ANTIMICROBIAL PROPERTIES OF SILK FIBROIN-CARRAGEENAN FILMS INCORPORATING GRAPE SEED EXTRACT

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ABSTRACT

ANTIMICROBIAL PROPERTIES OF SILK FIBROIN-CARRAGEENAN FILMS INCORPORATING GRAPE SEED EXTRACT

In this study antimicrobial edible films were developed by incorporation of grape seed extract into silk fibroin-carrageenan films. Developed films were subjected to instrumental analysis such as scanning electron microscopy, atomic force microscopy, X-ray diffractometer, and Fourier transform infrared spectrometer for the characterization of the film. The antimicrobial activity of silk fibroin-carrageenan films on different bacteria including *Listeria innocua*, *Staphylococcus aureus*, *Staphylococcus carnasus*, *Bacillus amyloliquefaciens*, *Escherichia coli*, *Pseudomonas fluorescens*, *Escherichia coli* O157:H7, and *Salmonella* Typhimurium, on raw chicken breast meat and beef sausages were tested. Application of silk fibroin-carrageenan films incorporating grape seed extract and/or Na_2EDTA on chicken breast meat and beef sausages demonstrated the efficacy of these films to enhance the microbial quality of the products. Increasing the concentration of grape seed extract in the films increased the antimicrobial activity of the films in food applications. It is found that silk fibroin-carrageenan films incorporating grape seed extract could be used to control the growth of *S. aureus* on beef sausages during 28 days of storage at 4 ºC. The use of these films on beef sausages had significant effect on moisture content of the sausages as well as the textural properties of the sausages. This study indicated the potential of using silk fibroin-carrageenan films incorporating grape seed extract to be used as an antimicrobial edible food packaging.

ÖZET

ÜZÜM ÇEKİRDEĞİ ÖZÜTÜ İÇEREN İPEK FİBROİN-KARRAGENAN FİLMLERİN ANTİMİKROBİYAL ÖZELLİKLERİ

Gerçekleştirilmiş olan bu çalışmada ipek fibroin-karragenan filmlere üzüm çekirdeği özütü ilave edilerek antimikrobiyal yenebilir filmler geliştirilmiştir. Geliştirilen filmler taramalı elektron mikroskobu, atomik kuvvet mikroskobu, Fourier transform infrared spektroskopisi ve X-ışınları kırınım cihazları aracılığıyla karakterize edilmiştir. Filmlerin antimikrobiyal özellikleri *Listeria innocua*, *Staphylococcus aureus, Staphylococcus carnasus, Bacillus amyloliquefaciens*, *Escherichia coli, Pseudomonas fluorescens*, *Escherichia coli* O157:H7 ve *Salmonella* Typhimurium gibi bakteriler, taze tavuk göğüs eti ve dana sosisleri üzerinde test edilmiştir. Üzüm çekirdeği özütü ve/veya Na_2EDTA içeren ipek fibroin–karragenan filmlerin tavuk eti ve dana sosislerine uygulanmasının bu ürünlerin mikrobiyal kalitelerinin iyileştirilmesinde etkili olduğu belirlenmiştir. Üzüm çekirdeği özütü konsantrasyonun artırılması gıda uygulamalarında kullanılan filmlerin antimikrobiyal aktivitelerini arttırmıştır. Üzüm çekirdeği özütü içeren filmlerin 28 gün boyunca 4 ºC'da depolanan dana sosislerideki *S. aureus*'un gelişimini kontrol etmek için kullanılabileceği belirlenmiştir. Bu filmlerin dana sosislerine uygulanması sosislerin nem içeriklerini ve aynı zamanda tekstürel özelliklerini istatistiksel olarak önemli düzeyde etkilemiştir. Bu çalışma üzüm çekirdeği özütü içeren ipek fibroin-karragenan filmlerin antimikrobiyal yenebilir gıda ambalajı olarak kullanılmasında potansiyele sahip olduğunu ortaya koymuştur.

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CHAPTER 1

INTRODUCTION

Packaging is today indispensible vehicle to maintain the quality of foods during storage, transport, and handling. Packaging protects food between processing and usage by consumer. Food packaging must be removed in an environmentally responsible manner (Marsh and Bugusu 2007). Silk fibroin (SF), *Bombyx mori*, is a natural fibrous polymer. SF has become one of the most extensively studied materials among the natural biopolymers due to no toxicity, no irritation, biodegradability and good biocompatibility. In addition, silk can be considered as a food material because it contains about 6 % essential amino acids. Based on the good chemical and physical properties of silk fibroin, it is possible to prepeare fibroin based materials, such as film, powder, spongens and gels. However, SF films are very brittle and unsuitable for practical use. Properties of SF films can be improved by blending with natural polymer like carrageenan or synthetic polymer (Li, et al. 2000, Bayraktar, et al. 2005, Dai, et al. 2002, Luo, et al. 2003). Proteins and polysaccharides have good film forming properties and can be used alone or in combination to form edible films (Turhan, et al. 2007). The main objectives of this study are; (1) to develop and characterize silk fibroin carrageenan films incorporating grape seed extract, (2) to test antimicrobial activity of the developed films on different pathogenic and spoilage bacteria, (3) to test the effects of developed films on microbial quality of raw chicken breast meat, and (4) to determine the ability of these films to inhibite the growth of *S.aureus* on inoculated sausages coated with silk fibroin-carrageenan films incorporating grape seed extract as well as to enhance the quality of the products in terms of moisture loss and textural properties.

CHAPTER 2

LITERATURE REVIEW

2.1. Packaging

Packaging is the technology, science and art of enclosing or protecting products for storage, distribution, use, and sale. Packaging also refers to the process of production, design, and evaluation of packages. Packaging can be described as a coordinated system of preparing goods for transport, logistics, warehousing, sale, and end use (Wikipedia 2008). Packaging is today indispensible vehicle to maintain the quality of foods during storage, transport and handling. Packaging protects food between processing and usage by the consumer. Food packaging must be removed in an environmentally responsible manner. Packaging technology must thus balance food protection with other issues, including material and energy costs, heightened social and environmental consciousness, and strict regulations on disposal of municipal solid waste and pollutants (Marsh and Bugusu 2007). Food packaging is the largest growing sector, within the plastic packaging market. Today, packaging materials are estimated at more than 180 million tons per year, with demand and growth increasing annually (Cutter 2006).

2.2. Active Packaging

The main aim of food packaging is to protect the food from chemical and microbial contamination, light, water vapor, and oxygen. Active packaging is an innovative food packaging; it has been introduced as a response to the continuous changes in current market trends and consumer demands. It has been defined as “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”. Active food packaging can provide several functions that do not exist in conventional packaging systems. The active functions may include antimicrobial activity, moisture, scavenging of oxygen, and emission of ethanol and flavours (Quintavalla and Vicini 2002). “Active” packaging can control and even react to, events taking place inside the package. Therefore, it provides a barrier to outside influences. Active packaging employs a packaging material that interacts with the internal gas environment to extend the shelf-life of a food. New technologies modify the gas environment (and may interact with the surface of the food) by removing gases from or adding gases to the headspace of a package. Table 2.1 presents the application of active packaging systems to different foods.

Table 2.1. Uses of active packaging
(Source: Food Science 2008)

USES OF ACTIVE PACKAGING	
Active Packaging System	*Application*
Oxygen scavenging	Most food classes
Carbon dioxide production	Most food affected by moulds
Water vapor removal	Dried and mould-sensitive foods
Ethylene removal	Horticultural produce
Ethanol release	Baked foods (where permitted)

2.3. Antimicrobial Food Packaging

Safety of processed foods and environmental pollution have become a major concern in recent years. Researchers have focused on edible films and several antimicrobial compounds incorporated into edible food packaging. Antimicrobial compounds are directly mixing with food, as opposed to this, their incorporation into film could localize functional effect at the food surface. There are a number of traditional techniques for preserving foods from the effect of microbial growth include thermal processing, drying, refrigeration, freezing, high-pressure processing, low-temperature processing, modified atmosphere packaging, irradiation, and adding antimicrobial agents or salts. However, some of these methods cannot be applied to some food products, such as ready-to-eat products and fresh meats (Quintavalla and Vicini 2002).

Antimicrobial packaging is a form of active packaging. In order to obtain a desired outcome, active packaging interacts with the product or the headspace between the package

and the food system. Similarly, antimicrobial food packaging acts to retard, reduce or inhibit the growth of microorganisms that may be present in the packed food or packaging material itself (Appendini and Hotchkiss 2002). The aim of food packaging is to preserve the safety and quality of the food. Likewise, important function of packaging is to protect the product from chemical, physical or biological damage. The most well-known packaging materials, which have been in use by the food industry for over 50 years, are polyethylene- or co-polymer based materials. They are safe, inexpensive, versatile, and flexible (Cutter 2006).

2.3.1. Types of Antimicrobial Food Packaging

The antimicrobial packaging can be applied by different methods. Five main types of antimicrobial packaging were introduced (Appendini and Hotchkiss 2002). These are;

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

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

Sachets are the most successful commercial application of antimicrobial packaging that are enclosed loose or attached to the interior of a package. Oxygen absorbers, moisture absorbers and ethanol vapor generators are the three predominated forms. Moisture and oxygen absorbers are used primarily in pasta, bakery produce and meat packaging to prevent oxidation and water condensation. Although oxygen absorbers are not an

antimicrobial agent, a reduction in oxygen inhibits the growth of aerobes, especially molds. Moisture absorbers can reduce water activity (a_w), which also indirectly affects microbial growth (Appendini and Hotchkiss 2002).

2.3.1.2. Incorporation of Volatile and Non-Volatile Antimicrobial Agents Directly into Polymers

Antimicrobial agents may be incorporated into packaging materials by two methods. These methods are addition of antimicrobials into polymers either in the melt or by solvent compounding the polymer. Thermal and melting polymer processing methods, extrusion and injection molding, may denature heat sensitive compounds. When thermostable antimicrobials (mostly chemical preservatives) are used in film making, melt forms of polymers are preferred (Appendini and Hotchkiss 2002, Han 2000, Suppakul, et al. 2003).

The antimicrobial agents used in packaging may be volatile or non-volatile substances. If they are non-volatile, antimicrobial packaging materials must contact the surface of the food so that the antimicrobial agents can diffuse to the surface. Thus, surface characteristics and diffusion kinetics become crucial. If the incorporated antimicrobial agents are volatile (e.g.chlorine dioxide, sulfur dioxide, carbon dioxide and allyl isothiocyanate), packaging materials do not need to contact the surface of the food. Using volatile antimicrobials has an advantage compared to non-volatile antimicrobial, that is, they can penetrate the bulk matrix of the food and that the polymer needs not necessarily directly contact the product (Suppakul, et al. 2003, Appendini and Hotchkiss 2002).

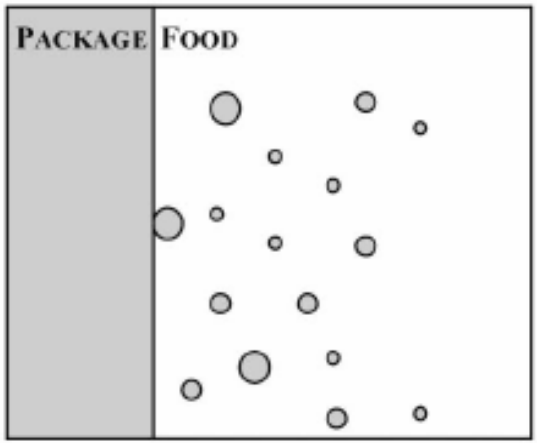


Figure 2.1. Diffusion of antimicrobial from package to food
(Source: Han 2000)

2.3.1.3. Antimicrobials Coated onto Polymer Surfaces

Antimicrobials which are sensitive to high temperatures cannot be used in polymer processing. Therefore, they are often coated onto the material after forming or are added to cast films. For example, cast edible films, have been used as carriers for antimicrobials and applied as coatings onto packaging materials and/or foods (Figure 2.2). Proteins have an increased capacity for adsorption due to their amphiphilic structure (Appendini and Hotchkiss 2002).

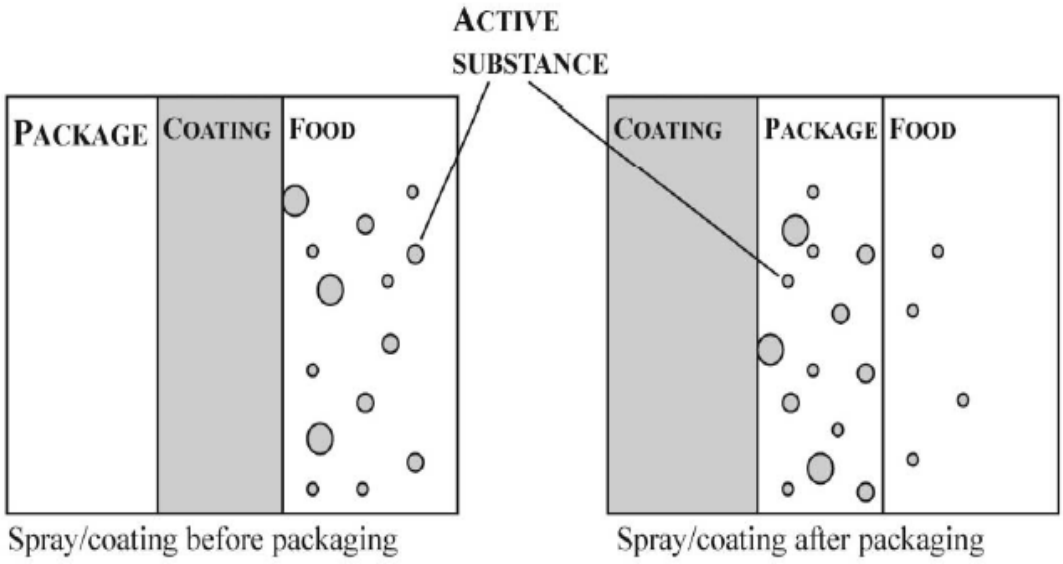


Figure 2.2. Different types of antimicrobial coatings applied to polymeric films (Source: Quintavalla and Vicini 2002)

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

In order to suppress the microbial growth, covalently immobilized antimicrobial substances have been used in antimicrobial packaging systems (Suppakul, et al. 2003). This type of immobilization occurs when both antimicrobial agent and the polymer have functional groups. Peptides, enzymes, polyamines and organic acids are potential examples for antimicrobials with functional groups. There are also some examples of polymers used for food packaging that have functional groups. These are stated in Table 2.2

Table 2.2. Antimicrobials covalently/ionically immobilized in polymer supports (Source: Appendini and Hotchkins 2002)

Functional support	Antimicrobials
Ionomeric films	Benomyl
	Benzoyl chloride
	Bacteriocin
Polystyrene	Lysozyme
	Synthetic antimicrobial peptides
Polyvinyl alcohol	Lysozyme
Nylon 6,6 resins	Lysozyme

2.3.1.5. Use of Polymers that are Inherently Antimicrobial

Cationic polymers such as chitosan and poly-L-lysine are inherently antimicrobial and have been used in films and coatings. These polymers interact with negative charges on the cell membrane and the interaction reduces the membrane integrity of bacteria and causes the leakage of their intracellular constituents (Appendini and Hotchkiss 2002).

2.4. Antimicrobial Packaging Systems

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

2.4.1. Package/Food Systems

In package/food systems, the packaging material contacts with the solid, low viscosity or liquid food without any headspace. Antimicrobials incorporated into the packaging material migrate to food through diffusion and partitioning at the interface (Figure 2.3). Individually wrapped cheese and ready-to-eat meat products, aseptic brick packages and "sous-vide" cooked products can be given as an example for this kind of packaging (Quintavalla and Vicini 2002).

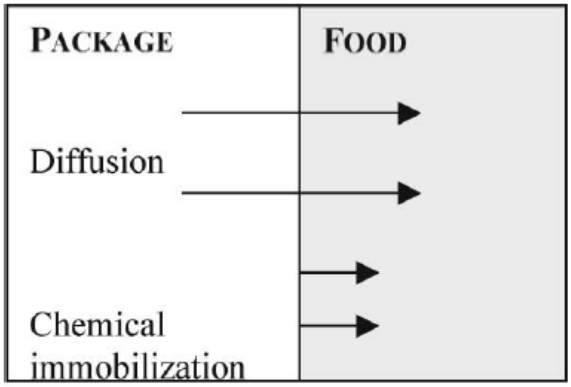


Figure 2.3. Package/food systems
(Source: Han 2000)

2.4.2. 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 (Figure 2.4). The migration of antimicrobial in these systems also occurs from food-package contact surfaces by diffusion. Examples of package/headspace/food systems are flexible packages, bottles, cans, cups, and cartons (Quintavalla and Vicini 2002).

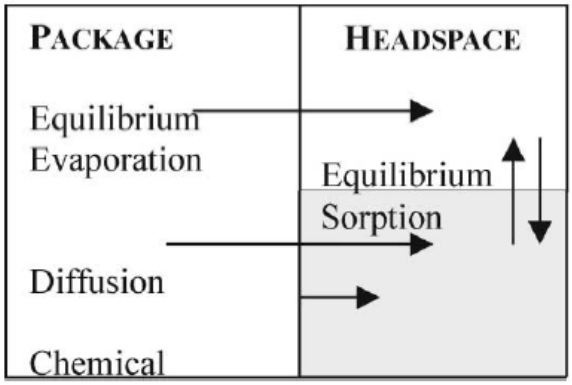


Figure 2.4. Package/headspace/food systems
(Source: Han 2000)

2.5. Bio-Based Polymers and Biopolymers

Biopolymers or bio-based polymers are developed from renewable resources. Polysaccharides (starch, alginates, pectin, carrageenans, chitosan/chitin), proteins (casein, whey, collagen, gelatin, corn, soy, wheat, etc.) and lipids (fats, waxes, or oils, etc) are the examples of renewable resources used in the manufacture. Polymers, such as polylactate (PLA) or polyesters, synthesized from biologically-derived monomers. Cellulose, curlan, xanthan, or pullulan are the example of polymers which can be produced by microorganisms. Biopolymers categorized based on the ability to be compostable or biodegradable. It is essential to note that bio-based packaging materials could be biodegradable since, not all biodegradable materials are bio-based. Innovations in biopolymer production, environmentally-friendly packaging is consumer demand (Cutter 2006).

2.5.1. Edible Coating and Biodegrable

"Biodegrable" means that the material is capable of being broken down by the action of living things such as microorganisms. "Edible" means that the material is safe to eat. "Coating" is a layer of one substance covering another, in this case, covering a food product. The purpose of coating of a food product are to improve quality of the food and extend the shelf life of products by acting as a barrier (gas and/or moisture) or providing gloss (shine) (Hang-wan, et al. 2007). Edible packagings have functional properties, which are selective and active properties. Selective properties of edible films and coatings are illustrated in Figure 2.5.

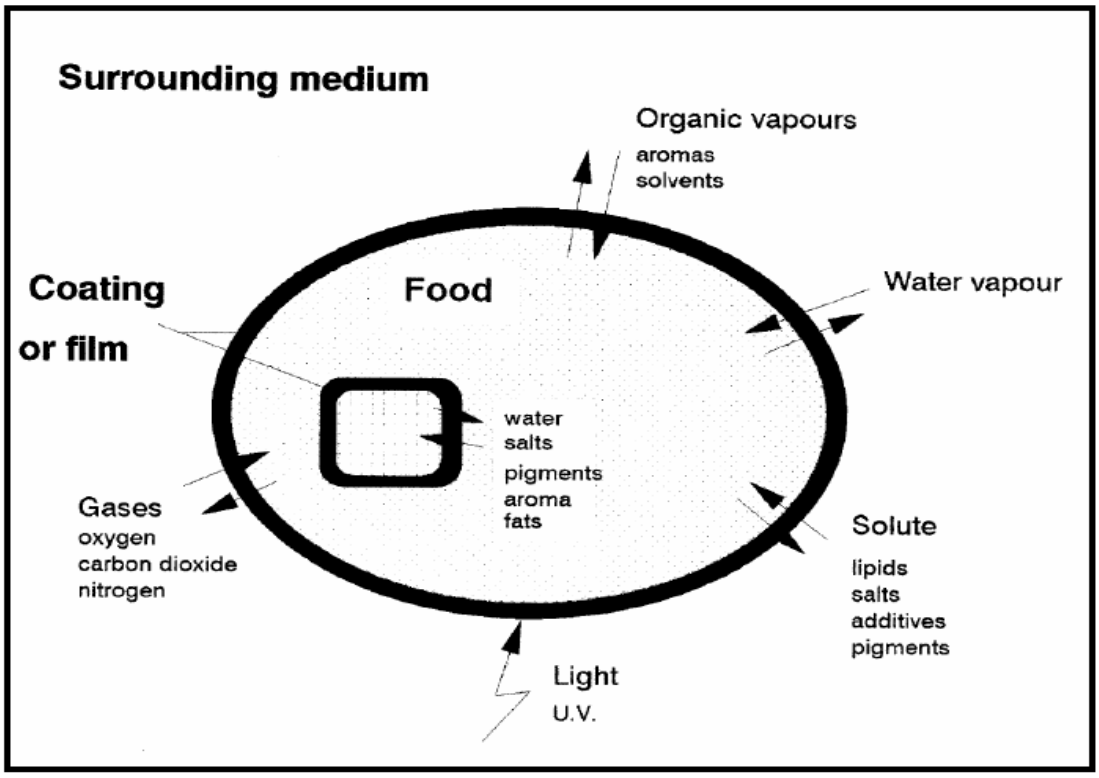


Figure 2.5. Selective functions of edible films and coatings
(Source: Debeaufort, et al. 1998)

Table 2.3. Some of the Application of Edible Films on Various Food Studies in the Literature (Source: Joerger 2007)

Authors	Film type	Additive(s)	Test medium
Theivendran et al. 2006	Soy protein isolate	Grape seed extract/ Green tea extract/ Nisin	Turkey frankfurters
Cutter et al. 2001	Polyethylene	Nisin/EDTA	Beef tissue
Janes et al. 2002	Zein/ Propylene Glycol	Nisin/Ca propionate	Chicken
Lungu and Johnson 2005	Zein/ Ethanol	Potassium sorbate/ Nisin	Turkey frankfurters
Natrajan and Sheldon 2000	Polyvinyl chloride/ Nylon/Linear low-density polyethylene	Nisin/EDTA/ Citric acid /Tween 80	Chicken drumsticks
Ercolini et al. 2006	Polythene	Bacteriocin from *L. curvatus*	Frankfurters
Ghalfi et al. 2006	Polyethylene	Bacteriocin from *L. curvatus*	Cold-smoked salmon
Mauriello et al. 2004	Polythene	Bacteriocin from *L.curvatus*	Pork steak
Ming et al. 1997	Cellulose	Pediocin powder	Ham
Cagri et al. 2002	Whey Protein Isolate	Sorbic acid	Bologna
Garcia et al. 2001	High Amylase Product	Sorbitol	Strawberries
Garcia et al. 1998	Starch	Sorbitol	Strawberries
Lungu and Johnson 2005	Zein/Propylene Glycol	Potassium Sorbate	Turkey frankfurters

(Cont. on next page)

Table 2.3. (Cont.) Some of the Application of Edible Films on Various Food Studies in the Literature (Source: Joerger 2007)

Authors	Film type	Additive(s)	Test medium
Jagannath et al. 2006	Casein	Tumeric	Carrots
Lee et al. 1998	Low- Density Polyethylene	Grapefruit seed extract	Curled lettuce
Oussalah et al. 2004	Milk Proteins	Oregano essential oils, Pimento essebtial oils	Beef
Ouattara et al. 2002	Caseinate	Thyme, Rosemary, Sage	Ground beef
Gill and Holle 2000	Gelatin	EDTA/Lysozyme /Nisin	Ham and bologna
Min et al. 2006	Whey Protein	Lactoperoxidase systerm	Smoked salmon
Zivanovic et al. 2005	Chitosan	Oregano essential oil	Bologna
Caillet et al. 2006	Calcium caseinate, Whey protein isolate, Carboxymethyl Cellulose, Glycerol, Pectine,	trans-Cinnamaldehyde	Peeled carrots
Ha et al. 2001	Polyethylene	Grape fruit seed extract	Ground beef
Ouattara et al. 2000	Chitosan	Acetic acid	Cooked ham

2.5.2. Edible Gels, Films and Coatings

2.5.2.1. Definition and Historical Background of Edible Film

Edible films or coatings are defined as continuous matrices, they made from natural biopolymers, such as proteins, lipids, and polysaccharides. The use of edible films in food products may seems new, food products were first covered by edible films and coatings long years ago. During the 15th century, the first free-standing edible films was developed and used for food preservation in Japan from soymilk by Yuba. Edible coatings date back even further for food products; in order to retard water loss, waxes were applied to oranges and lemons, during the 12th century in China and in order to control moisture loss, food products were coated with fat during the 16th century. Fresh vegetables and fruits have been coated with oil-in-water emulsions and carnauba wax since 1950. Edible films are considered as a packaging that should fulfill a number of requirements, such as high barrier, good sensory quality and mechanical efficiencies, biochemical, physicochemical, and microbial stability, simple, non-toxic, non-polluting, and low cost. Edible films and coatings have been used in various applications, including casings for sausages and chocolate coatings for nuts and fruits. Currently, edible films and coatings are used in various food applications, mostly fruits, vegetables, candies, and some nuts (Cagrı, et al. 2004, Cutter 2006, Debeaufort, et al. 1998).

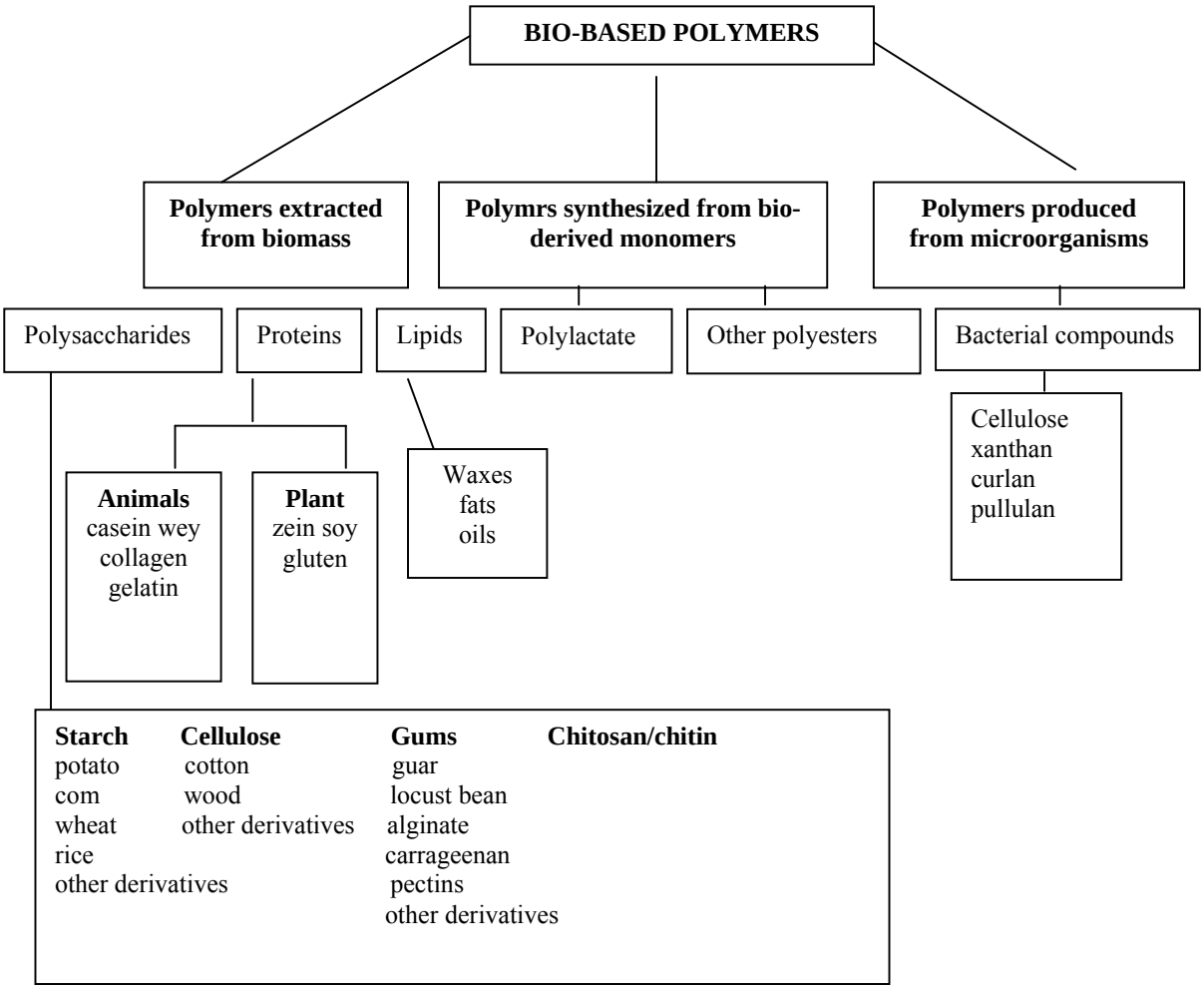


Figure 2.6. Different categories of bio-based materials
(Source: Cutter 2006)

2.5.2.2. Film Application Techniques

A number of methods for application of edible films to foods have been employed, including but not limited to casting, foaming, dipping, spraying, brushing, wrapping or rolling. Dipping, casting and spraying techniques are more common techniques. Spraying technique provides a thinner and a more uniform film required for certain surfaces. Early coating procedures involved sprays, with further distribution over food surfaces via roller or brushes, followed by tumbling to spread the coating. Casting is useful for forming free-

standing film and by this technique, film thickness can be controlled. Dipping provides a uniform coating on an irregular surface but by dipping it is hard to control film thickness. Dipping is the commonly used method for fruits, vegetables and meat products. Food product is directly dipped into the composite coating formulation (in aqueous medium). After dipping, the excess coating usually drips off and the remaining material is allowed to set or solidify on food with air dry, whereby a thin film is formed over the food surface. (Cutter and Sumner 2002, Donhowe and Fennema 1994, Tharanathan 2003).

2.5.3. Lipid-Based Coatings

Lipid based coatings have number of advantages for coating of foods. Lipids are impart hydrophobicity, cohesiveness, and flexibility and due to the tightly packed crystalline structure of lipids, they make excellent moisture barriers. Beeswax, carnauba, and candelilla waxes also have been used to coat frozen meat pieces and extend storage without substantial dehydration. Despite these advantages, lipid-based films may exhibit lower permeability to gases at higher storage temperatures. Lipid-based films also are lack of structural integrity and poor adherence to hydrophilic surfaces, subjected to oxidation, cracking, flaking and retention of off-flavors are disadvantages of these films (Cutter 2006).

2.5.4. Polysaccharide Films

Starch, alginate, cellulose ethers, chitosan, carageenan, or pectins films are examples of polysaccharide films. Polysaccharides impart hardness, compactness, crispness, viscosity, adhesiveness, thickening quality, and gel-forming ability to a variety of films. In general, their hydrophilic nature makes them poor barriers for water vapor and due to their polymer chains, polysaccharide films can exhibit low gas permeability (Cutter 2006, Gennadios, et al. 1997).

2.5.4.1. Starch

Starch is composed of amylose and amylopectin. Amylose is known to form coherent, free-standing, relatively strong films. High amylose starch films are water soluble, flexible, oil resistant, oxygen impermeable and heat-sealable, in contrast to amylopectin films which are brittle and noncontinuous. Starch-based films have physical characteristics similar to plastic films. They can be odorless, colorless, non-toxic, resistant to passage of oxygen and semi-permeable to carbon dioxide (Cutter 2006, Gennadios, et al. 1997).

2.5.4.2. Alginate

Alginate, extracted from brown seaweed, is a salt of alginic acid which is a linear polymer of β-D-mannuronic acid and α-L-guluronic acid (Olivas, et al. 2008). Na-alginate is a naturally occurring non-toxic polysaccharide, a water soluble salt of alginic acid (Cha 2002). Gelling agents, divalent cations (calcium, magnesium, manganese, aluminum, or iron) are used in alginate film formation and calcium appears to be more effective in gelling alginates than magnesium, manganese, aluminium, ferrous, and ferric ions. Films produced by evaporation of water from a thin layer of alginate solution are impervious to oils and greases, but they, as with other hydrophilic polysaccharides, have high water vapor permeabilities. For the application of aqueous sodium alginate solutions on products by dipping method, the food is firstly dipped into a sodium alginate solution and then crosslinked with a solution containing calcium salt solution to induce gelation (fixing) and makes the alginate polymeric network insolublize. Several calcium salts can be used for alginate gel coating formation such as calcium chloride, calcium gluconate, nitrate, or propionate. Alginates are possess good film-forming properties that make them particularly useful in food applications (Cutter 2006, Gennadios, et al. 1997, Krochta, et al. 2002).

2.5.4.3. Cellulose Ethers

Cellulose, the structural polysaccharide of plants, is composed of D-glucose units linked through ß-1,4 glycosidic linkages. Native cellulose is a crystalline cold water-insoluble high molecular weight polymer. The reactivities of the three hydroxyl groups at positions 2, 3, and 6 on the glucosyl units of cellulose are utilized for making useful derivatives. Cellulose ethers are polymer substances obtained by partial substitution of hydroxyl groups in cellulose by ether functions. Several cellulose derivatives are widely produced commercially, most commonly methylcellulose (MC), hydroxypropyl cellulose (HPC), hydroxypropyl methylcellulose (HPMC), and carboxymethylcellulose (CMC) are water soluble ethers possessing good film-forming properties by solubilizing in aqueous or aqueous-ethanol solution. Cellulose based edible films are generally transparent, flexible, odorless, tasteless, water soluble, and resistant to oil and fats (Lacroix and Tien 2005). Their relative hydrophilicities increase in the order of HPC <MC<HPMC<CMC .Cellulose is a non-digestible component of plant cell walls. In the manufacture of edible films, cellulose-based films tend to be water soluble, resistant to fats and oils, tough, and flexible. Coatings made with ethylcellulose and lipids were transparent and readily peelable, prevented desiccation, and extended shelf life of beef steaks (Cutter 2006, Gennadios, et al. 1997).

2.5.4.4. Chitin/Chitosan

Chitosan is an edible and biodegradable polymer derived from chitin, the major organic skeletal substance in the exoskeleton of arthropods, including insects, crustaceans, and some fungi. When compared to chitin, chitosan is more soluble and has better antimicrobial activity due to the positive charge on the C-2 of the glucosamine monomer at pH 6 and below (Lacroix and Tien 2005). Due to its high molecular weight and solubility in acidic aqueous solutions, chitosan can form film (Han, et al. 2005). Chitosan forms films having good oxygen and carbon dioxide permeability, as well as excellent mechanical

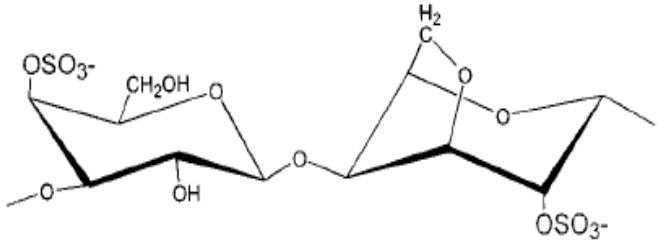
properties without the addition of additives. One of the disadvantages of chitosan is its high sensitivity to moisture. Chitosan not only exhibits antimicrobial activity against bacteria yeasts, and molds, but also acts as a chelator in biological systems (Vartiainen, et al. 2004). Long positively charged chitisan molecules interact with negatively charged bacteria membranes causing disruption and death of cell (Cutter 2006). Antimicrobial and functional properties of chitosan depend on several factors including characteristics of chitosan molecule, its molecular weight, degree of deacetylation, concentration in solution, and pH.

2.5.4.5. Carrageenan

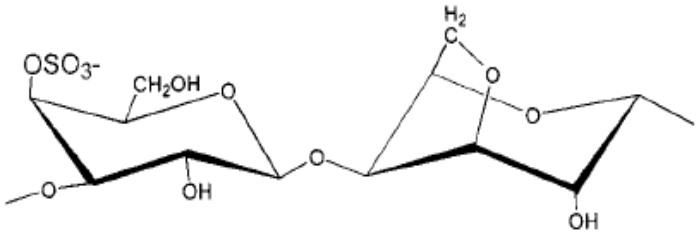
Carrageenans are water-soluble-sulphated polysaccharides extracted from the cell walls of various red seaweeds. These hydrocolloids are linear polymers of about 2500 galactose residues. They are used as a high value functional ingredient for gelation thickening and stabilisation in foods, in the dairy industry, in cosmetics and pharmaceuticals. Carrageenans are classified as κ, ι and λ carrageenans, according to the number and position of sulfate groups. Due to their property to produce thermoreversible gels on cooling below the critical temperature, only κ and ι are used as thickener or gelling agents and also as film forming materials dependent upon their ion environment., Iota carrageenan molecules, in aqueous solutions, are composed of altering α (1,3)-D-galactose-4-sulfated and β (1,4)-3,6- anhydro-D-galactose-2-sulfate, undergo a coil to helix transition on cooling that leads to the formation of a elastic and clear gel as a result of right-handed double helix association. Mechanism of gelation with a conformation change from a disordered state to an ordered state is strongly based on the promotion by the presence of cations such as potassium, calcium, sodium, and polymer concentration. The latter induce formation of associations between double helices through electrostatic interactions to form an infinite network. Carrageenan film formation includes gelation mechanism during moderate drying, result in a three-dimensional network formed by polysaccharide double-helices and to a solid film after solvent evaporation. Carrageenan coatings exhibit poor

water barrier properties as most of the polysaccharides (Karbowiak, et al. 2007, Morris, et al. 1980).

Carrageenan-based coatings have been used to prolong the shelf life of a variety of muscle foods including poultry and fish. Antioxidants, such as ascorbic or gallic acids or lecithin, antibiotics or salt, can be added to the coatings to improve the microbiological stability and quality of muscle foods (Cutter 2006). Cha et al. (2002) prepared Na-alginate and κ-carrageenan based antimicrobial films. Lysozyme, nisin, and grape fruit seed extract (GFSE) were used as antimicrobial agents and they were incorporated into the films, both alone and in combination. Na-alginate-based films exhibited larger inhibitory zones compared to κ-carrageenan-based films even within similar combinations and levels of antimicrobial agents.



Iota Carrageenan



Kapa Carrageenan

Figure 2.7. Different types of carrageenans
(Source: Hossain, et al. 2001)

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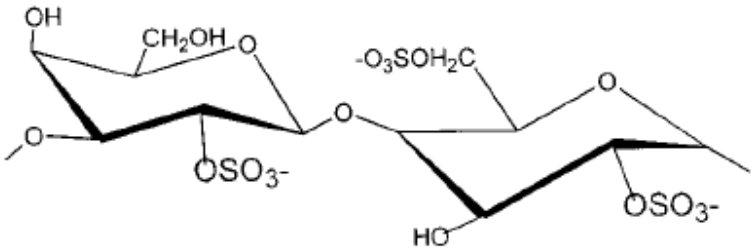

Lambda Carrageenan

Figure 2.7. (Cont.) Different types of carrageenans
(Source: Hossain, et al. 2001)

Carrageenan coatings were also applied on poultry meat. Fresh chicken meat were dipped into a 40 g/L aqueous solution of carrageenan at 64 °C. During storage at 2 °C, shelf-life of coated chicken meat slightly increased. Spoilage was further retarded by incorporation of water soluble antibiotics (chlortetracycline, oxytetracycline) into carrageenan coatings (Gennadios, et al. 1997).

2.5.5. Protein Based Films and Coatings

Silk fibron, casein, whey protein, gelatin/collagen, fibrinogen, wheat gluten, soy protein, egg albumen, and corn zein have been processed into edible films. Protein-based films adhere well to hydrophilic surfaces, provide barriers for carbon dioxide and oxygen, but do not resist water diffusion.

2.5.5.1. Gelatin

Gelatin (also called gelatine) is prepared by the thermal denaturation of collagen, isolated from animal skin and bones, with very dilute acid. It can also be extracted from fish skins. Gelatin is a heterogeneous mixture of single or multi-stranded polypeptides, each with extended left-handed proline helix conformations and containing between 50-1000 amino acids. Gelatin is primarily used as a gelling agent forming transparent elastic thermoreversible gels on cooling below about 35°C (Isbu 2009). Gelatin produces clear, strong, flexible and oxygen-impermeable films when cast from aqueous solution in the presence of plasticizers. They have good gas and oil barrier properties but poor water barrier property due to their hydrophilic nature (Lacroix and Cooksey 2005).

Edible films also may serve as gas and solute barriers, thereby improving the quality and shelf life of muscle foods. One example of such a film is gelatin which is reported to have better oxygen barrier properties when combined with other types of films (Gennadios, et al. 1997). In one study, Villegas et al. (1999) dipped the cooked ham and bacon to gelatin dips (2 %, 4 %, and 6 %), packaged them in oxygen permeable or vacuum packaging films, and stored them under frozen conditions for 7 months.

2.5.5.2. Soy Protein

Soy protein can be used to produce edible antimicrobial film to apply antimicrobial agents on the surface of food products. Soy protein films are usually formed by the polymerization of 11S and 7S protein by disulfide linkages. Soy protein mainly consists of globulins 2S, 7S, 11S, and 15S (Eswarandam, et al. 2004). Soy protein used in film formation is classified as soy protein concentrates and soy protein isolates. Commercially soy protein concentrate contains about 80 % protein and is obtained by removing alcohol-soluble nonprotein compounds from defatted meal with 60-80 % aqueous alcohol. Soy protein isolate contains more than 90 % protein and is obtained by alkali extraction followed by acid precipitation (pH 4.5) (Cho, et al. 2007). Soy protein is a renewable

resource for producing environmentally safe industrial products. Plasticizer is necessary to produce soy protein films that have the required mechanical strength for handling. The potential for use of biodegradable soy protein films as packaging materials depends on their mechanical and barrier properties although these films have poor mechanical and moisture barrier properties (Eswarandam, et al. 2004, Park, et al. 2000). Theivendran et al. (2006) demonstrated that combination of nisin with grape seed extract or green tea extract in soy protein based edible films suppressed the growth of *L. monocytogenes* on full-fat turkey stored at 4 °C and 10 °C approximately by 2.8 and 2.3 log CFU/mL, and improve the quality of ready-to-eat meat.

2.5.5.3. Whey Protein Isolate (WPI)

Whey is an abundant, inexpensive and readily available by product of cheese industry (Banerjee and Chen 1995). Whey protein isolate (WPI) is a highly purified protein product (90 % to 95 % protein, dry basis) that can be made into edible films and coatings, both in the denatured and the native state of whey-proteins (McHugh, et al. 1994, Perez-Gago, et al. 1999). Native whey proteins are globular proteins, containing most of the SH and hydrophobic groups hidden in the interior of the molecule. Formation of whey protein films has mainly involved heat denaturation of whey proteins in aqueous solutions. Heating modifies the 3-dimensional structure of the protein, exposing internal SH and hydrophobic groups, which promote intermolecular S-S and hydrophobic bonding upon drying. McHugh et al. (1994) studied the optimization of whey protein film-forming conditions and found that heat treatment was necessary (for example, 90 °C for 30 min) for the formation of intact whey-protein-based edible films. Whey protein films were characterized by their water insolubility, which can be beneficial in maintaining film and food integrity. Whey protein has excellent functional and nutritional properties and the ability to form films. Whey protein has been shown to produce transparent, flexible, and water-based edible films as well as provide excellent oxygen, aroma and oil barrier properties. On the other hand, whey protein films provide a poor moisture barrier **(**Perez-Gago and Krochta 2001).

Cagri et al. (2002) reported that whey protein films containing sorbic acid and p-amino benzoic acid clearly inhibited the growth of *Listeria monocytogenes*, *E. coli* O157:H7, and *S.* Typhimurium DT104 on both bologna and summer sausage slices. Moreover, percent elongation of the film increased as a result of contact with bologna and summer sausage while tensile strength sharply decreased.

2.5.5.4. Zein

Zein is a water-insoluble prolamine protein extracted from corn gluten and has generally recognized as safe (GRAS) status for use in food products. Zein coating proteins only dissolve in organic solvents and form hard and glossy coatings. Corn zein films are characterized by their ability to form though, hard, glossy, grease proof coatings (Lungu and Johnson 2005). Zein without any plasticizer is resulted in a very brittle film. To increase film flexibility, plasticizers such as glycerol and sorbitol are needed to be incorporated into the film (Paramawati, et al. 2001). Zein is presently used to coat candy, dried fruits, and nut meats because these films are good barriers to oxygen and lipid (Janes, et al. 2002).

Lungu and Johnson (2005) developed zein coating containing nisin and potassium sorbate and investigated its antimicrobial effect against *L. monocytogenes* on turkey frankfurters at 4 °C. Inoculated frankfurters treated with the different solvents (ethanol, glycerol, and propylene glycol) used to dissolve zein had counts that were significantly lower than the control samples at day 28.

2.5.5.5. Silk Protein

Silk, generally defined as protein polymers, is spun into fibers by some lepidoptera larvae such as silkworms, spiders, scorpions, flies and mites. Silk proteins are usually produced within specialized glands after biosynthesis in epithelial cells, followed by secretion into the lumen of these glands where the proteins are stored prior to spinning into fibers. Silks differ widely in structure, composition and properties depending on the specific source (Altman, et al. 2003). The most extensively characterized silks are form of the domesticated silkworm, *Bomyx mori*, which is the most abundant, obtained easily and cheaply.

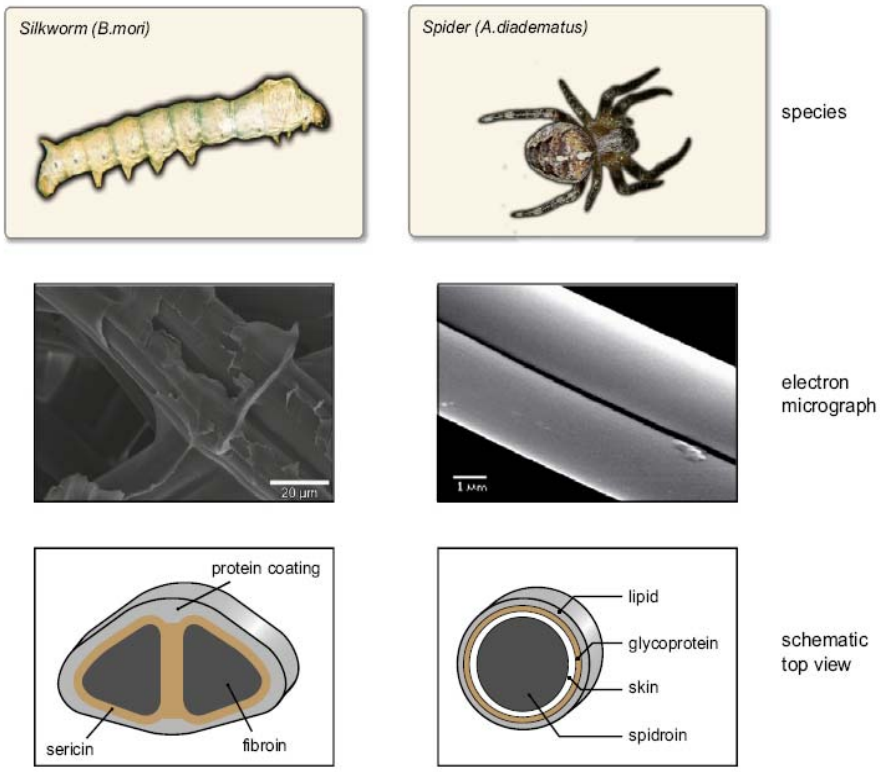

Figure 2.8. Examples of silk fibers produced by silkworms and spiders and a schematic Illustration (Source: Hardy, et al. 2008)

Silk synthesized by *B. mori* consists of two kinds of protein, fibroin and sericin. Sericin is the water-soluble glue-like protein, surrounds and binds the fibroin fibers. Fibroin is the structural fibrous protein and composed of 70 % of the intact silk and 5 % others, like carbohydrates and lipid (Magoshi, et al. 1996). The inner protein is fibroin and the outer protein surrounding fibroin is sericin. Fibroin is water insoluble and real silk fiber for textiles. Sericine is water soluble and removed in thread-making process from the cocoon. Glycine, alanine, serine and tyrosin are the major amino acids of fibroin (Fineco 2009).

Silk fibroin is a kind of native fibrous polymer. Silk fibroin has many unique chemical and physical properties and good biological compatibility that is especially attractive. In addition to being used as food additives, silk fibroin is used in nontextile fields, surgical sutures, and cosmetics industries. Silk fibroin has been studied as enzyme-immobilization materials, antithromboplastic materials, wound covering materials, dialysis membranes and soft contact lenses in recent years. Drug-delivery carriers, cell culture substrates, and artificial skins are the practical application of silk fibroin gel and porous materials in biomedical fields. The results of various clinical and animal experiments with fibroin membrane used as wound protective materials indicated that silk fibroin has no toxicity or irritation, and is of good biocompatibility (Li 2002). Based on the good physical and chemical properties of silk fibroin, it is possible to prepare porous silk fibroin materials through controlling the preparation conditions, with required fine structure, morphological structure, physical, and chemical properties. Aqueous silk solutions represent a good starting material for the preparation of different kinds of fibroin-based materials, such as film, powder, gel, and membranes. Recently, many researchers have investigated SF as one of the promising resources of biomedical and biotechnological materials due to its unique properties including good biodegradability, biocompatibility, and minimal inflammatory reaction (Bayraktar, et al. 2005).

Fibroin consists of many micro fibrils, and it has 65 % amorphous and 35 % crystal in structure (Fineco 2009). There is a highly repeated hydrophobic and crystallizable sequence in the fibroin primary structure: Gly-Ala-Gly-Ala-Gly–X, where X represents Tyr or Ser, alternated with more amorphous and hydrophilic chain segments, in order that the protein develops a micellar structure in aqueous environments (Servoli, et al. 2005).

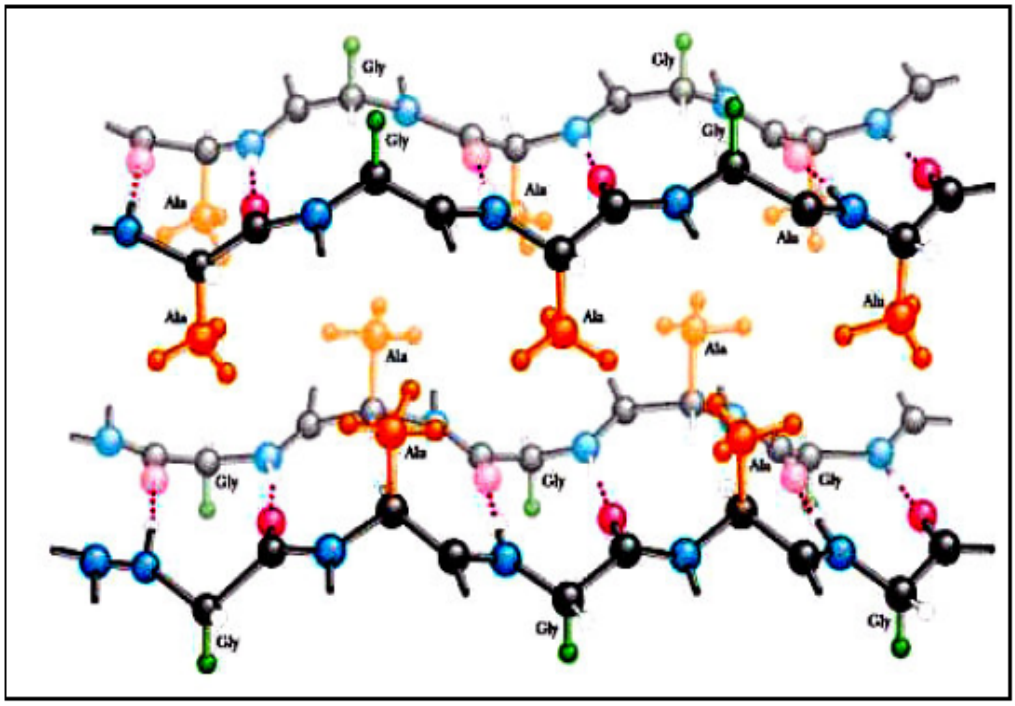

Figure 2.9. Structure of silk fibroin
(Source: University of Florida 2008)

The domesticated silkworm (*B. mori*) silk fibroin fibers are about 10–25 mm in diameter and composed of two proteins, present in a 1:1 ratio and linked by a single disulfide bond with heavy chain (~390 kDa) and a light chain (~26 kDa). Silk fibroin is purified from hydrophilic protein sericins (20–310 kDa) by boiling silk cocoons in an alkaline solution. Sericin is removed during the de-gumming process and consist of 25-30 % of the silk cocoon mass (Vepari and Kaplan 2007). The heavy chain is consist of 12 repetitive domains whose typical compositions are clusters of oligopeptides Gly-Ala-Gly-Ala-Gly-Ser, [Gly-Ala]*n*-Gly-Tyr, and [Gly-Val]*n*-Gly- Ala (*n*) 1-8) and are separated by 11 amorphous regions in which peptides are present mainly as Gly-Ala-Gly-Ser and Gly-Ala-Gly-Ala-Gly-Ser. The heavy chain contains a considerable number of hydrophobic amino acid residues. However, it gives affinity to water presence of hydroxyl residues of Ser and Tyr along the chain.

Glutamic acid (Glu) and asparatic acid (Asp) are the two charged amino acid residues of distributed in two chain ends, and the amorphous region may endow a polyelectrolyte nature to the heavy chain. The light chain does not have such a repetitive region is hydrophilic in nature, and characteristics of higher contents of Glu and Asp residues (Hossain, et al. 2003).

Silk polymorphs, including the glandularstate prior to crystallization (silk I), the spun silk state which composed of the β-sheet secondary structure (silk II), and an air/ water assembled interfacial silk (silk III, with a helical structure). The silk I structure is the water-soluble state and upon exposure to heat or physical spinning easily converts to a silk II structure. The silk I structure is observed *in vitro* in aqueous conditions and converts to a β–sheet structure when exposed to potassium chloride or methanol. The β-sheet structures are asymmetrical with one side occupied with the methyl side chains from the alanines that populate the hydrophobic domains and the other occupied with hydrogen side chains from glycine. The β-sheets are arranged in order that the methyl groups and hydrogen groups of opposing sheets interact to form the intersheet stacking in the crystals. Hydrogen bonds and strong van der Waals forces generate a thermodynamically stable.The inter- and intra-chain hydrogen bonds form between amino acids perpendicular to the axis of the chains and the fiber. The silk II structure excludes water and insoluble in several solvents including mild acid conditions and alkaline, and several chaotropes (Vepari and Kaplan 2007).

In the silk fiber, the intermolecular hydrogen bonding is sufficiently strong to prevent the separation of the molecules and hence to resist their dissolution to pure water. It easily redissolves in water with very concentrated amounts of a chaotropic salt such as lithium thiocyanate (LiSCN), lithium bromide (LiBr), sodium thiocyanate (NaSCN), and calcium chloride ($CaCl_2$). LiSCN can dissolve fibroin to a concentration of 3 % or more at room temperature. LiBr can dissolves fibroin at room temperature. Dissolution by $CaCl_2$ (Ajisawa's reagent) and NaSCN requires heating process to 78 ºC (Yamada, et al. 2001). The degradation of fibroin and resulting molecular weights of the protein by various treatments have been investigated, and it was found that the $CaCl_2$ treatment does not cause appreciable degradation.

In practice, calcium chloride is usually used for dissolving the silk fibroin because of its low cost. It is possible to dissolve 10-15 g of fibroin in 100 mL aqueous calcium chloride solution. Fibroin was boiled in 50 % calcium chloride solution to dissolve it, instantly. Then the solution was dialysed for 2-3 days against tap water. The pure solution obtained was colorless, tasteless and odorless (Luo, et al. 2003).

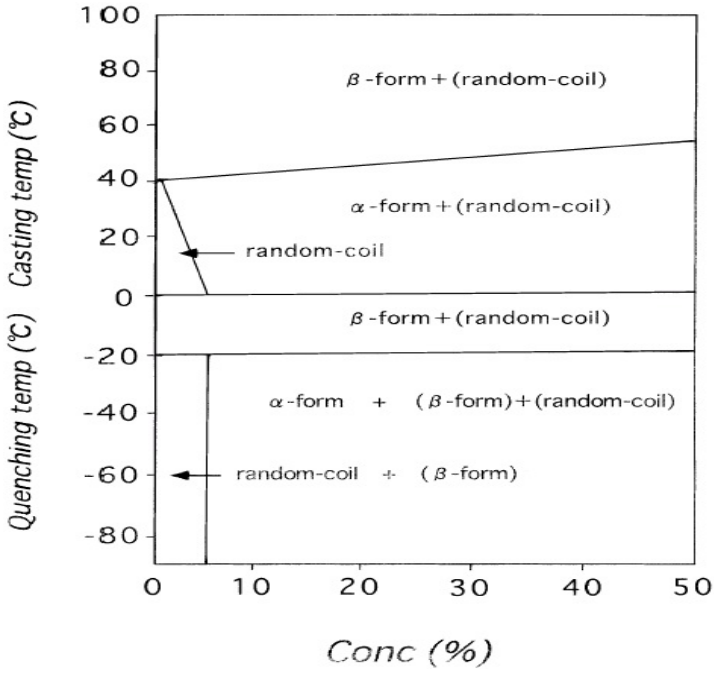

Figure 2.10. The relation between conformation, quenching or casting temperature and (starting) concentration of silk fibroin, *B. mori* (Source: Magoshi, et al. 2000).

Silk fibroin was crystallized as the water evaporated during the process of drying. The crystal structure was changed from the α- to β-form depending on the initial silk fibroin concentration in water and drying temperature (Magoshi, et al. 2000). The molecular weight of fibroin is about 350 kDa. This molecular weight of fibroin could be reduced to several tens of thousands daltons with calcium chloride treating. Further reduction of the fibroin molecular weight can be achieved with enzyme treatment. Acid hydrolysis would give much lower ordered macromolecules of fibroin, the molecular weight is reduced to several hundreds daltons in the latter case.

The intestinal enzyme and bacteria of humans or animals might decompose the fibroin into oligo-peptides and amino acids. These might be absorbed in the body easily. Silk contains about 6 % essential amino acids and it can be considered as a food material. Furthermore, there are some other reasons for eating silk fibroin. The amino acids which fibroin consists of, such as glycine (45 %), alanine (30 %), serine (12 %), and tyrosine (5 %) have some special properties. According to recent experiments, alanine has been found to be an agent helping to get relief of symptoms caused by excessive alcohol consumption. Silk major component glycine has been found to be an effective amino acid which could reduce the blood cholesterol level. The utilization of fibroin as food materials could prevent the diseases, such as blood pressure and apoplexy. Fortunately, silk has 6 % tyrosine, tyrosine reacts with hydrated enzyme we get dopa which could cure Parkinson's disease. Alanine has positive effect on intestinal activity; glycine reduces the blood cholesterol level; Dementia Praecox can be cured by tyrosine. Food materials made of silk would practically play an important role in the preparation of meals, especially for aged people and patients because silk fibroin can be used to prevent many adult diseases. Silk fibroin is mainly consists of proteins for that reason it can be used as a raw material in tonic manufacturing industries. Silk amino acids recommend the adaptability of silk as a food material; moreover, fibroin as a food material has a bright future (Luo, et al. 2003). Silk fibroin film is too brittle to be used by itself. The poor mechanical properties of silk fibroin could be improved by blending it with other natural or synthetic polymers (Park, et al. 1999). Such as chitosan, poly(vinyl alchol)(PVA), gelatin, cellulose, poly(ethylene oxide), polyacrylamide, poly(ethylene glycol), polyallylamine, sodium alginate and carrageenan have been studied to improve the mechanical or thermal or membrane properties of silk films.

Table 2.4. Some of the Silk Fibroin blends studies in the literature

References	Blend/Composite/Gel
Arai et al. 2002	Silk Fibroin and Polyallylamine Composites
Dai et al. 2002	Poly(vinyl alchol)/Silk Fibroin Blends
Freddi et al. 1999	Silk Fibroin/Polyacrylamide Blend Films
Freddi et al. 1995	Silk Fibroin/Cellulose Blend Films
Gotoh et al. 1997	Poly(ethylene-glycol)-Silk Fibroin Conjugate Films
Kweon et al. 2000	Fibroin/ chitosan blend film
Li et al. 2002	Silk fibroin–poly(vinyl alcohol) gel
Liang and Hirabayashi 1992	Fibroin Membranes/Sodium Alginate
Park et al. 1999	Silk Fibroin/Chitosan Blends
Yamaura et al. 1990	Silk Fibroin/Syndiotactic-Rich Poly(vinyl alcohol)
Jin et al. 2004	Silk Fibroin with Poly(ethylene oxide)

2.6. Antimicrobials Used in Edible Films and Coatings

Incorporating antimicrobial compounds into coatings or edible films provides a novel means for enhancing the shelf life and safety of foods (Cagrı, et al. 2001). An edible film containing a preservative can be used as an active, edible film on food surfaces to improve microbial stability (Ozdemir and Floros 2003). Edible films and coatings can also carry food ingredients, improve mechanical integrity of foods, and reduce the packaging material required for food products (Perez-Gago and Krochta 2001). Sorbic acid, *p*-aminobenzoic acid, lactic acid, and acetic acid are stated as GRAS food preservatives (Cagrı, et al. 2001). Antimicrobial compounds such as organic acids (acetic, propionic, benzoic, sorbic, lactic, lauric), potassium sorbate, bacteriocins (nisin, lacticin), grape seed extracts, spice extracts (thymol, *p*-cymene, cinnamaldehyde), thiosulfinates (allicin),

enzymes (peroxidase, lysozyme), proteins (conalbumin), isothiocyanates (allylisothiocyanate), antibiotics (imazalil), fungicides (benomyl), chelating agents (EDTA), metals (silver), or parabens (heptylparaben) could be added to edible films to reduce bacteria in solution, on culture media, or on foods (Cutter 2006). Examples of potential antimicrobial agents for antimicrobial food packaging systems are given in Table 2.5.

Table 2.5. Examples of potential antimicrobial agents for antimicrobial food packaging systems (Source: Han 2000)

Classifications	Antimicrobial agents
Organic acids	Acetic acid, benzoic acid, lactic acid, citric acid, malic acid, propionic acid, sorbic acid, succinic acid, tartaric acid, mixture of organic acids
Acid salts	Potassium sorbate, sodium benzoate
Acid anhydrides	Sorbic anhydride, benzoic anhydride
Para benzoic acids	Propyl paraben, methyl paraben, ethyl paraben
Alcohol	Ethanol
Bacteriocins	Nisin, pediocin, subtilin, lacticin
Fatty acids	Lauric acid, palmitoleic acid
Fatty acid esters	Glycerol mono-laurate
Chelating agents	EDTA, citrate, lactoferrin
Enzymes	Lysozyme, glucose oxidase, lactoperoxidase
Metals	Silver, copper, zirconium
Antioxidants	BHA, BHT, TBHQ, iron salts
Antibiotics	Natamycin
Fungicides	Benomyl, Imazalil, sulfur dioxide

(Cont. on next page)

Table 2.5. (Cont.) Examples of potential antimicrobial agents for antimicrobial food packaging systems (Source: Han 2000)

Classifications	Antimicrobial agents
Polysaccharide	Chitosan
Plant volatiles	Allyl isothiocyanate, cinnamol dehyde, eugenol, linalool, Terpineol, thymol, carvacrol, pinene
Plant/spice extracts	Grape seed extract, grapefruit seed extract, hop beta acid, Brassica erucic acid oil, rosemary oil, oregano oil, other herb/spice extracts and their oils
Probi otics	Lactic acid bacteria
Phenolics	Catechin, crysol, hydroquinone
Sanitizing gas	Ozone, chlorine dioxide, carbon monoxide, carbon dioxide
Sanitizers	Cetyl pyridinium chloride, acidified NaCl, triclosan

2.7. Plasticizers

Plasticizer is defined as "a substantially nonvolatile, high boiling, nonseparating substance, which when added to another material changes the physical and/or mechanical properties of material" (Banker 1966). Films prepared from pure polymers tend to be brittle and often crack upon drying. Addition of food-grade plasticizers to film-forming solution cope with this problem (McHugh, et al. 1994). The plasticizer improves flexibility and reduces brittleness of the film. Plasticizers reduce intermolecular forces, improve flexibility of the films, and increase mobility of biopolymer chains, preventing them from cracking or chipping during their preparation, storage and handling. However, they also increase

intermolecular spacing while reducing internal hydrogen bonding. This results in reducing the vapor, gas and solute barrier properties of the films. The amount of plasticizer added can cause adverse effects on film properties such as increasing mass transfer through the films. When large amounts of plasticizers were introduced into the formulation, significant film properties were changed (increases in flexibility and extensibility, decreases in mechanical resistance, elasticity). When plasticizer is incorporated into the polymer matrix, a competition for hydrogen bonding between polymer–plasticizer and polymer–polymer occurs. As a consequence, direct interactions between polymer chains are reduced partly because of hydrogen bond formation with plasticizer. The concentration of plasticizer also significantly increased the hydrogen bond formation (Turhan, et al. 2007). Polyethylene glycol, glycerol, propylene glycol, and sorbitol are the most commonly used plasticizers in edible film production.

2.8. Phytochemicals

The "phyto-" of the word phytochemicals is derived from the Greek word *phyto*, which means plant. Thus, phytochemicals can be defined as plant chemicals. Phytochemicals are bioactive plant compounds in fruits, vegetables, grains, and other plant foods. Although a large percentage still remain unknown and need to be identified, it is estimated that 5000 individual phytochemicals have been identified in fruits, vegetables, and grains.

Phytochemicals can be classified as phenolics, carotenoids, alkaloids, nitrogen-containing compounds, and organosulfur compounds. The most studied phytochemicals are the phenolics and carotenoids (Liu 2004).

2.8.1. Phenolic Compounds

Phenolic compounds are secondary metabolites, derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants (Balasundram, et al. 2006). Phenolic compounds or polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with more than 8000 phenolic structures currently. Polyphenols are products of the secondary metabolism of plants. They arise biogenetically from two main synthetic pathways: the shikimate pathway and the acetate path (Bravo 1998). These compounds, one of the most widely occurring groups of phytochemicals, are of considerable physiological and morphological importance in plants. These compounds play an important role in growth and reproduction, providing protection against pathogens and predators, besides contributing towards the color and sensory characteristics of fruits and vegetables. Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, antioxidant, vasodilatory and cardioprotective effects. Phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerised compounds with this structural diversity, the group of compounds are referred to as "polyphenols". Polyphenols can be divided into 10 different classes depending on their basic chemical structure. Simple phenols C_6, benzoquinones C_6, phenolic acids C_6-C_1, acetophenones C_6-C_2, phenylacetic acids C_6-C_2, hydroxycinnamic acids C_6-C_3, phenylpropenes C_6-C_3, coumarins, isocoumarins C_6-C_3, chromones C_6-C_3, naftoquinones C_6-C_4, xanthones C_6-C_1-C_6, stilbenes C_6-C_2-C_6, anthraquinones C_6-C_2-C_6, flavonoids C_6-C_3-C_6, lignans, neolignans $(C_6\text{-}C_3)_2$, lignins $(C_6\text{-}C_3)_n$ (Bravo, 1998). Phenolic compounds, whether simple or complex, are present in all plants and exhibit three major chemical properties. Firstly, they are acidic and partially dissociate in water. Secondly, the phenolic hydroxyl groups can form hydrogen bonds by intermolecular and/or intramolecular interaction. The formation of hydrogen bonds between or within molecules affects the chemical and physical properties of the molecule. Thirdly, the phenolic hydroxyl group can form complexes with metal ions, in particularly aluminium and iron (Ribereau-Gayon 1972).

The antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations. The structure of phenolic compounds is a key determinant of their radical scavenging and metal chelating activity, and this is referred to as structure–activity relationships (SAR). In the case of phenolic acids, for example, the antioxidant activity depends on the numbers and positions of the hydroxyl groups in relation to the carboxyl functional group. Phenolic acids consist of two subgroups, hydroxybenzoic and hydroxycinnamic acids (Balasundram, et al. 2006). Flavonoids, phenolic acids, and tannins are the major categories of phenolic compounds.

2.8.1.1 Phenolic Acids

Phenolic acids are the form of another large class of phenolic compounds. Hydroxybenzoic acids and hydroxycinnamic acid derivatives are commonly found in plants. Phenolic acids are a range of substituted benzoic acid derivatives and present naturally in many plants and fruits. Phenolic acids have the general structure of C_6-C_1 referring gallic acid structure and usually occur in conjugated or esterified forms (Ribereau-Gayon 1972). Phenolic acids contain two main groups;

1.Hydroxybenzoic acids: Hydroxybenzoic acids include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which in common have the C_6–C_1 structure (Balasundram et al. 2006).

2. Hydroxycinnamic acids: Hydroxycinnamic acids are aromatic compounds with a three-carbon side chain (C_6–C_3), with caffeic, ferulic, *p*-coumaric and sinapic acids being the most common (Balasundram et al. 2006). Hydroxycinnamic acids contain a double bond, therefore can exist in two isomeric forms, *cis* and *trans*. Naturally occurring hydroxycinnamic acids exist in the more stable *trans* isomeric form. Derivatives of hydroxycinnamic acids including glycosides and sugar esters may be found covalently bonded to other phenolic compounds such as anthocyanins (Ribereau-Gayon 1972).

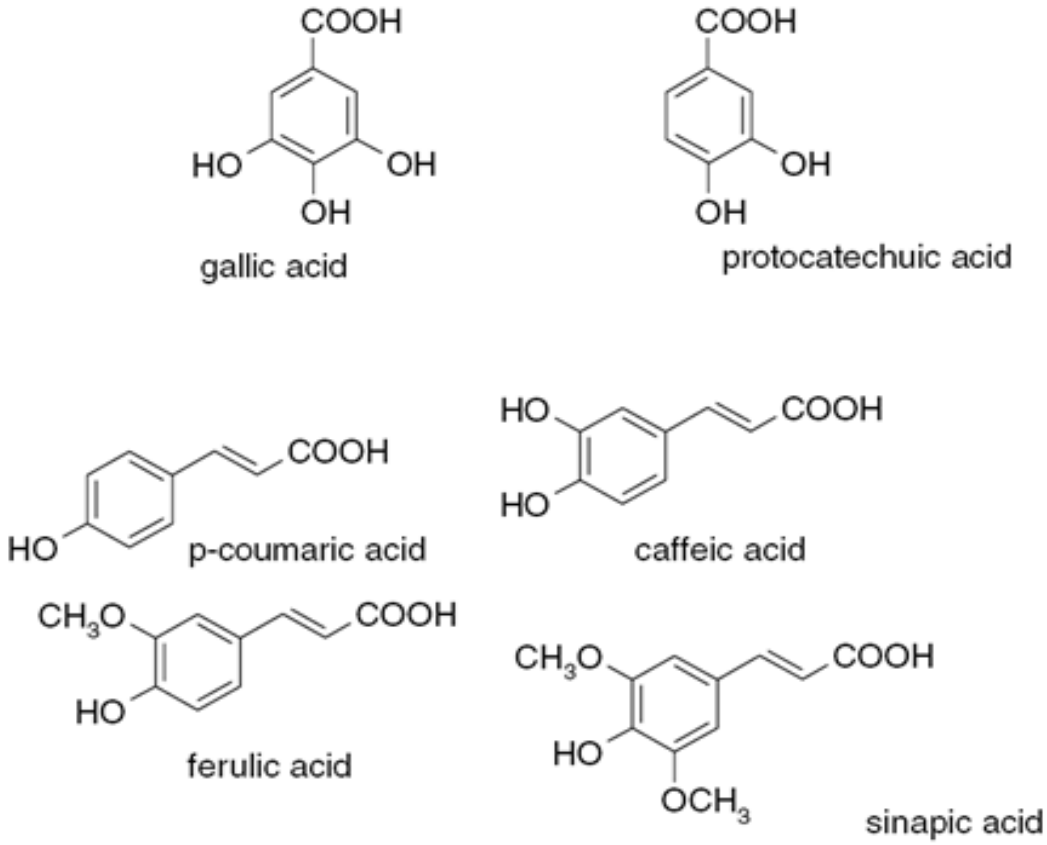

Figure 2.11. Examples of hydroxybenzoic and hydroxycinnamic acids
(Source: Balasundram, et al. 2006)

2.8.1.2. Flavonoids

Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds. Flavonoids may be divided into 8 different classes (flavonols, flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols, and chalcones) based on differences in molecular backbone structure. Flavonoids are low molecular weight compounds, consisting of 15 carbon atoms, arranged in a C_6–C_3–C_6 configuration.

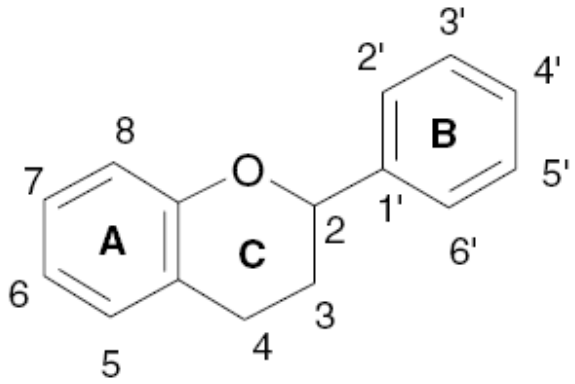

Figure 2.12. Generic structure of a flavonoid molecule
(Source: Balasundram, et al. 2006)

Structure consists of two aromatic rings A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring, C. The aromatic ring A is derived from the acetate/malonate pathway, while ring B is derived from phenylalanine through the shikimate pathway. Variations in substitution patterns to ring C result in the major flavonoid classes, i.e., flavonols, flavanones, flavones, isoflavones, flavanols (or catechins), flavanonols, and anthocyanidins. Flavonoids are basically divided into two groups; anthocyanins and anthoxanthins. Anthocyanins have some color pigments such as red, purple, and blue. Anthoxanthins possess colorless or white to yellow molecules (flavonols, flavones, isoflavones) (King and Young 1999). Anthocyanins have the same structure with the degree of hydroxylation and methylation of the benzene rings differentiating them. Substitutions to rings A and B give rise to the different compounds within each class of flavonoids. These substitutions may include oxygenation, alkylation, glycosylation, acylation, and sulfation (Balasundram, et al. 2006).

Monomeric flavan-3-ols are flavonoid compounds frequently found in plant tissue where they can be found in monomeric or polymeric forms. The most important of these compounds are the isomers of catechin and epicatechin. They have the structure C_6-C_3-C_6 and unlike other classes of flavonoids are not generally glycosylated or esterified (Ribereau-Gayon 1972), with the exception of the epicatechin-3-*O*-gallate identified in grapes. Monomeric flavan-3-ols with a trihydroxylated B ring exist as gallocatechin and epigallocatechin. Flavan-3-ols are frequently found in a polymerised form, condensed tannin.

Table 2.6. Major dietary flavonoids and examples
(Source: Yılmaz 2006)

Flavonoid	Examples
Anthocyanidins	Delphinidin, cyanidin, petunidin, peonidin, and malvidin
Flavonols	Quercetin, kaempferol, and quercetagetin
Flavanols	Catechin, epicatechin, epicatechin gallate, and epigallocatechin-3-gallate
Isoflavonoids	Isoflavones (e.g. genistein, diadzein, formononetin, and biochanin A), and coumestans (e.g. coumestrol)
Flavones	Rutin, apigenin, luteolein, and chrysin
Flavonones	Myricetin, hesperidin, naringin, and naringenin

Flavonol aglycones have the general structure C_6-C_3-C_6, but also exist as glycosides in which the C_6-C_3-C_6 aglycone part of the molecule is esterified with a number of different sugars. The most commonly found flavonols are kaempferol, myricetin, and quercetin (Ribereau-Gayon 1972). Flavonoids are generally more complicated than hydroxycinnamic and hydroxybenzoic acids due to the relative complexity of the flavonoid molecules. Some of the structural features and nature of substitutions on rings B and C which determine the antioxidant activity of flavonoids include the following:

(i) The degree of hydroxylation and the positions of the –OH groups in the B ring, in particular an *o*-dihydroxyl structure of ring B (catechol group) results in higher activity as it confers higher stability to the aroxyl radical by electron delocalisation, or acts as the preferred binding site for trace metals.

(ii) The presence of hydroxyl groups at the 3'-, 4'-, and 5'-positions of ring B (a pyrogallol group) has been reported to enhance the antioxidant activity of flavonoids compared to those that have a single hydroxyl group. However, under some conditions, such compounds may act as pro-oxidants, thus counteracting the antioxidant effect.

The conversion of the 3',4 '-dihydroxyphenyl to 3' ,4',5 '-trihydroxylphenyl increases the antioxidant activity for anthocyanidins but decreases the activity for catechins.

(iii) A double bond between C-2 and C-3, conjugated with the 4-oxo group in ring C enhances the radical scavenging capacity of flavonoids.

(iv) A double bond between C-2 and C-3, combined with a 3-OH, in ring C, also enhances the active radical scavenging capacity of flavonoids, as seen in the case of kaempferol . Substitution of the 3-OH results in increase in torsion angle and loss of coplanarity, and subsequently reduced antioxidant activity.

(v) Substitution of hydroxyl groups in ring B by methoxyl groups alters the redox potential, which affects the radical scavenging capacity of flavonoids (Balasundram et al. 2006).

2.8.1.3. Tannins

Tannins, the relatively high molecular weight compounds which constitute the third important group of phenolics, may be subdivided into hydrolysable and condensed tannins.

1. Hydrolyzable tannins: They include a central core of polyhydric alcohol such as glucose and hydroxyl groups. They are esterified partially or wholly by gallic acid (gallotannins) or hexahydroxy-diphenic acid (ellagitannins).

2. Condensed tannins: They are more common and have more complex structures than the hydrolyzable tannins. They consist of oligomers and polymers of catechins. Condensed tannins or proanthocyanidins consist of two classes of polymers, procyanidins and prodelphinidins. Procyanidins consist of catechin and epicatechin and prodelphinidins consist of epigallocatechin and gallocatechin. Plant phenolics, tannins are compounds of intermediate to high molecular weight with a molecular mass of up to 30 kDa.

Tannins are highly hydroxylated molecules and can form insoluble complexes with carbohydrates and protein. This function of plant tannins is responsible for the astringency of tannin-rich foods due to the precipitation of salivary proteins. The term "tannin" comes

from the tanning capacity of these compounds in transforming animal hides into leather by forming stable tannin-protein complexes with skin collagen (Bravo 1998).

2.9. Mode of Antimicrobial Action of Phenolic Compounds

Phenolic compounds have antimicrobial activity against a range of microorganisms including Gram-positive and Gram-negative bacteria. Gram-positive bacteria are more susceptible to the action of biocides than Gram-negative bacteria. Gram-positive bacteria have a different cell envelope when compared to Gram-negative bacteria. Gram-positive bacteria have an inner cell membrane consisting of a lipid bilayer, and an outer cell wall consisting chiefly of peptideoglycan. Gram-negative bacteria have an inner cell membrane, an outer cell wall containing little peptideoglycan, and an outer membrane composed of lipoprotein, lipopolysaccharide and other macromolecules. The lack of an outer membrane and the permeable nature of the cell wall of Gram-positive bacteria allows the penetration of biocides into the bacteria. The outer membrane of Gram-negative bacteria could provide a protective layer preventing the diffusion of hydrophobic compounds and large hydrophilic compounds (MW >600), whereas, the Gram-positive cell wall allows access to antimicrobial molecules up to 30 to 57 kDa (Lambert 2002). The Gram-positive bacteria are generally more sensitive to biocides such as phenols, alcohols, aldehydes, quaternary ammonium compounds and bisbiguanides, which penetrate the wall with ease. The resistance of Gram-negative bacteria was explained in part by the barrier function of the Gram-negative outer membrane and the presence of negatively charged lipopolysaccharides at the exterior of the outer membrane, which reduced the binding of the biocide to the bacterial cell. Antimicrobial peptides such as nisin (3354 Da) and defensins (3000–3500 Da) are able to penetrate the wall to interact with the cytoplasmic membrane (Friedrich, et al. 2000). Lysozyme (14 400 Da) can reach the peptidoglycan in the cell wall and secreted phospholipase A2 (14 000 Da) which can penetrate the cell membrane to reach its phospholipid target, phosphatidyl glycerol (Lambert 2002).

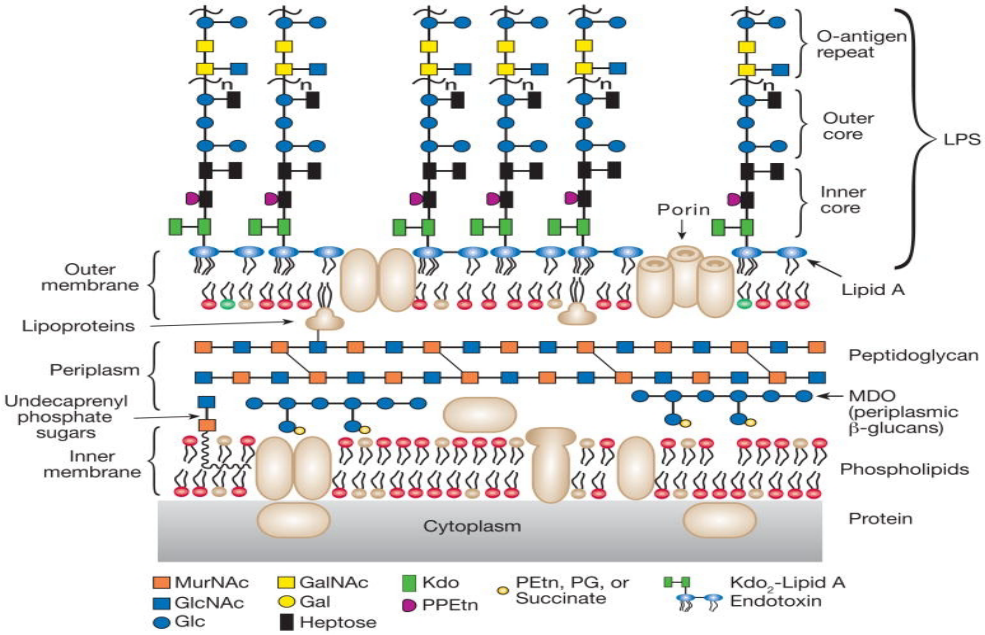

Figure 2.13. Cell wall of gram-positive bacteria
(Source: Varki, et al. 2009)

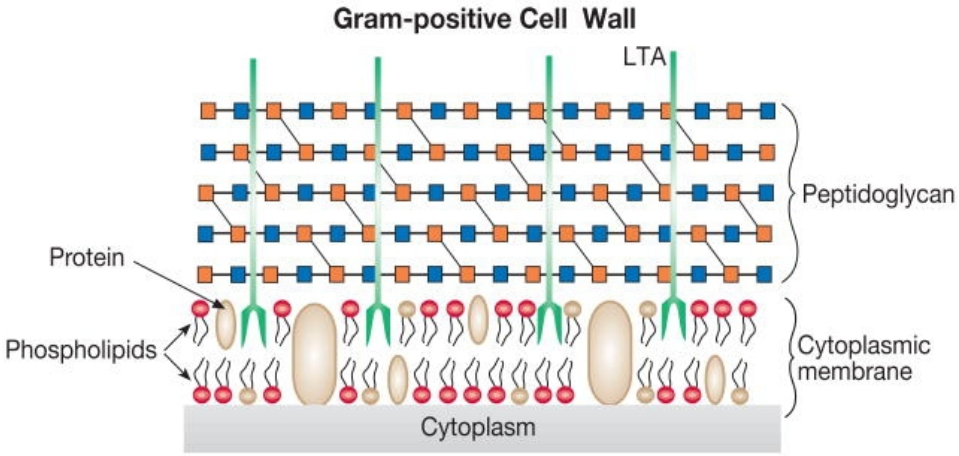

Figure 2.14. Cell wall of gram-positive bacteria
(Source: Varki, et al. 2009)

2.10. Phenolics in Grape

Wine industry wastes, mainly consist of solid by-products include pomace, marcs and stems, and almost 30 % (w/w) of the grapes used for wine production. All these products may bear a considerable burden of phenolic components, depending on the type of grape (white or red), the part of the tissue (skins, seeds, etc.), in addition to the processing conditions (e.g., pomace contact). Over the past few years, agricultural wastes of plant origin have attracted considerable attention as potential sources of bioactive phenolics. They can be used for various purposes in the food, pharmaceutical and cosmetics industries (Markis, et al.2007).

A general composition of the grape consists of 2–6 % stems, 5–12 % skins, 80–90 % juice and 0–5 % seeds. Chemically, one of the important constituents is phenolic substances (frequently called polyphenols). Grape seeds, although they make up a small percentage of the weight of grapes, contain two-thirds of the extractable phenols. The seeds are highest in phenol content and may contain up to 5–8 % phenols by weight that are essentially all flavonoids. They are also referred to as monomeric flavan-3-ols, which joined together is known as oligomeric procyanidins. Oligomeric proanthocyanidins (OPCs) are a class of polyphenolic biflavanoids and found in fruits and vegetables. The highest concentration of these is found in the seeds of grapes. The percentage of the total extractable polyphenols in grape tissues are: 10 % or less in the pulp, 60–70 % in the seeds and 28–35 % in the skin. The polyphenol content of seeds may range from 5 to 8 wt % (Kar, et al. 2006, Nawaz, et al. 2006).

Grape seeds and skins are good sources of polyphenolic tannins that provide the astringent taste to wine. The phenolic acid, gallic acid and monomers, epicatechin, catechin are the main phenolic compounds in grape seeds. These are also the major flavonoids present in grape skins in addition to various anthocyanins. Terminal units of polymeric procyanidins of grape skins contain 67 % (+)-catechin, whereas extension units contain 60 % (-)-epicatechin (Yılmaz and Toledo 2004). The most common phenolic acids in grape are cinnamic acids (coumeric, caffeic, ferulic, chlorogenic and neochlorogenic acids) and benzoic acids (p-hydroxybenzoic, protocathecuic, vanillic and gallic acids). Flavonoids in

grape consist of flavan-3-ols (catechin, epicatechin, their polymers and their ester forms with glucose), flavanones (the most common one being quercetin) and anthocyanins.

Anthocyanins are a class of flavonoids and have the general structure C_6-C_3-C_6. There are five anthocyanidins in grapes: delphinidin, petunidin, malvidin, cyanidin, and peonidin. These anthocyanidin aglycones also exist as glycosides (anthocyanins): 3 monoglucosides and 3,5-diglucosides, and as acylated heterosides (Ribereau-Gayon 1974).

In grape seed, the content of highly polymerised procyanidins is generally more abundant than that of the oligomers. Nevertheless, they are still poorly characterized, due basically to them being difficult to isolate and identify (Garcia-Marino, et al. 2006).

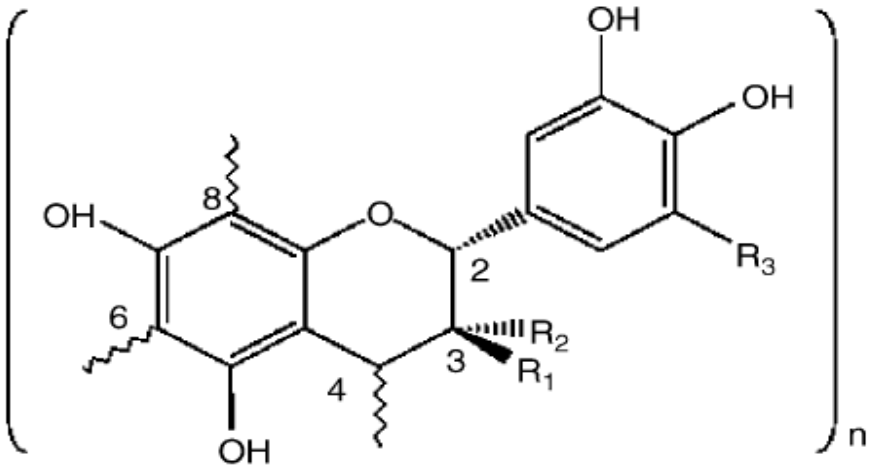

Figure 2.15. General scheme of grape proanthocyanidins: *n*, degree of polymerization; winding lines indicate C4–C6 or C4–C8 interflavanic linkages. Main constitutive units are as follows: (R1 = OH, R2 =H, R3 = H), (+)-catechin; (R1 =H, R2 = OH, R3 = H), (−)-epicatechin; (R1 =H, R2 =*O*-galloyl, R3 = H), (−)- epicatechin-3-*O*-gallate; (R1 =H,R2 = OH, R3 = OH), (−)-epigallocatechin (Source: Garcia-Marino, et al. 2006)

2.11. Extraction and Production of Grape Seed

Numerous methods of extraction have been developed with the objective of obtaining extracts with higher yields and lower costs. Principally, there are three principle techniques that may be used: (1) solvent extraction, (2) solid-phase extraction, (3) supercritical CO_2 extraction. The extraction of polyphenols depends on two actions, the dissolution of each polyphenolic compound at the cellular level in the plant material matrix, and their diffusion in the external solvent medium. The basic system to extract the phenolic substances is the solvent extraction. Conventionally, organic solvents such as methanol, acetone, acetonitrile, ethyl acetate, and others are used to extract polyphenols from grape seeds and the extracts are evaporated under vacuum to remove the solvent (Garcıa-Marino, et al. 2006, Nawaz, et al. 2006). Supercritical extraction is reliable and safest method to extract desired material from the solid matrices. However, the cost of this system is too high that, in industry, it is not preferably chosen.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

The bacterial strains used in this study, *Listeria innocua* (NRRL B-33314), *Bacillus amyloliquefaciens* (NRRL NRS-762), *Escherichia coli* (NRRL B-3008), *Pseudomonas fluorescens* (NRRL B-253), *Staphylococcus carnosus* (NRRL B-14760), were supplied from the United States Department of Agriculture, Microbial Genomics and Bioprocessing Research Unit, Peoria, Illinois. In addition to these strains, *Escherichia coli* O157:H7 (ATCC 700728, Dr. Ali Aydın, Department of Food Hygiene and Technology, Faculty of Veterinary, İstanbul University, Turkey), *Salmonella* Typhimurium (CCM 5445, Dr. A. Handan Baysal, Department of Food Engineering, İzmir Institute of Technology, Turkey) and *Staphylococcus aureus* (RSKK 95047, Dr. Gülsün Evrendilek, Department of Food Engineering, Abant Izzet Baysal University, Turkey) were used in this study.

Commercial grape seed extract (GSE) was obtained from Polyphenolics (CA, USA). Raw chicken breast meat was obtained from a local supermarket (İzmir, Turkey). Sausages used in this study were produced by Pınar Et A.Ş. (İzmir, Turkey). Glycerol, ferric ammonium sulfate, hydrochloric acid, n-butanol and ethanol (absolute GR for analysis) were supplied from Merck (Darmstadt, Germany). Disodium EDTA.$2H_2O$, calcium chloride-2-hydrate were purchased form Riedel-de haën (Sigma-Aldrich Laborchemikalien, Seelze, Germany). Dialysis tubing (MW Cut-off: 12,000-14,000) was obtained from Sigma (St. Louis, MO, USA). Silk fibroin (SF) was obtained from Silk Biochemical Company (Silk Biochemical Co., Ltd., China). The Folin-Ciocalteu reagent was obtained from Fluka.

3.2. Methods

3.2.1. Preparation of Aqueous Fibroin Solution

Silk fibroin (SF) aqueous solution was prepared by the universally applied Ajisawa's method. SF (1.5 g) was added to 30 mL Ajisawa's reagent ($CaCl_2$ $2H_2O$/ethanol/water, in weight, mole ratio=1:2:8) in a Schott bottle with a volume of 100 mL. The mixture was stirred at 120 rpm at 78 °C in a water bath to form a clear solution for 2 h. SF solution was dialyzed against deionized water for 3 days at 4 °C in cellulose tubing with a molecular weight cutoff value of 12-14 kDa to remove the neutral salts. The water change was done for half-an-hour intervals for the first 3 h and then for 12 h intervals for the rest of the 3 days. Dialysis was accomplished in 2 L erlenmeyer flasks. Then the solution was concentrated using a rotary evaporator (Heidolph Laborota 4000, Germany) at 37 °C and 150 rpm. The pure aqueous SF solution with a concentration of 2.5 % (w/v) was obtained.

3.2.2. Preparation of Silk Fibroin-Carrageenan Films

Carrageenan (0.4 %) was dissolved in the aqueous silk fibroin solution (0.375 g silk fibroin) having a concentration of 2.5 % (w/v). The mixture was stirred at 120 rpm and 80 °C for 30 min in a water bath and then the mixture was cooled to room temperature. Glycerol was added as a plasticizer to all solutions at 50 % (protein to glycerol ratios of 2:1). GSE and/or $Na_2EDTA.2H_2O$ were incorporated into film forming solutions by a homogenization method. Film forming solution was homogenized at 1000 rpm for 10 min. Film forming solutions were degassed under vacuum by using speed Vac concentrator (Thermo SPD121P, North America) for 15 min to remove air bubbles in solutions. The films forming solution (11 g) were casted on high-density polyethylene plates (HDPE).

Then they were dried at 25 °C and 40 % RH for 24 h in an environmental chamber (Angelontoni-ACS environmental chambers, Italy).

3.2.3. Determination of Total Phenol and Total Proanthocyanidin Contents of Grape Seed Extract

3.2.3.1. Folin- Ciocalteu's (F-C) Method

The total phenolic content of GSE used in the SFC films was determined using the Folin-Ciocalteu's method with a modification of Lako (Lako, et al. 2007). GSE (0.0125 g) was dissolved in 25 mL water. GSE (500 μL) was mixed with 2.5 mL of 1/10 diluted Folin-Ciocalteu's reagent and the mixture was kept at room temperature for 5 min to allow for the Folin-Ciocalteu's reagent to react completely with the oxidizable substances or phenols. Then 2 mL of 7.5 % (w/v) sodium carbonate (Na_2CO_3) solution was added to the mixture. After 1 h incubation at room temperature in a dark place, the absorbances of the solutions were measured at 725 nm by a spectrophotometer (Shimadzu UV-2450, UV-Visible Spectrophotometer, Japan). Total phenol content of GSE was determined using the gallic acid calibration curve and the results were expressed as mg/L gallic acid equivalents (GAE) per gram fresh weight. Calibration curve of gallic acid is given in appendix A.

3.2.3.2. Porter Assay

Total proanthocyanidin (condensed tannins) content of GSE was determined using the HCl/butan-1-ol assay with a slight modification of Bahorun (Bahorun, et al. 2004). GSE aqueus solution (0.5 mL) was added to 3 mL of 95 % solution of *n*-butanol/HCl (95:5 v/v) in a test tube, followed by 0.1 mL of a solution of ferric reagent ($NH_4Fe(SO_4)_2 \cdot 12H_2O$ in

2M HCl) and the mixture in the tube was vortexed. The tubes were placed in a water bath at 100°C for 60 min. Then, the tubes were cooled and absorbance was recorded at 550 nm using a spectrophotometer. The absorbance of the blank sample (sample which was not heated) was also measured.

3.2.4. Determination of Total Proanthocyanidin Content Released from the Silk Fibroin-Carrageenan Films

The release test was performed in a refrigerated incubator at 4 °C. SFC films with GSE (4 x 4 cm) were placed in a glass Petri dish containing 80 mL distilled water (4 °C). The Petri dish was then covered with a parafilm in order to prevent moisture loss and was incubated at 4 °C for 1440 min with a continuous stirring at 160 rpm using an orbital shaker. Total proanthocyanidin content in the release test solution was monitored by taking 0.5 mL aliquots in triplicate from the release test solution at different time intervals. 0.5 mL of aqueus solution was added to 3 mL of *n*-butanol/HCl (95:5 v/v) in a test tube, followed by 0.1 mL of a solution of ferric reagent ($NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2M HCl) and the tube was vortexed. The tube was put in a water bath at 100°C for 60 min. Then, the tubes were cooled and absorbance was measured at 550 nm using a spectrophotometer (Shimadzu UV-2450, UV-Visible Spectrophotometer, Japan).

3.2.5. Characterization of Silk Fibroin-Carrageenan Films

SFC films (control film) and SFC films incorporated with GSE were characterized by instrumental analysis techniques. Scanning electron microscopy (SEM), atomic force microscopy (AFM), X-ray diffractometer (XRD), and Fourier transform infrared (FTIR) spectrometer were used to analyze the film characteristics. Mechanical properties of films were also determined.

SEM (Philips XL 30S FEG) analyses were performed to determine the morphological changes in the films and the film thickness was also measured. Films were cut into 1 x 1 cm pieces using a sharp razor for the cross-section observation. The surface of films was coated with a gold palladium (100-200 Å thickness) for 2 min in a Magnetron Sputter Coating Instrument to eliminate charge effect, and then observed by SEM.

The roughness of the films was determined by AFM (Digital Instruments MMAFM-2/1700EXL). Films were cut into 1 x 1 cm pieces using a sharp razor. The contact mode was used and 10 μm scales were used for the area scanned.

The changes in the crystalline state were monitored by XRD (Philips X'pert Pro, The Netherlands) with CuKα radiation for 2θ from 5 to 70°.

Infrared spectra of the films were obtained in 4000–650 cm^{-1} range with a Perkin Elmer Spectrum 100 FTIR spectrometer (Perkin Elmer Inc., Wellesley, MA, USA) equipped with a deuterated tri-glycine sulphate (DTGS) detector. A horizontal attenuated total reflectance (HATR) was used to collect the spectral data of the films. The resolution was set at 4 cm^{-1} and the number of scans collected for each spectrum was 64.

Tensile strength (TS), Young's modulus (YM) and elongation (E %) of films were measured according to ASTM Method D882 (1996) by a texture analyzer (TA.XT.plus, Stable Instruments, UK). The films were conditioned at 40% RH and at 25 °C for 24 h prior to the measurement of mechanical properties. Films were cut into 5 mm x 50 mm strips. Five kg load cell was used for all films. Initial grip separation was 50 mm and head speed was set to 50 mm min^{-1}. The stress–strain curves were analyzed using the software provided with the texture analyzer (Texture Expert Exceed 2.3, Stable Micro Systems). Six replicates were performed in each case.

3.2.6. *In Vitro* Antimicrobial Activity of Silk Fibroin-Carrageenan Films

The inhibitory effect of SFC films (Table 3.1) against spoilage and pathogenic microorganisms was tested on agar media. Test of antimicrobial activity was conducted by using *S. carnasus* (NRRL B-14760), *S. aureus* (RSKK 95047), *L. innocua* (NRRL B-

33314), *E. coli* (NRRL B-3008), *E. coli* 0157:H7 (ATCC 700728), *S*. Typhimurium (CCM 5445), B. *amylaliquefaciens* (NRRL NRS-762) and P. fluerescens (NRRL B-253). *L.innocua*, *E.coli*, *E.coli* 0157:H7, *S. carnasus*, *S. aureus*, and *S.* Typhimurium were incubated using nutrient broth at 37 °C 24 h, it was sub-cultured and incubated for 8 h. *B. amylaliquefaciens* was incubated using nutrient broth at 30 °C for 24 h, it was sub-cultured and incubated for 8 h. *P.fluorescens* was incubated using nutrient broth at 26 °C for 24 h, it was sub-cultured and incubated for 8 h. For antimicrobial tests 12 discs (1.3 cm in diameter) were cut by a sterile cork borer under aseptic conditions. Four discs were randomly selected and placed onto the surface of the inoculated (0.1 mL of inoculum) nutrient agar in the Petri plates. The Petri plates for *S. carnasus*, *S. aureus*, *L. innocua*, *E.coli*, *E.coli* 0157:H7, and *S.* Typhimurium were incubated at 37°C/24 h, the plates for *B. amylaliquefaciens* was incubated at 30°C/24 h, and the plates for *P. fluorescens* were incubated at 26 °C/24 h. The area of the fully formed zones (ffz) observed was determined by measuring the zone diameter with a caliper. The zones formed on only one side of the discs were designated as partially formed zones (pfz) and their numbers were recorded. The number of negative zone (nz) was also counted and reported.

Table 3.1. Films used for antimicrobial activity determination

1) SFC film(without GSE and EDTA.2H_2O_2 (w/v))
2) SFC film with EDTA.2H_2O_2 (w/v)
3) SFC film with 0.5% GSE (w/v)
4) SFC film with 0.5% GSE and EDTA.2H_2O_2 (w/v)
5) SFC film with 1% GSE (w/v)
6) SFC film with 1% GSE and EDTA.2H_2O_2 (w/v)
7) SFC film with 2% GSE (w/v)
8) SFC film with 2% GSE and EDTA.2H_2O_2 (w/v)
9) SFC film with 3% GSE (w/v)
10) SFC film with 3% GSE and EDTA.2H_2O_2 (w/v)

3.2.7. Antimicrobial Activity of Silk Fibroin-Carrageenan Films on Raw Chicken Breast Meat

Raw chicken breast meat was obtained from a local supermarket (İzmir, Turkey). Raw chicken breast meat was cut into pieces weighing approximately 10 g and they were coated with SFC films by dipping the samples into the film solution. The samples were divided into 7 groups (control and treated samples). Seven different coating treatments applied were as follows: Control (without film); SFC film (without GSE and EDTA.$2H_2O_2$(w/v); SFC film with EDTA.$2H_2O_2$(w/v); SFC film with 2% GSE (w/v); SFC film with 2% GSE and EDTA.$2H_2O_2$ (w/v); SFC film with 3% GSE (w/v); SFC film with 3 % GSE and EDTA.$2H_2O_2$ (w/v). Samples were placed into sterile Petri dishes, stored at 4°C, and analyzed at day 0, 1, 3, and 5. The experiment was performed in triplicate. For microbiological analyses, samples (10 g) were aseptically mixed with 90 mL of 0.1% peptone water and homogenized using a stomacher (Bagmixer® 400, Interscience, France). Mixtures were serially diluted (1:10) in 0.1% peptone water. Sample dilutions were plated and incubated at 30 ºC/48 h to determine the total viable bacteria counts using plate count agar (PCA) and at 37 ºC/24 h to determine the coliform counts using violet red bile agar (VRBA). Lactic acid bacteria were enumerated using DeMan, Rogosa and Sharp (MRS) agar and Petri plates were incubated at 37ºC/48 h in a CO_2 incubator (5% CO_2 and 50% RH). Experiments were performed in triplicate. The microbial counts were expressed as $\log_{10}$ colony forming units (CFU) per g of sample.

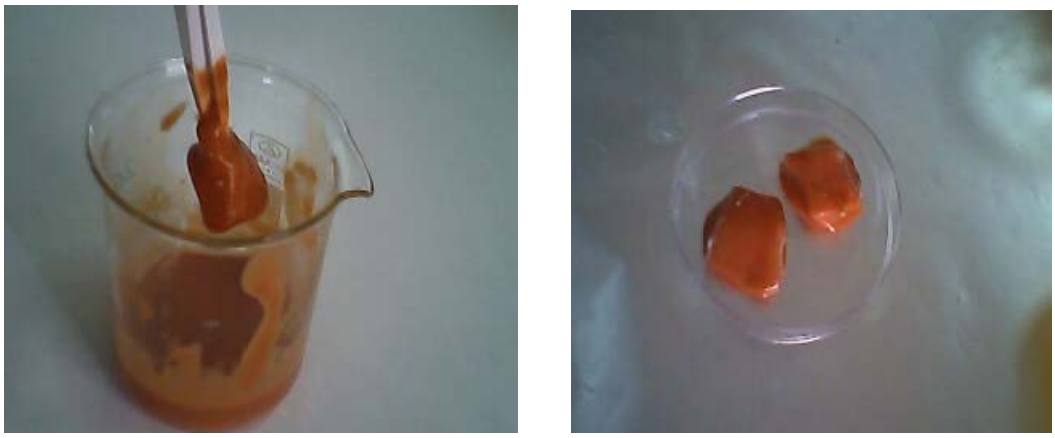

Figure 3.1. Raw chicken breast meat samples coated with SFC films incorporating GSE

3.2.8. pH Analysis of Raw Chicken Breast Meat

The pH of raw chicken breast meat (1 g meat /10 mL deionized water) samples was measured in duplicate by a pH meter (Hanna Instruments, Portugal).

3.2.9. Antimicrobial Activity of Silk Fibroin-Carrageenan Films on Beef Sausages

Frozen stock culture of *S. aureus* (RSKK 95047) was obtained from Dr. Gülsün Evrendilek (Department of Food Engineering, Abant Izzet Baysal University, Turkey). The bacterial culture was grown for 24 h at 37 °C in nutrient broth and subcultured at 5 h later. Ten mL of two subcultered broth mixed and cells of strain in 20 mL was collected by centrifugation (4500*g*, 10 min). The cocktail was diluted with 10 mL peptone water (OD 1.07; log 7.66 CFU/mL). The inoculum volume was 100 mL (10 mL of *S. aureus strains* + 90 mL buffered peptone water) and the concentration of inoculum was 6.37 log CFU/mL.

Beef sausages used in this study was obtained from Pınar Et A.Ş. (İzmir, Turkey). Sausage samples pieces weighing approximately 7 g. Beef sausage pieces were divided into 5 groups. Five different coating treatments applied were as follows: the first two group without coating; 1) without inoculum and without coating; 2) with inoculum and without the coating, the second three group with inoculum and coated with SFC film solution with dipping method; 1) SFC film solution without GSE; 2) SFC film with 2 % GSE (w/v); 3) SFC film with 3 % GSE (w/v). Samples were placed into sterile Petri dishes, stored at 4 °C, and analyzed at day 0, 7, 14, 21 and 28. The experiment was performed in triplicate. Samples (7 g) were aseptically mixed with 90 mL of buffered peptone water and homogenized using a stomacher (Bagmixer® 400, Interscience, France). Mixtures were serially diluted (1:10) in buffered peptone water. Sample dilutions were plated and incubated at 37 ºC/48 h to determine *S. aureus* counts using Baird-Parker Agar (BPA) supplemented with egg yolk tellurite. Experiments were performed in triplicate. Microbial counts were expressed as $\log_{10}$ colony forming units (CFU) per g of sample.

3.2.10. pH Analysis of Beef Sausages

The pH of beef sausages was measured as it was described in section 3.2.8.

3.2.11. Moisture Analysis of Beef Sausages

Moisture content of control and treated samples were analyzed in using the oven method at 105 ± 2 °C at day 0, 7, 14, 21, and 28 (AOAC, 1999).

3.2.12. Texture Profile Analysis of Beef Sausages

The texture profile analysis (TPA) was done to the samples with a slight modification of Choi and Chin (2002) using a texture analyzer (TA.XT.plus, Stable Instruments, UK). Beef sausage samples were cut into pieces (1 cm thick, 1.5 cm diameter) and subjected to two cycle compression test. Five kg load cell was compressed twice at the test speed of 2 mm/min. The TPA parameters were as follows: hardness (N), maximum force required to compress the sample; springiness was determined as the height that the sample recovered during the time elapsed between the end of first compression and the start of second compression; cohesiveness was the extent to which the sample could be deformed prior to rupture (A2/A1, where A1 was the total energy required for the first compression and A2 the total energy required for the second compression); gumminess(N) was calculated as the products of hardness x cohesiveness; and chewiness(N) was calculated as the products of gumminess x springiness.

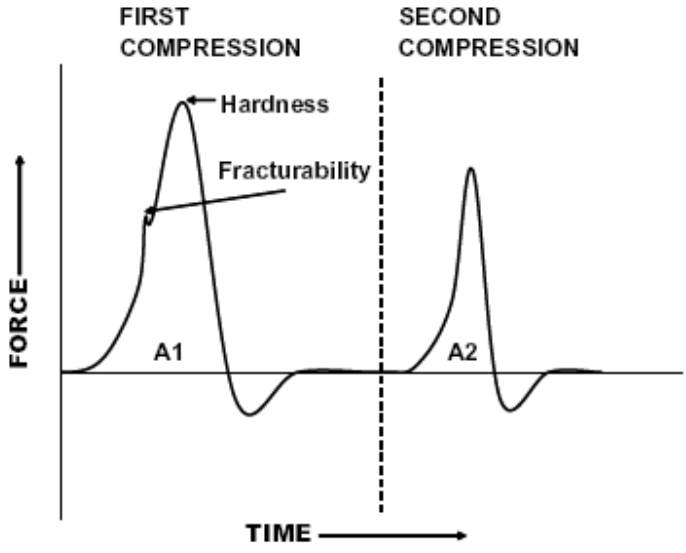

Figure 3.2. A typical texture profile analysis force–time obtained from the TA.XT.plus Texture Analyzer (Source: Martinez, et al. 2004)

3.2.13. Statistical Analysis

Data were analyzed by ANOVA using Design Expert 7.0 (trial version) and Minitab 15. ANOVA was used to evaluate treatment and storage as fixed effects, for the microbiological, moisture, color, and texture profile analysis studies. Means with a significant difference ($p < 0.05$) were compared using the Tukey's multiple range tests.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Total Phenol and Total Proanthocyanidin Contents of Grape Seed Extract

Total phenol content of GSE used in this study was analyzed using Folin-Ciocalteu method. This is a colorimetric oxidation/reduction assay that measures all phenolic molecules with no differentiation between gallic acid, monomers, dimers and larger phenolic compounds. Total phenol content of GSE is shown in Table 4.1.

phenolics+alkaline+FC reagent+heat = blue colored product, Abs 755 nm (4.1)

Total proanthocyanidin content of GSE was analyzed using porter assay. The butanol-HCl-iron method is widely used for the measurement of extractable condensed tannins (proanthocyanidins) in feeds and foods. The Porter assay is a colorimetric test based on acid hydrolysis. Dimer and larger molecules are converted to anthocyanidins by acid hydrolysis (Activin 2009). Total proanthocyanidin content of GSE is given in Table 4.1.

Proanthocyanidins +NH4Fe(SO4)2*12H2O+HCl/n-butanol+heat=cyanidin (4.2)

(dimers & larger) Abs 550 nm

Table 4.1. Total phenol and total proanthocyanidin contents of GSE

	Folin-Ciocalteu's method	Porter Assay
	mg gallic acid / g GSE	mg proantosiyanidin / g GSE
Grape seed extract	752.35	666.11

4.2. Total Proanthocyanidin Content Released from the Silk Fibroin-Carrageenan Films

The principle action of antimicrobial films is based on the release of antimicrobial agents. Properties of antimicrobial agents play an important role in preventing spoilage and these agents should enhance microbial safety. The active antimicrobial compounds need be released at a controlled rate over prolonged period of time and active agents can be effectively released from the packaging material to the food products. The GSE is composed of 89.3 % proanthocyanidins, which contained 6.6 % dimers, 5.0 % trimers, 2.9 % tetramers and 74.8 % oligomers and polymers larger than pentamer, 6.6 % monomeric flavanols (2.5 % (+)-catechin, 2.2 % (−)-epicatechin, 1.4 % (−)-epigallocatechin and 0.5 % (−)-epigallocatechin gallate), 2.24 % moisture, 0.8 % ash, and 1.06 % protein (Yamakoshi et al. 2002).

Absorption, immobilization and release systems are the three typical systems for determining the activity of antimicrobial agent. The release system allows the migration of the antimicrobial agent (solute or gas) into the food. Food stuffs are comprised of a complex mixture of substances such as proteins, water, carbohydrates, fats, lipids, vitamins, fibers, and minerals. Due to this complex matrix, it is difficult to measure the migration of an active agent into the food. For this reason, migration studies are usually performed using food simulants. In current European food packaging regulations (European Standard EN 1186-1, 1999), various food simulants that can be used for migration testing have been identified. These include: water (simulant A), 3 % (v/v) acetic acid in water (simulant B);

15 % (v/v) ethanol in water (simulant C); olive oil; sunflower oil; and synthetic fat simulant HB 307 (simulant D) where each simulant is representative of a particular type of a food (Mistry 2006, Dopico, et al. 2003).

Maximum total proanthocyanidin contents released from films containing 1 % and 2 % GSE at 120 min were 0.44 and 0.63 mg proanthocyanidin/cm^2 of film, respectively. Films having the highest GSE (3 %) concentration, which was used in the food application studies, had the maximum total proanthocyanidin content release of 1.04 mg proanthocyanidin/cm^2 at 1440 min (Figure 4.1).

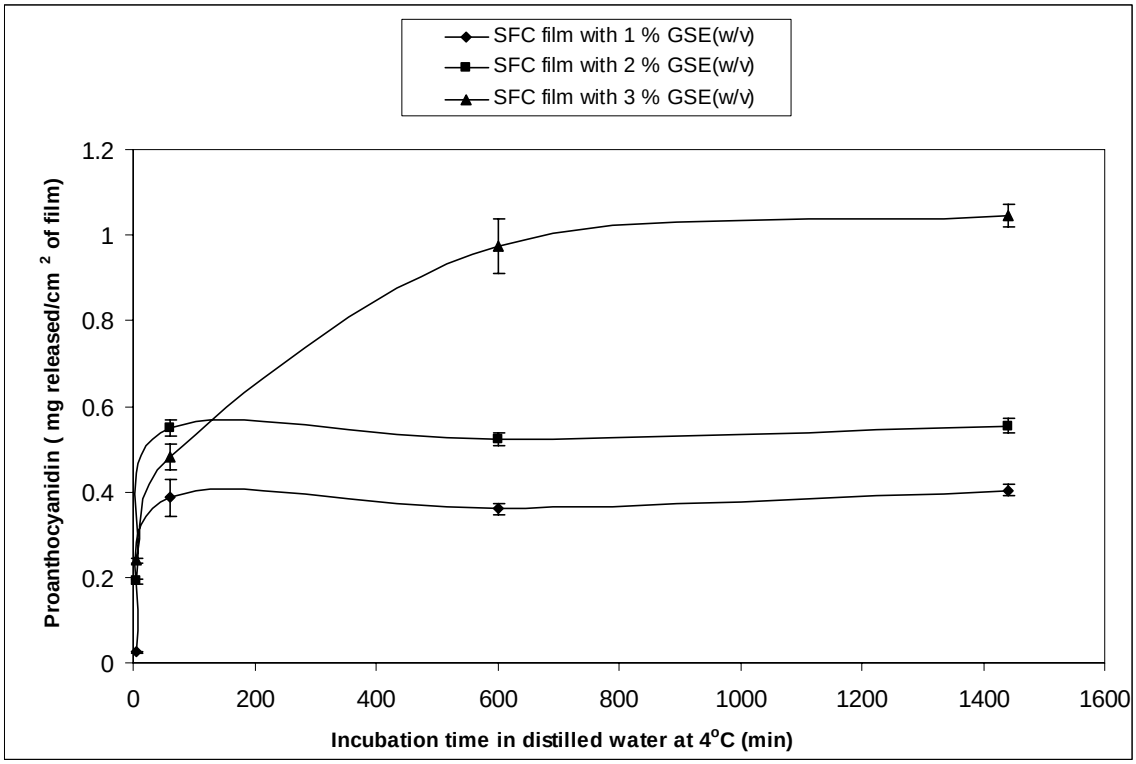

Figure 4.1. Total proanthocyanidin contents released from silk fibroin-carrageenan films incorporating different concentrations of GSE

4.3. Characterization of Silk Fibroin-Carrageenan Films Incorporated with Grape Seed Extract

Silk fibroin (SF), *Bombyx mori*, is a natural fibrous polymer. SF has become one of the most extensively studied materials among the natural biopolymers due to its non-toxicity, non-irritation and biodegradability. In addition, silk can be considered as a food material since it contains about 6 % essential amino acids. Based on the good chemical and physical properties of SF, it is possible to prepare fibroin based materials, such as films, powders, and gels. However, SF films are very brittle and unsuitable for practical use. The properties of SF films can be improved by blending SF with other natural and/or synthetic polymers (Li, et al. 2000, Bayraktar, et al. 2005, Dai, et al. 2002, Luo, et al. 2003). Proteins and polysaccharides have good film forming properties and can be used alone and in combination to form the edible films. Incorporation of the plasticizers like glycerol into the polymer matrix increases the film flexibility and reduces the intermolecular forces. The use of glycerol prevents them from cracking or chipping during their preparation, handling, and storage (Turhan, et al. 2007). In this study, edible films incorporated with grape seed extract (GSE) were prepared by using silk fibroin, carrageenan. The characterization of these films along with their *in vitro* antimicrobial activities and potential food applications were studied.

4.3.1. FT-IR Analysis

The FT-IR spectra of SFC films with and without GSE, silk fibroin film and carrageenan film were recorded to study the possible structural changes of SFC film with the addition of GSE. The possible interactions between the phenolic components of grape seed extract and biopolymers (silk fibroin and carrageenan) were also studied by means of infrared spectroscopy (Figure 4.2, 4.3 and 4.4).

In literature, characteristic bands of silk at 1655-1660 cm^{-1} for amide I (-CO- and –CN- stretching), 1531-1542 cm^{-1} for amide II (secondary -NH- de-formation and C−N stretching), 1230 cm^{-1} for amide III (-CN- stretching) were attributed to random coil (silk I conformation). On the other hand, β –sheets (silk II conformation) show characteristic bands at 1620-1630 cm^{-1} for amide I, 1515-1530 cm^{-1} for amide II and 1240 cm^{-1} for amide III (Ayutsede, et al. 2005, Freddi, et al. 1999, Wang, et al. 1997, Yamada, et al.2003, Tsuboi, et al. 2001).

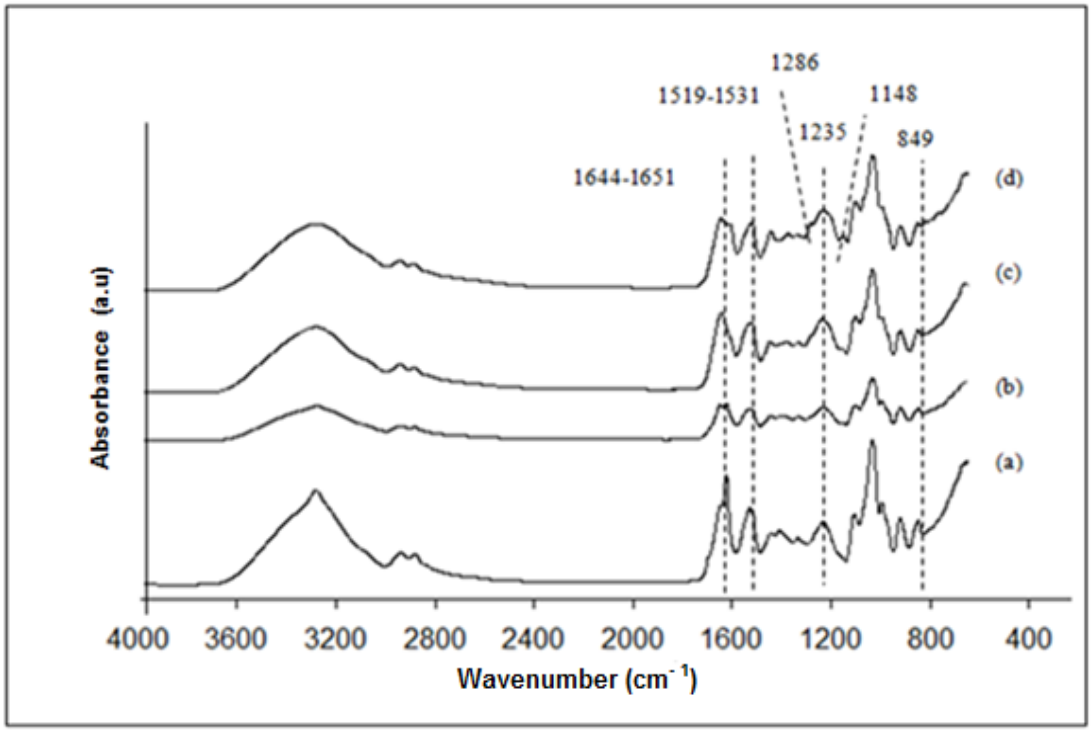

Figure 4.2. ATR-IR spectra of the casted SFC films; a) Control film (without GSE), b) SFC film with 0.5 % GSE, c) SFC film with 1 % GSE, d) SFC film with 2 % GSE

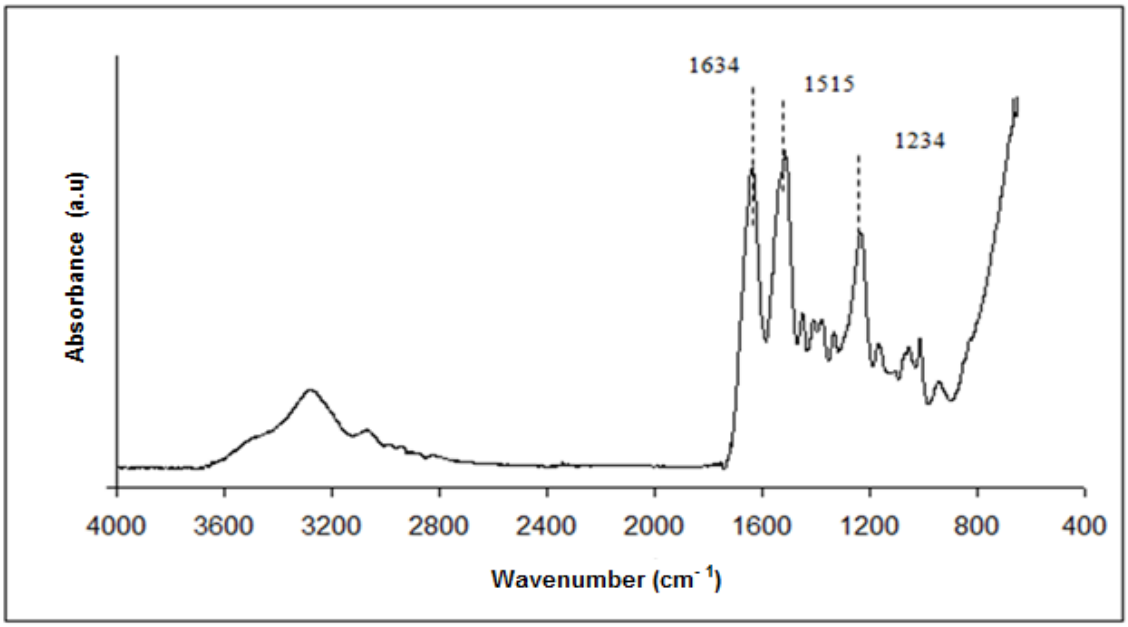

Figure 4.3. ATR-IR spectra of casted silk fibroin film

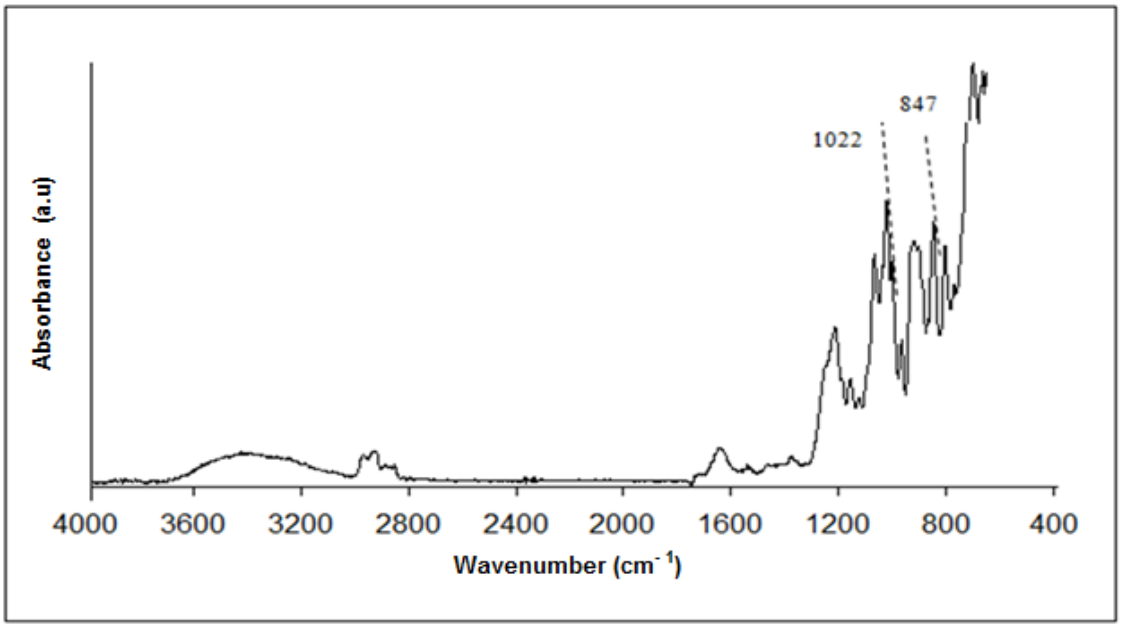

Figure 4.4. ATR-IR spectra of casted carrageenan film

In literature, for carrageenan substantial levels of sulfate ester have characteristic bands at 1230–1240 cm^{-1}. The diagnostic region (800–950 cm^{-1}) of the spectrum of the native preparation resembled that of τ-carrageenan with absorption bands at 935 cm^{-1} (attributable to AnGal residues), 855 cm^{-1} (indicative of axial sulfateester at O-4 of 3-linked Gal), and 810 cm^{-1} (indicative of axial sulfate ester at O-2 of 4-linked AnGal*p*) (Chiovitti, et al. 2004).

The general spectral pattern of the polymeric phenolic fraction and the procyanidin polymers reveals a number of peaks in common. In the literature, the peaks appear at 1520 cm^{-1}, 1449 cm^{-1}, 1341 cm^{-1}, 1287 cm^{-1}, 1233 cm^{-1}, 1157 cm^{-1}, and 1116 cm^{-1} can be contributed to polymeric phenolic fraction in GSE (Foo 1981). In this study, the peaks appeared at 1525 cm^{-1}, 1450 cm^{-1}, 1375 cm^{-1}, 1288 cm^{-1}, 1234 cm^{-1}, 1157 cm^{-1}, and 1112 cm^{-1} were therefore contributed to polymeric phenolic fraction in GSE. These results were in accordance with literature results reported previously. Silk fibroin in SFC film in the presence of GSE revealed characteristic bands at 1644-1651 cm^{-1}(amide I), 1519-1531 cm^{-1} (amide II), 1235 cm^{-1} (amide III). Fibroin in the structure of SFC film without GSE has shown characteristic bands at 1622 cm^{-1} (amide I), 1530 cm^{-1} (amide II), 1233 cm^{-1} (amide III). The appearance of strong peak of SFC film with 2 % GSE (Figure 4.2. d) at 1519 cm^{-1} represented the β-sheet dominant conformation, when compared to that of SFC film with SFC film 0.5 % (Figure 4.2. b) GSE and SFC Film 1 % GSE (Figure 4.2. c) showed 1531 cm^{-1} absorption band of the silk fibroin from of random coil conformation. The silk fibroin film showed strong absorption bands at 1634 (amide I), 1515 (amide II), and 1234 cm^{-1} (amide III), represented the β-sheet dominant conformation (Figure 4.3). Addition of GSE into SFC film caused the structural changes of fibroin from a random coil conformation to β-sheet structure. Increasing the GSE concentration further induced the formation of β-sheet structure of silk fibroin present in SFC films. Characteristic bands belonging to polymeric phenolic fractions of GSE present in SFC films were observed at 1148, 1286 cm^{-1}, this means that some of the polymeric phenolic fractions of GSE interacted with biopolymers while some of them did not. The intensities of these specific bands increased with increasing GSE content of SFC films. Nevertheless, some characteristic bands of GSE and carrageenan disappeared or shifted therefore, it might have participated in a specific interaction between GSE and biopoymers. The presence of glycerol could also induce the

occurrence of intermolecular interactions between these two biopolymers mainly due to hydrogen bonding of hydroxyl groups of glycerol and amide groups of SF (Dai, et al. 2002). Tannins are highly hydroxylated molecules and can form insoluble complexes with carbohydrates and protein (Bravo 1998).

4.3.2. X-Ray Diffraction Analysis

X-ray diffraction (XRD) analysis is a powerful technique for characterizing crystalline materials. It reveals information about the crystallinity, chemical composition, and physical properties of materials and thin films. XRD method has been mainly used to study crystalline structure, which affects various properties in solid state.

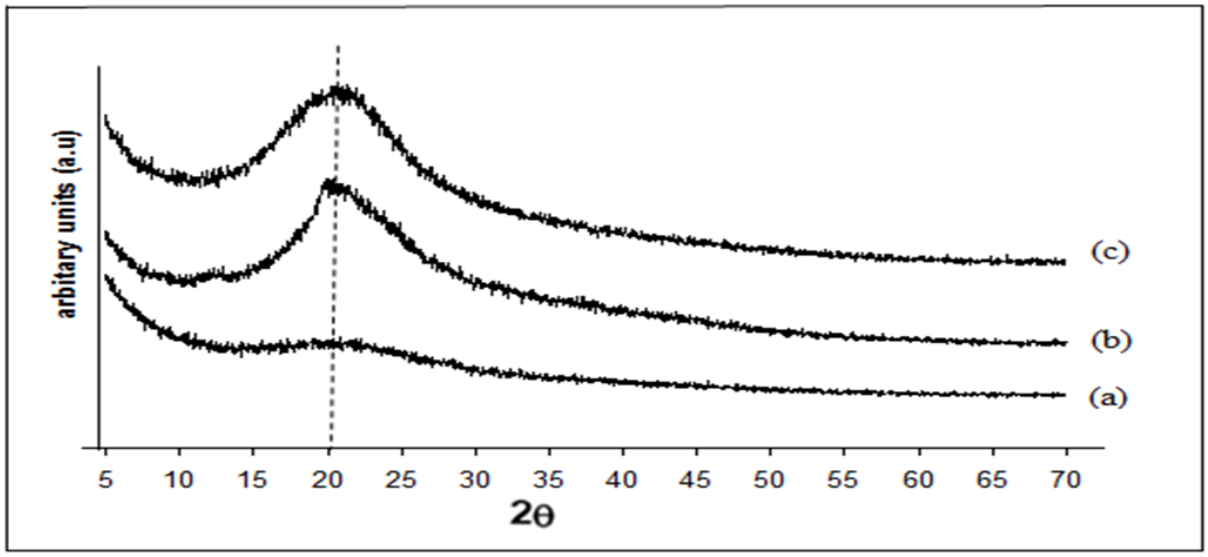

Figure 4.5. X-ray diffraction (XRD) analyses; a) Silk Fibroin Film, b) SFC Film 0.5 % GSE, c) SFC Film 2 % GSE

The main diffraction peaks of silk in literature silk I crystal are given at 12.2°, 19.7°, 24.7°, 28.2° and silk II at 9.1°, 18.9° and 20.7° (Li, et al. 2002). There is a relation between regularity of molecular structure and crystallizability. Typical crystalline polymers are chemically and geometrically regular in structure. Carrageen powder showed peaks at 2θ=13.8°, 2θ= 23.1°. SFC with 0.5 % GSE showed a peak at 2θ=21.08°. SFC film incorporated with 2 % GSE showed a peak at 2θ=21.3° (Figure 4.5). The molecular orientation and/or the crystallinity of silk fibroin can be improved by the addition of GSE. However, all the films were amorphous. The SFC film with 2 % GSE has an ordered structure compared to other films.

4.3.3. SEM Analysis

SFC films with and without GSE were casted and dried at 25°C and 40% RH. The cross section images were obtained by scanning electron microscope (SEM) (Figure 4.6). The average thicknesses of the films determined by SEM were 54.12 ± 2.96 (control film), 42.61 ± 0.98 (0.5 % GSE film), 50.15 ± 0.32 (1 % GSE film), and 43.33 ± 0.68 μm (2 % GSE film).

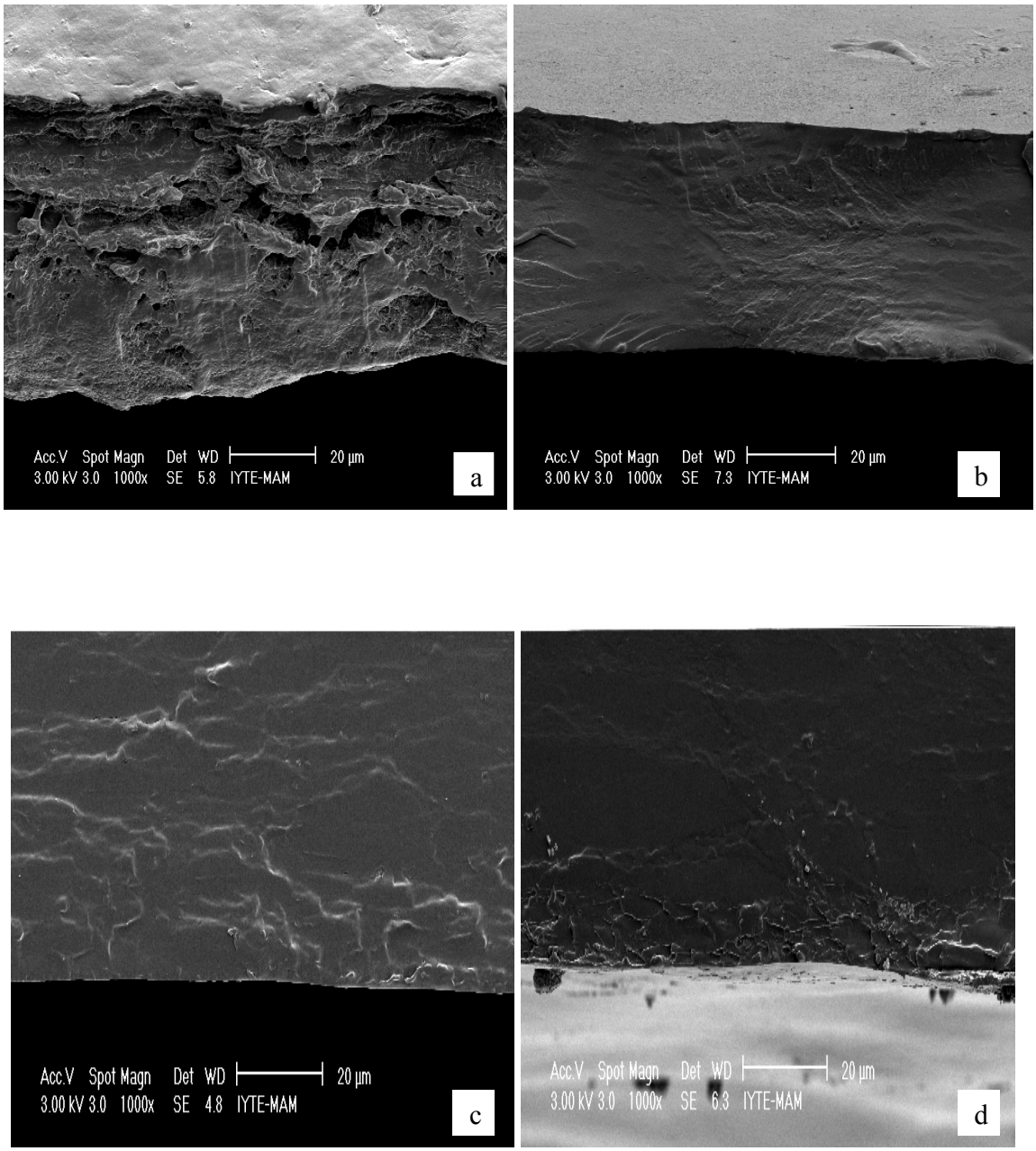

Figure 4.6. Cross section images of the SFC film with and without GSE obtained by scanning electron microscope (SEM) magnified at 1000x; a) Control film, b) 0.5 % GSE film, c) 1 % GSE film, d) 2 % GSE film

From SEM observations, it could be concluded that at high concentrations of GSE a uniform distribution was observed in blend films. Microscopic phase separation and cracks were not occurred in these blend films. SEM images of SFC films with increasing GSE concentration revealed dense film structure where as control film without GSE has shown a porous structure.

4.3.4. AFM Analysis

Surface morphologies were also examined by means of atomic force microscopy (AFM), in contact mode. The atomic force microscope is an ideal technique for quantitatively measuring the nanometer scale surface roughness. The AFM images revealed the surface roughnesses of different SFC films with and without GSE (Figure 4.7).

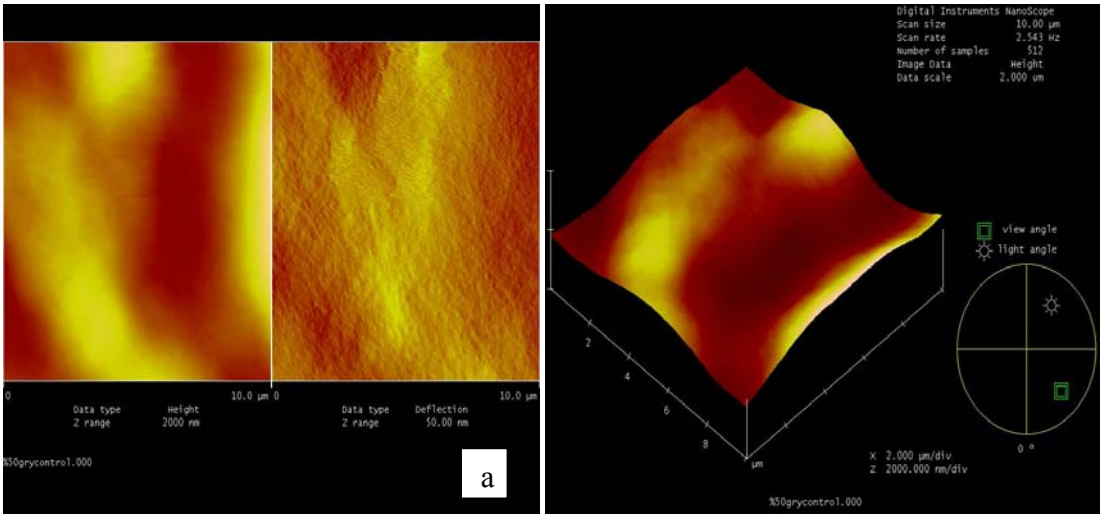

Figure 4.7. AFM images of the SFC Film with and without GSE, with deflection and 3D height view for 10x10 μm^2; a) control film

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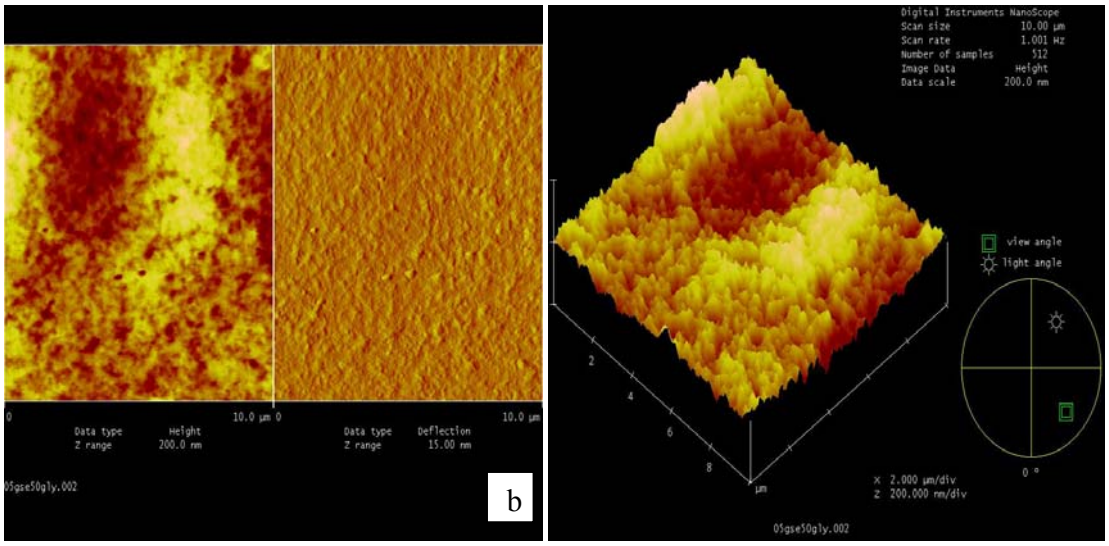

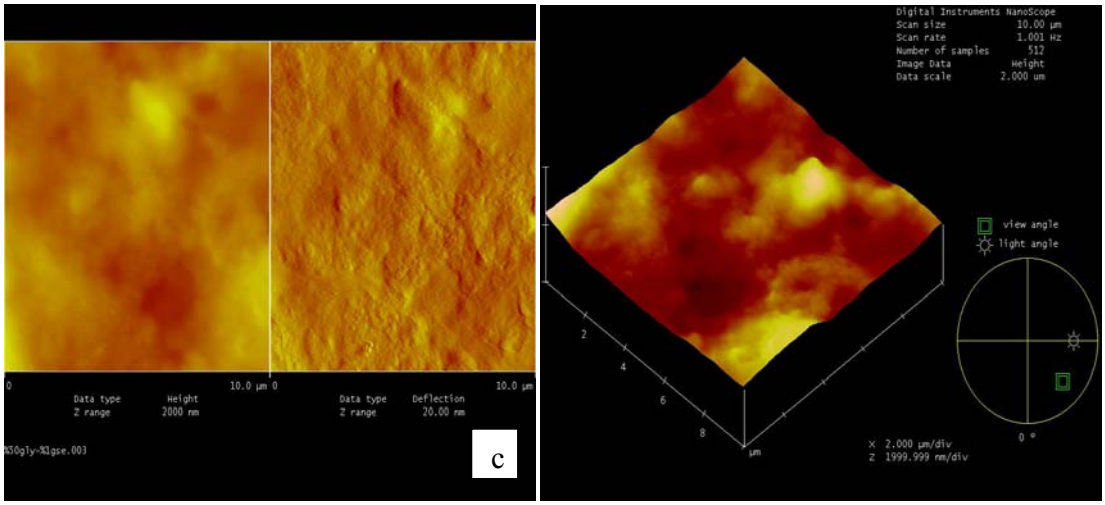

Figure 4.7. (Cont.) AFM images of the SFC Film with and without GSE, with deflection and 3D height view for 10x10 μm^2; b) 0.5 % GSE film, c) 1 % GSE film

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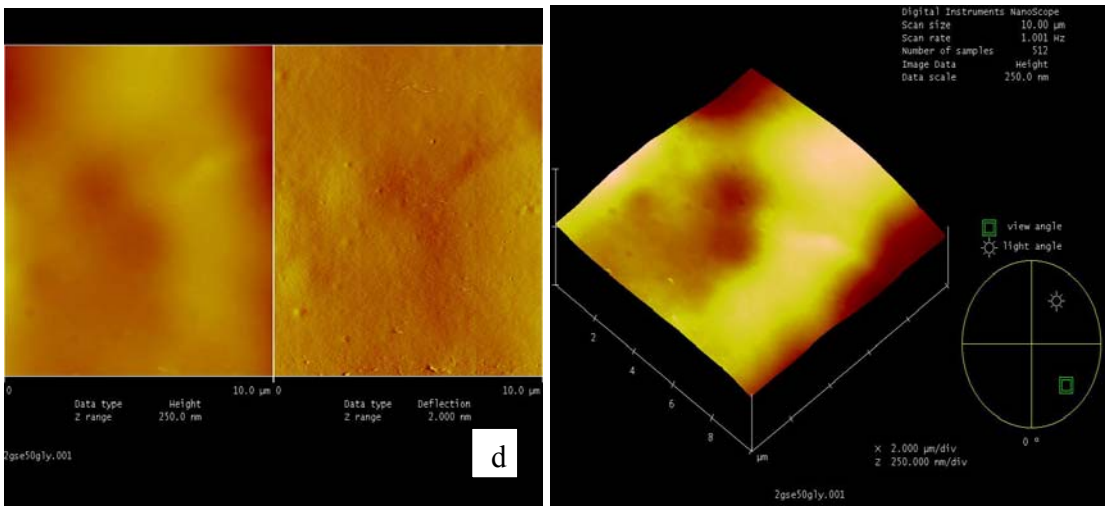

Figure 4.7. (Cont.) AFM images of the SFC Film with and without GSE, with deflection and 3D height view for 10x10 μm^2; d) 2 % GSE film

Average surface roughnesses for SFC film without GSE, SFC films with % 0.5, % 1, 2 % GSE were found to be 204, 21.25, 68.45 and115.27 nm, respectively. SFC films had a smooth texture. No crack formation was observed for the films dried at 25°C and 40% RH. AFM results were in accordance with the results obtained from SEM analysis.

4.3.5. Mechanical Properties of Films

The study of the mechanical properties is of primary importance for determining the performance of a material that is expected to undergo various kinds of stresses during use. According to the published results the pure SF film displayed the typical behavior of brittle materials, with low strength (2.1 kg/mm^2) and elongation (0.7%) values (Freddi, et al. 1999). The tensile properties of the SFC films are given in Table 4.2. The addition of GSE to SFC film is effective in inducing significant changes in the mechanical properties of the films. The addition of GSE to the SFC film, when compare to SFC without GSE film, seems to play a role in decreasing the tensile strength and Young's modulus, in contrary,

increasing the elongation of the films. SFC film having 2 % GSE had the lowest tensile strength (6.78 Pa) compared to the rest of the samples and Young's modulus of this film was 0.74 Pa. This result was in good agreement with FTIR and XRD results.

Table 4.2. Mechanical properties of SFC film with and without GSE

Films	Mechanical Properties		
	Tensile Strength (Pa)	Elongation (%)	Young's Modulus (Pa)
Control film	10.95 ± 2.0^{a}	18.95 ± 5.9^{b}	1.39 ± 0.4^{a}
%0.5 GSE Film	9.93 ± 1.2^{a}	34.58 ± 2.8^{a}	0.75 ± 0.3^{b}
1 % GSE Film	7.57 ± 1.6^{b}	44.13 ± 10.3^{a}	0.63 ± 0.3^{b}
2 % GSE Film	6.78 ± 1.8^{b}	21.25 ± 11.6^{b}	0.74 ± 0.5^{b}

[a-b]: Means having different letters within each column denote significant difference at $p<0.05$

4.4. *In Vitro* Film Antimicrobial Activity

The zone of inhibition assay on agar media was used for the determination of the antimicrobial effects of films against *L. innocua, S. carnasus, S. aureus, E. coli, E. coli* 0157:H7*, S.* Typhimurium*, B. amylaliquefaciens* and *P. fluerescens.* The results of the antimicrobial tests of SFC films incorporated with different concentrations of GSE against different bacteria are given in Tables 4.3 and 4.4.

Table 4.3. Antimicrobial effects of GSE incorporated SFC films against selected bacteria by disc diffusion test

Test Bacteria	**GSE % concentration (w/v) in film solution**	**Average area of fully formed zones (cm^2)**	**Number of fully formed zone(ffz) , negative zone (nz) and partially formed zone (pfz)**
L. innocua	-	0	12nz
	0.5% GSE	0.97±0.26	12ffz
	1% GSE	2.24±0.16	12ffz
	2% GSE	3.31±0.30	12ffz
	3% GSE	4.10±0.24	12ffz
S . aureus	-	0	12nz
	0.5% GSE	0.14±0.03	4ffz /8pfz
	1% GSE	0.34±0.10	12ffz
	2% GSE	0.94±02.0	12ffz
	3% GSE	2.26±0.25	12ffz
S . carnasus	-	0	12nz
	0.5% GSE	0	12nz
	1% GSE	1.01.±0.21	12ffz
	2% GSE	2.14±0.30	12ffz
	3% GSE	2.32±0.26	12ffz
B. amylaliquefaciens	-	0	12nz
	0.5% GSE	0	12nz
	1% GSE	0	12nz
	2% GSE	1.14±0.16	12ffz
	3% GSE	1.63±0.19	12ffz

Table 4.4. Antimicrobial effects of GSE and/or disodium EDTA incorporated SFC films against selected bacteria by disc diffusion test

Test Bacteria	GSE % concentration (w/v) in film solution	Disodium EDTA (w/ v)	Average area of fully formed zones (cm^2	Number of fully formed zone(ffz) , negative zone (nz) and partially formed zone (pfz)
P. fluorescens	-	200	0	12nz
	0.5% GSE	200	0	12nz
	1% GSE	200	0	12nz
	2% GSE	200	0.93±0.24	12ffz
	3% GSE	200	1.53±0.16	12ffz

SFC films having different concentrations of GSE were tested against selected microorganisms for the zone of inhibition area. The zone of inhibition areas for the films against microorganisms are shown in Figure 4.8. Gram-positive bacteria, including *S. carnasus, S. aureus, L. innocua, B. amylaliquefaciens* were more susceptible to GSE than the Gram-negative bacteria *P. fluerescens.* Higher molecular weight polyphenols were found to be more inhibitory than lower molecular weight polyphenols and GSE was more effective against Gram-positive bacteria than Gram-negative bacteria. The active compound for the inhibition of *E. coli* and *Salmonella enteritidis* was identified as gallic acid. Structural activity of correlation assays revealed that three hydroxyl groups of the compounds were effective for antibacterial activity and all the substituents of the benzene rings were effective against *S. aureus*. The wide antimicrobial spectrum might also explain the difference in extraction solvent concentration and the concentration of phenolic compounds extracted (Jayaprakasha, et al. 2003). Ahn et al. (2004) found that the commercial GSE (ActiVin) had antimicrobial activity against *L. monocytogenes, E. coli,* and *S.* Typhimurium. *L .innocua* was found to be more sensitive than *P. fluorescens.* SFC films having 200µg (w/v) disodium EDTA and without disodium EDTA were used in order to test the antimicrobial effects of GSE against Gram–negative bacteria. The zone of

inhibition was not observed at any GSE concentration against *E. coli, E. coli* 0157:H7 and *S.* Typhimurium. However, the zone of inhibition was observed at SFC film with 2 % GSE + Na_2EDTA and 3% GSE + Na_2EDTA concentration against *P. fluorescens.* Gram–positive bacteria have a different cell envelope when compared to Gram-negative bacteria. Gram-positive bacteria have an inner cell membrane consisting of a lipid bilayer, and an outer cell wall consisting of peptideoglycan and lack of outer membrane. Gram-negative bacteria have an inner cell membrane and an outer cell wall containing peptideoglycan and outer membrane composed of lipopolysaccharide, lipoprotein, and other macromolecules (Lambert 2002). Sivarooban et al. (2008) reported that the GSE, nisin and EDTA incorporated soy protein edible film was effective to variable degrees in inhibiting the growth of *L. monocytogenes, E. coli* and *S.* Typhimurium. The GSE had high total phenolics. The extent of the inhibitory effects of grape seed extract could be attributed to their phenolic composition (Baydar, et al. 2006). Baydar et al. (2004) also determined that the GSE had antimicrobial activities against fourteen bacteria (*A. hydrophila, B. brevis, B. cereus, B. megaterium, B. subtilis, E. faecalis, E. coli, K. pneumoniae, L. monocytogenes, M. smegmatis, P. vulgaris, P. aeruginosa, S. aureus, E. aerogenes*). Gram-positive bacteria such as *S. aureus, B. cereus and B. subtilis* were inhibited more easily than the Gram-negative ones such as *P. aeruginosa* and *E. coli.* The highest antimicrobial effect was found against *L. monocytogenes*. Phenolics were the most important compounds active against bacteria and gallic acid as the most active compound for inhibition of bacteria. The effects of GSE on the bacteria depend on the concentration of GSE and bacterial species.

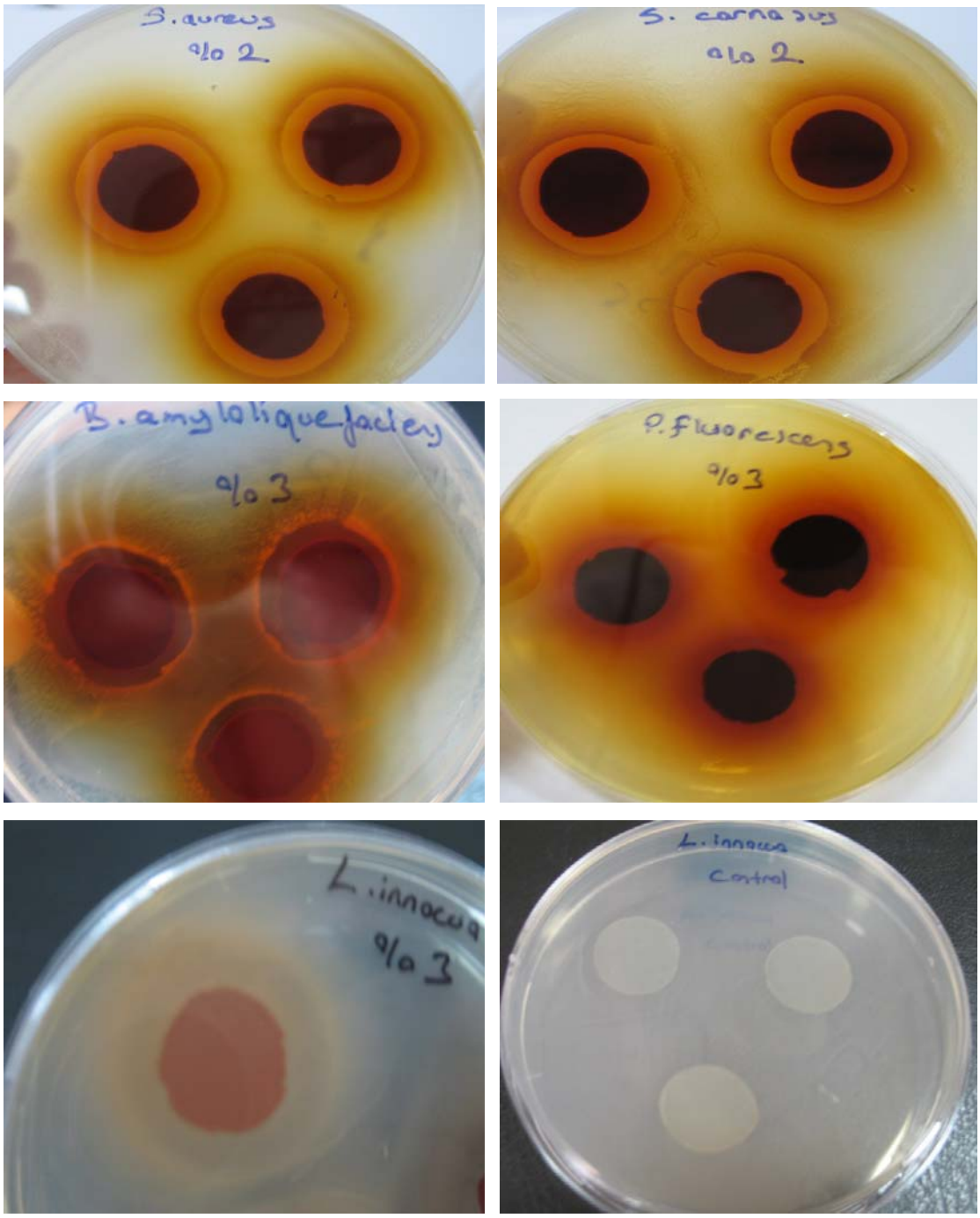

Figure 4.8. Antimicrobial activity of SFC film incorporated with different concentrations of GSE

4.5. Antimicrobial Activity of Silk Fibroin-Carrageenan Films on Raw Chicken Breast Meat

Microorganisms have unfavorable effects on safety, quality and shelf life of foods. The use of antimicrobial additives into the packaging materials is one of the procedures employed to prevent spoilage. For this reason, SFC films having GSE as an antimicrobial agent were developed to enhance the safety of raw chicken breast meat. Antimicrobial activity of SFC films incorporated with 2 % and 3 % GSE (w/v) with/without 200 μg/cm^2 (w/v) disodium EDTA on total viable bacteria count (TVC) (Figure 4.9), total coliform count (TCC) (Figure 4.10), and lactic acid bacteria count (LAB) (Figure 4.11) of raw chicken breast meat was investigated. The microbial growth has decreased for the samples coated with the films incorporating GSE compared to control samples. However, the total viable counts for all samples exceeded the spoilage limit which was 10^6-10^7 CFU/g. Different concentrations of GSE showed significant effect on total viable, coliform and lactic acid bacteria counts of raw chicken breast meat treated with different concentrations of GSE during 5 days of storage at 4 ºC. During storage total viable, coliform, and lactic acid bacteria counts were decreased significantly about approximately 1-1.5, 0.4-0.5 and 0-0.3 log (at day 5), respectively ($p<0.05$) (Tables 4.5, 4.6, and 4.7). The results clearly indicated that using of 2 % GSE, 2 % GSE and EDTA, 3% GSE and 3% GSE and EDTA had the beneficial effect in controlling the microbial load of raw chicken breast meat during 5 days of storage at 4ºC. The pH of the samples varied from 6.93±0.04 to 6.05±0.07.

Table 4.5. Total viable counts of raw chicken breast meat coated with different SFC films

Total Viable Count (log CFU/g)				
	Storage Time at 4°C (days)			
Treatments	**0**	**1**	**3**	**5**
Uncoated raw chicken breast meat				
	4.86±0.15 ^{A}	6.41±0.22 ^{B,b}	8.23±0.13 ^{C,b}	10.11±0.10 ^{D,b}
Raw chicken breast meat coated with SFC film				
		6.07±0.04 ^{B,b}	8.11±0.02 ^{C,b}	10.02±0.04 ^{D,b}
Raw chicken breast meat coated with SFC film incorporated with Na_2EDTA				
		6.02±0.04 ^{B,cb}	7.90±0.06 ^{C,cb}	9.19±0.03 ^{D,cb}
Raw chicken breast meat coated with SFC film incorporated with 2 % GSE				
		5.67±0.1 ^{B,ac}	7.65±0.5 ^{C,ac}	9.01±0.07 ^{D,ac}
Raw chicken breast meat coated with SFC film incorporated with 2 % GSE and Na_2EDTA				
		5.70±0.05 ^{B,ac}	7.70±0.06 ^{C,ac}	8.70±0.13 ^{D,ac}
Raw chicken breast meat coated with SFC film incorporated with 3 % GSE				
		5.68±0.03 ^{B,ac}	7.62±0.03 ^{C,ac}	8.91±0.02 ^{D,ac}
Raw chicken breast meat coated with SFC film incorporated with 3 % GSE and Na_2EDTA				
		5.58±0.1 ^{B,a}	7.59±0.05 ^{C,a}	8.52±0.55 ^{D,a}

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

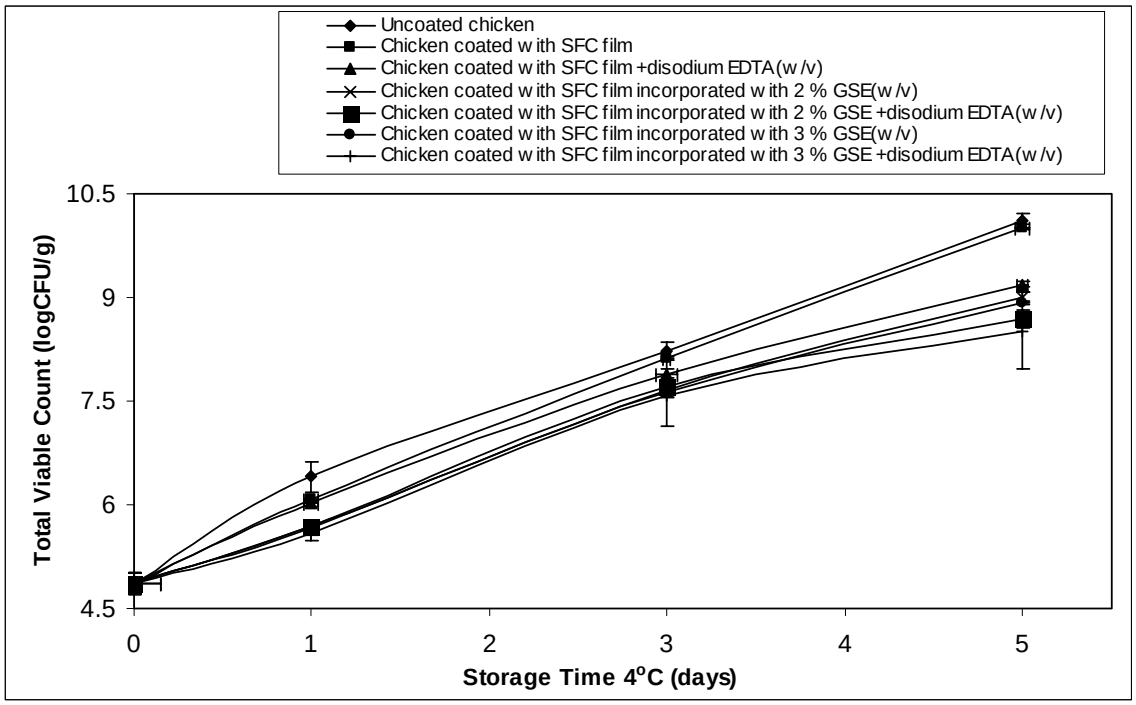

Figure 4.9. Total viable counts of raw chicken breast meat coated with different SFC films

Table 4.6. Total coliform counts of raw chicken breast meat coated with different SFC films

Coliform Counts (log CFU/g)				
	Storage Time at 4ºC (days)			
Treatments	**0**	**1**	**3**	**5**
Uncoated raw chicken breast meat	3.04±0.14 ^{A,b}	5.12±0.18 ^{B,b}	5.98±0.09 ^{C,b}	8.23±0.02 ^{D,b}
Raw chicken breast meat coated with SFC film		4.98±0.09 ^{B,bc}	5.93±0.18 ^{C,bc}	7.99±0.05 ^{D,bc}
Raw chicken breast meat coated with SFC film incorporated with Na_2EDTA		4.40±0.55 ^{B,ac}	5.91±0.2 ^{C,ac}	7.81±0.06 ^{D,ac}
Raw chicken breast meat coated with SFC film incorporated with 2 % GSE		4.63±0.09 ^{B,ac}	5.89±0.09 ^{C,ac}	7.80±0.02 ^{D,ac}
Raw chicken breast meat coated with SFC film incorporated with 2 % GSE and Na_2EDTA		4.48±0.14 ^{B,a}	5.81±0.06 ^{C,a}	7.75±0.04 ^{D,a}
Raw chicken breast meat coated with SFC film incorporated with 3 % GSE		4.45±0.18 ^{B,a}	5.5±0.08 ^{C,a}	7.68±0.1 ^{D,a}
Raw chicken breast meat coated with SFC film incorporated with 3 % GSE and Na_2EDTA		4.41±0.2 ^{B,a}	5.5±0.04 ^{C,a}	7.80±0.03 ^{D,a}

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

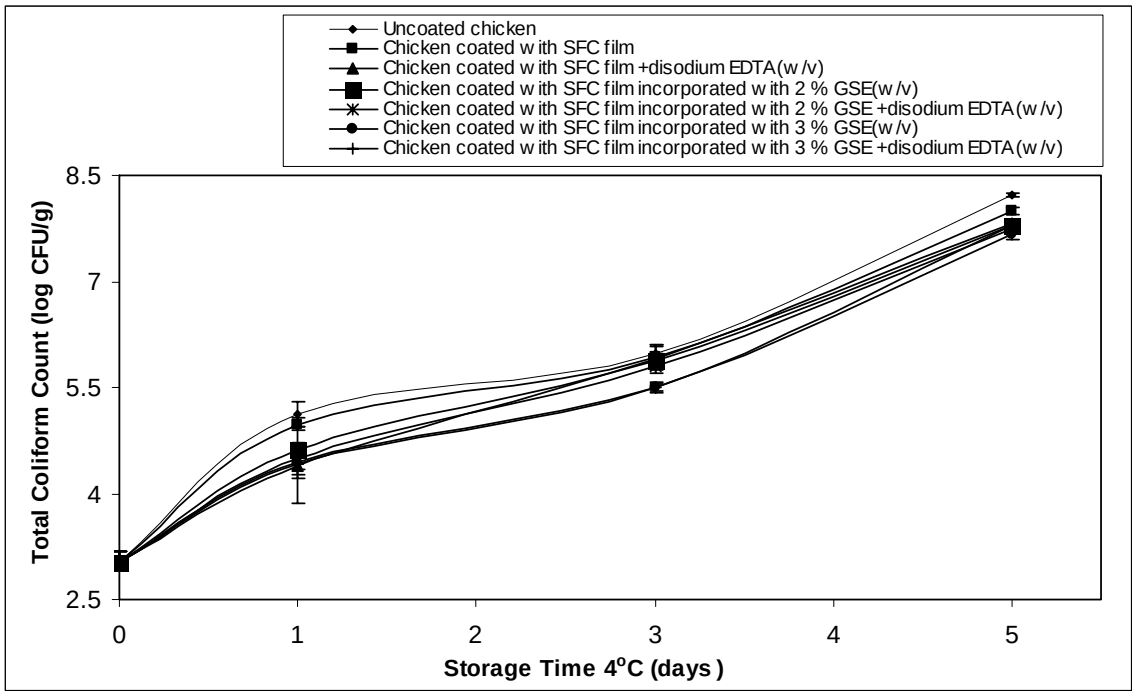

Figure 4.10. Total coliform counts of raw chicken breast meat coated with different SFC films

Table 4.7. Lactic acid bacteria counts of raw chicken breast meat coated with different SFC films

Lactic Acid Bacteria Counts (log CFU/g)				
	Storage Time at 4ºC (days)			
Treatments	**0**	**1**	**3**	**5**
Uncoated raw chicken breast meat	3.81±0.12 ^{A,cb}	3.82±0.11 ^{B,cb}	3.77±0.18 ^{B,cb}	3.79±0.06 ^{B,cb}
Raw chicken breast meat coated with SFC film		3.61±0.37 ^{B,ab}	3.71±0.04 ^{B,ab}	3.39±0.26 ^{B,ab}
Raw chicken breast meat coated with SFC film incorporated with Na_2EDTA		3.75±0.11 ^{B,cb}	3.78±0.07 ^{B,cb}	3.6±0.26 ^{B,cb}
Raw chicken breast meat coated with SFC film incorporated with 2 % GSE		3.28±0.3 ^{B,a}	3.27±0.03 ^{B,a}	3.49±0.01 ^{B,a}
Raw chicken breast meat coated with SFC film incorporated with 2 % GSE and Na_2EDTA		3.56±0.31 ^{B,b}	3.75±0.06 ^{B,b}	3.80±0.03 ^{B,b}
Raw chicken breast meat coated with SFC film incorporated with 3 % GSE		3.25±0.02 ^{B,ba}	3.59±0.04 ^{B,ba}	3.58±0.28 ^{B,ba}
Raw chicken breast meat coated with SFC film incorporated with 3 % GSE and Na_2EDTA		3.27±0.34 ^{B,ab}	3.25±0.26 ^{B,ab}	3.64±0.33 ^{B,ab}

a-c : Means having different letters within each treatment denote significant difference at $p<0.05$.
A-B: Means having different letters within each storage time (row) denote significant difference at $p<0.05$.
Data are mean values ± S.D. (n=3)

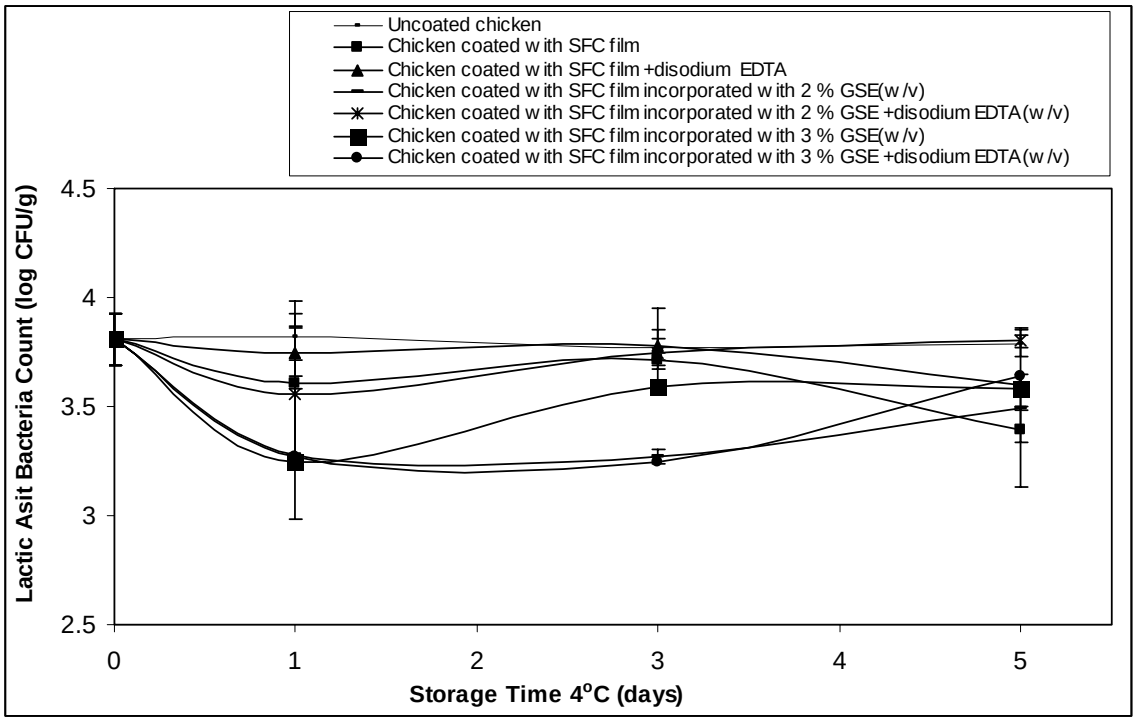

Figure 4.11. Lactic acid bacteria counts of raw chicken breast meat coated with different SFC films

4.6. Antimicrobial Activity of Silk Fibroin-Carrageenan Films on Beef Sausages

Pathogens such as *Staphylococcus aureus*, *Salmonella* spp. and *Escherichia coli* are frequently found in meat products. *S. aureus* is considered the second or third most common pathogen causing outbreaks of food poisoning, after *Salmonella* and *Clostridium perfringens*. Meat preparations are exposed to microbiological risk due to their chemical-physical characteristics. *Staphylococcus aureus* is frequently found in fermented sausages and in raw meat at low levels. Above 10^5 CFU/g *Staphylococcus aureus* enterotoxins produced are sufficient to cause food poisoning. In Spain, *S. aureus* was the third cause of

food-borne disease outbreaks from 1993 to 1998, responsible for 228 outbreaks out of a total 5517 detected. *S. aureus* is poor competitor under anaerobic conditions, at low pH values and low temperatures (Ananou, et al. 2005).

Antimicrobial activity of SFC films incorporated with 2 % and 3 % GSE (w/v) on *S. aureus* (Figure 4.12) inoculated beef sausages was investigated during 28 days of storage at 4 °C. To exert antimicrobial activity, GSE should be released on the surface of beef sausages and inhibit the growth of the bacteria. Release of GSE from the film was confirmed by the inhibition of *S.aureus* by *in vitro* studies and total proanthocyanidin content determined during release tests. Results demonstrated a significant reduction ($p<0.05$) of *S. aureus* level coated with SFC film containing GSE after 28 days of storage. The pH of the samples varied from 6.39±0.014 to 6.68±0.021.

Table 4.8. *S. aureus* counts on the beef sausages coated with different SFC films incorporated with GSE during 28 days of storage

	***S. aureus* (log CFU/g)**				
	Storage Time at 4ºC (days)				
Treatments	**0**	**7**	**14**	**21**	**28**
Uncoated sausage without inoculum					
	$0^{A,c}$	$0^{B,c}$	$0^{B,c}$	$0^{C,c}$	$0^{D,c}$
Uncoated sausage with inoculum					
	$4.89\pm0.16^{A,b}$	$4.43\pm0.09^{B,b}$	$4.39\pm0.15^{B,b}$	$3.51\pm0.04^{C,b}$	$3.09\pm0.06^{D,b}$
Sausage coated with SFC film with inoculum					
	$4.89\pm0.1^{A,b}$	$4.32\pm0.06^{B,b}$	$4.11\pm0.06^{B,b}$	$3.34\pm0.08^{C,b}$	$3.05\pm0.03^{D,b}$
Sausage coated with SFC film incorporated with 2% GSE with inoculum					
	$4.65\pm0.02^{A,ab}$	$4.28\pm0.01^{B,ab}$	$3.99\pm0.04^{B,ab}$	$3.29\pm0.09^{C,ab}$	$2.90\pm0.08^{D,ab}$
Sausage coated with SFC film incorporated with 3% GSE with inoculum					
	$4.64\pm0.003^{A,a}$	$4.09\pm0.02^{B,a}$	$3.71\pm0.04^{B,a}$	$3.17\pm0.06^{C,a}$	$2.67\pm0.01^{D,a}$

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

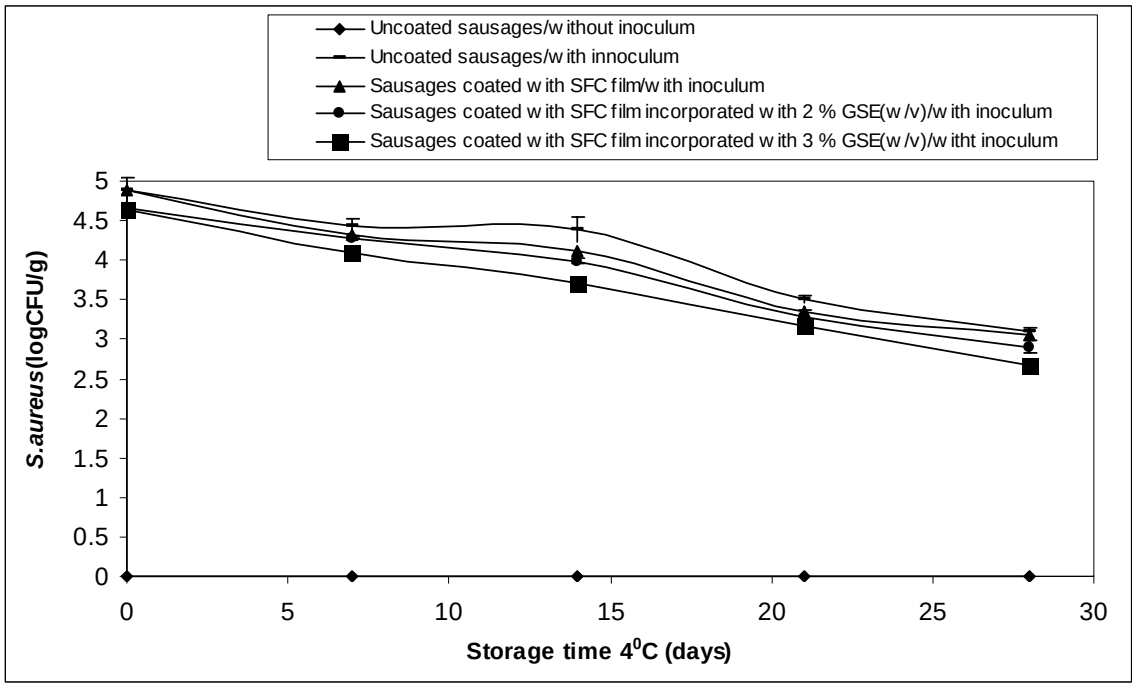

Figure 4.12. *S.aureus* counts on the beef sausages coated with different SFC films incorporated with GSE during 28 days of storage

4.7. Moisture Analysis of Beef Sausages

The moisture content of beef sausages coated with SFC films incorporating GSE was investigated. Initial moisture content was about 64.36 % and it decreased during the 28 days of storage. SFC films incorporating GSE are effective on moisture content of sausage ($p<0.05$). The moisture contents during storage are given in Table 4.9 and Figure 4.13.

Table 4.9. Moisture content of beef sausages treated with different SFC film coatings incorporating GSE during 28 days of storage

Moisture Content (%)					
	Storage Time at 4ºC (days)				
Treatments	**0**	**7**	**14**	**21**	**28**
Uncoated sausage	64.36±0.30 ^{A,b}	65.29±0.3 ^{A,b}	63.69±0.69 ^{A,b}	63.63±0.14 ^{A,b}	60.86±0.07 ^{B,b}
Sausage coated with SFC film	67.91±0.52 ^{A,a}	68.78±0.06 ^{A,a}	67.71±0.72 ^{A,a}	68.40±0.6 ^{A,a}	66.03±1.11 ^{B,a}
Sausage coated with SFC film incorporated with 2 % GSE	68.84±0.95 ^{A,a}	69.01±0.83 ^{A,a}	69.58±0.01 ^{A,a}	69.48±0.33 ^{A,a}	66.42±1.02 ^{B,a}
Sausage coated with SFC film incorporated with 3 % GSE	67.99±1.8 ^{A,a}	71.33±3.8 ^{A,a}	68.21±1.15 ^{A,a}	68.57±1.11 ^{A,a}	66.35±2.74 ^{B,a}

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

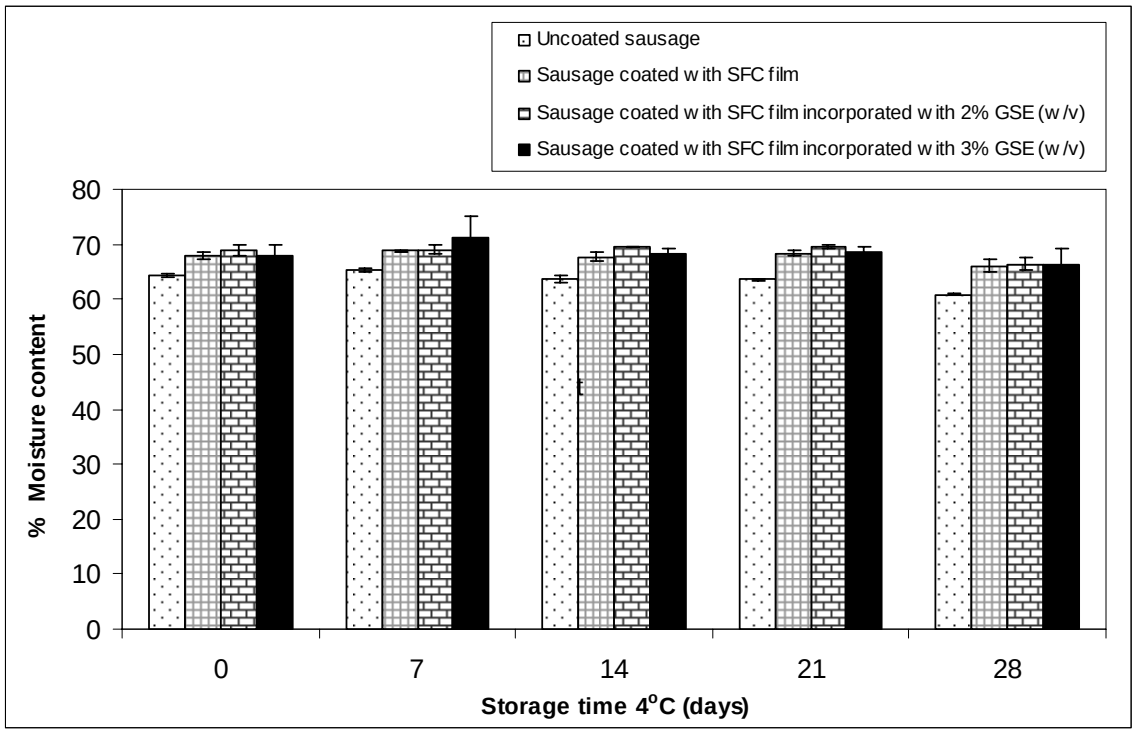

Figure 4.13. Moisture contents of beef sausages coated with different SFC films incorporating GSE during 28 days of storage.

4.8. Texture Profile Analysis of Beef Sausages

The texture profile analysis of beef sausages coated with different SFC films incorporating GSE was performed. The results of the texture profile analysis are given in (Tables 4.10, 4.11, 4.12, 4.13, and 4.14). It was found that storage time and GSE concentration x storage time had a significant effect on hardness of beef sausages ($p<0.05$). GSE, storage time and GSE x storage time interactions had a significant effect on cohesiveness, gumminess and chewiness of beef sausages ($p<0.05$), time is only effective on springiness of sausages ($p<0.05$). Increased level of GSE had significant effect on cohesiveness, gumminess, and chewiness.

Table 4.10. Hardness (N) of beef sausages coated with different SFC films incorporating GSE during 28 days of storage

Texture Profile Analysis / Hardness (N)					
	Storage Time at 4ºC (days)				
Treatments	**0**	**7**	**14**	**21**	**28**
Uncoated sausage					
	31.16±10.98^{A}	25.36±7.86 ^{A}	19.35±5.63^{AB}	9.40±5.21 ^{AB}	5.93±2.01^{B}
Sausage coated with SFC film					
	18.16±6.20 ^{A}	12.39±4.2 ^{A}	12.99±3.96^{AB}	17.54±4.43^{AB}	15.07±4.67 ^{B}
Sausage coated with SFC film incorporated with 2 % GSE					
	15.62±5.69 ^{A}	21.99±5.49^{A}	17.95±2.33^{AB}	20.74±6.21^{AB}	17.29±3.68^{B}
Sausage coated with SFC film incorporated with 3 % GSE					
	17.37±6.43 ^{A}	16.41±3.63 ^{A}	13.00±2.03^{AB}	17.42±7.63^{AB}	17.49±5.43 ^{B}

$^{A-B}$: Means having different letters within each storage time (row) denote significant difference at $p<0.05$. Data are mean values ± S.D. (n=5)

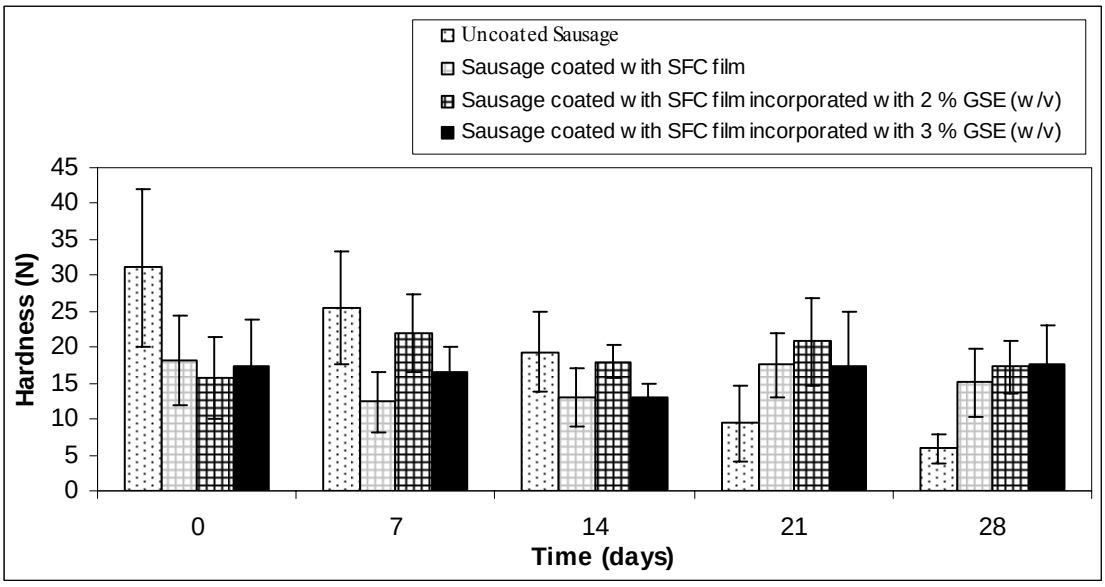

Figure 4.14. Hardness (N) of beef sausages coated with different SFC films incorporating GSE during 28 days of storage

Table 4.11. Cohesiveness of beef sausages coated with different SFC films incorporating GSE during 28 days of storage

Texture Profile Analysis / Cohesiveness					
	Storage Time at 4ºC (days)				
Treatments	**0**	**7**	**14**	**21**	**28**
Uncoated sausage	$0.73\pm0.29^{A,b}$	$0.69\pm0.045^{B,b}$	$0.77\pm0.077^{AD,b}$	$0.80\pm0.16^{C,b}$	$0.72\pm0.13^{CD,b}$
Sausage coated with SFC film	$0.42\pm0.22^{A,a}$	$0.31\pm0.27^{B,a}$	$0.59\pm0.10^{AD,a}$	$0.68\pm0.07^{C,a}$	$0.68\pm0.02^{CD,a}$
Sausage coated with SFC film incorporated with 2 % GSE	$0.37\pm0.18^{A,a}$	$0.21\pm0.098^{B,a}$	$0.46\pm0.14^{AD,a}$	$0.72\pm0.036^{C,a}$	$0.64\pm0.069^{CD,a}$
Sausage coated with SFC film incorporated with 3 % GSE	$0.37\pm0.19^{A,a}$	$0.13\pm0.12^{B,a}$	$0.47\pm0.216^{AD,a}$	$0.66\pm0.07^{C,a}$	$0.70\pm0.04^{CD,a}$

a-b : Means having different letters within each treatment denote significant difference at $p<0.05$.
A-D: Means having different letters within each storage time (row) denote significant difference at $p<0.05$.
Data are mean values ± S.D. (n=5

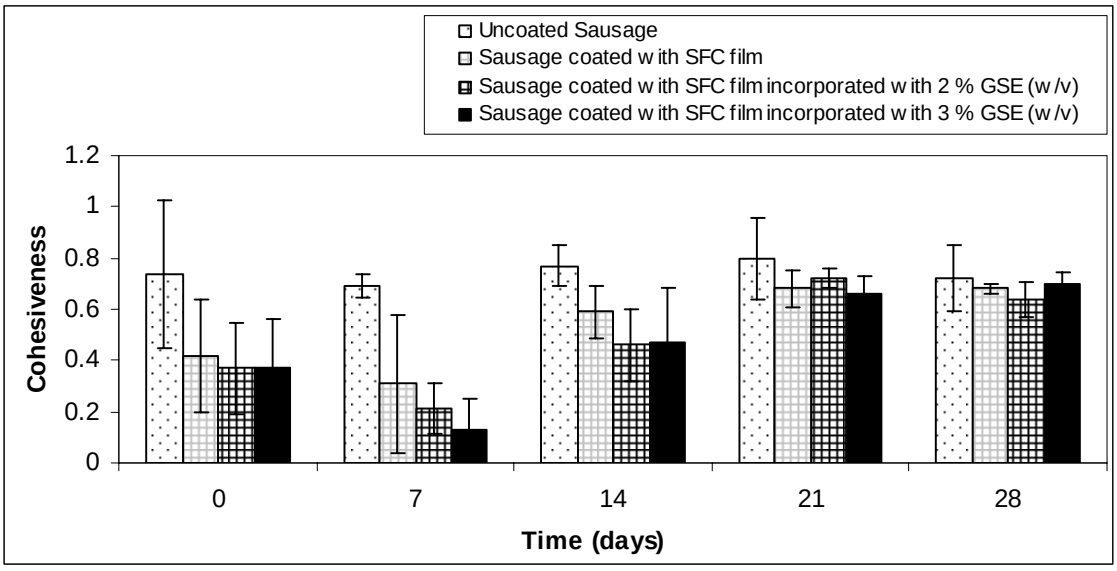

Figure 4.15. Cohesiveness of beef sausages coated with different SFC films incorporating GSE during 28 days of storage

Table 4.12. Springiness of beef sausages coated with different SFC films incorporating GSE during 28 days of storage

Texture Profile Analysis / Springiness					
	Storage Time at 4ºC (days)				
Treatments	**0**	**7**	**14**	**21**	**28**
Uncoated sausage					
	0.96±0.66^{AB}	1.01±0.02 ^{A}	0.99±0.022 ^{AB}	1.00±0.02 ^{B}	1.00±0.035 ^{B}
Sausage coated with SFC film					
	1.00±0.03^{AB}	0.98±0.04 ^{A}	0.98±0.011 ^{AB}	1.00±0.02 ^{B}	0.98±0.01 ^{B}
Sausage coated with SFC film incorporated with 2 % GSE					
	0.98±0.05^{AB}	0.93±0.08 ^{A}	1.003±0.02 ^{AB}	0.99±0.01 ^{B}	1.00±0.011 ^{B}
Sausage coated with SFC film incorporated with 3 % GSE					
	1.021±0.01^{AB}	0.91±0.07 ^{A}	1.01±0.02 ^{AB}	1.00±0.016 ^{B}	0.99±0.010 ^{B}

$^{A-B:}$ Means having different letters within each storage time (row) denote significant difference at $p<0.05$. Data are mean values ± S.D. (n=5)

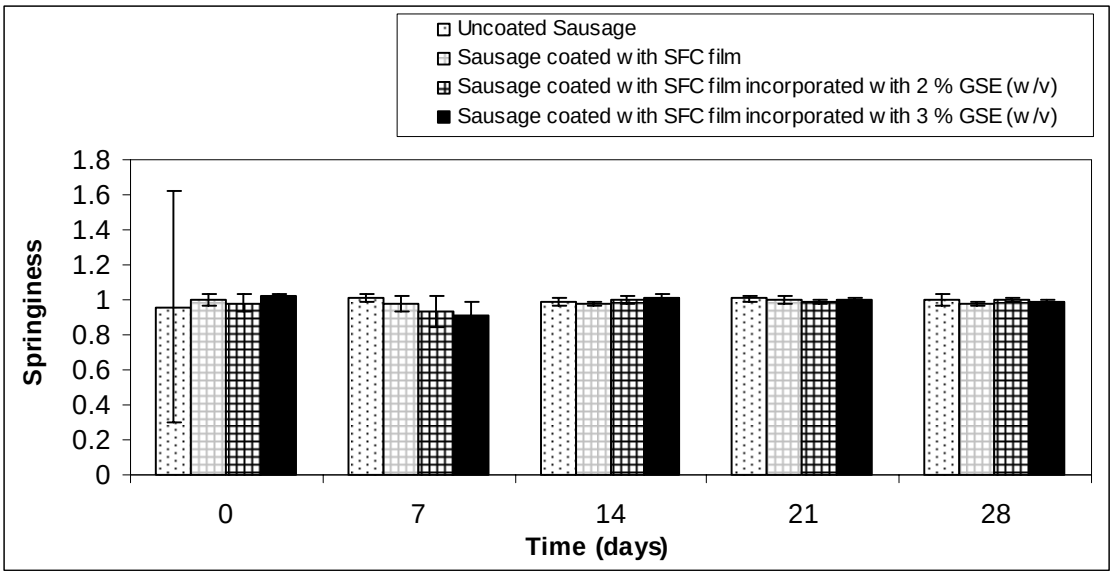

Figure 4.16. Springiness of beef sausages coated with different SFC films incorporating GSE during 28 days of storage

Table 4.13. Gumminess (N) of beef sausages coated with different SFC films incorporating GSE during 28 days of storage

Texture Profile Analysis / Gumminess (N)					
	Storage Time at 4ºC (days)				
Treatments	**0**	**7**	**14**	**21**	**28**
Uncoated sausage	$20.77\pm7.01^{AB,b}$	$17.62\pm5.52^{A,b}$	$15.07\pm5.53^{AB,b}$	$8.48\pm4.98^{B,b}$	$4.27\pm1.66^{AB,b}$
Sausage coated with SFC film	$8.48\pm6.05^{AB,a}$	$4.61\pm4.61^{A,a}$	$7.95\pm3.47^{AB,a}$	$11.96\pm2.78^{B,a}$	$10.36\pm3.28^{AB,a}$
Sausage coated with SFC film incorporated with 2 % GSE	$5.85\pm3.82^{AB,a}$	$5.05\pm3.00^{A,a}$	$8.36\pm2.55^{AB,a}$	$14.93\pm4.07^{B,a}$	$11.21\pm2.63^{AB,a}$
Sausage coated with SFC film incorporated with 3 % GSE	$7.47\pm5.99^{AB,a}$	$2.23\pm2.55^{A,a}$	$6.30\pm3.17^{AB,a}$	$11.89\pm5.61^{B,a}$	$12.20\pm3.80^{AB,a}$

a-b : Means having different letters within each treatment denote significant difference at $p<0.05$.
A-B: Means having different letters within each storage time (row) denote significant difference at $p<0.05$.
Data are mean values ± S.D. (n=5)

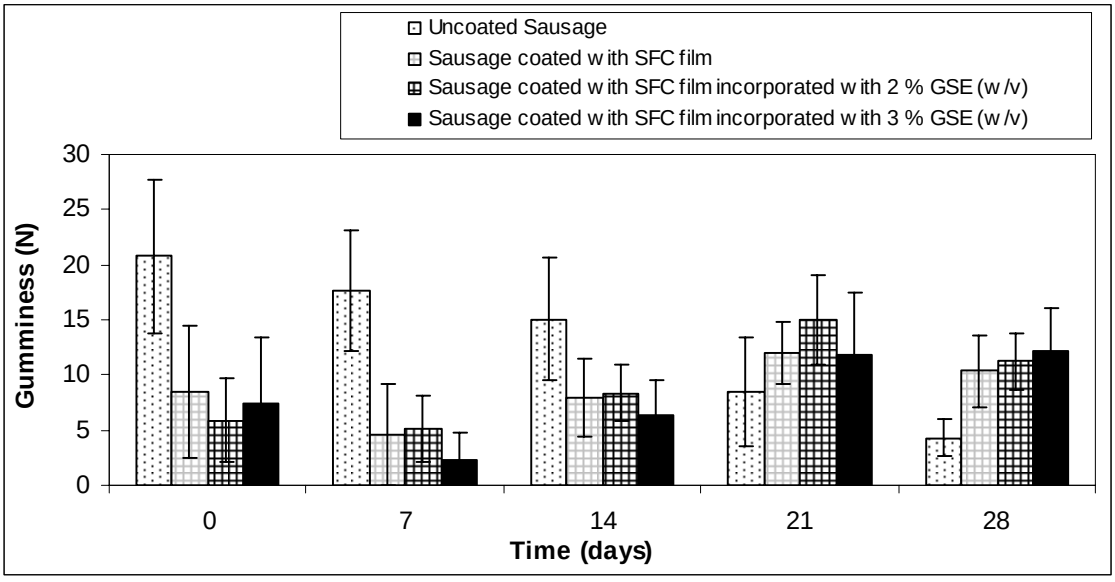

Figure 4.17. Gumminess (N) of beef sausages coated with different SFC films incorporating GSE during 28 days of storage

Table 4.14. Chewiness (N) of beef sausages coated with different SFC films incorporating GSE during 28 days of storage

Texture Profile Analysis / Chewiness (N)					
	Storage Time at 4ºC (days)				
Treatments	**0**	**7**	**14**	**21**	**28**
Uncoated sausage					
	19.75±5.59 ^{B,b}	17.81±5.58^{A,b}	14.85±5.17^{AB,b}	8.50±4.88 ^{B,b}	4.31±1.70 ^{AB,b}
Sausage coated with SFC film					
	8.64±6.27 ^{AB,a}	4.62±4.64 ^{A,a}	7.82±3.45 ^{AB,a}	12.06±2.92^{B,a}	10.24±3.26^{AB,a}
Sausage coated with SFC film incorporated with 2 % GSE					
	5.81±3.81 ^{AB,a}	4.93±3.19 ^{A,a}	8.38±2.57 ^{AB,a}	14.89±4.37^{B,a}	11.23±2.58^{AB,a}
Sausage coated with SFC film incorporated with 3 % GSE					
	7.59±6.04 ^{AB,a}	2.14±2.61 ^{A,a}	6.36±3.16 ^{AB,a}	11.84±5.52^{B,a}	12.15±3.76^{AB,a}

$^{a-b:}$ Means having different letters within each treatment denote significant difference at $p<0.05$.
$^{A-B:}$ Means having different letters within each storage time (row) denote significant difference at $p<0.05$.
Data are mean values ± S.D. (n=5)

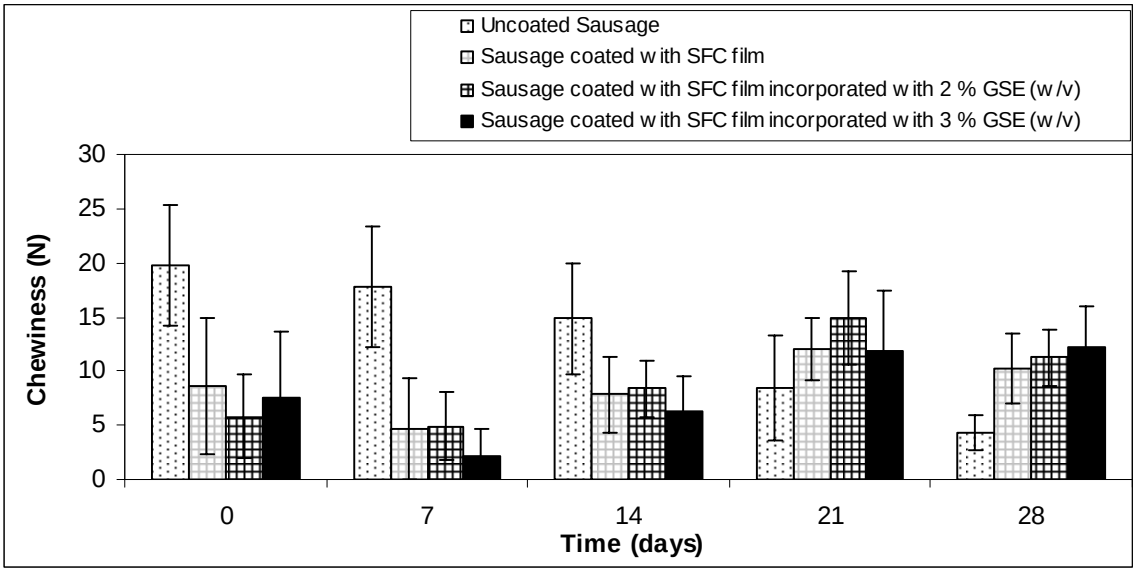

Figure 4.18. Chewiness (N) of beef sausages coated with different SFC films incorporating GSE during 28 days of storage

CHAPTER 6

CONCLUSION

In this study, natural polymers, silk fibroin, carrageenan, and grape seed extract were chosen to produce biodegradable material, and studies were performed to carry out the characterization of silk fibroin-carrageenan film incorporated with grape seed extract, its *in vitro* antimicrobial activity and its food applications. The casted silk fibroin carrageenan films incorporating grape seed extract dried at 25°C and 40% RH had a homogeneous texture and no crack formation was observed. Addition of GSE into SFC film caused the structural changes of fibroin from a random coil conformation to β-sheet structure. Increasing the GSE concentration further induced the formation of β-sheet structure of silk fibroin present in SFC films and increased the ordered structure of film and also increased the average surface roughness of films. The addition of GSE to the SFC film, when compare to SFC without GSE film, seemed to play a role in decreasing strength and Young's modulus, in contrary, increasing the elongation of the films. The films also showed antimicrobial activity against *L. innocua, S. carnasus, S. aureus, B. amyloliquefaciens*, and *P. fluorescens*. The zone of inhibition was not observed at any GSE concentration against *E. coli, E. coli* 0157:H7, and *S.* Typhimurium. Bacterial contamination is the main factor that determines food quality loss and shelf-life reduction. Food application studies also demonstrated the efficacy of silk fibroin-carrageenan films containing grape seed extract and/or Na_2EDTA on microbial quality of raw chicken breast meat. Release of GSE from the film was confirmed by the inhibition of *S. aureus* and total proanthocyanidin content released from the film during the release tests. *S. aureus* was inoculated on the surface of sausage and the growth was monitored during 28 days storage at 4 °C. Results suggested that grape seed extract could be used to control the growth of *S. aureus* on beef sausages. SFC films (control films) and SFC films incorporating different

concentrations of GSE had significant effect on moisture content of sausages as well as on cohesiveness, gumminess and chewiness of sausages. However, GSE did not have a significant effect on springiness of sausage.

This research clearly demonstrated that the grape seed extract could be incorporated into silk fibroin-carrageenan films in order to obtain antimicrobial edible films and they could be applied to meat products. Further studies are needed to demonstrate the effectiveness of these films against other pathogenic microorganisms such as *Listeria monocytogenes* in food applications.

REFERENCES

Ahn, J., Grün, I.U. and Mustapha, A. 2004. Antimicrobial and antioxidant activities of natural extracts *in vitro* and in ground beef. *Journal of Food Protection* 67: 148-155.

Ajisawa, A. 1969. *J Sericuet Sci Jpn*. 38(4):340.

Altman, G.H., Diaz, F., Jacuba, C., Calabro, T., Horan, R.L., Chen, J., Lu, H., Richmound, J. and Kaplan, D.L. 2003. Silk-Based Biomaterials. *Biomaterials* 24:401-416.

Ananou, S., Maqueda, M., Martinez-Bueno, M., Galvez, A. and Valdivia, E. 2005. Control of *Staphylococcus aureus* in sausages by enterocin AS-48. *Meat Science* 71:549–556.

AOAC, 1999. Official Methods of Analysis of AOAC Internatinal. 16th ed. 5th revision.

Appendini, P. and Hotchkiss, J.H. 2002. Review of Antimicrobial Food Packaging. *Innovative Food Science & Emerging Technologies* 3:113-126.

Aria, T., Wilson, D. L., Kasai, N., Freddi, G., Hayaska, S. and Tsukda, M. 2002. Preperation of Silk Fibroin and Polyallylamine Composites. *Journal of Applied Polymer Science* 84:1963-1970.

Ayutsede, J., Gandhi, M., Sukigar, S., Micklus, M., Chen, H.E. and Ko, F. 2005. Regeneration of Bombyx mori sil by Electrospinning. Part3: Characterization of Electrospun Non woven Mat. *Polymer* 46: 1625-1634.

Bahorun, T., Luximon-Ramma, A., Crozier, A. and Aruoma, O. 2004. Total phenol, flavonoid, proanthocyanidin and vitamin C levels and antioxidant activities of Mauritian vegetables. *J Sci Food Agric* 84:1553–1561.

Balasundram, N., Sundram, K. and Saman, S. 2006. Phenolic compounds in plants and agri- Industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemisty* 99:191-203.

Banerjee, R. and Chen, H. 1995. Functional Properties of Edible Films Using Whey Protein Concentrate. *J Dairy Sci.* 78:1673-1683.

Banker, G.S. 1966. Film coating theory and practice. *J. Pharma Sci.* 55:81-89.

Baydar , N.G. Sagdic, O., Ozkan, G. and Cetin, S. 2006. Determination of antibacterial effects and total phenolic contents of grape (Vitis vinifera L.) seed extracts. *International Journal of Food Science and Technology* 41;799–804.

Baydar, N.G., Ozkan, G. and Sagdic, O. 2004. Total phenolic contents and antibacterial activities of grape (Vitis vinifera L.) extracts. *Food Control* 15:335–339.

Bayraktar, O., Malay, O., Ozgarip, Y. and Batıgün, A. 2005. Silk Fibroin as a Novel Coating Material for Controlled Release of Theophylline. *European Journal of Pharmaceutics and Biopharmaceutics* 60:373-381.

Biomaterial. http://www.fineco.net/
(accessed July 27, 2009)

Bravo, L. 1998. Polyphenols: Chemistry, Dietary Sources, Metabolism, and Nutritional Significance. *Nutrition Reviews* Vol. 56,No. 11.

Cagri, A., Ustunol, Z. and Ryser, E.T. 2001. Antimicrobial, Mechanical, and Moisture Barrier Properties of Low pH Whey Protein-based Edible Films Containing Aminobenzoic or Sorbic Acids. *Journal of Food Science* Vol. 66, No. 6.

Cagri, A., Ustunol, Z. and Ryser, E.T. 2002. Inhibition of Three Pathogens on Bologna and Summer Sausage Using Antimicrobial Edible Films. *Food Microbiology and Safety* 67(4):833-848.

Cagri, A.,Ustunol, Z. and Ryser, E.T. 2004. Antimicrobial Edible Films and Coatings. *Journal of food Protection* 67(4):833-848.

Cha, D.S. and Chinnan, M.S. 2004. Biopolymer-Based Antimicrobial Packaging: A Review. Critical. *Reviews in Food Science and Nutrition* 44:223-237.

Cha, D. S., Choi, J. H., Chinnan, M.S. and Park, H. J. 2002. Antimicrobial Films Based on Na-alginate and Carrageenan. *Lebensmittel Wissenschaft and Technologie* 35(8): 715-719.

Chiovitti, A., Bacic, A., Craik, D. J., Kraft, G.T. and Liaob, M.L. 2004. A nearly idealized 6'-*0*-methylated ι-carrageenan from th Australian red alga *claviclonium ovatum* (Acrotylaceae, Gigartinales). *Carbohydrate Research* 339:1459-1466.

Cho, S.Y., Park, J.-W., Batt, H.P. and Thomas, R. 2007. Edible Films Made from Membrane Processed Soy Protein Concentrates. *LWT* 40:418-423.

Choi, S.H. and Chin, K.B. 2003. Evaluation of sodium lactate as a replacement for conventional chemical preservatives in comminuted sausages inoculated with *Listeria monocytogenes. Meat Science* 65:531–537.

CSIRO Food and Nutritional Sciences Fact Sheet. http://www.foodscience.csiro.au/.htm (accessed September 1, 2009).

Cutter, C.N. and Summer, S.S. 2002. Applications of Edible Film Coatings on Muscle Foods. In: *Protein-Based Films and Coatings*, edited by Gennadios, Aristippos. Boca Raton, New York:CRC Pres.

Cutter C.N. 2006. Opportunities for bio-based packaging technologies to improve the quality and safety of fresh and further processed muscle foods. *Meat Sci.*; 74:131–142.

Dai, L., Li, J. and Yamada, E. 2002. Effect of glycerin on structure transition of PVA/SF blends. *Journal of Applied Polymer Science* 86:2342-2347.

Danhowe, I. Greener and O. Fenema. 1994. Edible Film and Coatings: Characteristics,Formation, Definations, and Testing Methods. In: *Edible Coatings and Films to Improve Food Quality*, edited by Krochta, John M., Elizabeth A. Baldwin and Myrna Nisperos-Carriedo. Baco Raton, New York: CRC Press.

Debeaufort, F., Quezada-Gallo, J.A. and Voilley, A. 1998. Edible Films and Coatings: Tomorrow's Packagings: A Review. *Critical Reviews in Food Science* 38(4):299-313.

Dopico, M.S., Lopez-Vilarino, J.M. and Gonzalez-Rodriguesz, M.V. 2003. Determination of antioxidant migration levels from low density polyethylene film into food simulants. *Journal of Chromatography* 1018:53–62.

Dry Creek Nutrition, Incorporated. http://www.activin.com/TestingGSE.html (accessed September 3, 2008).

Eswaranandam, S., Hettiarachchy, N.S. and Johnson, M.G. 2004. Antimicrobial Activity of Citric, Lactic, Malic, or Tartaric Acids and Nisin-Incorporated Soy Protein Film against *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella gaminara*. *Food Microbiology and Safety* 69(3):FMS79-FMS84.

Foo, L.Y. 1981. Proanthocyanidins: gross chemical structures by infrared spectra. *Phytochemistry* 20 1397E1402.

Freddi, G., Tsukada, M. and Beretta, S. 1999. Structure and Physical Properties of Silk Fibroin/Polyacrylamide Blend Films. *Journal of Applied Polymer Science* 71:1563-1571.

Freddi, G., Romano, M., Massafra, M.R. and Tsukada, M. 1995. Silk Fibroin/Cellulose Blend Films: Preparation, Structure, and Physical Properties. *Journal of Applied Polymer Science* 56:1537-1545.

Friedrich, C.L., Moyles, D., Beveridge, T.J. and Hancock, R.E. 2000. Antibacterial action of structurally diverse cationic peptides on gram-positive bacteria. *Antimicrobial Agents and Chemotherapy* 44: 2086–2092.

Garcıa-Marino, M., Rivas-Gonzalo, J.C., Ibanez, E. and Garcıa-Moreno, C. 2006. Recovery of catechins and proanthocyanidins from winery by-products using subcritical water extraction. *Analytica Chimica Acta* 563 :44–50.

Gennadios, A., Hanna, M.A. and Kurth, L.B. 1997. Application of Edible Coatings on Meats, Poultry and Seafoods: A Review. *Lebensm Wiss*. *U*.-*Technol* 30:337-350.

Gotoh, Y., Tsukada, M., Baba, T. and Minoura, N. 1997. Physical Properties and Structure of Poly(ethylene glycol)-Silk Fibroin Conjugate Films. *Polymer* 38:487-490.

Han, J.H. 2000. Antimicrobial Food Packaging. *Food Technology* 54(3):56-65.8.

Han, C., Lederer, C., Mcdaniel, M. and Yanyun, Z. 2005. Sensory Evaluation of Fresh Strawberries (Fragaria ananassa) Coated with Chitosan-Based Edible Coatings. *Sensory and Nutritive Quality of Foods* 70(3):S172-S178.

Hang-wan, V.C., Lee, C.M. and Lee, S.Y. 2007. Understanding Consumer Attitudes On Edible Films and Coatings: Focus Group Findings. *Journal of Sensory Studies* 22: 353–366.

Hardy, J.G., Römer, L.M. and Scheibel, T.R. 2008. Polymeric materials based on silk proteins. *Polymer* 49:4309–4327.

Hossain, K.S., Ohyama, E., Ochi, A., Magoshi, J. and Nemoto, N. 2003. Dilute-Solution Properties of Regenerated Silk Fibroin. *J. Phys. Chem. B*,107 (32), 8066-8073.

Hossain, K.S., Miyanaga, K., Maeda, H. and Nemoto, N. 2001. Sol-Gel Transition Behavior of Pure ι-Carrageenan in Both Salt-Free and Added Salt States. *Biomacromolecules* 2:*442-449.*

Janes, M.E., Kooshesh, S. and Johnson, M.G. 2002. Control of *Listeria monocytogenes* on the Surface of Refrigerated, Ready-to-eat Chicken Coated with Edible Zein Film Coatings Containing Nisin and/or Calcium Propionate *Journal of Food Science* 67:7.

Jayaprakasha, G.K., Selvi, T. and Sakariah, K.K. 2003. Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. *Food Research International.* 36: 117-122.

Jin, H.J., Park, J., Valuzzi, R., Cebe, P. and Kaplan, D.L. 2004. Biomaterial Films of *Bombyx mori* Silk Fibroin with Poly(ethylene oxide). *Biomacromolecules* 5:711-717.

Joerger, R.D. 2007 . Antimicrobial Films for Food Applications: A Quantitative Analysis of Their Effectiveness. *Packag. Technol. Sci.* 20: 231–273.

Kar, P., Laight, D., Shaw, K.M. and Cummings, M. H. 2006. Flavonoid-rich grapeseed extracts: a new approach in high cardiovascular risk patients? *J Clin Pract* 60:11, 1484–1492.

Karbowiak, T., Debeaufort, F. and Voilley, A. 2007. Influence of thermal process on structure and functional properties of emulsion-based edible films. *Food Hydrocolloids* 21:879–888.

King, A.G. and Young, G. 1999. Characteristics and occurrence of phenolic phytochemicals. *Journal of the American Dietetic Association* 99:213-218.

Krochta, John M. 2002. Proteins as a Raw Materials for Films and Coatings: Definations, Current Status, and Opportunities. In: *Protein-Based Films and Coatings*, edited by Gennadios, Aristippos. Boca Raton, New York:CRC Pres.

Kweon, S., Woo, O. and Park, Y. H. 2001. Effect of heat treatment on the structural and conformational changes of regenerated Antheraea pernyi silk fibroin films. *J. Appl. Polym. Sci* 81-2271.

Lacroix, Monique and Kay Cooksey. 2005. Edible Films and Coatings from Animal-Origin Proteins. In: *Innovations in Food Packaging*, edited by Han, Jung H. SanDiego, Calif.: Academic Press.

Lacroix, Monique and Chan Lee Tien. 2005. Edible Films and Coatings from Non-Starch Polysaccharides. In: *Innovations in Food Packaging*, edited by Han, Jung H. San Diego, Calif.:Academic Press.

Lako, J., Trenerry, V.C., Wahlqvist, M., Wattanapenpaiboon, N., Sotheeswaran, S. and Premier, R. 2007. Phytochemical Flavonols, Carotenoids, and the Antioxidant Properties of a Wide Selection of Fijian Fruit, Vegetables and Other Readily Available Foods. *Food Chemistry* 101:1727-1741.

Lambert, P.A. 2002. Cellular impermeability and uptake of biocides and antibiotics in Grampositive bacteria and mycobacteria. *Journal of Applied Microbiology.* 92, 46S-54S.

Lee, S.D., Hwang, Y.I. and Cho, S.H. 1998. Developing antimicrobial packaging film for curled lettuce and soybean sprouts. *Food Sci. Biotechnol.*; 7: 117–121.

Liang, C.X. and Hirabayashi, K. 1992. Improvements of the Physical Properties of Fibroin Membranes with Sodium Alginate. *Journal of Applied Polymer Science* 45:1937-1943.

Li, M., S, Lu., Wu, Z., Yan, H., Mo, J. and Wang, L. 2001. Study on Porous Silk Fibroin Materials. I. Fine Structure of Freeze Dried Silk Fibroin. *Journal of Applied Polymer Science* 79:2185–2191.

Li, M., Lu, S., Wu, Z., Tan, K., Minoura, N. and Kuga, S. 2002. Structure and properties of silk fibroin-poly(vinyl alcohol) gel. *International Journal of Biological Macromolecules* 30:89–94.

Liu, R.H. 2004. Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J. Nutr.* 134: 3479S–3485S.

Lungu, B. and Johnson, M.G. 2005. Potassium Sorbate Does Not Increase Control of Listeria monocytogenes When Added to Zein Coatings with Nisin on the Surface of Full Fay Turkey Frankfurter Pieces in a Model System at 4 °C. *Food Microbiology and Safety* 70(2):M95-M99.

Luo, J., Chen, K., Xu, Q. and Hirabayashi, K. 2003. Study on Floodization of Fibroin and Its Functionality. (The New Silk Road, Inc.).

Magoshi, J., Magoshi, Y. and Becker-Nakamura, S. 1996. Polymeric Materials Encyclopedia, edited by J.C. Salamone, CRC Press, New York, p. 667.

Magoshi, J., Magoshi, Y., Becker, M.A., Kato, M., Han, Z., Tanak, T., Inoue, S. and Nakamura, S. 2000. Crystallization of Silk Fibroin from Solution, *Thermochimica Acta* 352-353:165-169.

Makris, D.P., Boskou, G. and Andrikopoulos, N.K. 2007. Polyphenolic content and in vitro antioxidant characteristics of wine industry and other agri-food solid waste extracts. *Journal of Food Composition and Analysis* 20 :125–132.

Mareci, T.M. University of Florida. Introduction to Protein Structure, Lecture 3. http://faraday.ufbi.ufl.edu/~thmareci/bch4024/lecture3.pdf (accessed June 25, 2008).

Marsh, K. and Bugusu, B. 2007. Food Packaging—Roles, Materials, and Environmental Issues. *Journal of Food Science* 72(3):1750-3841.

Martin, Chaplin. 2009 Water structure and science. http://www.lsbu.ac.uk/water/hygel.html (accessed May 21, 2009)

Martinez, O., Salmeron, J., Guillen, M.D. and Casas, C. 2004. Texture profile analysis of meat products treated with commercial liquid smoke flavourings. *Food Control* 15:457–461.

McHugh, T.H., Aujard, J.F. and Krochta, J.M. 1994. Plasticized whey protein edible films:water vapor permeability properties. *J Food Sci* 59(2):416-423.

Mistry, Y., Master of Thesis. Development of LDPE-based Antimicrobial Films for Food Packaging Packaging and Polymer Research Unit, School of Molecular Sciences, Faculty of Health, Engineering and Science,Victoria University, July, 2006.

Morris, E.R., Rees, D.A. and Robinson, G. 1980. Cation-specific aggregation of carrageenan helices: Domain model of polymer gel structure. *Journal of Molecular Biology*. 138(2):349–362.

Natrajan, N. and Sheldon, B.W. 2000. Inhibition of *Salmonella* on Poultry Skin Using Protein- and Polysaccharide-Based Films Containing a Nisin Formulation. *Journal of Food Protection* 63(9):1268-1272.

Nawaz, H., Shi, J., Mittal, G.S. and Kakuda, Y. 2006. Extraction of polyphenols from grape seeds and concentration by ultrafiltration. *Separation and Purification Technology* 48 : 176–181.

Northwestern University. Keck Interdisciplinary Surface Science Center Nuance. http://www.nuance.northwestern.edu/keckii/ftir1.asp (accessed December 12, 2008).

Olivas, G.I. and Barbosa-Canovas, G.V. 2008. Alginate-Calcium Films: Water Vapor Permeability and Mechanical Properties as Affected by Plasticizer and Relative Humidity. *LWT* 41:359-366.

Oussalah, M., Caillet, S., Salmieri, S., Saucier, L. and Lacroix, M. 2004. Antimicrobial and antioxidant effects of milk protein-based film containing essential oils for the preservation of whole beef muscle. *J. Agric. Food Chem.*; 52: 5598–5605.

Ozdemir, M. and Floros, J. D. 2001. Analysis and Modeling of Potassium Sorbate Diffusion through Edible Whey Protein Films. *Journal of Fod Engineering* 47:149-155.

Packaging and labeling. http://en.wikipedia.org/wiki/Packaging (accessed February 12, 2008).

Paramawati, R., Yoshino, T. and Isobe, S. 2001. Properties of Plasticized-Zein Film as Affected by Plasticizer Treatments. *Food Sci. Technol. Res.* 7(3):191-194.

Park, S.K., Bae, D.H. and Rhee, K.C. 2000. Soy Protein Biopolymers Cross-Linked with Glutaraldehyde. *JAOCS* 77:8 .

Park, S.J., Lee, K.Y., Ha, W.S. and Park, S.Y. 1999. Structural Changes and Their Effect on Mechanical Properties of Silk Fibroin/Chitosan Blends. *Journal of Applied Polymer Science* 74:2571–2575 .

Perez-Gago, M.B., Nadaud, P. and Krochta, J.M. 1999. Water vapor permeability, solubility, and tensile properties of heat-denatured versus native whey protein films. *J Food Sci.* 64(6):1034-1037.

Perez-Gago, M.B and Krochta, J.M. 2001. Denaturation Time and Temperature Effects on Solubility, Tensile Properties, and Oxygen Permeability of Whey Protein Edible Films. *Journal of Food Science* 66:5.

Quintavalla, S. and Vicini, L. 2002. Antimicrobial Food Packaging in Meat Industry. *Meat Science* 62:373-380.

Ribereau-Gayon, P. 1972. *Plant Phenolics*. pp. 1-53. Edinburgh: Oliver & Boyd.

Saitoh, H., Ohshima, K., Tsubouchi, K., Takasu, Y. and Yamada, H. 2004. X-Ray Structural Study of Noncrystalline Regenerated *Bombyx mori* Silk Fibroin. *International Journal of Biological Macromolecules* 34:317-323.

Servoli, E., Maniglio, D., Motta, A., Predazzer, R. and Migliaresi, C. 2005. Surface Properties of Silk Fibroin Films and Their Interaction with Fibroblasts. *Macromol. Biosci*. 5, 1175–1183.

Sivarooban, T., Hettiarachchy, N.S. and Johnson, M.G. 2008. Physical and antimicrobial properties of grape seed extract, nisin, and EDTA incorporated soy protein edible films. Food Research International 41 : 781–785.

Suppakul, P., Miltz. J., Sonneveld, K. and Bigger, S.W. 2003. Active Packaging Technologies with an Emphasis on Antimicrobial Packaging and Its Applications. *Concise Reviews and Hypotheses in Food Science* 68(2):408-420.

Tharanathan, R.N. 2003. Biodegradable Films and Composite Coatings: Past, Present and Future. *Trends in Food Science & Technology* 14:71-78.

Theivendaran, S., Hettiarachchy, N.S. and Johnson, M.G. 2006.Inhibition of *Listeria monocytogenes* by nisin combined with grape seed extract or green tea extract in soy protein film coated on turkey frankfurters. *J. Food Sci.*; 71: M39–M44

Tsuboi, Y., Ikejiri, T., Shiga, S., Yamada, K. and Itaya, A. 2001. Light can transform the secondary structure of silk protein. Appl. Phys. *A Mater. Sci. Process* 73:637–640.

Turhan, K. N., Erdohan-Sancak, Z. Ö., Ayana, B. and Erdoğdu, F. 2007. Optimization of glycerol effect on the mechanical properties and water vapor permeability of whey protein methylcellulose films. *Journal of Food Process Engineering* 30:485–500.

Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W., Etzler, M.E. 2009. Essentials of Glycobiology. La Jolla, California. CSH Press.
http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=glyco2&part=ch20)
(accessed May 8, 2009).

Vartiainen, J., Motion, R., Kulonen, K., Ratto, M., Skytta, E. and Advenainen, R. 2004. Chitosan-coated paper: effects of nisin and different acids on the antimicrobial activity. *Journal of Applied Polymer Science* 94:986–993.

Veparia, C. and Kaplan, D. L. 2007. Silk as a biomaterial. *Progress in Polymer Science*. 32 :991–1007.

Villegas, R., O-Connor, T.P., Kerry, J.P. and Buckley, D.J. 1999. Effect of gelatin dip on the oxidative colour stability of cooked ham and bacon pieces during frozen storage. *International Journal of Food Science & Technology* 34:385–389.

Wang, H., Li, W., Lu, Y. and Wang, Z. 1997. Studies on Chitosan and Poly(acrylic acid) Interpolymer Complex. I. Preparation, Structure, pH-Sensitivity, and Salt Sensitivity of Complex Forming Poly(acrylic acid) Chitosan Semi Interpenetrating Polymer Network. *Journal of Applied Polymer Science* 65:1445-1450.

Yamada, H., Tsuboi, Y. and Itaya, A. 2003. AFM Observations of Silk Fibroin on Mica Substrates: Morphologies Reflecting the Secondary Structures. *Thin Solid Films*, 440:208-216.

Yamaura, K., Kuranuki, S., Suzuki, M., Tanigami, T. and Matsuzawa, S. 1990. Properties of Mixtures of Silk Fibroin/Syndiotactic-Rich Poly(vinyl alcohol). *Journal of Applied Polymer Science* 41:2453-2461.

Yılmaz, Y. and Toledo, R.T. 2004. Major Flavonoids in Grape Seeds and Skins: Antioxidant Capacity of Catechin, Epicatechin, and Gallic Acid. *Journal of Agricultural and Food Chemistry* 52:255-260.

Yılmaz, Y. 2006. Novel uses of catechins in foods. *Trends in Food Science & Technology.* 17:64–71.

APPENDIX A

CALIBRATION CURVES OF MICROORGANISMS

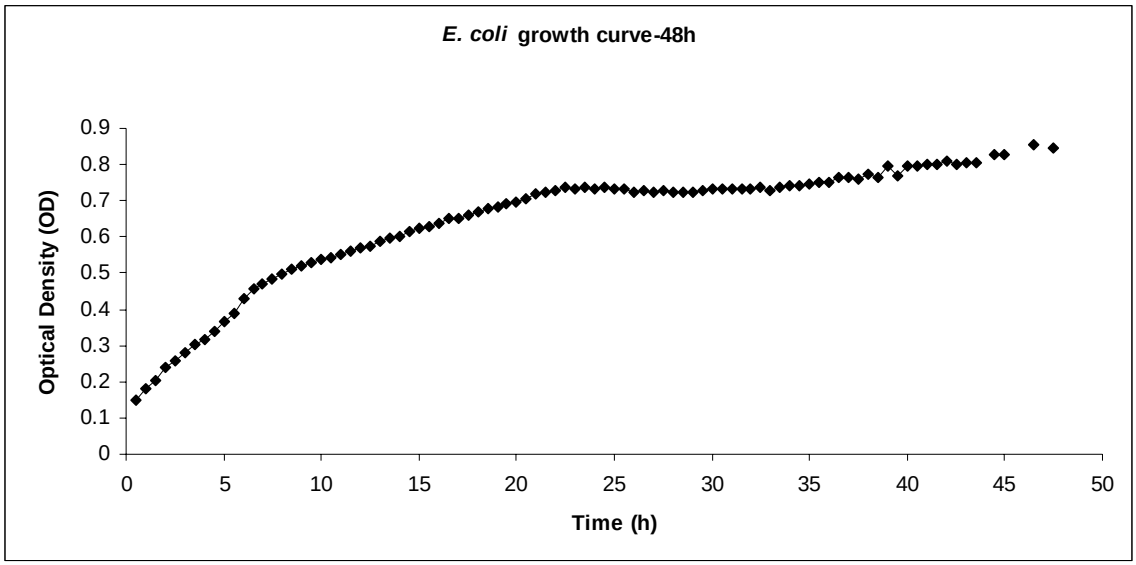

Figure A.1. The growth curve of *E. coli*

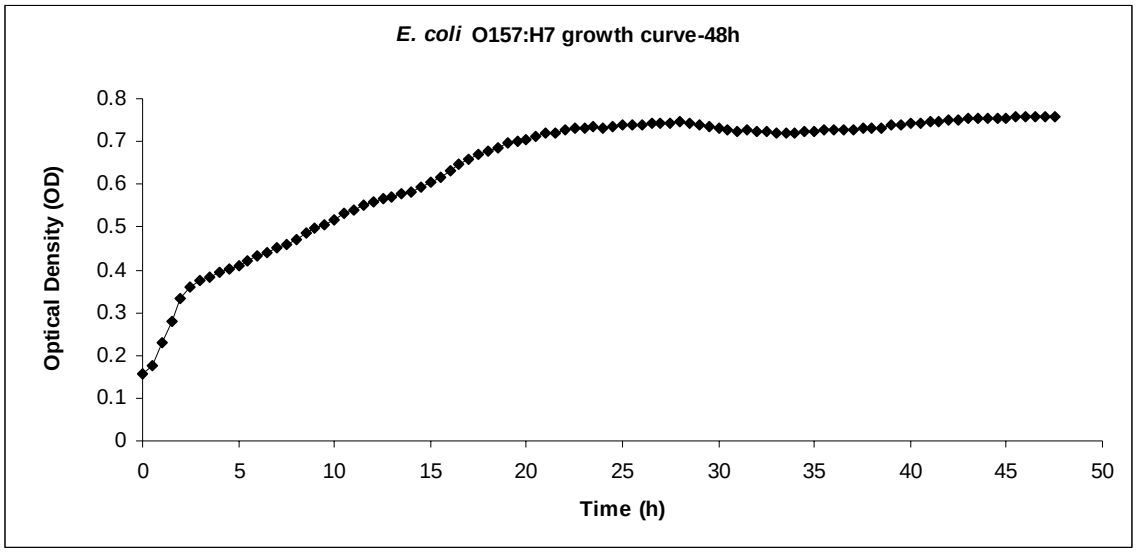

Figure A.2. The growth curve of *E. coli* O157:H7

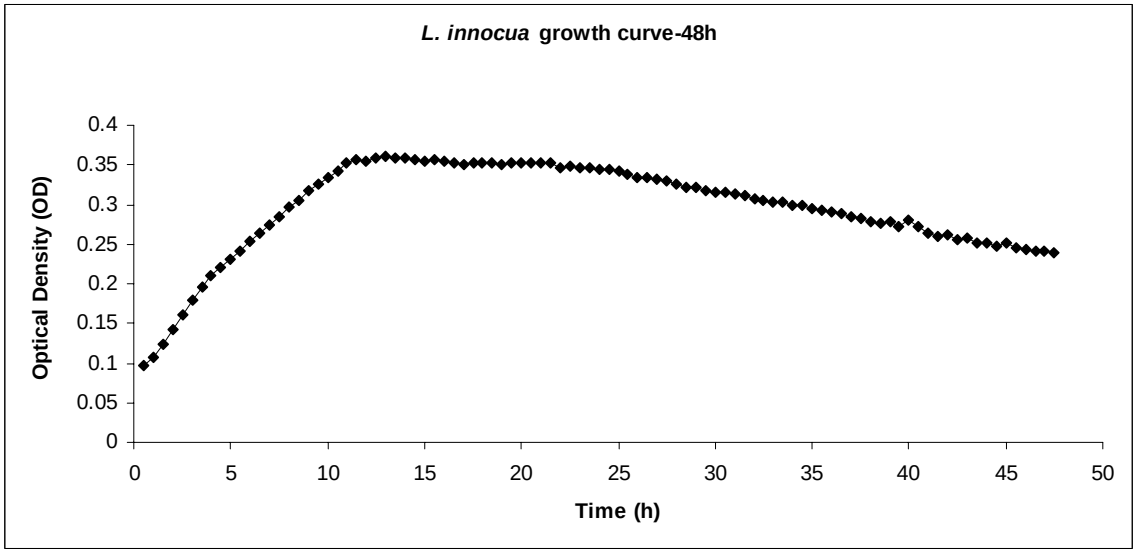

Figure A.3. The growth curve of *L. innocua*

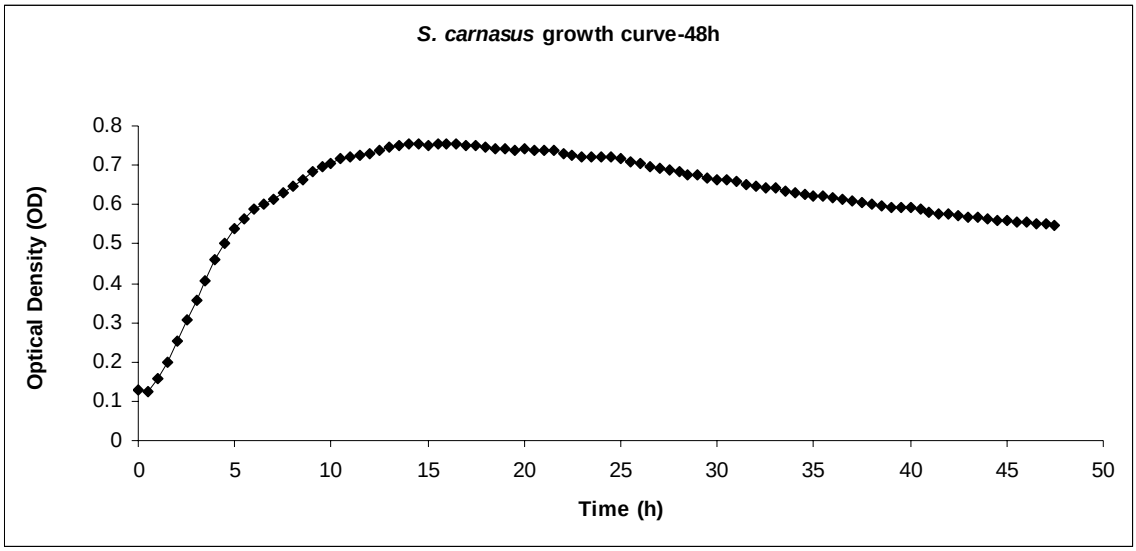

Figure A.4. The growth curve of *S. carnasus*

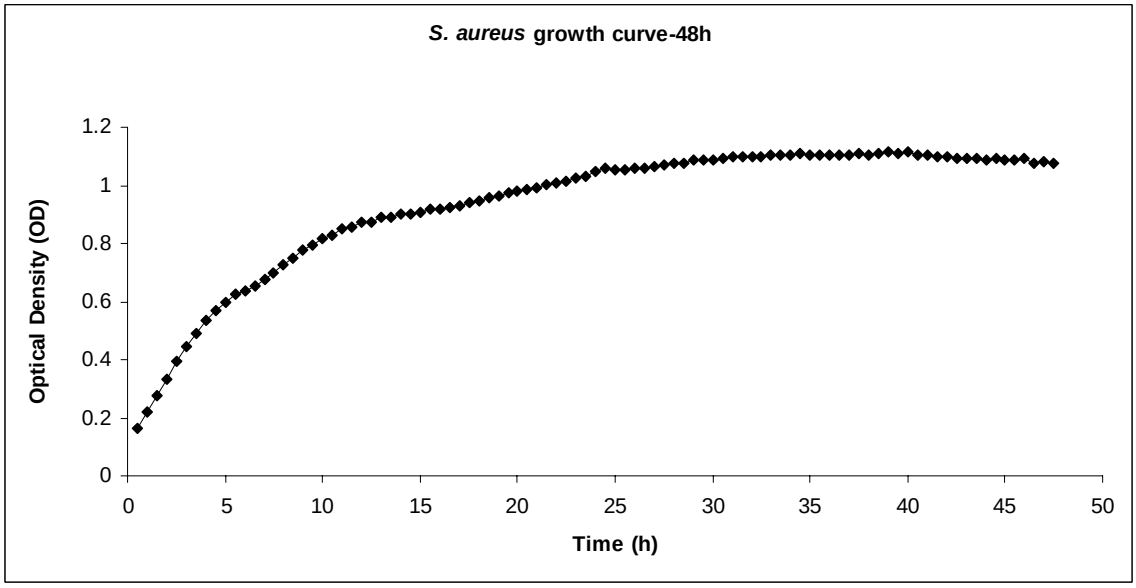

Figure A.5. The growth curve of *S. aureus*

APPENDIX B

CALIBRATION CURVE OF GALLIC ACID

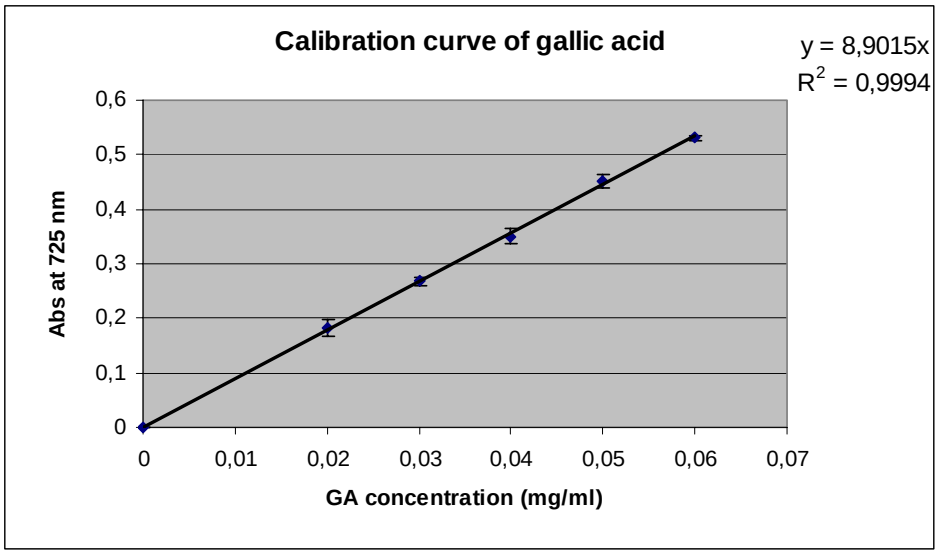

Figure B.1. Calibration curve for Folin-Ciocalteu's method

GAEq(mg GA/g sample)=[A*DF*Vsolv(mL)]/[slope of calib. curve*sample amount(g)]

A: Absorbance
DF:Dilution Factor: 500 df
V: Volume of Solvent: 1 mL
Sample : 0.1 g

APPENDIX C

CALIBRATION CURVE OF PORTER ASSAY

Calibration curve for Porter Assay was obtained from Grape Seed Extract (Polyphenolics (CA, USA)) .

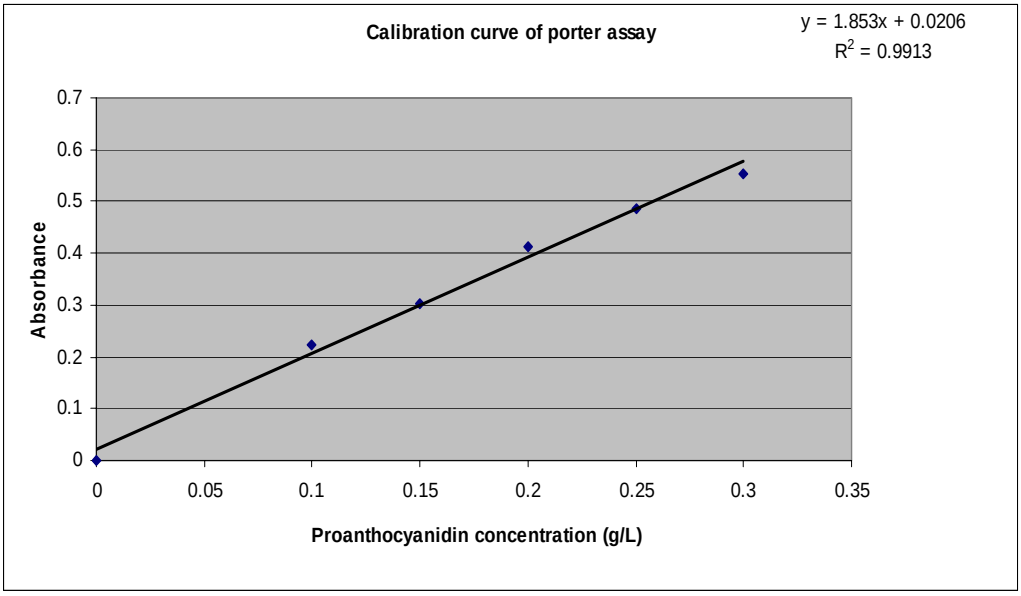

Figure C.1. Calibration curve of Porter Assay