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Antimicrobial Properties of Wheat Gluten-Chitosan Composite Film in Intermediate-Moisture Food Systems

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Abstract Wheat gluten-chitosan composite film (WGCCF) can prevent moisture migration and enhance the antimicrobial properties of gluten in intermediate-moisture foods like sandwiches. To mimic the structure of actual sandwich-type products we developed multi-layer food models, where moisture content and water activity differ. Water activity gradients direct moisture migration and therefore determine product characteristics and product stability. A 10% wheat gluten film-forming solution was mixed with chitosan film-forming solution (0-3%, *w/w*) and evaporated to generate WGCCF. Addition of 3% chitosan enhanced the mechanical properties of the film composite, lowered its water vapor permeability, and improved its ability to protect against both, *Streptococcus faecalis* and *Escherichia coli*, in a 24 hr sandwich test (reduction of 1.3 and 2.7 log cycles, respectively, compared to controls). Best barrier and antimicrobial performance was found for 3% chitosan WGCCF at pH 5.1. Film of this type may find application as barrier film for intermediate-moisture foods.

Keywords: gluten-chitosan film, antimicrobial property, mechanical property, barrier property, moisture migration

Introduction

Edible protein film can prevent quality changes in foods by acting as a barrier against moisture migration, oxygen and carbon dioxide transfer, lipid oxidation, and loss of volatile flavors and aromas. Protein film has better mechanical properties compared to other film from polysaccharides and lipids. Edible film is primarily used for intermediate-moisture foods to prevent or delay moisture migration. It can be applied inside heterogeneous ingredients at the interfaces between different layers of components, and can function as a carrier for anti-microbiotic agents. A wide variety of edible compounds have attracted attention due to their film-forming ability (1-8).

In the past, refrigeration, dehydration, and preservatives have been the most effective way to extend shelf-life and to guarantee the food safety. Today, the food industry is faced with increasing consumer demands for the safety of convenient snack food products. Application of antimicrobial edible film is another potential food safety strategy for reducing the risks of bacterial contamination in snack food. We here report the development of prototypic edible film utilizing wheat protein and chitosan. Our data demonstrate that wheat gluten protein film has excellent antimicrobial properties and improved oxygen/moisture barrier characteristics when medium molecular weight chitosan (~450 kDa) is used. Wheat gluten protein film forms when heat-denatured proteins polymerize via disulfide and hydrophobic bonds into a network.

The moisture blocking capability of wheat gluten-chitosan film makes it an ideal candidate for applications where extended shelf-life of intermediate-moisture foods (e.g. sandwiches, sushi, and *kimbab*) is desired. Incorporation of natural antimicrobials into the film could additionally extend the food's allowable storage period. Antimicrobially enhanced packaging film has great potential for ensuring the safety of food through controlled release of antimicrobiotic substances from the carrier film structure to the food's surface (4, 5, 9). Antimicrobial compounds and their incorporation into food packaging materials have been previously reviewed (10, 11).

Chitosan is a renewable, non-toxic polymer with excellent biocompatibility with other substances (12). As the deacetylated derivative of chitin (β-[1-4]-poly-N-acetyl-D-glucosamine), chitosan is an abundant byproduct of seafood processing. Because of the high density of amino and hydroxyl groups in its polymer structure, chitosan has good film-forming properties, broad antimicrobial activity, and excellent compatibility with other substances (13-15), making it a highly attractive but underappreciated biopolymer for the development of new applications.

In this study, chitosan was used as a film-forming polymeric matrix to incorporate wheat gluten protein. Our objective was to develop a wheat gluten-chitosan composite film (WGCCF) that could be used between layer interfaces in heterogeneous foods to provide antimicrobial and mechanical protection.

Materials and Methods

Materials Shrimp-derived chitosan of medium (450 kDa) molecular weight [1%(*w/w*) in acetic acid at 25°C, 11 centipoises viscosity, and 89.9% deacetylation] was purchased from Nutrapol and used without further purification (Gwangju, Korea). Spray-dried water-soluble wheat gluten was obtained from Midwest Grain Products (Atchison, KS, USA). Acetic acid, sodium hydroxide, and glycerol were purchased from Fisher Scientific (Pittsburg, PA, USA). All chemicals were of reagent grade. Typical Gram-positive cocci (*Streptococcus faecalis*, ATCC

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14508) and Gram-negative bacili, (*Escherichia coli* B) were used as test microorganisms to evaluate the antimicrobial properties of the edible film. Brain heat infusion (BHI) broth, Man-Rogosa-Sharpe broth (MRS), and agar were purchased from Difco (Benton, Dickson and Co., Sparks, MD, USA).

Preparation of edible composite film Wheat gluten-chitosan film-forming solution (WGCFS) was prepared by dissolving 2% chitosan in 1% acetic acid at pH 5.1 and adding 25% glycerol (*w/w*) to the mixture. Wheat gluten film solution (WGFS) was prepared by dissolving wheat gluten in 120 mL of water to 10% and adding 25% glycerol (*w/w*) to achieve the same plasticizer level as in WGCFS. Subsequently, WGFS was mixed with chitosan solution to a final concentration of 0, 1, 2, or 3% (gluten dry weight per chitosan dry weight). The solution was homogenized with a Virtishear Tempest homogenizer (VirTis, Gardiner, NY, USA) for 30 min. Additionally, WGCFS was adjusted to pH 5.1 with 6 N NaOH. All sample solutions were mesh-filtered to remove insoluble residues and thereafter degassed with a GAST vacuum pump (Benton Harbor, MI, USA). Fifteen-milliliter aliquots of degassed WGCFS were poured onto 75 cm^2 polystyrene plates to approximately 75 μm thickness. The plates were dried at room temperature (24±2°C and 40±5 % relative humidity) for 2 days, the film was removed and cut into pieces. Prior to all measurements, all film pieces were stored at 23°C and 55% relative humidity (maintained by 12 N sulfuric acid) in 4-shelf desiccators (50×35×30 cm) until further evaluation (2, 16, 17).

Physical properties After two conditioning days at 25 °C and 50% RH, film thickness was measured with an electronic digital micrometer (Cole-Parmer Instruments, Vernon Hills, IL, USA). Strips of film were placed between the jaws of the micrometer and the gap reduced until first contact was noted. The mean of measurements at six different locations was used to calculate barrier and mechanical properties of the film. Moisture content was determined gravimetrically by drying the film samples at 105°C for 18 hr in a forced-air oven (Precision Scientific, Chicago, IL, USA) and expressed in percent wet basis. Color values of the film were measured with a ColorGard System/05 Gardner Colorimeter (Pacific Scientific, Silverspring, MD, USA).

Mechanical properties Mechanical properties were measured with a TA.XT2 texture analyzer (Texture Technologies, New York, NY, USA). Sample preparation and handling for texture analyses were carried out according to standard ASTM D 882-91 methods (18). Briefly, film strips measuring 100×25 mm were mounted onto the texture analyzer, pulled 7.5 mm apart at 2 mm/sec in tension mode, and the tensile strength (unit: MPa) calculated by dividing the peak load by the film cross sectional area. Puncture strength was measured as follows: circular film samples with a 6.5 cm diameter were mounted onto a cup and secured between a metal rim and a rubber gasket by six screws placed symmetrically around the cup's circumference. The film was then punctured with a cylindrical 3 mm probe in compression mode and puncture strength (unit: newton) was recorded at the point of rupture. Elongation at break (ETB), or a film's ability to stretch, was measured by the percentile change in length due to pulling stress at the break point.
Percent ETB (% ETB) was calculated as follows:

$$\% \text{ ETB} = (D_0 - D_1) / D_0 \times 100$$

where, D_0 is initial distance and D_1 is distance at the break point.

Measurement of water vapor permeability Water vapor permeability (WVP) was determined using a cup at 25°C with a 50-100% relative humidity gradient, following ASTM E96 procedures (19). Circular film samples of about 12 cm diameter were placed over the open mouth of aluminum cups (33 cm^2) and secured between a metal rim and a rubber gasket. Measurements were taken in a chamber at 22°C and 50% relative humidity. Air velocity was approximately 163 m/min over the surface of the cups to remove the permeating water vapor. Distilled deionized water was placed in the cups with an air gap of 1.4 cm above the water surface. The cup assembly was weighed every 30 min for a minimum of 10 hr and weight loss was plotted against time. Linear regression-derived slopes were used to estimate the water vapor transmission rate and WVP was expressed in g/m·s·Pa (16, 20).

Assessment of antimicrobial properties in multi-layer food models Artificial multi-layer food models were designed to mimic the structure of sandwich-type products, where moisture content and water activity vary. Water activity gradients are the driving force for moisture migration and are hence critical to the product's characteristics. To prevent moisture, lipid migration, and microbial contamination, WGCCF were placed between bread and sliced ham and tested for their effectiveness with a 5 day storage at 10°C. A1.5 g sample of each sandwich was dissolved in Butterfield's Buffer (Hardy Diagnostics, Santa Maria, CA, USA) and shaken with 50 rpm at room temperature. One milliliter of this culture was then added to 99 mL of the same broth. The bacterial culture broth for *E. coli* and *S. faecalis* was a mixture of BHI agar and MRS. Ten mL samples were spread on 85-mm petri dishes; cultures without bread samples were used as controls. The dishes were incubated at 37°C for 48 hr before the colony count. All tests were done in triplicates.

Film morphology The surface and internal structure of the film was evaluated using a 3300FE field emission scanning electron microscope (AmRay, Bedford, MA, USA). The film pieces were fractured in liquid nitrogen, mounted on aluminum stubs, and coated with gold-palladium alloy using an S150B sputter coater (BOC Edwards Vacuum, Manor Royal Crawley West Sussex, UK). Each coated sample was examined using at 5 kV.

Statistical analysis Thickness, as well as tensile and puncture strength were averaged from six measurements. Barrier properties were averaged from three measurements. Statistics on a completely randomized design were determined using the general linear model procedures of

SAS program (1994) to determine the influence of the various variables on the film properties. Least significance differences at the 5% level were used to compare treatments.

Results and Discussion

Film formation and its basic physical properties Integration of chitosan into wheat gluten solution never resulted in precipitation, suggesting that acetic acid-dissolved chitosan solution is miscible with water-soluble gluten. Because we used a carefully calculated amount of WGCFS, all dried film, once removed from the casting plates, had a thickness of 72 ± 11 μm to ensure a homogeneous population (p>0.05) without artifactual moisture content differences. Appearance and color are important properties of edible film, as they potentially affect the consumer's acceptance of such film in food applications. Total color differences (ΔE) and color values L, a, and b of the film are listed in Table 1. Film (WGCCF) with a 1% chitosan content was generally clearest (highest L value) and most uniform. The chitosan concentration in WGCCF also greatly affected the color properties of the film.

Moisture content tended to decline with increasing amounts of chitosan. This may be caused by wheat gluten and chitosan containing a large number of -OH and -NH_2 groups that use hydrogen bonding as the main binding force. Wheat gluten, the base of the film, improved water barrier properties due to its large number of non-polar amino acids and covalent disulfide bonds that give rise to mostly hydrophobic interactions (21). Addition of chitosan chains into wheat gluten molecules may alter the structural configuration of the chitosan molecules by an increased number of hydrogen bonds and van der Waals interactions. During film formation, the wheat gluten hydrophobic core may form by hydrophilic amino acid side chains protruding toward the aqueous WGCFS due to hydrophobic interactions that play an important role in the folding of wheat gluten (8, 21, 22). It is conceivable that addition of wheat gluten increases the number of hydrophobic side chains in the film matrix sufficiently to decrease the moisture content of WGCCF. However, more studies are needed to fully understand the interactions between chitosan and wheat gluten. Figure 1 shows a typical SEM micrograph of the WGCCF surface, which appears compact and uniform. All film samples had a homogeneous appearance with continuous structures bare of any pores or cracks in the matrix. Micrographs of 1% chitosan

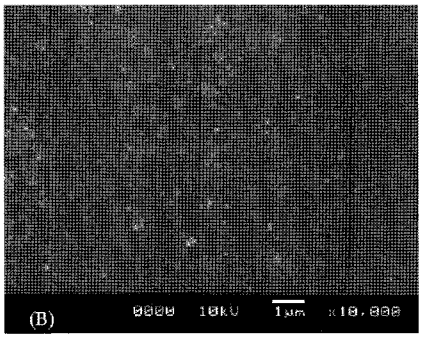

Fig. 1. Scanning electron micrographs of the wheat gluten-chitosan composite film surface (3% of chitosan) at a magnification of 5,000× (A) and 10,000× (B).

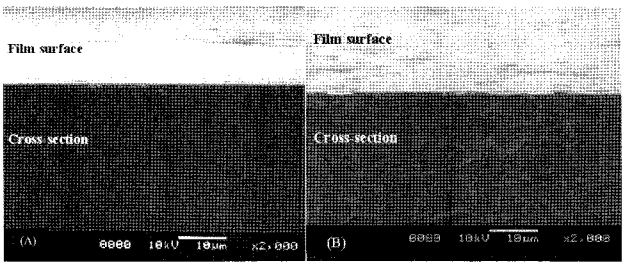

Fig. 2. Scanning electron micrographs of the internal structure of wheat gluten-chitosan composite film without chitosan (A, control) and containing 3% chitosan (B).

WGCCF showed bright marbling on the film's surface, which was regularly distributed and increased with the chitosan content. White areas found in the marbling may be chitosan microparticle deposits in the wheat gluten film matrix. Uniform distribution of chitosan throughout the matrix is speculative, but supported by the homogeneous crosssection appearance of highly chitosan-incorporated film. Also, crosssectional micrographs showed no vertical cracks or phase separation between wheat gluten and chitosan (Fig. 2).

Mechanical properties The effects of chitosan on the mechanical film properties are shown in Table 2. These properties are good indicators of the film's integrity under conditions of stress, such as processing, handling, and storage. Maximal tensile strenght and ETB were achieved with a chitosan concentration of 3% (w/w against total

Table 1. Effect of chitosan on the color of wheat gluten composite film

Chitosan content (%)	L	a	b	% ΔE
0	46.34±1.17^b	-4.28±1.24^b	7.21±0.11^b	48.95±0.36^b
1	53.21±1.34^b	-3.96±0.11^b	6.72±0.12^b	41.14±1.34^b
2	49.23±1.47^b	-5.68±1.04^a	7.62±0.28^a	52.64±1.18^a
3	48.26±1.61^b	-4.56±0.04^c	6.93±0.21^b	49.97±0.54^b

$^{a-c}$Same superscript indicate no statistically significant difference (p< 0.05).

Table 2. Effect of chitosan on the mechanical properties of wheat gluten composite film

Chitosan content (%)	TS[1] (MPa)	PS[2] (MPa)	ETB[3] (%)
0	5.5±0.8^c	5.9±0.7^c	18.2±1.6^c
1	6.4±0.9^b	8.5±0.7^a	24.2±0.9^a
2	6.8±1.1^b	8.2±0.5^b	20.3±0.9^b
3	8.3±1.3^a	9.6±0.6^a	26.7±1.2^a

$^{a-c}$Different superscripts indicate p<0.05.
[1)]Tensile strength.
[2)]Puncture strength.
[3)]Elongation at break.

wheat gluten). Control ETB was 18.2%. Puncture strength of 1, 2, and 3% chitosan WGCCF was 8.5, 9.2, and 9.6 MPa, respectively. These results indicate that addition of chitosan generally enhanced the stability and mechanical strength of the film. This strength may be in part based on polycations of the chitosan binding to predominantly negatively charged amino acid side chains of the wheat gluten protein at a pH higher than the isoelectric point (8, 9, 15), and thus, increasing denser three-dimensional networks in the composite film. The increase in protein hydrophobicity may be attributed to cations forming crosslinks between negatively charged carboxyl groups on polypeptide chains, which prevent interaction with water and give the film its rigid structure (23, 24).

Water vapor permeability Chitosan-treated WGCCF had a WVP lower than the control due to its homogeneous structure (Table 3). Addition of chitosan improved the mechanical strength and the water barrier properties of WGCCF by reacting with wheat gluten constituents to yield insoluble proteinates and altered film consistency. These inter- and intra-structural bridges between the protein and chitosan may have contributed to the formation of denser three-dimensional networks, and thus, the lower WVP in WGCCF compared to control film. Polycationic groups in the chitosan solution may have maximized the interactions between negatively charged molecules resulting in an improved protein network and stability. Furthermore, ionic crosslinking likely not only reduced protein segmental mobility, but also protein solubility in water, thus reducing the WVP throughout the protein matrix (16, 20).

Antimicrobial properties in multi-layer food models Inhibitory effects of WGCCF against *S. faecalis* and *E. coli* in 5 day/10°C food models are shown in Table 4.

Table 3. Effect of chitosan on the water vapor permeability of wheat gluten composite film

Chitosan content (%)	Thickness (μm)	WVP[1)] (g·mm/m^2·hr·kPa)
0	72.3±0.8^c	2.64±0.32^a
1	76.6±0.5^a	2.28±0.46^c
2	79.4±0.6^a	2.04±0.28^b
3	83.3±0.4^b	1.98±0.41^c

$^{a-b}$Different superscripts indicate $p< 0.05$.
$^{1)}$Water vapor permeability.

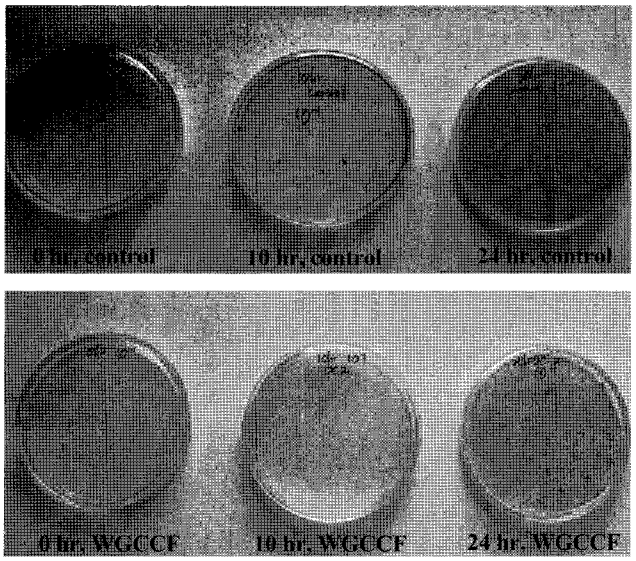

Fig. 3. Photographs showing the growth of *S. faecalis* (blue) and *E. coli* (red) in sandwich samples containing wheat gluten-chitosan composite film (WGCCF) with 2% chitosan incubated at 37°C for 48 hr before the colony count.

Among the naturally occurring microorganisms, *S. faecalis* and *E. coli* were chosen as index bacteria for personal hygiene, because they are typical fecal microorganisms. Maximum population density of these test microorganisms was reached within the first 24 hr. Addition of 2% chitosan to WGCCF enhanced its antimicrobial properties against both, *S. faecalis* and *E. coli*. Compared to the controls, it achieved 1.3 and 2.7 log cycle reductions for *S. faecalis* and *E. coli*, respectively, in the first 24 hr of storage. This finding demonstrates that chitosan film is more effective against microbial contamination than bacteriocin (4, 25). However, growth of *S. faecalis* was not inhibited by WGCCF as effectively as that of *E. coli*, because viable *S. faecalis* slightly recovered during 24 hr of storage (Fig. 3). In general, chitosan showed bactericidal action against Gram-negative *E. coli*, but little inhibitory effects on the growth of Gram-positive *S. faecalis*. Although the exact antimicrobial mechanism of chitosan is still unclear, several mechanisms have been proposed. One of the most convincing is that the polycationic nature of chitosan reacts with the negatively charged residues of macromolecules at the cell surface (26), which could alter the permeability characteristics of

Table 4. Antimicrobioal properties of wheat gluten-chitosan composite film in 10 °C food models with variable chitosan content

Chitosan contents of WGCCF (%)	Average population of bacteria (CFU/sandwich sample)									
	S. faecalis					*E. coli*				
	10 hr	24 hr	48 hr	72 hr	120 hr	10 hr	24 hr	48 hr	72 hr	120 hr
0	3.98×10^2	5.62×10^4	4.13×10^4	4.92×10^4	5.27×10^4	1.32×10^2	2.42×10^5	2.83×10^5	3.03×10^5	3.21×10^5
1	42.5×10	4.76×10^3	3.54×10^3	4.78×10^3	4.92×10^3	<10	6.31×10^3	7.24×10^3	6.92×10^3	7.37×10^3
2	<10	2.82×10^3	3.25×10^3	3.36×10^3	3.92×10^3	<10	4.82×10^2	6.59×10^2	7.38×10^2	7.52×10^2
3	<10	3.35×10^3	3.57×10^3	4.68×10^3	4.42×10^3	<10	3.86×10^2	5.24×10^2	6.26×10^2	6.49×10^2

chitosan, resulting in chitosan-induced leakage of bacterial cell components, such as proteins (27) and glucose (28). WGCCF have improved antimicrobial properties especially against Gram-negative representative bacteria without altering the moisture barrier characteristics of the film. This enhancement may open up new applications for these renewable natural materials, such as ensuring food safety and quality. Typically, the film 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 the product's shelf-life. We hence conclude that chitosan/wheat gluten film may effectively extend the shelf-life of layered intermediate-moisture foods such as sandwiches and *kimbabs*.

Acknowledgments

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