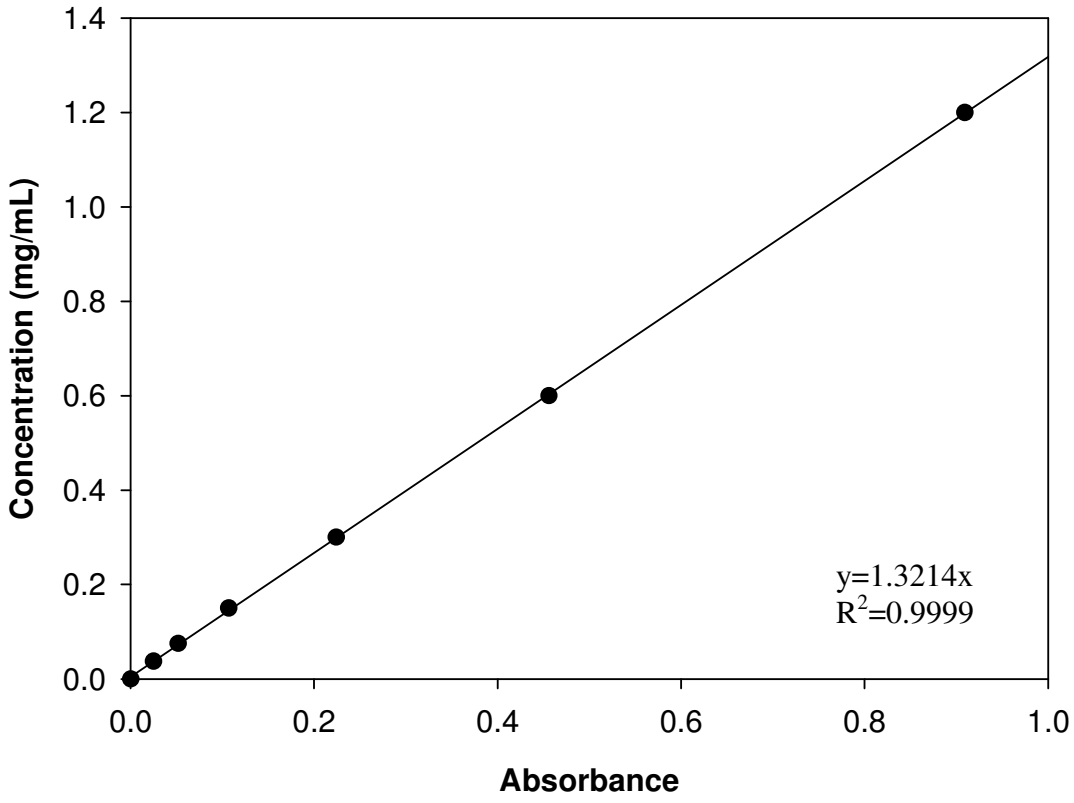
WEB_7, 2007. Inchem, 19/06/2007. http://www.inchem.org/documents/cicads/cicads-/cicad26.htm

WEB_8, 2007. Science toys, 19/06/2007. http://sci-toys.com/ingredients/-sorbic_acid.html

Yam K.L., Saba R.G., and Ho Y.C. 1999. "Packaging: Materials," in *Encyclopedia of Food Science and Technology*, edited by F.J. Francis, (John Wiley & Sons, New york), pp. 1824-1829.

APPENDIX A

CALIBRATION CURVE OF LYSOZYME VS ABSORBANCE AT 280 NM



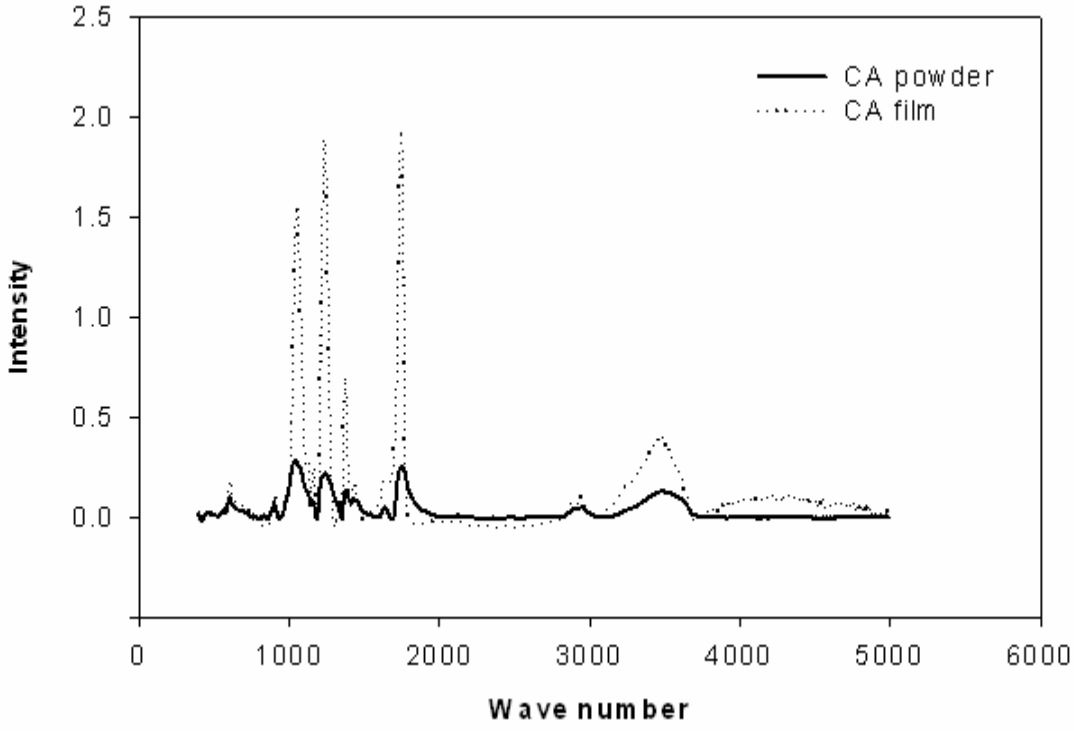
APPENDIX B

PARTIAL PURIFICATION OF LYSOZYME FROM HEN EGG WHITE

Volume (mL)	Total Activity (U)	Total Protein (mg)	Spesific Activity (U/mg)	Recovery (%)	Purity (fold)
Dilution of egg white 3-fold with 0.05M NaCl					
600	22875000±1176902	32629±4260	701	100	1.0
pH adjustment + 30% ethanol					
1066	24899983±1565181	3125±213	7968	109	11.4
21 h dialysis					
1500	18212500±1609590	3148±192	5785	80	8.3

APPENDIX C

FTIR SPECTRUM OF CA POWDER AND CA FILM



FTIR spectrum of CA powder and CA film have indicated that no acetone is left in the films used in the experiments.