

# Functional Properties of Antimicrobial Lysozyme–Chitosan Composite Films

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**ABSTRACT:** Lysozyme–chitosan composite films were developed for enhancing the antimicrobial properties of chitosan films. A 10% lysozyme solution was incorporated into 2% chitosan film-forming solution (FFS) at a ratio of 0%, 20%, 60%, and 100% (w lysozyme/w chitosan). Films were prepared by solvent evaporation. Lysozyme release from the film matrix, the antimicrobial activity of films against *Escherichia coli* and *Streptococcus faecalis*, and basic film properties were investigated. The lysozyme release proportionally increased with increasing initial concentration of lysozyme in the film matrix, and the amount of released lysozyme was in natural log relationship with time. The films with 60% lysozyme incorporation enhanced the inhibition efficacy of chitosan films against both *S. faecalis* and *E. coli*, where 3.8 log cycles reduction in *S. faecalis* and 2.7 log cycles reduction in *E. coli* were achieved. Water vapor permeability of the chitosan films was not affected by lysozyme incorporation, whereas the tensile strength and percent elongation values decreased with increased lysozyme concentration. Scanning electron microscopy images revealed that lysozyme was homogeneously distributed throughout the film matrix. This study demonstrated that enhanced antimicrobial activity of lysozyme–chitosan composite films can be achieved by incorporating lysozyme into chitosan, thus broadening their applications in ensuring food quality and safety.

**Keywords:** Lysozyme–chitosan composite films, antimicrobial activity, microstructure, water vapor permeability, mechanical property

## Introduction

Antimicrobial-enhanced packaging films have great potential for ensuring the safety of food surfaces through controlled release of antimicrobial substances from the carrier film structure to food surface. The antimicrobial compounds and their incorporation into packaging materials have been well reviewed (Han 2000; Appendini and Hotchkiss 2002).

Lysozyme is a lytic enzyme found in many natural systems. It is a small and stable enzyme whose dimensional structure and sequence were completely analyzed (Blake and others 1965). Lysozyme has high potential in food preservation because of its stability over a wide range of pH and temperature (Proctor and Cunningham 1988). However, its limited antimicrobial efficacy against Gram-negative bacteria restricts its application in the food industry. Masschalck and Michiels (2003) recently reviewed several methods of extending the antimicrobial spectrum of lysozyme to Gram-negative bacteria, including denaturation of lysozyme, modification by attachment of other compounds to lysozyme, and the use of membrane-permeabilizing agents with lysozyme. A number of studies have demonstrated that the antimicrobial spectrum of lysozyme could be enhanced when it is used with other substances, such as hydrogen peroxide and ascorbic acid (Miller 1969), ethylenediamine tetra-acetic acid (EDTA) (Padgett and others 1998), caffeic acid (Valenta and others 1998), and chitosan (Song and others 2001). The addition of lysozyme in a polymeric supporting matrix by immobilization, absorption, or entrapment has been reported in pharmaceutical and food-related areas. The lytic efficacy of lysozyme toward *Micrococcus lysodeikticus* or *Micrococcus luteus* has

been evaluated after immobilizing lysozyme into synthetic or natural polymeric structure, such as cellulose and polyacrylamide (Datta and others 1973), chitosan, silica gel, glass beads, and polystyrene divinylbenzene matrix (Crapisi and others 1993), polyvinyl alcohol beads, nylon pallets, and cellulose triacetate films (Appendini and Hotchkiss 1997). In addition, lysozyme was incorporated into soy protein and corn zein (Padgett and others 1998), and alginate and carrageenan (Cha and others 2002) based natural polymers, and the antimicrobial activities of those lysozyme-containing films were reported.

Chitosan is the deacetylated derivative of chitin ( $\beta$ -[1-4]-poly-N-acetyl-D-glucosamine), an abundant by-product of seafood processing. Chitosan has been well known for its good film-forming property, broad antimicrobial activity, and excellent compatibility with other substances by the presence of the high density of amino groups and hydroxyl groups in chitosan polymer structure (Li and others 1992; Shahidi and others 1999). Antimicrobials, antioxidants, nutrients, colorants, and flavors can be possibly carried by chitosan-based films and released in a controlled manner (Park and Zhao 2004). Like lysozyme, chitosan may be combined with other active antimicrobial substances for enhancing its antimicrobial efficacy. Chen and others (1996) incorporated food preservatives, such as potassium sorbate and sodium benzoate, into chitosan film matrix and compared their inhibitory effects on microbial growth. Lee and others (2003) reported enhanced microbial stability of milk and orange juice, which were exposed to paperboard coated with chitosan and nisin. Furthermore, synergistic effects of chitosan covalently bonded with lysozyme have been reported by Song and others (2001). Chitosan is a renewable and nontoxic polymer with excellent biocompatibility with other substances. Inherent antimicrobial properties of chitosan and its high film-forming and entrapping ability could be the primary driving force in the development of new applications for this underused biopolymer. In this study,

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chitosan was used as a film-forming polymeric matrix to incorporate hen egg white lysozyme. Our objectives were to develop semi-permeable lysozyme–chitosan composite films and to evaluate their antimicrobial activities and basic film properties including water vapor permeability and mechanical properties.

## Materials and Methods

### Materials

Shrimp-derived chitosan from Vanson Inc. (Redmond, Wash., U.S.A.; 11 centipoises viscosity of a 1% w/w aqueous acetic acid solution at 25 °C and 89.9% deacetylation) was used without further purification. Hen egg white lysozyme was obtained from Eiprodukte GmbH and Co., Germany. United States pharmacopoeia-grade glycerol from EM Science (Darmstadt, Germany) and reagent-grade glacial acetic acid from J. T. Baker (Phillipsburg, N.J., U.S.A.) were used.

*Streptococcus faecalis* ATCC 14508 and *Escherichia coli* B were used as test microorganisms to evaluate antimicrobial properties of films. Lyophilized *M. lysodeikticus* (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was used for lysozyme release measurement. Brain heat infusion (BHI) broth; Man, Rogosa, Sharpe (MRS) broth; and agar used in this study were purchased from Difco (Benton, Dickson and Co., Sparks, Md., U.S.A.).

### Preparation of film-forming solutions

Film-forming solutions (FFSs) were prepared by dissolving 2% chitosan in a 1% acetic acid solution with addition of 25% glycerol (w/w chitosan) in the mixture. Lysozyme stock solution was prepared by dissolving 10% lysozyme in distilled water with addition of 25% glycerol (w/w lysozyme) to achieve the same plasticizer level in FFS after mixing lysozyme solution with chitosan solution. Lysozyme solution was then mixed into chitosan solution in concentrations of 0%, 20%, 60%, and 100% (percent dry weight of lysozyme per dry weight of chitosan). The solution mixtures were homogenized using a homogenizer (PT 10-35, Kinematica, Switzerland) at 3000 rpm for 60 s, and the pH of the FFSs was adjusted to 5.2 with 5 N sodium hydroxide. All sample solutions were filtered through nylon mesh to remove insoluble residues and degassed under vacuum using a vacuum pump (Model 0211-P204, Gast Mfg. Corp., Benton Harbor, Mich., U.S.A.).

### Film formation

A calculated amount of each degassed FFS, in the range of 117 to 195 mL, was cast on a leveled Teflon-coated glass plate with an area of 260 × 260 mm to achieve a uniform film thickness of about 70 μm. After drying at room condition (24 ± 2 °C and 40% ± 5% RH) for 2 d, dried films were removed from the plates and cut into pieces for the measurement of functional properties. Film segments of 25 × 25 mm were used for density and moisture content evaluation, 25 × 86 mm for the mechanical test, and 70 × 70 mm for water vapor permeability test. Before all measurements, film pieces were conditioned in an environmental chamber (T10RS, Tenney Environmental, Williamsport, Pa., U.S.A.) set at 25 °C and 50% RH for at least 2 d.

### Measurement of film thickness, density, and moisture content

After conditioning at 25 °C and 50% for 2 d, the thickness of the films was measured at 5 random locations for each film specimen using a caliper micrometer (Nr 293-766-30, Mytutoyo Manufacturing Co. Ltd., Japan). Film density was calculated by dividing film weight with film volume. Moisture content of the films was deter-

mined gravimetrically by drying film samples at 105 °C for 18 h in a forced-air oven (Precision Scientific Inc., Chicago, Ill., U.S.A.). The percentage of moisture content was calculated on wet basis.

### Lysozyme release assay

Lysozyme release was measured using the spectrophotometric turbidity assay described by Daeschel and others (2002). About 0.03 g of lysozyme–chitosan film specimen was submerged into 20 mL of 0.15 M phosphate buffer (pH 6.2) in vials and shaken at 50 rpm using a shaker (New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.) at room temperature. A stock substrate solution of lyophilized *M. lysodeikticus* was prepared in 0.15 M phosphate buffer solution (absorbance of 0.65 at 450 nm). A portion (1 μL) of each cell suspension shaken with film specimens was taken at time intervals of 0, 0.25, 1, 4, 12, 24, and 48 h, mixed with 2.5 mL of *M. lysodeikticus* substrate into a cuvette, and then immediately read for 40 s at 25 °C using a Shimadzu UV-Vis 2100 spectrophotometer (Shimadzu Co., Japan). Activity rates of lysozyme were determined by measuring the decrease in solution absorbance at 450 nm, which reflects the hydrolysis of the cell wall substrate. Decreasing optical density was expressed as a change in milliabsorbance units per min (Mabs/min). Activity units were converted to mg/L lysozyme based on regression lines with standardized lysozyme concentrations in 0.15 M phosphate buffer, the amount of released lysozyme was then reported as grams of lysozyme released per gram of dry film.

### Antimicrobial activity

*E. coli* and *S. faecalis* were grown at 37 °C overnight in BHI broth and MRS broth, respectively. One milliliter of culture was diluted with 99 mL of the same broth to get approximately 10<sup>7</sup> colony-forming units (CFU)/mL. About 0.03 g of each film specimen was placed in a petri dish (85-mm dia), in which 10 mL of culture was added. Culture without film was used as a control. The petri dishes were shaken at 50 rpm at room temperature, and the samples were taken at 0, 6, 12, and 24 h, diluted with diluent (Dilu-Lock II™ Butterfield's Buffer, Hardy Diagnostics, Santa Maria, Calif., U.S.A.) and plated for enumeration in duplicate. Brain heat infusion agar and MRS agar were used for *E. coli* and *S. faecalis*, respectively. Plates were incubated at 37 °C for 48 h before counting the number of colonies.

### Measurement of water vapor permeability

Water vapor permeability (WVP) was determined using a cup method at 25 °C and 100%/50% RH gradient, following ASTM E 96 (ASTM 2000). Eleven milliliters of distilled water was placed in each test cup made of Plexiglas® with a 57-mm inside dia and a 15-mm inner depth. The distance between water and the film was 10.7 mm, and the effective film area was 25.5 cm<sup>2</sup>. Test cup assemblies were placed in the environmental chamber (25 °C and 50% RH). Each cup assembly was weighed every hour for 6 h using the electronic balance (0.0001 g accuracy) to record moisture loss over time. Water vapor permeability was then corrected for resistance of the stagnant air gap between the film and the surface of water using the WVP correction method (Gennadios and others 1994).

### Mechanical properties

Mechanical properties of the films were determined using a texture analyzer (TA.XT2i, Texture Technologies Corp., Scarsdale, N.Y., U.S.A.). All property measurements were performed immediately after removing film specimens from the chamber to minimize moisture content variability. ASTM D882 method (ASTM 2001) with some modifications was used for measuring tensile strength (TS)

and percent elongation at break (EL) as reported in our previous study (Park and Zhao 2004). Each film specimen was mounted between the grips (TA 96) of the texture analyzer and tested with initial grip separation of 50 mm and crosshead speed of 1 mm/s. Tensile strength values were reported as measured maximum load (N) divided by film cross-sectional area (mm<sup>2</sup>) with a unit of MPa. Elongation values were obtained by recording elongation at break divided by the initial length of the specimen and multiplied by 100.

### Microstructure

The surface and internal structure of the films were evaluated using a Scanning Electron Microscopy (AmRay 3300FE field emission SEM, AmRay, Bedford, Mass., U.S.A.). The film pieces were fractured in liquid nitrogen, mounted on aluminum stubs, and coated with gold-palladium alloy using a sputter coater (Edwards model S150B Sputter Coater; BOC Edwards Vacuum Ltd., U.K.). Each coated sample was examined using a voltage of 5 kV.

### Statistical analysis

All experiments were replicated 3 times. In each replication, 2 film specimens were used for lysozyme release and antimicrobial activity measurements, 5 film samples for density, moisture content, and WVP measurements, and 10 film specimens for mechanical property measurements. The general linear models (GLM) procedure was applied in testing differences among different films using the SAS (SAS Inst., Cary, N.C., U.S.A.). PROC GLM for analysis of variance (ANOVA) was performed for all treatments. PROC REG was performed to find fitting regression model for measured responses. Duncan's multiple-range test was used for the multiple means comparisons. Significance of difference was defined at  $P < 0.05$ .

## Results and Discussion

### Film formation and basic physical properties

When integrating lysozyme into chitosan solutions, no precipitation was observed. This suggested that an acetic acid–dissolved chitosan solution is miscible with water-soluble lysozyme. All dried films were easy to remove from the casting plates, and the thickness of all types of films was carefully controlled in a range of  $72 \pm 11 \mu\text{m}$  by casting calculated amount of FFSs so that no significant differences in film thickness were observed ( $P < 0.05$ ).

Table 1 lists the density and moisture content of all films. Pure chitosan film (L0) had the highest values in both density and moisture content, but the density was not statistically different among all films. Moisture content tends to decrease with increased lysozyme concentration. This may be because both chitosan and lysozyme contain a large number of -OH and -NH<sub>2</sub> groups so that the hydrogen bonding is the main attraction force among these groups. The addition of lysozyme molecules into chitosan chains may alter the structural configuration of chitosan molecules by increasing interactions between chitosan and lysozyme molecules, such as hydrogen bonding and van der Waals interactions. Meanwhile, lysozyme contains both hydrophilic and hydrophobic amino acids. During film formation, the lysozyme hydrophobic core may be formed with the hydrophilic amino acid side chains protruding toward the aqueous FFS by the hydrophobic interactions that play an important role in the folding of lysozyme (Proctor and Cunningham 1988). The increased hydrophobic side chains in the film matrix by the addition of lysozyme may be responsible for the decreases in moisture content of lysozyme–chitosan composite films. However, more studies are needed to fully understand the interactions between chitosan and lysozyme.

**Table 1—Composition and physical properties of lysozyme–chitosan composite films<sup>a</sup>**

Film type	Composition			Moisture content (%)
	Lysozyme (% w/w chitosan)	Glycerol (% w/w) <sup>b</sup>	Density (g/mL)	
L0	0%	25	1.34 ± 0.09a	23.6 ± 2.4a
L20	20%	25	1.30 ± 0.05ab	21.7 ± 3.0a
L60	60%	25	1.30 ± 0.04b	19.0 ± 3.5b
L100	100%	25	1.31 ± 0.06ab	18.8 ± 2.8b

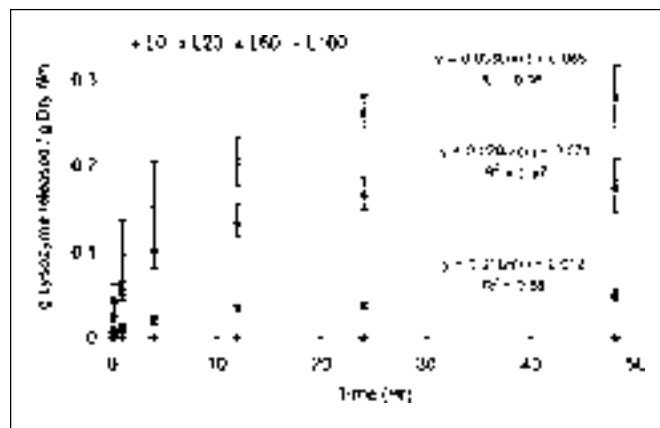
<sup>a</sup>Means within the same column with same letters are not significantly different ( $n = 15$ ;  $P < 0.05$ ). Film thickness is in a range of  $72.6 \pm 8.2 \mu\text{m}$ .

<sup>b</sup>Percentage of glycerol weight per the sum of chitosan and lysozyme in film-forming solutions.

### Release of lysozyme

The amount of lysozyme released from the film matrix is illustrated in Figure 1. The released amount of lysozyme as a function of time follows a natural log pattern. Chitosan films without lysozyme (L0) did not display any enzymatic activity, whereas these activities increased with increased concentration of lysozyme incorporated into the film matrix. The percentage of release amount of lysozyme from each film specimen (the release amount of lysozyme in Figure 1 divided by the amount of lysozyme in each dry film specimen) after 48 h was approximately 65%, 79%, and 76% for L20, L60, and L100 films, respectively. When lysozyme–chitosan composite films were placed in the phosphate buffer solution, the films were swollen as a result of the diffusion of water molecules into the polymeric film structure, leading to the release of incorporated lysozyme into aqueous environment from film matrix. Buonocore and others (2003) developed a mathematical model to describe the release kinetics of lysozyme from a highly swellable polyvinylalcohol film matrix using 2 kinetic models: the water uptake kinetic model and the lysozyme release kinetic model. In our results, lysozyme released from the film matrix in a natural log relationship with time  $t$ . The release of lysozyme at time  $t$  was linearly correlated with the initial lysozyme concentration in the polymeric matrix.

Keeping desired levels of antimicrobials on food surface with controlled and delayed diffusion could be beneficial for extending shelf life of foods because microbial growth occurs mainly on the



**Figure 1—The amount of released lysozyme from lysozyme–chitosan composite film matrix to 0.15 M phosphate buffer as a function of time ( $n = 6$ ). L0 = 0% lysozyme (w/w chitosan); L20 = 20% lysozyme (w/w chitosan); L60 = 60% lysozyme (w/w chitosan); L100 = 100% lysozyme (w/w chitosan).**

surface of food. This study demonstrated that lysozyme incorporated into chitosan films can be released from the film matrix in controlled manner with retained lytic activity against bacterial cell wall substrate.

### Antimicrobial activity

Figure 2 shows the survival of *E. coli* and *S. faecalis* in broth treated with lysozyme–chitosan films. The antimicrobial efficacies of lysozyme–chitosan composite films against *E. coli* increased with increased lysozyme concentrations, except the L100 films which inhibited the growth up to 6 h followed by the recovery of cell population. After 24 h of incubation, cell numbers were reduced about 1.8, 2.3, and 2.7 log cycles in BHI broth with L0, L20, or L60 films, respectively, whereas about 0.1 and 2.3 log cycles increments occurred in L100 treatment and control (film-free broth). The growth of *S. faecalis* was not effectively inhibited by the lysozyme–chitosan composite films containing low concentration of lysozyme (L0 and L20 films), where viable *S. faecalis* were slightly recovered during 24 h of exposure. However, 3.3 and 3.8 log reductions of *S. faecalis* were observed in broth containing L60 or L100 films, respectively. The increase in antimicrobial activity with increased lysozyme concentration against Gram-positive *S. faecalis* may represent that lysozyme is the primary contributor of Gram-positive bacteria inhibition. However, it is difficult to establish the general antimicrobial trends with a single representative strain. Initial microbial concentrations above  $10^7$  CFU/mL are very unusual in most food prod-

ucts. These high initial microbial numbers inoculated in nutritionally rich broth in our tests may be a reason of limited bactericidal efficacies of lysozyme–chitosan composite films.

Pure chitosan film (L0) showed bactericidal action against Gram-negative *E. coli*, but little inhibition effects on the growth of Gram-positive *S. faecalis*. The most steady synergistic inhibition trend against both bacteria was observed in L60 films. The recovery of *E. coli* at L100 films is suspicious, and may be explained from the following arguments. Along with the release of lysozyme from the film matrix (Figure 1), chitosan molecules will be simultaneously released to the inoculum's suspension that will stack up on cell surface or interact with lysozyme to form lysozyme–chitosan complex. When excess amount of lysozyme is exposed to the cell suspension, the chance of lysozyme–chitosan interactions may increase to interfere with the reactions between chitosan and Gram-negative cell surface.

Although the exact antimicrobial mechanism of chitosan is still unclear, several mechanisms have been proposed. One of the major proposals is that the polycationic nature of chitosan reacts with the negatively charged residues of macromolecules at the cell surface (Young and Kauss 1983), which could alter the permeability characteristics of chitosan, resulting in chitosan-induced leakage of bacterial cell components, such as proteins (Young and others 1982) and glucose (Tsai and Su 1999). Lysozyme is a well-known antimicrobial enzyme. It hydrolyzes the glycosidic bonds in bacterial cell wall components, specifically the  $\beta(1-4)$  linkages between C-1 of N-acetylmuramic acid and C-4 of N-acetyl-D-glucosamine (Masschalck and Michiels 2003). Lysozyme could be effective against Gram-negative bacteria when outer membranes of bacteria are disrupted or uncovered by chitosan. The outer membranes of *E. coli* treated with 0.01% chitosan was frayed and wrinkled, and the significant separation between cell membrane and the cell wall was previously observed in morphology studies of *E. coli* (Hwang and others 1998).

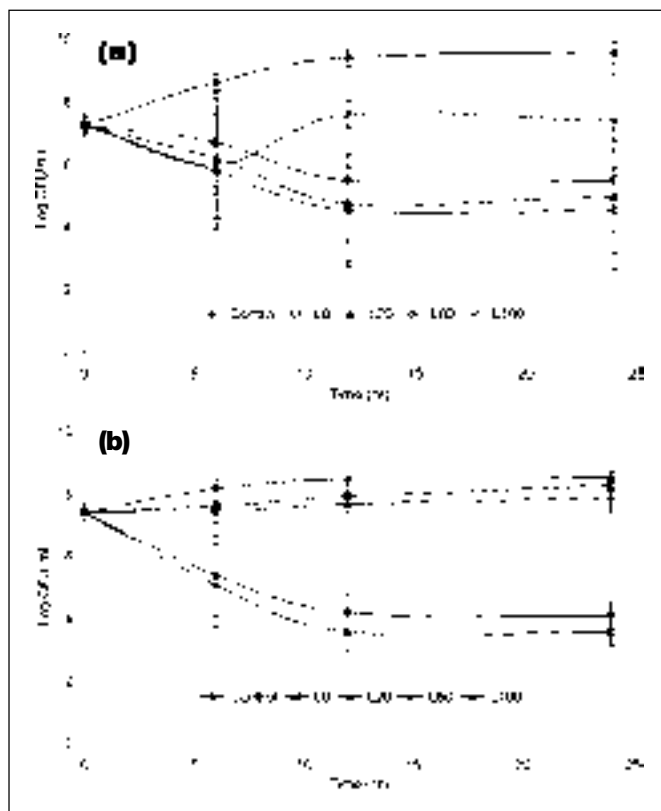
In our results, the strongest antimicrobial activity against *S. faecalis* was exhibited in chitosan films containing the highest lysozyme concentration (L100), whereas for *E. coli* it was in the films with 60% lysozyme concentration (w/w chitosan). These results suggest that the antimicrobial activity of chitosan can be enhanced with incorporation of lysozyme into chitosan film matrix and the ratio of lysozyme to chitosan molecules is an important factor affecting the antimicrobial mechanisms of lysozyme–chitosan composite films.

### Water vapor permeability

Water vapor barrier property of the films was not significantly affected by the incorporation of lysozyme in tested concentration levels ( $P < 0.05$ ) (Table 2). This result may be explained by the similar mechanisms described in the moisture content evaluations. Chitosan films, like many other protein or polysaccharide edible films, exhibited relatively low water barrier characteristics due to their high hydrophilic nature. Because lysozyme contains hydrophobic amino acid side chains, the hydrophilicity of lysozyme–chitosan composite films may decrease with lysozyme addition. However, the compact structure of chitosan, especially crystalline parts, may be disrupted by lysozyme molecule resulting in increased WVP through film matrix. These 2 contradicting effects with the addition of lysozyme may offset or minimize the differences in WVP characteristics of lysozyme–chitosan composite films.

### Mechanical properties

Tensile strength (TS) and percent elongation at break (EL) of the films significantly decreased ( $P > 0.05$ ) with the addition of



**Figure 2—Effects of lysozyme–chitosan composite films against *Escherichia coli* in brain heart infusion broth (a) and *Streptococcus faecalis* in MRS broth (b) ( $n = 6$ ). Control = no film; L0 = 0% lysozyme (w/w chitosan); L20 = 20% lysozyme (w/w chitosan); L60 = 60% lysozyme (w/w chitosan); L100 = 100% lysozyme (w/w chitosan).**

**Table 2—Water vapor permeability of lysozyme–chitosan composite films<sup>a</sup>**

Film type <sup>b</sup>	Thickness (μm)	WVP (g mm/m <sup>2</sup> d kPa)
L0	72.5 ± 13.4 a	177.2 ± 47.4 a
L20	68.8 ± 6.6 a	157.4 ± 26.5 a
L60	70.9 ± 4.4 a	160.0 ± 23.9 a
L100	69.0 ± 9.8 a	166.2 ± 22.2 a

<sup>a</sup>Means within the same column with same letters are not significantly different ( $P < 0.05$ ). WVP = water vapor permeability.

<sup>b</sup>L0 = 0% lysozyme (w/w chitosan); L20 = 20% lysozyme (w/w chitosan); L60 = 60% lysozyme (w/w chitosan); L100 = 100% lysozyme (w/w chitosan).

**Table 3—Mechanical properties of lysozyme–chitosan composite films<sup>a</sup>**

Film type <sup>b</sup>	Thickness (μm)	Tensile strength (MPa)	Elongation (%)
L0	74.9 ± 15.5a	17.4 ± 4.6a	60.3 ± 16.2a
L20	70.8 ± 9.2a	14.4 ± 3.4b	53.8 ± 9.0b
L60	73.0 ± 8.9a	9.5 ± 2.3c	39.3 ± 11.7c
L100	69.9 ± 9.2a	7.4 ± 1.5d	29.1 ± 8.2d

<sup>a</sup>Means within the same column with same letters are not significantly different ( $n = 30$ ;  $P < 0.05$ ).

<sup>b</sup>L0 = 0% lysozyme (w/w chitosan); L20 = 20% lysozyme (w/w chitosan); L60 = 60% lysozyme (w/w chitosan); L100 = 100% lysozyme (w/w chitosan).

lysozyme (Table 3). Both TS and EL reductions with increased lysozyme concentration can be described by following linear equations under the testing condition of 25 °C and 50% RH:

$$TS = -0.10 \cdot C_l + 16.70, R^2 = 0.96$$

$$weEL = -0.32 \cdot C_l + 59.89, R^2 = 0.99$$

where  $C_l$  is the percent concentration of lysozyme in lysozyme–chitosan film matrix (% w/w chitosan). In addition, it was found that there is a linear relationship between EL and TS under the same testing condition:

$$EL = 3.07 \cdot TS + 8.30, R^2 = 0.98$$

One may keep in mind that many other parameters affect TS and EL values; equations reported here may only apply for the films and testing conditions given in this study.

At 1:1 chitosan and lysozyme ratio (L100), there were 43% and 48% reductions in TS and EL values, respectively. Decreases on TS and EL of Na-alginate and κ-carrageenan-based films with lysozyme incorporation were reported by Cha and others (2002), in which about 30% reduction in TS and about 40% reduction in EL were observed with a 5% and 10% lysozyme incorporation in film matrix, respectively.

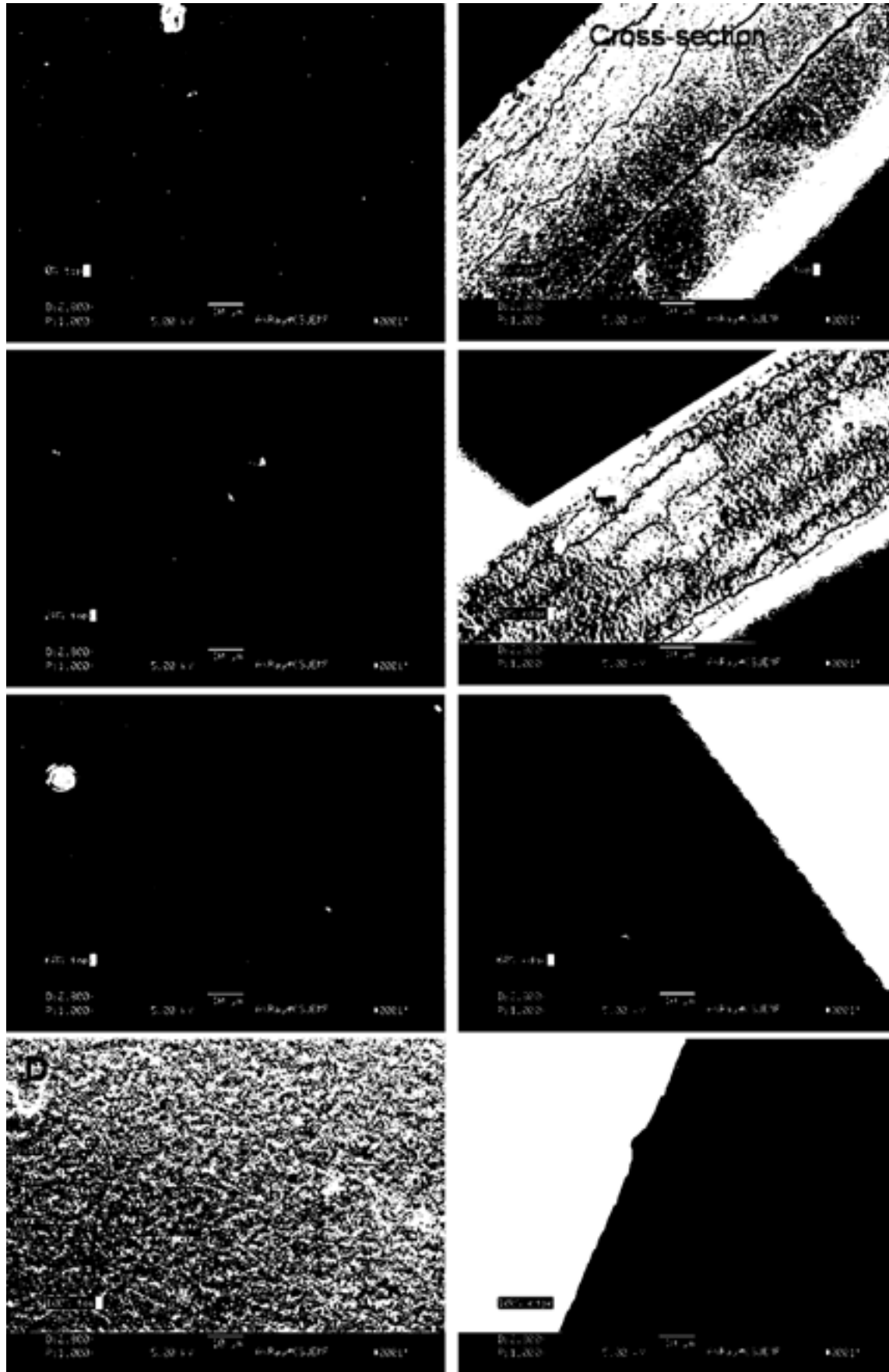
Chitosan is an excellent film-forming linear polymer with a rigid backbone structure, whereas lysozyme is a positively charged enzyme with less film-forming capacity. The reductions in both TS and EL indicate that the lysozyme incorporation weakened the film structure and integrity. Lysozyme molecules in FFS possibly disrupt the crystalline structure formation during the drying process and weaken intermolecular hydrogen bonding among chitosan molecules. The decreased TS and EL values may also be attributed to the degradation of chitosan molecules by lysozyme. In the presence of water, chitosan molecules could be hydrolyzed by lysozyme during film formation, resulting in the degradation of chitosan molecules and the formation of smaller molecular chitosan structure. Hwang and others (1998) reported that the dissolution of chitosan membranes into phosphate buffer (pH 6) increased with increased lysozyme concentration in buffer. Lysozyme could hydrolyze the β(1-4) linkages between *N*-acetyl-D-glucosamine units in chitosan which consists of mostly D-glucosamine units and small amount of *N*-acetyl-D-glucosamine units (Pangburn and others 1982; Nordtveit and others 1996). The degradation of chitosan molecules by lysozyme can be reduced by using highly deacetylated chitosan. In addition, the mechanical properties and water barrier properties of lysozyme–chitosan composite films may be improved by the use of cross-linking agents, such as glutaraldehyde and formaldehyde (Uragami and others 1994).

## Microstructure

Figure 3 shows the outer surface (left) exposed to air during film formation and cross-section (right) at a magnification of 1000×. The surface structures of the films were compact and uniform. All films have homogeneous appearances and continuous structures without any pores and cracks in the matrix. The microphotographs of L60 (c) and L100 (d) films show the bright marbling on the film surface. The marbling was uniformly distributed and increased with increasing lysozyme level. These white areas could represent the deposit of lysozyme micro-particles in the chitosan matrix. The uniform distribution of lysozyme through the film matrix could be speculated by homogeneous appearances in cross-sections of highly lysozyme incorporated films (c and d). As shown from the micrographs of cross-sections, there were no vertical cracks or phase separation between chitosan and lysozyme. The cross-section structures were compact and continuous except some cracks parallel to surface, which are possibly formed during the cryofracture process. Foreign materials fallen from the upper surface of film were captured in film L20 (b). This is a disadvantage of the solvent evaporation film-forming method, especially when films are cast and dried in the open air environment. Compact structure with a smooth contour of pure chitosan has been reported by Wong and others (1992). Our micrographs showed similar compact structure in pure chitosan film (L0) and other lysozyme–chitosan composite films. It could be evidence of good miscibility between chitosan and lysozyme. For fully understanding the impact of lysozyme on the microstructure of chitosan films, additional film microstructure may be studied using X-ray diffraction and FTIR (Fourier Transform Infrared) spectroscopy in the future works.

## Conclusions

This study demonstrated that a chitosan-based film matrix can effectively carry high concentration of lysozyme to develop Lysozyme–chitosan composite films. Lysozyme is released from the polymeric chitosan film matrix in controlled manner and retained its lytic activity against bacterial cell wall substrate. Such composite films have enhanced antimicrobial functions against both Gram-positive and Gram-negative representative bacteria in contrast to chitosan-alone films without altering moisture barrier characteristics of the films. Excellent biocompatibilities between chitosan and lysozyme and homogeneous distribution of lysozyme throughout the chitosan matrix were confirmed by scanning electron micrographs. Enhanced antimicrobial activities of lysozyme–chitosan composite films can broaden the applications of these renewable natural materials in ensuring food safety and quality. Typically, the films may be used to wrap foods that are highly susceptible to microbial growth or directly used as a surface coating on perishable fruits and vegetables to enhance microbial safety and extend shelf life of the products.



**Figure 3—**Scanning electron micrographs of the surface (left) and cross-section (right) of lysozyme-chitosan composite films viewed at a magnification of 1000x; L0 (a), L20 (b), L60 (c), L100 (d). L0 = 0% lysozyme (w/w chitosan); L20 = 20% lysozyme (w/w chitosan); L60 = 60% lysozyme (w/w chitosan); L100 = 100% lysozyme (w/w chitosan).

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