PRODUCTION OF FUNCTIONAL PACKAGING MATERIALS BY USE OF BIOPRESERVATIVES

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ABSTRACT

In this study, partially purified lyophilized lysozyme (LSZ) and lactoperoxidase (LPS) from chicken egg white and bovine whey were incorporated into zein and alginate films, respectively. The LSZ showed very low affinity to zein films. Thus, when zein films incorporated with 63-455 μg/cm^2 (187-1318 U/ cm^2) LSZ was exposed to release tests in distilled water, the enzyme released rapidly from the films with release rates of 9 to 29 U/cm^2/min. The ethanol used in film making caused significant activation of the LSZ. Therefore, the released LSZ activity from zein films was mostly 130-300 % higher than the activity incorporated into films. On the other hand, the LPS incorporated into alginate films showed a very high affinity to these films and immobilized.The incorporation of 0.53 or 1.06 mg/cm^2 proteins such as crude thermally processed chick-pea proteins (TP-CP) and sericine in combination with LSZ and LPS into zein or alginate films increased the antioxidant activity of edible films significantly. In zein films, the incorporation of antioxidant proteins reduced the total activity of LSZ released from the films between 50 % and 75 %, but increased the immobilized enzyme activity 3.5-15 fold. On the other hand, incorporation of antioxidant proteins into alginate films enhanced LPS activity slightly to moderately. The results of this study clearly showed the good potential of edible films and natural biopreservatives in active packaging.

ÖZET

Bu çalışmada, sırasıyla yumurta akı ve peynir altı suyundan kısmi saflaştırma ile elde edilmiş ve liyofilize edilmiş lisozim (LSZ) ve laktoperoksidaz (LPS) enzimleri zein ve alginat filmlere katılmıştır. LSZ enziminin ilave edildiği zein filmlere olan afinitesini oldukça düşük olup, 63-455 µg/cm^2 (187-1318 U/ cm^2) LSZ ilave edilmiş ve destile su içerisine yerleştirilmiş filmlerden suya 9 ile 29 U/cm^2/dak gibi yüksek hızlarda aktivite geçişi olmaktadır. Ayrıca zein film üretimi sırasında kullanılan etanolun aktive edici etkisi nedeniyle, bu filmlerden suya geçen aktivite düzeyleri filmlere başlangıçta ilave edilenin % 130-300'ü kadar yüksek olmaktadır. Diğer yandan LPS enzimi alginat filmlere oldukça yüksek bir afinite göstermekte ve bu filmler içerisinde tutuklanabilmektedir. Alginat ve zein filmler içerisine LSZ ve LPS ile birlikte 0.53 veya 1.06 mg/cm^2 kadar termal işlem görmüş ham nohut proteinleri (TP-CP) veya serisin proteini katılması filmlerin antioksidan aktivitesini önemli düzeyde arttırmaktadır. Zein filmlere antioksidan protein katılması filmlerden salınan toplam LSZ aktivitesinde %50 ile %75 arasında azalmalara yol açmıştır, ancak bu filmlerdeki tutuklanmış LSZ aktivitesinin 3.5-15 kat kadar artmasına neden olmuştur. Diğer yandan antioksidan protein ilavesi alginat filmlerde LPS aktivitesinin belirli ölçüde artmasına neden olmaktadır. Gerçekleştirilen bu çalışma yenebilir filmler ve biyoprezervatiflerin aktif paketleme alanında oldukça önemli bir potansiyel oluşturabileceğini doğrulamaktadır.

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CHAPTER 1

INTRODUCTION

The increased demand on easily prepared minimally processed fresh produce and the related increase in food-borne microbial outbreaks (De Roever 1998) have intensified the research on antimicrobial packaging technologies (Suppakul et al. 2003). The antimicrobial packaging is conducted by (1) the addition of antimicrobial containing sachets or pads into food packages; (2) the coating, immobilization or direct incorporation of antimicrobials into food packaging materials or (3) the use of packaging materials that are inherently antimicrobial (Appendini and Hotchkiss 2002).

Different chemicals such as organic or inorganic acids, metals, alcohols, ammonium compounds or amines can be incorporated into packaging materials as antimicrobials (Appendini and Hotchkiss 2002, Suppakul et al. 2003). However, because of the health concerns of the consumers, producers are now highly interested in the use of biopreservatives in antimicrobial packaging. The biopreservatives suggested for antimicrobial packaging include bacteriocins such as nisin, pediocin and lacticin and antimicrobial enzymes such as lysozyme, lactoperoxidase, chitinase and glucose oxidase (Labuza and Breene 1989, Suppakul et al. 2003). Because of the environmental concerns and technological problems such as denaturing effects of thermal polymer processing methods, extrusion and injection molding, the incorporation of biopreservatives into biodegradable films is more suitable than their incorporation into plastic films (Appendini and Hotchkiss 2002, Suppakul et al. 2003, Han 2000). Most of the biodegradable films are edible and their film formation occurs under mild conditions. Different edible films incorporated with biopreservatives include films from cellulose derivatives, carragenan, alginate, and whey proteins (Han 2000, Cha et al. 2002, Quintavalla and Vicini 2002, Suppakul et al. 2003). Recently, a particular interest has also been focused on the incorporation of different biopreservatives such as nisin and lysozyme (Padgett et al. 1998, Hoffman et al. 2001, Dawson et al. 2000, Janes et al. 2002, Teerakarn et al. 2002) into zein films. Zein may be applied as food coating or its pre-cast films may be used for wrapping of foods (Herald et al. 1996, Janes et al. 2002).

The pre-cast zein films have also been used successfully for the modified atmosphere packaging of vegetables (Rakotonirainy et al. 2001).

Lysozyme (LSZ) is one of the most frequently used biopreservatives in antimicrobial packaging (Han 2000, Quintavalla and Vicini 2002). This enzyme shows antimicrobial activity mainly on gram-positive bacteria by splitting the bonds between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan in their cell walls. Because of their protective outer membrane surrounding the peptidoglycan layer, LSZ does not show antibacterial activity against gram-negative bacteria. However, when it is combined with EDTA, the outer membranes of gram-negative bacteria are destabilized by this agent and the antimicrobial spectrum of LSZ increases significantly (Padgett et al. 1998, Branen and Davidson 2004). In studies related to antimicrobial films, most of the workers used commercial LSZ obtained by the classical repeated salt crystallization method which may require a week until the enzyme is obtained with sufficient purity (Chang et al. 2000). The commonly used commercial LSZs are quite pure. They are reported to contain only 1-6 % (w/w) protein impurities (Judge et al. 1998), and they have a very high enzyme activity (between 20000-100000 U/mg). However, for the application of LSZ in food industry, the use of cheaper partially purified LSZ preparations obtained by some faster methods may be economically more feasible. For this reason, some fast partial purification procedures have recently been developed based on heat induced precipitation of non-LSZ protein impurities in presence of reductants (Chang et al. 2000) or selective precipitation of LSZ with anionic surfactants (Shin et al. 2003).

Another antimicrobial enzyme which may be used in antimicrobial packaging is lactoperoxidase (LPS). Recently, different studies have also been conducted related to use of LPS-thiocyanate-H_2O_2 antimicrobial system in food preservation. The LPS system is part of the natural preservation system exists in milk. Thus, addition of thiocyanate and /or H_2O_2 to milk to activate naturally occurring LPS is used to improve microbial quality of milk and cheese (Seifu et al. 2004, Seifu et al. 2005). The addition of LPS and other components of this antimicrobial system to thermally processed skim milk (Zapico et al. 1998) and meat products (Elliot et al. 2004, Kennedy et al. 2000) and prevention of the development of pathogenic bacteria have also been studied. The studies related to use of LPS in antimicrobial packaging, on the other hand, are very limited. In their detailed review Suppakul et al. (2003) suggested the use of LPS in antimicrobial packaging. Recently, Min and Krochta (2005) studied the inhibition of

P*enicillium commune* by LPS impregnated whey protein films and showed the good potential of this enzyme for use in antimicrobial packaging. The mechanism of the antimicrobial action of LPS is based on conversion of thiocynate (SCN^-) to antimicrobial products such as hipothiocyanite ($OSCN^-$) ion, hypothiocyanous acid (HOSCN) and some other highly reactive and short lived oxidation products in presence of H_2O_2 (Pruitt et al. 1982). This system generally shows bactericidal effect on gram-negative bacteria and bacteriostatic effect on gram-positive bacteria (Seifu et al. 2005). Also, it has antifungal (Min and Krochta 2005, Jacob et al. 2000) and antiviral (Pakhanen and Aalto 1997, Seifu et al. 2005) activities. The synergetic effect of LPS with nisin has also been demonstrated (Zapico et al. 1998, Boussouel et al. 2000, Dufour et al. 2003). Thus, the use of LPS alone or in combination with nisin may give unique biopreservation systems that are effective on most pathogens. The good potential of LSP as a natural food preservative encouraged different workers to develop the production methods of this enzyme. Thus, it is now possible to obtain LPS directly from bovine whey by using some cation-exchange chromatographic methods (Hahn et al. 1998, Ye et al. 2000).

Besides antimicrobial packaging, the incorporation of antioxidants into food packaging materials to maintain the sensory properties, nutritional value and color of food also attracts a great interest (Madhavi and Salunkhe 1996). The main antioxidants used in food packaging are synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Madhavi et al. 1996, Herald et al. 1996, Han 2000, Moore et al. 2003). Although these antioxidants are used in food systems intensively there have been serious health concerns related to their application (Madhavi et al. 1996). Thus, the use of natural antioxidants such as vitamin C and E and phenolic compounds in food packaging gained a great importance (Vermeiren et al. 1999, Moore et al. 2003, Ou et al. 2005). Recently, the use of inherently antioxidant milk protein based coatings has also successfully been tested (Le Tien et al. 2001). However, studies related to use of antioxidant proteins in active packaging are scarce.

In this study, we have developed functional edible films by incorporation of antimicrobial enzymes such as LSZ and LPS alone or in combination with antioxidant proteins such as crude chick-pea proteins, sericin and bovine serum albumin. The main objectives of this study were the determination of effects of film making on antimicrobial enzyme activity, characteristics and release rates. The effects of incorporation of different antioxidant proteins on antimicrobial enzyme activity and

antioxidant activities of the films were also determined. The antimicrobial enzymes used in this study were produced with some fast, simple and practical methods to obtain more applicable active packaging systems for food industry.

CHAPTER 2

ACTIVE PACKAGING

2.1. Packaging

Packaging is one of the most important factors affecting the quality of foods during storage, transportation and end use (WEB_1 1999). It is an essential technique for minimizing food wastage and use of chemical additives and stabilizers, and preserving food quality by protecting it from physical, chemical and biological hazards such as moisture, light, gases, aromas, microorganisms, rodents and insects (Lindstrom et al. 1992, WEB_3 2001). Food packaging materials also provide useful information such as period of consumption, description of food contents, nutritional values and manufacturer's name and address.

2.2. Active Packaging

Active packaging is "a type of packaging that changes the condition of the packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food" (Quintavalla and Vicini 2002). To improve their functionality, various kinds of substances can be incorporated into packaging materials. The extra functions that can be provided by the incorporated active substances include antimicrobial activity, antioxidant activity and scavenging or emission of different gases, aroma compounds, respiration metabolites etc.

2.2.1. Antimicrobial Food Packaging

Microbial contamination reduces the shelf-life of food and may cause food borne illnesses. For preserving food from the effects of microbial growth, there are traditional methods such as thermal processing, freezing, drying, refrigeration, irradiation and adding antimicrobial agents or salts (Quintavalla and Vicini 2002).

Antimicrobial food packaging is one of the particular applications of active packaging conducted by incorporating antimicrobial agents into packaging materials. Such an application can help controlling the microbial population by limiting their growth rate or extending their lag phase. There are many antimicrobial agents that are suitable for incorporation into packaging materials. Some of these agents were given in Table 2.1.

Table 2.1. Antimicrobial agents used in packaging materials (Suppakul et al. 2003, Han 2000, Appendini and Hotchkiss 2002).

Class	**Antimicrobial agent**	**Packaging material**
Alcohol	Ethanol	Silica gel sachet
Amine	Hexamethylenetetraamine	LDPE
Bacteriocin	Nisin	Edible films, LDPE
	Pediocin	
	Lacticin	
Chelating agents	EDTA	Edible films
	Lactoferrin	
Fungicide	Benomyl	Ionomer
	Imazalil	LDPE
Metals	Silver	various polyolefins
Organic acid	Potassium sorbate	LDPE
	-	MC/palmitic acid
	-	MC/HPMC/fatty acid
	-	MC/chitosan
	-	Starch/glycerol
	Calcium sorbate	CMC/paper
	Propionic acid	Chitosan
	Acetic acid	Chitosan
	Sodium benzoate	MC/chitosan
	Sorbic acid anhydride	PE
	Benzoic acid anhydride	PE

(cont. on next page)

Table 2.1 (cont.)

Parabens	Propyl paraben	Clay coated cellulose
Peptide/protein/enzyme	Lysozyme	PVOH, nylon
	-	Cellulose acetate
	-	Soy protein isolate film
	-	Corn zein film
	Glucose oxidase	Alginate

(LDPE=low-density polyethylene; MC=methyl cellulose; HPMC=hydroxypropyl methyl cellulose; PE=polyethylene; CMC=carboxy methyl cellulose; EDTA=ethylene diamine tetra acetic acid; PVOH=polyvinylalcohol)

2.2.1.1. Types of Antimicrobial Packaging

The antimicrobial packaging can be applied by different methods. The five main types of antimicrobial packaging were introduced below.

2.2.1.1.1. Addition of Antimicrobial Containing Small Sachets into Packages

The addition of antimicrobial containing small sachets into food packages is the most successful commercial application of antimicrobial packaging. These types of sachets may contain some chemicals that generate vapors of antimicrobial agents. Also, they may scavenge moisture or scavenge or emit some gases to modify the package atmosphere and create an unsuitable air composition for the development of microorganisms. The main types of sachets used commercially are as follows;

Oxygen Scavenging Systems: These systems reduce headspace oxygen in the package and partially protect food against aerobic spoilage microorganisms. They also slow down rancidity of food by limiting oxygen. Oxygen scavenging by this method is used to control growth of aerobic bacteria and molds in dairy and bakery products (Han 2000, WEB_2 2000). There are many different patented oxygen scavenging systems available. However, the most popular commercialized oxygen scavenging system is the "Ageless" which is in use since 1987. The Ageless system basically contains iron

which oxides to the ferric state and may reduce the package oxygen content to a level of 0.01 % (Labuza and Breene 1989).

Moisture Absorbers: Moisture absorbers are used to reduce moisture content of package atmosphere. By this way they indirectly affect the water activity of food and microbial growth on the foodstuff. The most common application of moisture absorber involves the use of NaCl containing sachets (Yemenicioğlu and Cemeroğlu 1996).

Ethanol Vapor Generators: In this system, sachets containing encapsulated or absorbed ethanol release ethanol vapor to the package headspace. The system is particularly effective on growth of yeasts and molds. Thus, it is suitable to control microbial spoilage of intermediate moisture foods, cheeses and bakery products (Suppakul et al. 2003, Labuza and Breene 1989).

2.2.1.1.2. Incorporation of Antimicrobial Agents into Packaging Materials

The antimicrobial agents may be incorporated into packaging materials by two ways; (1) addition of antimicrobial into the melt form of polymer, (2) addition of antimicrobial into solvents containing also the polymer (solvent compounding). Since melting and thermal polymer processing methods, extrusion and injection molding, may denature heat sensitive compounds, the addition of antimicrobials to melt forms of polymers is preferred when thermostable antimicrobials (mostly chemical preservatives) are used in film making (Suppakul et al. 2003, Han 2000, Appendini and Hotchkiss 2002). For example, silver substituted zeolites used in antimicrobial packaging can withstand to temperatures up to 800 ºC (Appendini and Hotchkiss 2002). In contrast, the solvent compounding is very suitable for using biopolymers and heat sensitive antimicrobial ingredients such as proteins, peptides and enzymes in film making.

The antimicrobial agents used in packaging may be volatile or non-volatile substances. If they are non-volatile, antimicrobial packaging materials must contact to surface of food to enable the diffusion of antimicrobial (Fig. 2.1). At this point, surface characteristics of food and diffusion kinetics become very important. The diffusion of antimicrobial from the film should not occur very rapidly, since the diffused

antimicrobial starts to migrate to the interior parts of food from the surface, the most potential site for contamination. The release rate should not also be very slow and it should be at a suitable rate to maintain the minimum inhibitory concentration of antimicrobial at the food surface. On the other hand, if the antimicrobial agent is volatile, there is no need for contact of polymer directly with the food surface. Volatile antimicrobials can penetrate the bulk matrix of the food. Examples of volatile substances used in antimicrobial packaging are chlorine dioxide, sulfur dioxide, carbon dioxide and allyl isothiocyanate (Suppakul et al. 2003, Appendini and Hotchkiss 2002).

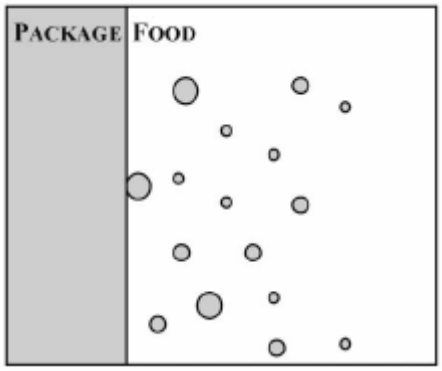
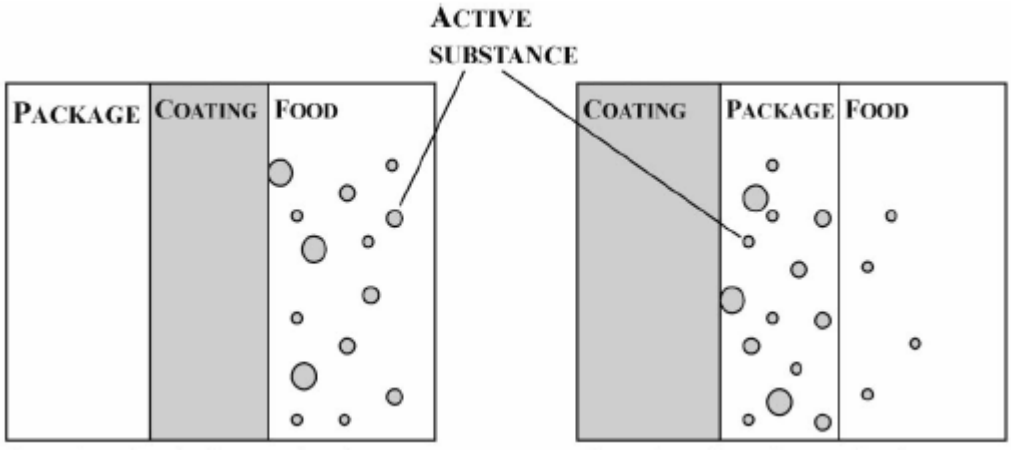


Figure 2.1. Diffusion of antimicrobial from package to food (Quintavalla and Vicini 2002)

2.2.1.1.3. Coating of Packaging Materials

Since they degraded during the application of thermal polymer processing methods such as extrusion and injection molding, heat sensitive antimicrobials are often coated onto pre-cast polymeric films. Nisin/methylcellulose coating for polyethylene films is a good example of developing this type of packaging materials (Appendini and Hotchkiss 2002). Another example of obtaining antimicrobial packaging by this method is coating of LDPE films with a mixture of polyamide resin in i-propanol/n-propanol and a bacteriocin solution (Suppakul et al. 2003). The coating may be applied to the food contact surface or it may be applied to their outer surface. The location of coating may affect the diffusion of antimicrobial agent significantly (Fig. 2.2).



Spray/coating before packaging Spray/coating after packaging

Figure 2.2. Different types of antimicrobial coatings applied to polymeric films (Quintavalla and Vicini 2002)

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

Immobilization occurs when antimicrobials and polymers have some chemical groups capable to form interactions and/or bonding (Table 2.2). The immobilization may be formed as a result of extensive H bonding, hydrophobic attraction or charge-charge attraction between antimicrobials and packaging materials (Figure 2.3). For example, antimicrobial enzyme lysozyme shows a high affinity to hydrophobic surfaces (Wertz and Santore 2002). Thus, it may be possible to immobilize this enzyme on hydrophobic films. However, there may be more than one factors (interactions or bonding) involved in the immobilization of antimicrobials. For example, it was reported that in hydrophobic CTA membranes, the lysozyme is absorbed mainly because of the cation-exchange properties of these membranes (Murata and Tonioka 1997).

2.2.1.1.5. Use of Polymers that are Inherently Antimicrobial

Some polymers such as chitosan and poly-L-lysine possess antimicrobial properties naturally. Chitosan is obtained from shells of crustaceans by washing in alkaline and acid solutions for several times (Cooksey 2001). Both chitosan and poly-L-lysine owe their antimicrobial effect to charged amines in their structure. These cationic polymers can interact with negative charges at the bacterial cell surface. Such an

interaction reduces the membrane integrity of bacteria and causes the leakage of their intracellular components.

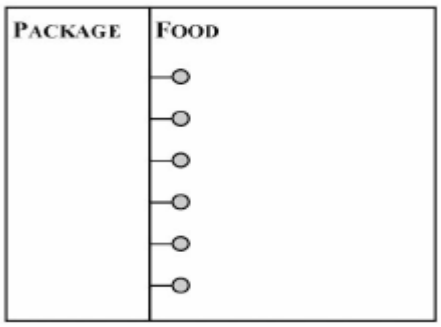


Figure 2.3. Immobilization of antimicrobial agents onto food packaging materials (Quintavalla and Vicini 2002)

Table 2.2. Antimicrobials ionically/covalently immobilized onto polymer supports (Scannel et al. 2000, Appendini and Hotchkiss, 2002)

Packaging material	Antimicrobial
Ionomeric films	Benomyl, benzoyl chloride, bacteriocins
Polystyrene	Lysozyme, Synthetic antimicrobial peptides
Cellulose triacetate	Lysozyme
Polyethylene/polyamide films	Nisin

2.2.1.2. Antimicrobial Packaging Systems

Antimicrobial food packaging systems consist of package/food systems and package/headspace/food systems. In these systems the migration of antimicrobial agent from packaging material to food occurs by different mechanisms.

2.2.1.2.1. Package/Food Systems

In this system, packaging material contacts with the solid, low viscosity or liquid food without headspace (Han 2000). Antimicrobials incorporated into the packaging material migrate to food through diffusion and partitioning at the interface (Fig. 2.4). Individually wrapped cheese and ready-to-eat meat products, aseptic brick packages and

'sous-vide' cooked products are examples of this system (WEB_2 2000), Quintavalla and Vicini 2002).

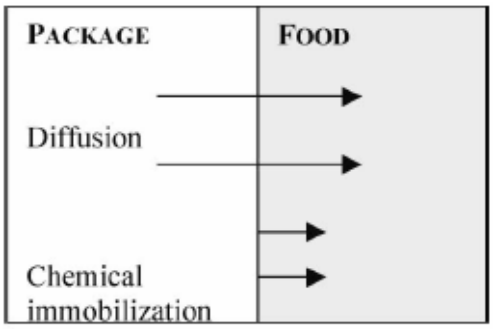


Figure 2.4. Package/food systems (Quintavalla and Vicini 2002).

2.2.1.2.2. Package/Headspace/Food Systems

In such systems, the migration of a volatile antimicrobial substance into food occurs through the headspace and air gaps between the package and the food (Fig. 2.5). The migration of antimicrobial in these systems also occurs from food-package contact surfaces by diffusion. Foods packed in flexible packages, bottles, cans, cups, and cartons are examples of package/headspace/food system (WEB_2 2000, Quintavalla and Vicini 2002).

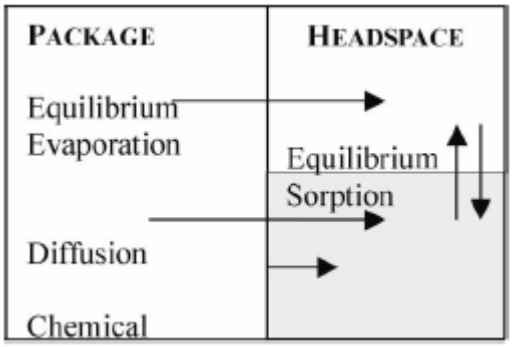


Figure 2.5. Package/headspace/food systems (Quintavalla and Vicini 2002).

2.2.1.3. Important Factors to be Considered during Production and Application of Antimicrobial Films

2.2.1.3.1. Processes Applied During Film Production

The activity of antimicrobial agents used for antimicrobial packaging can change during and/or after film formation. For example, the chemical stability of incorporated antimicrobial agents may reduce during the extrusion processing in which high temperatures, shearing forces and pressures are used (Suppakul et al. 2003). Converting operations such as lamination, printing, and drying as well as the adhesives and solvents used in the process may also affect the antimicrobial activity of the agents. Moreover, loss of volatile antimicrobial agents during casting and storage causes reduction of antimicrobial activity (Suppakul et al. 2003, Han 2002). Thus, it is essential to select suitable film materials, processing methods and antimicrobials to obtain sufficient residual antimicrobial activity after film making.

2.2.1.3.2. Interactions of Antimicrobial Agents with Film Matrix

Polarity, molecular weight and ionic charge of antimicrobial agents are the major considerations during selection of a suitable polymeric material to use in antimicrobial film production. It is suggested that the antimicrobials with low polarity should be used with non-polar polymeric materials (Suppakul et al. 2003). Some agents can bind to films because of hydrophobic interactions, whereas some others can bind as a result of ion exchange reactions and H bonding. For example, as reported above, antimicrobial enzyme lysozyme binds to cellulose triacetate films strongly because of the cation-exchange properties of these membranes (Murata and Tonioka 1997). Such a high affinity of antimicrobial agent to film material may be undesirable if this prevents diffusion and reaching of the critical concentration at the food surface.

Han (2001) reported that the incorporated antimicrobial agents are mostly low molecular weight compounds compared to the film forming polymeric materials and they do not affect the mechanical strength of the polymeric material, since they position themselves in the amorphous structural regions of the film (WEB_3 2001). However, this assumption is not valid when large molecular weight antimicrobial enzymes are

incorporated into films. For example, Padgett et al. (1998) determined significant structural changes in edible zein films when they incorporated lysozyme enzyme into films at high concentrations.

2.2.1.3.3. Properties of Antimicrobial Agents and Foods

Foods have different chemical properties such as water activity, pH and acidity. Also, there may be significant differences in the microbial flora of food. Thus, the antimicrobial agents used should be selected very carefully by considering the food properties. For example, because of their association/dissociation properties antimicrobial agents such as sorbic, benzoic and propionic acids show their antimicrobial activity mainly in acidic food (Cemeroglu and Acar 1986). In their detailed review, Suppakul et al. (2003) reported that the diffusion of potassium sorbate through polysaccharide films increases with increased water activity. Also, the use of nisin, an antimicrobial peptide, in fresh meat products is not very successful since this agent forms an inactive complex with glutathione (Rose et al. 1999). Similarly, the antimicrobial enzyme lysozyme, effective mainly on gram-positive bacteria, should not be used alone in a food system where the major concern is spoilage or poisoning due to gram negative bacteria.

2.2.1.3.4. Storage Temperature of Packed Foods

The storage temperature of foods is highly effective on the impacts of antimicrobial packaging on microorganisms. Suppakul et al. (2003) reported that at low storage temperatures the little amounts of an antimicrobial may be more effective on microorganisms than its high concentrations at room temperature. An increase in storage temperature increases also the diffusion of antimicrobial agent onto food surface. Thus, rapid diffusion of antimicrobial and its subsequent migration through inner parts of food may leave the food surface unprotected against the growth of surviving microorganisms.

In the literature, most of the food applications of antimicrobial packaging have been conducted as a part of a hurdle concept. In most cases, the antimicrobial packaging is supported with refrigeration (Scannell et al. 2000, Nattress and Baker 2002, Janes et

al. 2002, Cagri et al. 2002). Thus, during film production it is essential to consider the release rates of antimicrobials at refrigeration temperatures.

2.2.1.4. Methods for Testing the Effectiveness of Antimicrobial Packaging

The three main methods to test the antimicrobial effects of films are given below (Appendini and Hotchkiss 2002).

2.2.1.4.1. Minimum Inhibitory Concentrations (MIC) Method

MIC is the minimum concentration of an antimicrobial in a polymer that completely inhibits the growth of a test microorganism. In this method, films containing different concentrations of antimicrobials were placed into tubes containing growth medium inoculated with the target microorganism. The tubes are then incubated for a period of time to determine films containing sufficient antimicrobial to inhibit the turbidity formation which is related with microbial growth. The lowest concentration that prevents the turbidity formation is the MIC value.

2.2.1.4.2. Dynamic Shake Flask Test

In this method, flasks containing liquid growth media, antimicrobial polymer and target microorganism are incubated with mild agitation. Samples are taken at different time intervals and enumerated to determine the reduction in growth rate. This method gives detailed information on antimicrobial kinetics.

2.2.1.4.3. Agar Plate Test

In this test, antimicrobial film is placed onto a solid medium inoculated with the target test microorganism. The agar plates are then incubated to observe the growth of microorganism. A clear zone formed around a film indicates that the antimicrobial diffused from the film and inhibited the microorganisms (Fig. 2.6 and 2.7). The diameter of clear zones can be measured to make this method quantitative. The lack of

growth under the films may also show the antimicrobial activity if conformed with appropriate controls. However, this type of inhibition suggests that the antimicrobial agent binds to films or its diffusion rate is very slow.

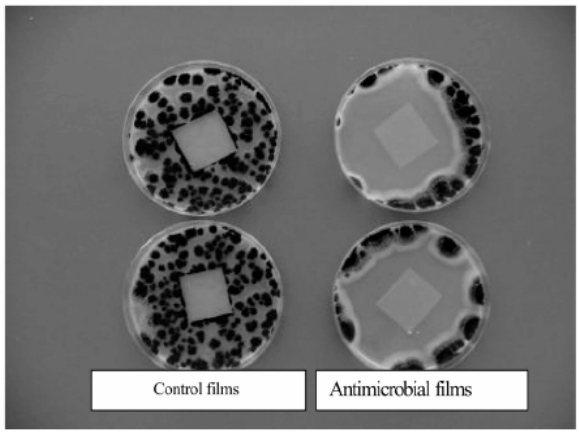


Figure 2.6. Effect of an antimicrobial plastic film on *Aspergillus niger* (Appendini and Hotchkiss 2002)

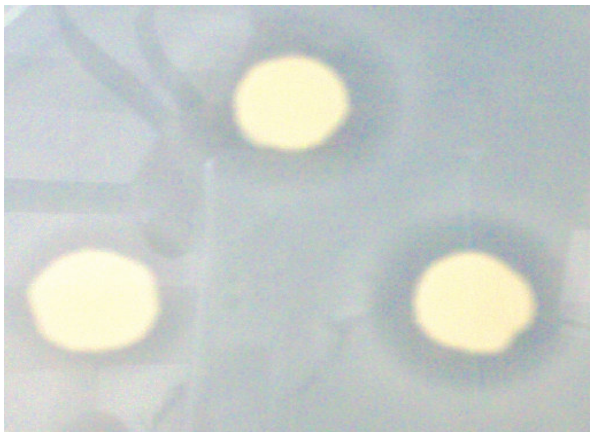


Figure 2.7. Effect of an antimicrobial edible zein film on *E. coli* (Mecitoğlu et al. 2005)

2.2.2. Antioxidant Incorporated Food Packages

In foods the oxidation of lipids causes loss of nutritional value, production of potential toxic compounds and generation of off-flavors (Nakamura et al. 1998). Thus, although much of the studies related to active packaging are focused on antimicrobial packaging, there is also a considerable interest in incorporation of different antioxidants into packaging materials. The U.S Food and Drug Administration define antioxidants as

"preservatives that specifically retard deterioration, rancidity or discoloration due to oxidation (Specchio 1992)". Antioxidants can be categorized into two main classes, free radical scavengers and metal chelators. Free radical scavengers react with free radicals to create less reactive elements. Butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), propyl gallate (PG), tocopherols, and ascorbic acid are examples of this group. Metal chelators exhibit their antioxidant activity by precipitating a metal or suppressing its reactivity by occupying all coordination sites. This class includes phosphates, EDTA, citric acid, lactoferrin and phosvitin (Satuè-Gracia et al. 2000, Nakamura et al. 1998, Specchio 1992).

2.2.2.1. Synthetic Antioxidants

The commonly used synthetic free radical scavenger antioxidants for active packaging are BHA and BHT (Rajalakshmi and Narasimhan 1996). These phenolic type synthetic antioxidants are steam-volatile and lipid soluble. Thus, when incorporated into packaging materials they easily diffuse into food lipid layers and slow down oxidation effectively. The BHA and BHT incorporated packaging materials are currently in use for high-fat candies, bakery products and breakfast cereals (Madhavi and Salunkhe 1996). There are also some studies to incorporate these antioxidants into packages of meat and meat products. For example, Herald et al. (1996) incorporated BHA into edible zein films and used these films successfully to control oxidative changes in cooked turkey. Moore et al. (2003) also tested BHA incorporated LDPE packaging materials on fresh beef and maintained the desired "cherry red" of this product.

2.2.2.2. Natural Antioxidants

Although BHA and BHT can effectively be used in active packaging there are significant concerns related to toxicological aspects of these antioxidants. For example, BHA was reported to induce forestomach papillomas and carcinomas in rats and hamster (Madhavi et al. 1996). The humans do not have forestomach, but they have similar cells in other organs such as esophagus. The major toxic effects of BHT, on the other hand, are adverse effects on blood clothing mechanism, liver hyperplasia and carcinogenesis. Thus, extensive research has recently been conducted to find some

alternative natural antioxidants in place of synthetic ones. Some of the natural antioxidants were given in Table 2.3. The natural antioxidants are readily accepted by the consumers and they are not considered as chemicals. Also, they do not require any toxicity tests if they are in GRAS (Generally Recognized as Safe) status.

The most common natural antioxidants are vitamin C, vitamin E and β-carotene. From these antioxidants vitamin C is water soluble, whereas vitamin E and β-carotene are oil soluble. Vitamin C is the L form of ascorbic acid and it has both biological and antioxidant activity. However, it is very expensive. Therefore, in the food industry D-izoascorbic acid (erythorbic acid) having antioxidant activity but lacking any biological activity is produced and used commercially. Ascorbic acid is one of the few water soluble antioxidants that may be used to prevent enzyme catalyzed oxidative changes that cause browning of fruits and vegetables. In past, sulfites had been used extensively for this purpose. But due to their adverse health effects, ascorbic or erythorbic acids have recently been employed in place of sulfites (Yemenicioglu 2002). Ascorbic acid and its derivatives are less effective in preventing enzymatic browning reactions catalysed by polyphenol oxidase. Also, in presence of metal atoms such as copper and iron, ascorbic acid acts as a prooxidant by converting Fe^{+3} and Cu^{+2} to Fe^{+2} and Cu^{+}, respectively. Such a conversion is undesirable since the metal dependent oxidative changes accelerate significantly in presence of Fe^{+2} and Cu^{+} atoms.

In food industry, there has also been an interest in use of natural phenolic compounds as antioxidants. The use of phenolics may effectively prevent the oxidative changes in most food. However, most of the phenolics are not compatible with the color and flavor of the products. Also, interaction of phenolic compounds with food proteins may mask the antioxidant activity of phenolic compounds partially (Arts et al. 2002). From the phenolic compounds, rosemary extract has a great potential in use for antimicrobial packaging. The odorless and tasteless rosemary extracts, reach in phenolic compounds, are effective in lard, chicken fat, sunflower oil, corn oil and soybean oil (Madhavi et al. 1996). Moore et al. (2003) also used rosemary extract incorporated LDPE packaging materials to maintain the desired “cherry red” of fresh beef. Although these workers found BHA more effective than rosemary extract for this purpose, beefs packed by rosemary extract incorporated films preserve the desired color better than the controls.

Recently, great efforts have also been spent to use proteins, peptides and amino acids as antioxidants (Rajalakshmi and Narasimhan 1996). These natural compounds

are generally less effective than phenolic compounds. However, compared to phenolic compounds, the odorless proteins are more compatible with most food products including meat and meat products and diary products. Also, the proteins may show both radical scavenging and metal chelating activity and they may have additional functions.

Table 2.3. Some natural antioxidants that may be suitable to incorporate into packaging materials

Source	Antioxidant compounds	Reference
Broad beans	Water soluble proteins	Okada and Okada, (1998)
Bovine whey	High molecular weight proteins	Tong et al (2000)
Bovine serum	Albumin protein	Kouoh et al (1999)
Silk warm cocoon or raw silk	Sericin protein	Yamada et al (2000)
Egg yolk	Phosvitin protein	Maheswari et al (1997)
Turmeric	Turmerin peptide	Rajalakshmi and Narasimhan (1996)
Skeletal muscles	Carnosine peptide	Rajalakshmi and Narasimhan (1996)
Bovine whey	Lactoferrin protein	Satuè-Garcia et al (2000)
Soy bean	Protein hydrolysate	Chen et al (1995)
Rosemary extract	Phenolic compounds	Moore et al (2003)
Black tea	Phenolic compounds	Yıldırım et al (2000)
Red beet	Betalains	Kanner et al (2001)
Species and herbs	Phenolic compounds	Madhavi et al (1996)

For example, casein protein shows both antioxidant and emulsifying capacity (Hu et al. 2003). Thus, it is very suitable to use this protein as both antioxidant and emulsifying agent in oil-in-water emulsions. In literature, there are very limited studies that use protein antioxidants in films. However, recently Le Tien et al. (2001) found that

coatings of caseinate and whey proteins can successfully prevent oxidative browning in sliced potatoes and apples.

2.2.3. Other Active Packaging Applications

Besides antimicrobials and antioxidants there are some other particular agents that can be used for special food applications. For example, Del Nobile et al. (2003) produced naringinase enzyme incorporated PVOH films for reduction of naringin content in grapefruit juices. Aldehyde scavenger films produced by incorporating polyethylene imine (PEI) as active ingredient into high density polyethylene films are also commercially available. Such films are used to reduce the undesirable volatile oxidation products such as hexanal formed in food packages (Del Nobile 2002).

CHAPTER 3

EDIBLE FILMS

3.1. Definition and History of Edible Films

An edible film is a thin layer of edible material formed on a food as a coating or placed (pre-formed) on or between food components (Krochta and Johnston 1997). It may be consumed along with food and can improve food quality and extend shelf-life by acting as a selective barrier to external factors such as moisture, oil, and vapor (Ryu et al. 2002, Guilbert et al. 1996, Choi et al. 2003). Since they may be consumed along with food, the composition of edible films must be compatible with the regulations applied to food products. Some of the many advantages of using edible films are as follows; (1) they can provide additional nutrients; (2) they may be incorporated with antimicrobials or antioxidants to improve food safety and quality; (3) due to their biodegradable nature, they decrease environmental pollution; (4) they may increase sensory characteristics of food by preventing loss of volatile flavors and aromas (Ryu et al. 2002, Padgett et al. 2000, Guilbert et al. 1996, Choi et al. 2003).

The use of edible films to protect food dates back to 1800s (Guilbert et al. 1996). Wax was the first edible coating used on fruits. Although Chinese applied wax coatings to oranges and lemons in the 12^{th} and 13^{th} centuries, they didn't realize all functions of edible coatings. They only found that wax-coated fruits could be stored longer than non-waxed fruits. In 1930s, hot-melt paraffin waxes and oils (mineral oil, vegetable oil) have been commercially available for fresh fruits such as apples and pears (Park 1999, Gennadios et al. 1997). In 1950s, edible films from alginates, fats, gums, and starches have been used for frozen meat, poultry, and sea food to prolong the shelf life of foods (Guilbert et al. 1996).

3.2. Major Components of Edible Films

Edible films generally consist of at least 2 major components: a high-molecular-weight film forming polymer and a plasticizer. A plasticizer is a small molecule with a

low volatility. The chemical nature of a plasticizer generally resembles to that of film forming polymer. Without a plasticizer an edible film may not have the desired flexibility. Also, it may be hard to process edible films which do not contain a plasticizer. The physical, mechanical, and barrier properties of films are mainly affected by the structure of film forming material that may be a polysaccharide, lipid or protein. However, the type and amount of plasticizer also changes the moisture sorption, mechanical properties, and water and oxygen barrier properties of the film.

Edible films should provide some specific functional requirements such as moisture barrier, solute and/or gas barrier, water or lipid solubility, color and appearance, mechanical and rheological characteristics, non-toxicity etc. To increase their functional properties plasticizers, cross-linking agents, antimicrobials, antioxidant agents, and texturizing agents can be incorporated into edible films (Guilbert and Gontard 1995). Edible films and coatings may provide barriers towards moisture, oxygen, carbondioxide, aromas, and lipids. However, because of their hydrophilic nature, most edible films are quite moisture sensitive. In fact, that's why composite films that contain hydrophobic materials such as fatty acids and waxes are sometimes required as edible films or coatings (Krochta and Johnston 1997).

In general, oxygen permeability of edible films is quite low. Especially protein films have lower oxygen permeability than non-ionic polysaccharide films. This may be because of their more polar nature and more linear structure (Miller and Krochta 1997, Krochta and Johnston 1997). The mechanical properties of edible films depend on the film forming polymeric material's structure, molecular length, geometry, molecular weight distribution and position of lateral groups. Although the mechanical properties of edible polymeric films are not as good as synthetic polymeric films, they are adequately durable coatings or wraps on food products (Krochta and Johnston 1997, Guilbert and Gontard 1995). Different edible films and their applications on various foods were given in Table 3.1.

Table 3.1. Different applications of edible films/coatings in food (Haugaard et al. 2001).

Product	Critical functions of packaging	Value added Function	Examples of materials
Meat products			
Fresh meat Cured meat	moisture barrier oxygen barrier	Antioxidant effect Antimicrobial effect	
Casings	Carbon dioxide barrier	-	
Cooked meat	frying oil barrier	-	alginate, carragenan, cellulose, gelatin and soy proteins
Beef	adhesion, mechanical protection, inhibit microbial growth	-	
Seafood			
Fish	oxygen barrier moisture barrier	Antioxidant effect	whey protein and acetylated monoglycerides
Ready meals			
Pizza base/sauce	moisture barrier	Thickening and Moisture control	alginate, whey protein
Fruits and Vegetables			
Mushrooms	oxygen barrier moisture barrier	-	alginate
Cabbage	oxygen barrier	Reduction of browning	sucrose fatty esters
Strawberries	moisture barrier	Improved sensory Quality, retardation of senescence	Cellulose based with poly ethylene glycol and incorporated with stearic, palmitic and lauric acid

3.3. Types of Edible Films

Edible films can be formed by using proteins, polysaccharides or lipids. Below each type of edible films were discussed in details.

3.3.1. Protein Films

With their availability, different molecular properties and chemical functions proteins are very suitable sources to obtain edible films. The edible films composed of proteins generally have good gas barrier properties and suitable mechanical and optical properties. However, they are highly sensitive to moisture and show poor water vapor barrier properties (Ryu et al. 2002, Peterson et al. 1999, Guilbert et al. 1996).

3.3.1.1. Zein Films

Zein, the major storage protein of corn, is a by-product of corn-refining industry. It is water insoluble prolamine protein that comprises nearly 45-50 % of the corn proteins. It may also be produced commercially form maize which contain zein in endosperm as small round particles, almost 1 μm in diameter (Shukla and Cheryan 2001, Lai and Padua 1997). Zein is also particularly rich in glutamic acid (21-26%), leucine (20%), proline (10%), and alanine (10%), but it has low amount of basic and acidic amino acids. In fact, zein owes its insolubility in water to its high amount of non-polar amino acid residues and deficiency of basic and acidic amino acids (Shukla and Cheryan 2001, Janes et al. 2002).The zein may contain α, β and γ fractions. However, commercial zein contains usually the α fraction that has a molecular weight between 21000 and 25000 (Lai and Padua 1997).

Since zein is accepted as a GRAS (Generally Recognized As Safe) substance, it can be safely used in food products. Zein can only dissolve in organic solvents such as ethanol and form glossy, hydrophobic, grease-proof films that are resistant to microbial attack (Shukla and Cheryan 2001, Padgett et al. 1998, Janes et al. 2002). Plasticizers such as glycerol, oleic acid are required to improve the flexibility of zein films since unplasticized films are too brittle for most applications (Padgett et al. 1998, Kim et al. 2004). Since it serves as a barrier for oxygen and lipid, zein is used in the formulation of coatings for dried fruits, candy, nut meats, and pharmaceutical tablets (Lai and Padua 1997, Janes et al. 2002). Also, it may be applied for coating of fresh fruits to prolog their storage by minimizing their respiration rate (Janes et al. 2002). Recently, a particular interest has also been focused on the incorporation of different natural antimicrobials such as nisin and lysozyme (Teerakarn et al. 2002, Padgett et al. 1998,

Janes et al. 2002, Hoffman et al. 2001, Dawson et al. 2000) and antioxidants such as BHA (Herald et al. 1996) into zein films. Such films may be applied as food coating or their pre-cast films may be used for wrapping of foods (Janes et al. 2002, Herald et al. 1996). Recently, the pre-cast zein films have also been used successfully for the modified atmosphere packaging of vegetables (Rakotonirainy et al. 2001). Thus, it is considered that the zein films will gain a particular importance for the future active packaging applications.

3.3.1.2. Whey protein films

Whey is the by product of cheese making industry. Liquid whey is produced in large quantities and much of this whey is not used. Therefore, it causes pollution and waste disposal problems (Ozdemir and Floros 2001, Lin and Krochta 2003).Whey proteins, account for 20% of milk protein, have very good functional properties and excellent nutritional value. Whey proteins are water soluble and they form hydrophilic edible films, having good oxygen and aroma barrier properties depending on relative humidity of the environment.

Whey protein based films are quite brittle. However, by use of food grade plasticizers the brittleness of these films can be reduced and they can be made more flexible. However, the use of plasticizers increases film permeability and this is undesirable for food quality. Thus, it is essential to use plasticizers at minimum effective concentration (Lin and Krochta 2003, Cisneros-Zevallos and Krochta 2003).

In dairy industry, there is a great interest in evaluation of whey proteins. Thus, many studies have been conducted related to developing novel uses of whey protein films in packaging industry. For example, Kim and Ustunol, (2001) produced heat sealable whey protein isolate/lipid emulsion edible films. Hong and Krochta (2003) applied heat denatured whey proteins to coat polypropylene films and this treatment successfully increased the barrier properties of these films against oxygen. This application showed the potential of whey protein coatings as an alternative to existing synthetic oxygen-barrier coating polymers. Whey protein coatings can also be used in paperboards to obtain a grease barrier packaging material (Lin and Krochta 2003). Different studies have also been conducted to apply whey proteins for coating of fresh fruits. For example, by using whey protein coatings, Vachon et al. (2003) reduced the

molding of strawberries while Le Tien et al. (2001) successfully prevented enzymatic browning of apple and potato slices. Cisneros-Zevallos and Krochta (2003) coated apples with whey proteins. However, further studies should be conducted to optimize the quality of coated apples that depend on slowing down the respiration without causing unaerobiosis. The whey protein films were also used in antimicrobial packaging applications. Examples of antimicrobial packaging applications related to whey protein films include the use of whey protein isolate films incorporated with p-aminobenzoic acid (PABA) and/or sorbic acid or potassium sorbate (Ozdemir and Floros 2001, Cagri et al. 2002).

3.3.1.3. Collagen Casings

In late 1950s, the current extrusion process for fabricating collagen casings was developed. Collagen is obtained from corium layer of bovine hide which is more than 90% collagen on a dry basis. To obtain casings, the insoluble collagen is decalcified and then acidified for swelling and finally dispersed into a very viscous suspension of collagen fibers through high shear (Lindstrom et al. 1992). To obtain casings with stronger mechanical properties, a small amount of cellulose and carboxymethyl cellulose may be added at this point. The acidified collagen dispersion that contains nearly 4% solid is then turned into the casing shape by extrusion into a coagulation bath of brine and ammonium. Finally, the casings are washed and plasticized with plasticizers such as glycerol or sorbitol (Lindstrom et al. 1992). Collagen casings have been used for sausages.

3.3.2. Polysaccharide Films

Due to their hydrophilic nature, similar to protein based films, carbohydrate films generally show limited water vapor barrier properties. However, they have good mechanical and optical properties. The polysaccharides and their derivatives used as edible films and coatings include starch, cellulose ethers, pectin, alginate, carragenan and chitosan.

3.3.2.1. Starch Films

Starch is a polysaccharide that can be isolated from grains such as corn and wheat and from tubers such as potato and tapioca (Shogrun 1998). Starch remains the most promising of the available polysaccharides for food packaging. It is widely abundant, inexpensive, and relatively easy to handle (Peterson et al. 1999, Guilbert et al. 1996). Starch is composed of two structurally different polymer of D-glucose: linear amylose and branched amylopectin. Linear polymers have better film forming properties than the branched forms. That's why films obtained from amylose are coherent, relatively strong and free-standing when compared to amylopectin films that are brittle and non continous (Gennadios et al. 1997). Since linear polymers are insoluble in water in their pure form, high temperatures and pressures or chemical modification of the amylose to form more soluble hydroxypropyl amylose are needed for the formation of a film. Amylose solution with plasticizers and emulsifiers are generally used for coating of candies, fruits and nuts. Both plasticized and unplasticized high amylose films exhibit no measurable oxygen permeability at relative humidity below 100%. In fact, Forssell et al (2002) found that under ambient humidity starch films are as good oxygen barriers as EVOH. On the other hand, moderate oxygen permeability is observed for these films at 100% relative humidity (Lindstrom et al. 1992, Gennadios et al. 1997).

3.3.2.2. Cellulose Ether Films

Cellulose is the structural polysaccharide of plants and it exists in wood, cotton, cereal straws etc. (Gilbert and Kadla 1998, Gennadios et al. 1997). It is composed of D-glucose units that are linked through β-(1→4) glycosidic linkages. There are three hydroxyl groups on the glycosyl units of cellulose which are used for making cellulose derivatives. By partial substitution of hydroxyl groups in cellulose by ether, polymeric substances called cellulose ethers are obtained. Methylcellulose, hydroxypropyl cellulose, hydroxypropylmethyl cellulose, carboxymethyl cellulose, ethylcellulose are ethers which are used for the formation of edible films (Gennadios et al. 1997). The cellulose derivatives are generally utilized in the matrix of composite films. For example, combination of ehtylcellulose, acetylated monoglyceride and calcium stearate

and application of extrusion gives films with good moisture barrier properties and thermal stability (Lindstrom 1992). The methyl cellulose films are highly hydrophobic. Thus, they may be suitable components for lipid films to provide moisture barrier films. Also, methyl cellulose based edible films may be used as fruit coatings to reduce the respiration rate and maturation. For example, Maftoonazad and Ramaswamy (2005) successfully delayed the maturation of avocadoes coated with methyl cellulose based coatings. Hydroxypropyl methylcellulose is also tested for coating of fresh apples. The obtained results with these films indicated the possibility of controlling internal gas composition in apples by coating (Cisneros-Zevallos and Krochta 2003). However, further studies are needed to optimize the thickness of hydroxypropylmethyl cellulose coatings and obtain optimum gas composition in treated apples. Another application of cellulose ether films involves the coating of vegetables before frying. It is reported that the potato sticks coated by carboxymethyl cellulose derivatives before frying showed little oil absorption (Rimac-Brncic et al 2004).

3.3.2.3. Pectin Films

Pectin mainly consists of α-(1→4) linked D-galacturonic acid and its methyl esters. Pectin is a water soluble complex polysaccharide found in the middle lamella of plant cells. It forms edible films in pure form or with other polysaccharides such as starch (Kester and Fennema 1986, Fishman and Coffin 1998).

Low methoxy pectins, having divalent cation gelling mechanism, are obtained by hydrolyzation of methyl esters in pectin. The coating of foods with pectin is achieved, by firstly dipping into a low methoxy pectin solution, then secondly dipping into a calcium chloride solution for cross-linking. The ionic calcium functions to bridge free carboxyl groups on adjacent polymer molecules. Pectin coatings can be applied to almonds, candied fruits and dried fruit (Kester and Fennema 1986, Lindstrom 1992).

3.3.2.4. Alginate Films

Alginic acid is a linear heteropolymer of D-mannuronic acid and L-guluronic acid found in seaweeds (Lindstrom et al. 1992, Huang et al. 1999). It has carboxy groups in each of its constituent residue (Figure 3.1). Carboxylic acid groups have

ability to form ionic cross links between polyvalent metal cations, especially with calcium ions, and they produce strong gels (Rhim 2004, Lindstrom 1992). Alginate gel network formation by different metal ions is given in Figure 3.2. Sodium alginate is a salt of alginic acid which is used for food applications. It is a non-toxic and water soluble polysaccharide (Cha et al. 2002). To form an alginate coating on food, as applied in low methoxyl pectin coating, the food is first treated with sodium alginate solution and then a solution of calcium chloride is applied for cross-linking and film formation. The main function of alginate films is the prevention of water loss from the food. The films have no barrier properties against moisture. However, since calcium alginate coatings have a very high water contend, they decrease moisture losses from the coated foods by acting as a water reservoir during evaporation. Alginate films are frequently used in coating of meat, poultry and fish (Lindstrom 1992).

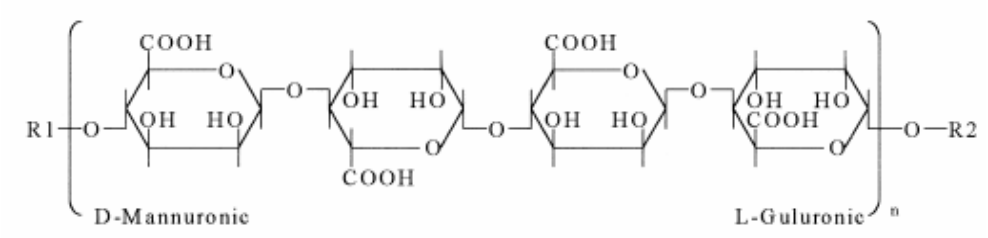


Figure 3.1.Chemical structure of alginic acid (Huang et al. 1999).

In the literature, there are very limited studies related to the use of alginate films in active packaging. However, Cha et al (2002) developed antimicrobial films by incorporation of lysozyme, nisin and EDTA into Na-alginate films. The films showed good antimicrobial effect on different bacteria including *E. coli, S. aureus, S. enteritidis* and *L. inocua*. Another antimicrobial packaging trial with alginate films, reported in the detailed review of Quintavalla and Vicini (2002), involves the incorporation of lactic acid into these films.

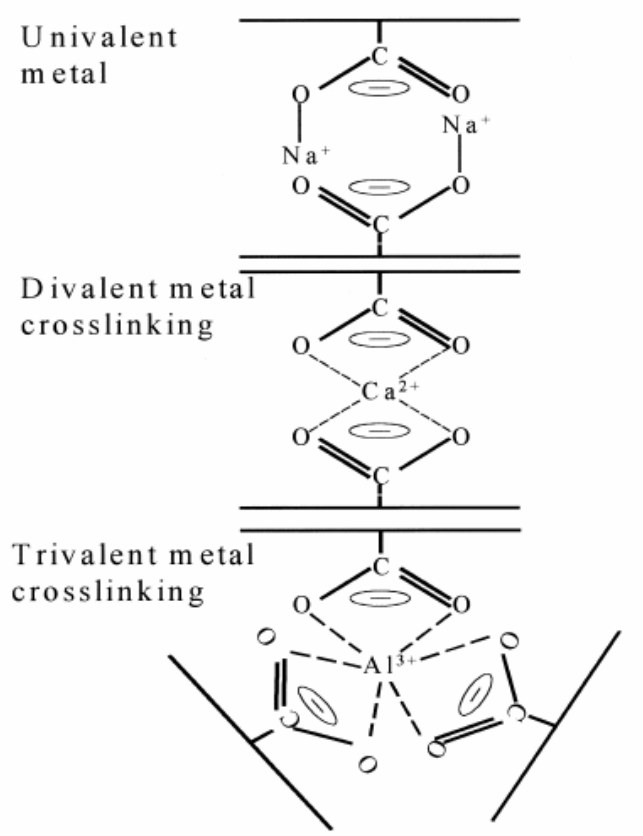


Figure 3.2. Alginate gel network formed by metal ions (Huang et al. 1999).

3.3.2.5. Carragenan Films

Carragenans are water soluble galactose polymers extracted from red seaweed (Cha et al. 2002). Carragenan is a mixture of different polymers called: ι, κ, λ, μ, and ν carragenan. ι, κ and λ-carragenan are used in food applications (Gennadios et al. 1997). ι-carragenan is highly sulfated and it forms gels with divalent cations like calcium. κ carragenan , a less sulfated molecule, forms thermo reversible gels.

Similar to alginate films, carragenan films also act as a reservoir of moisture and prevent moisture loss from the food (Lindstrom et al.1992). Frozen precooked meat, poultry, and fish have been coated with calcium carragenan coatings. In the literature, studies related to use of carragenan films in active packaging are scarce. Cha et al (2002) developed lysozyme incorporated carragenan films. However, it was reported that these films showed lower antimicrobial activity than lysozyme incorporated alginate films tested under the same conditions.

3.3.2.6. Chitosan Films

Chitin is the structural polysaccharide found in the exoskeletons of insects, crustaceans, fungi and algae. It is a linear polymer of β-(1→4) linked N-acetylglucose amine. However, since it is water insoluble it should be modified to chitosan, a water soluble form, by deacetylation before used as a packaging film material (Lindstrom et al. 1992). Due to its cationic properties chitosan films are inherently antimicrobial. The antimicrobial effect of chitosan is attributed to its interaction with the negatively charged residues of bacterial surface (Martiral-Gros et al. 2002).

Dip or spray coating with chitosan is applied in the coating of fresh fruits (Lindstrom et al. 1992). Such coatings reduce the maturation rate and they may also provide an antimicrobial effect. Recently, Park et al. (2004) developed some lysozyme incorporated chitosan composite films. These workers found that the inherent antimicrobial effect of chitosan films can be improved by the incorporation of lysozyme. Thus, chitosan films may be the most potential candidate for use in combination with biopreservatives.

3.3.3. Lipid Films

Lipid compounds prevent the transport of moisture. Thus, films made from lipids have good water vapor barrier properties. However, they are quite fragile, unstable and relatively inflexible. Also, lipids usually form opaque films (Ryu et al. 2002, Guilbert 1996).Wax and acetylated monoglycerides are given below as examples of lipid films.

3.3.3.1. Wax Coatings

Waxes consist of esters of long chain alcohols and fatty acids. They are widely used for coating of fruits and vegetables to prevent moisture loss since they prevent moisture transport better than most other lipid and non-lipid films. For example, the moisture loss can be reduced by 50% with waxes that are coated on apples, grapes, pears, prunes, tomatoes, peppers, and cucumbers, but wax coatings do not reduce decay

(Lindstrom et al. 1992). Some of the examples for waxes suitable for food coating include beeswax, candelilla wax and carnauba wax (Baldwin et al. 1997).

3.3.3.2. Acetylated Monoglycerides

Acetylated glycerides can be obtained by either through the reaction of glycerides with acetic anyhydride or through the catalyzed interesterification of a fat or oil with triacetin (Gennadios et al. 1997). Acetylated monoglycerides are waxy and relatively impermeable to moisture migration (Lindstrom et al. 1992, Kester and Fennema 1986). They have been applied to meat cuts and poultry to protect against dehydration during storage (Kester and Fennema 1986). To prevent moisture loss, dehydrated apricot pieces were also coated by dipping into molten acetylated monoglyceride (Lindstrom et al. 1992).

3.3.4. Composite Films

Different functional characteristics of several film elements can also be combined to form a single film or coating. Different kinds of polysaccharides, proteins, and lipids have been used, either alone or in mixtures, to obtain composite films. For example, Schultz et al. (1949) incorporated lipids into low-methoxy pectinate films to improve resistance to water vapor permeation (Kester and Fennema 1986). To maintain high concentration of sorbic acid at the surface of an intermediate-moisture cheese analog, Torres et al. (1985) also used a composite film composed of zein and acetylated monoglycerides and plasticized with glycerol (Kester and Fennema 1986). Emulsion coatings containing methyl cellulose, vegetable oil, beewax, and glycerol monooleate are also good examples of composite films (Lindstrom et al. 1992).

CHAPTER 4

BIOPRESERVATION AND BIOPRESERVATIVES

4.1. Biopreservation

In food technology, different chemicals such as organic or inorganic acids, metals, alcohols, ammonium compounds or amines can be used as antimicrobials (Suppakul et al. 2003, Appendini and Hotchkiss 2002). However, due to the health concerns of the consumers, producers are now highly interested in the use of biopreservatives to maintain food safety and quality. For food technologists and engineers the "biopreservation" is a new term. As reported by Hugas (1998) the term biopreservation refers to "extended storage life and enhanced safety of foods using their natural or controlled microflora and (or) their antibacterial products". The biopreservation may also be defined as "the use of active substances extracted from food or obtained by food grade microorganism fermentation to enhance food safety and quality" (Franchi et al. 2003).

4.2. Biopreservatives

As given in the definitions above, biopreservation is conducted by using some protective microorganisms and their antibacterial products or by some active compounds extracted from food. Different biopreservatives were discussed below.

4.2.1. Lactic Acid Bacteria

The use of lactic acid bacteria in food preservation have been practiced for a very long time. In fact, the fermentation is one of the oldest biopreservation methods used by humans and it involves the oxidation of carbohydrates by lactic acid bacteria to produce a range of antimicrobial products including organic acids such as lactic and acetic acid, hydrogen peroxide, bacteriocins etc. However, since the fermentation changes the sensory properties of food almost completely, the current trend of using lactic acid

bacteria in biopreservation involves the addition of viable bacteriocin-producing pure cultures to food for inhibition of pathogens and/or extension of the shelf-life while changing the sensory properties as little as possible (Vermeiren et al. 2004, Hugas 1998). Bacteriocins are ribosomally-produced antimicrobial polypeptides or proteins that may inhibit some pathogenic bacteria (Ross et al. 2002, Hugas 1998). Thus, the growth and bacteriocin production of added lactic acid bacteria may increase the safety of food and/or prolong its shelf life. However, the success of this application depends on the ability of the culture to grow and produce bacteriocin in the food under the environmental or technological conditions. For example, it was reported that the psychotropic bacteriocin-producing lactic acid bacteria have a good potential for use in biopreservation of meat and meat products (Hugas 1998). Recently, there have also been studies to characterize lactic acid bacteria capable to produce hydrogen peroxide under refrigeration temperatures (Villegas and Gilliland 1998, Jaroni and Brashears 2000). The hydrogen peroxide is a strong oxidant that is effective on both gram-positive and gram-negative bacteria. Thus, inoculation of pure cultures of such bacteria may be a novel approach for the use of lactic acid bacteria in biopreservation.

Besides addition of pure protective cultures, the use of original micro flora of food may also be employed in biopreservation. For example, chilled-storage of red meat under modified atmosphere triggers the change of natural micro flora from putrefactive gram negative bacilli to fermentative lactic acid bacteria. In such a case, due to the low carbohydrate content and strong buffering capacity of meat, the lactic acid bacteria conduct a mild fermentation without any significant change in the sensory characteristics of food and the shelf-life of product increases considerably (Hugas 1998).

4.2.2. Bacteriocins

As indicated above some lactic acid bacteria produce antimicrobial polypeptides or proteins called bacteriocins (Vermeiren et al. 2004, Ross et al. 2002, Hugas 1998). Thus, crude or pure bacteriocin preparations obtained from fermentation mediums of bacteriocin-producing lactic acid bacteria are potential antimicrobials for biopreservation of food. Some of the well known bacteriocins include nisin, pediocin and lacticin. However, nisin is the only bacteriocin that found a widespread commercial

use (Ross et al. 2002). Nisin composed of 34 amino acids is a polypeptide that shows antimicrobial activity mainly against gram positive bacteria. In diary products it shows strong antibotulinical, antilisterial and antistaphylococcal activity when used at suitable concentrations (Hugas 1998). However, the use of nisin in fresh meat products is not very successful since this agent forms an inactive complex with glutathione (Rose et al. 1999). It was reported that the complex formation reaction is catalyzed by Glutathione S-transerase enzyme in raw meat (Rose et al. 2002). Thus, it is suggested that the nisin should be used in cooked meat products which do not contain active enzyme.

4.2.3. Antimicrobial Enzymes

4.2.3.1. Lysozyme

Lysozyme (EC3.2.1.17) is a single peptide enzyme found in different sources including plants, animals and microorganisms (Ibrahim et al. 1996). It contains 129 amino acid residues and it has a molecular weight of 14400 (Takahashi et al. 2000, Ibrahim et al 1991). The enzyme shows lytic activity on bacteria by hydrolyzing glycosidic β-linkages between N-acetylhexosamines of pepdidoglycan (PG) in their cell walls. Therefore, different studies have been conducted to use of lysozyme for the biopreservation of food. Commercially the enzyme is obtained from hen egg white (Chang et al. 2000). Thus, most of the studies related to lysozyme have been focused on hen egg white lysozyme. The enzyme shows antibacterial activity mainly against gram-positive bacteria, but it is ineffective against gram-negative bacteria which PG layer is surrounded by a protective lipopolysaccharide (LPS) membrane (Nakamura et al. 1991, Ibrahim et al. 1991). Thus, native lysozyme alone is currently employed in food technology for some specific purposes such as prevention of the late blowing and off-flavor formation in cheeses by *Clostridium tyrobutiricum* (de Roos et al. 1998). Because of the absorption of lysozyme by casein micelles and retention of enzyme activity after absorption, the enzyme may be very suitable for the biopresevation of dairy products (de Roos et al. 1998). Also, there are some studies related to the control of malolactic fermentation in wines (Marchal et al. 2000) and reduction of specific pathogenic bacteria on meat surfaces by lysozyme (Korhonen et al. 1998). The sweet taste of the enzyme in solutions also encouraged some scientists to study the potential applications

of enzyme in beverage industry as both a sweetener and antimicrobial (Masuda et al. 2001). Moreover, the studies related to the incorporation of enzyme on plastic or edible films or film materials have also been conducted successfully (Padgett et al. 1998, Cha et al. 2002, Appendini and Hotchkiss 1997).

The extensive studies to increase the antimicrobial spectrum of lysozyme and make it a general purpose biopreservative are also quite promising. For example, it was found that combination of lysozyme with EDTA makes lysozyme highly effective on gram-negative bacteria. The increased antimicrobial spectrum of lysozyme in presence of EDTA is attributed to destabilizing effect of EDTA on the LPS layer which surrounds PG layer of gram-negative bacteria. The mechanism of the destabilization of LPS layer is not fully characterized. However, it was thought that it occurs by the chelating of the divalent cations such as Ca^{+2} and Mg^{+2} that are necessary for binding of LPS to PG (Branen and Davidson 2004). Nakamura et al. (1991, 1992) showed that 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 ^{o}C. These conjugates obtained naturally by maillard reaction also showed excellent emulsifying properties superior to those of commercial emulsifiers (Nakamura et al. 1992). The conjugates of lysozyme with glycolipids such as glucose stearic monoester can also be obtained naturally through maillard reaction to produce perfect emulsifiers (Takahashi et al. 2000). However, such conjugates show very little antimicrobial activity. The same problem was also observed in a genetically modified double glycosylated lysozyme which showed excellent emulsifying properties but no lytic action (Shu et al. 1998). By lipophilization of lysozyme with different fatty acids, Ibrahim et al. (1991, 1993) and Liu et al. (2000) produced some modified lysozymes which possess antimicrobial activity against gram-negative bacteria at room temperature. The main disadvantage of lipophilization is the low recovery of enzyme activity after this process. However, this may be overcome by stabilization of enzyme activity with glycosylation applied before lypophilization process (Liu et al. 2000). Also, Ibrahim et al (1996a, 1996b) found that when lysozyme is partially denatured by heating in pH 6.0 buffer at 80 ^{o}C for 20min, it gained a strong bactericidal activity against gram-negative bacteria such as *E. coli, S. enteritidis and P. auriginosa*. It was reported that the partially denatured enzyme also maintained its antimicrobial activity against gram-positive bacteria even when it was heated to lose its

catalytic activity towards PG completely. Thus, antimicrobial activity of heat treated lysozyme may not be determined only by its catalytic activity.

Although, the most probable candidate for food preservation is hen egg white lysozyme, there are some other lysozymes which naturally have broad antimicrobial activity. For example, Human lysozyme (HL) and bovine stomach lysozyme (BSL) are reported to have antimicrobial activity towards both gram-positive and gram-negative bacteria as well as fungi. Wilcox et al. (1997) showed that BSL genetically introduced to tobacco plant may be recovered and purified to 93 % homogeneity from the transgenic plants by an easily scalable process. This study showed the possibility of commercial BSL production from transgenic plants and increased the importance of this enzyme as hen egg white lysozyme alternatives.

4.2.3.2. Lactoperoxidase

The enzyme lactoperoxidase is a basic glycoprotein, containing a heme group with Fe^{+3}. It consists of a single polypeptide chain of 612 residues with a molecular mass of ~ 78.5 kDa and a carbohydrate content of about 10%. It is an oxidoreductase found in mammalian milk, saliva, and tear (Wolf et al. 2000, Pakkanen and Aalto. 1997, Jacob et al. 2000, Monzani et al. 1997) and it plays an important role in protecting the lactating mammary gland and the intestinal tract of new-born infants against pathogenic microorganisms. The mechanism of the antimicrobial action of LPS is based on conversion of thiocynate (SCN^-) to antimicrobial products such as hipothiocyanite ($OSCN^-$) ion, hypothiocyanous acid (HOSCN) and some other highly reactive and short lived oxidation products in presence of H_2O_2 (Pruitt et al. 1982). This system owing its antimicrobial effect to oxidation of sulphydryl (-SH) groups in microbial enzymes shows bactericidal effect on gram-negative bacteria and bacteriostatic effect on gram-positive bacteria (Seifu et al. 2005). Also, it has antifungal (Min and Krochta 2005, Jacob et al. 2000) and antiviral (Pakhanen and Aalto 1997, Seifu et al. 2005) activities. The synergetic effect of LPS with nisin has also been demonstrated (Zapico et al. 1998, Dufour et al. 2003, Boussouel et al. 2000). Thus, the use of LPS alone or in combination with nisin may give unique biopreservation systems that are effective on most pathogens.

Due to its broad antimicrobial spectrum and generally recognized as safe (GRAS) status (Elliot et al. 2003) different studies have recently been conducted related to use of lactoperoxidase (LPS)-thiocyanate-H_2O_2 antimicrobial system in biopreservation. Since LPS system is part of the natural preservation system exists in milk, the addition of thiocyanate and /or H_2O_2 to milk to activate naturally occurring LPS is used to improve microbial quality of milk and cheese (Seifu et al. 2004, Seifu et al. 2005). Commercially, the enzyme is produced from bovine whey (Ye et al. 2000, Hahn et al. 1998). Thus, the addition of LPS and other components of this antimicrobial system to thermally processed skim milk (Zapico et al. 1998) and meat products (Kennedy et al. 2000, Elliot et al. 2004) and prevention of the development of pathogenic bacteria have also been studied. The studies related to use of LPS in antimicrobial packaging, on the other hand, are very limited. In their detailed review Suppakul et al. (2003) suggested the use LPS in antimicrobial packaging. Recently, Min and Krochta studied the inhibition of P*enicillium commune* by LPS impregnated whey protein films and showed the good potential of this enzyme for use in antimicrobial packaging.

4.2.3.3. Glucose Oxidase

Although the lysozyme and lactoperoxidase are the most suitable enzymes to use in biopreservation, there are some other enzymes that may be used to form an antimicrobial mechanism. The enzyme glucose oxidase, gained a GRAS status in US in 1958 (Labuza and Breene 1989), has long been used to reduce glucose content of powdered egg (Petruccioli et al. 1999). The enzyme is an oxidoreductase and its antimicrobial activity is related mainly with its limitation of oxygen needed for the aerobic microorganisms and production of hydrogen peroxide. The mechanism of the enzyme depends on its removal of two hydrogen from the –CHOH groups of glucose with the formation of glucono-delta-lacton and hydrogen peroxide (Labuza and Breene 1989). By use of glucose oxidase-glucose system, it is possible to remove one mole of oxygen per mole of oxidized glucose. However, in presence of catalase, the effectiveness of this enzyme system is reduced since the enzymatically formed hydrogen peroxide is decomposed to water and oxygen. The enzyme is commercially produced as a by-product of the gluconic acid fermentation from *Aspergillus niger* or

from fermentation of *Penicillium amagasakiense* (Karmali et al. 2004). The catalase is a natural contaminant of the glucose oxidase enzyme preparations and the need of further purification to separate catalase makes the use of glucose oxidase system very expensive (Labuza and Breene 1989). Thus, currently the use of this enzyme in food packaging applications is very limited.

4.2.3.4. Chitinase

Another antimicrobial enzyme is chitinase. Chitinase is an enzyme capable to catalyze the hydrolysis of chitin to its oligomers and/or monomers. Thus, the antimicrobial effect of this enzyme is against fungi. The commercial production of enzyme is conducted by fermentation by using different microorganisms such as *Tricoderma harzainum, Streptomyces sp. and Penicillium sp.* (Navani and Kapadnis 2005, Binod et al. 2005). However, the studies to use this enzyme in food industry are scarce. One of the few studies conducted with this enzyme was that of Fenice et al. (1999) who used enzyme in combination with high hydrostatic pressure. These workers reported that the application of chitinase reduces the pressure necessary for inactivation of food spoiling mold *Mucor plumbeus*. Further studies are needed to increase the potential food applications of this enzyme.

4.2.4. Bioactive Proteins and Peptides

4.2.4.1. Lactoferrin

Lactoferrin is a member of transferrin family of non heme iron binding glycoproteins having a molecular weight of 80 kDa. It is found in colostrum, milk, and other exocrine secretions such as tears (Satuè-Gracia et al. 2000, Recio and Visser 2000, Pakkanen and Aalto 1997). The protein has bactericidal and/or bacteriostatic activities on both gram-positive and gram-negative bacteria, and fungistatic activity on fungi. The bacteriostatic and fungistatic activities of lactoferrin have been attributed mainly to its ability to bind and reduce the availability of iron for the microorganisms (Andersson et al. 2000), while its bactericidal activity is related to its direct interaction with cell membranes. Lactoferrin also exhibits an antiviral activity against some human intestinal

viruses which may be potential food contaminants (Superti et al. 1997, Marchetti et al. 1999). The mechanism of the antiviral activity of lactoferrin has not been fully understood, but it is attributed to its complex formation ability with virus particles or its interference with the viral attachment to cell receptors (Marchetti et al. 1999). Besides its broad antimicrobial spectrum, lactoferrin acts also synergistically with lysozyme (Ellison et al. 1991). Thus, combination of these two proteins may be used when higher antimicrobial activity is required in food systems. Pakkanen and Aalto (1997), on the other hand, reported that lactoperoxidase and lactoferrin show additive antibacterial effect against *Streptococcus mutans*. The hydrolysis of lactoferrin with pepsin generates a considerably more potent bactericidal peptide called lactoferricin. Lactoferricin corresponds to the 24 (17-41) or 25 (17-42) amino acid residues near the N terminus of lactoferrin (Recio and Visser 1999). This location is reported to be a region distinct from lactoferrin's iron-binding sites (Lucca and Walsh 1999). The peptide is also reported to have a strong antifungal activity (Lucca and Walsh 1999). Liceaga-Gesualdo et al. (2001) reported that lactoferricin effectively inhibits both spore germination and mycelial growth of *Penicillium spp* and suggested the use of peptide as an antifungal in bottled water. In contrast, Min and Krochta (2005) found whey protein films incorporated with lactoferrin and lactoferricin ineffective on *Penicillium commune*.

Besides its high potential as an antimicrobial, lactoferrin may also be used as an antioxidant in food systems. The antioxidant activity of the polypeptide is the result of its strong iron binding capacity. Lactoferrin has two globular lobes that bind one iron atom each. This protein-iron complex is very stable between pH 4 and 7 values (Ward et al. 1996). Also, the protein maintains its iron binding ability and solubility after mild pasteurization and UHT treatments (Mata et al. 1998). There are only limited studies about the antioxidant effect of lactoferrin in food systems. Huang et al (1999) showed the decreased prooxidant effect of iron in corn oil emulsions. Sauté-Garcia et al (2000) reported the concentration-dependent inhibition of oxidation by lactoferrin in whey-based and casein predominant infant formulas. Thus, lactoferrin may be used in a wide range of food systems as a bifunctional food additive.

Bovine milk contains approximately 0.1 mg/mL lactoferrin that is 22% saturated with iron (Satuè-Gracia et al. 2000). Thus, commercially lactoferrin is obtained form bovine whey, a by-product of dairy industry, by using standard column chromatographic procedures or modern membrane techniques (Ulber et al. 2001). Also, there is an increasing demand for the production of recombinant human lactoferrin (Hlf)

in microorganisms or in transgenic animals and plants (Spik and Theisen 2000, Mata et al. 1998). Mata et al (1998) found that Hlf and recombinanat Hlf from *Aspergillus awamori* can be survived from mild pasteurization (72 ^{o}C-20 sec) and UHT treatments (135 ^{o}C-8 sec). Thus, they proposed that these Hlfs would have been more suitable for food which will be subjected to heat treatment.

4.2.4.2. Phosvitin

Phosvitin is a phosphoglycoprotein found in egg yolk (Nakamura et al. 1998, Khan et al. 1998). Nearly 50 % of its amino acids are serine (Castellani et al. 2003a , Castellani et al. 2003b) and it is a highly phosphorylated protein containing approximately 10 % phosphorus (Lee et al. 2002, Khan et al. 1998). Phosvitin has been fractionated into α and β components that contain different phosphorus contents and molecular weights. The main components have molecular mass of 45000 for β-phosvitin, and 37500, 42000, and 45000 for α-phosvitin (Khan et al. 1998; Castellani et al. 2003b).

Phosvitin is used as a natural antimicrobial due to its ability to bind and limit iron necessary for bacterial growth. When combined with mild heating, due to its emulsifying properties, the protein showed high affinity particularly to gram-negative bacteria cell wall that consists of lipopolysaccharides (Khan et al. 2000). Phosvitin acts also as an antioxidant and inhibits metal catalyzed lipid-oxidation (Maheswari et al. 1997). Strong affinity of phosvitin to metal ions is due to its high phosphorus content (Guérin-Dubiard et al. 2002). Castellani et al. (2003a) investigated the influence of different factors on iron binding capacity of purified phosvitin found that pH 6.5 was the best pH for iron fixation of phosvitin. The maximum iron binding capacity of the protein was 115 μg of iron/mg of phosvitin and this corresponds to one iron per two phosphate groups (Castellani et al. 2003a). The antioxidant activity of phosvitin may be increased by modification. For example, Nakamura et al. (1998) modified the phosvitin by natural maillard reaction with galactomannan and successfully increased its antioxidant activity. Recently, Lee et al. (2002) showed the antioxidant activity of phosvitin in muscle food. However, according to these researchers, in muscle food phosvitin is not a very effective antioxidant and it should be combined with other

antioxidants. Further studies are needed to evaluate the real antimicrobial and antioxidant potential of phosvitin in food systems.

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

Whole milk used in LPS production was obtained in different time intervals from the same Holstein cow of a local farm in Urla, Izmir (Turkey). Fresh hen eggs used to produce LSZ were obtained from a supermarket in İzmir (Turkey). Toyopearl sulphopropyl (SP) cation exchanger (SP-550C, fast flow, size: 100 micron) was obtained from Supelco (Bellefonte, PA, USA). Corn zein, *Micrococcus lysodeikticus,* dialysis tubes (cut off: 12000 MW), dextran (from *Leuconostoc mesenteroides*, 73.200 MW), ABTS (2,2-Azino-bis-(3-Ethylbenz-Thiazoline-6-Sulfonic acid)) and sodium salt of alginic acid (from *Macrocystis pyrifera ,* viscosity of 2 % solution at 25 ^{o}C is 3500 cpc) were obtained from Sigma (St. louis, Mo., USA). Rennet was obtained from ICN Biomedicals Inc. (Aurora, Ohio, USA). Glycerol and ascorbic acid were obtained from Merck (Darmstadt, Germany). Sericin (Silk Biochemical Co. Ltd) was kindly donated by Assistant Professor Dr. Oğuz Bayraktar from Izmir Institute of Technology. The lyophilized preparations of crude thermally processed chick pea proteins (TP-CP) were provided by Research Assistant İskender Arcan from İzmir Institute of Technology. These crude preparations containing almost 51% protein were produced by modification of the method described by Geneuese and Lajolo (1998) according to Arcan (2005).

5.2. Methods

5.2.1. Production of Biopreservatives

5.2.1.1. Production of Lactoperoxidase (LPS)

The LPS was produced from bovine whey by the method given in Ye et al. (2000) with slight modifications. The skim milk was obtained by centrifugation of 1L of whole milk at 5000 x g for 20 min at 30 ºC. The skim milk was then filtrated from cheese-

cloth, warmed up to 37 ºC in an incubator and 90 mg rennet was added to 900 mL of the skim milk. After 1 h at 37 ºC, the precipitates in whey were separated by filtration from a cheese-cloth and centrifuged at 10000 x g for 25 min at 4 ºC. 400 mL of the clarified whey was then loaded to a SP Toyopearl column (height: 11.5 cm, diameter: 2.8 cm) previously equilibrated with 0.05 M Na-phosphate buffer at pH 6.5. The column was first washed with 500 mL of the equilibration buffer and then it was eluted with a linear gradient of 600 mL of 0-0.55 M NaCl prepared in the same buffer. 10 mL fractions collected from the column were assayed for their absorbance at 280 nm and LPS activity (see section 5.2.3). This study was conducted by using LPS from three different purification studies. In the first purification study, the activity profile of LPS eluted was monitored quantitatively whereas in the other studies it was monitored qualitatively by using the same reaction mixture. The enzyme containing fractions collected from the column were pooled and dialyzed 24 h at 4 ^{o}C. The dialyzed enzyme was then lyophilized in a Labconco freeze-dryer (FreeZone 6 liter, Kansas City, MO, USA), working approximately between -44 and -47 ^{o}C collector temperature and 50 x 10^{-3} and 100 x 10^{-3} mBar vacuum, after dissolving 250-300 mg dextran in the dialyzed enzyme extract as a supporting material. The sample container volume was two to three times the sample volume. The lyophilized enzymes used in film making were stored at – 18 ^{o}C and their activities were determined as U/mg before each film preparation.

5.2.1.2. Production of Lysozyme (LSZ)

The LSZ was produced by slightly modifying the partial purification step given in 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 LSZ, 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 LSZ was first dialyzed for 21 h at 4 ^{o}C by three changes of 2000 mL distilled water and then lyophilized as described above without using any supporting material. The lyophilized enzymes used in film making

were stored at – 18 ^{o}C and their activities were determined as U/mg before each film preparation.

5.2.2. Stability of the Produced LPS and LSZ in Lyophilized Form

The stability of the lyophilized LPS and LSZ was determined by monitoring of enzymes' activity during frozen storage at – 18 ^{o}C. The preparations were dissolved in distilled water and their activities were determined as described in sections 5.2.3 and 5.2.4.

5.2.3. Determination of LPS Activity

The activity of LPS was determined spectrophotometrically by using a Shimadzu (Model 2450, Tokyo, Japan) spectrophotometer equipped with a constant temperature cell holder working at 30 ^{o}C and using ABTS as substrate (Chávarri et al. 1998). The reaction mixture was formed by mixing 2.3 mL, 0.65 mM ABTS prepared in 0.1 M Na-phosphate buffer at pH 6.0, 0.1 mL enzyme solution and 0.1 mL, 0.2 mM H_2O_2 solution. All components of reaction mixture were brought to 30 ^{o}C before mixing. The increase in absorbance was monitored at 412 nm for 5 min and enzyme activity calculated from the slope of the initial linear portion of absorbance vs. time curve was expressed as Unit (0.001 absorbance change in one minute).

5.2.4. Determination of LSZ Activity

The activity of LSZ was determined spectrophotometrically at 660 nm and 30 ^{o}C by modification of the method given in Chang and Chang (2000). The reaction mixture was prepared by mixing 2.3 mL *Micrococcus lysodeikticus* cell suspension (at 30 ^{o}C) prepared 0.26 mg/mL in 0.05 M, pH 7.0 Na-phosphate buffer and 0.2 mL enzyme solution (incubated at 30 ^{o}C for 5 min). The reduction in absorbance was monitored for 5 min and enzyme activity was calculated from the slope of the initial linear portion of absorbance vs. time curve. The enzyme activity was determined as Unit (0.001 change in absorbance in one minute) or percent initial activity.

5.2.5. Preparation of Films

5.2.5.1. Preparation of Alginate Films

To prepare the films, 0.62-5.2 mg LPS preparation and/or 4 or 8 mg antioxidant protein (TP-CP proteins, sericin or bovine serum albumin) was dissolved per g of 2 % (w/v) alginic acid solution by mixing slowly with a magnetic stirrer (final concentrations of LPS in dried films: 80-690 µg/cm^2 [516-4325 U/cm^2]; final concentrations of antioxidant proteins in dried film: 0.53 or 1.06 mg/cm^2). After complete solubilization of the ingredients 10 g portions of film preparation solution were spread onto glass petri dishes (9.8 cm in diameter). The petri dishes were dried at room temperature for three days and 0.8 mL, 0.3 M $CaCl_2$ was pipetted into them to cross-link the dried films. The average thickness of dried control films prepared by this method is determined by scanning electron microscope (Philips XL 30S FEG, FEI Company, Eindhoven, Netherlands) as 12.8 µm.

5.2.5.2. Preparation of Zein Films

The zein films were produced as described in Padgett et al. (1998). Briefly, 1.4 g corn zein was dissolved with 8.1 mL of ethanol (97 %) by mixing slowly with a magnetic stirrer for 25 min. 0.39 mL glycerol was then added to medium and the temperature of the mixture was increased until it started to boil. The mixing was then ceased and the film solution was boiled for 5 min. After cooling to room temperature, 1.1-16.2 mg LSZ and/or 9-18 mg antioxidant protein (TP-CP proteins or bovine serum albumin) was added per g of film preparation solution (final concentrations of LSZ in dried films: 63-957 µg/cm^2 [187-2800 U/ cm^2]; final concentrations of antioxidant proteins in dried films: 0.53 or 1.06 mg/cm^2). The mixture was further stirred for 25 minute and 4.3 g of it was spread evenly onto a 8.5 x 8.5 cm glass plate, previously cleaned with ethanol. The plates were placed in an incubator (Memmert, 108 L, Model BE 500, Germany) and films were dried at room temperature for 24 h by keeping the incubator door half open. After drying, the films were peeled from the glass plates carefully and 6 x 6 cm pieces cut from the middle of films were used in all tests. The average thicknesses of films determined by a micrometer changed between 0.14 and

0.15 mm for control and LSZ incorporated zein films and 0.16 and 0.18 mm for LSZ and antioxidant protein incorporated films.

5.2.6. Studies Related to Alginate Films Incorporated with LPS

5.2.6.1. Monitoring of LPS Activity Release from Alginate Films

The release tests were conducted in a refrigerated incubator (Sanyo Model MIR-153, Japan). The films (containing 0.15 mg/cm^2 [941 U/cm^2 LPS] area: 75.4 cm^2) were placed in glass petri dishes containing 50 mL distilled water (4 ^{o}C). The petri dishes were then covered with nylon stretch films and their glass lids and incubated for 1800 min at 4 ^{o}C with continuous stirring at 200 rpm with a magnetic stirrer (2 cm long Teflon coated rod). The LPS activity in release test solution was monitored by taking 0.6 mL aliquots from the release test solution at different time intervals and conducting activity measurements for three times by using 0.2 mL of the taken aliquot in a single measurement. In release tests, the standard reaction mixture was changed as; 1.5 mL, 0.65 mM ABTS prepared in 0.1 M Na-phosphate buffer at pH 6.0, 0.2 mL release test solution and 0.5 mL 0.1 mM H_2O_2. This reaction mixture measures enzyme activity almost 4.3 fold higher than the standard reaction mixture given in section 5.2.3. Thus, it is quite suitable to measure residual amounts of LPS released from the films.

5.2.6.2. Determination of LPS Affinity to Alginate Films

The affinity of LPS to alginate films was determined by modifying the method used by Appendini and Hotchkiss (1997) who determined the affinity of lysozyme to different plastic films. This method was based on considering the amount of enzyme activity retained in films after repeated activity measurements. In this study, after each activity measurement, the films were additionally washed with cold distilled water. The films used in this study contained 3252 U/cm^2 (0.52 mg/cm^2) LPS. The activity determination was conducted for 7 times and after each activity determination, to remove the free enzyme (if existed) and oxidized substrate absorbed, the films were washed by placing firstly for 3 min into a beaker containing 100 mL of cold distilled water and then secondly for 15 min into another beaker containing 250 mL of cold

distilled water stirred magnetically. In these tests only, the activity measurements were conducted by a two point assay, by reading standard reaction mixture absorbance (at 412 nm) at 0^{th} and 30^{th} minutes of reaction (Activity = Abs_{30min}-Abs_{0min}). The activity was expressed as percent initial activity.

5.2.6.3. Determination of Immobilized LPS Activity in Alginate Films

Before assayed for LPS activity, the crosslinked films were peeled from the petri dishes and immersed into a beaker containing 50 mL distilled water for 30 seconds, to remove excessive $CaCl_2$ that causes some precipitations in reaction mixture. The films were then halved carefully with a clean razor and one of these halves (area: 37.7 cm^2) was placed into a glass petri dish containing 23 mL, 0.65 mM ABTS solution prepared in 0.1 M Na-phosphate buffer at pH 6.0 and 2 mL, 0.1-0.3 mM H_2O_2 solution brought to 30 ^oC before placing films. The petri dishes were kept in an incubator at 30 ^oC and stirred at 200 rpm with a magnetic stirrer. The activity monitored by measuring the reaction mixture absorbance at 412 nm at different time intervals was determined from the slope of the initial linear portion of absorbance vs. time curve. The measurements were repeated for the remaining halves of the films and average of two measurements was considered in all measurements. The activity was expressed as U (0.001 absorbance change in one minute) per cm^2 of the films.

5.2.6.4. Characterization of Immobilized LPS Activity in Alginate Films

In all characterization studies, the activities were determined with the standard method given in section 5.2.6.3 at 30 ^oC unless otherwise was indicated. All measurements were applied for two film halves seperately and averages were used in determination of an effect on LPS activity, expressed as U/cm^2 or percent initial activity.

Effect of LPS preparation concentration on LPS activity of alginate films

The effect of LPS preparation concentration on activity of this enzyme in films was determined by measuring activity of films containing LPS between 516 and 4325 U/cm^2 (0.08 and 0.69 μg/cm^2).

Effect of H_2O_2 concentration on LPS activity of alginate films

The effect of H_2O_2 concentration on LPS activity of films was determined by measuring activity of films containing 1200 U/cm^2 (0.24-0.28 mg/cm^2) LPS at different H_2O_2 concentrations (final concentrations in reaction mixture was between 2 and 24 μM).

Effect of temperature on LPS activity of alginate films

The effect of temperature on LPS activity of films was determined by measuring activity of films containing 998 U/cm^2 (0.23 mg/cm^2) LPS between 4 ° and 30 °C. The activation energy (E_a) for the activity was calculated by using Arrhenius plot formed by plotting natural logarithm of activity (U/cm^2) vs. reciprocal of temperature (1/T(^{o}K)). The temperature quotient (Q_{10}) of the enzyme activity was calculated from the equation of $k_2/k_1 = (Q_{10})^{T2-T1}$, where k is reaction rate (activity) and T is temperature (^{o}C).

Effect of pH on LPS activity of alginate films

The effect of pH on LPS activity of films was determined by measuring enzyme activity of films containing 1274 U/cm^2 (0.19 mg/cm^2) LPS in the reaction mixture of 23 mL, 0.65 mM ABTS solution prepared in 0.1 M acetate (at pH 3.1, 4.0 or 5.0) or Na-phosphate (at pH 6.0 or 7.0) buffers and 2 mL, 0.3 mM H_2O_2.

pH stability of LPS activity of alginate films

The pH stabilities of LPS activity of films were determined by incubating films containing 1114 U/cm^2 (0.19 mg/cm^2) LPS in 25 mL, 0.1 M acetate (at pH 3.1, 4.0 or 5.0) or 0.1 M Na-phosphate (at pH 6.0) buffers at 4 °C for 24 h. The remaining activity

of the films were determined at pH 6.0 by using the following reaction mixture, 23 mL 0.65 mM ABTS prepared in 0.1 M Na-phosphate buffer at pH 6.0 and 2 mL, 0.3 mM H_2O_2. The remaining LPS activity of films were given as percent of initial activity determined at pH 6.0 by using films which were not incubated for 24h.

5.2.7. Studies Related to Alginate Films Incorporated with LPS and Antioxidant Proteins

5.2.7.1. Determination of LPS and Antioxidant Activity in Alginate Films

In these experiments one half of a dried and cross-linked film was used for the determination of antioxidant activity whereas the other half was used for the determination of LPS activity as described in section 5.2.6.3. Before used in activity or antioxidant activity determination, the films were immersed into a beaker containing 50 mL distilled water for 30 seconds, to remove excessive $CaCl_2$ that causes precipitations in reaction mixtures. The water used in washing was not discarded and assayed for antioxidant activity as described in section 5.2.8.1. For the determination of antioxidant activity of films, a washed film half was placed in 25 mL, 7 mM ABTS radical cation ($ABTS^{\cdot+}$) solution (at 30 °C), produced by using potassium persulfate and diluted with 5 mM PBS (pH 7.4) to absorbance of ~0.75 at 734 nm (Re et al. 1999). The inhibition of ABTS radical was monitored at 734 nm and 30 ^{o}C and the percent inhibition at the end 3 or 15 minute was calculated from the following equation;

$$\%\ \text{inhibition} = \left(\left[1 - \frac{Abs_{(3or15\,\min)sample}}{Abs_{(0\,\min)\,sample}} \right] - \left[1 - \frac{Abs_{(3or15\,\min)\,control}}{Abs_{(0\,\min)control}} \right] \right) \times 100$$

Distilled water was used as control. The obtained inhibitions were also compared with those inhibitions of the standard antioxidant vitamin C tested under the same conditions and given as nmol vitamin C/cm^2 (see Appendix A).

5.2.8. Studies Related to Zein Films Incorporated with LSZ and/or Antioxidant Proteins

5.2.8.1. Monitoring of LSZ and Antioxidant Activity Release from Zein Films

The release tests were conducted in a refrigerated incubator at 4 ^{o}C. The films (6 x 6 cm) were placed in glass petri dishes containing 50 mL distilled water (4 ^{o}C). The petri dishes were then covered with nylon stretch films and their glass lids and incubated for 1800 min with continuous stirring at 200 rpm with a magnetic stirrer (2 cm long teflon coated rod).

The LSZ activity released from films was monitored by taking 0.6 mL aliquots from the release test solution at different time intervals and conducting activity measurements (see section 5.2.4.) for three times by using 0.2 mL of the taken aliquot in a single measurement. The activity was expressed as total units released per cm^2 of the films (U/cm^2) for a given time period. All calculations were corrected by considering the total activity removed during sampling. The monitoring of enzyme release was continued until the activity increase in release test solutions ended and a slight reduction in activity initiated. The release rates were calculated from the slopes of the best fit curves of initial portions of activity released vs. time curves as U/cm^2/min. The total activity released from a film was determined by considering the highest activity points in activity released vs. time curves. The percentage of activity recovered from a film in a release test was determined from the ratio of total activity released and incorporated activity, calculated by considering the activity and amount of partially purified LSZ added to film making solutions.

The antioxidant activity released from films was monitored by taking 0.6 mL aliquots from the release test solution at different time intervals and conducting antioxidant activity measurements for three times by using 0.2 mL of the taken aliquot in a single measurement. The antioxidant activity was determined by using the ABTS radical cation ($ABTS^{\cdot+}$) prepared and diluted as described in section 5.2.7.1. The reaction mixture was formed by mixing 0.2 mL sample and 1.9 mL ABTS radical solution. The inhibition of ABTS radical was monitored at 734 nm and 30 ^{o}C and the percent inhibition at the end of 3 or 15 minute was calculated from the equation given

in section 5.2.7.1. Distilled water was used as control. The total antioxidant activity released from the films at the end of release test was calculated as nmol vitamin C/cm^2 by considering the highest activities released from the films (see standard curve for vitamin C in Appendix B). During calculation of total antioxidant activity released, the antioxidant activity removed during sampling was also considered.

5.2.8.2. Determination of Immobilized LSZ Activity in Zein Films Remained after Release Tests

In release tests, the LSZ release from all films ended at 1380 min of the release test. Also, no measurable activity was determined in selected films exposed to a second release test following the first release test last for 1800 min. Thus, the enzyme retained in zein films after the first release test was designated as immobilized enzyme. To determine the immobilized enzyme activity, the films (6 x 6 cm) obtained from release tests (for 1800 min) were placed into glass petri dishes containing 25 mL *Micrococcus lysodeikticus* solution (at 30 ^{o}C) prepared 0.26 mg/mL in 0.05 M, pH 7.0 Na-phosphate buffer. The petri dishes were kept in an incubator at 30 ^{o}C and their contents' absorbance at 660 nm was monitored periodically. The LSZ activity of the films were determined from the slopes of the initial linear portions of absorbance vs. time curve and given as U/cm^2 after multiplication of the obtained result by the factor of ten. The factor was used to obtain comparable results with the measurements of standard reaction mixture of soluble enzyme that has 10 fold lower reaction volume (see section 5.2.4).

5.2.8.3. Determination of Retained Antioxidant Activity in Zein Films after Release Test

For the determination of retained antioxidant activity in films, following 1800 min release test, the films were transferred to glass petri dishes containing 25 mL, 7 mM ABTS radical solution (see section 5.2.7.1 for preparation) at 30 °C. The inhibition of ABTS radical was monitored at 734 nm and 30 ^{o}C and the percent inhibition was calculated from the equation given in 5.2.7.1. The obtained inhibitions were also compared with those inhibitions obtained for the standard antioxidant vitamin C tested

under the same conditions and given as nmol vitamin C/cm^2 (see standard curve for Vitamin C in Appendix A).

5.2.8.4. Stability of LSZ in Pre-cast Zein Films

For the determination of the stability of enzyme in pre-cast films, 374 U/cm^2 (112 µg/cm^2) LSZ and 180 µg/cm^2 disodium EDTA incorporated films were cold stored at 4 ^oC. In certain time periods, the films (6 x 6 cm) were placed into glass petri dishes containing 50 mL distilled water at 4 ^oC and stirred at 200 rpm as described in the release tests (see section 5.2.8.1). At the end of 1800 min, a period that enzyme release ended and a slight decline was observed in activity, the films were removed from distilled water and the total enzyme activity in release test solutions were determined. The activity was expressed as total units released per cm^2 of the films.

5.2.9. Protein Content

Protein content was determined according to Lowry procedure by using bovine serum albumin as standard (see Appendix C) (Harris 1987).

CHAPTER 6

RESULTS AND DISCUSSIONS

In this study, the biopreservatives produced by simple fractionation, dialysis or column chromotagraphy were incorporated into edible films. From the edible films, alginate films were used for incorporation of lactoperoxidase (LPS) and/or antioxidant proteins, whereas zein films were used for incorporation of lysozyme (LSZ) and/or antioxidant proteins. The antioxidant proteins, bovine serum albumin (BSA), thermally processed chick pea proteins (TP-CP) and sericin, were obtained from different chemical companies or provided by researchers specified in the materials section.

6.1. Production of Biopreservatives

6.1.1. Production of LPS

In different partial purification studies conducted, the LPS elution from SP-Toyopearl column initiated when salt concentration is between 0.21 and 0.26 M, and ended when salt concentration reached between 0.42 and 0.51 M. In all elutions the most active LPS fractions came between 0.3 and 0.4 M salt concentrations. In batch-1, a large protein peak containing two fractions and lacking LPS activity was detected before LPS elution (Fig. 6.1). The elution profile of LPS activity suggested the presence of two major LPS enzyme form in bovine whey (F1 and F2). In batch-2, conducted with another milk sample after several months, the LPS was not separated from the major protein peak eluted before LPS active fractions and several protein peaks were observed in the enzyme active region (Fig. 6.2). This increased the protein content of the collected LPS active fractions. However, because of the higher recovery of LPS activity following column chromatography of batch-2, the specific activity of enzyme and purification fold of separation were not significantly different from those of the same parameters in batch-1 (Table 6.1). In batch-3, as occurred in batch-1, the major portion of LPS active fractions eluted separated from the large protein peak coming before enzyme. The bovine milk used in batch-3 contained the highest LPS activity and its

specific activity and recovery after column chromatography was also highest. The results of these chromatographic separations are quite different from those of Ye et al. (2000) that used the same chromatographic procedure for the partial purification of LPS from bovine whey. These workers eluted LPS from the column between 0.16 and 0.19 M salt concentrations as a single peak. Also, they eluted a large protein fraction containing two protein peaks, reported as lactoferrin-a and lactoferrin-b by these workers, eluted from the column between 0.42 and 0.55 M salt concentrations. Özdemir and Floros (2001), on the other hand, determined two LPS fractions after cation exchange chromatography on CM-Sephadex C-50. However, the LPS gave a single band in SDS- PAGE electrophoresis.

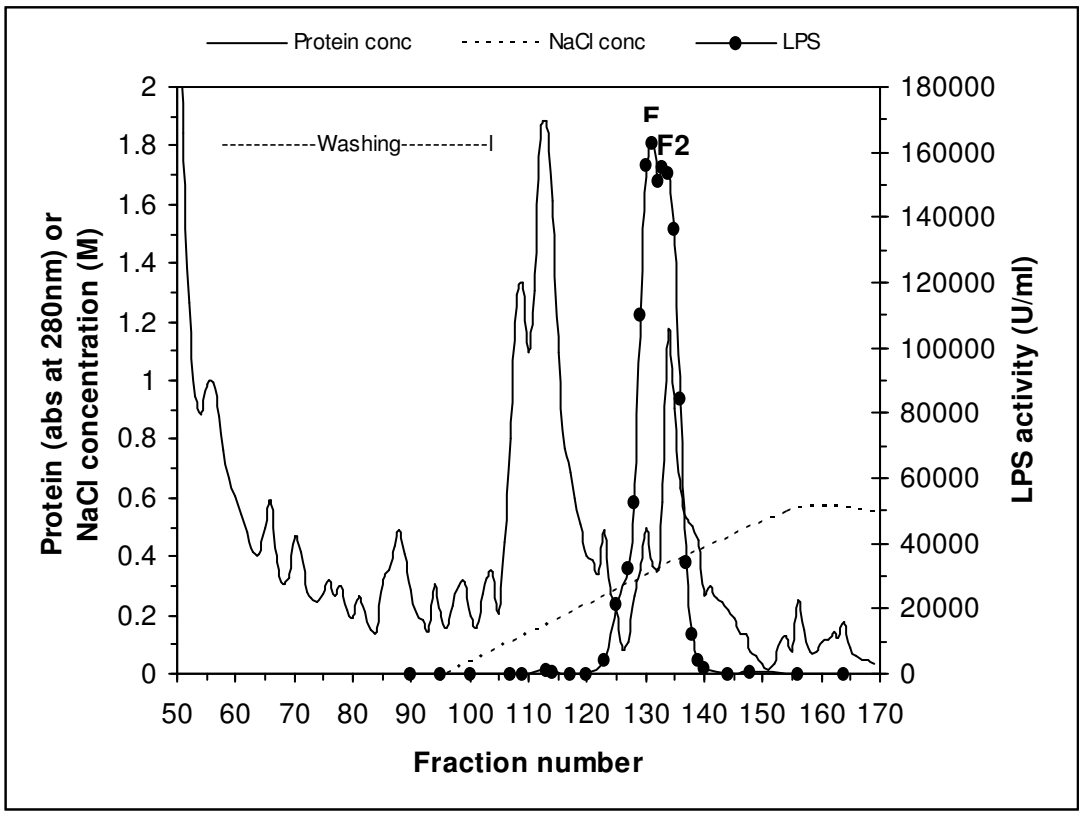


Figure 6.1. Protein and LPS activity profiles during Toyopearl-SP cation exchange chromatography of bovine whey (Batch no: 1)

Watanabe et al. (2000) explained the heterogenic profiles of LPS in disk-electrophoresis and ion-exhange chromatography by differences in the N-terminal amino acid residues of different LPS forms. However, as LPS is a glycoprotein that contains almost 10 % carbohydrate, such a heterogeneity may also be related with the type of carbohydrates attached to enzyme by glycosilation. In fact, Wolf et al. (2000)

reported that the glycosilation sites of LPS are fully occupied with different high mannose and complex structures and this may change molecular weight of LPS between 74 850 and 79 188 Da.

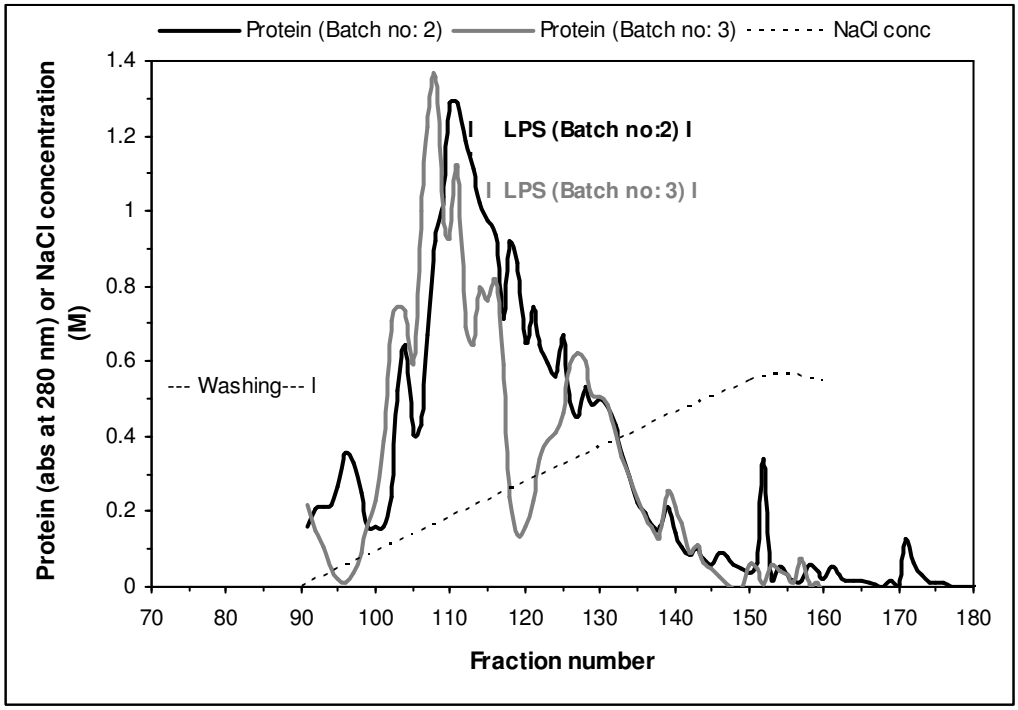


Figure 6.2. Protein profiles and active fraction regions during Toyopearl-SP cation exchange chromatography of bovine whey (Batch no: 2 and 3)

6.1.2. Production of LSZ

The partial purification of LSZ was based on the removal of egg white proteins other than the LSZ by ethanol precipitation (Jiang et al. 2001). During precipitation, the pH of the medium was set close to the isoelectric point (pI) of the major egg white protein ovalbumin (pI= 4.5). The purification fold and the recovery of LSZ from different batches of hen egg whites were given in Table 6.2 for the different stages of the partial purification process. The pH adjustment and the addition of ethanol caused the activation of the enzyme. Thus, the recovery of enzyme after 6h incubation period changed between 123-137 %. Jiang et al. (2001) applied this method with 8h ethanol incubation period and without dialysis as a partial purification procedure before column chromatography. However, although these workers attributed the high recovery of LSZ to the protective effect of ethanol they did not observe the activation of enzyme with the

ethanol treatment. Our preliminary studies conducted by measuring the enzyme activity at pH 7.0 following bringing extract pH to 4.0 showed that the pH adjustment applied during extraction did not cause any activation. Thus, it is clear that the activation is related to the ethanol treatment.

Table 6.1. Summary of partial purification studies of LPS from bovine whey

Batch ≠	Volume (mL)	T. activity (U)	T. protein (mg)	S. activity (U/mg)	Purity (fold)	Recovery (%)
1. Treatment of skim milk with rennet and centrifugation						
1	400	13708000	4374	3134	1	100
2	400	18824000	5032	3741	1	100
3	400	26614000	3632	7328	1	100
2. Toyopearl- SP cation exchange chromatography						
1	150	3651750	76	48049	11	27
2	330	9698700	182	53290	14	52
3	248	21588400	95	227246	31	81
3. 24 h dialysis at 4 ^{o}C						
1	nd[a]	nd	nd	nd	nd	nd
2	300	9447000	153	61745	17	50
3	267	16950495	74	229061	31	64

[a] not determined

The effect of ethanol on LSZ depends on its concentration (Liu et al. 2004, Bonincontro et al. 1997). For example, Bonincontro et al. (1997) reported that at very low ethanol concentrations at which mole fraction of ethanol in LSZ solution is 0.06, the α-helix content of the enzyme increases. At higher mole fractions of ethanol (between 0.06-0.25) the α-helix content approaches to that of its native form but the enzyme becomes more tightly folded. Liu et al. (2004) reported that ethanol concentrations between 0 and 20 % (v/v) enhance the LSZ solution stability. It was hypothesized that the stabilization occurs as a result of the binding of ethanol to the LSZ surface via hydrophobic interactions. This reduces the contacts among the hydrophobic patches and increases the local hydrophilicity due to the exposed OH groups of the bound ethanol (Liu et al. 2004). In fact, this is the basis of the partial purification of LSZ in the presence of 30 % ethanol used in this study. Following dialysis, on the other

hand, the loss of the activity gained by activation and the 28 to 55 % of initial activity suggested that the activation occurred could be reversible and part of the enzyme was destabilized in the presence of 30 % ethanol.

Table 6.2. Summary of partial purification studies of LSZ from hen egg white

Batch #	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purity (fold)	Recovery (%)
Dilution of egg white 3-fold with 0.05 M NaCl						
1	147	3049000	7218	422	1	100
2	147	3256000	7696	423	1	100
3	147	4010000	7999	501	1	100
pH adjustment + 6 hours incubation in presence of 30 % ethanol						
1	250	4167500	925	4505	10.7	137
2	267	3999660	720	5555	13.1	123
3	274	5123800	785	6527	13.0	128
21h dialysis at 4 ºC						
1	321	2208480	820	2693	6.4	72
2	375	1477500	680	2173	5.1	45
3	394	2458560	713	3448	6.9	61

In this study, the average recovery (59 %) of the partially purified and dialyzed LSZ is lower than the recovery (78 %) of the undialyzed partially purified LSZ obtained by heat denaturation of non LSZ proteins in hen egg white in the presence of ascorbic acid used as reductant (Chang et al. 2000). However, the average specific activity (2771 U/mg) of the dialyzed enzyme obtained in this study was almost equal to that of (2996 U/mg) the undialyzed enzyme reported by Chang et al. (2000). Since they found the destabilizing effect of ascorbic acid on LSZ during cold storage, Chang et al. (2000) also applied dialysis after the partial purification. However, the recovery and the specific activity of enzyme following dialysis were not reported by these workers. On the other hand, the average recovery of LSZ obtained in this study after dialysis was slightly higher than that of 50 % for the undialyzed partially purified LSZ obtained by

the selective precipitation with di-(2-ethylhexyl) sodium sulfosuccinate (AOT), an anionic surfactant, used by Shin et al. (2003).

6.1.3. Stability of the Produced LPS and LSZ in Lyophilized Form

6.1.3.1. Stability of LPS

In the literature, the general stabilizing effect of dextran on enzymes was reported by different workers (Wasserman 1984, Sasahara et al. 2003, de la Casa et al. 2002). As given in Table 6.3, different enzymes lyophilized by using dextran maintained almost 70-75 % of their initial activity after 1-2 months at -18 ^{o}C. Further storage of one of the enzymes for almost 5.4 months increased the loss in enzyme activity only slightly (16 %). Thus, it appears that the lyophilized LPS used in film making showed sufficient stability.

6.1.3.2. Stability of LSZ

The lysozyme is generally known with its high structural stability. However, Chang et al. (2000) found that the partially purified lyophilized lysozyme is not stable during cold storage without dialysis. These workers attributed the instability of undialyzed partially purified lysozyme to the inhibitory effects of reductants such as ascorbic acid, cysteine or cystine used during enzyme purification. The activities of different batches of lyophilized lysozymes used in this study varied between 2900 and 3351 U/mg and the enzymes showed a very high stability in lyophilized form at -18 ^{o}C (Table 6.4). For example, the initial activity of enzyme obtained in batch # 2 unchanged at the end of 8 months storage. These results clearly showed that the partially purified lysozyme obtained in this study may be a suitable source for food industry.

Table 6.3. Storage stability of partially purified and lyophilized LPS at -18 °C

Batch #	Storage time (days)	Activity	
		U/mg	% initial activity
1	0	6271 ±210	100
	28	4542 ±73	72
	48	4767 ±84	76
	161	3768 ±284	60
2	0	4363 ±107	100
	32	4424 ±134	101
	70	3005 ±178	69
3	0	6576 ±479	100
	20	5750 ±132	87
	33	4621 ±152	70

Table 6.4. Storage stability of partially purified and lyophilized LSZ at -18 ºC

Batch #	Storage time (days)	Activity	
		U/mg	% initial activity
1	0	3345 ±166	100
	10	3658 ±70	109
	80	3521 ±128	105
2	0	2900 ±325	100
	24	2979 ±234	103
	137	3179 ±236	110
	240	2941 ±71	101
3	0	3351 ±501	100
	45	3374 ±300	101

6.2. Studies Related to Alginate Films Incorporated with LPS

6.2.1. Release of LPS from Alginate Films

In release tests conducted with films containing 0.15 mg/cm^2 (941 U/cm^2) LPS we did not observe the release of any enzyme activity from the alginate films. This result clearly showed the strong binding of the added enzyme to films at this concentration. However, to determine the affinity of enzyme to alginate films in more details we also conducted some repeated activity measurements with high LPS containing films.

6.2.2. Affinity of LPS to Alginate Films

As seen in Figure 6.3, after two activity measurements conducted by 30 min reaction at 30 ^{o}C and two washings, the films containing 0.52 mg/cm^2 maintained most of their LPS activity. In characterization studies, the LPS content of films was equal almost half of the LPS content of films used in repeated activity measurements. Thus, very little or no LPS leakage is expected during characterization studies. The alginate films are composed of linear copolymers of D-mannuronic acid and L-guluronic acid that are cross-linked by the $CaCl_2$ (Lindstrom 1992). Because of its high isoelectric point (pI=9.6), the LPS is mainly positively charged in the pH values studied. Thus, the enzyme may bind to alginate films by some ion exchange reactions mediated by the negatively charged carboxylic acid groups on polymeric chains of alginate. In this study the LPS was prepared with dextran. Thus, the H-bonding of dextran to enzyme and alginate films can also make a contribution to the immobilization of enzyme. After 3rd and 4th measurements and washings, on the other hand, a moderate drop (almost 30 %) in enzyme activity was observed, whereas significant drops in activity occurred in the following measurements and washings. The significant reduction in enzyme activity after 5th measurement and washing might be related with the inhibitory effect of the substrate's oxidation products since washing was not very effective in removal of blue colored oxidized substrate absorbed by the alginate films.

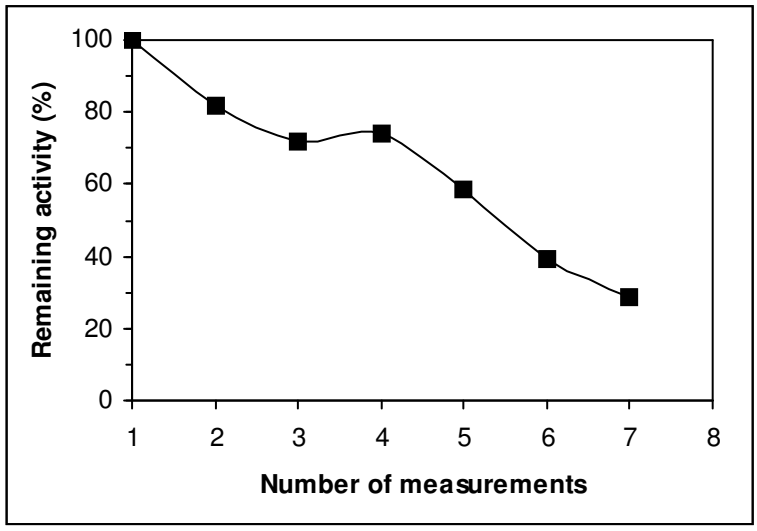


Figure 6.3. Stability of LPS activity of alginate films during repeated activity measurements and washings

6.2.3. Characterization of Immobilized LPS Activity in Alginate Films

6.2.3.1. Effect of LPS Preparation Concentration on LPS Activity of Alginate Films

Incorporation of LPS between 0.08-0.33 mg/cm^2 (516-2046 U/cm^2) did not change the LPS activity of alginate films considerably (Fig. 6.4). However, incorporation of LPS at greater concentrations increased the LPS activity of films significantly. The unexpected increase in activity at high LPS concentration may be due to LPS leakage into reaction mixture. In this study the characterization studies were conducted with films containing less than 0.30 mg/cm^2 LPS. Thus, it is once more confirmed that the characteristics of LPS in this study reflect mainly the characteristics of immobilized form of enzyme. In such films, the antimicrobial effect of LPS system is controlled by the diffusion of antimicrobial compounds enzymatically formed from the thiocyanate incorporated into films or transformation of thiocyanate added to food or naturally occurring in food.

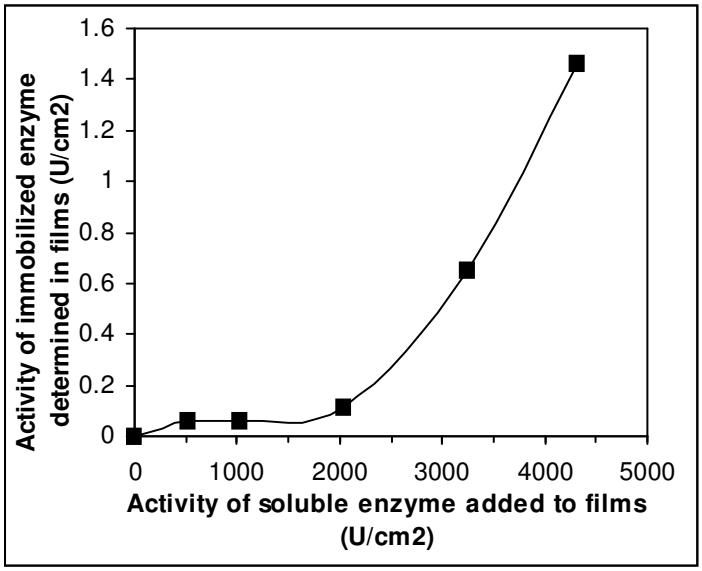


Figure 6.4. Effect of LPS preparation concentration on enzyme activity of alginate films

6.2.3.2. Effect of H_2O_2 Concentration on LPS Activity of Alginate Films

The effect of H_2O_2 on LPS activity immobilized in alginate films was determined between 2-24 μM H_2O_2 concentrations. As seen in Figure 6.5, increase of H_2O_2 concentration increased the LPS activity of alginate films linearly. In the H_2O_2 concentration range studied, the activities of films changed between 0.044 and 1.5 U/cm^2 and an almost two fold increase in LPS activity was observed when H_2O_2 concentration was increased two folds. Thus, these results showed that by regulation of H_2O_2 concentration, it is possible to form and control the rate of an antimicrobial mechanism in alginate films containing small amounts of immobilized LPS.

6.2.3.3. Effect of Temperature on LPS Activity of Alginate Films

The effect of temperature on LPS activity immobilized in alginate films was determined between 4 ^{o} and 30 ^{o}C, a temperature range that packaged food generally is exposed.

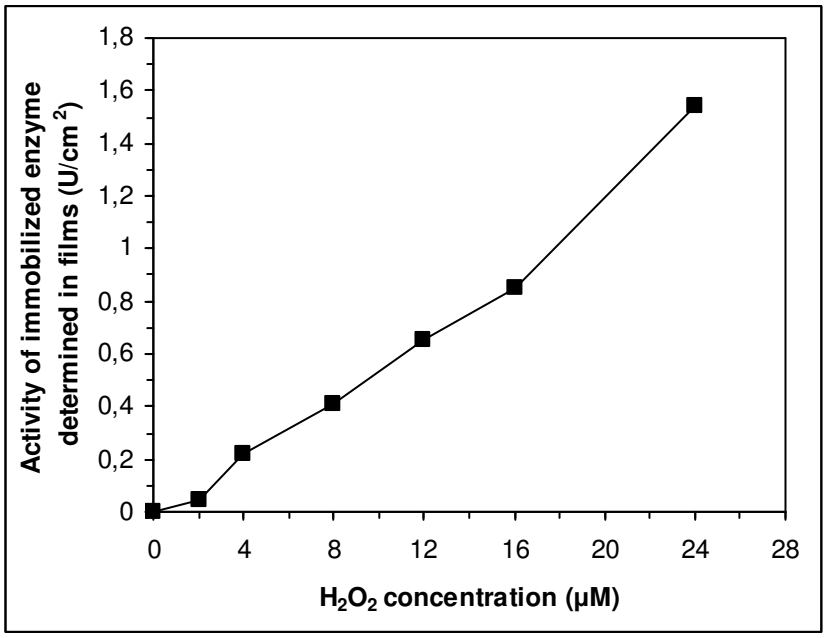


Figure 6.5. Effect of H_2O_2 concentration on LPS activity of alginate films

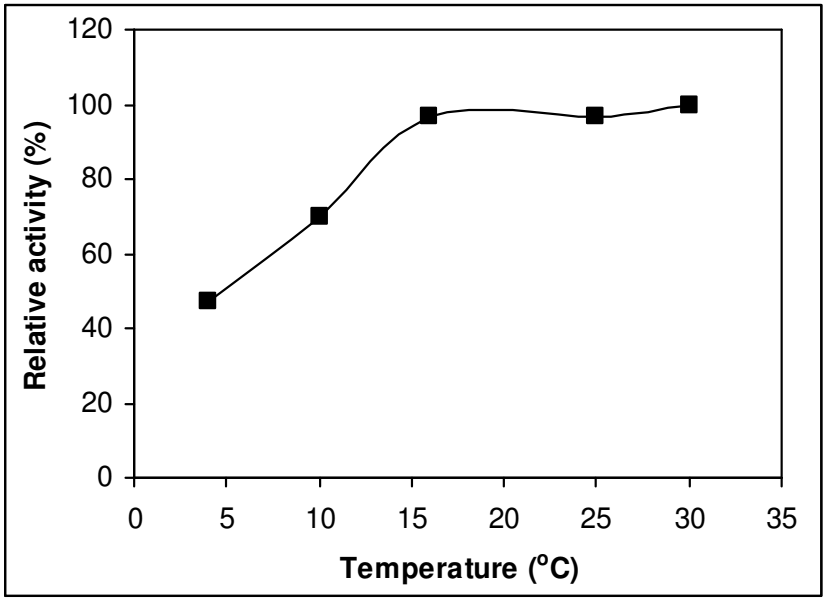


Figure 6.6. Effect of temperature on LPS activity of alginate films

As seen in Figure 6.6, between 4 ^{o} and 16 ^{o}C the increase of temperature increased the enzyme activity of films. In this temperature range a 10 ^{o}C change in temperature caused only a 0.26 fold (Q_{10}) change in enzyme activity. The activation energy of enzyme between 4 ^{o} and 16 ^{o}C was also calculated as 40.6 kJ/mol. These E_a and Q_{10} values indicate the low dependency of LPS activity in alginate films on temperature change. Between 16 ^{o} and 30 ^{o}C, on the other hand, the temperature had almost no

effect on LPS activity of films. The low temperature dependency of the immobilized LPS in alginate films is an advantage for the food applications of the enzyme when a certain amount of enzyme activity is desired in the product under variable process and/or storage temperatures.

6.2.3.4. Effect of pH on LPS Activity of Alginate Films

The effect of pH on LPS activity was determined between pH 3.0 and 7.0. The pH profile of LPS activity gave an interesting pattern (Figure 6.7). At pH 4.0 the activity of enzyme was minimum. However, the enzyme showed almost 30 % of its activity at this pH value. The optimum activity of enzyme was at pH 6.0, but the enzyme is also very active at pH 7.0. However, at pH 7.0 the alginate films were decomposed in reaction mixture during activity measurements. It is likely that the decomposition of films occurred because of the loosening of cross-linking interactions at this pH by the effect of oxidative changes catalyzed by LPS in reaction mixture. The enzyme, on the other hand, maintained minimum 50 % of its activity at pH 3.0 and 5.0. The broad pH spectrum of LPS suggests the suitability of using alginate films containing immobilized LPS as an antimicrobial coating system for different foods. The pH optimum and profile of immobilized LPS in alginate films is not significantly different from that of soluble LPS in bovine milk. For example, Blel et al (2001) showed that the LPS in milk maintained minimum 50 % of its activity between pH 6.0 and 8.0 and it showed optimum activity at pH 6.7. In their detailed review Seifu et al (2005) also reported the optimum pH of soluble bovine LPS between 5.0 and 6.0, depending on the concentrations of the substrates (ABTS and H_2O_2) used in the assay.

6.2.3.5. pH Stability of LPS Activity of Alginate Films

The stability tests showed that the LPS lost almost 90 % of its activity in 24 h at pH 3.0 (Fig. 6.8). However, the enzyme activity is very stable between pH 4.0 and 6.0, a range where most food fall in this category. The retention of most of the enzyme activity in alginate films in this pH range after 24 h once more showed the strong binding of LPS by the alginate films.

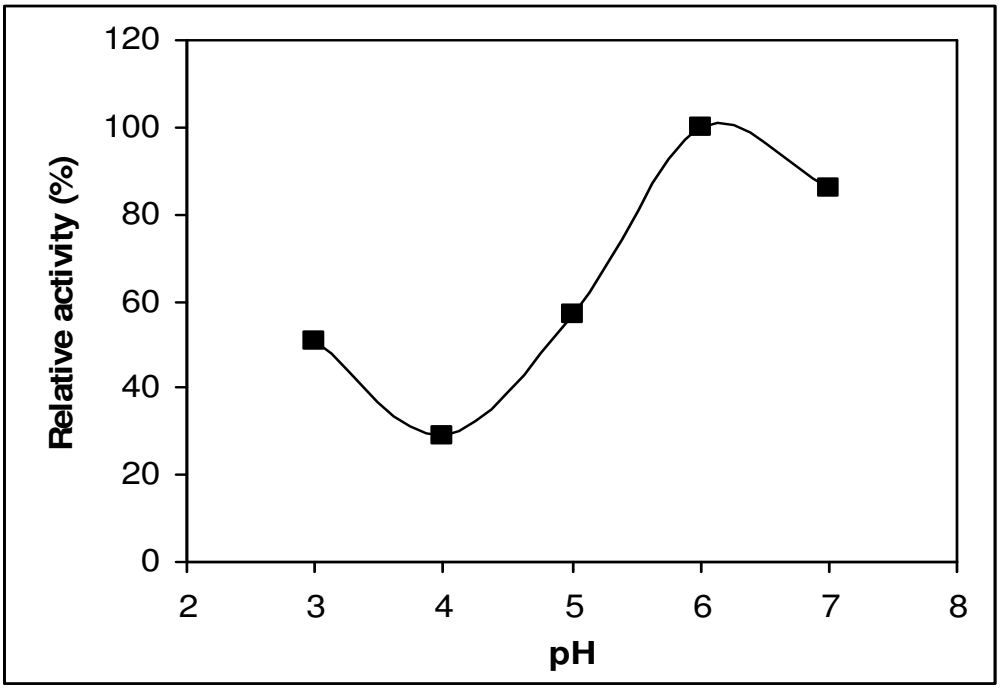


Figure 6.7. Effect of pH on LPS activity of alginate films

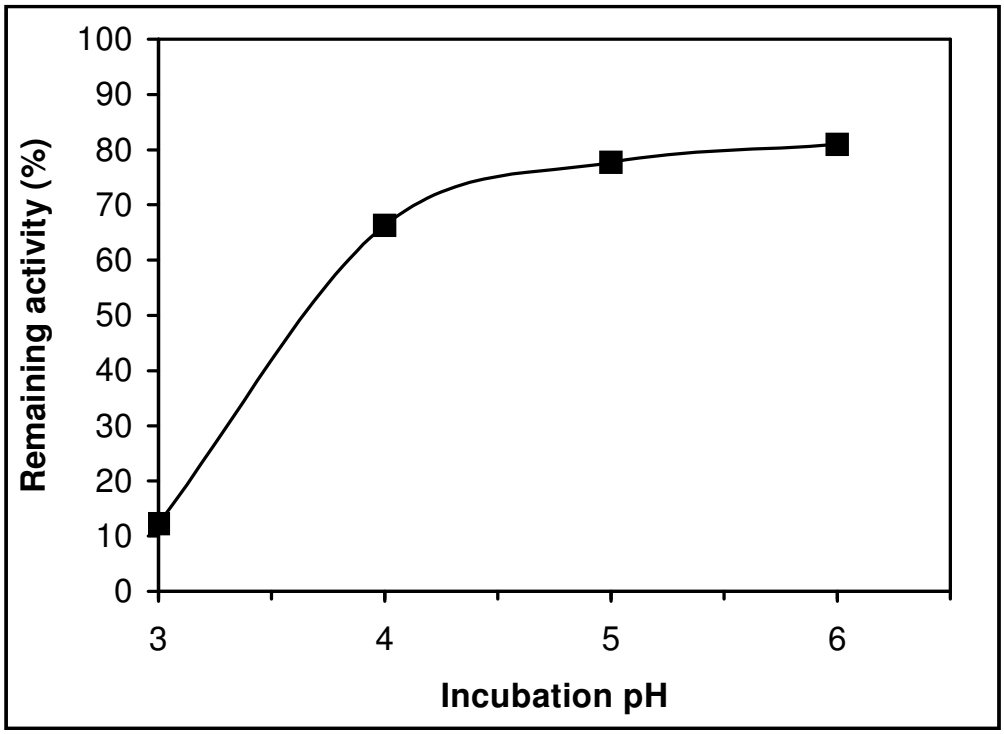


Figure 6.8. pH stability of LPS activity of alginate films incubated for 24h at 4 ^{o}C

6.3. Studies Related to Alginate Films Incorporated with LPS and Antioxidant Proteins

6.3.1. Antioxidant Activity of Alginate Films Incorporated with LPS and Antioxidant Proteins

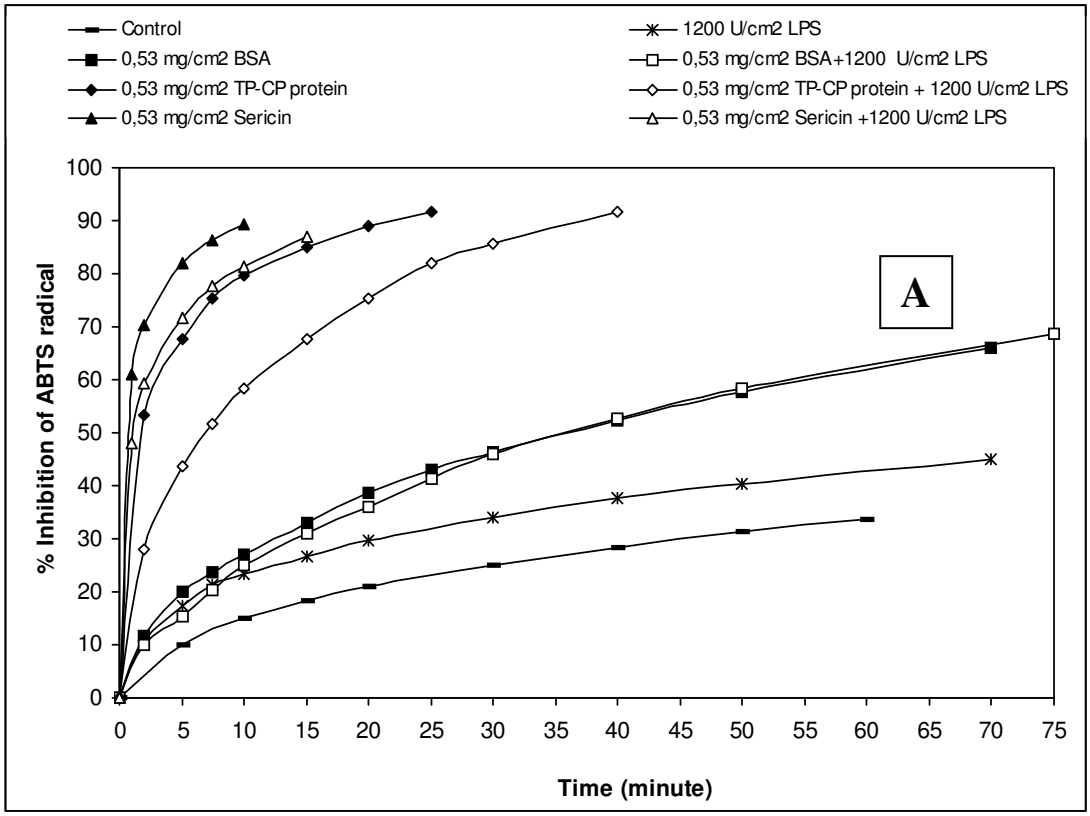
To produce alginate films with both LPS and antioxidant activity, in addition to enzyme, different antioxidant proteins were also incorporated into the films. As discussed above, when LPS was incorporated into alginate films alone, it bound and showed a high affinity to alginate films. Our results suggested that particularly at low LPS concentrations (0.08-0.33 mg/cm^2) the enzyme binds to films very tightly. However, compared to LPS, the antioxidant proteins were incorporated into alginate films at very high concentrations (0.53 and 1.06 mg/cm^2). Thus, we determined significant releases of antioxidant activities from the films during their washing following cross-linking (Table 6.5). Thus, it appears that after a food coating application the cross-linking of the alginate films containing high concentrations of proteins should not be conducted by dipping of food to $CaCl_2$ solutions. This can cause the significant leaching of the antioxidant proteins incorporated into the films. Thus, it is essential to spray $CaCl_2$ solutions instead of dipping into the solution of this cross-linking agent. Also, it is clear that the major portion of antioxidant activity in such alginate coatings will release at the initial stages of a packaging application.

On the other hand, the antioxidant activity released from the films by washing was significantly greater when sericin protein was incorporated instead of TP-CP proteins. The antioxidant activity of washed films incorporated with sericin was also greater than those of films incorporated with TP-CP proteins (Fig. 6.9A and B). For example, 0.53 mg/cm^2 sericin incorporated washed films exhibited the same antioxidant activity with 1.06 mg/cm^2 TP-CP protein incorporated washed films in two fold shorter time period. In 1.06 mg/cm^2 sericin and 1200 U/cm^2 LPS incorporated washed films, on the other hand, the antioxidant activity of 1.06 mg/cm^2 TP-CP protein and 1200 U/cm^2 LPS incorporated washed films was achieved in almost four fold shorter time period. Thus, it should be concluded that compared to TP-CP protein incorporated films, sericin incorporated films are more effective against active radicals.

Table 6.5. LPS and/or antioxidant activity of alginate films incorporated with LPS and/or antioxidant proteins

Incorporated concentrations in films		**LPS activity (U/cm^2)[a]**	**Antioxidant activity of washed films (nmol Vit C/cm^2)**	**Antioxidant activity released in washing water (nmol Vit C/cm^2)[c]**
Antioxidant protein (mg/cm^2)	**LPS (U/cm^2)**			
-	-	0	6.3 (60)[b]	-
-	1200	1.00	8.7 (70)	-
0.53 (TP-CP)	-	-	18.9 (25)	-
0.53 (TP-CP)	1200	0.96	18.8 (40)	-
1.06 (TP-CP)	-	-	18.5 (20)	24.5
1.06 (TP-CP)	1200	1.64	18.3 (15)	58.6
0.53 (BSA)	-	-	13.9 (75)	-
0.53 (BSA)	1200	0.78	13.3 (70)	-
0.53 (Sericin)	-	-	18.3 (10)	268
0.53 (Sericin)	1200	1.60	17.9 (15)	206
1.06 (Sericin)	-	-	18.0 (7.5)	318
1.06 (Sericin)	1200	1.15	18.1 (3.5)	353

[a] the final concentration of H_2O_2 in reaction mixture was 16 µM; [b] the time period (min) necessary to reach the indicated antioxidant activity; [c] before antioxidant activity determination, the films were washed in 50mL distilled water for 30 seconds to remove the cross-linking agent $CaCl_2$ which causes precipitations in reaction mixtures.



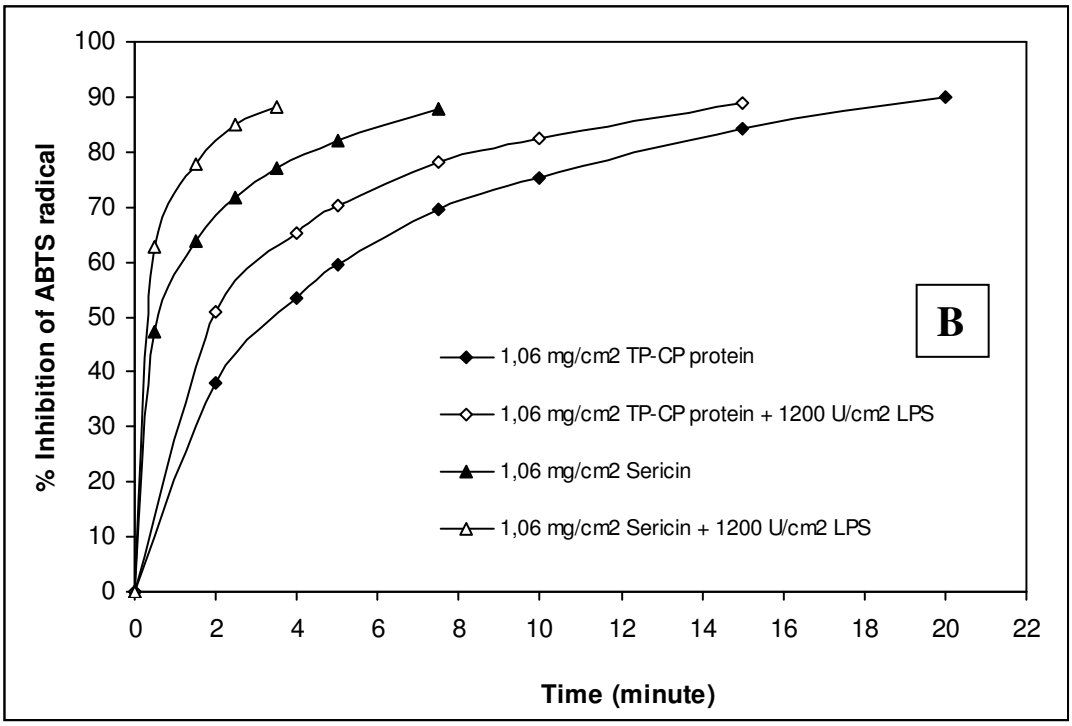


Figure 6.9. Antioxidant activities of different alginate films incorporated with LPS and/or antioxidant proteins (A: low protein concentrations; B: high protein concentrations)

6.3.2. LPS Activity of Alginate Films Incorporated with LPS and Antioxidant Proteins

The effect of incorporation of different antioxidant proteins on LPS activity of alginate films was also investigated (Figure 6.10). The incorporation of 1200 U/cm^2 LPS or 1200 U/cm^2 LPS and 0.53 mg/cm^2 TP-CP protein into alginate films did not cause any change in LPS activity of films (Table 6.5). However, a two fold increase in TP-CP protein concentration in alginate films caused the increase of the measured LPS activity in films almost 1.6 fold. The sericin and LPS containing films also showed higher LPS activity than films containing only LPS. Since the soluble enzymes are much more active than the bound enzymes, the increase in LPS activity in TP-CP protein and sericin containing films may be related with the solubilization of part of the LPS in these films during activity determination. In contrast, a slight reduction observed in LPS activity of BSA containing films suggests the release of a portion of enzyme activity in these films during washing step applied before activity determination.

6.3.3. Effect of Hydrogen Peroxide Concentration on LPS Activity of Alginate Films Incorporated with LPS and Antioxidant Proteins

The LPS activity curves of alginate films incorporated with LPS and different antioxidant proteins and assayed at different H_2O_2 concentrations were given in Figure 6.11. In alginate films incorporated with only 1221 U/cm^2 LPS, the activity of films increased almost 3 fold by the same fold increase in the concentration of H_2O_2 (from 8 to 24 µM) used in activity measurement (Table 6.6.). In TP-CP and sericin containing films, the increase of H_2O_2 concentration from 8 to 24 µM increased the activity of enzyme 3.6 and 2.1 fold, respectively. At 8 and 24 µM H_2O_2 concentrations, the films incorporated with TP-CP proteins gave 2 to 3 fold higher LPS activity than those films incorporated with only LPS or LPS and sericin. At 16 µM H_2O_2 concentration, 0.53 mg/cm^2 sericin and 1200 U/cm^2 LPS containing films showed higher activity (1.6 U/cm^2) (see Table 6.5). Thus, it seems that the increase in H_2O_2 concentration did not always cause an increase in LPS activity of antioxidant containing alginate films.

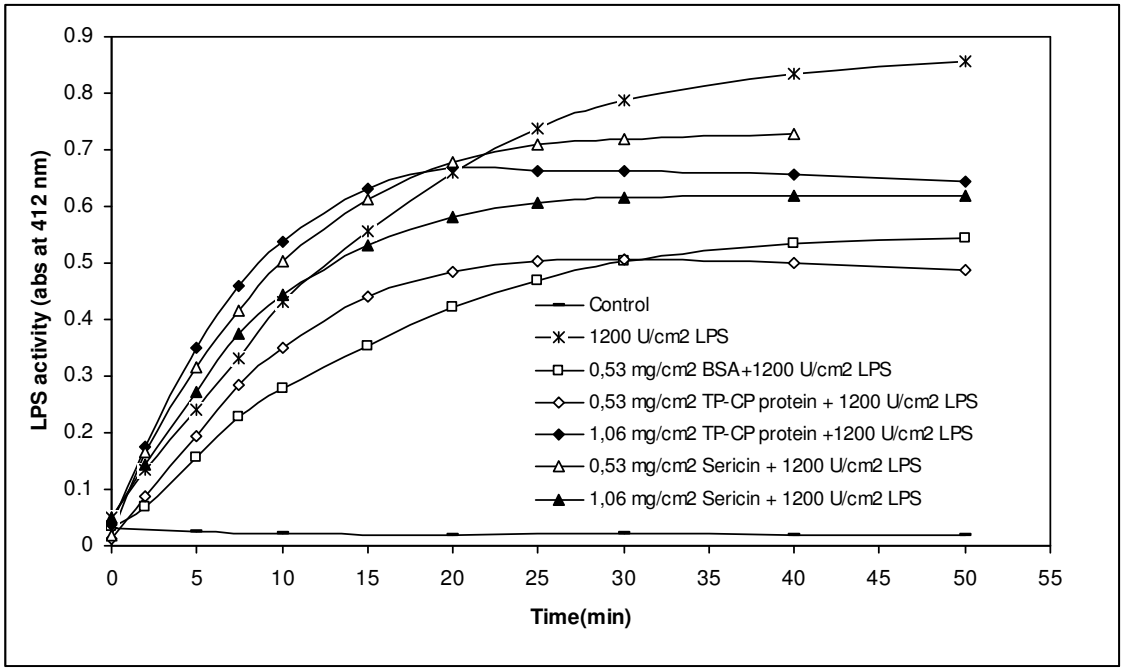


Figure 6.10. LPS activities of different alginate films incorporated with LPS and antioxidant proteins

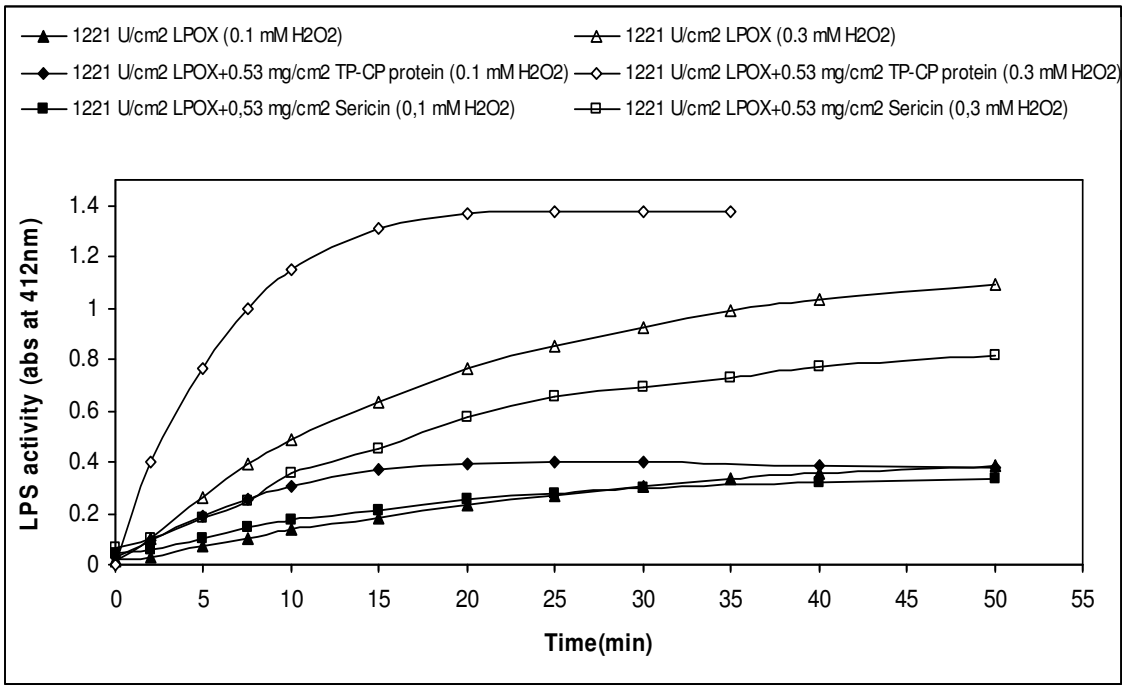


Figure 6.11. Effect of H_2O_2 concentration on LPS activity of alginate films incorporated with LPS and antioxidant protein

This result suggests that the differences in the LPS activity of antioxidant protein containing films may also be related with changes in the kinetics of enzyme or

properties of film reactive surface. Further studies are needed to determine the actual reasons of the varying reactivities of LPS in alginate films incorporated with different antioxidant proteins.

Table 6.6. Effect of type of antioxidant protein and H_2O_2 concentration on LPS activity of alginate films

Incorporated concentrations in films		**Final concentration of H_2O_2 in reaction mixture (µM)**	**Activity (U/cm^2)**	**Ratio[a]**
Antioxidant protein (mg/cm^2)	**LPS (U/cm^2)**			
-	1221	8	0.40	2.9
-	1221	24	1.16	
0.53 TP-CP	1221	8	0.95	3.6
0.53 TP-CP	1221	24	3.45	
0.53 Sericin	1221	8	0.44	2.1
0.53 Sericin	1221	24	0.91	

[a] the ratio of activity at 24 µM H_2O_2 and 8 µM H_2O_2

6.4. Studies Related to Zein Films Incorporated with LSZ

6.4.1. Release of LSZ from Zein Films

Unlike to LPS that bind strongly to alginate films, LSZ did not bind zein films strongly. Thus, significant amount of LSZ release was observed during release tests of zein films. For films incorporated with 187, 374 and 541 U/cm^2 LSZ, the initial release rates of enzyme changed between 7 and 9 U/cm^2/min (Table 6.7). In films incorporated with 374 U/cm^2 LSZ, the total activity released was close to the incorporated activity. However, in films incorporated with 187 and 541 U/cm^2 LSZ, the total activities released were 32 and 35 % greater than the incorporated activities in films, respectively. This showed the activation of the LSZ incorporated into zein films. As observed during partial purification studies, the activation may occur by the effect of ethanol used as film preparation solvent. However, the substantial increases (over 300 % of incorporated activity) of the activities recovered from films incorporated with 708 and 1318 U/cm^2 LSZ suggest that the enzyme concentration may also be effective on the

activation (Fig. 6.12). Also, it appears that the 97 % ethanol used in the film preparation had a stronger activating effect on LSZ than the 30 % ethanol used in the partial purification medium. At high concentrations, the ethanol is more effective on the protein conformation. In fact, the high concentrations of ethanol may denature or destabilize proteins (Liu et al. 2004). However, it seems that the conformational changes in LSZ induced by high ethanol concentration pronounced the activation that occurred less intensively at low ethanol concentration. Also, at high ethanol concentrations, the activity gained by activation was stable and not destabilized as occurred in dialysis applied during the partial purification. For example, during release tests of films incorporated with 708 and 1318 U/cm^2 LSZ, the activity loss of LSZ in the following 24h of maximal release in distilled water at 4 ^{o}C (between 360th and 1800th minutes of release test) were only 25 and 14 %, respectively.

For films incorporated with 708 and 1318 U/cm^2 LSZ, the enzyme release rates increased 3-4 folds. Particularly in films incorporated with 1318 U/cm^2 enzyme, almost 18 % of the total activity released freed immediately at the first 2.5th min of the release test. The rapid release occurred due to the structural changes in films at this high LSZ concentration and it caused an initial burst in the release curve. At high concentrations, the partially purified enzyme preparation showed very limited solubility in zein film solutions prepared with ethanol. Thus, after casting and drying, the excessive enzyme and the other proteins in enzyme preparation aggregated in the films. The aggregated proteins, formed some semi-transparent light yellow spots on the film surfaces (Fig. 6.13), are hydrophilic and their greater interaction with water might have caused the increases in enzyme release rates. The aggregates also existed in films incorporated with 541 U/cm^2 or less LSZ, but in these films they were small and rare. Following release tests (for 1800 min) the aggregates in the films were hardly perceptible. However, the surfaces of LSZ incorporated rehydrated films, particularly those incorporated with 1318 U/cm^2 LSZ, become rough. Moreover, following release tests, both control and LSZ incorporated rehydrated films were elastic and they maintained their integrity. However, when films were stretched, the eroded locations of aggregates in LSZ incorporated films appeared and films torn at these locations if further stretching was applied.

Table 6.7. Some kinetic parameters related to LSZ activity release from zein films at 4 ^{o}C

Incorporated LSZ activity in films (U /cm^2)	LSZ release rate (U/ cm^2/min)	Total LSZ activity released (U/cm^2)	LSZ activity recovered from films (%)[d]	Immobilized LSZ activity retained in films (U/cm^2)
187 (63)[a]	9 (0-10)[b]	246 ±60 (1380)[c]	132	1.1
374 (126)	8 (0-30)	323 ±25 (360)	86	4.6
541 (148)	7 (0-60)	731 ±85 (1380)	135	11
708 (244)	27 (0-60)	2229 ±71 (360)	315	5
1318 (455)	29 (0-120)	4034 ±37 (360)	306	5

[a]LSZ 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 highest activity released; [d] (total activity released / incorporated activity) x 100

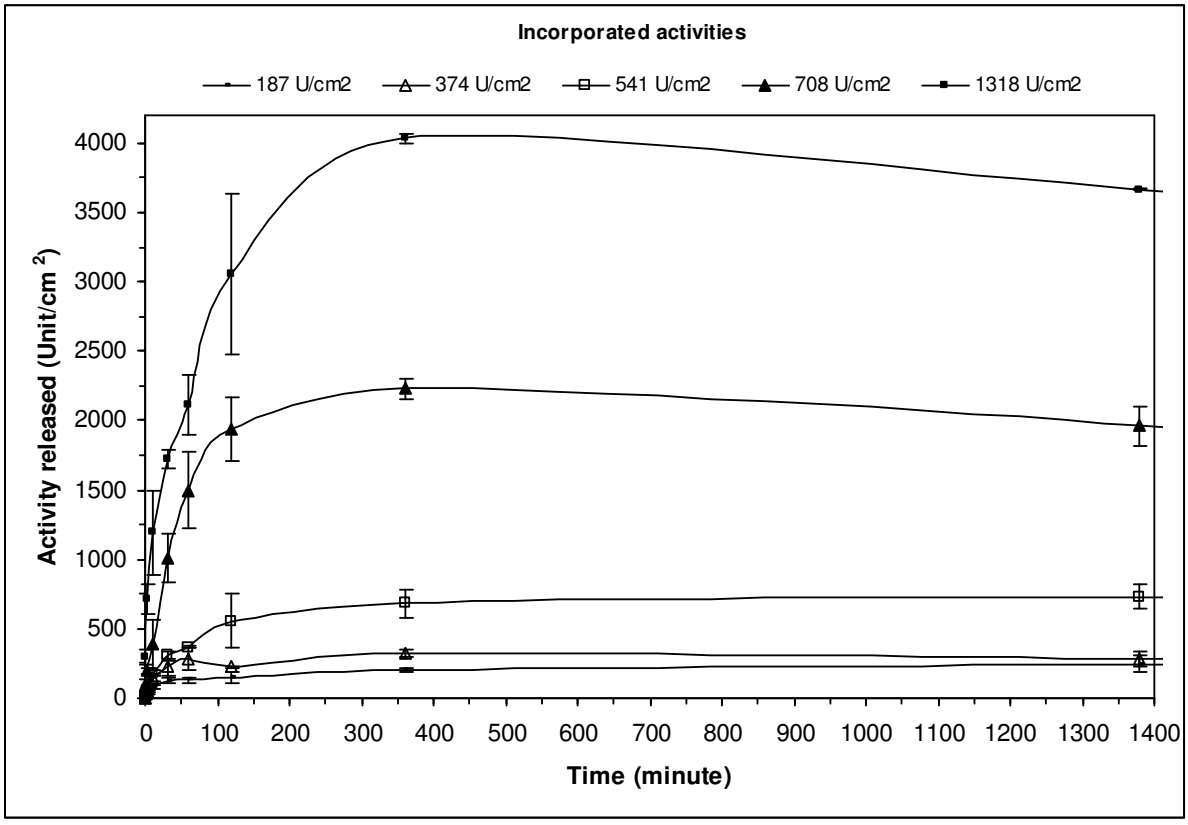


Figure 6.12. Release of LSZ from different zein films in distilled water at 4 ^{o}C

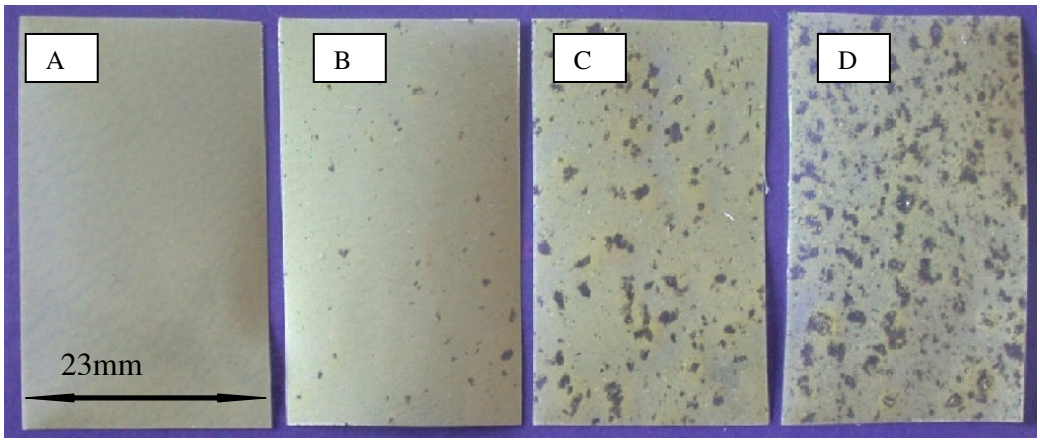


Figure 6.13. Photographs of zein films incorporated with LSZ (2678 U/mg) (incorporated activities: A: Control; B: 187 U/cm^2; C: 708 U/cm^2; D: 1318 U/cm^2; The background was dark to make the spots of light yellow semitransparent protein aggregates visible)

6.4.2. Immobilized LSZ Activity Retained in Zein Films

In packaging of foods, the retention of part of the LSZ in films may be beneficial to maintain the aseptic nature of films and to obtain greater antimicrobial effect at the food contact surfaces. In this study, following 1800 min release test in distilled water at 4 ^{o}C, the immobilized activity retained in films initially containing 187 U/cm^2 LSZ was insignificant (Table 6.7). However, the immobilized activity of films initially containing 374 and 541 U/cm^2 LSZ increased 4 and 10 fold, respectively (Figure 6.14). In films initially containing 708 and 1318 U/cm^2 LSZ, on the other hand, the activity of films dropped. It seems that the deformations occurred because of protein aggregation caused the release of most LSZ retained by these films. In the literature, reports related to affinity of different edible films to LSZ are scarce. However, Appendini and Hotchkiss (1997), investigated the immobilization of LSZ on different polymers, determined the high retention of enzyme on cellulose triacetate (CTA) films. These workers incorporated 10-300 mg LSZ (3x crystallized, Sigma Chem. Co.) per g dry weight of CTA used in film preparation and determined 0.6-5.5 U/cm^2 activity in 10 mL *Micrococcus lysodeikticus* cell suspension at pH 6.2 phosphate buffer. Although the LSZ incorporated CTA films were assayed for immobilized enzyme activity directly, the activities of these films are not significantly greater than those determined in this study for zein films exposed to 1800 min release test before activity measurements.

Thus, as these workers also specified, the measured activity of films does not reflect their LSZ content but it reflects only the activity of enzyme immobilized at the film surface. Due to its ability to bind on hydrophobic surfaces (Wertz and Santore 2002), LSZ in zein films that are also mainly hydrophobic may be retained by hydrophobic interactions. However, it was reported that in hydrophobic CTA membranes, the LSZ is absorbed mainly because of the cation-exchange properties of these membranes (Murata and Tonioka 1997). Further studies are needed to reveal the binding mechanism of LSZ on zein films and to show the contribution of immobilized LSZ activity to their antimicrobial effect.

6.4.3. Stability of Partially Purified LSZ in Pre-cast Zein Films

Since the combination of disodium EDTA with LSZ was suggested (Padgett et al. 1998, Mecitoğlu et al. 2005) to enhance the antimicrobial activity of enzyme against gram-negative microorganisms, the stability tests were conducted in presence of this additive. In zein films incorporated with 374 U/cm^2 LSZ and 180 µg/cm^2 disodium EDTA and cold stored at 4 ^{o}C for 15 days, the enzyme lost almost 15 % of its total activity determined in initial release tests. However, in the following months of storage the total activity released increased almost to the level of activity initially released from the films. The importance of some hydrophobic groups such as tryptophan-108 in maintaining LSZ activity and conformational stability has been shown recently (Inoue et al. 1992). Thus, the stability of the enzyme in zein films may be related with the protective effect of hydrophobic environment in these films.

Table 6.8. Stability of partially purified LSZ in pre-cast zein films[a] cold stored at 4 ^{o}C

Storage time	**LSZ activity**	
(days)	**U/cm^2**	**% initial activity**
0	382 ±87	100
15	321 ±56	84
45	378 ±81	99
120	417 ±87	109

[a] Incorporated with 374 U/cm^2 lysozyme and 180 µg/cm^2 disodium EDTA. In antimicrobial tests this combination was found effective on *E.coli* (Mecitoğlu et al. 2005)

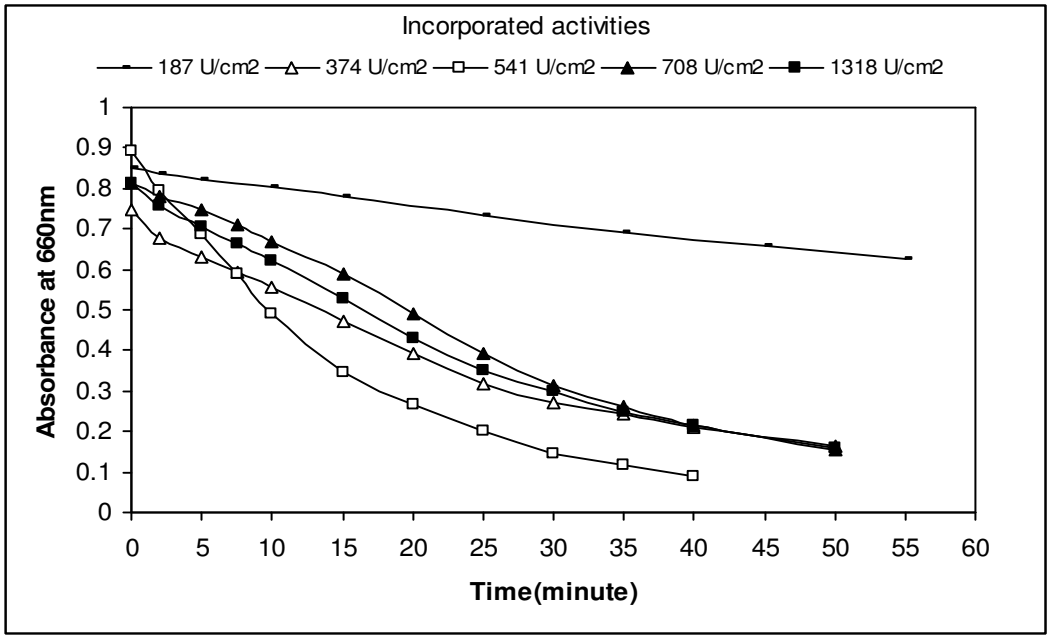


Figure 6.14. Immobilized LSZ activity retained in different zein films determined in *Micrococcus lysodeikticus* solutions after 1800 min release test in distilled water at 4 ^{o}C

The results of this study showed also the lack of any inhibitory effect of ingredients used in zein film preparation. Thus, it is clear that the ready to use pre-cast zein films incorporated with LSZ and disodium EDTA may be supplied commercially for packaging applications.

6.5. Studies Related to Zein Films Incorporated with LSZ and Antioxidant Proteins

6.5.1. Homogeneity of Zein Films Incorporated with LSZ and Antioxidant Proteins

As discussed in section 6.4.1, the incorporation of LSZ caused the formation of hydrophilic protein aggregates in zein films (compare Fig. 6.15A and B). The incorporation of LSZ into zein films with water soluble TP-CP proteins further increased the hydrophilic nature of the films. As seen in Figure 6.15C, the incorporation of 1400 U/cm^2 LSZ and 0.53 mg/cm^2 TP-CP proteins caused the increase of the tiny hydrophilic spots in films significantly. Since the LSZ preparation used in this study

was highly hydrophilic this caused a better distribution of LSZ on film surface. The increase of the LSZ content of films to 2800 U/cm^2 while keeping TP –CP protein concentration at 0.53 mg/cm^2, on the other hand, caused significant changes in film structure (Fig. 6.15D). The films became more transparent and more homogenous. Since the emulsifying activity is a well known function of legume proteins (Vliet et al. 2002, Horax et al. 2004), it is possible that an emulsion formed among LSZ, TP-CP proteins and zein. The further increase of TP-CP protein content to 1.06 mg/cm^2 and reduction of LSZ content to 1400 U/cm^2 gave similar films with those of 2800 U/cm^2 LSZ and 0.53 mg/cm^2 TP-CP protein containing films.

This study did not intent the characterization of the mechanical properties of the obtained films. However, it is observed that the incorporation of TP-CP proteins increased also the film elasticity. When control and LSZ containing films were kept several months at room temperature they became quite brittle. However, TP-CP protein containing films kept several months under the same conditions maintained their elasticity and did not brake up when folded. The incorporation of BSA, on the other hand, made similar effect with incorporation of LSZ alone. The BSA formed large and transparent spots on film surfaces and did not make contribution in distribution of hydrophilic aggregates in films. This may be due to the low emulsifying activity of BSA (Kang et al. 2003). Further studies are needed to characterize the mechanical properties of films incorporated with different proteins.

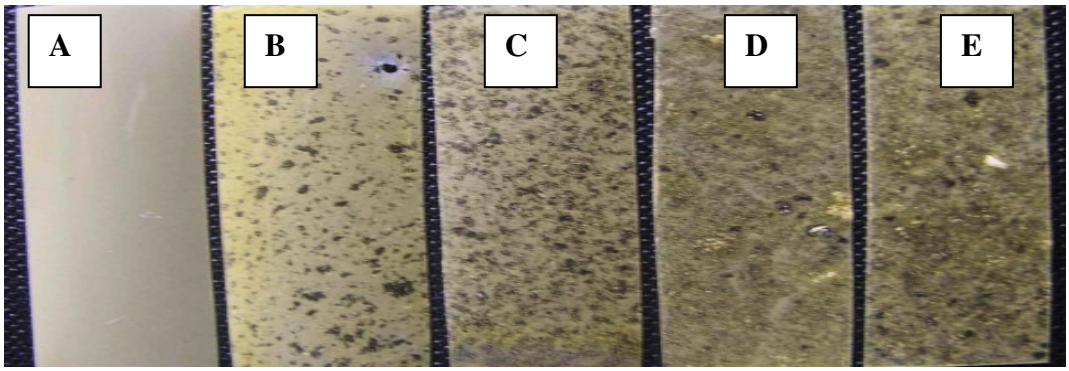


Figure 6.15. Photographs of zein films incorporated with LSZ and TP-CP proteins (incorporated activities: A: Control; B: 1400 U/cm^{2}LSZ; C: 1400 U/cm^2 LSZ + 0.53 mg/cm^2 TP-CP proteins; D: 2800 U/cm^2 LSZ + 0.53 mg/cm^2 TP-CP proteins; E: 1400 U/cm^2 LSZ + 1.06 mg/cm^2 TP-CP proteins. The background was dark to make the spots of light yellow semitransparent protein aggregates visible.

6.5.2. Release of Antioxidant Activity from Zein Films Incorporated with LSZ and Antioxidant Proteins

The results of the release tests conducted in distilled water at 4 ^{o}C were given in Figure 6.16. The incorporation of antioxidant TP-CP proteins or BSA did not change the initial release rate of antioxidant activity significantly (Table 6.9). The release tests also clearly showed the inherent antioxidant activity of zein films. In protein assays conducted in release test solutions significant amount of protein release was determined from the control films. Thus, it seems that the antioxidant activity of controls is due to the released soluble protein fractions in the zein films. The incorporation of LSZ and BSA did not affect the total released antioxidant activity from the films. However, the incorporation 0.53 or 1.06 mg/cm^2 TP-CP proteins increased the antioxidant activity released from the films almost 17 and 25 %, respectively. In this study due to the limitation of extensive sampling, the antioxidant activities during release tests were based on a 3 min inhibition test. Thus, it should be considered that the actual antioxidant activity of the films may be slightly greater than the reported values if test periods were extended over 3 min.

6.5.3. Retained Antioxidant Activity of Zein Films Incorporated with LSZ and Antioxidant Proteins

Following 1800 min release tests, the antioxidant activities retained in films were also determined (Table 6.9). As seen in Figure 6.17, the antioxidant activity retained in films incorporated initially with 0.53 mg/cm^2 BSA was slightly higher than that retained in control films. The retained antioxidant activity of 0.53 mg/cm^2 TP-CP incorporated films, on the other hand, was almost 1.5-2 fold higher than the retained antioxidant activity of control and BSA incorporated films. Thus, the results obtained in this study clearly showed the good potential of TP-CP proteins to increase both the homogeneity and the radical scavenging activity of zein films.

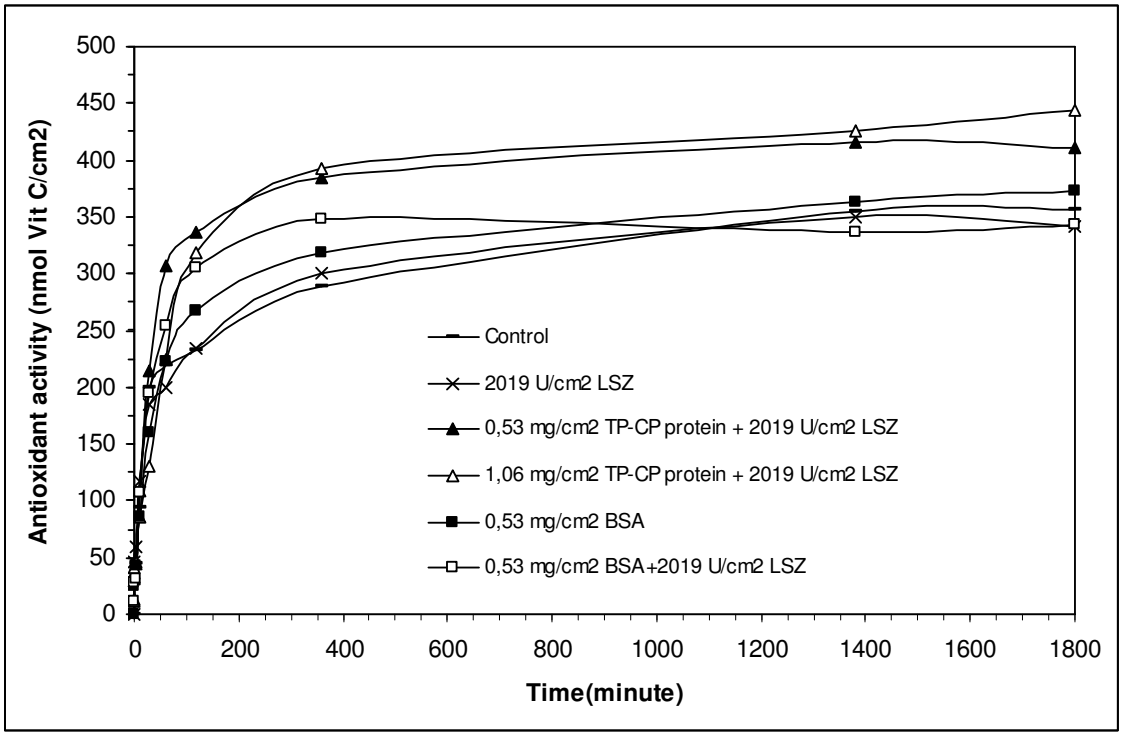


Figure 6.16. Antioxidant activity released from zein films incorporated with LSZ and/or different antioxidant proteins at 4 ^{o}C

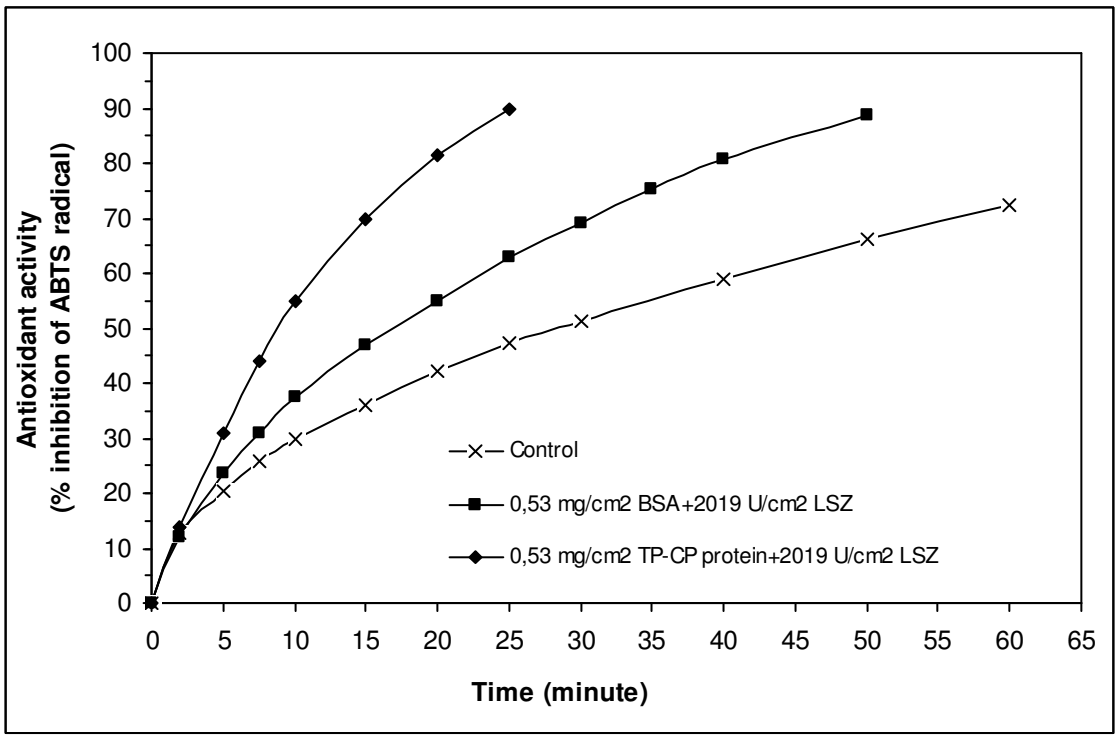


Figure 6.17. Antioxidant activity remained in films following 1800 min release test.

Table 6.9.Kinetic parameters related to antioxidant activity release from zein films at 4°C

Incorporated concentrations in films		Release rate of antioxidant activity	Total antioxidant Activity released From films	Antioxidant activity retained in films
Antioxidant protein (mg/cm^2)	**LSZ (U/cm^2)**	**(nmol Vit. C/ cm^2/min)**	**(nmol Vit. C/cm^2)**	**(nmol Vit.C/cm^2)**
-	-	4.4[b]	357 ±13 (1800)[c]	10.7 (25)[d] 15.0 (50)
-	2019 (546)[a]	4.1	349 ±4.4 (1380)	-
0.53 (TP-CP)	2019 (770)	5.7	416 ±8.6 (1380)	20.4 (25)
1.06 (TP-CP)	2019 (680)	3.2	444 ±21 (1800)	-
0.53 (BSA)	-	4.2	373 ±4.0 (1800)	-
0.53 (BSA)	2019 (670)	4.8	349 ±6.3 (360)	14.3 (25) 20.1 (50)

[a]LSZ incorporated into films as $\mu g/cm^2$; [b]The data in the first 60 min were used in best fit to calculate initial rates; [c]Release test periods (min) to achieve highest antioxidant activity released; [d]the time period necessary to reach the indicated antioxidant activity

6.5.4. Release of LSZ from Zein Films Incorporated with LSZ and Antioxidant Proteins

The release of LSZ from different films incorporated with LSZ and antioxidant proteins was seen in Figure 6.18. Similar to our previous release tests, a significant activity release was observed from films incorporated only with 2019 U/cm^2 LSZ. The activation of the enzyme in these films was minimum 227 % (Table 6.10). The activation was observed also in BSA containing films (234 %). In 2019 U/cm^2 LSZ and 0.53 mg/cm^2 TP-CP containing films, on the other hand, the released activity was

almost equal to incorporated activity. However, in all other TP-CP containing films, the released activity was lower than the incorporated activity. Thus, it seems that the incorporation of TP-CP protein prevented the activation of LSZ. Also, it is possible that the incorporation of TP-CP proteins increased the fraction of LSZ molecules bound to zein films.

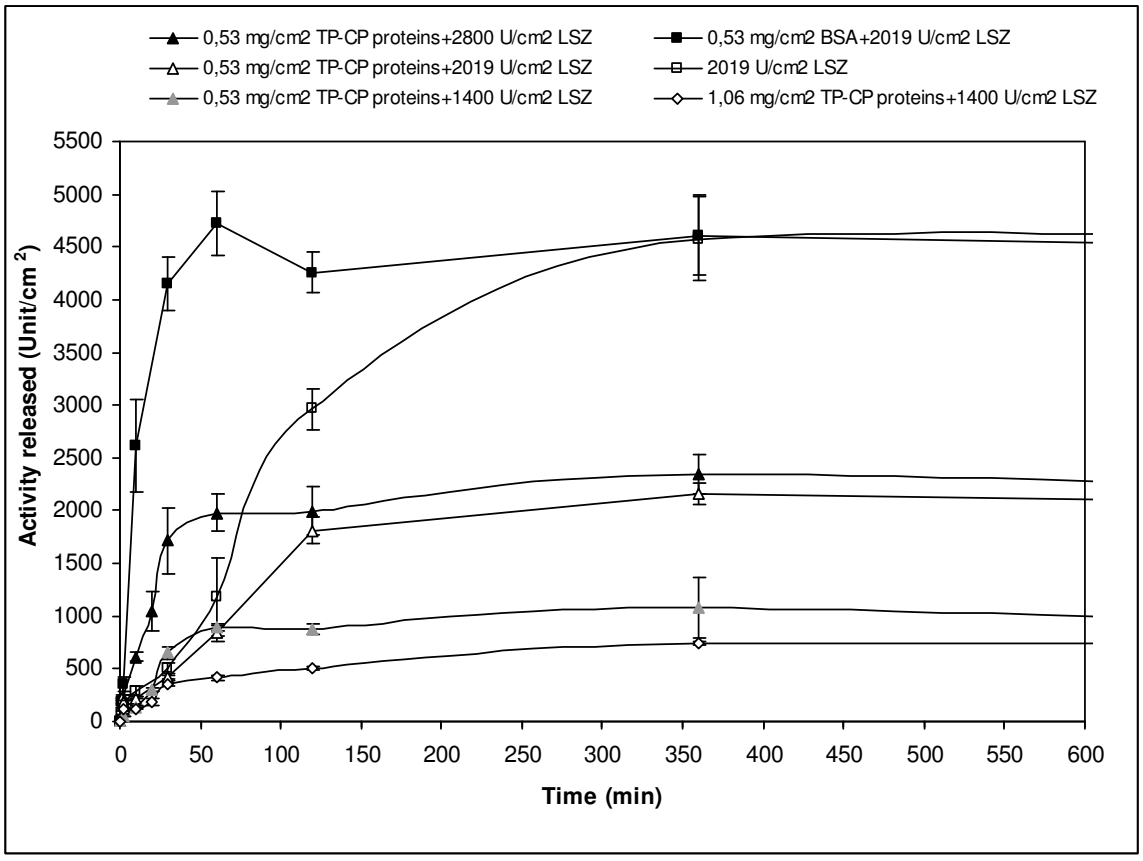


Figure 6.18. LSZ activity released from zein films incorporated with LSZ and/or antioxidant proteins at 4 ^{o}C

6.5.5. Immobilized LSZ Activity of Zein Films Incorporated with LSZ and Antioxidant Proteins

In 0.53 mg/cm^2 TP-CP containing films, the incorporation of 1400 or 2019 U/cm^2 LSZ did not change the initial release rate of LSZ. However, the immobilized LSZ activity of zein films incorporated with 0.53 mg/cm^2 TP-CP and 2019 U/cm^2 LSZ was almost two fold higher (Table 6.10). The increase of incorporated LSZ to 2800 U/cm^2 while keeping the concentration of incorporated antioxidant protein at 0.53 mg/cm^2, on

the other hand, increased the LSZ release rate and immobilized activity of LSZ 2-3 fold. Thus, it seems that the increase in LSZ concentration increased both the soluble and bound LSZ fractions. In this study, the lowest LSZ release rate was obtained for 1400 U/cm^2 LSZ and 1.06 mg/cm^2 TP-CP protein containing films. The low release rate and low enzyme activity recovered (53 %) from these films suggest the retention of most of the LSZ in these films. However, interestingly, the immobilized activity retained in these films was almost equal to that of 1400 U/cm^2 LSZ and 0.53 mg/cm^2 TP-CP protein containing films (Fig. 6.19).

Table 6.10. Kinetic parameters related to LSZ release from zein films at 4 ^oC

Incorporated concentrations in films		**LSZ release rate (U/ cm^2/min)**	**Total LSZ activity released from films (U/cm^2)**	**LSZ activity recovered from films (%)[d]**	**Immobilized LSZ activity retained in films (U/cm^2)**
Antioxidant protein (mg/cm^2)	**LSZ (U /cm^2)**				
-	2019 (670)[a]	23 (0-120)[b]	4577 ±400 (360)[c]	227	9.1
0.53 (TP-CP)	1400 (479)	16 (0-60)	1084 ±286 (360)	77	34
0.53 (TP-CP)	2019 (770)	15 (0-120)	2158 ± 101 (360)	107	62
0.53 (TP-CP)	2800 (958)	40 (0-60)	2344 ±186 (360)	84	141
1.06 (TP-CP)	1400 (479)	8.2 (0-60)	740 ±18 (360)	53	33
0.53 (BSA)	2019 (670)	95 (0-60)	4729 ±302 (60)	234	61

[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 highest activity released; [d] (total activity released / incorporated activity) x 100

The immobilized LSZ activities retained in 2019 U/cm^2 LSZ and 0.53 mg/cm^2 BSA containing films which released significant amount of LSZ activity and 2019 U/cm^2 LSZ and 0.53 mg/cm^2 TP-CP protein containing films that released two fold

lower LSZ activity were also same. These results once more confirmed that the immobilized enzyme activity of films does not reflect their retained enzyme content but it reflects the amount of enzyme immobilized at film surface and capable to show activity.

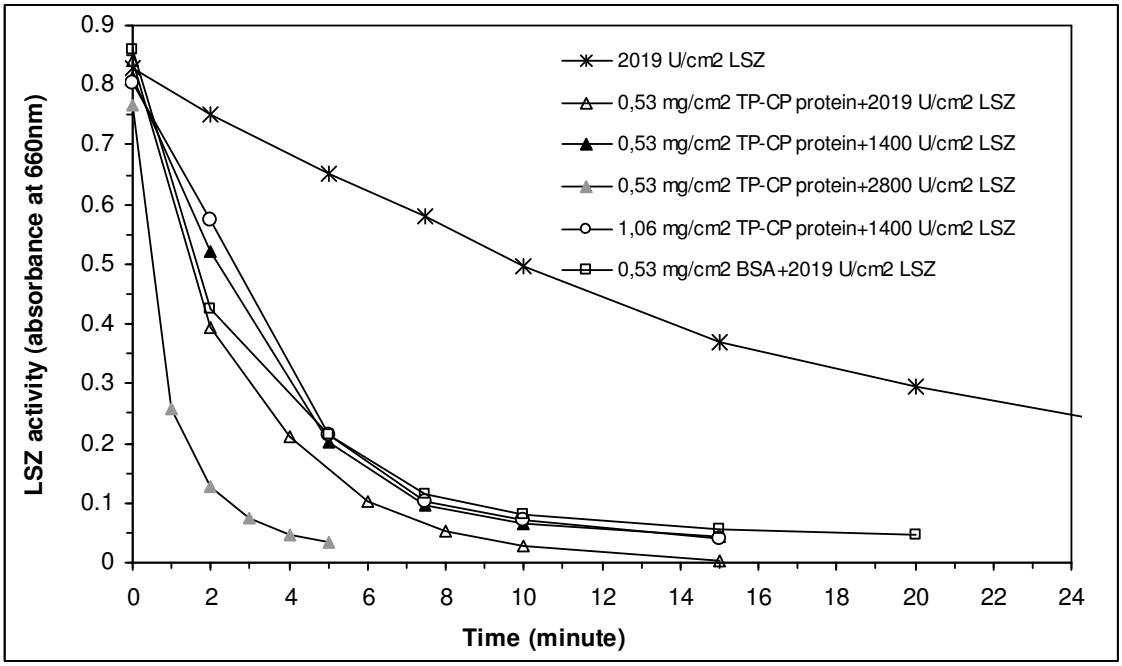


Figure 6.19. Immobilized LSZ activity retained in zein films incorporated with LSZ and/or antioxidant proteins and tested in *Micrococcus lysodeikticus* solutions after 1800 min release test in distilled water at 4 ^{o}C

CHAPTER 7

CONCLUSIONS

Production of Biopreservatives

- Partially purified LSZ obtained by alcohol and isoelectric pH precipitation of nonlysozyme proteins and dialysis showed very high storage stability in lyophilized form. The application of this method is very simple and practical. Also, it may easily be applied in industrial scale for the mass production of enzyme. Thus, the enzyme produced by this method may be a commercial source for the food applications of LSZ.
- Partially purified LPS may be obtained by direct application of bovine whey onto a SP-Toyopearl column and dialysis. Depending on the changes in enzyme molecular properties, the elution profiles of enzyme from the column may show variations. However, the enzyme elutes from the column in almost the same salt concentration range. The LPS showed a moderate stability in lyophilized form prepared with dextran as supporting material. Thus, further studies are needed to improve the long term storage stability of the enzyme.

Production of Alginate Films Incorporated with LPS

- The incorporation of LPS into alginat films causes the immobilization of enzyme. Thus, the potential antimicrobial effects of these films will be controlled by the diffusion of converted antimicrobial products of thiocyanate incorporated into films with LPS enzyme.
- The activity of immobilized enzyme showed a very low response to temperature changes. It has a broad pH spectrum and good pH stability between pH 4 and 6. Also, the activity of the immobilized enzyme may easily be controlled by regulation of H_2O_2 concentration. Thus, the antimicrobial mechanism of these films may be very suitable for various food systems having different pH and storage temperatures.

Production of Alginate Films Incorporated with LPS and Antioxidant Proteins

- The antioxidant activity of alginate films may be increased by incorporation of TP-CP and sericin protein into films.
- The antioxidant proteins did not show affinity to alginate films and released easily from the films in case of contact with water. Thus, it is essential to apply the cross-linking of food treated by these films with spraying of $CaCl_2$, instead of dipping into the solution of this cross-linking agent.
- The incorporation of TP-CP and sericin protein into alginate films enhanced the LPS activity.

Production of Zein Films Incorporated with LSZ

- The LSZ showed very low affinity to zein films and it released rapidly from the films.
- The ethanol used in the production of zein films activates LSZ significantly and this brings the advantage of obtaining a high enzyme activity by use of minimum amount of enzyme.
- The LSZ showed a very high stability in zein films during cold storage. Thus, pre-cast LSZ containing zein films may be supplied for commercial food applications.

Production of Zein Films Incorporated with LSZ and Antioxidant Proteins

- Incorporation of TP-CP proteins causes a better distribution of LSZ in films.
- The zein films have inherent antioxidant activity, since zein used in film production already contains antioxidant protein fractions. However, incorporation of TP-CP improves the antioxidant activity of films significantly.

- Incorporation of TP-CP proteins reduces the release rate and total activity of LSZ recovered from the films. However, the films incorporated with antioxidant proteins showed higher immobilized enzyme activity.

Current Status of the Project

- The antimicrobial effects of zein films incorporated with LSZ or LSZ and antioxidant proteins were fully characterized by using *E. coli, B. subtilis and L. plantarum.*
- The tests for determination of antimicrobial effects of LPS incorporated alginate films are still continuing.
- The test of obtained zein films in real food systems is still continuing. The applications is concentrated more on meat products.

Future Plans

- Test of obtained films on different foods.
- Characterization and improvement of mechanical properties of film.

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APPENDIX A

Vitamin C Standard (1) for ABTS Radical Cation Discoloration Method

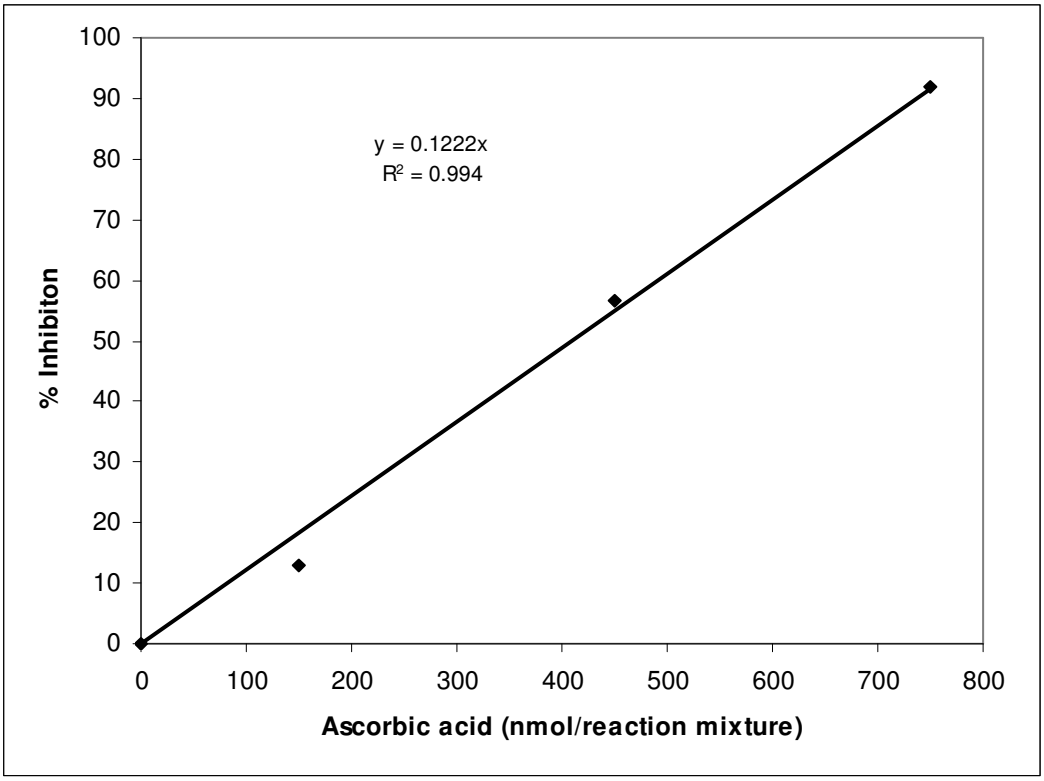

Figure A.1. Standard curve (1) for vitamin C

Reaction Mixture = 1 mL Vitamin C + 24 mL $ABTS^{\cdot+}$ solution (prepared as described in section 5.2.7.1.)

APPENDIX B

Vitamin C Standard (2) for ABTS Radical Cation Discoloration Method

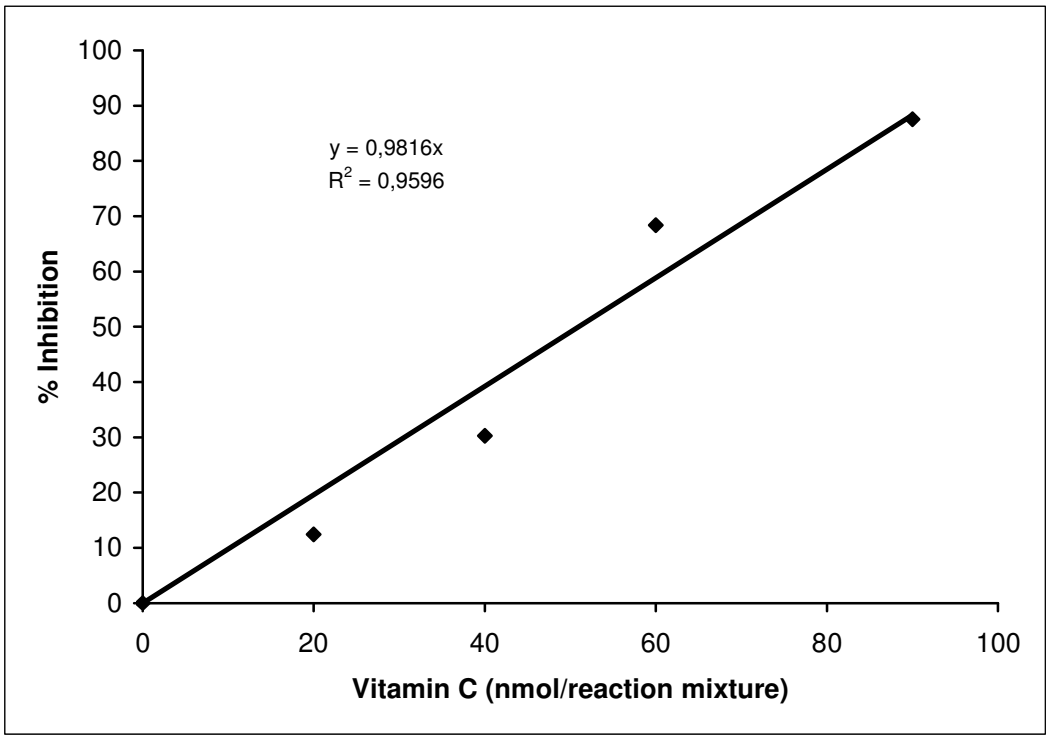

Figure B.1. Standard curve (2) for vitamin C

Reaction mixture = 0.2 mL Vitamin C+1.9 mL $ABTS^{\cdot+}$ solution (prepared as described in section 5.2.7.1.)

APPENDIX C

Protein Standard for Lowry Method

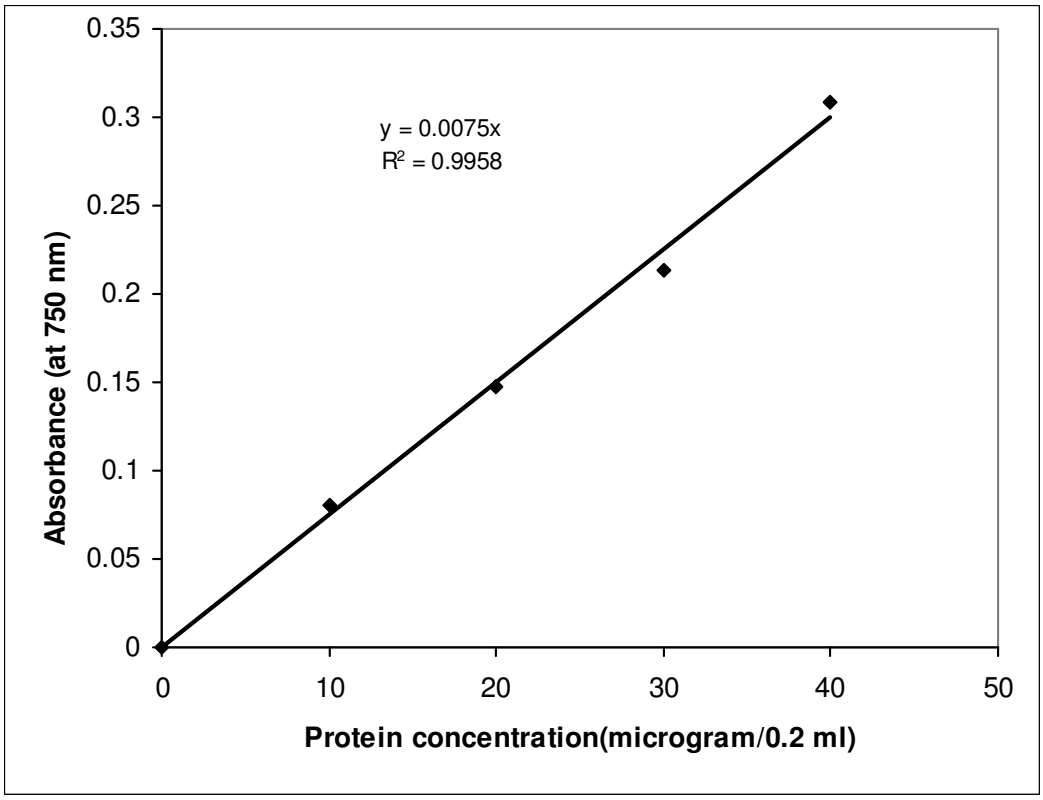

Figure C.1. Protein standard curve for Lowry method