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นายเอกสิทธิ ลีฬหาวงศ์

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PHYSICAL AND ANTIMICROBIAL PROPERTIES OF CHITOSAN FILM CONTAINING CINNAMALDEHYDE

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Food Technology
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เอกสิทธิ ลีพหาวงศ์ : สมบัติทางกายภาพและการยับยั้งการเจริญของจุลินทรีย์ของฟิล์มไคโตซานผสมซินนามาลดีไฮด์ (PHYSICAL AND ANTIMICROBIAL PROPERTIES OF CHITOSAN FILM CONTAINING CINNAMALDEHYDE) อ. ที่ปรึกษา : ผศ.ดร. อุบลรัตน์ สิริภัทราวรรณ, อ. ที่ปรึกษาร่วม : Prof. MUNEHIKO TANAKA, อ. ที่ปรึกษาร่วม : ผศ.ดร. รมณี สงวนดีกุล, 82 หน้า.

งานวิจัยนี้ได้พัฒนาฟิล์มป้องกันแบคทีเรียโดยเติมซินนามาลดีไฮด์ลงในฟิล์มไคโตซาน ฟิล์มไคโตซานเตรียมได้โดยละลายผงไคโตซาน (95% degree of deacetylation) 1 g กับซอร์บิทอลหรือกลีเซอรอล (20 40 และ 60 %โดยน้ำหนัก) ในสารละลายกรดอะซิติกหรือแลกติกความเข้มข้น 1 %โดยปริมาตร ในงานวิจัยนี้ทดสอบสมบัติทางกายภาพ ได้แก่ ค่าความหนา (thickness) ค่าความต้านทานแรงดึงขาด (tensile strength, TS) ค่า % การยืดตัวเมื่อขาด (elongation at break, EAB) ค่าการซึมผ่านของไอน้ำ (water vapor permeability, WVP) ค่าสีของผิวหน้า (surface color) และค่าความโปร่งใส (transparency) พบว่า ฟิล์มที่ได้จากการขึ้นรูปโดยใช้กรดอะซิติกและกรดแลกติกมีลักษณะที่มองเห็นได้ด้วยตาไม่แตกต่างกัน อย่างไรก็ตามการขึ้นรูปฟิล์มที่เตรียมจากสารละลายกรดแลกติกทำได้ยากโดยเฉพาะเมื่อมีการเติมสารพลาสติไซเซอร์ จึงทำให้ไม่สามารถทดสอบสมบัติทางกายภาพต่างๆ ได้ จากการศึกษาชนิดและปริมาณสารพลาสติไซเซอร์ พบว่า เมื่อความเข้มข้นของซอร์บิทอลหรือกลีเซอรอลเพิ่มขึ้น ค่า TS จะลดลงในขณะที่ค่า EAB และ WVP เพิ่มขึ้น ฟิล์มที่ใช้ซอร์บิทอลเป็นพลาสติไซเซอร์มีค่า WVP ต่ำกว่าฟิล์มที่ใช้กลีเซอรอลเป็นพลาสติไซเซอร์ และจากการพิจารณาสมบัติทางกายภาพ พบว่า ซอร์บิทอลความเข้มข้น 40 %โดยน้ำหนัก เหมาะสมสำหรับการขึ้นรูปฟิล์มที่จะใช้ศึกษาในขั้นต่อไป ในการศึกษาผลของปริมาณซินนามาลดีไฮด์ (cinnamaldehyde) ที่ความเข้มข้น 50 100 และ 150 µl/g พบว่า เมื่อปริมาณซินนามาลดีไฮด์เพิ่มขึ้น ฟิล์มไคโตซานมีค่า TS และ EAB เพิ่มขึ้นอย่างมีนัยสำคัญ ($p \leq 0.05$) ในขณะที่ค่า WVP ลดลงอย่างมีนัยสำคัญ การเพิ่มขึ้นของค่า TS และ EAB เกิดจากปฏิกิริยาระหว่างหมู่ฟังก์ชันของไคโตซานและซินนามาลดีไฮด์ ซึ่งสามารถพิสูจน์ได้จากผลการทดลองในส่วนของ Fourier transform infrared (FT-IR) spectra การทดลองสมบัติในการยับยั้งจุลินทรีย์ (*S. aureus*, *B. lichenformis*, *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. putrefaciens*) พบว่า ฟิล์มไคโตซานผสมซินนามาลดีไฮด์สามารถสร้างบริเวณยับยั้งการเจริญของ *S. aureus* ในขณะที่สามารถยับยั้งจุลินทรีย์ชนิดอื่นได้เพียงบริเวณที่ฟิล์มสัมผัส การเติมสารซินนามาลดีไฮด์สามารถปรับปรุงสมบัติทางกายภาพของฟิล์มไคโตซานได้แก่ TS EAB และ WVP แต่เนื่องจากปฏิกิริยาที่เกิดขึ้นระหว่างหมู่ฟังก์ชันของไคโตซานและซินนามาลดีไฮด์ทำให้ซินนามาลดีไฮด์ถูกปลดปล่อยออกจากฟิล์มไคโตซานได้ในปริมาณเพียงเล็กน้อย การยับยั้งการเจริญของจุลินทรีย์จึงเกิดได้ดีเฉพาะบริเวณที่ฟิล์มสัมผัสเท่านั้น

ภาควิชาเทคโนโลยีทางอาหาร ลายมือชื่อนิสิต.....
สาขาวิชาเทคโนโลยีทางอาหาร ลายมือชื่ออาจารย์ที่ปรึกษา.....
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KEY WORD: CHITOSAN FILM/ CINNAMALDEHYDE / PHYSICAL PROPERTIES / ANTIMICROBIAL PROPERTY

AKASITH LEERAHAWONG : PHYSICAL AND ANTIMICROBIAL PROPERTIES OF CHITOSAN FILM CONTAINING CINNAMALDEHYDE. THESIS ADVISOR : ASST. PROF. UBONRAT SIRIPATRAWAN, THISIS COADVISOR : PROF. MUNEHIKO TANAKA, THESIS COADVISOR : ASST. PROF. ROMANEE SANGUANDEEKUL, 82 pp.

This research aims to produce antimicrobial film by incorporating cinnamaldehyde into chitosan film. The films were prepared by dissolving chitosan powder (95% degree of deacetylation) into 1 %v/v acetic or lactic acid solution and blending with glycerol or sorbitol at different concentration (20, 40 and 60 %w/w of chitosan powder) as plasticizer. Physical properties including thickness, tensile strength (TS), elongation at break (EAB), water vapor permeability (WVP) surface color and transparency were examined. Films from acetic acid and lactic acid solution showed similar visual appearance. However, chitosan film-forming solution from lactic acid was difficult to form into film, especially when the plasticizers were added, and thus physical property measurements were not possible. The results showed that an increase in amount of the plasticizers resulted in significant ($p \leq 0.05$) increase in thickness, decrease in mechanical resistance (decrease in TS) and increase in extensibility (increase in EAB). TS decreased but EAB and WVP increased when the concentration of sorbitol and glycerol increased from 20 to 60 %w/w of chitosan powder. Moreover, film plasticized with sorbitol had lower WVP than those with glycerol at each concentration. The results suggested that 40 %w/w sorbitol was the optimum concentration of plasticizer for forming the chitosan film and thus used for further studies. In further studies, cinnamaldehyde was incorporated into chitosan film-forming solution (plasticized with sorbitol 40 % w/w) at different concentrations (50, 100 and 150 µl/g of chitosan powder). Addition of cinamaldehyde led to a significant increase in both TS and EAB, but a significant decrease in WVP of the films. Increasing of TS and EAB could be due to the interaction between fuctional group of chitosan and cinnamaldehyde. This could be proved by FT-IR spectra results. Antimicrobial property of chitosan film containing cinnamaldyhyde was tested against target microorganisms (*S. aureus*, *B. lichenformis*, *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. putrefaciens*). The film was found to have small inhibition zone against *S. aureus* while inhibited other target microorganisms only on the contact surface. Addition of cinnamaldehyde could improve physical properties of chitosan film in terms of TS, EAB and WVP. However, only small amount can release from chitosan matrix due to the interaction between functional groups of chitosan and cinnamaldehyde.

Department Food Technology — Student's signature.....

Field of study Food Technology — Advisor's signature.....

Academic year 2007 — Co-advisor's signature.....

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INTRODUCTION

The growing interests in edible and biodegradable films come from various sources. Consumers and processors alike are committed to reducing the environmental problems associated with packaging waste (Kim *et al*., 2006). There has been an increasing research interest in edible and biodegradable packaging films during the last decade, possibly due to their numerous advantages over synthetic packaging films (Srinivasa *et al*., 2007).

Many substances with antimicrobial properties are directly incorporated into food products to increase the shelf life and limit the development of pathogens and food-spoiling microorganisms. Recently, antimicrobial packaging materials have been developed to improve storability of foodstuffs especially sensitive to microbial growth (Siragusa and Dickson, 1992; Ouattara *et al*., 2000; Coma *et al*., 2001; Sebti *et al*., 2002). The use of antimicrobial packaging film based on antimicrobial polymer could prove to be more efficient by maintaining high concentrations on food surface with a low migration of active substances.

Annually, Thailand exports large amounts of frozen marine food products, especially shrimp, crab and squid. The amount of exported products indicates the generation of large quantities of marine waste from the process such as shrimp shell, crab shell, and squid pen. These wastes have been sold at a very low price for animal feed. Therefore, value-added products from marine waste are of great interest. Marine wastes can be modified to value-added products such as chitin and chitosan. Chitosan is of great interest as a potential edible film component because of its good oxygen and carbon dioxide barrier properties (Hosokawa *et al*., 1990). Chitosan has been proved to be nontoxic, biodegradable, biofunctional, and has antimicrobial characteristics (Wang, 1992; Darmadji and Izumimoto, 1994). The antimicrobial property of chitosan is due to its positively charged amino group which interacts with negatively charged microbial cell membrane, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms (Shahidi *et al*., 1999). A number of studies on the antimicrobial characteristics of chitosan films have been carried out earlier (Chen *et al*.,

1996; Ouattara *et al*., 2000; Coma *et al*., 2002; Yingyuad *et al*., 2006). However, since chitosan film is in a solid form, therefore, only organisms in direct contact with the active sites of chitosan are inhibited (Srinivasa et al., 2007).

An attempt to incorporate natural antimicrobial agents as additives into packaging materials has increased markedly due to their potential safety advantages. Essential oils such as garlic oil and cinnamon oil are proved to be able to inhibit microbial growth although different results are observed depending on test conditions, target microorganisms, and the property of the antimicrobial compound. Cinnamaldehyde was among the most active components against Gram's positive and Gram's negative bacteria. However, there are only a few studies on the possibility of incorporation of cinnamaldehyde into chitosan film.

1.1 Objectives

This research aimed to develop a chitosan film containing cinnamaldehyde to be used as an antimocribial film for food packaging, to study the effect of acid type, plasticizer type and concentration on physical properties of chitosan film and to study the effect of cinnamaldehyde on physical and antimicrobial properties of chitosan film.

CHAPTER II
LITERATURE REVIEW

2.1 Edible films and coatings

In the past approximately 50 years, impressive advances have been made in the production of synthetic polymer films designed to protect foods, pharmaceuticals, and other products and to perform other functions such as mulching. With the increasing population and stress on limited resources and the environment, uses of renewable resources to produce edible and biodegradable films that can improve product quality and/or reduce waste disposal problems are being explored. (Krochta, 2002)

2.1.1 Definition and function

Films versus coating

Films are normally regarded as stand-alone, being formed separate of any eventual intended use. These stand-alone films also are used as testing structures for determination of barrier, mechanical, solubility, and other properties provided by a certain film material. Such films can be used as covers, wraps, or separation layers; and they can be potentially formed into casings, capsules, pouches, and bags. Related products include molded items of greater thickness. Coatings involve formation of films directly on the surface of the object they are intended to protect or enhance in some manner. In this sense, coatings become part of the product and remain on the product through use and consumption.

Edible versus biodegradable

Films and coatings based on proteins are edible and/or biodegradable, depending on formulation, formation method, and modification treatments. As long as food-grade proteins and other food-grade additives (e.g., plasticizers, acid or base, salts, and enzymes) are used and only protein changes due to heating, pH modification, salt addition, enzymatic modification, and water removal occur, the resulting film or coating is edible (Krochta and De Mulder-Johnston, 1997).

Because edible films and coatings can normally support microbial growth, proper attention must be paid to water activity, pH, temperature, atmosphere,

and time. Addition of antimicrobials to edible films can protect the films and coatings, as well as the related foods, from microbial growth.

Edible films and coatings also are biodegradable. However, edibility is lost when the film-forming material is reacted with other chemicals before or during film or coating formation (e.g., chemical grafting or chemical cross-linking), or when non-edible components are added to the film or coating. Biodegradable films and coatings for food packaging applications must be shown safe for such use (Krochta and De Mulder-Johnston, 1997). The challenge to biodegradable films and coatings for food packaging and other uses is that the film or coating must serve its function safely and effectively for the time needed. Only after the intended functional use has ended should biodegradation proceed.

Edible film

Edible films are defined as thin layer of material which is edible and can provide a barrier to moisture, oxygen and solute movement for the product (Guilbert, 1986). The growing interests in edible and biodegradable films come from various sources. Consumers and processors alike are committed to reducing the environmental problems associated with packaging (Kim *et al*., 2006). There has been an increasing research interest in edible and biodegradable packaging films during the last decade, possibly due to their numerous advantages over synthetic packaging films (Srinivasa *et al*., 2007). The advantages of edible films over other traditional non edible polymeric packaging material are summarized by Gennadios and Weller (1990) as follows:

1. They can be consumed with the packaged products.
2. There is no package to dispose of even if the films are not consumed they can still contributed to the reduction of environmental pollution.
3. The films are produced exclusively from renewable, edible ingredients and therefore are anticipated to degrade more readily than polymeric materials.
4. The films could be enhanced for the organoleptic properties of packaged foods provided that various components (flavorings, colorings, sweeteners) are also incorporated.
5. The films could be supplemented for the nutrition value of the foods.

6. The films could be used for individual packaging of small portion of food, particularly for such products that currently are not individually packed for practical reasons such as pears, beans, nuts and strawberries.
7. The films could be applied inside heterogeneous foods at the interfaces between different layers of component moisture and solute migration in foods such as pizzas, pie and candies.
8. The films could be functioned as carriers for antimicrobial and antioxidant agents. In a similar application they also can be used at the surface of food to control the diffusion rate of preservative substances from the surface to the interior of the food.
9. The films could be very conveniently used for microencapsulation of food flavoring and leavening agents to efficiently control their additional and released into the interior of food.
10. Another possible application for edible films could be their uses in multilayer food packaging materials together with non edible films. In this case, the edible films would be the internal layers in direct contact with food materials.

Production of edible films causes less waste and pollution, however, their permeability and mechanical properties are generally poorer than synthetic films (Kester and Fennema, 1986). Materials which can be used to form edible films include proteins, polysaccharides, lipids (waxes), and their composites (Conca and Yang, 1993).

Functions of edible films and coatings

Most commonly, edible films and coatings are intended to function as a barrier to moisture, oxygen, flavor, aroma, and/or oil, thus improving food quality and shelf life. An edible film or coating may also provide some mechanical protection for a food, reducing bruising and breakage and thus improving food integrity. When an edible film or coating provides a moisture, flavor, aroma, or oil barrier between food components of different water activity, flavor, aroma, and/or oil content in a heterogeneous food, the quality and shelf life of the food are increased. When an edible film or coating prevents exchange of moisture, oxygen, aroma, or oil between the food and the environment, the quality and shelf life of the food also are increased. However,

when functioning in this manner, edible films and coatings are not normally intended to eliminate the need for non-edible protective packaging. Rather, they are intended to work with conventional packaging to improve product quality and shelf life. However, the amount of conventional protective packaging may be reduced (source reduction); and the remaining, simpler package may be more recyclable. In addition, after the package is opened, an edible film or coating can continue to protect the product. The protective function of edible films and coatings may be enhanced with addition of antioxidants or antimicrobials to the film or coating. Depending on the nature of the food, an edible coating may also carry flavors, nutrients, etc., to enhance the quality of the food. Finally, an edible coating can provide additional important sensory attributes to foods, including gloss, color, and non-greasy, non-sticky, or non-color-bleeding surface. The various functions of edible films and coatings are summarized in Table 2.1.

Table 2.1 Possible functions for films and coatings

Uses	Edible	Biodegradable
Barrier to moisture, oxygen, aroma, oil, etc,	X	X
Carrier of antimicrobial, antioxidant, etc,	X	X
Carrier of flavor, color, nutrients	X	
Resistance to mechanical forces	X	X
Product appearance enhancer (gloss, color, etc,)	X	

Source: Krochta (2002)

2.2 Film and coating composition

Materials available for forming films and film coatings fall generally into the categories of proteins, polysaccharides, lipids, and resins. A plasticizer must often be added to reduce film or coating brittleness. Other constituents can include antioxidants and antimicrobials to enhance the film or coating effectiveness. The U.S. Code of Federal Regulations provides the status of protein, polysaccharide, lipid, resin, plasticizer, emulsifier, preservative, and antioxidant materials related to acceptable use (Baldwin, 1999).

2.2.1 Proteins

Proteins cover a broad range of polymeric compounds that provide structure or biological activity in plants or animals. Proteins are distinguished from polysaccharides because they are based on approximately 20 amino acid monomers, rather than just a few or even one monomer, such as glucose in the case of cellulose and starch. The amino acids are similar in containing an amino group ($-NH_2$) and a carboxyl group (-COOH) attached to a central carbon atom. However, each amino acid has a different side group attached to the central carbon that lends unique character to that amino acid. The side group can be non-polar (hydrophobic), polar uncharged (hydrophilic), positively charged at pH 7, or negatively charged at pH 7 (Cheftel *et al*., 1985). Most proteins contain 100–500 amino acid residues. Depending on the sequential order of the amino acids (primary structure of the protein), the protein will assume different structures along the polymer chain (secondary structure of the protein), based on Van der Waals, hydrogen bonding, electrostatic, hydrophobic, and disulfide cross-link interactions among the amino acid units (Cheftel *et al*., 1985). The tertiary protein structure reflects how the secondary structures organize relative to each other, based on the same types of interactions, to form overall globular, fibrous, or random protein structure. Finally, quaternary structure occurs when whole proteins interact with each other into associations to provide unique structure or biological activity. The secondary, tertiary, and quaternary structures of proteins can be modified by various physical and chemical agents, including heat, mechanical treatment, pressure, irradiation, lipid interfaces, acids and alkalis, and metal ions (Cheftel *et al*., 1985). Such agents are often used in the formation of protein films and coatings to optimize protein configuration, protein interactions, and resulting film properties.

Protein film-forming materials derived from animal sources include collagen, gelatin, fish myofibrillar protein, keratin, egg white protein, casein, and whey protein. Protein film-forming materials derived from plant sources include corn zein, wheat gluten, soy protein, peanut protein, and cottonseed protein.

2.2.2 Lipids

Edible lipids include beeswax, candelilla wax, carnauba wax, triglycerides (e.g., milkfat fractions), acetylated monoglycerides, fatty acids, fatty alcohols, and sucrose fatty acid esters. Edible resins include shellac and terpene resin. Because lipid and resin materials are not polymers, they do not generally form cohesive stand-alone films. However, along with often providing desirable gloss, they can be used to coat a food or drug surface to provide a moisture barrier or to provide the moisture-barrier component of a composite film. Composite films can consist of a lipid layer supported by a protein or polysaccharide layer, or lipid material dispersed in a protein or polysaccharide matrix (Krochta, 1997).

2.2.3 Polysaccharides

Polysaccharide film-forming materials include starch and starch derivatives, cellulose derivatives, alginate, carrageenan, chitosan, pectinate, and various gums. Proteins can be combined with polysaccharides to modify film mechanical properties (Arvanitoyannis *et al*., 1996, 1997, 1998a, 1998b; Arvanitoyannis and Biliaderis, 1998).

2.2.3.1 Chitosan

Chitosan is of interest as a potential edible film component because of its good oxygen and carbon dioxide barrier properties (Hosokawa *et al*., 1990). Chitosan has been proved to be nontoxic, biodegradable, biofunctional, and has antimicrobial characteristics (Wang, 1992; Darmadji and Izumimoto, 1994). Chitosan is a linear polysaccharide composed of randomly distributed β-(1,4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (Fig 2.1). Chitosan is produced commercially by deacetylation of chitin. When the degree of deacetylation of chitin reaches about 50% (depending on the origin of the polymer), it becomes soluble in aqueous acidic media and is called chitosan. The solubilization occurs by protonation of the $-NH_2$ functional group on the C-2 position of the D-glucosamine repeat unit, whereby the polysaccharide is converted to a polyelectrolyte in acidic media. Chitosan is the only pseudonatural cationic polymer and thus, it finds many applications that follow from this unique character (flocculants for protein recovery, depollution, etc.). Being soluble in aqueous solutions, it is largely used in different applications as solutions, gels, or films and fibers (Goosen, 1997).

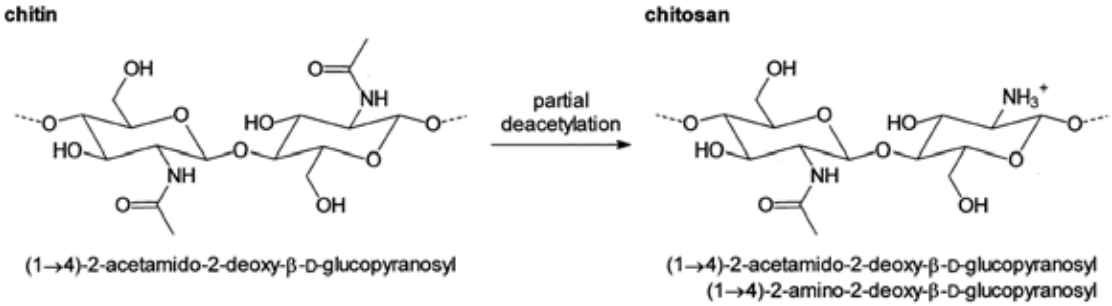

Fig 2.1 Structure of chitin and chitosan (Goosen, 1997).

Chitosans are described in terms of the degree of deacetylation and average molecular weight and their importance resides in their antimicrobial properties in conjuction with their-forming properties (Muzzarelli, 1996). Chitosan could form semi-permeable coatings, which could modify the internal atmosphere, thereby delaying ripening and decreasing transpiration rates in fruits and vegetables. Films from aqueous chitosan were clear, tough, flexible and good oxygen barrier (Sanford, 1989; Kaplan *et al*. 1993). Butler *et al*. (1996) observed that films from chitosan were rather stable and mechanical and barrier properties changed slightly during storage. Chitosan coating has been used with antimicrobial purpose for shelf life extension of fruit and vegetable products such as strawberries, cucumbers, bell peppers (El Ghaouth *et al*., 1991a and b).

The antimicrobial property of chitosan is due to its positively charged amino group which interacts with negatively charged microbial cell membrane, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms (Shahidi *et al*., 1999). A number of studies on the antimicrobial characteristics of chitosan films have been carried out earlier (Chen *et al*., 1996; Ouattara *et al*., 2000; Coma *et al*., 2002; Yingyuad *et al*., 2006). However, chitosan film is in a solid form, therefore, only organisms in direct contact with the active sites of chitosan are inhibited (Srinivasa *et al*., 2007).

2.3 Film Additives

2.3.1 Plasticizer

Various materials can be incorporated into edible films to influence mechanical, protective, sensory, or nutritional properties. Generally, two types of plasticizers were distinguished. First, Internal plasticization is a result of modifications to the chemical structure of the polymer, for example, by copolymerization or selected hydrogenation or transesterification in the case of edible fats or similar. Second, external plasticization is obtained by adding an agent which modifies the structure and energy within the three-dimensional arrangement of the film polymer (Banker, 1966). A plasticizer may be defined as a compound, when added to another material and under given conditions, modifies certain physical and mechanical properties of material. The addition of plasticizer to films produces films, which are less likely to break and more flexible and stronger. The reduction of the intermolecular bonds between the polymer chains, and thus the overall cohesion, facilitated elongation of the films and reduced its glass transition temperature. This is manifested by a reduction in the barrier properties to gases, vapors, and film solutes (Banker, 1966).

Plasticizing agents are essential generally to overcome the brittleness of the chitosan films. Srinivasa *et al*. (2007) reported that chitosan blended with sorbitol gave better tensile strength than those with glycerol, polyethylene glycol (PEG) and fatty acid.

Glycerol and PEG were found to be the most effective plasticizers for methyl cellulose (MC) (Donhowe and Fennema, 1993). Park *et al*. (1993) studied the effect of three plasticizer comprising PEG, propylene glycol (PG), Glycerin (G) at 4 level concentrations. They found a decrease in tensile strength (TS) and an increase in elongation (E) when plasticizer content increased.

2.3.2 Antimicrobial agents

There are many antimicrobial agents that exist and are widely used. To be able to use antimicrobial agents in the foods, pharmaceuticals and cosmetic products, the industry must follow the guidelines and regulations of the country that they are going to use them in, for example, FDA and/or EPA in the United States. This implies that new antimicrobial packaging materials may be developed using only agents which are approved by the authorization agencies as examples of FDA-approved or notified-to-use

within the concentration limits for food safety enhancement or preservation. Various antimicrobial agents may be incorporated in the packaging system, which are chemical antimicrobials, antioxidants, biotechnology products, antimicrobial polymers, natural antimicrobials and gas (Table 2.2).

Table 2.2 Antimicrobial agents and packaging systems

Antimicrobials	Packaging materials	Foods	Microorganisms
Organic acids			
Benzoic acids	PE	Tilapia fillets	Total bacteria
	Ionomer	Culture media	*Penicillium spp., Aspergillus niger*
Parabens	LDPE	Simulants	Migration test
	PE coating	Simulants	Migration test
	Styrene-acrylates	Culture media	*Saccharomyces cerevisiae*
Benzoic & sorbic acids	PE-co-met-acrylates	Culture media	*Aspergillus niger, Penicillium spp.*
Sorbates	LDPE	Culture media	*Saccharomyces cerevisiae*
	PE, BOPP, PET	Water, cheese	Migration test
	LDPE	Cheese	Yeast, mould
	MC/palmitic acid	Water	Migration test
	MC/HPMC/fatty acid	Water	Migration test
	MC/chitosan	Culture media	
	Starch/glycerol	Chicken breast	
	WPI	Culture media	*Saccharomyces cerevisiae, Aspergillus niger, Penicillium roqueforti*

Table 2.2 Antimicrobial agents and packaging systems (continued)

Antimicrobials	Packaging materials	Foods	Microorganisms
	CMC/paper	Cheese	
Sorbic anhydride	PE	Culture media	*Saccharomyces cerevisiae*, moulds
Sorbates & propionates	PE/foil	Apples	Firmness test
Acetic, propionic acid	Chitosan	Water	Migration test
Enzymes			
Lysozyme, nisin, EDTA	SPI, zein	Culture media	*Escherichia coli, Lactobacillus plantarum*
Lysozyme, nisin	WPI	Culture media	*Listeria monocytogenes*
EDTA, propyl paraben			*Salmonella typhimurium, Escherichia coli, Bacillus stearothermophilus, Staphylococcus aureus*
Immobilised lysozyme	PVOH, nylon, cellulose acetate	Culture media	Lysozyme activity test
Glucose oxidase		Fish	

Table 2.2 Antimicrobial agents and packaging systems (continued)

Antimicrobials	Packaging materials	Foods	Microorganisms
Nisin	PE	Beef	*Bacillus stearothermophilus*
	HPMC	Culture media	*Listeria monocytogenes*, *Staphylococcus aureus*
	Corn zein	Shredded cheese	Total aerobes
Nisin, lacticins	Polyamide/LDPE	Culture media	*Micrococcus favus*, *Listeria monocytogenes*
Nisin, lacticin, salts	Polyamide/LDPE	Culture media	*Micrococcus flavus*
Nisin, EDTA	PE, PE-co-PEO	Beef	***Brocothrix*** *thermosphacta*
Nisin, citrate, EDTA	PVC, nylon, LLDPE	Chicken	*Salmonella typhimurium*
Nisin, organic acids mixture	Acrylics, PVA-co-PE	Water	Migration test
Nisin, lauric acid	Zein	Simulants	Migration test
Nisin, pediocin	Cellulose casing	Turkey breast, ham, beef	*Listeria monocytogenes*
Fungicides			
Benomyl	Ionomer	Culture media	
	Imazalil LDPE	Bell pepper	
	PE	Cheese	Moulds

Table 2.2 Antimicrobial agents and packaging systems (continued)

Antimicrobials	Packaging materials	Foods	Microorganisms
Polymers			
Chitosan	Chitosan/paper	Strawberry	*Escherichia coli*
Chitosan, herb extract	LDPE	Culture media	*Lactobacillus plantarum, Escherichia coli, Saccharomyces cerevisiae, Fusarium oxysporum*
UV/excimer laser irradiated nylon	Nylon	Culture media	*Staphylococcus aureus, Pseudomonas fluorescens, Enterococcus faecalis*
Natural extract			
Grapefruit seed extract	LDPE, nylon	Ground beef	Aerobes, coli-forms
	LDPE	Lettuce, soy-sprouts	*Escherichia coli, Staphylococcus aureus*
Clove extract	LDPE	Culture media	*Lactobacillus plantarum, Escherichia coli, Fusarium oxysporum, Saccharomyces cerevisiae*
Herb extract, Ag-Zirconium	LDPE	Lettuce, cucumber	*Escherichia coli ,Staphylococcus aureus, Lactobacillus mesenteroides, Saccharomyces cerevisiae, Aspergillus spp, Penicillium spp.*

Table 2.2 Antimicrobial agents and packaging systems (continued)

Antimicrobials	Packaging materials	Foods	Microorganisms
	LDPE	Strawberry	Firmness test
Eugenol, cinnamaldehyde,	Chitosan	Bologna, ham	Enterobacteriaceae, *lactic acid bacteria*, *Lactobacillus sakei* *Serratia spp.*
Horseradish extract	Paper	Ground beef	*Escherichia coli* 0157: H7
Allyl isothiocyanate	PE film/pad	Chicken, meats, smoked salmon	*Escheriachia coli*, *Salmonella enteritidis, Listeria monocytogenes*
Oxygen absorber			
Ageless	Sachet	Bread	Moulds
BHT	HDPE	Breakfast cereal	
Gas			
Ethanol	Silicagel sachet	Culture media	
	Silicon oxide	Bakery	
	(Ethicap) sachet		

Table 2.2 Antimicrobial agents and packaging systems (continued)

Antimicrobials	Packaging materials	Foods	Microorganisms
Hinokithiol	Cyclodextrin/plactic (Seiwa) sachet	Bakery	
$C10_2$	Plastic films		Migration test
Others			
Hexamethylenetehtramine	LDPE	Orange juice	Yeast, lactic acid bacteria
Silver zeolite, silver nitrate	LDPE	Culture media	*Saccharomyces cerevisiae*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Vibrio parahaemolyticus*
Antibiotics	PE	Culture media	*Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Klebsiella neumoniae*

MC: methyl cellulose; HPMC: hydroxypropyl methyl cellulose; WPI: whey protein isolate; CMC: carboxyl methyl cellulose; SPI: soy protein isolate

Source: Han (2000)

Chemical antimicrobial agents are the most common substances used in the industry. They include organic acids, fungicides, alcohols and antibiotics. Organic acids and their derivatives such as benzoic acids, parabens, sorbates, sorbic acid, propionic acid, acetic acid, lactic acid, medium-size fatty acids and their mixture possess strong antimicrobial activity and have been used as food preservatives, food contact substances and food contact material sanitizers. Benomyl and imazalil had been incorporated in plastic films and demonstrated antifungal activity. Ethanol has strong antibacterial and antifungal activity, however, it is not sufficient to prevent the growth of yeast. Ethanol may enhance some volatile flavor compounds but also causes a strong undesirable chemical odor in most food products. Some antibiotics can be incorporated into animal feedstuffs for the purpose of disease treatment, disease prevention or growth enhancement as well as human disease curing. The use of antibiotics as package additives is not approved for the purpose of antimicrobial functions and is also controversial due to the development of resistant microorganisms. However, antibiotics may be incorporated for short-term use in medical devices and other non-food products. Antioxidants are effective antifungal agents due to the restrictive oxygen requirement of moulds. Food grade chemical antioxidants could be incorporated into packaging materials to create an anaerobic atmosphere inside packages, and eventually protect the food against aerobic spoilage (Smith *et al.*, 1990). Since the package did not contain oxygen, the partial pressure difference of oxygen is formed between the outside and inside of packaging materials. Therefore, in order to maintain the low concentration of oxygen inside the package, the packaging system requires high oxygen barrier materials such as EVOH, PVDC or aluminum foil that prevent the permeation of oxygen. Besides the antioxidants, a multi-ingredient oxygen scavenging system, such as commercial oxygen-absorbing sachets, can be used to reduce oxygen concentration inside the package. Various bacteriocins that are produced by microorganisms also inhibit the growth of spoilage and pathogenic microorganisms. These fermentation products include nisin, lacticins, pediocin, diolococcin, and propionicins (Daeschul, 1989; Han, 2002). These biologically active peptides possess strong antimicrobial properties against various bacteria. Other non-peptide fermentation products such as reuterin also demonstrate antimicrobial activity.

Besides the above food grade bacteriocins, other bacteriocins would be utilized for the development of antimicrobial packaging systems.

Some synthetic or natural polymers also possess antimicrobial activity. Ultraviolet or excimer laser irradiation can excite the structure of nylon and create antimicrobial activity. Among natural polymers, chitosan (chitin derivative) exhibits antimicrobial activity. Short or medium size chitosan possesses quite good antimicrobial activity, while long chain chitosan is not effective. Chitosan has been approved as a food ingredient from FDA recently; therefore, the use of chitosan for new product development as well as a natural antimicrobial agent would become equally feasible and more popular.

The interest in the development and application of natural antimicrobial agents as additives in packaging materials has increased markedly due to their potential safety advantages. Essential oils such as garlic oil and cinnamon oil are proved to be able to inhibit microbial growth although different results are observed depending on test conditions, microorganisms, and the source of the antimicrobial compounds. The study by Pranoto *et al*. (2005) indicated that the films containing antimicrobial agents including garlic oil, potassium sorbate and nisin could enhance antimicrobial activity. Garlic oil incorporated into chitosan film led to an increase in its antimicrobial efficacy, and had little effect on physical properties of chitosan film as it did not have any interaction with the functional groups of chitosan. This is evidence that spice extract has potential to be used as an antimicrobial agent to enhance antimicrobial quality of chitosan film.

2.3.2.1 Cinnamaldehyde

Cinnamic aldehyde or cinnamaldehyde or *trans*-3-phenyl-2-propenal (more precisely trans-cinnamaldehyde, the only naturally-occurring form) (Fig. 2.2) is the chemical compound that gives a specific spicy aroma and flavor to cinnamon. Cinnamaldehyde occurs naturally in the bark of cinnamon trees and other species of the genus Cinnamomum like camphor and cassia. These trees are the natural source of cinnamon, and the essential oil of cinnamon bark contains about 90% cinnamaldehyde (Senanayake and Wijesekera, 2004). Sanla-Ead *et al*. (2006) found that cinnamaldehyde was among the most active components against Gram's positive and

Gram's negative bacteria. Minimum inhibitory concentration of cinnamaldehyde and zone of inhibition of 50 µl/ml cinnamaldehyde are shown in Table 2.3 and 2.4, respectively.

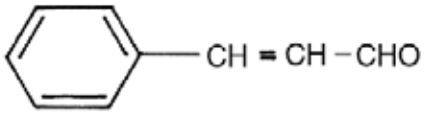

Figure 2.2 Cinnamaldehyde.

Table 2.3 Minimum inhibitory concentration of cinnamaldehyde

Microorganism	MIC (µl/ml)
Gram's positive bacteria	
Bacillus cereus	3.12
Enterococcus faecalis	0.78
Listeria monocytogenes	6.25
Micrococcus luteus	6.25
Staphylococcus aureus	1.56
Gram's negative bacteria	
Aeromonas hydrophila	0.78
Escherichia coli	12.5
Escherichia coli O157: H7	6.25
Pseudomonas aeruginosa	12.5
Salmonella enteridis	6.25

Source: Sanla-Ead *et al*. (2006)

Table 2.4 Zone of inhibition of cinnamaldehyde

Microorganism	Zone of inhibition (mm)
Gram's positive bacteria	
Bacillus cereus	22.95
Enterococcus faecalis	27.76
Listeria monocytogenes	30.09
Micrococcus luteus	22.35
Staphylococcus aureus	28.93
Gram's negative bacteria	
Aeromonas hydrophila	22.27
Escherichia coli	23.45
Escherichia coli O157: H7	21.08
Pseudomonas aeruginosa	12.66
Salmonella enteridis	22.76

Source: Sanla-Ead *et al*. (2006)

CHAPTER III
MATERIALS AND METHODS

3.1 Modification of chitosan-based film

3.1.1 Effect of acid type on physical properties of chitosan film

Chitosan film-forming solutions were prepared by dissolving commercial grade chitosan (95% degree of deacetylation) powder (Seafresh Chitosan (LAB) Co., Ltd., Bangkok, Thailand) into 1 %v/v acetic or lactic acid solution. The film-forming solutions were then filtered through silk screen (320 mesh) and air bubbles were removed using Hybrid Mixer (HM-500, Kyence Co., Tokyo, Japan). The prepared film-forming solutions (4 ml) were cast onto a rimmed silicone plate (50×50 mm) and dried at 25 °C in electronic low temperature chamber (Advancetec TE-203A, Toyo Seisakusho Kaisha Ltd., Chiba, Japan) for 24 h. The resulting films were manually peeled off. All film samples were conditioned in ventilated oven (EYELA KCL-2000, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at 25 °C and 50 %RH for 24 h before physical properties determination.

3.1.1.1 Film physical properties testing

3.1.1.1.1 Mechanical properties

Film thickness was measured using a micrometer (Dial Pipe Gauge, Peacock Co., Tokyo, Japan) at six random locations of the film. After conditioned for 24 h, tensile strength (TS) and elongation at break (EAB) were determined using a Tensipresser® (TTP-508X II, Taketomo Electric Inc., Tokyo, Japan) according to the ASTM D 882-22 (ASTM, 1989) (Appendix A.1).

3.1.1.1.2 Water vapor permeability (WVP)

WVP ($g \cdot m/m^2 \cdot sec \cdot Pa$) was measured using modified ASTM method reported by Gontard *et al*. (1992) (Appendix A.2).

3.1.1.1.3 Surface color

Color values (Hunter L, a, b) were measured using a color reader (CR-13, Konica Minolta Sensing Inc., Tokyo, Japan) at three random locations of the film.

3.1.1.1.4 Transparency

The transparency of the films was measured using a UV-vis spectrophotometer (UV-160, Shimadzu Co., Kyoto, Japan) following the ASTM method D 1746-92 (ASTM, 1987) with slight modification. The transparency was calculated as Eq. (1):

$$\text{Transparency} = \frac{A_{600}}{x} \text{ or } \frac{(-\log T_{600})}{x} \quad (1)$$

where A_{600} is the absorbance at 600 nm, T_{600} is the transmittance at 600 nm, and x is the film thickness (mm) (Yildirim and Hettiarachchy, 1998).

3.1.1.2 Statistical analysis

Completely randomized design (CRD) was used in this experiment. The effect of organic acid type on physical properties of chitosan film was statistically analyzed using ANOVA test. The statistical differences between mean values were established at $p \leq 0.05$ with the Duncan's New Multiple Range Test (DNMRT) (Cochran and Cox, 1992).

3.1.2 Effect of plasticizer type and concentration on physical properties of chitosan film

Glycerol or sorbitol (20, 40 and 60 %w/w of chitosan powder) (Wako Pure Chemical Industries Ltd., Tokyo, Japan) were added into film-forming solution as plasticizer. After stirred for 1 h, the plasticized film-forming solution was filtered through silk screen and air bubbles were removed. The plasticized film-forming solution were cast and dried as described in section 3.1.1

3.1.2.1 Film physical properties testing

After conditioned for 24 h, the films were examined for their physical properties by the same method as described in section 3.1.1.1.

3.1.2.2 Statistical analysis

Factorial (2×3) in CRD was used in this experiment. The effect of plasticizer type and concentration on physical properties of chitosan film was statistically analyzed using ANOVA test. The statistical differences between mean values were established at $p \leq 0.05$ with the Duncan's New Multiple Range Test (DNMRT).

3.2 Effect of cinnamaldehyde on physical and antimicrobial properties of chitosan film

Cinnamaldehyde (*trans*-3-phenyl-2-propenal) (Wako Pure Chemical Industries Ltd., Tokyo, Japan) at different concentrations (50, 100 and 150 µl/g chitosan) was incorporated into optimum chitosan film-forming solution obtained from section 3.1 (1% w/v chitosan powder in 1 %v/v acetic acid and plasticized with sorbitol 40 %w/w). The chitosan-plasticizer-cinnamaldehyde solution was stirred for 1 h prior to casting, drying and conditioning as described in section 3.1.

3.2.1 Film physical properties testing

After conditioned for 24 h, the films were examined for their physical properties by the same method as described in section 3.1.1.1.

3.2.2 Statistical analysis

CRD was used in this experiment. The effect of cinnamaldehyde concentration on physical properties was statistically analyzed using ANOVA test. The statistical differences between mean values were established at $p \leq 0.05$ with DNMRT.

3.2.3 Interactions of cinnamaldehyde with chitosan

The spectra of chitosan films (control and those incorporated with cinnamaldehyde) were recorded using attenuated total reflection (ATR) in a Digilab **Fourier Transform Infrared (**FT-IR) spectrometer (excaliber series, FTS 3000, Randolph, MA, USA) at room temperature. Light source of transmittance was in the middle infrared 500-4000 cm^{-1}. The FT-IR was operated in a reflection mode with 256 consecutive scan at 2 cm^{-1} resolution were average for duplicate measurements for each spectrum. The spectra obtained were used to determine the possible interactions between functional groups of chitosan with cinnamaldehyde.

3.2.4 Glass transition temperature

Glass transition temperature (T_g) of chitosan films (control and those incorporated with cinnamaldehyde) was characterized using differential scanning calorimeter (DSC, Shimadzu Co., Kyoto, Japan) according to method of Kristo and Biliaderis (2006) and Quijada-Garrido *et al*. (2007) with slight modification (Appendix A.3).

3.2.5 Antimicrobial property of chitosan film containing cinnamaldehyde

Chitosan film containing different concentration of cinnamaldehyde were tested for their antimicrobial property against the selected Gram's positive bacteria (*Staphylococcus aureus*, *Bacillus licheniformis* and *Bacillus subtilis*) and Gram's negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Shewanella putrefaciens* using double layer agar diffusion method as described by Hamakuchi (2006). Positive control was done using sterilized paper discs containing the same amount of cinnamaldehyde as chitosan film sample.

3.2.5.1 Culture preparation

Gram's positive bacteria and Gram's negative bacteria cultures in glycerol stock were obtain from Laboratory of Applied Microbiology in Tokyo University of Marine Science and Technology. All cultures were grown in 10 ml of tryptic soy broth (TSB) (Bacto™, Becton, Dickinson and Company, Sparks, USA) for 24 or 48 h at 30 °C and set at the absorbance of 0.2 at 630 nm (ca. 10^5-10^6 cfu/ml) using Micro plate reader BIO-RAD, Model 550, Tokyo, Japan.

3.2.5.2 Determination of anitomicrobial property of cinnamaldehyde

The solid media consisted of two layers. The lower layer was prepared by pouring 15 ml of tryptic soy agar (TSB with 1.4 %w/v agar) on plastic petri dish. The upper layer was prepared by pouring 5 ml of tryptic soy agar (TSB with 0.8 %w/v agar) inoculated with bacterial cultures previously set at 0.2 (absorbance at 630nm) on to the lower layer. Sterilized paper discs containing cinnamaldehyde, prepared by dropped 100 µl of cinnamaldehyde homogenized solution (50, 100 and 150 µl/ml) into the paper discs (0.1 g) and dried at 25 °C for 24h, were placed on the upper layer and incubated at 30 °C for 24 h. The petri dishes were examined for clear zone, and the diameter of the zone was measured using vernier caliper (Model 530-101 N15, Mitutoyo Co., Kawasaki, Japan). No growth underneath the paper disc indicated inhibitory effect on the contact surface and the inhibition zone was calculated by subtracting diameter of the clear zone around the paper disc by the diameter of paper disc. In the case of inhibitory zone above 25 mm, antimicrobial testing was repeated by using only one sterilized paper disc containing cinnamaldehyde in one petri dish.

3.2.5.3 Determination of antimicrobial property of chitosan film containing cinnamaldehyde

The film disc samples (8.6 mm) with positive control (sterilized paper disc containing 150 µl/g cinnamaldyhyde) were place on the upper layer of double layer agar. Inhibition zone and inhibitory effect on the contact surface were determined as described in section 3.2.5.2. No growth underneath the film disc indicated inhibitory effect on the contact surface and the inhibition zone was calculated by subtracting diameter of the clear zone around the film disc by the diameter of the film disc (8.6 mm).

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Modification of chitosan-based film

In this study, commercial grade chitosan with 95% degree of deacetylation was used as raw material of the film in order to gain high efficiency on antimicrobial property. There are many researchers who have studied the effect of acid type, plasticizer type and concentration on physical properties of chitosan film, however, the innate properties (molecular weight and degree of acetylation) of chitosan also have an effect on physical properties of the film.

4.1.1 Effect of acid type on physical properties of chitosan film

Chitosan films from acetic acid and lactic acid solution showed similar visual appearances as shown in Figure 4.1. Shrinkage and breakage of the films were experienced while peeling off from the silicone resin plate as well as during determination of their properties.

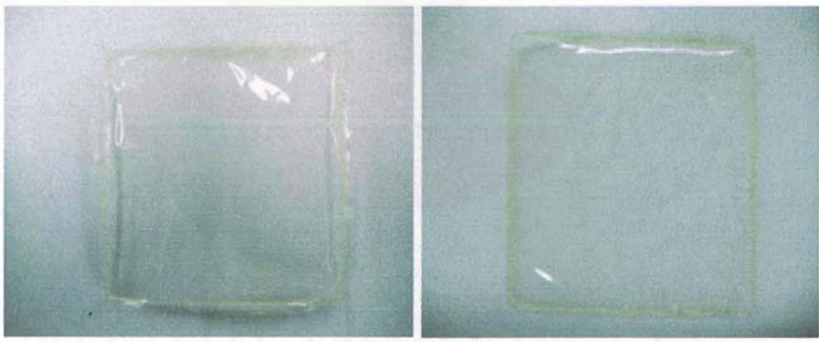

Figure 4.1 Unmodified chitosan film cast with acetic acid (left) and lactic acid (right).

Film-forming solutions with varying acid type of acetic and lactic acid were studied. The effect of acid type on films thickness, tensile strength, elongation at break and water vapor permeability was presented in Figures 4.2 A, 4.2 B, 4.2 C and 4.3, respectively. There were no significant differences in TS and WVP values, while thickness and EAB were significantly different ($p \leq 005$). The films prepared using lactic acid solution showed higher thickness and elongation at break than those with acetic

acid. Bégin and Van Calsteren (1999) have suggested that an increase in thickness and elongation at break is related to the molecular volume of the counter ion (acid used as solvent). These could be further explained by examining the crystal structure of chitan (Appendix D.1). The acetyl group is located between two parallel chains of the polysaccharide. The molecular volume of lactic acid (around 70 °A) cannot occupy the space between polymer chains without interfering crystallinity formation. Since, resistance of the film to deformation depends on the nature and formation of crystallinity (Billmeyer, 1984), it could be assumed that the larger the molecular volume of counter ion in the range of 50-70 °A, the more stretchable the film becomes.

The surface color of chitosan films was affected by type of acid, films from acetic acid solution showed significantly higher ($p \leq 0.05$) b value (Fig. 4.5 C) while transparency, L value and a value of the films were not significantly different as shown in Figures 4.4, 4.5 A and 4.5 B, respectively. The surface color of the films prepared using acetic acid was more yellowish than those prepared using lactic acid. Zeng *et al*. (2007) studied the browning pigment formation of chitooligomer (COS), chitosan with degree of polymerization less than 20, which can be achieved by depolymerization of chitosan, by examining the absorbance at 278 nm. They indicated that COS undergo browning reaction upon heating. They also found that among pH in acidic range, the browning pigment formation was highest at pH 4. Therefore, It could be inferred that during dissolving chitosan powder in acid solution, browning reaction of film-forming in acetic acid solution (pH 4.0) could occur more when compared to lactic acid (pH 3.3) (data not shown).

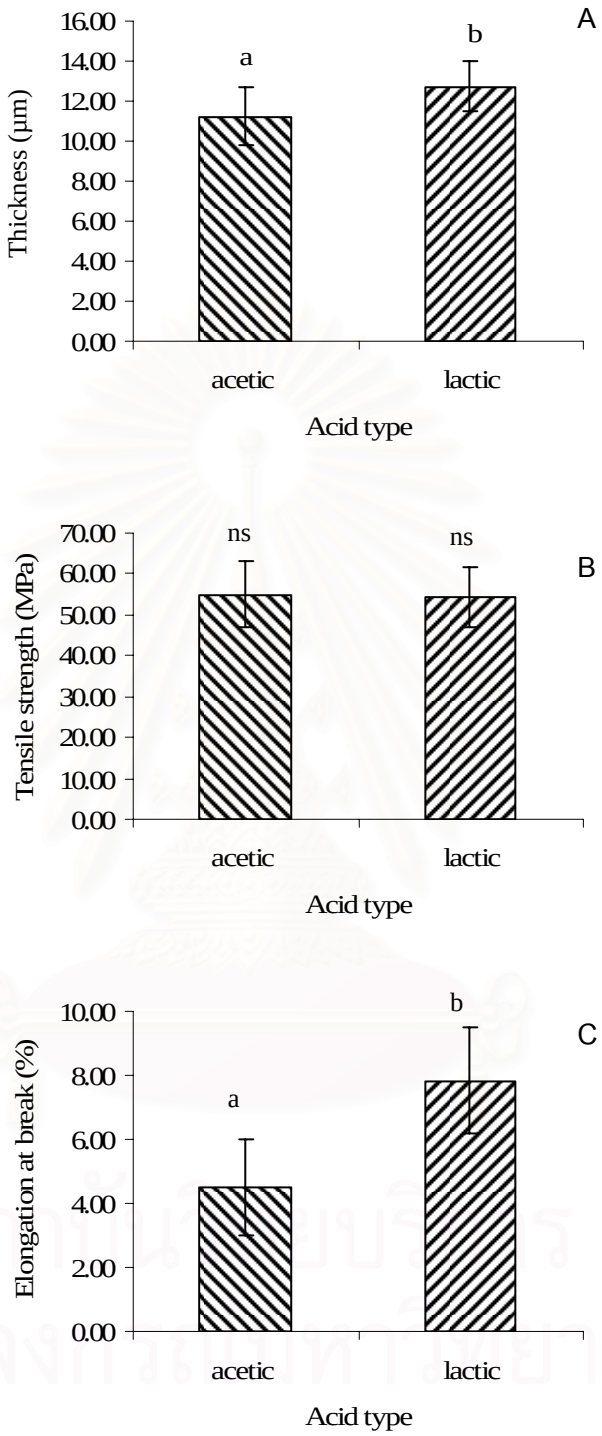

Figure 4.2 Effect of acid type on thickness (A), tensile strength (B) and elongation at break (C) of chitosan films.

Means with different letters represent significant differences ($p \leq 0.05$). ns indicates no significant differences ($p > 0.05$)

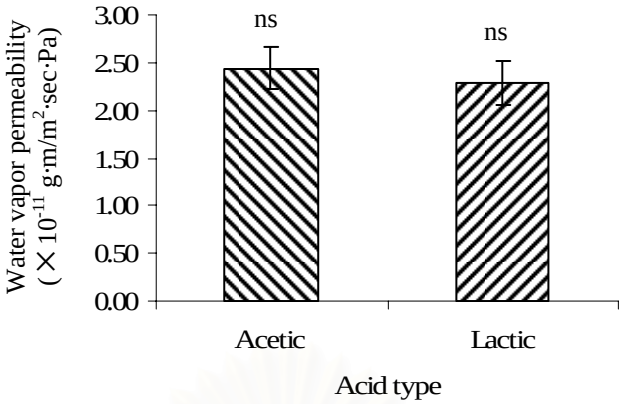

Figure 4.3 Effect of acid type on water vapor permeability of chitosan films. ns indicates no significant differences ($p > 0.05$)

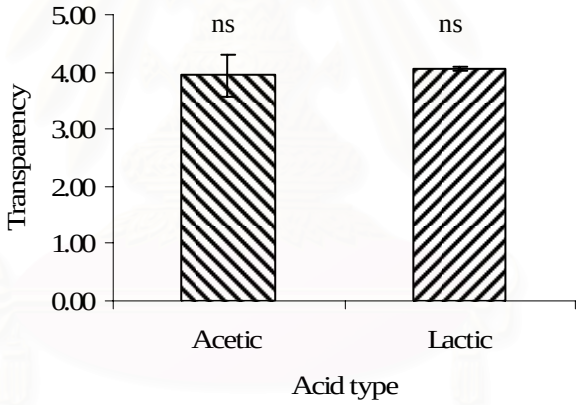

Figure 4.4 Effect of acid type on transparency of chitosan films. ns indicates no significant differences ($p > 0.05$)

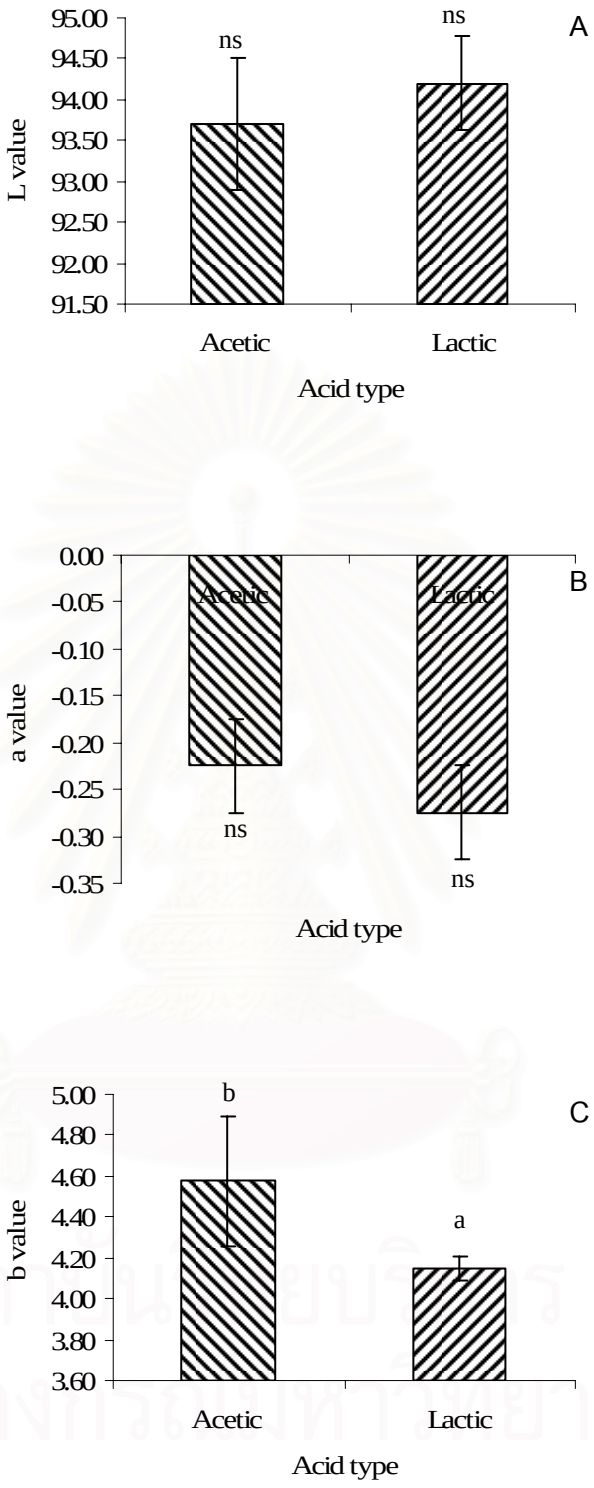

Figure 4.5 Effect of acid type on L (A), a (B) and b value (C) of chitosan films. Means with different letters represent significant differences ($p \leq 0.05$). ns indicates no significant differences ($p > 0.05$).

4.1.2 Effect of plasticizer type and concentration on physical properties of chitosan film

4.1.2.1 Mechanical properties

The results in section 4.1.1 demonstrated that films without plasticizer were relatively brittle and torn easily when peeled off the silicone resin plate. Therefore, desirable mechanical properties of the films were improved by addition of plasticizers (glycerol and sorbitol) at different concentration (20, 40 and 60 %w/w of chitosan powder). The mechanical properties of films plasticized by glycerol or sorbitol, at different concentration were determined by measuring their thickness, tensile strength and elongation at break.

The results showed that chitosan film-forming in lactic acid solution and plasticized with glycerol or sorbitol was difficult to form into film, and thus physical property measurements were not possible (Fig. 4.6). The results showed that an increase in amount of the plasticizers resulted in significant ($p \leq 0.05$) increase in thickness, decrease in mechanical resistance (decrease in tensile strength) and increase in extensibility (increase in elongation at break). Tensile strength decreased from 76.13 to 28.39 and 48.69 to 24.22 MPa (Fig. 4.7 B) when sorbitol and glycerol concentration increased from 20 to 60 %w/w, while elongation at break increased from 5.76 to 51.06 and 28.73 to 53.18 %, respectively (Fig. 4.7 C). Changes in mechanical properties as affected by hydrophilic plasticizers were observed for various hydrocolloid-based films (Gontard *et al*., 1993; Butler *et al*., 1996; Caner *et al*., 1998; Srinivasa *et al*., 2007).

Tensile strength and elongation at break are inversely correlated. The latter showed an increasing trend with the addition of plasticizers. Sorbitol and glycerol are low molecular weight hydrophilic molecules that could fit into chitosan chains and established hydrogen bonding with reactive groups of chitosan. By reducing internal hydrogen bonding between polymer chains, the density of intermolecular interaction in material decrease and the free volume between polymer chains increase (Cuq *et al*., 1997). The films mechanical properties changed due to decrease in density and reversibility of intermolecular and intramolecular interaction occurring in the films.

The mechanical properties of sorbitol and glycerol plasticized film were statistically compared (Fig. 4.7). The sorbitol plasticized film had significantly ($p \leq 0.05$) higher tensile strength and lower elongation at break than glycerol plasticized film at 20 %w/w while there are no significant difference among mechanical properties of the film with different type of plasticizer at concentration of 40 %w/w.

Figure 4.6 Chitosan film cast from lactic acid solution plasticized with 20, 40 and 60 %w/w sorbitol (from left to right).

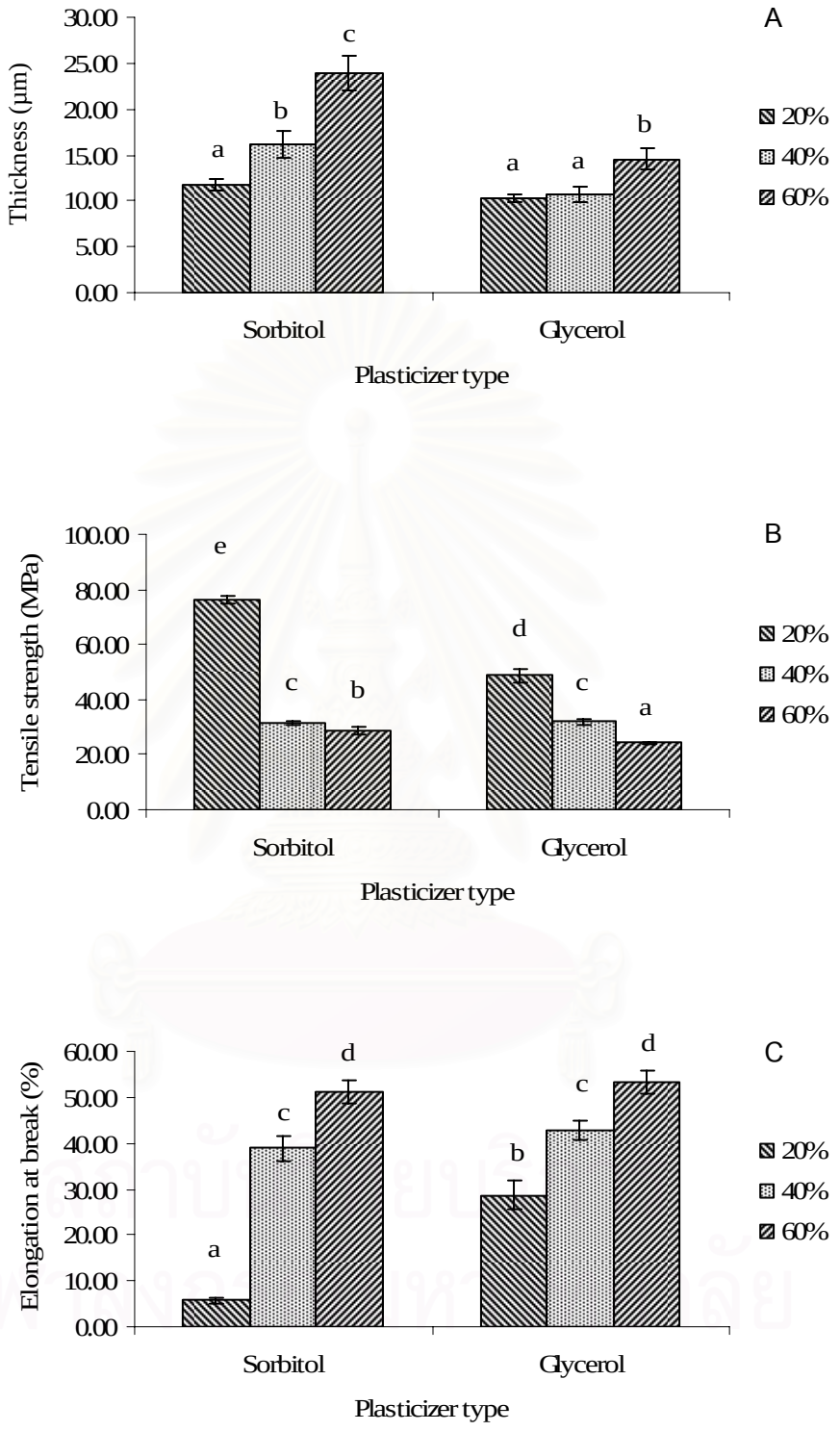

Figure 4.7 Effect of plasticizer type and concentration on thickness (A), tensile strength (B) and elongation at break (C) of chitosan films prepared by acetic acid.

Means with different letters represent significant differences ($p \leq 0.05$).

4.1.2.2 Water vapor permeability

Water vapor permeability of films from chitosan with different type and concentration of plasticizer was examined. The water vapor permeability increased significantly ($p \leq 0.05$) with addition of plasticizer (Fig. 4.8). The water vapor permeability increased from 2.09×10^{-11} to 4.32×10^{-11} and 3.03×10^{-11} to 4.94×10^{-11} g·m/m^2·sec·Pa, respectively, when the concentration of sorbitol and glycerol increase from 20 to 60 %w/w (Fig. 4.8). This change could be explained by structural modifications of the chitosan network. The incorporation of plasticizers modified the molecular arrangement of the chitosan network, with an increase in free volume. The network becomes less dense and, as a consequence, more permeable (Ashley, 1985). Water vapor permeability increased with plasticizer concentration and this could be related to hydrophilicity of plasticizer molecules. Incorporating hydrophilic plasticizers was reported to increase the water vapor permeability of hydrocolloid-based films (Gontard *et al*., 1993; McHugh *et al*., 1994; Srinivasa *et al*. 2007; Talja *et al*., 2007).

Comparison of the water vapor permeability for each plasticized film is shown in Figure 4.8. Films plasticized with sorbitol had lower water vapor permeability than those with glycerol at each concentration due to the fact that sorbitol has less ability to bind water than glycerol (McHugh *et al*. 1994).

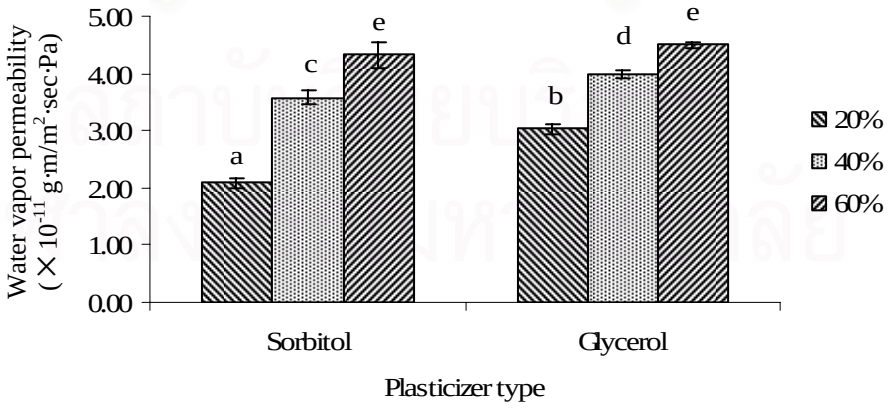

Figure 4.8 Effect of plasticizer type and concentration on water vapor permeabilty of chitosan films prepared by acetic acid.

Means with different letters represent significant differences ($p \leq 0.05$).

4.1.2.3 Surface color and transparency

The effects of plasticizer type and concentration on film surface color and transparency are shown in Figures 4.9 and 4.10, respectively. b value increased significantly ($p \leq 0.05$) with addition of plasticizers (Fig. 4.9). b value significantly increased from 4.63 to 5.20 and 4.77 to 6.27 (Fig. 4.9 C), respectively, when sorbitol and glycerol concentration increased from 20 to 60 %w/w. The surface color of the films was also affected by the type of plasticizer. Increasing in yellowness (b value) occurred greater when glycerol was used. Cuq *et al*. (1996) also observed a relatively slight yellow color in glycerol-plasticized protein-based film. Labuza and Saltmarch (1981) reported that glycerol might participate in the browning reaction, but no specific mechanism was proposed. The films were lighter (greater + L value) (Fig. 4.9 A) and more transparent (lower transparency value) (Fig 4.10) with incorporation of sorbitol. It could be concluded that, at the concentration of 40 %w/w, chitosan film plasticized with sorbitol has better appearance in terms of surface color and transparency than the one cast with glycerol.

Based on physical properties, the results represented that 40% w/w sorbitol was the optimum concentration in that it gave the film with the best physical properties and was suitable for further experiment.

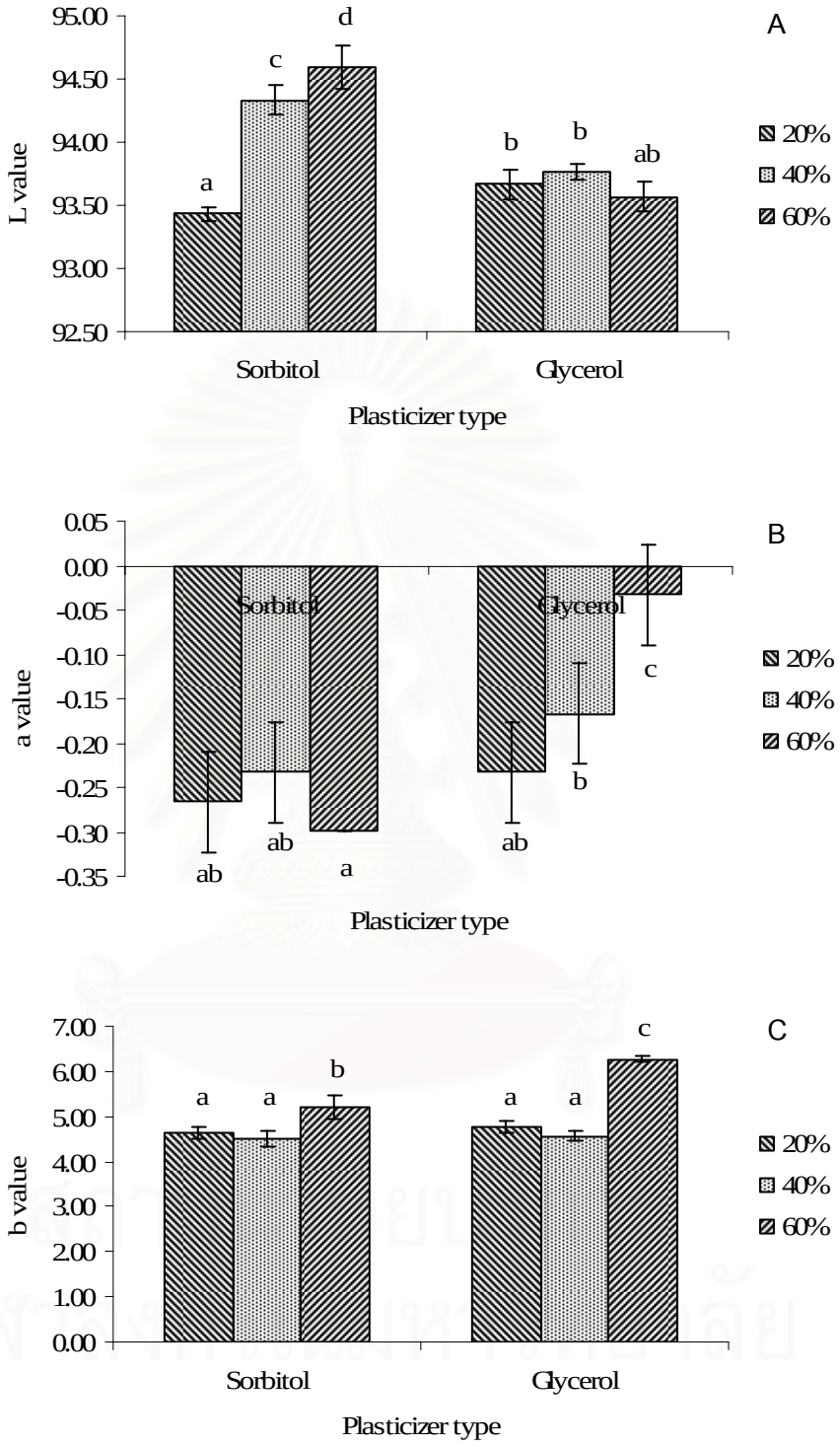

Figure 4.9 Effect of plasticizer type and concentration on L (A), a (B) and b value (C) of chitosan films prepared by acetic acid.

Means with different letters represent significant differences ($p \leq 0.05$).

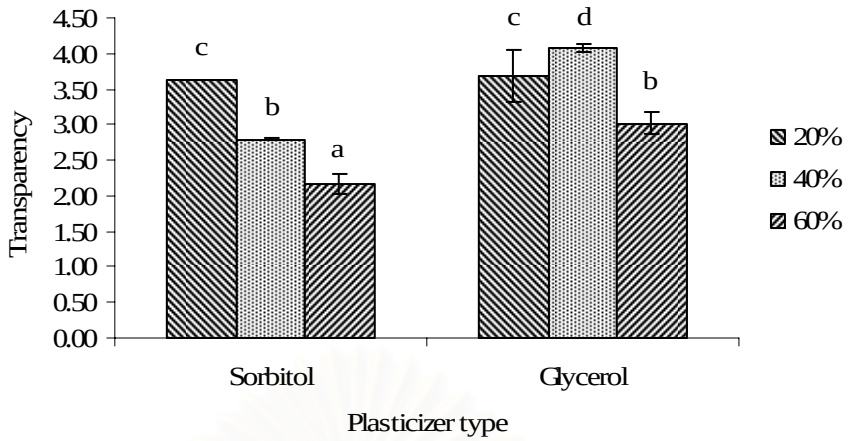

Figure 4.10 Effect of plasticizer type and concentration on transparency of chitosan films prepared by acetic acid.

Means with different letters represent significant differences ($p \leq 0.05$).

4.2 Effect of cinnamaldehyde on physical and antimicrobial properties of chitosan film

Cinnamaldehyde at different concentrations (50, 100 and 150 µl/g chitosan) was incorporated into the optimum film-forming solution obtained from section 4.1 (1% w/v chitosan powder in 1 %v/v acetic acid plasticized with 40 %w/w of chitosan powder sorbitol). The effects of the incorporation of this antimicrobial agent on physical and antimicrobial properties of chitosan film were investigated.

4.2.1 Mechanical properties

The effects of cinnamaldehyde on film thickness, tensile strength and elongation at break are shown in Figures 4.11 A, B and C, respectively. Film thickness significantly ($p \leq 0.05$) decreased with the addition of cinnamaldehyhyde (50 µl/g), whereas no significant difference was observed upon further addition (Fig. 4.11 A). Incorporation of cinnamaldehyde led to a significant increase in both tensile strength and elongation at break. Tensile strength and elongation at break increased from 39.54 to 48.18 MPa (Fig. 3.11 B) and 37.90 to 48.97 % (Fig. 3.11 C), respectively, when the concentration of cinnamaldehyde increased from 50 to 150 µl/g. The effect of cinnamaldehyde on mechanical properties did not show the same trend as the addition

of plasticizing agent. Therefore, an increase in both tensile strength and elongation at break could be due to the interaction between functional groups of chitosan and cinnamaldehyde. The interaction between chitosan and cinnamaldehyde can be further explained in section 4.2.4.

At the concentration of 150 µl/g, the mechanical properties of chitosan film incorporated with cinnamaldehyde are close to the mechanical properties of oriented polypropylene (OPP) (Appendix D.2). However, this amount of cinnamaldehyde addition significantly affected the surface color and transparency of the films (section 4.2.3).

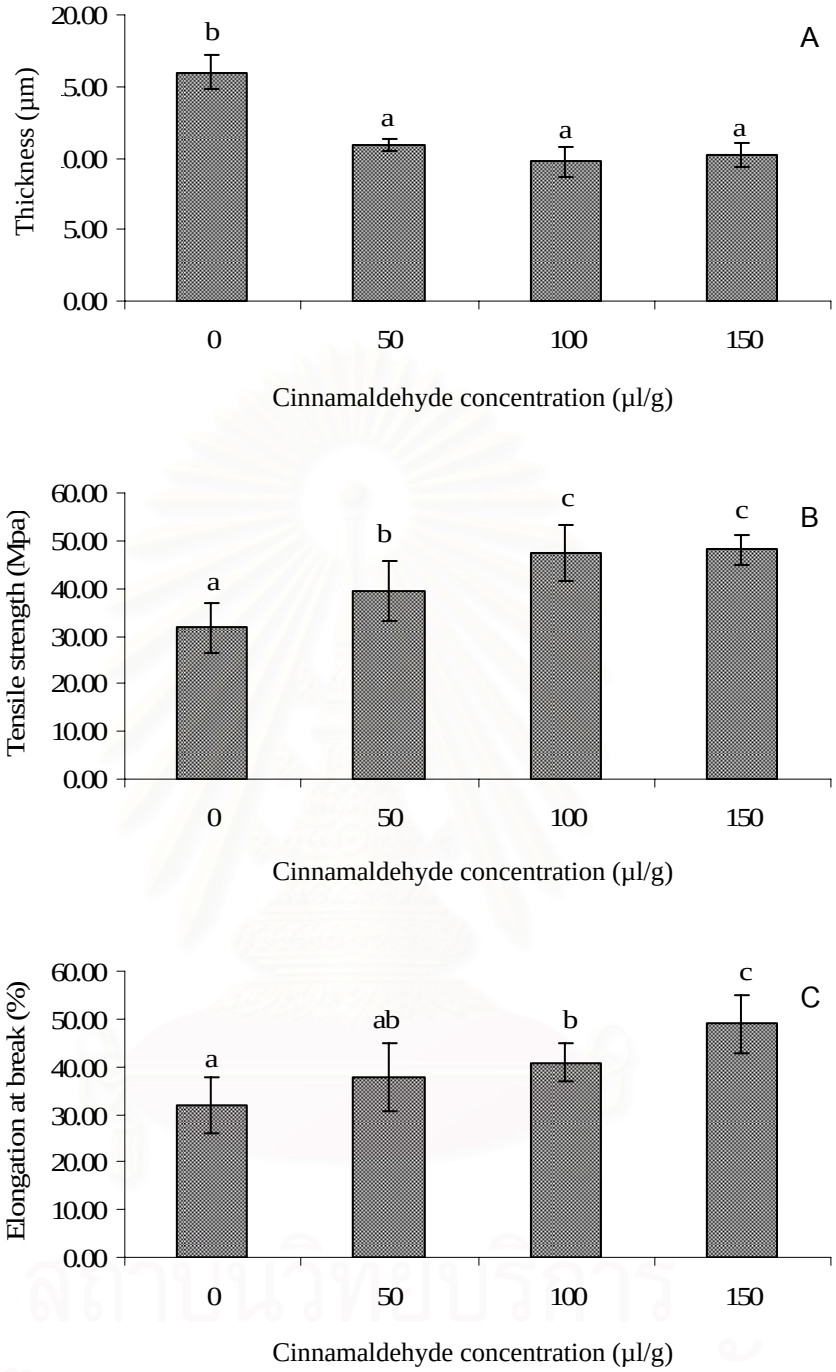

Figure 4.11 Effect of cinnamaldehyde concentration on thickness (A), tensile strength (B) and elongation at break (C) of chitosan films. Means with different letters represent significant differences ($p \leq 0.05$).

4.2.2 Water vapor permeability

The effect of cinnamaldehyde concentration on water vapor permeability of the films is shown in Figure 4.12. The water vapor permeability decreased significantly ($p \leq 0.05$) upon addition of cinnamaldehyde. The water vapor permeability decreased from 3.07×10^{-11} to 2.47×10^{-11} g·m/m^2·sec·Pa, when the concentration of cinnamaldehyde increased from 50 to 150 µl/g (Fig. 4.12). Incorporation of cinnamaldehyde showed the same effect on water vapor permeability of alginate–apple purée edible films (Rojas-Graü *et al*., 2007). Hernandez (1994) indicated that water vapor transfer generally occurred through the hydrophilic portion of the film and depends on the hydrophilic-hydrophobic ratio of the film components. Cinnamaldehyde can slightly dissolve in water. Decreasing in hydrophilic-hydrophobic ratio can cause by increasing in amount of cinnamaldehyde. Thus, incorporation of cinnamaldehyde can decrease water vapor permeability of chitosan film.

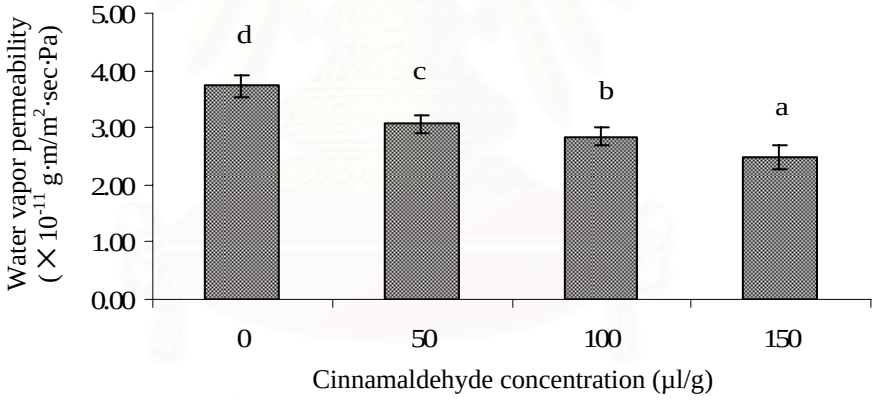

Figure 4.12 Effect of cinnamaldehyde concentration on water vapor permeability of chitosan films.

Means with different letters represent significant differences ($p \leq 0.05$).

4.2.3 Surface color and transparency

The effect of cinnamaldehyde concentration on film surface color and transparency are shown in Figures 4.13 and 4.14, respectively. There were no significant differences in L but some differences in a values (Fig. 4.13 A and B, respectively). The films were more yellow (greater + b value) with addition of cinnamaldehyde (50 µl/g of chitosan powder) but no significant differences ($p > 0.05$) were observed with further addition of cinnamaldehyde (Fig. 4.13 C). An Increase in b value could be due to cinnamaldehyde own yellowish color. The results in Figure 4.14 showed that the films became significantly less ($p \leq 0.05$) transparent (higher transparency value) with incorporation of cinnamaldehyde. It could be due to the insoluble portion in the film-forming solution which increased with further addition of cinnamaldehyde. With addition of 150 µl/g cinnamaldehyde, the color of the film-forming solution became milk-like after stirring for 1 h in the films preparation process.

Overall, cinnamaldehyde can improve physical properties of chitosan film. Tensile strength and elongation at break increased while water vapor permeability decreased upon the incorporation of cinnamaldehyde, however, this incorporation has disadvantage in terms of surface color and transparency. The films became more yellow and more opaque with addition of cinnamaldehyde.

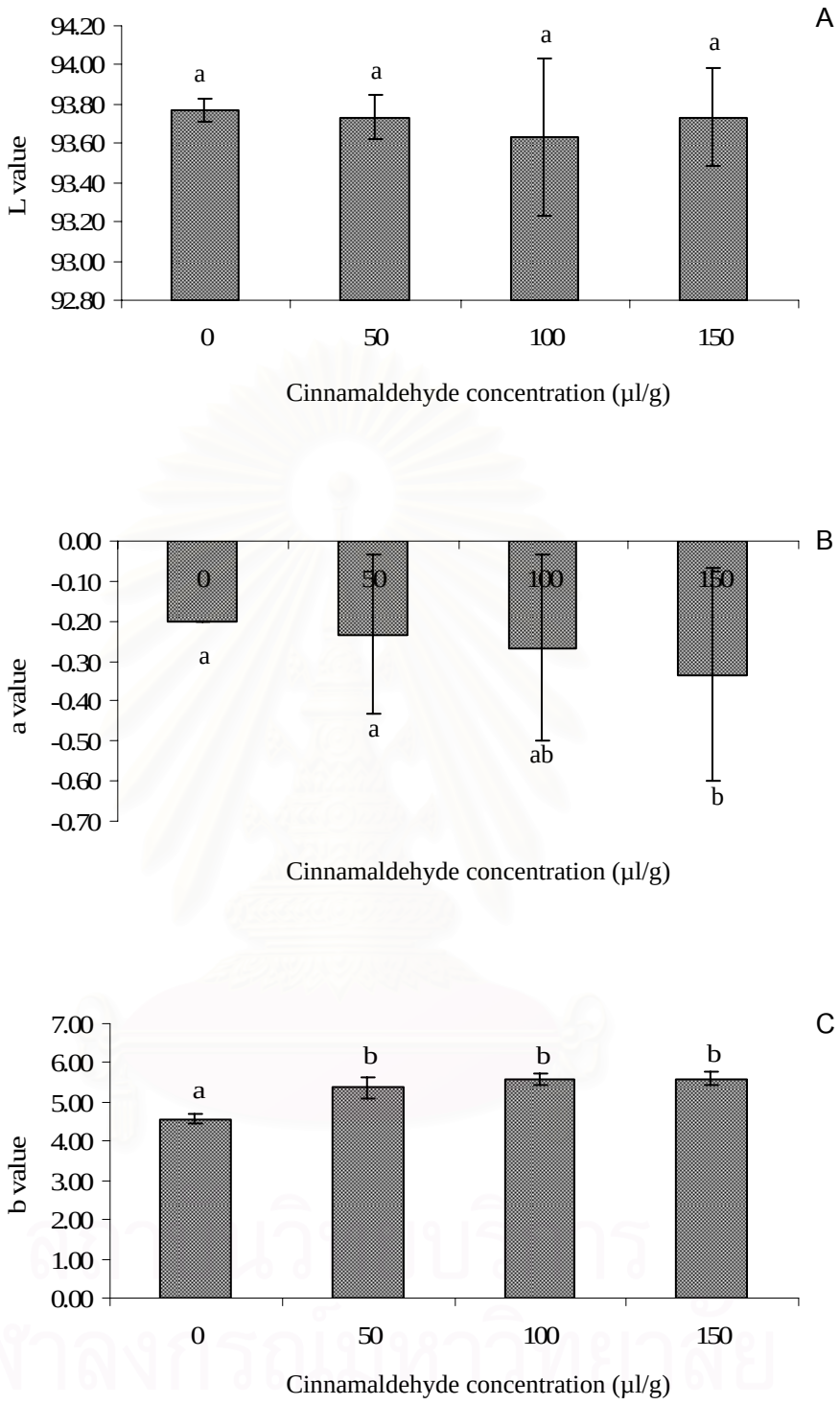

Figure 4.13 Effect of cinnamaldehyde concentration on L (A), a (B) and b value (C) of chitosan films.

Means with different letters represent significant differences ($p \leq 0.05$).

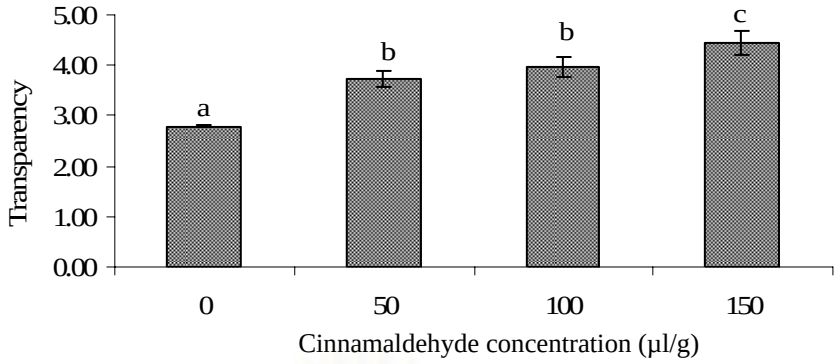

Figure 4.14 Effect of cinnamaldehyde concentration on transparency of chitosan films. Means with different letters represent significant differences ($p \leq 0.05$).

4.2.4 Interaction of cinnamaldehyde with chitosan

The possible interaction between functional group of chitosan and cinnamaldehyde was observed using FT-IR spectrometry. Figure 4.15 showed the spectra of chitosan films (with and without incorporation of cinnamaldehyde). All spectra showed similar pattern with the peak around 3500 cm^{-1}. Absorption in this area indicates stretching vibration of hydroxyl group (-OH) and indicated intermolecular hydrogen bonding of chitosan molecules (Kim *et al*., 2006). They are also overlapped in the same region of an NH stretching (Nunthanids *et al*., 2001). The carbonyl, C=O, amine, NH_2 and ammonium, NH_3^+ bands were situated in the region between 1400 cm^{-1} and 1700 cm^{-1}. A change in the band at 1649 cm^{-1} was observed. It was smoother and then disappeared when the amount of cinnamaldehyde increased from 0 to 150 µl/g. This indicated some interaction between amine group of chitosan and carbonyl group of cinnamaldehyde. In addition, C=N absorption band around 1600 cm^{-1} appeared when 150 µl/g cinnamaldehyde was incorporated. From these spectral results, the possible interaction between functional group of chitosan and cinnamaldehyde could be due to imine formation (see Appendix D.3 for further information).

The infrared spectral data support mechanical data (section 4.2.1) of chitosan films incorporated with cinnamaldehyde. When chitosan films are incorporated with cinnamaldehyde, there is a modification on the functional groups of chitosan. There is thus a significant change on the mechanical properties (increased in both tensile strength and elongation at break).

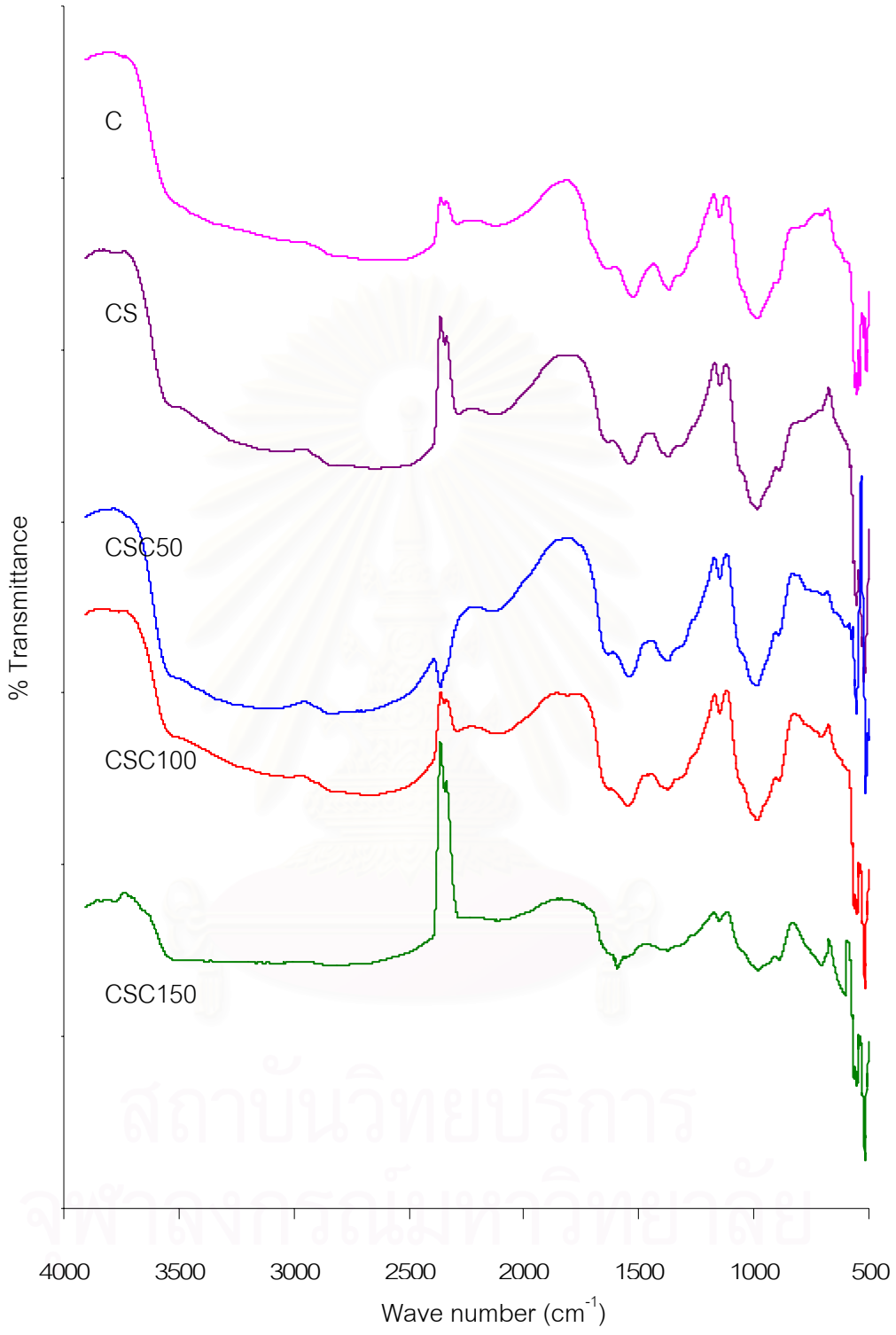

Figure 4.15 FT-IR spectra of control chitosan film (C), chitosan film plasticized with sorbitol (CS) and plasticized chitosan film incorporated with cinnamaldehyde (50 (CSC50), 100 (CSC100) and 150 (CSC150) µl/g.

4.2.5 Glass transition temperature

The effect of cinnamaldehyde on glass transition temperature (T_g) of chitosan film was determined using differential scanning calorimeter. Figure 4.16 presents DSC thermograms of chitosan film (C), plasticized chitosan film plasticized with sorbitol (CS) and plasticized chitosan film incorporated with cinnamaldehyde at 50 µl/g (CSC50), 100 µl/g (CSC100), and 150 µl/g (CSC150). The temperature range used on the determination was from 0 to 180 °C. The maximum temperature of 180 °C was selected in order to limit possible chitosan degradation. Water content of the samples ranged from 10 to 14% and, according to the literature (Qu *et al*., 2000), at this water content, only nonfreezable water is present. An onset of broad endothermic peak close to 100 °C was observed on the curve of control film (Fig. 4.16) attributed to evaporation of residual water, from the sample. The same endothermic peak of chitosan-based material was also observed by Mucha and Pawlak (2005) and Neto *et al*. (2005). From the results, T_g of control film was observed around 77.8 °C. T_g of chitosan at this point was also reported by Toffey and Glasser (2001) and Lazaridou and Biliaderis (2002). The T_g of chitosan is still a subject of controversy. The main reason may be that, being a natural polymer, some properties like crystallinity, molecular weight and deacetylation degree, can present wide variations according to the source and/or method of extraction and these factors will influence the T_g.

Addition of sorbitol led to a decrease of T_g of the films. T_g decreased to around 68.0 °C (Fig. 4.16) when 40 %w/w sorbitol was added due to the plasticizing effect of sorbitol. Many researchers have reported a decrease in T_g values of hydrocolloid-based films with an addition of sorbitol (Lazaridou and Biliaderis, 2002; Kristo and Biliaderis, 2006; Talja *et al*., 2007). Incorporation of cinnamaldehyde slightly affected the T_g, probably due to the fact that cinnamaldehyde has no plasticizing effect on the film. Moreover, an interaction between amine group of chitosan and carbonyl group of cinnamaldehyde eliminates water molecule, consequently led to a decrease of T_g of the film.

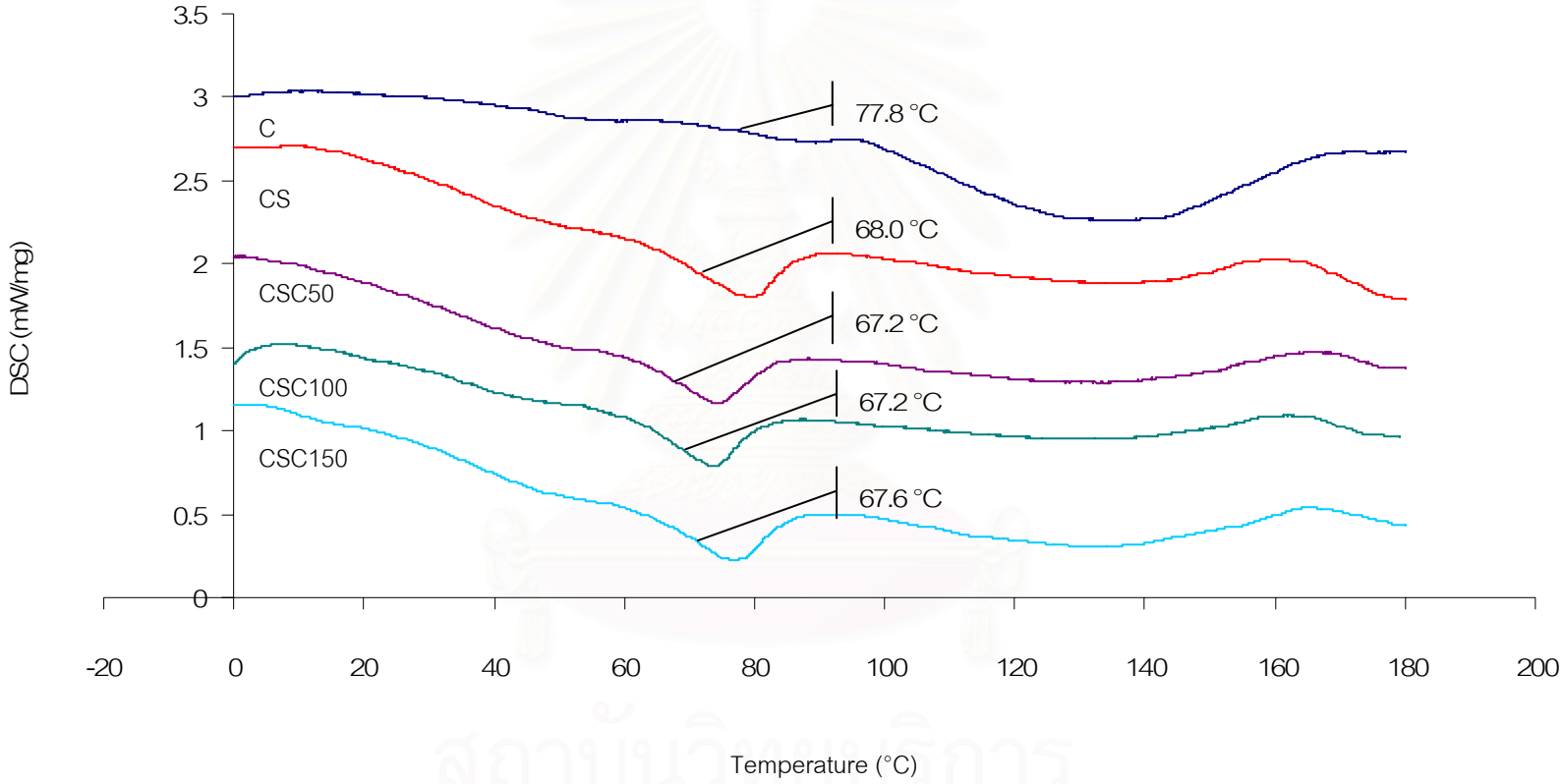

Figure 4.16 DSC thermograms of control chitosan film (C), chitosan film plasticized with sorbitol (CS) and plasticized chitosan film incorporated with cinnamaldehyde (50 µl/g (CSC50), 100 µl/g (CSC100) and 150 µl/g (CSC150))

4.2.6 Antimicrobial property of chitosan film containing cinnamaldehyde

The antimicrobial property of chitosan film incorporated with cinnamaldehyde against pathogenic bacteria and spoilage microorganisms in general including Gram's positive (*Staphylococcus aureus*, *Bacillus licheniformis* and *Bacillus subtilis*) and Gram's negative (*Escherichia coli*, *Pseudomonas aeruginosa* and *Shewanella putrefaciens*) bacteria are shown in Table 4.1 and 4.2, respectively. All tested microorganisms were selected based on general food products contaminants. *Staphylococcus aureus* and *Escherichia coli* are pathogenic bacteria commonly found in food products (Walderhaug, 1992a, 1992b). *Bacillus subtilis* is not considered a human pathogen; it produces the proteolytic enzyme subtilisin. *Bacillus subtilis* spores can survive the extreme heating that is often used to cook food, and it is responsible for causing ropiness in spoiled bread dough. *Bacillus licheniformis* is part of subtilis group. These bacteria are commonly known to cause food poisoning and food spoilage. *Bacillus licheniformis* is also known for contaminating dairy products, cooked meats and processed baby foods (Walderhaug, 1992c). *Shewanella putrefaciens* is chief marine products spoiler (Khashe and Janda, 1998)

Inhibitory zone was calculated by subtracting overall clear zone by diameter of the film disc (Seydim snd Sarikus, 2006). If there is no clear zone surrounding the film disc, it is assumed that there is no inhibitory zone. Contact area was used to evaluate growth inhibition underneath the film discs in direct contact with target microorganisms in agar (Pranoto *et. al*., 2005). From the results, chitosan films without incorporation of cinnamaldehyde (control film) showed antimicrobial effect on contact surface especially against Gram's positive bacteria (Table 4.1) while there are no inhibitory effect on the contact surface against all tested Gram's negative microorganisms (Table 4.2). The results also showed that control film limited growth of *Bacillus licheniformis* and *Bacillus subtilis* underneath chitosan film discs (Table 4.1). This could be due to the innate antimicrobial characteristic of chitosan (Wang, 1992; Darmadji and Izumimoto, 1994). The antimicrobial effect of chitosan occurred without migration of active agents (Brody *et al*., 2004, cited in Pranoto *et al*., 2005). As chitosan is in a solid form, therefore, only organisms in direct contact with the active sites of chitosan is inhibited. Liu *et al*. (2004) indicated that chitosan increased the permeability of the outer membrane and inner

membrane and ultimately disrupted bacterial cell membranes, with the release of cellular contents. This damage was likely caused by the electrostatic interaction between NH_3^+ groups of chitosan and phosphoryl groups of phospholipid components of cell membranes.

In terms of inhibitory zone, the control films did not show inhibitory effect against all tested microorganisms (Table 4.1 and 4.2). On the other hand, inhibitory zone of chitosan film incorporated with cinnamaldehyde was markedly observed against *Staphylococus aureus* (Table 4.1). In addition, inhibitory effect on contact surface of the films incorporated with cinnamaldehyde was observed against all tested microorganisms.

Comparison between the inhibitory effect of film sample (Table 4.1 and 4.2) and inhibitory effect of cinnamaldehyde (Table 4.3 and 4.4) revealed that only small amount of cinnamaldehyde can released from chitosan matrix. This could be concluded that cinnamaldehyde cannot release properly due to the interaction between their functional group. The interaction could be further explained as in section 4.2.4. On the other hand, there is a possibility that small inhibitory effect of chitosan film containing cinnamaldehyde could be due to small amount of the active compound in the film. The original concentrations of cinnamaldehyde in the film-forming solutions are 50, 100 and 150 µl/g of chitosan powder. However the tested films weighed only around 0.03 g. Therefore, the tested films contained cinnamaldehyde around 30 times less than the original concentration.

All antimicrobial testings revealed that incorporation of cinnamaldehyde into chitosan edible film improves antimicrobial efficacy of chitosan film especially on the contact surface of the films. Therefore, this film could be used as antimicrobial wrapping film, coating or inner layer of multilayer packaging film for food products to inhibit microbial growth on the contact surface.

Table 4.1 Inhibitory effect of chitosan films containing cinnamaldehyde against Gram's positive bacteria

Cinnamaldehyde concentration (µl/g chitosan)	*S. aureus*		*B. licheniformis*		*B. subtilis*	
	Inhibitory zone (mm)	Contact surface	Inhibitory zone (mm)	Contact surface	Inhibitory zone (mm)	Contact surface
Control	0	-	0	+, -	0	+
50	1.85	+	0	+	0	+
100	2.50	+	0	+	0	+
150	3.65	+	0	+	0	+

- indicates growth in the area, + indicates no growth;

Control is a plain film disc without cinnamaldehyde incorporation.

Table 4.2 Inhibitory effect of chitosan films containing cinnamaldehyde against Gram's negative bacteria

Cinnamaldehyde concentration (µl/g chitosan)	*E. coli*		*P. aeruginosa*		*S. putrefaciens*	
	Inhibitory zone (mm)	Contact surface	Inhibitory zone (mm)	Contact surface	Inhibitory zone (mm)	Contact surface
Control	0	-	0	-	0	-
50	0	+	0	+	0	+
100	0	+	0	+	0	+
150	0	+	0	+	0	+

- indicates growth in the area, + indicates no growth;

Control is a plain film disc without cinnamaldehyde incorporation.

Table 4.3 Inhibitory effect of sterilized paper discs containing cinnamaldehyde against Gram's positive bacteria

Cinnamaldehyde concentration (µl/g chitosan)	*S. Aureus*		*B. licheniformis*		*B. subtilis*	
	Inhibitory zone (mm)	Contact surface	Inhibitory zone (mm)	Contact surface	Inhibitory zone (mm)	Contact surface
Control	0	-	0	-	0	-
50	42.55	+	20.55	+	21.30	+
100	51.15	+	21.00	+	22.70	+
150	52.20	+	23.55	+	24.60	+

- indicates growth in the area, + indicates no growth;

Control is a plain paper disc without cinnamaldehyde incorporation.

Table 4.4 Inhibitory effect of sterilized paper discs containing cinnamaldehyde against Gram's negative bacteria

Cinnamaldehyde concentration (µl/g chitosan)	*E. coli*		*P. aeruginosa*		*S. putrefaciens*	
	Inhibitory zone (mm)	Contact surface	Inhibitory zone (mm)	Contact surface	Inhibitory zone (mm)	Contact surface
Control	0	-	0	-	0	-
50	30.75	+	15.50	+	11.45	+
100	32.05	+	21.00	+	11.90	+
150	33.35	+	21.50	+	14.45	+

- indicates growth in the area, + indicates no growth;

Control is a plain paper disc without cinnamaldehyde incorporation.

CHAPTER V
CONCLUSIONS

The larger molecular volume of counter ion (acid used as solvent) in the range of 50 °A (acetic acid) to 70 °A (lactic acid), the more stretchable the film becomes. From the results, the film cast with lactic acid was more stretchable, however, the physical properties revealed that acetic acid was more suitable for further studies. Addition of plasticizer led to an increase in elongation at break and water vapor permeability of the films while decreased tensile strength. At concentration of 40 %w/w, the films plasticized with sorbitol had better physical properties in terms of water vapor permeability, surface color and transparency.

Incorporation of cinnamaldehyde (50-150 µl/g) into chitosan film caused an increase in both tensile strength and elongation at break while a decrease in water vapor permeability of the films. Thus, incorporation of cinnamaldehyde could improve physical properties of chitosan film in terms of tensile strength, elongation at break and water vapor permeability. It gave the film with higher tensile strength, elongation at break and water barrier property.

The interaction between functional group of chitosan and cinnamaldehyde was revealed by FT-IR spectra. According to the FT-IR spectra, the possible reaction was imine formation. The infrared spectral data support mechanical as well as antimicrobial properties data of chitosan films incorporated with cinnamaldehyde. When chitosan films are incorporated with cinnamaldehyde, there is a modification on the functional groups of chitosan. There is thus a significant change on the mechanical properties (increased in both tensile strength and elongation at break). The active compounds are not free to inhibit microorganisms in the antimicrobial test due to the proposed interaction. The presence of interaction between chitosan and cinnamaldehyde led to a lower inhibitory effect as observed in antimicrobial assay. The interaction between functional group of chitosan and cinnamaldehyde was also responsible for a slight decrease of T_g due to elimination of water molecule.

Chitosan films show an antimicrobial effect on contact surface especially against Gram's positive bacteria. All tested microorganisms in direct contact were inhibited by

chitosan film containing cinnamaldehyde. However, cinnamaldehyde cannot release properly from chitosan metrix caused by the interaction between the functional group of chitosan and cinnamaldehyde. Therefore, chitosan film containing cinnamaldehyde could possibly used as wrapping film, coating or inner layer of food packaging in order to inhibit microbial growth on the contact surface.

CHAPTER VI
SUGGESTIONS

Edible films are usually considerably more expensive than synthetic polymers. Improvement in production practice, economic of scale and increasing source of by products could all be necessary to produce more favorable economic situation for biodegradable polymer.

The sorption isotherms of the films vary, depending on their water vapor permeability. In addition, the sorption isotherm is a necessary parameter to predict the properties of the films at different environments, thus it is important to study sorption isotherm of chitosan film containing cinnamaldehyde.

The amount of residual of cinnamaldehyde in the films and kinetics of migration of cinnamaldehyde from the films are the important factors to determine the successful usage of antimicrobial film, therefore both of them is necessary to be studied.

All antimicrobial testings revealed that incorporation of cinnamaldehyde into chitosan edible film improves antimicrobial efficacy of chitosan film especially on the contact surface of the films. It is interesting to study chitosan and cinnamaldehyde as an edible coating.

Although cinnamaldehyde is slightly soluble in water, it is interesting to study more amount of addition into the films in form of emulsion film.

Other film properties such as oxygen permeability, heat sealability, and releasing of cinnamaldehyde from the film should be studied to determine the possible application of chitosan film containind cinnamaldehyde.

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APPENDICES

APPENDIX A
RESEARCH METHODOLOGY

A.1 Tensile strength and elongation at break

Tensile strength (TS) and elongation at break (EAB) were determined according to the ASTM standard method D 882-22 (ASTM, 1989)

1. Two rectangular strips (width 20 mm; length 45 mm) were prepared from each film to determine their mechanical properties.
2. Initial grip separation and mechanical crosshead speed were set at 30 mm and 0.5 mm/s, respectively.
3. TS (MPa) was calculated as Eq. (1)

$$TS = \frac{f}{A} \qquad (1)$$

where f is the maximum load (N) necessary to pull the sample apart and A is cross-sectional area of the sample film (m^2).

4. EAB (%) was calculated as Eq. (2)

$$EAB = \frac{\Delta L}{L} \times 100 \qquad (2)$$

where ΔL is the elongation at the moment of rupture (mm) and L is the initial grip length (30 mm). A total of 12 samples were tested for each film type.

A.2 Water vapor permeability

WVP was measured using modified ASTM method reported by Gontard *et al*. (1992).

1. Sample film (50 ×50 mm) was sealed on a glass permeating cup containing silica gel (0% RH) with silicone vacuum grease and a plastic band to hold the film in place.
2. The cups were placed in a desiccator with distilled water (100% RH) at 30 °C.
3. The cups were weighed at 1 h intervals over 8 h periods.
4. WVP ($g \cdot m/m^2 \cdot sec \cdot Pa$) of the films was calculated as Eq. (3)

$$WVP = \frac{(w \cdot x)}{A \cdot t \cdot (P_2 - P_1)} \qquad (3)$$

where w is the weight gain of the cup (g), x is film thickness (m), A is the area of exposed film (m^2), t is the storage time (s), and (P_2-P_1) is the vapor pressure differential across the film (Pa).

A.3 Glass transition temperature

Glass transition temperature (T_g) of chitosan films (control and those incorporated with cinnamaldehyde) was characterized using differential scanning calorimeter (DSC-50, Shimadzu Co., Kyoto, Japan) according to modified method of Kristo and Biliaderis (2006) and Quijada-Garrido *et al*. (2007)

1. Film discs for DSC were punched off the chitosan film.
2. Film discs were collected and stored in a desiccator containing dry silica gel for 72 h.
3. Film disc (15 mg approximately) was placed onto DSC cell and hermetically sealed.
4. Alpha-alumina (not less than two times of film sample weight) was used as reference material.
5. Samples were analyzed at a heating rate of 5 °C/min under a purge of dry nitrogen at a rate of 20 ml/min during data collection.
6. Second scan were done after quench cooling of sample by liquid nitrogen.
7. T_g of the sample was determined from the midpoint of the observed change in heat capacity.

APPENDIX B

STATISTICAL ANALYSIS

B.1 Effect of acid type on physical properties of chitosan film

Table B.1.1 Effect of acid type on thickness of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Acid type	9.000	1	9.000	4.667*	0.049
Error	27.000	14	1.929		
Total	36.000	15			

* indicates significant differences ($p \leq 0.05$)

Table B.1.2 Effect of acid type on tensile strength of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Acid type	1.224	1	1.224	0.021^{ns}	0.887
Error	824.050	14	58.861		
Total	825.274	15			

ns indicates no significant differences ($p > 0.05$)

Table B.1.3 Effect of acid type on elongation at break of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Acid type	44.634	1	44.634	17.852*	0.001
Error	35.002	14	2.500		
Total	79.635	15			

* indicates significant differences ($p \leq 0.05$)

Table B.1.4 Effect of acid type on water vapor permeability of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Acid type	0.089	1	0.089	1.791^{ns}	0.202
Error	0.696	14	0.050		
Total	0.785	15			

ns indicates no significant differences ($p > 0.05$)

Table B.1.5 Effect of acid type on L value of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Acid type	0.500	1	0.500	1.027^{ns}	0.350
Error	2.920	6	0.487		
Total	3.420	7			

ns indicates no significant differences ($p > 0.05$)

Table B.1.6 Effect of acid type on a value of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Acid type	0.005	1	0.005	2.000^{ns}	0.207
Error	0.015	6	0.003		
Total	0.020	7			

ns indicates no significant differences ($p > 0.05$)

Table B.1.7 Effect of acid type on b value of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Acid type	0.361	1	0.361	6.827*	0.040
Error	0.318	6	0.053		
Total	0.679	7			

* indicates significant differences ($p \leq 0.05$)

Table B.1.8 Effect of acid type on transparency of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Acid type	0.033	1	0.033	.496^{ns}	0.508
Error	0.393	6	0.066		
Total	0.426	7			

ns indicates no significant differences ($p > 0.05$)

B.2 Effect of plasticizer type and concentration on physical properties of chitosan film

Table B.2.1 Effect of plasticizer type and concentration on thickness of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Type (A)	132.140	1	132.140	93.046*	0.000
Concentration (B)	215.877	2	107.939	76.004*	0.000
AB	48.536	2	24.268	17.088*	0.000
Error	17.042	12	1.420		
Total	413.595	17			

* indicates significant differences ($p \leq 0.05$)

Table B.2.2 Effect of plasticizer type and concentration on tensile strength of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Type (A)	487.821	1	487.821	223.812*	0.000
Concentration (B)	4557.451	2	2278.726	1045.477*	0.000
AB	667.650	2	333.825	153.159*	0.000
Error	26.155	12	2.180		
Total	5739.077	17			

* indicates significant differences ($p \leq 0.05$)

Table B.2.3 Effect of plasticizer type and concentration on elongation at break of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Type (A)	422.872	1	422.872	70.766*	0.000
Concentration (B)	3801.497	2	1900.748	318.082*	0.000
AB	399.454	2	199.727	33.423*	0.000
Error	71.708	12	5.976		
Total	4695.531	17			

* indicates significant differences ($p \leq 0.05$)

Table B.2.4 Effect of plasticizer type and concentration on water vapor permeability of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Type (A)	0.255	1	0.255	17.560*	0.001
Concentration (B)	10.634	2	5.317	366.378*	0.000
AB	1.380	2	0.690	47.548*	0.000
Error	0.174	12	0.015		
Total	12.443	17			

* indicates significant differences ($p \leq 0.05$)

Table B.2.5 Effect of plasticizer type and concentration on L value of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Type (A)	0.934	1	0.934	73.087*	0.000
Concentration (B)	1.071	2	0.536	41.913*	0.000
AB	1.231	2	0.616	48.174*	0.000
Error	0.153	12	0.013		
Total	3.389	17			

* indicates significant differences ($p \leq 0.05$)

Table B.2.6 Effect of plasticizer type and concentration on a value of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Type (A)	0.067	1	0.067	24.200*	0.000
Concentration (B)	0.021	2	0.011	3.800^{ns}	0.053
AB	0.048	2	0.024	8.600*	0.005
Error	0.033	12	0.003		
Total	0.169	17			

* indicates significant differences ($p \leq 0.05$)

ns indicates no significant differences ($p > 0.05$)

Table B.2.7 Effect of plasticizer type and concentration on b value of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Type (A)	0.802	1	0.802	33.581*	0.000
Concentration (B)	5.071	2	2.536	106.140*	0.000
AB	0.938	2	0.469	19.628*	0.000
Error	0.287	12	0.024		
Total	7.098	17			

* indicates significant differences ($p \leq 0.05$)

Table B.2.8 Effect of plasticizer type and concentration on transparency of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Type (A)	2.420	1	2.420	79.373*	0.000
Concentration (B)	3.780	2	1.890	61.997*	0.000
AB	1.190	2	0.595	19.516*	0.000
Error	0.366	12	0.030		
Total	7.756	17			

* indicates significant differences ($p \leq 0.05$)

B.3 Effect of cinnamaldehyde on physical properties of chitosan film

Table B.3.1 Effect of cinnamaldyhyde concentration on thickness of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Concentration	151.809	3	50.603	58.686*	0.000
Error	17.245	20	0.862		
Total	169.054	23			

* indicates significant differences ($p \leq 0.05$)

Table B.3.2 Effect of cinnamaldyhyde concentration on tensile strength of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Concentration	1067.258	3	355.753	12.665*	0.000
Error	561.801	20	28.090		
Total	1629.059	23			

* indicates significant differences ($p \leq 0.05$)

Table B.3.3 Effect of cinnamaldyhyde concentration on elongation at break of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Concentration	901.174	3	300.391	8.573*	0.001
Error	700.766	20	35.038		
Total	1601.940	23			

* indicates significant differences ($p \leq 0.05$)

Table B.3.4 Effect of cinnamaldyhyde concentration on water vapor permeability of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Concentration	5.046	3	1.682	50.681*	0.000
Error	0.664	20	0.033		
Total	5.710	23			

* indicates significant differences ($p \leq 0.05$)

Table B.3.5 Effect of cinnamaldyhyde concentration on L value of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Concentration	0.030	3	0.010	0.164^{ns}	0.917
Error	0.487	8	0.061		
Total	0.517	11			

ns indicates no significant differences ($p > 0.05$)

Table B.3.6 Effect of cinnamaldyhyde concentration on a value of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Concentration	0.029	3	0.010	3.889^{ns}	0.055
Error	0.020	8	0.003		
Total	0.049	11			

ns indicates no significant differences ($p > 0.05$)

Table B.3.7 Effect of cinnamaldyhyde concentration on b value of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Concentration	2.103	3	0.701	21.564*	0.000
Error	0.260	8	0.033		
Total	2.363	11			

* indicates significant differences ($p \leq 0.05$)

Table B.3.8 Effect of cinnamaldyhyde concentration on transparency of chitosan film

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Concentration	4.351	3	1.450	45.556*	0.000
Error	0.255	8	0.032		
Total	4.605	11			

* indicates significant differences ($p \leq 0.05$)

APPENDIX C

ANTIMICROBIAL PROPERTY TESTING

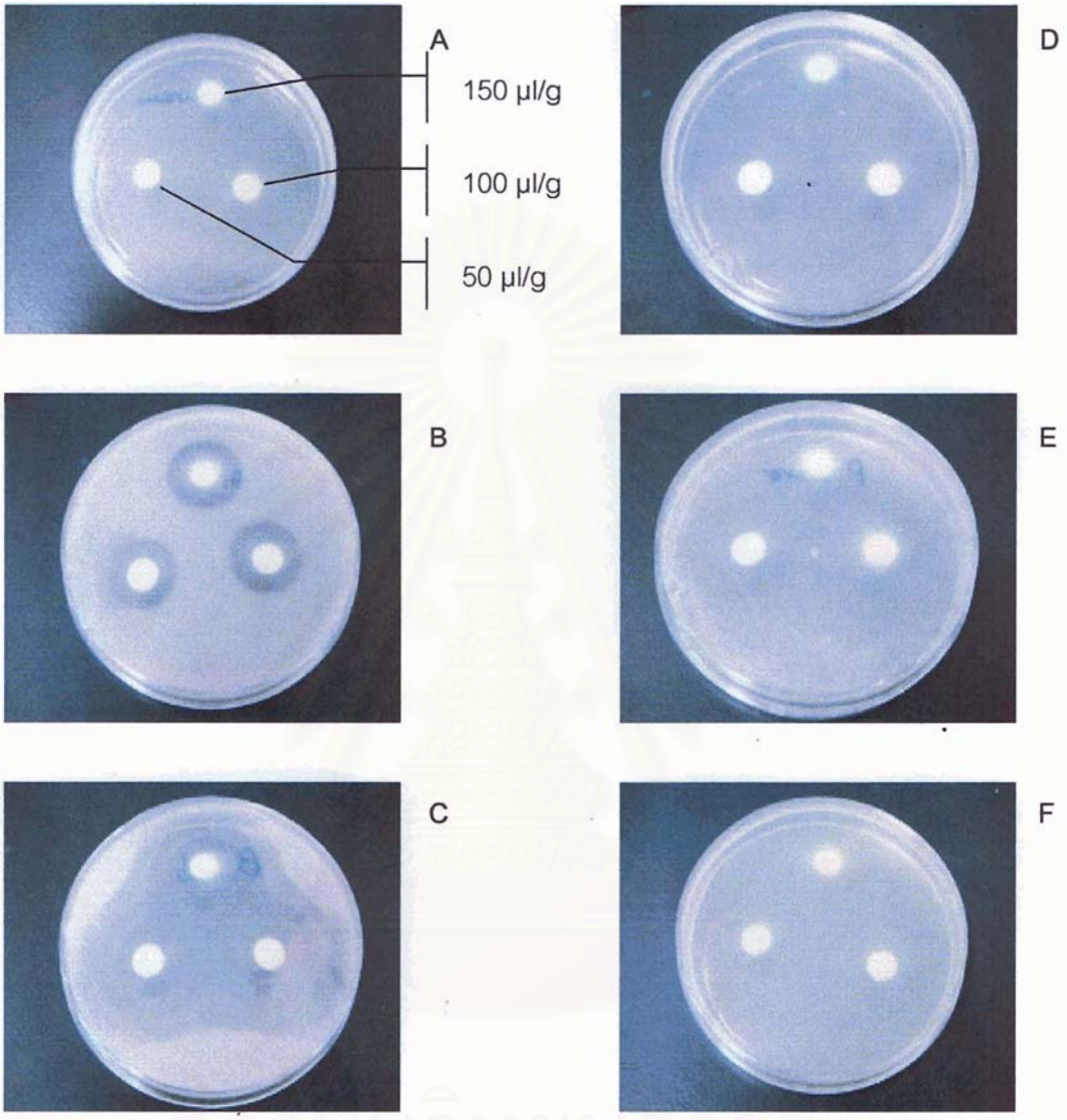

Figure C.1 Antimicrobial property of cinnamaldehyde (50, 100 and 150 µl/g) against *Staphylococcus aureus* (A), *Bacillus licheniformis* (B), *Bacillus subtilis* (C), *Escherichia coli* (D), *Pseudomonas aeruginosa* (E) and *Shewanella putrefaciens* (F).

APPENDIX D
FURTHER INFORMATION

D.1 The crystal structure of chitan

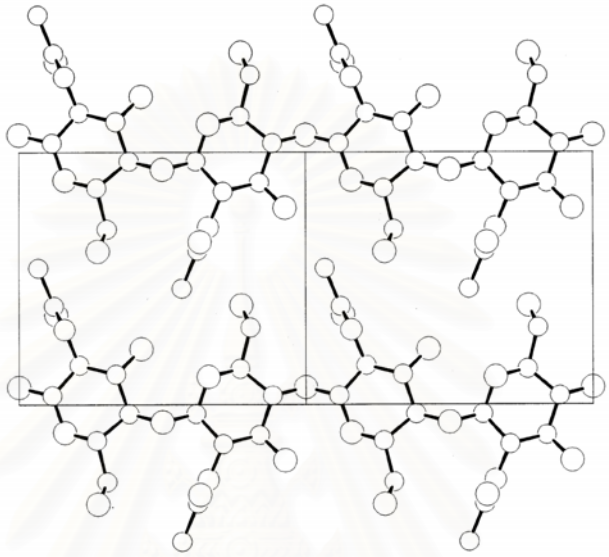

Figure D.1 The crystal structure of chitan down the *b* axis. H atoms are not visible (Bégin and Van Calsteren, 1999).

D.2 Tensile strength, elongation at break and water vapor permeability of synthetic plastic films

Table D.1 Tensile strength, elongation at break and water vapor permeability of synthetic plastic films

Plastic film	TS (MPa)	EAB (%)	WVP ($\times 10^{-10}$ g·m^{-1}·s^{-1}·Pa^{-1})
LDPE	16.5 ± 0.9	>100	0.020 ± 0.006
OPP	50.7 ± 8.2	73 ± 27	0.038 ± 0.035
PE	81.2 ± 3.2	19 ± 6	0.198 ± 0.015
PVDC	65.6 ± 10.8	18 ± 5	0.002 ± 0.000

LDPE: low density poly ethylene, OPP: oriented polypropylene, PE: polyethylene, PVDC: polyvinylidene chloride

D.3 Imine formation (Solomons, 1997)

Aldehydes react with primary amines (RNH_2) to form compounds with a carbon-nitrogen double bond called imines (RCH=NR or R_2C=NR). The reaction is acid catalyzed.

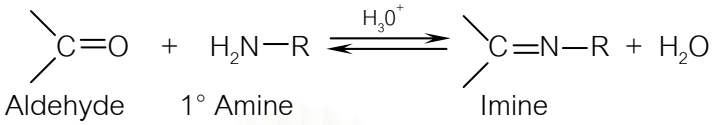

Figure D.2 Imine formation

Imine formation is slow at very low and at very high pH and generally takes place fastest between pH 4 and pH 5.

D.4 Conference proceeding

EFFECT OF ACIDS AND PLASTICIZERS ON PHYSICAL PROPERTIES OF CHITOSAN FILMS

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Introduction

Chitosan, the derivative of chitin, is a cationic heteropolysaccharide composed mainly of β-(1,4)-2-deoxy-2-amino-D-glucopyranose (D-glucosamine) units and partially of β-(1,4)-2-deoxy-2-acetamido-D-glucopyranose (N-actetyl-D-glucosamine) (Goosen, 1997). Chitosan has been proved to be nontoxic, biodegradable, biofunctional, biocompatible and has antimicrobial characteristics (Wang, 1992; Darmadji & Izumimoto, 1994). Chitosan is of interest as a potential edible film component because of its good oxygen and carbon dioxide barrier properties (Hosokawa et al., 1990). However, the type of acid used for film preparation significantly affects the film properties. Plasticizing agents are essential generally to overcome the brittleness of the chitosan films. Plasticizers by reducing the intermolecular forces soften the rigidity of the film structure and increase the mobility of the biopolymeric chains. Films with sorbitol were more flexible compare to those with glycerol and polyethylene glycol while the films with glycerol showed the lowest WVP value (Srinivasa *et. al*, 2007). In the present work the effect of acids and plasticizers on physical properties of the film was, therefore, studied.

Materials and methods

1. Film preparation

Chitosan film-forming solutions were prepared by dissolving commercial grade chitosan powder (95% degree of deacetylation) into 1% acetic or lactic acid solution and using 0.2, 0.4 and 0.6 %w/v glycerol or sorbitol (Wako Pure Chemical Industries Ltd., Tokyo, Japan) as plasticizer.

The prepared film-forming solutions were cast onto rimmed silicone plate (50×50 mm) and dried. All film samples were conditioned in ventilated oven (EYELA KCL-2000, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at 25 °C 50 %RH for 24 h. before physical properties determination.

2. Film thickness

Film thickness was measured using micrometer (Dial Pipe Gauge, Peacock Co., Tokyo, Japan) at six random locations of the film.

3. Mechanical Properties

TS and %EAB were determined using Tensipresser® (TTP-508X II, Taketomo Electric Inc., Tokyo, Japan) according to the ASTM standard method D882-22 (ASTM, 1989).

4. Water vapor permeability

WVP ($g \cdot m/m^2 \cdot sec \cdot Pa$) was measured using modified ASTM method reported by Gontard *et al*. (1992).

5. Color measurement

Color values (L, a, b) were measured using color reader (CR-13, Konica Minolta Sensing Inc., Tokyo, Japan).

6. Transparency

The transparency of the films was measured by modified method of ASTM method D 1746-92 (ASTM, 1987).

7. Statistical analysis

Completely randomized design and factorial (2×3) in completely randomized design were used in this experiment. The effect of organic acid type, plasticizer type and concentration on physical properties were statistically analyzed using ANOVA test. The statistical differences between mean values was established at $p < 0.05$ with the Duncan's New Multiple Range Test (DNMRT) (Cochran and Cox, 1992) in the general linear model of the SPSS statistical package (SPSS Inc. Version 14.0, Chicago, IL)

Results and discussion

1. Effect of acid type on physical properties of chitosan film

Table 1 show the effect of acid type on film thickness, TS, EAB and WVP respectively. There was no significant difference in TS and WVP values, while thickness and %EAB were significantly different ($p < 0.05$). The films prepared from lactic acid solution showed higher thickness and %EAB value than those from acetic acid solution. Bégin and Van Calsteren (1999) suggested that the increase in %EAB is related to the molecular volume of the acid used as solvent.

Table 2 show the mean value and standard deviation of surface color and transparency of the films. The results indicated that chitosan films prepared from acetic acid solution had less lightness but more yellowness than those from lactic acid solution.

Table 1 Effect of acid type on thickness, TS, EAB and WVP

Acid type	Thickness (µm)	TS (MPa)	EAB (%)	WVP ($g.m/m^2.sec.Pa$)
Acetic acid	11.25*±1.48	55.00 ±8.00	4.48*±1.49	$2.44 \pm 0.22 \times 10^{-11}$
Lactic acid	12.75*±1.28	54.45 ±7.32	7.82*±1.66	$2.29 \pm 0.22 \times 10^{-11}$

*Mean values in the same column represent significant difference ($p < 0.05$)

Table 2 Effect of acid type on surface color and transparency.

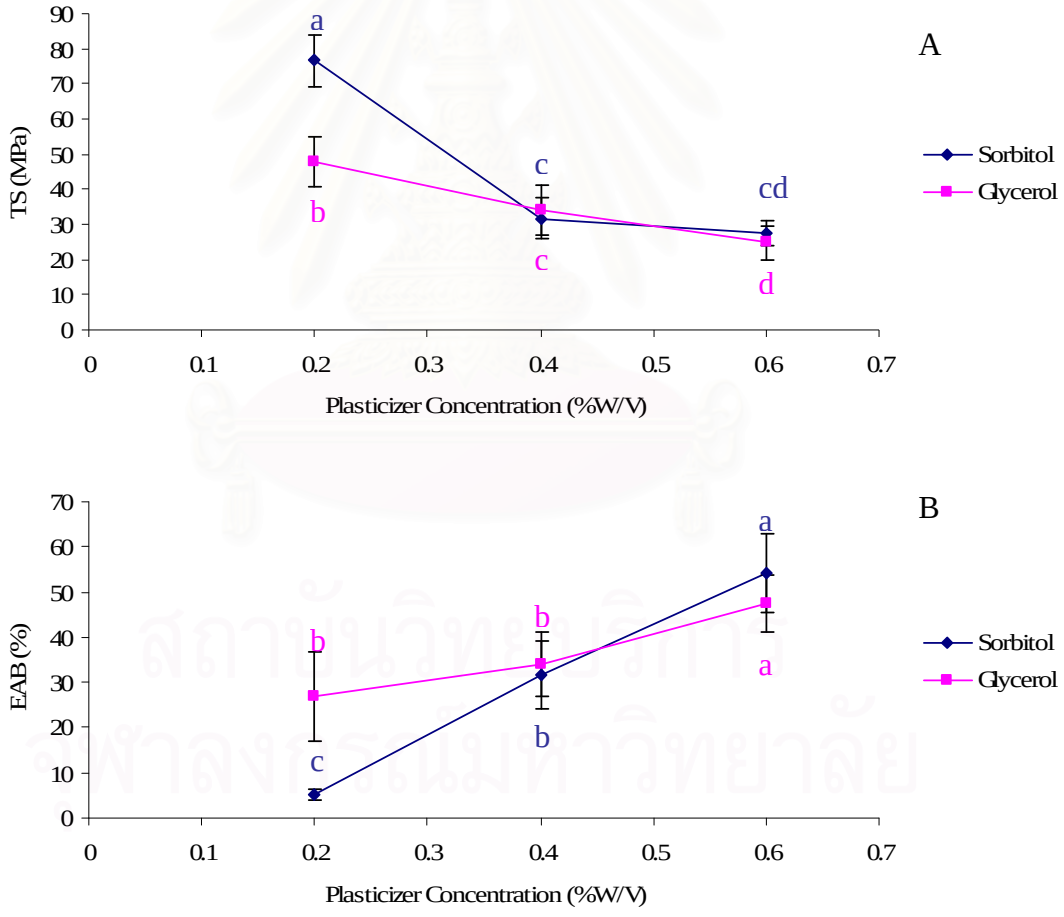
Acid type	L	a	b	Transparency
Acetic acid	93.70*±0.80	-0.23*±0.05	4.58±0.32	3.94±0.36
Lactic acid	94.20*±0.58	-0.28*±0.05	4.15±0.05	4.07±0.03

*Mean values in the same column represent significant difference ($p < 0.05$)

2. Effect of plasticizer type and concentration on physical properties of chitosan film

Sorbitol and glycerol were blended with the film forming solution using different concentrations (0.2, 0.4 and 0.6 %W/V). Chitosan solution prepared using lactic acid was difficult to form into film, especially when the plasticizers were added, and thus physical property measurements were not possible.

Effect of plasticizers on TS and %EAB is shown in fig. 1A and 1B respectively. Addition of plasticizers showed the same trend as many researchers have reported (Srinivasa *et al*., 2007; Canner *et al*., 1998; Butler *et al*., 1996). The TS and EAB are inversely correlated. The latter showed an increasing trend with the addition of plasticizers. WVP increased upon the addition of plasticizers. However, Incorporation of plasticizers did not significantly affect the WVP of chitosan film (fig. 1C). Srinivasa *et al*. (2007) and Arvanitoyannis *et al*. (1997) reported an increased WVP with the addition of sorbitol and glycerol respectively.



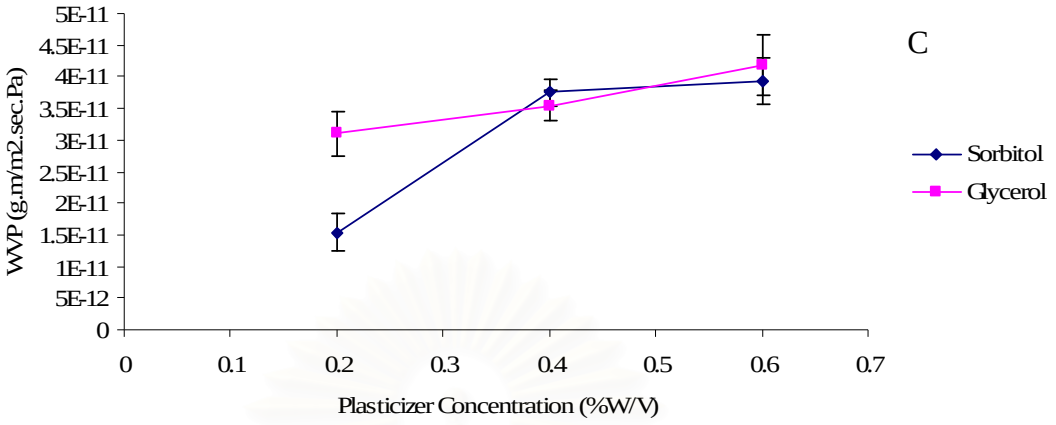


Figure 1 Effect of plasticizers on TS (A), %EAB (C) and WVP (B) (Means with different letters represent significant difference (p<0.05))

At the concentration of 0.4 %w/v, films with sorbitol showed similar physical properties as those with glycerol. However, films with sorbitol had better appearance in terms of surface color and transparency than those with glycerol (table 3). Therefore, 0.4 %w/v was selected as optimum concentration for further studies.

Table 3 Effect of plasticizer type and concentration on surface color and transparency

Plasticizer type	Conc. (%w/v)	L	a	b	Transparency
Sorbitol	0.2	93.48^{bc}±0.10	-0.27^{cd}±0.05	4.82^{c}± 0.15	3.78^{a}±0.31
	0.4	94.35^{bc}±0.10	-0.22^{bc}±0.05	4.70^{cd}±0.28	2.71^{b}±0.17
	0.6	94.07^{c}±0.51	-0.30^{d}±0.00	6.22^{a}±0.10	2.11^{c}±0.16
Glycerol	0.2	93.72^{c}±0.15	-0.22^{bc}±0.05	4.60^{cd}±0.12	3.81^{a}±0.39
	0.4	93.72^{a}±0.10	-0.17^{b}±0.05	4.45^{d}±0.17	4.03^{a}±0.12
	0.6	93.60^{ab}±0.12	-0.02^{a}±0.05	5.12^{b}±0.26	2.94^{b}±0.20

$^{a,b,\cdots}$Means with different letters in the same column represent significant difference (p<0.05)

Conclusion

Chitosan films from lactic acid solution produced softer film than those from acetic acid solution but became difficult to form into film when plasticizers were added. The TS of the chitosan films added with plasticizers decreased while %EAB and WVP increased. At the concentration of 0.4 %w/v, there is no significant difference between TS, %EAB and WVP of the films with sorbitol and those with glycerol. However, addition of sorbitol gave better appearance in terms of surface color and transparency.

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VITAE

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