Development and characterization of antimicrobial edible films from crawfish chitosan

Kandasamy Nadarajah
Louisiana State University and Agricultural and Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations

Part of the Life Sciences Commons

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_dissertations/1630

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Doctoral Dissertations by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
DEVELOPMENT AND CHARACTERIZATION OF
ANTIMICROBIAL EDIBLE FILMS FROM CRAWFISH CHITOSAN

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Food Science

by
Kandasamy Nadarajah
B.Sc., University of Peradeniya, 1994
M.Sc., University of Peradeniya, 1997
May 2005
Dedicated to
My parents
Mr. and Mrs. A. Kandasamy
ACKNOWLEDGEMENTS

First, I would like to express my deepest gratitude to my academic advisor, Dr. Witoon Prinyawiwatkul for his invaluable enthusiasm, guidance, and encouragement, and for giving me the opportunity to complete my studies at my own pace.

I express my warmest gratitude to Professor Hong Kyoong No, for sharing his long experience in the field of chitosan and for his suggestions and advice. I am especially thankful to Dr. Marlene Janes for allowing me to use her Microbiology Lab and for guiding me with microbiological techniques throughout this study.

I am thankful to Dr. Joan King, Dr. Frederick Shih, and Dr. Kevin Carman for their constructive comments and suggestions for the improvement of this dissertation.

I am most grateful to Dr. Subramaniam Sathivel who has been a guide to me throughout my career. Without his enthusiasm and help, this dissertation would not be materialized.

I am thankful to the whole staff of food science department providing me with the most pleasant and convenient working environment.

Finally, my warmest thanks go to my parents and wife, Radha, for their everlasting unconditional support.

Nada, February 2005
# TABLE OF CONTENTS

- **DEDICATION** ......................................................................................................................... ii
- **ACKNOWLEDGEMENTS** ........................................................................................................ iii
- **LIST OF TABLES** .................................................................................................................. vii
- **LIST OF FIGURES** ................................................................................................................ ix
- **ABSTRACT** ............................................................................................................................. x

## CHAPTER 1. INTRODUCTION
1.1 Introduction ......................................................................................................................... 2
1.2 References .......................................................................................................................... 4

## CHAPTER 2. LITERATURE REVIEW
2.1 Chitosan ............................................................................................................................... 8
2.2 Characterization of Chitosan
   2.2.1 Degree of Deacetylation of Chitosan ............................................................................ 9
   2.2.2 Molecular Weight .......................................................................................................... 11
   2.2.3 Viscosity ....................................................................................................................... 11
2.3 Sources of Chitosan
   2.3.1 Crawfish Shell Waste as a Source of Chitosan ............................................................. 13
   2.3.2 Extraction of Chitosan from Crawfish ........................................................................ 13
2.4 Film-forming Ability of Chitosan
   2.4.1 Film-forming Methods ................................................................................................. 16
   2.4.2 Film-forming Mechanisms ......................................................................................... 17
   2.4.3 Film Morphology and Defects .................................................................................... 17
   2.4.4 Function of Plasticizers in Film Formation .................................................................. 18
2.5 Properties of Chitosan and Chitosan Films
   2.5.1 Safety of Chitosan Films ............................................................................................. 18
   2.5.2 Biodegradation of Chitosan and Chitosan Films .......................................................... 19
   2.5.3 Mechanical Properties of Chitosan Films .................................................................. 19
   2.5.4 Transport Properties of Chitosan Films ...................................................................... 20
   2.5.5 Antimicrobial Properties of Chitosan and Chitosan Films ......................................... 22
2.6 Applications of Chitosan Films
   2.6.1 Applications of Chitosan and Chitosan Films in Foods ............................................. 26
2.7 References ............................................................................................................................ 30

## CHAPTER 3. PHYSICOCHEMICAL PROPERTIES OF EDIBLE CHITOSAN FILMS DEVELOPED FROM CRAWFISH SHELL WASTE
3.1 Introduction ........................................................................................................................... 42
3.2 Materials and Methods ....................................................................................................... 43
  3.2.1 Preparation of Chitosan from Crawfish Shell Waste .................................................. 44

iv
3.2.2 Characterization of Crawfish Chitosan .......................................................... 45
3.2.3 Preparation of Crawfish Chitosan Films ...................................................... 46
3.2.4 Thickness and Density of Crawfish Chitosan Films ...................................... 46
3.2.5 Color of Crawfish Chitosan Films ............................................................... 46
3.2.6 Transparency of Crawfish Chitosan Films .................................................. 47
3.2.7 Swelling of Crawfish Chitosan Films ......................................................... 47
3.2.8 Solubility of Crawfish Chitosan Films ......................................................... 48
3.2.9 Microstructure of Crawfish Chitosan Films ................................................ 48
3.2.10 Statistical Analysis ..................................................................................... 48
3.3 Results and Discussions .................................................................................. 49
  3.3.1 Characteristics of Crawfish Chitosan ......................................................... 49
  3.3.2 Film-forming Ability of Crawfish Chitosans ............................................. 50
  3.3.3 Thickness and Density of Crawfish Chitosan Films .................................... 51
  3.3.4 Color of Crawfish Chitosan Films ............................................................. 53
  3.3.5 Transparency of Crawfish Chitosan Films ................................................ 55
  3.3.6 Swelling of Crawfish Chitosan Films ......................................................... 56
  3.3.6 Solubility of Crawfish Chitosan Films ....................................................... 58
  3.3.7 Microstructure of Crawfish Chitosan Films .............................................. 59
3.4 Conclusions ...................................................................................................... 61
3.5 References ........................................................................................................ 62

CHAPTER 4. SORPTION AND WATER PERMEABILITY BEHAVIORS OF CRAWFISH
CHITOSAN FILMS ............................................................................................... 65
4.1 Introduction ....................................................................................................... 66
4.2 Materials and Methods .................................................................................... 67
  4.2.1 Preparation of Chitosan from Crawfish Shell Waste .................................. 67
  4.2.2 Characterization of Crawfish Chitosans .................................................... 68
  4.2.3 Preparation of Crawfish Chitosan Films .................................................... 69
  4.2.4 Sorption Isotherm Experiments ................................................................. 69
  4.2.5 Water Vapor Permeability of Crawfish Chitosan Films ............................. 71
  4.2.6 Statistical Analysis ..................................................................................... 71
4.3 Results and Discussion ..................................................................................... 71
  4.3.1 Characteristics of Crawfish Chitosan ......................................................... 71
  4.3.2 Film-forming Ability and Film Characteristics .......................................... 72
  4.3.3 Sorption Isotherms of Crawfish Chitosan Films ....................................... 73
  4.3.4 Sorption Model Analysis ......................................................................... 78
  4.3.5 Water Vapor Transmission Rate ............................................................... 83
4.4 Conclusions ...................................................................................................... 88
4.5 References ........................................................................................................ 89

CHAPTER 5. MECHANICAL PROPERTIES OF CRAWFISH CHITOSAN FILMS AS
AFFECTED BY CHITOSAN PRODUCTION PROTOCOLS AND FILM
CASTING SOLVENTS ........................................................................................ 94
5.1 Introduction ...................................................................................................... 95
  5.1.1 Objective ................................................................................................... 96
5.2 Materials and Methods .................................................................................... 97
LIST OF TABLES

3.1  Physicochemical properties of chitosans extracted from crawfish shell ............... 49
3.2  Film-forming ability of unplasticized crawfish chitosan ..................................... 52
3.3  Thickness and density of unplasticized crawfish chitosan films .......................... 53
3.4  Color attributes of crawfish chitosan films .......................................................... 54
3.5  Solubility of unplasticized crawfish chitosan films .............................................. 59
4.1  Isotherm models used for fitting experimental data .............................................. 70
4.2  Constants of sorption models for chitosan acetate films ..................................... 79
4.3  Constants of sorption models for chitosan formate films .................................. 80
4.4  Constants of sorption models for chitosan citrate films .................................... 81
4.5  Water vapor transmission rate and water vapor permeability at 25°C and 50% RH gradient. ........................................................................................................... 85
4.6  Water vapor permeability of chitosan, edible and plastic films .............................. 87
5.1  Tensile measurements of crawfish chitosan films ............................................... 101
5.2  Comparison of tensile strength and percent elongation values for selected biopolymer and synthetic polymer films ................................................................. 105
5.3  Comparison of tensile properties of chitosan films with organic acid ................ 106
5.4  Puncture strength of crawfish chitosan films ....................................................... 108
6.1  Effect of chitosan films on selected food pathogenic bacteria. ............................... 122
6.2  Recovery of *Listeria monocytogenes* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours. ............................... 127
6.3  Log reduction of *Listeria monocytogenes* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours. ....................... 128
6.4  Recovery of *Staphylococcus aureus* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours. ................................. 130
6.5 Log reduction of *Staphylococcus aureus* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours. ........................................ 130

6.6 Recovery of *Salmonella typhimurium* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours. ........................................ 131

6.7 Log reduction of *Salmonella typhimurium* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours. ........................................ 132

6.8 Recovery of *Shigella sonnei* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours. .................................................. 133

6.9 Log reduction of *Shigella sonnei* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours. .................................................. 134
LIST OF FIGURES

2.1 Chemical structure of cellulose, chitin and chitosan ................................................................. 8
2.2 Conversion of chitin into chitosan .................................................................................................. 10
2.3 Scheme for chitosan production (Modified from No and Meyers 1995)................................. 15
3.1 Transparency of unplasticized crawfish chitosan films ............................................................... 56
3.2 The % swelling of crawfish chitosan films formed with acetic and formic acids. .... 57
3.3 The unplasticized DPMA chitosan film formed with formic acid............................................. 57
3.4 The SEM of crawfish chitosan films. ......................................................................................... 60
4.1 Moisture sorption isotherms of representative crawfish chitosan films at 25ºC. ...... 74
4.2 Moisture sorption isotherms of chitosan acetate films at 25ºC................................................. 75
4.3 Moisture sorption isotherms of chitosan formate films at 25ºC. ............................................. 76
4.4 Effect of water activity on clustering of water molecules in chitosan acetate films... 84
5.1 Stress-strain curves of crawfish chitosan films with organic acid solvents................. 100
5.2 Tensile strength of films made with different crawfish chitosans and acids.. ........... 102
5.3 Young's modulus of films made with different crawfish chitosans and acids........... 103
6.1 Inhibition of food pathogenic bacteria by chitosan citrate films. ........................................ 124
6.2 Inhibition zones produced by crawfish chitosan films.. ......................................................... 125
Inherent antibacterial/antifungal properties and film-forming ability of chitosan make it ideal for use as a biodegradable antimicrobial packaging material. This study was attempted to develop antimicrobial films from crawfish chitosan. Traditional chitosan production involves: deproteinization (DP), demineralization (DM), decolorization (DC), and deacetylation (DA). Modification of chitosan production affects film properties. Effects of chitosan production protocols, film-casting solvents, and plasticizer contents on physicochemical, mechanical and antibacterial properties were investigated. Four chitosans were prepared from traditional (DPMCA) and modified processes [excluding either DP, DC or both DP and DC]. Chitosan (1%w/v) was dissolved in 1% acetic, ascorbic, citric, formic, lactic and/or malic acid, and cast with and without glycerol (a plasticizer) at a ratio of 1:0.1, 1:0.2, 1:0.3, 1:0.4 and 1:0.5 (chitosan:glycerol, w/w) to form films.

Flexible and transparent films could be prepared from chitosans with acetic, formic or citric acid without a plasticizer. DMCA acetate films showed higher tensile strength (135.8 MPa), but poor antibacterial properties. DPMCA formate films with tensile strength of 76.8 MPa reduced microbial loads of *Staphylococcus aureus*, *Salmonella typhimurium*, and *Shigella sonnei* by more than 2.5 log CFU/mL in 24 hours. DMA citrate films showed tensile strength of 29.3 MPa and reduced *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Shigella sonnei* by more than 4.4 log CFU/mL in 24 hours. This study demonstrated the feasibility of developing antimicrobial edible films from crawfish chitosans. Some critical factors required for desirable film properties were identified.
1.1 INTRODUCTION

Chitosan is a carbohydrate polymer that can be derived from crustacean seafood wastes such as shells of crabs, shrimps and crawfish. Chitosan has a wide range of applications in diverse fields ranging from medical sutures and seed coatings to dietary supplements and coagulants for waste treatment. Physicochemical properties of chitosans and their functionalities are affected by their sources (Rhazi and others 2004) and the methods employed to extract them (Brine and Austin 1981). Hence, different physicochemical properties and functionalities can be expected from chitosans derived from crawfish shell by different extraction protocols.

Physicochemical properties of different chitosans derived from crawfish shell waste were reported earlier (Rout 2001). However, their application potentials in food and other fields are yet to be investigated. Recently, the potential of developing flexible and transparent edible films and packaging materials from crawfish chitosan was reported by Nadarajah and Prinyawiwatkul (2002). Development of antimicrobial edible films and packaging materials from crawfish chitosans may expand their applications in food systems. The possibility of producing crawfish chitosans with varying physicochemical properties presents a niche for selecting the most suitable chitosans for the development of antimicrobial films and packaging materials.

Contamination of food products by pathogenic bacteria has emerged as a serious public concern. Bacteria such as Listeria monocytogenes, Bacillus cereus, Escherichia coli (O157:H7), Staphylococcus aureus, Salmonella typhimurium, and Shigella sonnei are identified as the most potent pathogens associated with food born illnesses in U.S.A (Mead 1999). Antimicrobial packaging is one promising approach to prevent both contamination of pathogens and growth of spoilage microorganisms on the surface of food. Direct addition of antimicrobial substances into food formulations or onto food surfaces may not be sufficient to prevent the growth of
pathogenic and spoilage microorganisms as antimicrobial substances applied could be partially
inactivated or absorbed by the food systems (Ouattara and others 2000). Antimicrobial films
render sustained release of antimicrobial substances onto the food surface and compensate for
the partial inactivation or absorption of them by food systems (Siragusa and Dickson 1992).

Chitosan is an ideal biopolymer for developing such antimicrobial films due to their non-
toxicity (Hirano and others 1990), biocompatibility (Muzzarelli 1993), biodegradability
(Shigemasa and others 1994), film-forming ability (Averbach 1978) and inherent antimicrobial
properties (Sudarshan and others 1992). Moreover, antimicrobial properties of chitosan can be
enhanced by irradiation (Matsuhashi and Kume 1997), ultraviolet radiation treatment, partial
hydrolyzation (Davydova and others 2000), chemical modifications (Nishimura and others
1984), synergistic enhancement with preservatives (Roller and others 2002), synergistic
enhancement with antimicrobial agents (Lee and others 2003), or in combination with other
hurdle technologies.

One of the simplest and most economical ways of producing antimicrobial films is to
incorporate antimicrobial substances into films (Weng and Hotchkiss 1993). Various organic
acids that naturally occur in fruit and vegetables and possess general antimicrobial activity such
as acetic, lactic, malic, and citric, sorbic, benzoic and succinic acids can be used for this purpose
(Beuchat 1998). Further, since chitosan needs to be dissolved in slightly acidic solutions, the
production of antimicrobial films from chitosan with organic acids is straightforward (Begin and
Calsteren 1999). However, the interaction between the preservatives and the film-forming
material may affect film casting, release of preservative and mechanical properties (Chen and
others 1996).
Antimicrobial edible films developed with several organic acids have demonstrated their effectiveness in reducing bacterial levels on meat products. Baron (1993) showed that edible corn starch films with potassium sorbate and lactic acid inhibited *S. typhimurium* and *E. coli* (O157:H7) on poultry. Siragusa and Dickson (1992) reported that organic acids were more effective against *L. monocytogenes*, *S. typhimurium* and *E. coli* (O157:H7) on beef carcass when immobilized in edible film than when applied directly. Recently, chitosan films containing acetic and propionic acids controlling *Enterobacteriaceae* and *Serratia liquefaciens* on bologna, regular cooked ham, and pastrami were reported (Ouattara and others 2000). These results indicate potentials for developing antimicrobial edible films from crawfish chitosan.

This dissertation study was intended to investigate the possibility of producing antibacterial edible films from crawfish chitosans by (1) extracting various chitosans from crawfish shell waste by employing different extraction protocols, (2) formulating films with different organic acids, and (3) screening (i) physicochemical properties, (ii) sorption behaviors, (iii) water permeability characteristics, (iv) mechanical properties, and (v) antibacterial activities of the chitosan films to identify properties best suited to develop antimicrobial films from crawfish chitosan.

1.2 REFERENCES


Baron JK. 1993. Inhibition of *S. typhimurium* and *E. coli* O157:H7 by an antimicrobial containing film [MSc thesis]. Lincoln, Nebr.: University of Nebraska.


CHAPTER 2

LITERATURE REVIEW
2.1 CHITOSAN

Chitosan is a modified natural carbohydrate polymer derived from chitin which has been found in a wide range of natural sources such as crustaceans, fungi, insects and some algae (Tolaimate and others 2000). The primary unit in the chitin polymer is 2-acetamido-2-deoxy-β-D-glucose. These units are combined by 1-4 glycosidic linkages, forming a long chain linear polymer without side chains. Chitin is chemically identical to cellulose, except that the secondary hydroxyl group on the alpha carbon atom of the cellulose molecule is substituted with acetoamide groups (Figure 2.1).

Removal of most of the acetyl groups of chitin by treatment with strong alkali yields chitosan (Peniston and Johnson, 1980) which is 2-amino-2-deoxy-β-D-glucose. A sharp nomenclature with respect to the degree of N-deacetylation has not been defined between chitin and chitosan (Muzzarelli 1977). In general, chitin with a degree of deacetylation of above 70% is considered as chitosan (Li and others 1997a).

Chitosan is insoluble in water but soluble in acidic solvents below pH 6. Organic acids such as acetic, formic and lactic acids are used for dissolving chitosan, and the most commonly used solvent is 1% acetic acid solution. Solubility of chitosan in inorganic acid solvent is quite limited. Chitosan is soluble in 1% hydrochloric acid but insoluble in sulfuric and phosphoric acids. Chitosan solution's stability is poor above pH 7 due to precipitation or gelation that takes place.

Figure 2.1: Chemical structure of cellulose, chitin and chitosan
place in alkali pH range. Chitosan solution forms a poly-ion complex with anionic hydrocolloid and provides gel.

2.2 CHARACTERIZATION OF CHITOSAN

Chitosan can be characterized in terms of its quality, intrinsic properties such as purity, molecular weight, viscosity, and degree of deacetylation and physical forms (Sanford 1989). The quality and properties of chitosan product may vary widely because many factors in the manufacturing process can influence the characteristics of the final chitosan product (Li and others 1992).

2.2.1 Degree of Deacetylation of Chitosan

Among many characteristics, the degree of deacetylation is one of the more important chemical characteristics, which influences the performance of chitosan in many of its applications (Muzzarelli 1977; Li and others 1992; Baxter and others 1992). In addition, the degree of deacetylation, which reveals the content of free amino groups in the polysaccharides (Li and others 1992), can be used to differentiate between chitin and chitosan. In general, chitin with a degree of deacetylation of above 70% is considered as chitosan (Li and others 1997a). In the process of deacetylation, acetyl groups from the molecular chain of chitin are removed to form amino groups (Figure 2.2).

Variables such as temperature or concentration of sodium hydroxide solution affect the removal of acetyl groups from chitin, resulting in a range of chitosan molecules with different properties and hence its applications (Baxter and others 1992; Mima and others 1983). Since the degree of deacetylation depends mainly on the method of purification and reaction conditions (Baxter and others 1992; Li and others 1997), it is essential to characterize chitosan by determining its degree of deacetylation prior to its utilization.
A number of methods have been used to determine the degree of deacetylation, such as linear potentiometric titration (Ke and Chen 1990), infrared spectroscopy (Baxter and others 1992), nuclear magnetic resonance spectroscopy (Hirai and others 1991), pyrolysis-mass spectrometry (Nieto and others 1991), first derivative UV-spectrophotometry (Muzzarelli and Rocchetti 1985), and titrimetry (Raymond and others 1993). Some of the methods are either too tedious, too costly for routine analysis (e.g., nuclear magnetic resonance spectroscopy), or destructive to the sample (Khan and others 2002). From the literature, the degree of deacetylation values of chitosan appear to be highly associated with the analytical methods employed (Khan and others 2002). However, one of the most frequently used methods is infrared spectroscopy because of its simplicity.
2.2.2 Molecular Weight

The molecular weight of chitosan varies depending on the raw material sources and preparation methods (Li and others 1992). Most commercial chitosans have a degree of deacetylation that is greater than 70% and a molecular weight ranging between 100,000 Da and 1.2 million Da (Li and others 1997; Onsoyen and Skaugrud 1990). Various factors, such as temperature, dissolved oxygen concentration, and shear stress can cause degradation of chitosan.

The molecular weight of chitosan can be determined by methods such as chromatography (Bough and others 1978), light scattering (Muzzarelli 1977), and viscometry (Maghami and Roberts, 1988). Among many methods, viscometry is a simple and rapid method for the determination of molecular weight. The intrinsic viscosity of a polymer solution is related to the polymer molecular weight according to the Mark-Houwink equation:

\[ [\eta] = KM^a \]

where \([\eta]\) is the intrinsic viscosity, \(M\) the viscosity-average molecular weight, and \(K\) and \(a\) are constants for a given solute-solvent system and temperature.

2.2.3 Viscosity

As with molecular weight and degree of deacetylation, viscosity is an important characteristic of chitosan. Viscosity of chitosan is highly dependent on the degree of deacetylation, molecular weight, concentration of solution, ionic strength, pH, and temperature. The processes involved in the extraction of chitosan also affect the viscosity of chitosan. For instance, chitosan viscosity decreases with an increased time of demineralization (Moorjani and others 1975). Bough and others (1978) found that elimination of the demineralization step in the chitin preparation decreased the viscosity of the final chitosan products. Moorjani and others (1975) reported that bleaching chitosan with acetone or sodium hypochlorite at any stage of the
extraction process leads to considerable reduction in viscosity. No and others (1999) demonstrated that chitosan viscosity is considerably affected by physical (grinding, heating, autoclaving, ultrasonication) and chemical (ozone) treatments, except for freezing, and decreases with an increase in treatment time and temperature.

### 2.3 SOURCES OF CHITOSAN

Chitosan is converted from chitin, which is a structural polysaccharide found in the skeleton of marine invertebrates, insects and some algae. Chitin is perhaps the second most important polysaccharide after cellulose and is an abundantly available renewable natural resource. The aquatic species that are rich in chitinous material (10-55% on a dry weight basis) include squids, crabs, shrimps, cuttlefish and oysters. Mucoraceous fungi, which are known to contain chitin and the deacetylated derivate, chitosan, in cell walls (22 to 44%), have been used for commercial chitin production (Muzzarelli 1977; Muzzarelli and others 1994). However, in comparison with marine sources, which yield more than 80,000 metric tons of chitin per year (Muzzarelli 1977; Subasingle 1995), chitin production from fungal waste is negligible.

Depending on the sources, the physicochemical properties and functionalities of chitosan differ (Rhazi and others 2004). For example, chitosan prepared from squid contains β-chitin (amine group aligned with the OH and CH₂OH groups) and those prepared from crustaceans contain α-chitin (anti-parallel chain alignment) (Shepherd and others 1997; Felt and others 1999). Despite a wide range of available sources, chitosan is commercially manufactured only from crustaceans (crab, shrimp, krill, and crayfish) primarily because a large amount of crustacean exoskeleton is available as a byproduct of food processing. Disposal of crustacean shell waste has been a challenge for seafood processors. Therefore, production of value-added products, such as chitin, chitosan and their derivatives, and utilization of these value added
products in different fields are of utmost interest to food industries. Continual use of new raw materials as a source of chitin would enable production to be significantly increased. Major progress is being made in the development of profitable technology for isolation of chitin and its derivatives (Rashidova and others 2004). However, commercial extraction has been hampered by the corrosive nature of the strong acids and bases used in the manufacture of chitosan, which destroys equipment, requires careful handling by workers, and presents potential environmental hazards (Peniston and Johnson 1980; Leffler 1997).

2.3.1 Crawfish Shell Waste as a Source of Chitosan

Louisiana is the world’s largest producer of crawfish. Crawfish consumed in Louisiana belongs to two species, the red swamp crawfish and the white river crawfish. The red swamp crawfish, *Procambarus clarkii*, is most common, accounting for 60% of the catch in the Atchafalaya river basin (No and Meyers 1995). Louisiana produces crawfish with an average annual harvest exceeding 100 million pounds. Since the edible portion of crawfish accounts for only 15%, about 85 million pounds of crawfish shell waste is produced in Louisiana annually. The crawfish shell waste contains 23.5% chitin, which can be converted into chitosan (No and Meyers 1992). The peeling waste has been used as animal feed with low economic value, although it is an inexpensive source of the biopolymer chitosan (Prinyawiwatkul and others 2002). Various applications of crawfish chitosan have been reported. However, commercialization of such findings is yet to be realized.

2.3.2 Extraction of Chitosan from Crawfish

Chitosan can be extracted from chitin sources by conventional chemical extraction or using enzymes. Depending on procedures and sources, resultant chitosans differ in their chemical and physical properties and functionality (Rhazi and others 2004). The conventional
chitosan extraction method involves, deproteinization to remove proteinous materials by an alkali treatment, demineralization to remove calcium carbonate and calcium phosphate by an acid treatment, decoloration to remove pigments by solvent extraction and bleaching, and deacetylation to convert chitin into chitosan by an alkali treatment.

No and Meyers (1995) established a procedure to extract chitin from crawfish shell waste and to convert chitin into chitosan by an alkali treatment with 50% NaOH (solid: solvent, 1:10, w/v) for 30 minutes at 100°C in air or 121°C at 115 psi (Figure 2.3). Rout and Prinyawiwatkul (2001) investigated properties of chitosan extracted from crawfish shell using different extraction processes and reported that the deacetylation time in air has to be increased by another 30 minutes (a total of 60 minutes) to obtain chitosan with film-forming ability. They also suggested that deprotenization and decoloration steps could be excluded since the harsh deacetylation process denatures any protein and removes most of pigments from crawfish shell. Nadarajah and Prinyawiwatkul (2002) investigated the degree of deacetylation of crawfish chitosan versus film-forming ability and reported that crawfish chitosan obtained by 60 minutes deacetylation led to a lower degree of deacetylation and poor film formation. Further, they suggested that a 90 minutes of the deacetylation process yielded chitosan with a higher degree of deacetylation which serve as good film formers. However, chitosan with similar film-forming ability can be obtained with an autoclaving process at a shorter time of 30 minutes (No and others 2000) (Figure 2.3). Apart from these studies, information on film-forming ability of crawfish chitosan and their functionalities is lacking. It is envisaged that chitosan obtained from modified or simplified extraction processes and film formation with different solvents could yield a film with elicited antimicrobial activity while bringing down the production cost.
Wet crawfish shell

Washing and drying

Grinding and sieving

**Deproteinization**

Deproteinized shell

Washing

**Demineralization**

Washing

**Extraction with Acetone**

Drying

**Bleaching**

Washing and Drying

**Deacetylation**

Washing and Drying

Chitosan

---

**3.55 % NaOH (w/v) for 2 h at 65°C solid: solvent (1:10, w/v)**

**1 N HCl for 30 min at room temp. solid: solvent (1:15, w/v)**

**0.315% NaOCl (w/v) for 5 min at room temp. solid:solvent (1:10, w/v)**

**50% NaOH for 30 min at 115 psi / 121°C, solid:solvent (1:10, w/v)**

---

Figure 2.3: Scheme for chitosan production (Modified from No and Meyers 1995).
2.4 FILM-FORMING ABILITY OF CHITOSAN

Chitosans with higher molecular weight have been reported to have good film-forming properties as a result of intra- and intermolecular hydrogen bonding (Muzzarelli 1977). A patent was granted to G.W. Rigby in 1936 for the earliest attempt to form films from chitosan. These films were described as flexible, tough, transparent, and colorless with a tensile strength of about 9,000 psi and prepared by a solvent casting method. Chitosan films prepared by similar methods were reported later by Muzzarelli and others (1974), Averbach (1978), Butler and others (1996), Caner and others (1998) and Wiles and others (2000). These films were described to have good gas barrier and mechanical properties. The chitosan film characteristics, however, varied from one report to another. Differences in the sources of chitin used to produce chitosan, chitosan properties, solvents used, methods of film preparation, and types and amounts of plasticizers used affect the quality of the chitosan films (Lim and Wan 1995; Remuñán-López and Bodmeier 1996; Begin and Calsteren 1999; Nunthanid 2001). The film-forming ability of chitosan extracted from crawfish has been reported by Nadarajah and Prinyawiwakul (2002).

2.4.1 Film-forming Methods

Edible films are formed by either a wet- or dry-process mechanism. The wet-process-mechanism is based on a film-forming dispersion or solution in which polymers are first dispersed or solubilized into a liquid phase, and then dried. Freeze drying is employed to obtain sponge-type scaffolds used in tissue engineering. The wet process is often preferred as it permits the application of films as coatings in a liquid form directly onto food products by dipping, brushing or spraying (Peressini and others 2004). Some edible films, such as starch films, can be prepared using a dry-process, such as thermoplastic extrusion. This extrusion process is based on
the thermoplastic properties of polymers when plasticized and heated above their glass transition
temperature under low water content-conditions (Warburton and others 1993; Psomiadou and
others 1997; Arvanitoyannis and Billiaderis 1998).

2.4.2 Film-forming Mechanisms

Polymeric solutions form films through a series of phases. When the polymer solution is
cast on a surface, cohesion forces form a bond between the polymer molecules (Banker 1996).
When the cohesive strength of the polymer molecules is relatively high, continuous surfaces of
the polymer material coalesce. Coalescence of an adjacent polymer molecule layer occurs
through diffusion. Upon evaporation of water, gelation progresses and allows the polymer chains
to align in close proximity to each other and to get deposited over a previous polymer layer
(Harris and Ghebre-Sellassie, 1997). When there is adequate cohesive attraction between the
molecules, sufficient diffusion, and complete evaporation of water, polymer chains align
themselves to form films (Harris and Ghebre-Sellassie 1997).

2.4.3 Film Morphology and Defects

Polymeric films should be uniform and free from defects for their applications.
Uniformity of the films is critical for their functionalities. The processing variables involved in
conversion of chitin into chitosan, especially the uniformity of particle size of shells used as a
starting material, greatly influence the properties of chitosan (No and others 1999), and hence the
uniformity of films produced. During the film-forming process, shrinkage of the films due to
evaporation of water or rapid drying often causes defects such as cracks or curling in the films
(Obara and McGinity 1995). Addition of plasticizers such as glycerol or sorbitol is often used to
reduce such defects.
2.4.4 Function of Plasticizers in Film Formation

Films prepared from pure polymers tend to be brittle and often crack upon drying. Addition of food-grade plasticizers to film-forming solution alleviates this problem (McHugh and Krochta, 1994). When a plasticizer is added, the molecular rigidity of a polymer is relieved by reducing the intermolecular forces along the polymer chain. Plasticizer molecules interpose themselves between the individual polymer chains, thus breaking down polymer-polymer interactions, making it easier for the polymer chains to move past each other. The plasticizer improves flexibility and reduces brittleness of the film. Polyethylene glycol, glycerol, propylene glycol, and sorbitol are the most commonly used plasticizers in edible film production (Aydinli and Tutas 2000).

The amount of plasticizer added can cause adverse effects on film properties such as increasing mass transfer through the films. Hence, plasticizers must be used with caution. When the plasticizer concentration exceeds its compatibility limit in the polymer, it causes phase separation and physical exclusion of the plasticizer (Aulton and others 1981). This leads to development of a white residue on edible films which has been referred to as “blooming” (Aulton and others 1981) or “blushing” (Sakellariou and others 1986). The amount of plasticizer used in film formation should also be small enough to avoid probable toxic effects (Nisperos-Carriedo 1994).

2.5 PROPERTIES OF CHITOSAN AND CHITOSAN FILMS

2.5.1 Safety of Chitosan Films

Chitosan is non-toxic and safe to domestic animals (Hirano and others 1990). According to Rao and Sharma (1997), chitosan films were non toxic and free from pyrogens. Many medical and pharmaceutical applications of chitosan films require sterility of films. Chitosan films can be
sterilized by irradiation (Lim and others 1998) and autoclaving (Rao and Sharma 1995), although these processes lead to some degradation of the films.

2.5.2 Biodegradation of Chitosan and Chitosan Films

Many studies have shown that chitin and chitosan are biodegradable polymers. Davies and others (1969) reported that chitosan is most susceptible to hydrolysis by lysozyme at pH 5.2, and the optimum range of pH value is between pH 5.2 and 8.0 (Davies and others 1969; Shigemasa and others 1994).

Pangburn and others (1982) studied the effect of deacetylation on susceptibility of chitin and chitosan to lysozyme and found that pure chitin (0% deacetylation) was most susceptible to lysozyme, while pure chitosan (100% deacetylation) was not degraded by lysozyme. Sashiwa and others (1990) studied the relative rates of degradation of six chitosans varying in degree of deacetylation (45%, 66%, 70%, 84%, 91%, and 95%), and reported that 70% deacetylated chitosan degraded most quickly.

Shigemasa and others (1994) investigated the effects of preparation methods on chitosan degradation. They found that for the same molecular weight and degree of deacetylation, homogeneously prepared chitosans were more susceptible to hydrolysis by lysozyme than those heterogeneously prepared.

2.5.3 Mechanical Properties of Chitosan Films

For edible films to be employed as a food packaging material, they should satisfy the requirement of being durable, stress resistant, flexible, pliable, and elastic. Thus, they should possess desirable tensile properties which could bear stresses exerted during various handling processes. Only limited literature is available on mechanical properties of chitosan films. There are variations of physical property values of chitosan films reported in the literature due to
different chitosans and testing conditions used. Films produced with low molecular weight chitosan at 3% w/w in 1% acetic acid, with glycerol as a plasticizer at 0.25 and 0.50 mL/g of chitosan, were reported to have tensile strength (TS) of 15 to 35 MPa and percent elongation at break (%E) of 17 to 76 (Butler and others 1996). Caner and others (1998) reported that films produced by a similar method but with different solvents (acetic, formic, lactic and propionic acid) at 1% and 7.5% concentrations exhibited the TS value range of 12 to 32 Mpa and %E value range of 14 to 70 with an exception of the film made with 7.5% lactic acid having the lowest TS value of 6.85 MPa and the highest %E value of 51. They also reported that increasing the plasticizer content decreased TS and increased %E. Kittur and others (1998) reported a much higher TS value of 70.3 MPa and a lower %E value of 6.2 for a film made of 2% w/w chitosan in 1% acetic acid. Variations in mechanical strength of chitosan films are due to the type of chitosan and concentration used, the type of plasticizer and its content, and solvent. The TS values of chitosan films are comparable to those of commercial DDPE and LDPE films but, their %E values are significantly lower than the commercial films (Briston 1988). However, compared to films made of other biopolymers (wheat gluten, corn zein protein and soy protein isolate), chitosan films exhibited significantly higher TS values (Cunningham and others 2000).

2.5.4 Transport Properties of Chitosan Films

In general, edible films and coatings provide the potential to control transport of moisture, oxygen, aroma, oil, and flavor compounds in food systems, depending on the nature of the edible film-forming materials (Donhowe and Fennema 1993; Krochta 1997; Krochta and De Mulder-Johnston 1997). However, when films are formed using biopolymers alone, they are very brittle. To lessen brittleness and to make flexible films, plasticizers are used. However, plasticizers increase the film permeability (Gontard and others 1993), especially for plasticized
hydrophilic films. Increased permeability of edible films is undesirable for food applications, so there is a need to minimize the use of plasticizers. Another potential approach to increase film flexibility is to reduce polymer molecular weight, thus reducing intermolecular forces along polymer chains and increasing polymer chain end groups and polymer free volume (Sears and Darby, 1982). This approach may permit a decrease in the required amount of added plasticizer in films; consequently, it may minimize permeability of films while producing needed film flexibility (Sothornvit and Krochta 2000).

Chitosan films exhibit gas barrier properties. Oxygen permeability of chitosan is as low as many conventional plastic films such as poly vinylidene dichloride (PVdC) and ethyl vinyl alcohol (EVOH) (Webber 2000). Since chitosans obtained from various sources and methods vary in their characteristics, barrier properties of film made of various chitosans also vary. Muzzarelli and others (1974) reported a water vapor transmission rate of 1200 g/m²/d measured at 100 °F and 90% relative humidity for chitosan membranes with 20 µm thickness. Wong and others (1992) reported a water vapor permeability (WVP) value of 0.41 g mm/m²/d/mmHg for chitosan and chitosan-lipid films cast from 1% chitosan solution using formic acid. Butler and others (1996) reported that chitosan films made with plasticizer (glycerol) levels of 0.25 and 0.50 ml/g had a mean WVP of $2.89 \times 10^{-4}$ g/m/d/mmHg at 25 ºC between 0% to 11% RH.

Manufacturing biopolymer based films with adequate water barrier properties is a major challenge as many of the biopolymers are hydrophilic by nature (Webber 2000). Butler and others (1996) stated that their chitosan films were extremely good barriers to oxygen, while having higher water vapor barrier properties because of their hydrophilic nature. They also reported that increasing plasticizer concentrations negatively affected barrier properties but improved formation, mechanical, and handling properties. Caner and others (1998) prepared chitosan films
using various acid and plasticizer concentrations and reported water vapor permeability coefficients ranging from $1.74 \times 10^{-5}$ to $7.04 \times 10^{-4}$ g/m/d/mmHg at 25°C between 50% to 100% RH. They also suggested that storage time had no effect on barrier properties of chitosan films.

Attempts to improve vapor barrier properties of chitosan films yielded only limited success. Wong and others (1992) used lipid to form chitosan-lipid composite films to improve moisture barrier properties. Hoagland and Parris (1996) developed a chitosan/pectin-laminated film to alter water vapor permeability and water solubility. Tual and others (2000) produced chitosan films with improved barrier properties by crosslinking chitosan with glutaraldehyde. However, these films were reported to be brittle due to formation of chemical junctions.

2.5.5 Antimicrobial Properties of Chitosan and Chitosan Films

Microbial growth on the surface of food is a major cause of food spoilage and food-borne illness. Therefore, the concept of using edible active coating to inhibit spoilage and pathogenic microorganism has received considerable interest (Rico-Pena and Torres 1991; Weng and Hotchkiss 1992, 1993; Ouattara and others 2000; Coma and others 2001). Development of antimicrobial plastics with added antimicrobial agents is less preferred as their releasing rate is unsatisfactory and there is growing environmental concern. Direct application of antimicrobial agents onto food surfaces by spraying, dipping or coating has proven to be less effective as there is loss of activity because of leaching onto the food, enzymatic activity, and reaction with other food components (Jung and others 1992; Ray 1992; Ouattara and others 2000). Hence, use of packaging films or coating as a matrix to deliver antimicrobial agents may provide an alternative approach to prevent food spoilage and food-borne illness. Such packaging or coating can maintain a high concentration of antimicrobial agents on the food surface and allow low migration into food (Torres and others 1985; Siragusa and Dickson 1992; Ouattara and others
Chitosan films having the ability for controlled release of added substances would help in this context.

Chitosan possesses unique properties that make it an ideal ingredient for development of antimicrobial edible film. Chitosan possesses film-forming properties (Averbach 1978), greater and broader spectra of antibacterial activity compared to disinfectants, a higher bacterial/fungal killing rate, and lower toxicity toward mammalian cells (Franklin and Snow, 1981; Takemono and others 1989). Further, Rhoades and Roller (2000) reported that the interaction (binding or chelation) of chitosan with endotoxins of gram-negative bacteria decreased their acute toxicity. Because of the strong chelating ability of chitosan, external chelating agents such as EDTA may not be required, when antimicrobial agents such as nisin are added to chitosan to control gram-negative bacteria.

Antimicrobial properties of chitosan have been reported by many investigators. Chitosan's ability to inhibit a wide variety of bacteria (Sudarshan and others 1992; Yalpani and others 1992), fungi (Allan and Hadwiger 1979; Stossel and Leuba 1984; Kendra and others 1989; Fang and others 1994), yeasts (Ralston and others 1964), and viruses (Kochkina and others; 1995; Pospieszny 1997; Chirkov 2002) make it a broad spectrum antimicrobial agent. A variety of research has been conducted to assess inhibitory effects of chitosan in a solution state or its oligosaccharides in terms of minimum inhibitory concentration (MIC). Chitosan is more effective in inhibiting bacteria than chitosan oligomers (Jeon and others 2001). Antimicrobial activity of chitosan is influenced by its molecular weight, degree of deacetylation, concentration in solution, and pH of the medium (Lim and Hudson 2003). No and others (2002b), reported that chitosan with different organic acid solvents exhibited varying inhibitory effects on bacteria. In general, acetic acid, lactic acid, and formic acids were more effective in inhibiting bacterial
growth than propionic and ascorbic acids. Chitosan shows stronger antimicrobial activity for gram-positive than gram-negative bacteria (Jeon and others 2001). Chitosan has been observed to act more quickly on fungi and algae than on bacteria (Cuero, 1999); however, like other properties of chitosan, this activity may be dependent on the type of chitosan, chitosan molecular weight, and degree of deacetylation, among other factors influencing the environment in which the chitosan is stored.

Antimicrobial property of chitosan can be enhanced by irradiation (Matsuhashi and Kume 1997), ultra violet radiation treatment, partial hydrolyzation (Davydova and others 2000), using different organic solvents (No and others 2002a, 2002b), chemical modifications (Nishimura and others 1984; Tanigawa and others 1992), synergistic enhancement with preservatives (Chen and others 1996; Roller and others 2002), synergistic enhancement with antimicrobial agents (Lee and others 2003; Song and others 2002), or in combination with other hurdle technologies.

Several mechanisms were proposed for the antimicrobial activity of chitosan. One mechanism is that the polycationic nature of chitosan interferes with the negatively charged residues of macromolecules at the cell surface, presumably by competing with Ca$^{2+}$ for electronegative sites on the membrane without conferring dimensional stability, rendering membrane leakage (Young and Kauss 1983). The other mechanism is that oligomeric chitosan penetrates into the cells of the microorganism and prevents the growth of cells by prohibiting the transformation of DNA into RNA (Hadwinger and others 1986). Tokura and others (1997) suggested that antimicrobial activity is related to the suppression of the metabolic activity of the bacteria by blocking nutrient penetration through the cell wall rather than the inhibition of the transcription from DNA.
Although several papers on the antimicrobial properties of chitosan have been published, relatively little work has been reported on the antimicrobial properties of chitosan films. Coma and others (2002) investigated antibacterial properties of chitosan acetate films on *Listeria monocytogenes* and *Listeria innocua* using Emmental cheese as a model system. The authors reported 100% inhibition of *Listeria monocytogenes* for 8 days but decreased antibacterial activity with time which was attributed to decreased availability of amino groups. Chen and others (1996) incorporated food preservatives, such as potassium sorbate and sodium benzoate, into a chitosan film matrix and compared their inhibitory effects on microbial growth. They reported that chitosan films made in dilute acetic acid solutions were able to inhibit the growth of *Rhodotorula rubra* and *Penicillium notatum* by direct application of the film on the colony-forming organism. Lee and others (2003) reported enhanced microbial stability of milk and orange juice that were exposed to paperboard coated with chitosan and nisin. Coma and others (2003) assessed antimicrobial activity of chitosan coating on the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes*. They reported that chitosan coating could be used to increase the microbial lag phase while decreasing the maximum density of selected microorganisms, and could have potential applications for dairy product preservation. Rodriguez and others (2003) reported that the use of chitosan in acetic acid as edible coating for precooked pizza (0.079 g/100 g pizza) delayed the growth of *Alternaria sp*, *Penicillium sp*, and *Cladosporium sp* (Deuteromycetes).

### 2.6 APPLICATIONS OF CHITOSAN FILMS

Chitosan can be used in vastly diverse fields ranging from flocculant for seed coating, toiletry components, controlled drug delivery systems (Graham 1990), membrane based transdermal drug delivery systems (Thacharodi and Rao 1993, 1995), eye contact lens (Felt and
others 1999), wound-healing, dressing material and artificial skin (Biagini and others 1992; Ueno and others 2001), various medical supplies including surgical dressing, sanitary cottons, gauzes, bandages, plasters, and sanitary pads, separation membranes, matrix for immobilization of biomolecules such as peptides (Bernkop-Schnurch and Kast 2001) and genes (Borchard 2001), bioseparation, support for bio sensors (Ng and others 2001), and bioadhesive to increase retention at the site of application (He and others 1998; Calvo and others 1997).

2.6.1 Applications of Chitosan and Chitosan Films in Foods

Chitosan possesses many desirable properties for use in food systems. The film-forming ability of chitosan and gas-barrier properties of chitosan film favor its use as an edible food packaging material. Their inherent antimicrobial properties along with non-toxicity and biocompatibility offer their use as antimicrobial additives. Further, as an additive chitosan can offer a variety of functionalities.

To date, only a few attempts have been made to assess the effects of chitosan films and coatings in real foods. Most of the work has been centered on the antimicrobial properties of chitosan. Agulló and others (1998) evaluated the capacity of chitosan films to extend the shelf-life of precooked pizzas. The study showed that increased shelf-life was mainly due to antifungal properties of chitosan instead of its action as a water vapor barrier. Rodriguez and others (2003) demonstrated that chitosan in acetic acid as an edible coating (0.079 g/100 g pizza) delayed the growth of *Alternaria* sp, *Penicillium* sp, and *Cladosporium* sp (Deuteromycetes) in precooked pizza. They also demonstrated that the use of chitosan in the dough is not effective because the biopolymer loses its antimicrobial capacity due to the Maillard reaction. Skonberg and Gillman (2000) reported extension of shelf life of fresh salmon and haddock fillets by chitosan coating. Nadarajah and others (2003) reported the use of chitosan coating on catfish fillets for retaining
color, inhibiting lipid oxidation, and retarding microbial spoilage. They demonstrated that the shelf life of refrigerated catfish fillets could be prolonged up to 8 days with high molecular weight (1,100 kDa) chitosan at 1% concentration. Srinivasa and others (2002) investigated the effect of modified atmosphere packaging by chitosan film on the quality of mango fruits and reported that mangos stored in chitosan-covered boxes showed an extension of shelf-life of up to 18 days without any microbial growth and off flavor.

Only limited literature is available on direct application of chitosan as an antimicrobial additive in foods. Darmadji and Izumimoto (1994) investigated the effect of chitosan on development of spoilage in minced beef patties stored at 30°C for 2 days and at 4°C for 10 days. At higher storage temperature, a reduction of one to two log cycles of total bacteria, pseudomonads, Staphylococci, coliforms, Gram-negative bacteria and Micrococci was observed in the presence of 1% chitosan; at lower storage temperature, similar reductions in spoilage flora were reported after 10 days. Fang and others (1994) investigated the use of chitosan as an antimicrobial agent against mold spoilage in candied kumquat. The authors reported that a concentration of 6 g/L of chitosan was required to maintain a mold-free shelf life of 65 days when the sugar concentration in the syrup was reduced from the traditional 65° Brix to 61.9° Brix at pH 4. No and others (2002a) reported inhibition of growth of bacteria isolated from spoiled tofu by chitosan and the possibility of using chitosan to extend the shelf life of tofu. They also showed that antibacterial activity of chitosan was higher than chitosan oligomers. Oh and others (2001) used mayonnaise as a complex food model and demonstrated that addition of chitosan can inhibit the growth of spoilage microorganisms *Lactobacillus fructivorans* and *Zygosaccharomyces bailii* when stored at 25°C. Roller and Covill (1999) found that chitosan at a rate of 1-5 g/L on apple juice reduced the growth rate of *Mucor racemosus* and *Byssochlamys*
spp. Coma and others (2002) reported use of chitosan film to inhibit growth of *Listeria monocytogenes* on Emmental cheese. Antimicrobial activity of chitosan on bacterial strains isolated from fish meat paste (Cho and others 1998) was also reported. Chitosan is frequently used as a preservative in solid foods in Japan in products such as kamaboko, noodles, soy sauce. However, as reported by Roller and Covill (1999), these reports are lacking in details so that the conditions/formulations would be difficult to replicate and verify (Li and others 1997a; Hirano 1997).

Chitosan offers various functionalities to foods. It has been considered as dietary fiber, which is recognized to reduce apparent fat digestibility (Deuchi and others 1994; Kanauchi and others 1995) and, therefore, it would be a promising approach for dietary supplementation when applied to foods. Kim and others (2000) inferred that absorption of fat in the human body from high fat foods such as whipped cream can be reduced by addition of chitosan without compromising sensory qualities. Austin (1982) reported that chitosan can be used to reduce lactose intolerance. Kataoka and others (1998) found that addition of 1.5% chitosan to walleye pollock surimi in combination with setting at 20°C resulted in a twofold increase in gel strength. Similarly, Benjakul and others (2000) reported that incorporation of chitosan, particularly in the presence of CaCl₂ greatly improved the gelling properties of surimi from barred garfish without changes in color.

Chitosan is an ideal preservative coating for fresh fruit and vegetables because of its film-forming and biochemical properties. Chitosan has a particular adhesiveness towards biological surfaces because of its positive charge and the negative charge of biological membranes (Henriksen and others 1996) and, therefore, is capable of forming stable films (Romanazzi and others 2002).
Chitosan has been shown to regulate gas exchange, decrease transpiration losses and maintain the quality of harvested fruits. Chitosan coating prolongs storage life and controls decay of strawberries (El Ghaouth and others 1991; Zhang and Quantick 1998), litchi (Zhang and Quantick 1997), apples (Du and others 1998), peach (Li and Yu 2000), mango (Srinivasa and others 2002), and table grapes (Romanazzi and others 2002).

It was reported that chitosan, as a semi-permeable coating, could delay the ripening of strawberries (El Ghaouth and others 1991), tomatoes (El Ghaouth and others 1992a), cucumbers and bell peppers (El Ghaouth and others 1992b), apples (Hu and Zou 1998; Zheng and others 1996), and pears (Zheng and others 1996; Li and Yu 2000) by slowing down the production of anthocyanin and ethylene. Chitosan helps retain the fruits’ firmness, fresh weight, titratable acidity, soluble carbohydrates and vitamin C. A polymeric coatings made of chitosan (Nutri-Save composed of N, O-carboxymethyl chitosan) has also shown promise in delaying ripening of pears (Meheriuk and Lau 1998).

Chitosan reduces the growth of many phytopathogenic bacteria and fungi (Allan and Hadwiger 1979). Moreover, it elicits phytoalexin formation (Reddy and others 1999), and induces the production of antifungal hydrolases (Zhang and Quantick 1998; Hirano 1999) and an increase of phenylalanine ammonia-lyase (PAL) activity (Romanazzi and others 2002). Chitosan has generally been applied in postharvest treatments (Baldwin and others 1997), and there are very few examples of preharvest application (Reddy and others 2000; Romanazzi and others 2000, 2002). Romanazzi and others (2002) suggested that pre-harvest spray of chitosan is best for commodities such as grapes since exposure to postharvest liquid-based treatments is not advisable for commodities as it could cause damage. However, since the effect of preharvest fungicide spraying is often inefficient because of the heavy foliage which obstructs full coverage
(Sholberg and Gaunce 1996) and the development of fungicide resistant isolates of postharvest pathogens (Hong and Michailides 1996), postharvest spraying of chitosan may be preferable and more advantageous.

2.7 REFERENCES


CHAPTER 3

PHYSICOCHEMICAL PROPERTIES OF EDIBLE CHITOSAN FILMS
DEVELOPED FROM CRAWFISH SHELL WASTE
3.1 INTRODUCTION

Naturally renewable biopolymers have attracted much research interest in recent years because of their potential use as edible and biodegradable films and coatings for food packaging. Chitosan, which can be derived from abundantly available chitin sources such as crustacean shell wastes, has excellent film-forming ability and inherent antimicrobial properties suitable for development of edible antimicrobial films (Ralston and others 1964; Allan and Hadwiger, 1979; Stossel and Leuba, 1984; Kendra and others 1989; Sudarshan and others 1992; Yalpani and others 1992; Fang and others 1994; No and others 2002). Moreover, the possibility of enhancing antimicrobial properties of chitosans by irradiation (Matsuhashi and Kume 1997), partial hydrolyzation (Davydova and others 2000), using different organic solvents (No and others 2002), chemical modifications (Nishimura and others 1984; Tanigawa and others 1992), synergistic enhancement with preservatives (Chen and others 1996; Roller and others 2002), or by combing with other hurdle technologies, make them ideal for use as antimicrobial edible packaging materials.

Very limited literature is available on physicochemical properties of chitosan films derived from crawfish shell waste. Chitosan derived from crawfish shell wastes was used to form various flexible and transparent films resembling plastic packaging materials (Nadarajah and Prinyawiwatkul 2002). Properties of chitosan films are reported to be affected by the chitosan extraction process (Rout 2001; Nadarajah and Prinyawiwatkul 2002). Furthermore, various organic acids used to dissolve chitosan affect resultant film properties (Kienzle-Sterzer and others 1982; Caner and others 1998; Rhim and others 1998; Park and others 1999; Park and others 2002). Therefore, a variety of crawfish chitosan films can be developed using various chitosan extraction methods and organic acids. The knowledge of physicochemical properties of
such films would allow more efficient selection of crawfish chitosan films for their specific and suitable applications.

Therefore, the primary objectives of this study were to develop a variety of crawfish chitosan based films and to identify their physicochemical and functional properties. Proper understanding of the physical and chemical fundamentals underlying the functional properties of crawfish chitosan films would help us develop process protocols suitable to prepare antimicrobial edible films. Variations in physicochemical properties and functionalities were intended to be achieved by using (1) different crawfish chitosans based on their extraction method, (2) different film casting (organic acid) solvents, and (3) different amounts of added plasticizer (glycerol).

3.2 MATERIALS AND METHODS

3.2.1 Preparation of Chitosan from Crawfish Shell Waste

All chemicals used in this study were analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO). Crawfish shell waste collected from a local seafood processing facility was washed with warm tap water to remove foreign materials and remaining muscle particles. The shells were then dried at 60°C overnight, ground with a centrifugal grinding mill (Retsch/Brinkmann ZM-1, Westbury, NY), and shell particles between a mesh size of 20 (0.841 mm) and 40 (0.420 mm) were used as starting material. The methods established to extract chitin from crawfish shell waste by No and Meyers (1995) and further processing of chitin into chitosan through autoclaving (No and others 2000) were used to prepare different chitosan samples for this study.

Dried crawfish shell particles were treated with 1 N NaOH at 65°C for 1 hour at a solid:solvent ratio of 1:10, w/v for deproteinization (DP). Following a washing step, deproteinized
shell particles were treated with 1 N HCl at room temperature for 30 minutes at a solid: solvent ratio of 1:1.5, w/v for demineralization (DM). Particles were then treated with acetone at 1: 10 w/v concentration, washed, and bleached with 0.315% NaOCl at a 1:10 w/v ratio for 5 minutes for decoloration (DC). Resultant chitin was treated with 50% NaOH at a 1:10 w/v ratio at 121°C/15 psi for 30 minutes for deacetylation (DA). Subsequent washing and drying steps yielded chitosan. Four different chitosan samples were prepared using the traditional method involving all above steps (DPMCA), the traditional method excluding deproteinization (DMCA), the traditional method excluding decoloration (DPMA), and the traditional method excluding both deproteinization and decoloration (DMA).

3.2.2 Characterization of Crawfish Chitosan

The viscosity average molecular weight of crawfish chitosans was determined by the intrinsic viscosity using the Mark-Houwink equation ([η] = KMα) where [η] is the intrinsic viscosity, M is the molecular weight, K=1.81 x 10⁻³ and α=0.93 at 25°C (Maghami and Roberts 1988). Crawfish chitosan films deprotonated by methanolic ammonia was used to determine the degree of deacetylation with IR spectroscopy (M2000 FTIR spectrophotometer, Midac Corp., Irvine, CA, USA) and the equation proposed by Sabnis and Block (1997) was used to calculate degree of deacetylation values. Viscosity was determined for 1% chitosan in 1% acetic acid (w/v) using the Brookfield viscometer at 50 rpm and 25°C with a spindle number RV5.

The moisture content (%) of the chitosans was determined gravimetrically in triplicate by drying samples at 100°C for 48 hours. Nitrogen (%) was determined by the combustion method using the Leco CHN analyzer (Model # FP-428, Leco Corporation, St. Joseph, Michigan, USA). The ash content (%) was determined according to the standard method # 923.03 (AOAC 1990).
3.2.3 Preparation of Crawfish Chitosan Films

Four types of crawfish chitosans (DPMCA, DPMA, DMCA, and DMA) were separately dissolved with 1% of film casting solvents (acetic, ascorbic, lactic, formic, citric or malic acids). Each solution was vigorously agitated for 30 minutes, immersed in boiling water for 10 minutes, cooled down to room temperature, and filtered through glass-wool to remove undissolved particles. Resultant film casting solution was divided into two batches. One batch contained glycerol as a plasticizer at a ratio of 0.10, 0.20, 0.30, 0.40, and 0.50 (chitosan: glycerol, w/w), and the other batch was without a plasticizer. The film casting solution (300 mL) was cast onto a 31 × 31 cm Teflon coated plate and allowed to dry at room temperature (23°C) for 48 hours. Dried films were carefully peeled out and stored in desiccators containing saturated NaBr until further tested.

3.2.4 Thickness and Density of Crawfish Chitosan Films

A micrometer (Model 293-766; Mitutoyo, Tokyo, Japan) was used to measure the film thickness to the nearest 0.001 mm. Thickness of each film (mm) was measured at room temperature (23°C and 45% RH) and expressed as an average of 10 random measurements and standard deviation. Weight of a 25 × 25 mm sample was taken to determine a density of each film (g/cm³), expressed as an average of 3 measurements and standard deviation.

3.2.5 Color of Crawfish Chitosan Films

Color of the film samples was measured using a portable Minolta spectrophotometer (Model CM-508d, Minolta Camera Co. Ltd., Osaka Japan) with 2° standard observer and D65 illuminant, and expressed as CIE color characteristics; L*, a* and b* values, where L* describes lightness (ranging from black to white), a* and b* describe the chromatic coordinates (ranging from –a: greenness, –b: blueness, +a: redness, +b: yellowness). Film specimens were placed on
the surface of a white standard plate (L*=92.91, a*=0.92, b*=0.03). A mean value of 10 measurements was reported for each color attribute. The total color difference (ΔE) was calculated by the following equation.

$$\Delta E = \sqrt{\left(\Delta L^*\right)^2 + \left(\Delta a^*\right)^2 + \left(\Delta b^*\right)^2}$$

The results were expressed as ΔE values with the acetate film made with DPMCA chitosan serving as a reference.

3.2.6 Transparency of Crawfish Chitosan Films

Transparency of crawfish chitosan films was measured according to the procedure of Han and Floros (1997) using a spectrophotometer (Spectronic Genesis 2, Thermo Spectronic, Rochester, USA). This method was modified from the ASTM method D1746-92, which is the standard test method for transparency of plastic sheeting (ASTM 1987). The transparency of the plastic film was determined from the following equation, based on film thickness and % transmittance (or absorbance) of the light at 600 nm (T_{600} or A_{600}).

$$Transparency = \frac{A_{600}}{b} \text{ or } \log \frac{T_{600}}{b}$$

where T_{600} is transmittance at 600 nm, A_{600} is absorbance, and b is the length of the light path through the medium (i.e., film thickness).

3.2.7 Swelling of Crawfish Chitosan Films

Water sorption capacities of chitosan films were determined by soaking them in phosphate buffered saline (PBS at pH 7.4) at room temperature. A known weight of chitosan film was placed in the PBS media for 30 minutes. The wet weight of the film was determined by first blotting the surface of the chitosan film with filter paper to remove excess water, and the
film weighed immediately. The percentage of water adsorption in the medium ($W_{sw}$) was calculated from the equation:

$$W_{sw} = \frac{W_{30} - W_0}{W_0} \times 100$$

where $W_{30}$ represents the weight of the chitosan film after 30 minutes of sorption and $W_0$ is the initial weight of the chitosan film. The $W_{sw}$ values were expressed as an average of 3 measurements and standard deviation.

### 3.2.8 Solubility of Crawfish Chitosan Films

The film solubility (S%) expresses the percentage of film's dry matter solubilized after immersion in water for 24 hours at 25°C, according to Gontard and others (1992). The experiment was performed under slow agitation. Film solubility (S%) was calculated from the following equation:

$$S\% = \left( \frac{W_{t_{initial}} - W_{t_{final}}}{W_{t_{initial}}} \right)$$

where $W_{t_{initial}}$ is the initial weight of chitosan film and $W_{t_{final}}$ is the weight of chitosan film after immersion.

### 3.2.9 Microstructure of Crawfish Chitosan Films

Scanning electron microscopy (SEM) was used to examine representative film surfaces. All films were equilibrated at 53% relative humidity prior to preparation for scanning. Film samples were sputter coated with silver and scanned using a FEI Quanta 200 ESEM (FEI Company, Hillsboro, Oregon, USA) with an accelerating beam voltage of 3.9 kV.

### 3.2.10 Statistical Analysis

All data from the physicochemical properties were analyzed using SAS (SAS 2002). The PROC GLM procedure with orthogonal contrasts was used to test the effect of film casting
solvents and chitosan extraction methods on physicochemical properties. Means were compared by the Tukey's studentized range test. Significance of differences was defined at $p < 0.05$.

### 3.3 RESULTS AND DISCUSSIONS

#### 3.3.1 Characteristics of Crawfish Chitosan

Chitosan extracted from crawfish shell was white or light pink in color depending on the extraction methods. Chitosans extracted without the decoloration process (DPMA and DMA chitosans) exhibited more pinkish color. This indicates that the harsh deacetylation process applied to convert chitin into chitosan was not sufficient to remove all pigments from crawfish shells.

Table 3.1 describes the general characteristics of chitosans extracted from crawfish shell waste. Molecular weight is one of the important factors governing the functional properties of chitosan. The viscometric method is the simplest and the most effective method for determining molecular weight of polymers (Muzzarelli 1985). Crawfish chitosans had molecular weight in the range of 3584-10168 Da. The traditional extraction method of chitosan (DPMCA) resulted in

<table>
<thead>
<tr>
<th>Chitosan Type$^a$</th>
<th>Molecular Weight (Da)</th>
<th>Viscosity (cP)</th>
<th>Deacetylation (%)</th>
<th>Moisture (%)</th>
<th>Ash$^b$ (%)</th>
<th>Nitrogen$^b$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPMCA</td>
<td>3584</td>
<td>8</td>
<td>83.06</td>
<td>2.19</td>
<td>0.35</td>
<td>7.35</td>
</tr>
<tr>
<td>DPMA</td>
<td>10168</td>
<td>48</td>
<td>82.43</td>
<td>2.74</td>
<td>0.25</td>
<td>7.20</td>
</tr>
<tr>
<td>DMCA</td>
<td>7269</td>
<td>16</td>
<td>81.87</td>
<td>2.16</td>
<td>0.31</td>
<td>7.22</td>
</tr>
<tr>
<td>DMA</td>
<td>7839</td>
<td>40</td>
<td>81.78</td>
<td>4.77</td>
<td>0.33</td>
<td>7.36</td>
</tr>
</tbody>
</table>

$^a$ DPMCA = Deproteinized + demineralized + decolorized + deacetylated, DPMA = Deproteinized + demineralized + deacetylated, DMCA = Demineralized + decolorized + deacetylated, DMA = Demineralized + deacetylated.

$^b$ Dry weight basis.
the lowest molecular weight of 3584 Da. The lowest molecular weight of the DPMCA crawfish chitosan can be attributed to the harsh autoclaving deacetylation procedure, which caused depolymerization. The viscosity measures correlated well with the molecular weight of crawfish chitosan. All crawfish chitosans showed similar physiochemical properties in terms of degree of deacetylation, ash, and nitrogen content.

3.3.2 Film-forming Ability of Crawfish Chitosans

Chitosan is known for its film-forming ability. Chitosan filmed with various organic acids vary in their physiochemical properties. Flexible and transparent films prepared from crawfish chitosan, resembling plastic films, were reported earlier (Nadarajah and Prinyawiwatkul 2002). The solvent casting method of film formation, in which chitosan is first dissolved in acidic solvent, cast on plates, and air dried to form films, is often used owing to its simplicity. In this study, all chitosans extracted from crawfish shell waste exhibited good film-forming ability. There was no apparent visual difference among films prepared from different crawfish chitosans, provided that films were cast with the same acid solvent. However, the film-forming ability of crawfish chitosan was greatly influenced by the casting solvents. The crawfish chitosan films formed with lactic or malic acids were highly sticky and hygroscopic. These films also exhibited a high degree of shrinking and deformed instantly upon peeling. Although the films made with lactic or malic acid were less sticky when a plasticizer was avoided, they still exhibited shrinkage and deformation upon peeling. All crawfish chitosans formed films with ascorbic acid; however, resultant films were very brittle. They became far too fragile to handle when no plasticizer was added. Furthermore, all the ascorbate films developed deep brown coloration, presumably due to browning reactions, and thus became unappealing for packaging purposes.
The film-forming ability of crawfish chitosans was desirable when acetic, formic, or citric acid was used, resulting in highly flexible and transparent films. However, all plasticized films with acetic, formic and citric acids exhibited high stickiness and hygroscopic nature, even in the presence of the lowest plasticizer content. The high degree of hydrophilicity of chitosan is attributed to the deacetylated amino groups present in the polymeric chain, favoring considerable migration of water molecules to these sites. Nadarajah and Prinyawiwatkul (2002) reported that plasticized crawfish chitosan films with acetic acid exhibited more hygroscopic nature compared to those films prepared with commercial chitosans. Addition of plasticizers minimizes or eliminates brittleness of the films. However, they also adversely affect film properties by making them more hygroscopic and contributing to greater absorption of moisture (Lawton 1992). Lawton (2004) demonstrated that addition of hydrophobic plasticizers can yield edible films that are minimally hydrophilic. This indicates the possibility of producing a variety of plasticized crawfish chitosan films if compatible hydrophobic plasticizers are identified.

Films made without plasticizers using acetic, formic and citric acids exhibited neither stickiness nor hygroscopic nature, and they resembled plastic films. However, the unplasticized films formed with citric acids were slightly brittle in nature. This suggests that flexible and transparent films that resemble plastic films can be produced from crawfish chitosans using acetic or formic acid without any plasticizers. The film-forming ability of unplasticized crawfish chitosans with different organic acids and their film properties are described in Table 3.2.

### 3.3.3 Thickness and Density of Crawfish Chitosan Films

We observed that the thickness of chitosan films was highly influenced by the type of film casting solvent used. Crawfish chitosan formed thicker films with citric acid compared to all other solvents used in the present study. This is in agreement with Begin and Calsteren (1999).
who reported that pronounced increase in thickness of film cast with lactic or citric acid. According to Begin and Calsteren (1999), the citric acid chitosan-film solution gelled before the molecules could align and pack, which resulted in a thicker film. They also stated that the ability of citric acid to form multiple salt bridges with amino groups could act as a reticulating agent to promote gel formation.

The thickness of films varied from 0.02 mm for acetate and formate films to 0.06 mm for citrate films (Table 3.3). There was no difference in thickness between acetic and formic acid films. Citrate films showed significantly (p<0.05) greater thickness than acetate and formate films. The density of films also varied significantly (p<0.05) depending on the acid used. The formate films had relatively higher density than citrate and acetate films. However, no correlation between thickness and density of chitosan films was evident.

Table 3.2: Film-forming ability of unplasticized crawfish chitosan with different organic acids†.

<table>
<thead>
<tr>
<th>Film casting solvent††</th>
<th>Film properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>A yellow tinted, flexible, transparent, non-sticky film with smooth shiny surface and slight acidic odor</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>A brown colored, highly brittle film</td>
</tr>
<tr>
<td>Citric acid</td>
<td>A yellowish, flexible, transparent, non-sticky film with slight brittle and grainier surface without any acidic odor</td>
</tr>
<tr>
<td>Formic acid</td>
<td>A yellow tinted, flexible, transparent, non-sticky film with smooth shiny surface without any acidic odor</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>Highly sticky films which shrink upon peeling, becoming a sticky mass/clump</td>
</tr>
<tr>
<td>Malic acid</td>
<td>Highly sticky films which shrink upon peeling, becoming a sticky mass/clump</td>
</tr>
</tbody>
</table>

† 1% chitosan in film casting solvent by w/v. †† 1% acid by w/v.
3.3.4 Color of Crawfish Chitosan Films

Color of films is an important attribute which influences its appearance, marketability, and their suitability for various applications. Clear edible films are typically desirable. The color attributes of un-plasticized crawfish chitosan films are described in Table 3.4. All crawfish chitosans formed transparent films with slightly yellowish tint. The yellowish tint was more
prominent in the citric acid films as shown by greater $b^*$ values (3.69 - 4.87). Chitosan films with similar yellow coloration were reported by Caner and others (1998) and Wiles and others (2000). The $a^*$ values obtained for all films were very low and close to 0 indicating that the films were more neutral in terms of red or green chromaticity. No difference in color lightness was visually evident among the films, although statistical analysis revealed some significant ($p<0.05$)

### Table 3.4: Color attributes of crawfish chitosan films†

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Chitosan Type††</th>
<th>$L^*$</th>
<th>$a^*$</th>
<th>$b^*$</th>
<th>$\Delta E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>DPMCA</td>
<td>88.05 ± 0.44$^{abcd}$</td>
<td>-0.54 ± 0.24$^g$</td>
<td>3.70 ± 0.90$^{bc}$</td>
<td>(Reference)</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>87.54 ± 0.38$^e$</td>
<td>0.75 ± 0.12$^{ab}$</td>
<td>4.37 ± 1.22$^{ab}$</td>
<td>2.01 ± 0.55$^a$</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>87.69 ± 0.30$^{de}$</td>
<td>-0.01 ± 0.15$^{de}$</td>
<td>3.28 ± 0.86$^{bc}$</td>
<td>1.04 ± 0.47$^b$</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>88.23 ± 0.13$^{ab}$</td>
<td>-0.04 ± 0.05$^{de}$</td>
<td>3.16 ± 0.16$^c$</td>
<td>1.02 ± 0.69$^b$</td>
</tr>
<tr>
<td>Formic acid</td>
<td>DPMCA</td>
<td>88.16 ± 0.33$^{ab}$</td>
<td>-0.35 ± 0.22$^{fg}$</td>
<td>3.31 ± 0.85$^{bc}$</td>
<td>1.20 ± 0.78$^{ab}$</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>88.33 ± 0.21$^a$</td>
<td>0.58 ± 0.07$^{bc}$</td>
<td>3.26 ± 0.67$^{bc}$</td>
<td>1.53 ± 0.67$^{ab}$</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>88.18 ± 0.40$^{ab}$</td>
<td>-0.21 ± 0.18$^{cf}$</td>
<td>3.15 ± 0.85$^c$</td>
<td>1.55 ± 1.06$^{ab}$</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>88.10 ± 0.35$^{abc}$</td>
<td>-0.01 ± 0.12$^{de}$</td>
<td>3.27 ± 0.80$^{bc}$</td>
<td>1.20 ± 0.83$^{ab}$</td>
</tr>
<tr>
<td>Citric acid</td>
<td>DPMCA</td>
<td>87.85 ± 0.21$^{bcde}$</td>
<td>0.08 ± 0.18$^d$</td>
<td>4.87 ± 0.93$^a$</td>
<td>1.79 ± 0.61$^{ab}$</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>87.91 ± 0.18$^{abcde}$</td>
<td>0.82 ± 0.02$^a$</td>
<td>3.69 ± 0.39$^{bc}$</td>
<td>1.58 ± 0.36$^{ab}$</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>87.69 ± 0.17$^{cde}$</td>
<td>0.15 ± 0.11$^d$</td>
<td>4.26 ± 0.52$^{abc}$</td>
<td>1.27 ± 0.42$^{ab}$</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>87.61 ± 0.38$^{de}$</td>
<td>0.48 ± 0.04$^c$</td>
<td>3.84 ± 0.22$^{abc}$</td>
<td>1.46 ± 0.45$^{ab}$</td>
</tr>
</tbody>
</table>

† Mean of 10 measurements and standard deviation.
†† DPMCA = Deproteinized + demineralized + decolorized + deacetylated, DPMA = Deproteinized + demineralized + deacetylated, DMCA = Demineralized + decolorized + deacetylated, DMA = Demineralized + deacetylated.
Means within the same column sharing same letters are not significantly different at $p \geq 0.05$. 

54
difference in L* values. Compared to acetic and citric acid films, the formic acid films exhibited slightly lighter color (higher L* values), especially the DPMA formate films. The total color difference (ΔE) gives better differentiation of color for comparison purposes (Francis 1983). The ΔE obtained for the chitosan films falling in a narrow range of 1.02 to 2.01 indicates that almost all the films look alike, in terms of color. It is generally known when ΔE values are less than 3.0, color differences cannot be easily detected by naked eyes (Francis 1983). Therefore, it can be inferred that crawfish chitosan type and organic solvents have minimal effect on the color attributes of films.

3.3.5 Transparency of Crawfish Chitosan Films

Transparency is one of the common optical properties of light permeable materials. Spectrophotometry is used to measure the transparency of a material by light-transmittance or absorbance using the Beer-Lambert’s law relating the amount of light absorbed or transmitted by a material to the nature of the light absorbing material (Chang 1981; Han and Floros 1997). Development of transparent packaging materials which allow product visibility is a general trend and requirement in packaging films.

The type of chitosan and acid used to form the film significantly (p<0.05) affected transparency of crawfish chitosan films (Figure 3.1). In general, the formate films were more transparent with a mean transparency value (absorbance/mm) of 197.7, followed by acetate films with 167.6 and citrate films with 84.1. The lowest transparency values obtained for the citric acid films can be attributed to the higher tint of yellowness (b* values) observed. However, compared to conventional low density polyethylene films with the transparency values of 20 - 30 (Han and Floros 1997), all crawfish films were exceptionally more transparent, thus may be used as see-through packaging or coating materials.
3.3.6 Swelling of Crawfish Chitosan Films

Chitosan, being a hydrophilic polymer, shows high affinity towards water. Hence, upon hydration, chitosan films absorb water and swell. The swelling behaviors of unplasticized crawfish chitosan film formed with acetic or formic acid are presented in Figure 3.2. The citrate films formed with all crawfish chitosans exhibited complete solubility in water and hence demonstrated no swelling effect. Figure 3.3 shows the formic acid film made with DPMA chitosan before and after swelling.

Crawfish chitosan films formed with acetic and formic acids showed higher degree of swelling ranging from 2797.68 to 18785.85 %. Chitosan films exhibiting such extreme swelling...
Figure 3.2: The % swelling of crawfish chitosan films formed with acetic and formic acids. Black bars indicate standard deviation. The citric acid film which dissolved upon hydration is indicated as 0 swelling.

Figure 3.3: The unplasticized DPMA chitosan film formed with formic acid; (a) before swelling and (b) after swelling.
was reported earlier (Bonina and others 2004; Bergera and others 2004). According to Bergera and others (2004), chitosan films exhibiting higher swelling are beneficial in biomedical applications since higher swelling allows absorption of water and/or bioactive compounds without dissolution and permits drug release by diffusion.

The formate film formed from DPMA and DMA chitosans had significantly (p<0.05) greater swelling than the acetic film. Despite their extreme swelling behavior, all acetate and formate films retained their integrity and showed no trend of dissolving in water, unlike the citrate films. Therefore, the crawfish chitosan films which showed lowest swelling (acetic and formic films with DPMCA or DMCA chitosan and acetic acid film with DPMA chitosan), are recommended for packaging purposes. The citrate films which dissolve in water may be a good candidate to develop edible films and packaging materials which require complete solubility upon hydration.

The crawfish chitosan films showing extremely high swelling (formic acid films with DPMA or DMA chitosan) may be desirable for development of absorbent pads such as those used in meat packages to absorb exudates from meat. In such case, the chitosan films can be directly used as absorbent pads without any plastic lining materials that are normally used to prevent desorption. The inherent antimicrobial property of chitosan films can also be beneficially utilized to improve shelf life of package contents. This would also help to replace currently used plastic wrapped absorbent pads.

3.3.6 Solubility of Crawfish Chitosan Films

Solubility of crawfish chitosan films is presented in Table 3.5. All citrate films were completely soluble and, hence, accounted for highest solubility. Formate films were more soluble than acetate films. Data showed that solubility of crawfish chitosan was influenced by
the type of acid used and not by the type of chitosan. An acetate film having lower solubility is favorable for packaging purpose.

### 3.3.7 Microstructure of Crawfish Chitosan Films

The scanning electron micrographs of crawfish chitosan films are given in Figure 3.4. The SEM revealed that the structure of crawfish chitosan films vary with the organic solvents used. Films formed with formic and citric acid were more homogeneous and continuous

<table>
<thead>
<tr>
<th>Table 3.5: Solubility of unplasticized crawfish chitosan films</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solvent</strong></td>
</tr>
<tr>
<td>Acetic acid</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Formic acid</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

* DPMCA = Deproteinized + demineralized + decolorized + deacetylated, DPMA = Deproteinized + demineralized + deacetylated, DMCA = Demineralized + decolorized + deacetylated, DMA = Demineralized + deacetylated.

Means within the same column sharing same letters are not significantly different at p≥ 0.05.

† Means of 3 measurements and standard deviation.
Figure 3.4: The SEM of crawfish chitosan films: (a) acetic acid film, (b) formic acid film, and (c) citric acid film
compared to those formed with acetic acid. The lamellar structure found in the acetate and formate films was not evident with citric acid films. Further research is required to deduce structure of citric acid films.

3.4 CONCLUSIONS

This study indicates that chitosans derived from crawfish shell waste by different methods can be used to formulate edible films with selected film casting solvents. All crawfish chitosans exhibited good film-forming ability. The film-forming ability was significantly affected more by the type of organic solvents and addition of a plasticizer than the type of chitosan used. Addition of plasticizers made the films more hydrophilic, sticky, and difficult to handle. The films formed with lactic and malic acids were highly hygroscopic with high shrinkage upon peeling. Films formed with ascorbic acids were highly brittle regardless whether plasticizer was added or not. Therefore, selection of an organic solvent and omission of plasticizers are important to obtain desirable films from crawfish chitosans.

The films formed with acetic, formic or citric acids without plasticizers were highly flexible and transparent, and resembled plastic films. No visual color difference was evident among these films and their transparency far exceeded that of conventional low density polyethylene. All citrate films exhibited high solubility in water and, hence, have potential for developing edible packaging material intended for easy solubility. The crawfish chitosan films (DPMA, DMA formic acid films and DMA acetic films) with higher swelling can be utilized to form absorbent pads. The acetate and formate films with DPMCA or DMCA chitosan and acetic acid film with DPMA chitosan exhibited lower swelling upon hydration and, therefore, are recommended for further research work on developing edible/biodegradable packaging materials.
3.5 REFERENCES


CHAPTER 4

SORPTION AND WATER PERMEABILITY BEHAVIORS OF CRAWFISH CHITOSAN FILMS
4.1 INTRODUCTION

In recent years, there has been an increasing interest in the development of antimicrobial packaging materials. The use of biodegradable and renewable polymers in an attempt to replace plastic packaging materials would reduce environmental pollution. With potent antibacterial (Sudarshan and others 1992; Yalpani and others 1992; No and others 2002a,b), and antifungal (Allan and Hadwiger, 1979; Stossel and Leuba, 1984; Kendra et al., 1989; Fang et al., 1994, Ralston et al., 1964) properties, chitosan is one of the most promising and abundant resources for this purpose. Chitosan possesses a greater and broader spectra of antibacterial activity compared to disinfectants, a greater microbial killing rate, and lower toxicity toward mammalian cells (Franklin and Snow, 1981; Takemono and others 1989).

Crawfish shell waste is an abundant, inexpensive and relatively unexploited resource for chitosan extraction. Since physicochemical properties of chitosan are greatly affected by the sources (Rhazi and others 2004) and the extraction methods (Rout 2001; Nadarajah and Prinyawiwatkul 2002), chitosans derived from crawfish shell waste by different extraction methods may differ in their functionalities. Chitosan dissolves in dilute organic acids. Consequently, the use of acids having antimicrobial properties such as acetic, lactic, formic, malic, and citric may offer a simple method to produce antimicrobial films from crawfish chitosan. Chitosan films formed with various organic acids such as acetic, formic, lactic, citric, propionic and malic acids have varying physicochemical properties (Butler and others 1996; Caner and others 1998; Rhim and others 1998; Park and others 1999; Park and others 2002).

Chitosan films exhibited selective permeabilities to gases (Wong and others 1992; Butler and others 1996) and possessed stronger texture. However, they lacked resistance to water transmission because of their hydrophilic nature. Hence, there is a major drawback in using
chitosans as packaging materials as they are far too humidity sensitive to be used in direct contact with food or for direct handling (Olbarrieta and others 2001). Hydrophilic films also lose their mechanical and barrier properties in high moisture conditions (Gontard and others 1994). Thorough understanding of sorption and water transmission behavior of chitosan film is necessary to formulate films that are less sensitive to humidity changes.

This study was conducted to determine the sorption and water transmission properties of crawfish chitosan films, in an attempt to select the films that are less sensitive to humidity and have better water barrier properties. Effects of chitosan types and film casting organic solvents on sorption and water transmission behaviors were studied. Models capable of describing moisture sorption behavior of crawfish chitosan films were identified.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of Chitosan from Crawfish Shell Waste

All chemicals used in this study were analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO). Crawfish shell waste collected from a local seafood processing facility was washed with warm tap water to remove foreign materials and remaining muscle particles. The shells were then dried at 60°C overnight, ground with a centrifugal grinding mill (Retsch/Brinkmann ZM-1, Westbury, NY), and shell particles between a mesh size of 20 (0.841 mm) and 40 (0.420 mm) were used as starting material. The method established to extract chitin from crawfish shell waste by No and Meyers (1995) and further processing of chitin into chitosan through autoclaving (No and others 2000) were used to prepare different chitosan samples for this study.

Dried crawfish shell particles were treated with 1 N NaOH at 65°C for 1 hour at a solid: solvent ratio of 1:10, w/v for deproteinization (DP). Following a washing step, deproteinized
shell particles were treated with 1 N HCl at room temperature for 30 minutes at a solid: solvent ratio of 1:1.5, w/v for demineralization (DM). Particles were then treated with acetone at 1: 10 w/v concentration, washed, and bleached with 0.315% NaOCl at a 1:10 w/v ratio for 5 minutes for decoloration (DC). Resultant chitin was treated with 50% NaOH at a 1:10 w/v ratio at 121°C/15 psi for 30 minutes for deacetylation (DA). Subsequent washing and drying steps yielded chitosan. Four different chitosan samples were prepared using the traditional method involving all above steps (DPMCA), the traditional method excluding deproteinization (DMCA), the traditional method excluding decoloration (DPMA), and the traditional method excluding both deproteinization and decoloration (DMA).

4.2.2 Characterization of Crawfish Chitosans

The viscosity average molecular weight of crawfish chitosans was determined by the intrinsic viscosity using the Mark-Houwink equation ([η] = KM^a) where [η] is the intrinsic viscosity, M is the molecular weight, K=1.81 x 10^-3 and a=0.93 at 25°C (Maghami and Roberts 1988). Crawfish chitosan films deprotonated by methanolic ammonia was used to determine the degree of deacetylation with IR spectroscopy (M2000 FTIR spectrophotometer, Midac Corp., Irvine, CA, USA) and the equation proposed by Sabnis and Block (1997) was used to calculate degree of deacetylation values. Viscosity was determined for 1% chitosan in 1% acetic acid (w/v) using the Brookfield viscometer at 50 rpm and 25°C with a spindle number RV5.

The moisture content (%) of the chitosans was determined gravimetrically in triplicate by drying samples at 100°C for 48 hours. Nitrogen (%) was determined by the combustion method using the Leco CHN analyzer (Model # FP-428, Leco Corporation, St. Joseph, Michigan, USA). The ash content (%) was determined according to the standard method # 923.03 (AOAC 1990).
4.2.3 Preparation of Crawfish Chitosan Films

Four types of crawfish chitosans (DPMCA, DPMA, DMCA, and DMA) were separately dissolved with 1% of film casting solvents (acetic, ascorbic, lactic, formic, citric or malic acids). Each solution was vigorously agitated for 30 minutes, immersed in boiling water for 10 minutes, cooled down to room temperature, and filtered through glass-wool to remove undissolved particles. The film casting solution (300 mL) was cast onto a 31 × 31 cm Teflon coated plate and allowed to dry at room temperature (23°C) for 48 hours. Dried films were carefully peeled out and stored in desiccators containing saturated NaBr until further tested.

A micrometer (Model 293-766; Mitutoyo, Tokyo, Japan) was used to measure the film thickness to the nearest 0.001 mm. Thickness of each film (mm) was measured at room temperature (23°C and 45% RH) and expressed as an average of 10 random measurements and standard deviation.

4.2.4 Sorption Isotherm Experiments

Moisture isotherms of chitosan films were determined in triplicate gravimetrically (Hatakcyama and Hatakcyama, 1998) at 25°C. In order to avoid possible curing effects that may arise due to heating, film samples were dried at 25°C for 3 weeks in hermetically sealed desiccators containing Drierite desiccant (W.A. Hammond Drierite Co. Ltd., Xena, OH).

Film samples were weighed in glass containers and placed inside a vacuum desiccator containing a saturated salt of known water activity (Robinson and Stokes, 1959). The range of water activities from 0.112 to 0.927 was studied using saturated salt solutions of lithium chloride ($a_w = 0.113$), magnesium chloride ($a_w = 0.327$), potassium carbonate ($a_w = 0.431$), sodium bromide ($a_w = 0.576$), potassium iodide ($a_w = 0.688$), sodium nitrate ($a_w = 0.742$), potassium chloride ($a_w = 0.843$), and potassium nitrate ($a_w = 0.935$). All chemicals were analytical grade
purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The desiccators with the samples were kept in a temperature controlled environment at 25°C. Equilibrium was considered to be reached after three weeks as our initial experiments revealed that sample weight did not change by more than 2 mg of water/g of chitosan film by dry weight. The amount of water adsorbed on the film sample was determined by re-weighing the containers and the contents.

Six sorption isotherm models: Guggenheim-Anderson-De Boer (GAB model) (Anderson 1946; De Boer 1953; Guggenheim 1966), Smith (1947), Halsey (1948), Caurie (1970), Oswin (1946), Henderson (1952) were used to fit the experimental sorption isotherm data (Table 4.1). GAB model parameters were calculated using a non-linear regression program developed by Professor T.P. Labuza, University of Minnesota, MN, USA.

Table 4.1: Isotherm models used for fitting experimental data

<table>
<thead>
<tr>
<th>Isotherm</th>
<th>Model</th>
<th>Equation No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oswin (1946)</td>
<td>( m = a \left( \frac{a_w}{1-a_w} \right)^n )</td>
<td>(1)</td>
</tr>
<tr>
<td>Smith (1947)</td>
<td>( m = m_b - m_w \times \ln(1 - a_w) )</td>
<td>(2)</td>
</tr>
<tr>
<td>Halsey (1948)</td>
<td>( \ln(m) = a + b \times \ln[-\ln(a_w)] )</td>
<td>(3)</td>
</tr>
<tr>
<td>Henderson (1952)</td>
<td>( \ln(m) = \frac{\ln\left( \frac{1}{(1-a_w)} \right)}{B} - \frac{\ln(A)}{B} )</td>
<td>(4)</td>
</tr>
<tr>
<td>Caurie (1970)</td>
<td>( \ln \left( \frac{1}{m} \right) = -\ln(c.m_o) + \frac{2c}{m_o} \ln \left( \frac{1-a_w}{a_w} \right) )</td>
<td>(5)</td>
</tr>
<tr>
<td>GAB</td>
<td>( \frac{a_w}{(1-ka_w)m} = \frac{1}{m_o Ck} + \left( \frac{C-1}{m_o C} \right) a_w )</td>
<td>(6)</td>
</tr>
</tbody>
</table>

Nomenclature:
- \( m \) = moisture content
- \( a_w \) = water activity
- \( a, b, m_o, m_b, A, B, c, C, k \) are constants
- \( m_o \) = monolayer moisture content
4.2.5 Water Vapor Permeability of Crawfish Chitosan Films

Water vapor permeability of crawfish chitosan films was measured in triplicate using the Permatran W3/31 modular system (Modern Controls, Inc., Minneapolis, MN) according to the American Society of Testing and Materials Standard Method E 96-95 (ASTM, 1995). All films were placed on a stainless steel mask with an open testing area of 5 cm². One side of the film was exposed to flowing nitrogen gas, and the other side was exposed to flowing water vapor at 25°C and 50 % RH.

The water vapor permeability, WVP, (ng·m/m²·s·Pa) of film was calculated as follows:

\[ WVP = \frac{WVTR}{\Delta P} \times L \]

Where WVTR is the measured water vapor transmission rate (ng/m²·s) through a film, L is the mean film thickness (m), and \( \Delta P \) was the partial water vapor pressure difference (Pa) across the 2 sides of the film.

4.2.6 Statistical Analysis

All experiments were carried out in triplicate and average values were reported. Linear regression analysis (PROC REG) was performed to derive regression models and to calculate the best fitted values of constants in the sorption isotherm equations using SAS (SAS, 2002). The suitability of the equations was evaluated and compared using the coefficient of regression (R²). PROC ANOVA with a nested factorial model was applied to test the differences among isotherms. Significance of differences was defined at \( P < 0.05 \).

4.3 RESULTS AND DISCUSSION

4.3.1 Characteristics of Crawfish Chitosan

The physicochemical properties of all chitosans used in this study are shown in Table 3.1. The chitosan extracted by the traditional method (DPMCA) had a lowest molecular weight due
to depolymerization of chitosan. The DPMCA chitosans also showed the lowest viscosity and highest degree of deacetylation. The DMA chitosan was subjected to least chemical treatments but had lower molecular weight than that of DPMA chitosan. However, considering the general range of molecular weight of commercial chitosans falling between 100,000 and 1,200,000 Daltons (Li and others, 1992), all crawfish chitosans produced in this study can be convincingly categorized as low molecular weight chitosans. Viscometric measurements agreed with the magnitude of molecular weight accounting lowest viscosity for DPMCA chitosan and highest viscosity for DPMA chitosan. The DMA chitosan contained about twofold moisture content of that of other chitosans.

4.3.2 Film-forming Ability and Film Characteristics

The film-forming ability of plasticized crawfish chitosans can be considered poor owing to the highly hygroscopic nature of the films, which makes them adsorb moisture from the atmosphere and makes the films sticky. Further, plasticized films formed with malic and lactic acids undergo shrinkage and deformation immediately after being peeled out from casting plates. The sticky and shrunk chitosan films are not desirable for handling and, therefore, not suitable for packaging applications. Nadarajah and Prinyawiwatkul (2002) reported that chitosan acetate films made from crawfish chitosan (deacetylated by a distillation process) with molecular weight 46,000 Da showed higher hygroscopic nature compared to the films made from commercially available shrimp chitosan. Although many have reported film-forming ability of chitosan (Muzzarelli and others, 1974; Averbach, 1978; Butler and others, 1996; Caner and others, 1998), the hygroscopic nature of plasticized chitosan films have not been reported and presumably not encountered with the chitosans used.
Among the unplasticized films, those made with malic and lactic acid showed stickiness similarly observed with plasticized films, although shrinkage of unplasticized films was not prominent. Regardless of the types of chitosans used (DPMCA, DMPA, DMCA, DMA), the films made with acetic or formic acid were highly transparent and flexible with slight yellow tint. All citrate films were yellowish and brittle in nature compared to acetate and formate films. Chitosan films possessing slight yellow tint was reported by Caner and others (1998) and Wiles and others (2000). All crawfish chitosan films showed the tendency of becoming yellowish during prolonged storage at higher humidity conditions. Similar observations were also reported by Wiles and others (2000) for chitosan acetate films. Apart from that, our crawfish chitosan films showed no apparent visual difference among them. Interestingly, the flexibility of chitosan acetate and formate films was not impaired at low water activity levels (a_w=0.113) despite the fact that unplasticized films tend to become brittle and lose their integrity at low water activity levels. However, the citrate films became more brittle at lower water activity levels and broken apart when handled. This reveals that flexible crawfish chitosan films can be formulated without added plasticizers and prepared with acetic and formic acids. Plasticizers are used to reduce brittleness of polysaccharide or protein-based films but their use entails decreased efficiency of barrier properties of edible packaging (Butler and others 1996; Shaw and others 2002). Further, the film-forming ability of crawfish DMA chitosan indicates the possibility of formulating more economical chitosan films because DMA chitosans undergo the least number of processing steps, chemicals, and time to produce.

4.3.3 Sorption Isotherms of Crawfish Chitosan Films

The moisture sorption characteristics of crawfish chitosan films are critical for ascertaining their use as a packaging material at varying humidity conditions. The sorption
isotherms of all (DPMCA, DPMA, DMCA, DMA) chitosan films showed a classical sigmoid curve described as the type II isotherm in BET classification (Brunanuer 1945) (Figure 4.1). Most biological products follow a sigmoid curve representing the type II isotherm BET classification (Labuza 1984).

![Figure 4.1: Moisture sorption isotherms of representative crawfish chitosan films at 25°C.](image)

The acetate and formate films exhibited similar adsorption curves. However, the adsorption curve of citrate films deviated from both acetate and formate films (Figure 4.1). The citrate films exhibited stickiness above water activity level of 0.74 and brittleness at below water
activity level of 0.33. However, the acetate and formate films retained their flexibility and non-sticking behavior throughout the water activity continuum.

The chitosan acetate films made from different crawfish chitosans exhibited identical sorption isotherms, despite the differences in physicochemical properties of chitosans (Figure 4.2). The PROC ANOVA procedure performed with nested water activity levels revealed that there was no significant difference (p>0.05) among the isotherms obtained from different types

Figure 4.2: Moisture sorption isotherms of chitosan acetate films at 25°C.
of crawfish chitosans. Thus, we can deduce that the extraction process of chitosan and their physicochemical properties have negligible influence on the sorption behavior of chitosan acetate films evaluated in this study.

Unlike chitosan acetate films, chitosan formate films made from different types of crawfish chitosans showed significantly different (p<0.05) isotherms (Figure 4.3). The PROC ANOVA with nested water activity levels revealed that the isotherm of DPMCA chitosan formate film was significantly different (p<0.05) from that of DMCA and DMA formate films.

![Figure 4.3: Moisture sorption isotherms of chitosan formate films at 25°C.](image-url)
No significant difference (p>0.05) in isotherms was found between DPMCA and DPMA chitosan formate films as well as between DPMA and DMCA chitosan formate films.

Comparison of isotherms of chitosan acetate with chitosan formate films indicated that the formate films were significantly different (p<0.05) from the acetate films, except for DPMCA acetate and DPMCA formate films (Figures 4.2 and 4.3). Chitosan citrate films were significantly (p<0.05) different from both acetate and formate films (Figure 4.1). Statistical analysis revealed that both the type of acids and chitosans used for film formation influenced the sorption behavior of crawfish chitosan films. However, the effect of chitosan type was less prominent.

At elevated water activity levels, a pronounced increase in sorption uptake was observed (Figures 4.2 and 4.3). Similar observations were also reported by Despond and others (2001). The pronounced increase in the equilibrium moisture content at elevated water activity levels indicates possible swelling effects in the films. Swelling would cause changes in the microstructure of the film and increase moisture sorption, and water vapor would act as a plasticizer inside the chitosan matrix (Wiles and others 2000; Rogers 1985). At water activity levels of 0.743 to 0.936, the amount of water absorbed by chitosan films increased by 138% (dry basis) for acetate films, by 192% (dry basis) for formate films, and by 285 % (dry basis) for citrate films. Since chitosan citrate film changes its nature from very brittle to very soggy at elevated water activity levels, it cannot be a good candidate for packaging purposes. Though both chitosan acetate and formate films retained their flexibility and integrity along the water activity continuum, the chitosan acetate films, which absorb less water and produce lower lying adsorption isotherm, are of better qualify for packaging purposes. However, apart from adsorption, the absorption and swelling behaviors of chitosan films have to be considered to
select more appropriate films. Accordingly, the chitosan acetate film with DMCA chitosan and the formate film with DPMCA chitosan, having lower swelling, qualify for packaging purposes.

4.3.4 Sorption Model Analysis

The isotherm models used in this study and the calculated constant values are summarized in Table 4.2, 4.3, and 4.4 for acetate, formate and citrate films, respectively. A good agreement between experimental and predicted data was found with the GAB models with the coefficient of determination ($R^2$) of 0.97 to 0.99 for all the films tested, regardless of the type of acids and chitosans used. Therefore, the GAB model is most appropriate for the prediction of equilibrium moisture content of crawfish chitosan acetate, formate, and citrate films. The GAB equation has been described to predict the moisture sorption of many foods and natural polymers with adequate accuracy (Joncquières and Fane 1998; Taoukis 1988). Despond and others (2001) reported the adequacy of the GAB model for water sorption of chitosan films.

Among other models tested, the Oswin and Caurie models can be used to predict sorption behavior of acetate and formate films with $R^2$ values of 0.93 to 0.98. However, except for the GAB model, most of the models tested did not accurately describe chitosan citrate films. This can be attributed to the deviation found in the sorption behavior of chitosan citrate films in which they adsorbed much lower and much higher moisture, respectively, at below and above the water activity level of 0.84.

The GAB model and the water clustering theory of Zimm and Lundberg (1956) were utilized to infer the water clustering phenomenon in chitosan films (Despond and other 2001). Zimm and Lundberg (1956) developed an equation to calculate a cluster integral from the sorption isotherm. The clustering function ($G_{ij}/v_i$) is a characteristic quantity that enables the
Table 4.2: Constants of sorption models for chitosan acetate films

<table>
<thead>
<tr>
<th>Isotherm</th>
<th>Chitosan Type*</th>
<th>Constants</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAB</td>
<td></td>
<td>$k$</td>
<td>$C$</td>
</tr>
<tr>
<td></td>
<td>DPMCA</td>
<td>0.90</td>
<td>16.63</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>0.93</td>
<td>27.77</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>0.87</td>
<td>9.61</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>0.88</td>
<td>13.25</td>
</tr>
<tr>
<td>Smith</td>
<td></td>
<td>$M_b$</td>
<td>$M_a$</td>
</tr>
<tr>
<td></td>
<td>DPMCA</td>
<td>0.021</td>
<td>0.134</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>0.036</td>
<td>0.111</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>0.021</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>0.025</td>
<td>0.118</td>
</tr>
<tr>
<td>Oswin</td>
<td></td>
<td>$a$</td>
<td>$n$</td>
</tr>
<tr>
<td></td>
<td>DPMCA</td>
<td>0.115</td>
<td>0.459</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>0.116</td>
<td>0.393</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>0.115</td>
<td>0.459</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>0.107</td>
<td>0.450</td>
</tr>
<tr>
<td>Halsey</td>
<td></td>
<td>$a$</td>
<td>$b$</td>
</tr>
<tr>
<td></td>
<td>DPMCA</td>
<td>-2.46</td>
<td>-0.60</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>-2.41</td>
<td>-0.52</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>-2.45</td>
<td>-0.59</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>-2.52</td>
<td>-0.59</td>
</tr>
<tr>
<td>Caurie</td>
<td></td>
<td>$C$</td>
<td>$m_o$</td>
</tr>
<tr>
<td></td>
<td>DPMCA</td>
<td>0.162</td>
<td>0.709</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>0.151</td>
<td>0.771</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>0.162</td>
<td>0.709</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>0.155</td>
<td>0.691</td>
</tr>
<tr>
<td>Henderson</td>
<td></td>
<td>$A$</td>
<td>$B$</td>
</tr>
<tr>
<td></td>
<td>DPMCA</td>
<td>14.41</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>24.18</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>109.86</td>
<td>2.17</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>14.02</td>
<td>2.21</td>
</tr>
</tbody>
</table>

*DPMCA = Deproteinized + demineralized + decolorized + deacetylated, DPMA = Deproteinized + demineralized + deacetylated, DMCA = Demineralized + decolorized + deacetylated, DMA = Demineralized + deacetylated.

b Refer to Nomenclature for constants
Table 4.3: Constants of sorption models for chitosan formate films

<table>
<thead>
<tr>
<th>Isotherm</th>
<th>Chitosan Type*</th>
<th>Constants byb</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k</td>
<td>C</td>
<td>m₀</td>
</tr>
<tr>
<td>GAB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPMCA</td>
<td>0.92</td>
<td>9.53</td>
<td>0.060</td>
</tr>
<tr>
<td>DPMA</td>
<td>0.92</td>
<td>14.08</td>
<td>0.066</td>
</tr>
<tr>
<td>DMCA</td>
<td>0.93</td>
<td>15.15</td>
<td>0.063</td>
</tr>
<tr>
<td>DMA</td>
<td>0.92</td>
<td>14.36</td>
<td>0.065</td>
</tr>
<tr>
<td>Smith</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPMCA</td>
<td>0.010</td>
<td>0.146</td>
<td>0.94</td>
</tr>
<tr>
<td>DPMA</td>
<td>0.016</td>
<td>0.159</td>
<td>0.94</td>
</tr>
<tr>
<td>DMCA</td>
<td>0.009</td>
<td>0.169</td>
<td>0.93</td>
</tr>
<tr>
<td>DMA</td>
<td>0.0131</td>
<td>0.161</td>
<td>0.93</td>
</tr>
<tr>
<td>Oswin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPMCA</td>
<td>0.111</td>
<td>0.501</td>
<td>0.95</td>
</tr>
<tr>
<td>DPMA</td>
<td>0.127</td>
<td>0.471</td>
<td>0.96</td>
</tr>
<tr>
<td>DMCA</td>
<td>0.125</td>
<td>0.483</td>
<td>0.94</td>
</tr>
<tr>
<td>DMA</td>
<td>0.127</td>
<td>0.460</td>
<td>0.94</td>
</tr>
<tr>
<td>Halsey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPMCA</td>
<td>-2.52</td>
<td>-0.65</td>
<td>0.93</td>
</tr>
<tr>
<td>DPMA</td>
<td>-2.37</td>
<td>-0.62</td>
<td>0.95</td>
</tr>
<tr>
<td>DMCA</td>
<td>-2.40</td>
<td>-0.64</td>
<td>0.94</td>
</tr>
<tr>
<td>DMA</td>
<td>-2.36</td>
<td>-0.61</td>
<td>0.95</td>
</tr>
<tr>
<td>Caurie</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPMCA</td>
<td>0.167</td>
<td>0.667</td>
<td>0.95</td>
</tr>
<tr>
<td>DPMA</td>
<td>0.173</td>
<td>0.734</td>
<td>0.96</td>
</tr>
<tr>
<td>DMCA</td>
<td>0.174</td>
<td>0.730</td>
<td>0.95</td>
</tr>
<tr>
<td>DMA</td>
<td>0.171</td>
<td>0.746</td>
<td>0.94</td>
</tr>
<tr>
<td>Henderson</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPMCA</td>
<td>79.00</td>
<td>1.99</td>
<td>0.95</td>
</tr>
<tr>
<td>DPMA</td>
<td>79.05</td>
<td>2.11</td>
<td>0.96</td>
</tr>
<tr>
<td>DMCA</td>
<td>73.67</td>
<td>2.06</td>
<td>0.94</td>
</tr>
<tr>
<td>DMA</td>
<td>86.20</td>
<td>2.17</td>
<td>0.94</td>
</tr>
</tbody>
</table>

*DPMCA = Deproteinized + demineralized + decolorized + deacetylated, DPMA = Deproteinized + demineralized + deacetylated, DMCA = Demineralized + decolorized + deacetylated, DMA = Demineralized + deacetylated.

b Refer to Nomenclature for constants.
Table 4.4: Constants of sorption models for chitosan citrate films

<table>
<thead>
<tr>
<th>Isotherm</th>
<th>Chitosan Type*</th>
<th>Constants by b</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>k</td>
<td>C</td>
<td>m₀</td>
</tr>
<tr>
<td>DPMCA</td>
<td>0.96</td>
<td>1.31</td>
<td>0.030</td>
</tr>
<tr>
<td>DPMA</td>
<td>0.89</td>
<td>0.38</td>
<td>0.083</td>
</tr>
<tr>
<td>DMCA</td>
<td>0.95</td>
<td>0.77</td>
<td>0.045</td>
</tr>
<tr>
<td>DMA</td>
<td>0.96</td>
<td>0.73</td>
<td>0.042</td>
</tr>
<tr>
<td>Smith</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mₘₜ</td>
<td>Mₘₐ</td>
<td></td>
</tr>
<tr>
<td>DPMCA</td>
<td>-0.008</td>
<td>0.080</td>
<td></td>
</tr>
<tr>
<td>DPMA</td>
<td>-0.035</td>
<td>0.124</td>
<td></td>
</tr>
<tr>
<td>DMCA</td>
<td>-0.046</td>
<td>0.138</td>
<td></td>
</tr>
<tr>
<td>DMA</td>
<td>-0.052</td>
<td>0.143</td>
<td></td>
</tr>
<tr>
<td>Oswin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>DPMCA</td>
<td>0.035</td>
<td>0.807</td>
<td></td>
</tr>
<tr>
<td>DPMA</td>
<td>0.036</td>
<td>0.912</td>
<td></td>
</tr>
<tr>
<td>DMCA</td>
<td>0.043</td>
<td>0.806</td>
<td></td>
</tr>
<tr>
<td>DMA</td>
<td>0.036</td>
<td>0.909</td>
<td></td>
</tr>
<tr>
<td>Halsey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>DPMCA</td>
<td>-3.85</td>
<td>-1.02</td>
<td></td>
</tr>
<tr>
<td>DPMA</td>
<td>-3.78</td>
<td>-1.11</td>
<td></td>
</tr>
<tr>
<td>DMCA</td>
<td>-3.52</td>
<td>-0.95</td>
<td></td>
</tr>
<tr>
<td>DMA</td>
<td>-3.49</td>
<td>-1.04</td>
<td></td>
</tr>
<tr>
<td>Caurie</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>m₀</td>
<td></td>
</tr>
<tr>
<td>DPMCA</td>
<td>0.119</td>
<td>0.294</td>
<td></td>
</tr>
<tr>
<td>DPMA</td>
<td>0.129</td>
<td>0.284</td>
<td></td>
</tr>
<tr>
<td>DMCA</td>
<td>0.131</td>
<td>0.326</td>
<td></td>
</tr>
<tr>
<td>DMA</td>
<td>0.128</td>
<td>0.283</td>
<td></td>
</tr>
<tr>
<td>Henderson</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>DPMCA</td>
<td>63.21</td>
<td>1.23</td>
<td></td>
</tr>
<tr>
<td>DPMA</td>
<td>37.08</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>DMCA</td>
<td>49.38</td>
<td>1.23</td>
<td></td>
</tr>
<tr>
<td>DMA</td>
<td>38.09</td>
<td>1.09</td>
<td></td>
</tr>
</tbody>
</table>

* DPMCA = Deproteinized + demineralized + decolorized + deacetylated, DPMA = Deproteinized + demineralized + deacetylated, DMCA = Demineralized + decolorized + deacetylated, DMA = Demineralized + deacetylated.

b Refer to Nomenclature for constants
calculation of tendency of the (water) molecules to cluster in the given polymer matrix. The clustering function is defined as the ratio:

$$\frac{G_{11}}{v_1} = -(1 - \phi_1) \left[ \frac{\partial \left( \frac{\alpha_1}{\phi_1} \right)}{\partial \alpha_1} \right]_{p,T} - 1$$  \hspace{1cm} (7)$$

where $G_{11}$ is the cluster integral, calculated from the molecular pair distribution, $v_1$ is the partial molar volume of the penetrant (e.g., water), $\phi_1$ is the volume fraction, and $a_1$ is the activity of component 1. For an ideal solution, the activity coefficient ($a_1/\phi_1$) does not vary with concentration, i.e., the activity is proportional to the volume fraction, and , therefore, eq. (7) becomes:

$$\frac{G_{11}}{v_1} = -1$$  \hspace{1cm} (8)$$

For nonrandom mixing solutions, however, the activity coefficient decreases with increasing $\phi_1$ so that $G_{11}/v_1$ is greater than -1. The extent of clustering in the solution is indicated by the extent to which $G_{11}/v_1$ exceeds -1. The quantity $\phi_1G_{11}/v_1$ is the mean number of excess water molecules in the neighborhood of a given water molecule. The average number of solvent molecules in a cluster can be described by the following equation:

$$N_c = \phi_1 \frac{G_{11}}{v_1} + 1$$  \hspace{1cm} (9)$$

where $N_c$ is the cluster number. For an ideal solution where there is no clustering of water, $N_c$ is equal to 1. The $N_c$ values greater than 1 represent clustering of water molecules. Positive $N_c$ values indicate that the solute increases the free volume of the polymer matrix, increasing the
sorption capacity, diffusivity, and permeability. By combining eqs. (6, Table 4.1), (7), and (9), $N_c$ can be expressed as (Zhang and others 1999):

$$N_c = -(1 - \phi_1) \times \left( \frac{\phi_1}{m_o C} \left( -2Ck_{w} + 2k_{w} + C - 2 \right) - 1 \right)$$

(10)

Where $m_o$ is the monolayer moisture content, and $k$ and $C$ are the GAB constants. To deduce this equation, the variable $m$ in eq. (6) (Table 4.1) was transformed from gravimetric to volumetric fraction $\phi_1$ using densities of crawfish chitosan films.

The $N_c$ values deduced from the equation 10 indicate that when water activity exceeds 0.57, clustering of water molecules takes place (Figure 4.4). At lower water activity levels, water was distributed mainly through the polymer matrix, probably absorbed on the active sites of hydrogen bonds. As water activity increased, water molecules predominately clustered on these hydrogen bonding sites, likely resulting in plasticization of the polymer. Water molecules that initially entered the polymer structure may have opened the structure to make it easier for subsequent water molecules to absorb in the neighborhood of the initially absorbed molecules (Despond and others 2001).

### 4.3.5 Water Vapor Transmission Rate

The water vapor transmission rates of crawfish chitosan films are shown in Table 4.5. Thickness of crawfish chitosan films was dependent of the type of chitosans and film casting solvents used. Chitosan citrate films had significantly ($p<0.05$) higher thicknesses than chitosan acetate and formate films. In general, chitosan acetate and formate films had similar film thickness. The variation in the film thickness observed in this study is in agreement with those described earlier by Caner and others (1998) and Begin and Calsteren (1999). Ideal polymeric films should not exhibit thickness effect on water vapor permeability. However, hydrophilic
films often, but not always, exhibit a positive slope relationship between thickness and water vapor permeability (Miranda and others 2004).

All citrate films showed significantly (p<0.05) lower WVTR compared to acetate and formate films. However, there was no significant difference in WVTR among all chitosan citrate films. The lower WVTR obtained by the citrate films can be attributed to the greater thickness.
compared to that of other films. In general, the chitosan formate films had higher WVTR than the acetate films. This observation is in agreement with that described by Caner and others (1998). The highest WVTR was obtained for DPMA formate films.

Table 4.5: Water vapor transmission rate and water vapor permeability at 25°C and 50% RH gradient.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Chitosan Type*1</th>
<th>Thickness*2 (mm)</th>
<th>WVTR*3 (g/m².day)</th>
<th>WVP (ng/m².s.Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>DPMCA</td>
<td>0.021d</td>
<td>244.35 ± 5.34c</td>
<td>0.038f</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>0.020d</td>
<td>217.12 ± 5.81d</td>
<td>0.032g</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>0.024cd</td>
<td>273.69 ± 2.58b</td>
<td>0.048c</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>0.026c</td>
<td>298.45 ± 0.63a</td>
<td>0.057b</td>
</tr>
<tr>
<td>Formic acid</td>
<td>DPMCA</td>
<td>0.027c</td>
<td>310.53 ± 2.71a</td>
<td>0.061a</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>0.020d</td>
<td>311.10 ± 1.35a</td>
<td>0.045cd</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>0.020d</td>
<td>272.87 ± 8.05b</td>
<td>0.040ef</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>0.021d</td>
<td>277.94 ± 11.91b</td>
<td>0.043de</td>
</tr>
<tr>
<td>Citric acid</td>
<td>DPMCA</td>
<td>0.059a</td>
<td>44.59 ± 3.12e</td>
<td>0.019h</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>0.050b</td>
<td>40.73 ± 4.36e</td>
<td>0.015hi</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>0.050b</td>
<td>38.36 ± 1.77e</td>
<td>0.014hi</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>0.052b</td>
<td>44.52 ± 5.23e</td>
<td>0.017hi</td>
</tr>
</tbody>
</table>

*DPMCA = Deproteinized + demineralized + decolorized + deacetylated, DPMA = Deproteinized + demineralized + deacetylated, DMCA = Demineralized + decolorized + deacetylated, DMA = Demineralized + deacetylated.

Means within the same column sharing same letters are not significantly different at p > 0.05. *2 Means of 10 random measurements and standard deviation. *3 Means of 3 measurement and standard deviation.

85
Water vapor permeability of a film is constant and independent of the driving force on water vapor transmission. However, in hydrophilic films, water molecules interact with polar groups in the film structure, causing plasticization or swelling, which, in turn, results in varying permeability (Miranda and others 2004).

Statistical analysis showed that both WVTR and WVP were significantly (p<0.05) influenced by the film casting solvents. Chitosan types showed significant (p<0.05) influence on WVTR of films. Hence, it can be deduced that both chitosan types and film casting solvent are critical in water transmission.

Prediction of water transport through chitosan films becomes complex due to films’ hydrophilic nature. The complexity is due to their nonlinear water sorption isotherms and water content dependent diffusivity. Chitosan, possessing high cationic and being hydrophilic in nature, leads to higher interaction with water molecules, which increases the permeation of water vapor. With increasing moisture, the films start swelling. Swelling would cause conformational changes in the microstructure of the film that not only increase moisture sorption, but also increases channels in the polymeric structure to allow the increase in permeant flow. Swelling results in deviation from Fickian behavior (Miranda and others 2004).

Table 4.6 shows a comparison of results obtained from our study with past literatures on chitosan films, common edible films and some plastic films. The WVP values obtained in the present study are in agreement with those obtained by Butler and others (1996) and Wong and others (1992). However, much higher WVR values were reported by Miranda and others (2004) and Caner and others (1998). The WVP of crawfish chitosan films is lower than that of many edible starch or protein based films, but is much higher than those of plastic films.
Table 4.6: Water vapor permeability of chitosan, edible and plastic films.

<table>
<thead>
<tr>
<th>Film</th>
<th>Water vapor permeability (ng.m/m².s.Pa)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crawfish chitosan films</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate films</td>
<td>0.032 - 0.057</td>
<td>Present study</td>
</tr>
<tr>
<td>Formate films</td>
<td>0.040 - 0.061</td>
<td>Present study</td>
</tr>
<tr>
<td>Citrate films</td>
<td>0.014 - 0.019</td>
<td>Present study</td>
</tr>
<tr>
<td><strong>Chitosan film from the literature</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitosan acetate&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.025</td>
<td>Butler and others (1996)</td>
</tr>
<tr>
<td>Chitosan acetate&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.078 - 0.125</td>
<td>Caner and others (1998)</td>
</tr>
<tr>
<td>Chitosan formate&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.107 - 0.135</td>
<td>Caner and others (1998)</td>
</tr>
<tr>
<td>Chitosan formate</td>
<td>0.0365</td>
<td>Wong and others (1992)</td>
</tr>
<tr>
<td>Chitosan acetate&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.345</td>
<td>Miranda and others (2004)</td>
</tr>
<tr>
<td><strong>Edible films</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxymethylated starch</td>
<td>1.2 - 2.1</td>
<td>Kim and others (2002)</td>
</tr>
<tr>
<td>Soybean protein</td>
<td>0.17</td>
<td>Ghorpade and others (1995)</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>0.14</td>
<td>Aydt and others (1991)</td>
</tr>
<tr>
<td>Rice bran</td>
<td>9.20</td>
<td>Gnanasambandam and others (1997)</td>
</tr>
<tr>
<td>Corn zein</td>
<td>0.12 - 0.33</td>
<td>Park and Chinnan (1995)</td>
</tr>
<tr>
<td><strong>Plastic films</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low density polyethylene</td>
<td>0.00055</td>
<td>Krochta and Johnson (1997)</td>
</tr>
<tr>
<td>High density polyethylene</td>
<td>0.00020</td>
<td>Krochta and Johnson (1997)</td>
</tr>
<tr>
<td>Polyvinyl chloride</td>
<td>0.00071</td>
<td>Park and Chinnan (1995)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Films made with 1% chitosan in 1% acetic acid (w/v). Test condition: 25°C and 50% RH gradient.

<sup>b</sup>Films made with 3% chitosan in 1% acetic acid (w/v). Test condition: 25°C and 50% RH gradient.

<sup>c</sup>Films made with 3% chitosan in 1% acetic acid (w/w). Test condition: 25°C and 50% RH gradient.

<sup>d</sup>Films made with 2% chitosan in 1% acetic acid (v/v). Test condition: 28 - 32°C and 95 - 16% RH gradient.
4.4 CONCLUSIONS

Crawfish chitosan forms flexible and transparent films when acetic or formic acid was used as a film casting solvent without a plasticizer. Chitosan extraction methods and film casting solvents significantly affected the sorption behavior of crawfish chitosan films although the effect was not apparent for those films made with acetic acid. The unplasticized crawfish chitosan acetate films, which are flexible, transparent and reaching lower moisture content at any water activity level compared to chitosan formate films, are good candidates for developing packaging. On average, chitosan formate films adsorbed more moisture than chitosan acetate or citrate films. While acetate and formate films maintained their flexibility, the citrate films exhibited brittleness to soggy nature along the water activity continuum and, are therefore, not suitable for packaging applications.

The sorption behavior of crawfish chitosan acetate and formate films can be best predicted by the GAB model ($R^2 = 0.97-0.98$) followed by the Oswin and Caurie models ($R^2 = 0.93-0.97$). The sorption behavior of chitosan citrate films, which showed much deviation from those of acetate and formate films, can only be best predicted by the GAB model ($R^2 = 0.98-0.99$). Therefore, the GAB model can be appropriately used to predict sorption behavior of all crawfish chitosan films. Furthermore, the GAB model provided information on clustering of water molecules at water activity exceeding 0.57.

The water vapor permeability of crawfish chitosan films was significantly influenced by the chitosan types and film casting solvents used. The chitosan citrate films showed the lowest WVP followed by chitosan acetate films. The highest WVP was obtained for DPMCA chitosan formate films.
4.5 REFERENCES


CHAPTER 5

MECHANICAL PROPERTIES OF CRAWFISH CHITOSAN FILMS AS AFFECTED BY CHITOSAN PRODUCTION PROTOCOLS AND FILM CASTING SOLVENTS
5.1 INTRODUCTION

Knowledge of mechanical properties is necessary for any packaging material to ensure integrity of it and its content. Developing edible films with mechanical properties comparable or superior to those of synthetic packaging materials has been a challenge. Most edible films lack mechanical properties appropriate for packaging purposes.

Chitosan is a natural polymer that is readily biodegradable by many chitosanase-producing bacteria. Chitosan possesses excellent film-forming properties (Muzzarelli and others 1974; Averbach 1978; Butler and others 1996; Caner and others 1998; Wiles and others 2000). Chitosan films are described as being tough, long lasting, flexible, and very difficult to tear (Butler and others 1996). Thus, most of their mechanical properties are comparable to many medium-strength commercial polymers (Butler and others 1996). However, the pursuit of making even better chitosan films with more strength and functionalities is in progress. Cross-linking chitosan with glutaraldehyde, divalent metal ions, polyeleetrolytes (Dutkiewicz and others 1992), and anionic polysaccharide (Hoagland and Parris 1996) or laminating chitosan with other polysaccharides such as pectin (Hoagland and Parris 1996) and methylcellulose (Chen and others 1996) are some of such attempts.

Chitosan films formed with various organic acids such as acetic, formic, lactic, citric or and malic acid have been reported to have varying physicochemical and mechanical properties (Kienzle-Sterzer and others 1982; Butler and others 1996; Caner and others 1998; Rhim and others 1998; Park and others 1999; Park and others 2002). Selection of organic acids that give maximum mechanical strengths to films is one straightforward approach to obtain high strength chitosan films. Various organic solvents (acetic, lactic, formic, citric, malic, and propionic acids) have been used for preparing chitosan films (Park and others 2002; Kienzle-Sterzer and others
1982; Butler and others 1996; Rhim and others 1998; Park and others 1999). The chitosan films formed without plasticizers were brittle films and not suitable for packaging applications.

Nadarajah and Prinyawiwatkul (2002) stated that crawfish chitosans extracted by different extraction methods affected film-forming ability of resultant chitosans and their film properties. They further reported that crawfish chitosans had excellent film-forming properties and can be used to produce flexible and transparent films, resembling plastic films. However, these films were so humidity sensitive, that makes them inappropriate for packaging purposes. We discovered that crawfish chitosan films that are less humidity sensitive can be formulated using different combinations of organic acids without any plasticizers and different types of chitosan obtained through modified chitosan extraction protocols. This study was attempted to investigate the effects of different film casting solvents and chitosan extraction protocols on mechanical properties of crawfish chitosan films.

5.1.1 Objective

The overall research objective was to formulate crawfish chitosan films with maximum mechanical properties by selecting appropriate casting solvent and chitosan extraction protocol. The specific objectives were:

1. to determine the effects of different organic acids on tensile and puncture strengths of crawfish chitosan films,
2. to determine the effects of crawfish chitosan extraction protocols on tensile and puncture strengths of crawfish chitosan films,
3. to establish an optimum combination of film casting solvent and chitosan extraction protocol to achieve maximum mechanical strength for crawfish chitosan films.
5.2 MATERIALS AND METHODS

5.2.1 Materials

Crawfish chitosans were derived from crawfish shell waste using the traditional and/or modified methods. The traditional method involves deproteinization (DP), demineralization (DM), decoloration (DC) and deacetylation (DA) (No and Meyers 1995; No and others 2000). Four different chitosan samples were prepared using the traditional method involving all above steps (DPMCA), the traditional method excluding deproteinization (DMCA), the traditional method excluding decoloration (DPMA), and the traditional method excluding both deproteinization and decoloration (DMA).

5.2.2 Characterization of Crawfish Chitosans

The viscosity average molecular weight of crawfish chitosans was determined by the intrinsic viscosity using the Mark-Houwink equation ([\eta] = KM^a) where [\eta] is the intrinsic viscosity, M is the molecular weight, \(K=1.81 \times 10^{-3}\) and \(a=0.93\) at 25°C (Maghami and Roberts 1988). Crawfish chitosan films deprotonated by methanolic ammonia was used to determine the degree of deacetylation with IR spectroscopy (M2000 FTIR spectrophotometer, Midac Corp., Irvine, CA, USA) and the equation proposed by Sabnis and Block (1997) was used to calculate degree of deacetylation values. Viscosity was determined for 1% chitosan in 1% acetic acid (w/v) using the Brookfield viscometer at 50 rpm and 25°C with a spindle number RV5.

The moisture content (%) s of the chitosans was determined gravimetrically in triplicate by drying samples at 100°C for 48 hours. Nitrogen (%) was determined by the combustion method using the Leco CHN analyzer (Model # FP-428, Leco Corporation, St. Joseph, Michigan, USA). The ash content (%) was determined according to the standard method # 923.03 (AOAC 1990).
5.2.3 Film Preparation

Four types of crawfish chitosans (DPMCA, DPMA, DMCA, and DMA) were separately dissolved with 1% of film casting solvents (acetic, ascorbic, lactic, formic, citric or malic acids). Each solution was vigorously agitated for 30 minutes, immersed in boiling water for 10 minutes, cooled down to room temperature, and filtered through glass-wool to remove undissolved particles. The film casting solution (300 mL) was cast onto a 31 × 31 cm Teflon coated plate and allowed to dry at room temperature (23°C) for 48 hours. Dried films were carefully peeled out and stored.

5.2.4 Conditioning of Films

All film specimens used for the mechanical tests were preconditioned for 48 hr at 25°C and 52.8% relative humidity inside desiccators containing saturated solutions of magnesium nitrate. To avoid post aging impact on films, films of 3 days old were used (Caner and others 1998).

5.2.5 Thickness of Films

A micrometer (Model 293-766; Mitutoyo, Tokyo, Japan) was used to measure the film thickness to the nearest 0.001 mm prior all tests. The film thickness was used for calculation of tensile and puncture strengths. For tensile tests, a mean of five measurements across each film specimen was used. For puncture tests, a mean was calculated from 10 random measurements made across each film.

5.2.6 Tensile Test

Tensile measurements for tensile strength, yield strength, elongation, and Young’s Modulus were performed with the Instron testing system (model 4411, Instron Engineering Corp., Canton, MA) following the ASTM standard D882 (ASTM 1998b). Uniform film
specimens of a 100 mm × 5 mm size were prepared from chitosan film samples. Film strips were placed in the pneumatic grips of the testing machine, which were set at an initial separation of 50 mm. The crosshead speed was set at 50 mm/min. At least 12 specimens for each film type were tested and an average of 8 measurements was reported.

5.2.7 Puncture Strength

Puncture strength (PS) of the chitosan film samples was determined, according to the ASTM standard D3763-97a (ASTM 1998a), using the texture analyzer system (TA.XT plus, Stable Micro System, Haslemere, Surrey, UK) equipped with a 5kg load cell and a film testing rig (TA-108). A square film sample of 100 mm × 100 mm was used. The puncture head was a cylindrical rod of 3 mm diameter (TA-53) and the puncture speed was set at 1 mm/s. The PS was measured 6 times for each sample and reported as N/mm (the peak force divided by the thickness of the film).

5.2.8 Statistical Analysis

Data from tensile, tear, and puncture tests were analyzed using SAS (SAS 2002). The PROC GLM procedure with orthogonal contrasts was used to test the effect of film casting solvents and chitosan extraction methods on tensile, tear, and puncture strengths. Significance of differences was defined at $P < 0.05$.

5.3 RESULTS AND DISCUSSIONS

5.3.1 Tensile Strength

The tensile strength curves of crawfish chitosan acetate and formate films followed typical stress-strain curve of LDPE films, except for the short elongation phase. However, the citrate films behaved differently and failed to exhibit any Hookian behavior (reversible
deformation zone) or true yield point before break (Figure 5.1). Similar observation was also reported by Begin and Calsteren (1999) for chitosan citrate films.

![Stress-strain curves of crawfish chitosan films with organic acid solvents.](image)

Figure 5.1: Stress-strain curves of crawfish chitosan films with organic acid solvents.

The tensile strength values of crawfish chitosan films prepared with different organic acids are shown in Table 5.1. Depending on the type of chitosan and film casting solvent, tensile strength of crawfish chitosan films varied widely ranging from 18.9 to 135.8 MPa. Among the acids used, acetic acid resulted in significantly (p<0.05) higher tensile strength, followed by formic and citric acids. This result agrees with that of Rhim and others (1998), Begin and Calsteren (1999), Khan and others (2000), and Park and others (2002), who found acetic acid formed films with highest tensile strength values compared to those formed with formic, malic, lactic, and citric acids. As per Billmeyer (1984), the acetic acid and formic acid films accounting
for tensile strength of 76.8 to 135.8 MPa and Young’s modulus in the range of 2400.3 to 4120.5 Mpa, can be considered as hard. The citric acid films, accounting for lower tensile strength and %E values, can be considered soft and brittle. According to Park and others (2000), in acetic acid solution, chitosan forms dimers indicating that the intermolecular interaction is relatively strong, which leads to tighter structure than those prepared with other acid solutions.

Table 5.1: Tensile measurements of crawfish chitosan films

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Chitosan Type*</th>
<th>Tensile Strength (MPa)</th>
<th>Yield Strength (MPa)</th>
<th>%Elongation</th>
<th>Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>DPMCA</td>
<td>82.0 ± 8.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>72.4 ± 4.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.0 ± 8.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3309.5&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>129.5 ± 4.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>73.7 ± 5.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.0 ± 6.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3120.6&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>135.8 ± 9.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92.1 ± 5.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.1 ± 3.1&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>3384.8&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>132.9 ± 9.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.3 ± 7.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.2 ± 3.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4120.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formic acid</td>
<td>DPMCA</td>
<td>76.8 ± 4.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>61.5 ± 5.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.5 ± 6.6&lt;sup&gt;ede&lt;/sup&gt;</td>
<td>2848.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>114.8 ± 16.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52.8 ± 7.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>54.9 ± 8.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2400.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>90.1 ± 4.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>71.0 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.2 ± 3.9&lt;sup&gt;ede&lt;/sup&gt;</td>
<td>3180.8&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>91.5 ± 12.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>56.0 ± 1.6&lt;sup&gt;ed&lt;/sup&gt;</td>
<td>40.0 ± 7.0&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>2858.4&lt;sup&gt;bce&lt;/sup&gt;</td>
</tr>
<tr>
<td>Citric acid</td>
<td>DPMCA</td>
<td>18.9 ± 4.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NTYP</td>
<td>5.9 ± 1.1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>504.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>24.4 ± 5.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NTYP</td>
<td>24.2 ± 8.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>666.8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>26.9 ± 3.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NTYP</td>
<td>13.0 ± 4.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>514.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>29.3 ± 7.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NTYP</td>
<td>26.7 ± 8.5&lt;sup&gt;ede&lt;/sup&gt;</td>
<td>285.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*DPMCA = Deproteinized + demineralized + decolorized + deacetylated, DPMA = Deproteinized + demineralized + deacetylated, DMCA = Demineralized + decolorized + deacetylated, DMA = Demineralized + deacetylated.

NTYP = No true yield point. Means within the same column sharing same letters are not significantly different at p> 0.05.
5.3.2 Effects of Acids and Chitosans on Tensile Properties

Results (Table 5.1) indicate that both the type of chitosans and acids used influence the tensile properties of crawfish chitosan films. However, effects of acids on tensile properties were more prominent. The influences of acids and chitosan type on tensile strength and Young's modulus are easily observed in Figure 5.2 and Figure 5.3, respectively.

![Bar chart showing tensile strength of films made with different chitosan types and acids](image)

**Figure 5.2:** Tensile strength of films made with different crawfish chitosans and acids. Refer to Table 5.1 for chitosan abbreviations.

Films made with different acids showed significant (p<0.05) difference in tensile strength values with few exceptions. The acetate films generally had significantly (p<0.05) higher tensile strength values than the formate and citrate films. The acetate and formate films possessed comparable %E while the citrate films possessed significantly lower %E than both films. The Young's modulus values of films formed with different acids were also significantly (p<0.05)
different. The acetate films also possessed relatively higher Young's modulus than formate films indicating that acetic acid films are tougher than formic acid films. Films formed with citric acid were weakest with significantly (p<0.05) lower tensile strength, %E and Young’s modulus compared to acetate and formate films. This suggests that acetic and formic acid films are suitable to form flexible and tough films with crawfish chitosans without any plasticizers.

![Figure 5.3](image)

Figure 5.3: Young's modulus of films made with different crawfish chitosans and acids. Refer to Table 5.1 for chitosan abbreviations.

The type of chitosans used to form films also significantly influenced tensile strength and %E of the films. The Young's modulus was not significantly influenced by the type of chitosans used for a given acid. There was significant difference (p<0.05) between tensile strength values of the films obtained from the traditional method (DPMCA) and the non-traditional methods (DPMA, DMCA, and DMA). The chitosan obtained by the traditional extraction (DPMCA)
formed significantly (p<0.05) weaker films in terms of tensile strength when acetic acid was used. The traditional extraction method yielded chitosan (DPMCA) with lower molecular weight of 3584 Da (refer to Table 3.1); this may contribute to the weaker films. Increasing tensile strength values with increasing Mw of chitosans was observed by Muzzarelli and others (1977) and Park and others (2002). Higher molecular weight chitosans offer large numbers of amino and hydroxyl groups, resulting in increased numbers of hydrogen bonding which is attributed for formation of higher tensile strength films (Park and others 2002). Chitosans produced by non-traditional methods (DPMA, DMCA, and DMA) showing no significant (p<0.05) difference in their tensile strength values can be attributed to their close molecular weight (Table 3.1). The type of chitosans used also significantly (p<0.05) affected the elongation of the films, similarly observed for tensile strength for acetate and formate films.

5.3.3 Comparison of Crawfish Chitosan Films with Selected Biopolymer

The tensile strength, %E, and thickness for selected biopolymer and synthetic polymer films are presented in Table 5.2. Most biopolymers are soft and brittle in nature with lower tensile strength, %E, and Young’s modulus values. Addition of plasticizers to biopolymer relieves their brittleness and makes them tougher. The films made in the present study, especially those made with acetic acid, without a plasticizer possess much higher tensile strength values than most biopolymers (Table 5.2). However, their %E is lower then some biopolymers, indicating that they are not tougher. This can be attributed to added plasticizers with the biopolymers which make them tougher. Compared to the synthetic films such as LDPE, HDPE, Saran®, and cellophane, the chitosan acetate and formate acid films are harder with higher tensile strength values. However, their %E is significantly lower than that of synthetic polymers,
except for that of cellophane. Nevertheless, the tensile strength and %E for crawfish chitosan films are adequate enough to form hard yet flexible packaging materials.

Table 5.2: Comparison of tensile strength and percent elongation values for selected biopolymer and synthetic polymer films

<table>
<thead>
<tr>
<th>Film Type</th>
<th>Tensile strength (MPa)</th>
<th>%E</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat Gluten:Gly</td>
<td>2.6</td>
<td>276.2</td>
<td>Gennadios and others (1993)</td>
</tr>
<tr>
<td>Whey Protein Isolate: Gly</td>
<td>13.9</td>
<td>30.8</td>
<td>McHugh and Krochta (1994)</td>
</tr>
<tr>
<td>Zein:Oleic acid</td>
<td>6.81</td>
<td>3.18</td>
<td>Lai and Padua (1997)</td>
</tr>
<tr>
<td>Soy Protein Isolate: Gly</td>
<td>5.23</td>
<td>90.27</td>
<td>Brandenburg and others (1993)</td>
</tr>
<tr>
<td>Sodium caseinate: Gly</td>
<td>36.9</td>
<td>18.0</td>
<td>Chen (1995)</td>
</tr>
<tr>
<td>Calcium caseinate: Gly</td>
<td>4.25</td>
<td>1.4</td>
<td>Benerjee and others (1994)</td>
</tr>
<tr>
<td>Cellophane</td>
<td>114.0</td>
<td>20.0</td>
<td>Aydt and others (1991)</td>
</tr>
<tr>
<td>Saran®</td>
<td>27.6 – 62.1</td>
<td>20 - 150</td>
<td>Karel (1975)</td>
</tr>
<tr>
<td>HDPE</td>
<td>17.3 - 34.6</td>
<td>300</td>
<td>Brinston (1988)</td>
</tr>
<tr>
<td>LDPE</td>
<td>8.6 - 17.3</td>
<td>500</td>
<td>Brinston (1988)</td>
</tr>
<tr>
<td>Chitosan</td>
<td>82.0-135.8</td>
<td>25.0 - 45.0</td>
<td>Present study</td>
</tr>
</tbody>
</table>

Gly = Glycerol plasticizer

5.3.4 Comparison of Tensile Properties of Chitosan Films

Chitosan films formed with various organic acids with or without plasticizers are presented in the Table 5.3. The variations found in the tensile strength values can be attributed to differences in the chitosan type, concentrations of chitosan and acids, plasticizer type and content, and various testing conditions adopted in their studies. The chitosan films made without
Table 5.3: Comparison of tensile properties of chitosan films with organic acid.

<table>
<thead>
<tr>
<th>Film casting solvent</th>
<th>Tensile strength (MPa)</th>
<th>%E</th>
<th>Modulus (Mpa)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid:PL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.67</td>
<td>32.19</td>
<td>NA</td>
<td>Caner and others (1998)</td>
</tr>
<tr>
<td>Acetic acid:PL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.6-18.7</td>
<td>45.9-76.0</td>
<td>NA</td>
<td>Butler and others (1996)</td>
</tr>
<tr>
<td>Acetic acid:UP&lt;sup&gt;d&lt;/sup&gt;</td>
<td>39.47</td>
<td>37.44</td>
<td>NA</td>
<td>Miranda and others (2004)</td>
</tr>
<tr>
<td>Acetic acid:UP&lt;sup&gt;e&lt;/sup&gt;</td>
<td>67.6 – 70.3</td>
<td>6.2 – 7.1</td>
<td>NA</td>
<td>Kittur and others (1998)</td>
</tr>
<tr>
<td>Acetic acid:UP&lt;sup&gt;f&lt;/sup&gt;</td>
<td>57.1</td>
<td>6.7</td>
<td>1741</td>
<td>Begin and others (1999)</td>
</tr>
<tr>
<td>Acetic acid:UP&lt;sup&gt;g&lt;/sup&gt;</td>
<td>67.1</td>
<td>21.35</td>
<td>NA</td>
<td>Khan and others (2000)</td>
</tr>
<tr>
<td>Acetic acid:UP&lt;sup&gt;h&lt;/sup&gt;</td>
<td>68.8-150.2</td>
<td>4.1-7.6</td>
<td>NA</td>
<td>Park and others (2004)</td>
</tr>
<tr>
<td>Formic acid:PL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.86</td>
<td>27.99</td>
<td>NA</td>
<td>Caner and others (1998)</td>
</tr>
<tr>
<td>Formic acid:UP&lt;sup&gt;f&lt;/sup&gt;</td>
<td>74.3</td>
<td>6.4</td>
<td>1741</td>
<td>Begin and others (1999)</td>
</tr>
<tr>
<td>Citric acid:UP&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.9</td>
<td>37.9</td>
<td>183</td>
<td>Begin and others (1999)</td>
</tr>
<tr>
<td>Citric acid:UP&lt;sup&gt;h&lt;/sup&gt;</td>
<td>6.7-17.4</td>
<td>41.9-117</td>
<td>NA</td>
<td>Park and others (2004)</td>
</tr>
<tr>
<td>Lactic acid:UP&lt;sup&gt;h&lt;/sup&gt;</td>
<td>17.1-62.6</td>
<td>19.6-31.1</td>
<td>NA</td>
<td>Park and others (2004)</td>
</tr>
<tr>
<td>Lactic acid:UP&lt;sup&gt;f&lt;/sup&gt;</td>
<td>22.2*</td>
<td>26.2</td>
<td>683</td>
<td>Begin and others (1999)</td>
</tr>
<tr>
<td>Lactic acid:UP&lt;sup&gt;g&lt;/sup&gt;</td>
<td>59.8</td>
<td>67.1</td>
<td>NA</td>
<td>Khan and others (2000)</td>
</tr>
<tr>
<td>Malic acid:UP&lt;sup&gt;h&lt;/sup&gt;</td>
<td>27.4-62.4</td>
<td>17.8-29.9</td>
<td>NA</td>
<td>Park and others (2004)</td>
</tr>
<tr>
<td>Propionic acid:PL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.56</td>
<td>24.57</td>
<td>NA</td>
<td>Caner and others (1998)</td>
</tr>
</tbody>
</table>

PL = Plasticized, UP = Unplasticized, * = Yield strength, NA=Not available

<sup>a</sup> 3% chitosan in 1% acid with polyethylene glycol at 0.25 or 0.50 mL/g chitosan as plasticizer.
<sup>b</sup> 2% chitosan in 1% acetic acid (v/v) with glycerol, sorbitol, polyethylene glycol, Tween 60 and Tween 80 at 0.3% and 0.6% w/v concentrations as plasticizers. Tensile strength variations due to plasticizer contents.
<sup>c</sup> 3% chitosan in 1% acetic acid (w/v) with glycerol at 0.25 or 0.50 g/g chitosan as plasticizer.
<sup>d</sup> 2% chitosan in 1% acetic acid (v/v) without any plasticizers.
<sup>e</sup> 2% chitosan in 1% acetic acid (v/v) without any plasticizers.
<sup>f</sup> 1% chitosan in 2% acid.
<sup>g</sup> 1.4% chitosan (w/v) in 2% acid (w/v).
<sup>h</sup> 2% chitosan (w/v) in 2% acid. Tensile strength variations due to different molecular weight of chitosans.
a plasticizer show higher tensile strength and lower %E compared to those plasticized films. Plasticizers are known to reduce tensile strength and increase %E and make the films more flexible with reduced toughness (Butler and others 1996; Banker 1966).

Kittur and others (1998) and Park and others (2004) reported unplasticized acetic acid film with higher tensile strength values ranging from 67.6 - 70.3 and 68.8 - 150.2 MPa, respectively. Although they used double concentration of chitosan in their studies, their tensile strength values are comparable to those obtained for our present study (82.0 – 135.8 MPa). However, their films were brittle and not suitable for packaging applications owing to lower %E values (<8.0%). The acetic acid film formed with crawfish chitosan possessed higher %E in the range of 25.0 – 45.0%, showing flexibility and toughness suitable for packaging applications.

Begin and Calsteren (1999) prepared formate films with a tensile strength value of 74.3 MPa and %E of 6.4. The low %E value suggests film brittleness. However, our formate films made from crawfish chitosan possessed comparable tensile strength values of 76.8 – 114.8 MPa, but much higher %E in the range of 31.5 - 54.9. This suggests that the formic acid films formed with crawfish chitosan were tougher than the former.

The citric acid films made with crawfish chitosans were soft and brittle. However, relative to previously reported tensile strength values of citric acid films, they presented higher tensile strength values. This indicates that citric acid films made with crawfish chitosan are not suitable for packaging applications.

5.3.5 Puncture Strengths of Crawfish Chitosan Films

Table 5.4 lists the puncture strength of chitosan films made with different types of crawfish chitosans and acids. All crawfish chitosan films exhibited variations in puncture strengths, depending on the type of acid and chitosan used. The puncture strengths of the films
made with different acids were significantly (p<0.05) different. In general, formate films
possessed highest puncture strength, followed by acetate and citrate films. Formate films had
higher puncture strength than acetate films but their tensile strength was lower.

Table 5.4: Puncture strength of crawfish chitosan films

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Chitosan Type*</th>
<th>Puncture (N/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>DPMCA</td>
<td>342.75&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>1409.46&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>1074.08&lt;sup&gt;ed&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>1105.00&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formic acid</td>
<td>DPMCA</td>
<td>571.48&lt;sup&gt;fg&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>1871.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>1191.19&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>1232.92&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Citric acid</td>
<td>DPMCA</td>
<td>510.76&lt;sup&gt;fg&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>727.80&lt;sup&gt;er&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>787.97&lt;sup&gt;def&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>956.19&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*DPMCA = Deproteinized + demineralized + decolorized + deacetylated, DPMA = Deproteinized + demineralized + deacetylated, DMCA = Demineralized + decolorized + deacetylated, DMA = Demineralized + deacetylated.

Means within the same column sharing same letters are not significantly different at p >0.05.

The films formed with all chitosan types were significantly (p<0.05) different in puncture
strength, except for DMCA and DMA films. The acetic and formic films which were tougher in
terms of tensile strength values produced a clean hole upon puncture. The citric acid films, which
were brittle in terms of tensile strength values, were splitted along radius upon puncture. This
again indicates that citric acid films are not suitable for packaging film formation.
5.4 CONCLUSIONS

Crawfish chitosans can be formulated into thin and flexible films that are suitable for packaging applications using acetic and formic acids without any plasticizers. Unplasticized films made with crawfish chitosans were sufficiently hard and tough enough for packaging applications. The type of chitosans and acids used to form the films significantly influenced the tensile properties of the films. While acetic and formic acids form hard and tough films with crawfish chitosans, citric acid forms weak and soft films that are unsuitable for packaging application. Among the crawfish chitosans used, those produced by the traditional extraction method (DPMCA) always resulted in poor mechanical strength.

Mechanical strength of crawfish chitosan films can be optimized by selecting the type of film casting solvent and chitosan. Chitosan extracted from crawfish shell by the traditional extraction method yielded highly degraded chitosan and thus mechanically weaker films. All chitosan extracted by the nontraditional method formed films with acetic and formic acids and had mechanical properties appropriate for packaging purposes. However, an unusually higher swelling phenomenon observed in crawfish chitosan films limits their suitability for packaging purposes. Since absorption of moisture and swelling would impair mechanical properties of crawfish chitosan films, those films exhibiting minimum swelling should only be selected for packaging applications. Hence, DMCA (chitosan extracted without deproteinization) chitosan with acetic acid and DPMCA chitosan with formic acid, which showed minimal swelling and adequate mechanical strengths, can be considered for packaging purposes.

The mechanical properties of crawfish chitosan films found to be superior to many biopolymers and comparable to synthetic polymers. Selection of acid solvents and chitosan extraction methods, and omission of plasticizers are critical to optimize mechanical properties of
crawfish chitosan film. If suitably scaled-up, the process may lead to the production of crawfish chitosan based biopolymeric films for packaging purposes.

5.5 REFERENCES


CHAPTER 6

EFFICACY OF ANTIMICROBIAL EDIBLE FILMS
MADE OF ORGANIC SALTS OF CRAWFISH CHITOSAN
6.1 INTRODUCTION

Chitosan, the deacetylated derivative of chitin, possesses numerous versatile properties suitable for antimicrobial edible films. The inherent antimicrobial activity against a variety of bacteria, fungi, and viruses, good film-forming property, excellent biocompatibility, and non-toxicity of chitosan are ideal for the development of antimicrobial edible films. In addition, chitosan can also possibly be used to carry active substances such as antioxidants, nutrients, colorants, and flavors to form multifunctional films. The ability of chitosan to release added active substances in a controlled manner (Zambito and Colo 2003) is advantageous to formulate active antimicrobial edible films.

Contamination of food products by pathogenic bacteria has emerged as a serious public concern. Bacteria such as *Listeria monocytogenes, Bacillus cereus, Escherichia coli* (O157:H7), *Staphylococcus aureus, Salmonella typhimurium, Shigella sonnei* and *Vibrio vulnificus* are identified as serious and most frequently occurring food-borne pathogens in U.S.A (Mead and others 1999). Antimicrobial packaging is one promising approach to prevent growth of contaminated food-borne pathogens and spoilage microorganisms on the surface of food.

Chitosan may be combined with other antimicrobial substances for enhancing its antimicrobial efficacy. Since chitosan dissolves in slightly acidic solutions, a simple and economical way of enhancing antimicrobial properties of chitosan films would be to dissolve it in organic acids that possess antimicrobial properties (Begin and Calsteren 1999). Various organic acids that naturally occur in fruits and vegetables and possess general antimicrobial activity such as acetic, lactic, malic, and citric, sorbic, benzoic and succinic acids can be used for this purpose (Beuchat 1998).
Addition of different organic acids and their interaction with chitosan affect the film properties such as permeability and mechanical strength (Chen and others, 1996). Further, different acids cause different levels of antimicrobial activity in the presence of chitosan. The types of chitosan used to form films also affect film properties (Rout and Prinyawiwatkul 2001; Nadarajah and Prinyawiwatkul 2002). Therefore, it is necessary to select a chitosan and organic acid combination that provides maximal antimicrobial activities without compromising their mechanical properties.

Chitosan films containing acetic and propionic acids were effective against Enterobacteriaceae and Serratia liquefaciens on bologna, regular cooked ham, and pastrami (Ouattara and others 2000). Coma and others (2002) reported complete inhibition of Listeria monocytogenes inoculated onto a chitosan film containing acetic acid for 8 days. Similarly, 100% inhibition of Staphylococcus aureus, and Listeria monocytogenes, and 70% inhibition of Pseudomonas aeruginosa on a model agar medium by a chitosan film with acetic acid were reported by Coma and others (2003). Rodriguez and others (2003) showed that chitosan film with acetic acid delayed growth of Alternaria sp, Penicillium sp, and Cladosporium sp (Deuteromycetes) in precooked pizza. Review of publications on chitosan films and their antimicrobial properties reveal that (1) studies focused on the effect of organic acids on the antimicrobial properties fail to address physicochemical changes of chitosan films due to addition of organic acids, and (2) studies focused on the effect of organic acids on physicochemical and other properties but not the antimicrobial properties that may be changed due to addition of organic acids.

This study was intended to determine the antibacterial effect of various crawfish chitosan film formulations on selected food pathogenic bacteria. Since many properties of chitosan films
such as antimicrobial, physicochemical, permeability and mechanical properties are affected by the type of chitosan and acid used, all these properties should be individually studied. Results obtained from such studies can help formulate crawfish chitosan films with greater antimicrobial activity and desirable packaging properties.

6.1.1 Objective

The overall objective of the research was to assess antibacterial properties of crawfish chitosan films, made of different organic acids and chitosans, on selected food pathogenic bacteria: *Listeria monocytogenes, Bacillus cereus, Shigella sonnei, Escherichia coli* (O157:H7), *Staphylococcus aureus, Salmonella typhimurium* and *Vibrio vulnificus*.

6.2 MATERIALS AND METHODS

6.2.1 Materials

All pathogenic bacteria *Listeria monocytogenes, Bacillus cereus, Shigella sonnei, Escherichia coli* (O157:H7), *Staphylococcus aureus, Salmonella typhimurium* and *Vibrio vulnificus* were obtained from the Food Microbiology Laboratory, Department of Food Science, Louisiana State University, LA, U.S.A. Microbiological media, brain heart infusion (BHI), University of Vermont Medium (UVM) Listeria enrichment broth and nutrient agar were purchased from Difco Laboratories (Deitroit, MI, U.S.A.). Salmonella Shigella (SS) agar was purchased from Troy Biologicals, Inc. (Troy, MI, U.S.A). Citric (anhydride), formic and acetic acids were purchased from Sigma Chemical Co. (St. Louis, MO). Nisin (Nisaplin®) was obtained from Alpin and Barrett Ltd. (Dorset, U.K.). Polyvenyl chloride wrapping film (Catalog number 15-610) was purchased from Fisher Scientific (Tustin, CA). Crawfish chitosans were derived from crawfish shell waste using the traditional method and/or modified methods. The traditional method involves deproteinization (DP), demineralization (DM), decoloration (DC)
and deacetylation (DA) (No and Meyers 1995; No and others 2000). Four different chitosan samples were prepared using the traditional method involving all above steps (DPMCA), the traditional method excluding deproteinization (DMCA), the traditional method excluding decoloration (DPMA), and the traditional method excluding both deproteinization and decoloration (DMA).

**6.2.2 Characterization of Crawfish Chitosan**

The viscosity average molecular weight of crawfish chitosans was determined by the intrinsic viscosity using the Mark-Houwink equation ([η] = KM^a) where [η] is the intrinsic viscosity, M is the molecular weight, K=1.81 x 10^-3 and a=0.93 at 25°C (Maghami and Roberts 1988). Crawfish chitosan films deprotonated by methanolic ammonia was used to determine the degree of deacetylation with IR spectroscopy (M2000 FTIR spectrophotometer, Midac Corp., Irvine, CA, USA) and the equation proposed by Sabnis and Block (1997) was used to calculate degree of deacetylation values. Viscosity was determined for 1% chitosan in 1% acetic acid (w/v) using the Brookfield viscometer at 50 rpm and 25°C with a spindle number RV5.

The moisture content (%) s of the chitosans was determined gravimetrically in triplicate by drying samples at 100°C for 48 hours. Nitrogen (%) was determined by the combustion method using the Leco CHN analyzer (Model # FP-428, Leco Corporation, St. Joseph, Michigan, USA). The ash content (%) was determined according to the standard method # 923.03 (AOAC 1990).

**6.2.3 Film Preparation**

Four types of crawfish chitosans (DPMCA, DPMA, DMCA, and DMA) were separately dissolved with 1% of film casting solvents (acetic, ascorbic, lactic, formic, citric or malic acids). Each solution was vigorously agitated for 30 minutes, immersed in boiling water for 10 minutes,
cooled down to room temperature, and filtered through glass-wool to remove undissolved
particles. The film casting solution (300 mL) was cast onto a 31 × 31 cm Teflon coated plate and
allowed to dry at room temperature (23°C) for 48 hours. Dried films were carefully peeled out
and stored in desiccators containing saturated NaBr until further tested.

A micrometer (Model 293-766; Mitutoyo, Tokyo, Japan) was used to measure the film
thickness to the nearest 0.001 mm. Thickness of each film (mm) was measured at room
temperature (23°C and 45% RH) and expressed as an average of 10 random measurements and
standard deviation.

6.2.4 Conditioning of Films

All film specimens used for the antimicrobial tests were preconditioned at 23°C and
57.6% relative humidity inside desiccators containing saturated solutions of sodium bromide.
Prior to their use, all films were exposed to UV light for only 5 minutes to sterilize them, as
prolonged exposure to UV light could affect physicochemical properties of chitosan (Andrady
and others 1996; Maria and Adam 2002). To avoid post aging impact on films, films of 3 days
old were used.

6.2.5 Organisms and Culture Maintenance

Pure cultures of *Listeria monocytogenes* strain V7 (serotype ½ a), *Shigella sonnei* (*P
6129), *Escherichia coli* (O157:H7), *Staphylococcus aureus*, *Salmonella typhimurium*, *Bacillus
cerous*, and *Vibrio vulnificus* were maintained at -70°C. The bacteria were transferred to 10 mL
BHI broth by a sterile inoculation loop and inoculated at 37°C for 24 hours. On the second day,
10 μL of the bacterial suspension was transferred into 10 mL BHI broth and incubated at 37°C
for 18 hours.
6.2.6 Zone Inhibition Assays

Zone inhibition assay was performed as a preliminary step to screen the antibacterial activity of all crawfish chitosan film formulations, in an effort to select film formulations with high antibacterial activity against majority of test pathogens.

Soft agar was prepared by mixing 0.8 g nutrient agar and 3.75 g BHI in 100 mL of distilled water. After boiling, 10 mL of solutions were dispensed into test tubes and autoclaved. Before using, tubes containing soft agar were melted in a boiling bath, then tempered to 46°C. A 10 µL of bacterial inoculum from the 2nd-day culture grown in BHI broth was mixed with 10 mL soft agar and poured onto a prepoured BHI plate (10 mL/plate). After hardening, circular film disks (6 mm diameter) cut from each chitosan formulation were placed onto the soft agar.

Based on the results of zone inhibition assay and physicochemical and mechanical properties, at least one film from each film casting organic acid was selected for quantitative microbial analysis. The selected film formulations were also further subjected to zone inhibition tests with controls before being tested quantitatively. Chitosan solution prepared with 1% chitosan and 1% acetic, formic or citric acids (w/v), an acid solution prepared with 1% acetic, formic, or citric acid (w/v), and the nisin solution (positive control) prepared with approximately 0.2 mg/mL concentration served as controls. A 88 µL volume of above controls, calculated based on the material requirement to form 6 mm diameter film, was spotted on the surface of bacterial lawns. A 6 mm diameter polyvenyle chloride film was also used as a control. All lawns were incubated at 37°C for 24 hours and the bacterial lawn was visually examined for inhibition of the inoculated pathogenic bacteria. The thickness of the inhibition zone around the film disks was measured using a micrometer (Model 293-766; Mitutoyo, Tokyo, Japan).
6.2.7 Direct Inoculation Assay

Crawfish chitosan films selected based on the preliminary zone inhibition assay were taken for quantitative analysis. Direct inoculation assays were performed with selected Gram positive food pathogenic bacteria; *Listeria monocytogenes* and *Staphylococcus aureus* and Gram negative food pathogenic bacteria; *Salmonella typhimurium* and *Shigella sonnei*. The 2nd day bacterial cultures were diluted 10-fold using a phosphate buffer solution (PBS) of pH 7 and 15 µL aliquot of the diluted culture was inoculated onto each film disk (9 mm diameter) that had been previously placed aseptically into stomacher bag. The control was 15 µL of the diluted culture placed into stomacher bag without any disk. The stomacher bags were incubated at 37°C for 0, 2, 4, 8, 24 hours. These stomacher bags were stomached for 2 minutes after adding with a 985 µL PBS (pH 7). The suspension formed in the stomacher was drawn and serially diluted with PBS (pH 7) for plating.

The selective media used during this study was UVM spread plates for *Listeria monocytogenes*, SS agar spread plates for *Salmonella typhimurium* and *Shigella sonnei* and 3M Petrifilm plates for *Staphylococcus aureus*. These plates were incubated at 37°C for 24 hours. The 3M Petrifilms were kept at 62°C for 2 hours, inserted with reactive disks and incubated at 37°C for another 2 hours. Inhibition of bacteria by chitosan film was measured by the log reduction numbers. Bacterial counts were obtained and expressed as log number of colony-forming units per mL (CFU/mL).

6.2.8 Statistical Analysis

All zone inhibition assays were carried out in duplication. All other experiments were repeated 3 times with 2 replications per experiment. Treatments were considered significantly different at P > 0.05 using the ANOVA procedure (SAS, 2002). Where significant differences
were detected, treatment means were separated using the Tukey’s studentized range test at $\alpha = 0.05$.

6.3 RESULTS AND DISCUSSIONS

6.3.1 Zone Inhibition Test

Antibacterial activity of crawfish chitosan film formulations against 7 pathogenic bacteria was expressed in terms of zone inhibition. The zone inhibition assay revealed primarily three types of observations namely; defaced films without any clear or inhibition zones which could be attributed to the absence of any inhibitory activity, clear zones without inhibitory zones which could be attributed to bacteriostatic activity, and clear inhibition zone representing bactericidal inhibition by films.

It was observed that the chitosan acetate and chitosan formate film disks placed on lawns absorbed moisture, swelled and expanded radially, which could easily be mistaken for an inhibition zone. Hence straightforward measurement of the inhibition zone thickness based on an outer diameter of the inhibition zone and an initial film diameter would give erroneous readings. Therefore, thickness of clear zone was measured using a micrometer with stringent visual observation and the inhibition zone thickness was measured from the edge of the film.

The thickness of the inhibition zones measured is given in Table 6.1.

Among the films studied, the chitosan acetate films showed minimum inhibitory activity. All chitosan acetate films were defaced with *Listeria monocytogenes*. This is in agreement with the report of Coma and others (2002) who stated that the chitosan acetate films tested on *Listeria monocytogenes* lawns failed to produce an inhibition zone. Also reported was the inability of chitosan solutions (chitosan in acetic acid) to produce inhibitory zones in agar medium containing *Listeria monocytogenes*, which was attributed to the limitation of chitosan solution to
Table 6.1: Effect of chitosan films on selected food pathogenic bacteria.

<table>
<thead>
<tr>
<th>Film casting solvent</th>
<th>Chitosan type</th>
<th>Thickness of inhibition zone (mm)</th>
<th>Listeria monocytogenes</th>
<th>Staphylococcus aureus</th>
<th>Bacillus cereus</th>
<th>Shigella sonnei</th>
<th>Salmonella typhimurium</th>
<th>Escherichia coli O157:H7</th>
<th>Vibrio vulnificus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>DPMCA</td>
<td>+</td>
<td>0.55</td>
<td>1.56</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>+</td>
<td>1.64</td>
<td>0.89</td>
<td>0.83</td>
<td>0.99</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>+</td>
<td>1.56</td>
<td>0.49</td>
<td>+</td>
<td>1.12</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>+</td>
<td>1.48</td>
<td>1.17</td>
<td>1.52</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Formic acid</td>
<td>DPMCA</td>
<td>+</td>
<td>2.26</td>
<td>1.33</td>
<td>1.40</td>
<td>1.03</td>
<td>1.03</td>
<td>0.78</td>
<td>6.00</td>
</tr>
<tr>
<td></td>
<td>DPMA</td>
<td>+</td>
<td>2.33</td>
<td>4.05</td>
<td>1.36</td>
<td>1.36</td>
<td>1.53</td>
<td>1.24</td>
<td>5.88</td>
</tr>
<tr>
<td></td>
<td>DMCA</td>
<td>+</td>
<td>1.85</td>
<td>4.13</td>
<td>1.52</td>
<td>0.52</td>
<td>1.19</td>
<td>1.11</td>
<td>5.77</td>
</tr>
<tr>
<td></td>
<td>DMA</td>
<td>+</td>
<td>1.75</td>
<td>5.75</td>
<td>1.01</td>
<td>1.54</td>
<td>1.31</td>
<td>1.04</td>
<td>5.74</td>
</tr>
</tbody>
</table>

a Values are means of duplicate analysis.
b DPMCA = Deproteinized + demineralized + decolorized + deacetylated, DPMA = Deproteinized + demineralized + deacetylated, DMCA = Demineralized + decolorized + deacetylated, DMA = Demineralized + deacetylated.
+ clear zone without any inhibition.
- no inhibition.
diffuse in agar medium. However, it was reported that 8% film-forming solution (chitosan in acetic acid) incorporated in agar medium (v/v) completely inhibited *Listeria monocytogenes*. Chitosan acetate films also defaced with *Bacillus cereus*, and *Vibrio vulnificus* lawns indicating they were ineffective in controlling these bacteria. However, all chitosan acetate films showed bacteriostatic effects against *Staphylococcus aureus*, *Shigella sonnei*, *Salmonella typhimurium*, and *Escherichia coli O157:H7* as indicated by their clear zones in lawns. The chitosan formate films were also ineffective in controlling *Bacillus cereus*, and *Vibrio vulnificus* as indicated by defaced films by these bacterial lawns. Nevertheless, the chitosan formate films showed inhibitory effects against *Staphylococcus aureus*, *Shigella sonnei*, *Salmonella typhimurium*, and *Escherichia coli O157:H7* and bacteriostatic activity against *Listeria monocytogenes*. However, compared to the chitosan citrate films, the inhibitory effect of chitosan formate films were lower and the thickness of the inhibitory zone was in the range of 0.49 to 1.64 mm compared to 0.78 to 6.0 mm of chitosan citrate films.

Among the crawfish chitosan films studied, all chitosan citrate films exhibited prominent inhibitory effect on all 7 pathogenic bacteria (Table 6.1). All chitosan citrate films showed distinctive inhibition zones against all pathogenic bacteria tested (Figure 6.1) and the inhibition zones were considerably thicker than those produced by chitosan formate films. Also, the inhibitory effects of chitosan citrate films were remarkably higher for *Staphylococcus aureus* and *Vibrio vulnificus* as indicated by thicker inhibition zones accounting for more than 4 mm (Figure 6.2). The chitosan citrate films were the only films with antimicrobial effects against *Bacillus cereus*, and *Vibrio vulnificus*. The higher inhibitory activity shown by all chitosan citrate films can be attributable to complete solubility of chitosan which could make them more reactive against bacterial cells.
Figure 6.1: Inhibition of food pathogenic bacteria by chitosan citrate films. In each lawn, DPMA (top left), DPMCA (top right), DMA (bottom left), and DMCA (bottom right) chitosan citrate films are shown.
Figure 6.2: Inhibition zones produced by crawfish chitosan films. In each bacterial lawn, top row – acetate films, middle row – citrate films, and bottom row – formate films. In each row from left to right DMA, DMCA, DPMA, and DPMCA chitosan films.
Based on higher antibacterial activity, lower swelling ratio and higher mechanical strengths, acetate films with DMCA chitosan, formate films with DPMCA chitosan and citrate films with DMA chitosan were selected for quantitative analysis of antimicrobial activity. The above selected films were also tested more with inhibition zone assays with more controls. The nisin spots used as the positive control produced more prominent clear zones with *Listeria monocytogenes*, and *Staphylococcus aureus* lawns representing the Gram positive bacteria and vague spots with *Shigella sonnei* and *Salmonella typhimurium* lawns representing the Gram negative bacteria. Regardless of the type of bacteria, controls such as chitosan solutions, acid solutions and the polyvinyl chloride plastic failed to produce any clear or inhibition zones indicating there were ineffective in inhibiting the above food pathogenic bacteria. This substantiates the claim that the direct application of antimicrobial agents, such as chitosan and acids solutions used in our studies, onto food surfaces is less effective due to loss of antimicrobial activity caused by leaching onto the food, enzymatic activity, and reaction with other food components (Jung and others 1992; Ray 1992; Ouattara and others 2000). Hence, use of packaging films or coating as a matrix to deliver antimicrobial agents becomes important. Such packaging or coating can maintain a high concentration of antimicrobial agents on a food surface and allows low migration into food (Torres and others 1985; Siragusa and Dickson 1992; Ouattara and others 2000).

### 6.3.2 Direct Inoculation Assay

Results of the direct inoculation study were in agreement with the inhibition zone assays. Table 6.2 shows the survivor log number CFU/mL of *Listeria monocytogenes* inoculated onto the surface of selected chitosan films (DMCA acetate, DPMCA formate and DMA citrate).
Listeria monocytogenes was more susceptible to chitosan citrate film than chitosan formate or chitosan acetate films. Chitosan citrate film reduced the bacterial count by 5.34 log CFU/mL within 4 hours of incubation (Table 6.3). Chitosan citrate films accounted for more than 4.47 log CFU/mL reduction of inoculum in 24 hours. Chitosan acetate films caused only marginal reduction of the inoculum, accounting for less than 1 log CFU/mL reduction over the entire 24-hour period incubation. The chitosan formate films caused about 1 log CFU/mL reduction of inoculum at 2 hours of incubation and maintained a 1 log CFU/mL reduction over 24 hours of incubation. The rate of reduction of microbial count was poor with both chitosan acetate and formate films as there was no significant difference in microbial count between 2 and 4-hours incubation and between 4 and 8-hour incubation as shown in Table 6.3.

Organic acids with smaller molecular weight have higher antimicrobial activity and undissociated smaller molecules of formic (46.03 Dalton) and acetic (60.05 Dalton) acids may...
enter into the bacterial cells easily to change the internal pH of the organisms (Eswaranandam and others 2004). Undissociated larger molecules of citric acid (192.13 Dalton) may not enter into the cells effectively. However, this trend was not observed in the present study and the result was in contrary. It indicates that chitosan films made of organic acids may behave as one entity rather than separate entities, i.e., as a career matrix containing an antimicrobial agent.

Table 6.3: Log reduction of *Listeria monocytogenes* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 Hour</th>
<th>2 Hour</th>
<th>4 Hour</th>
<th>8 Hour</th>
<th>24 Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>7.24 ± 0.10</td>
<td>7.48 ± 0.12</td>
<td>7.34 ± 0.05</td>
<td>7.15 ± 0.21</td>
<td>6.47 ± 0.08</td>
</tr>
<tr>
<td>Acetate film</td>
<td>0.10 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32 ± 0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.24 ± 0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.28 ± 0.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.03 ± 0.10&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formate film</td>
<td>0.15 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.15 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.08 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.09 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.65 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Citrate film</td>
<td>0.21 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.46 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt; 5.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt; 5.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt; 4.47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*All analyses were based on three separate experiments with each mean ± standard deviation being an average of three determinations. Means within each vertical column followed by the same letters are not significantly different (P> 0.05).
† Log survivors of *Listeria monocytogenes* inoculated at 7.24 ± 0.10 (mean ± standard error) CFU/mL without any film was used as control.

Several studies have demonstrated that antimicrobial edible films can reduce bacterial levels on meat products. Siragusa and Dickson (1992, 1993) showed that organic acids were more effective against *L. monocytogenes* on beef carcass tissue when immobilized in calcium alginate than when used as a spray or dip. In another study, Hoffman and others (2001) reported that zein films, impregnated with nisin, lauric acid and EDTA and tested with broth cultures of *L. monocytogenes*, reduced the bacterial counts over 5 logs after 48 hours. Janes and others (2002) reported zein films containing nisin produced a 4.5 to 5 log reduction on *L. monocytogenes*
inoculated onto chicken breast tenders refrigerated for 16 days. Ming and others (1997) impregnated the surface of meat casing with pediocin powder to produce a 1 to 3 log reduction of *L. monocytogenes* on ham, turkey breast, and beef compared to inoculated controls. In most of these studies, antibacterial activity against *L. monocytogenes* was attempted with added antimicrobials. Considering this, the chitosan citrate film producing more than a 4.4 log reduction in *L. monocytogenes* is a commendable achievement.

Effect of crawfish chitosan films on *Staphylococcus aureus* is shown in Table 6.4. As with the case *Listeria monocytogenes*, the chitosan citrate films showed higher antibacterial activity against *Staphylococcus aureus*. The chitosan citrate films produced more than a 5 log reduction in *Staphylococcus aureus* within 4 hours of incubation and maintained its inhibitory effect throughout the incubation period (Table 6.5). The chitosan acetate films produced a poor inhibition with less than 1 log reduction at 24 hours. The chitosan formate films maintained about 1 log reduction for up to 4 hours. At 24-hour incubation chitosan formate films produced more than a 5 log reduction similarly observed for chitosan citrate films.

Relatively very little research work has been dedicated to formulate edible films active against *Staphylococcus aureus*. Ha and others (2001) reported that polyethelene film containing grapefruit seed extract showed an inhibitory effect against *Staphylococcus aureus* as indicated by a 2.5 to 7.0 mm inhibition zone by the agar diffusion method. Scanell and others (2000) reported about 1.5 log and 2.8 log reduction of *Staphylococcus aureus* in cheese and ham by nisin-absorbed bioactive inserts. Coma and others (2001) reported that an edible packaging made of cellulosic esters, fatty acids and nisin produced up to 88 mm diameter inhibition zone on *Staphylococcus aureus*. Further, they reported that addition of fatty acid reduced the inhibitory activity. All these studies indicate the importance of having added antimicrobials in the films to
Table 6.4: Recovery of *Staphylococcus aureus* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log CFU/mL*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Hour</td>
</tr>
<tr>
<td>Control†</td>
<td>7.29 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetate film</td>
<td>7.24 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formate film</td>
<td>7.10 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Citrate film</td>
<td>6.81 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*All analyses were based on three separate experiments with each mean ± standard deviation being an average of three determinations. Means within each vertical column followed by the same letters are not significantly different (P> 0.05).

†An inoculum of *Staphylococcus aureus* at 7.29 ± 0.10 (mean ± standard error) CFU/mL without any film was used as control.

ND = Not detected.

Table 6.5: Log reduction of *Staphylococcus aureus* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log CFU/mL*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Hour</td>
</tr>
<tr>
<td>Control†</td>
<td>7.29 ± 0.10</td>
</tr>
<tr>
<td>Acetate film</td>
<td>0.06 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formate film</td>
<td>0.19 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Citrate film</td>
<td>0.48 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*All analyses were based on three separate experiments with each mean ± standard deviation being an average of three determinations. Means within each vertical column followed by the same letters are not significantly different (P> 0.05).

† Log survivors of *Staphylococcus aureus* inoculated at 7.29 ± 0.10 (mean ± standard error) CFU/mL without any film was used as control.
control *Staphylococcus aureus*. However, the crawfish chitosan citrate and formate films which contained no added antimicrobials could produce more than a 5 log reduction. Further, the inhibitory effect of chitosan citrate and formate films against *Staphylococcus aureus* was higher than that of *Listeria monocytogenes*.

Along with *Listeria monocytogenes*, *Salmonella typhimurium* has been considered as a microbiological hurdle for a long time. The effect of crawfish chitosan films against *Salmonella typhimurium* is shown in Table 6.6. As with *Listeria monocytogenes* and *Staphylococcus aureus*, a similar trend of inhibition was observed with *Salmonella typhimurium*. The chitosan citrate films produced more than 3.4 log reduction in *Salmonella typhimurium* within 2 hours of incubation, and reduction in counts reached 3.85 log at 4-hour and 4.83 log at 8-hour incubation (Table 6.7). The chitosan acetate films were less effective with about 1 log reduction at 24 hours.

Table 6.6: Recovery of *Salmonella typhimurium* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2 Hour</th>
<th>4 Hour</th>
<th>8 Hour</th>
<th>24 Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>6.97 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.91 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.83 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.76 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetate film</td>
<td>6.11 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.00 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.86 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.71 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formate film</td>
<td>4.26 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.05 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.86 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.06 ± 0.32&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Citrate film</td>
<td>3.47 ± 0.25&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.05 ± 0.40&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*All analyses were based on three separate experiments with each mean ± standard deviation being an average of three determinations. Means within each vertical column followed by the same letters are not significantly different (P> 0.05).
†An inoculum of *Salmonella typhimurium* at 6.98 ± 0.06 (mean ± standard error) CFU/mL without any film was used as control.
ND = Not detected.
There was no significant \((p>0.05)\) change in the *Salmonella typhimurium* count from 2-hour to 24-hour for chitosan acetate film. The chitosan formate films maintained about \(2.7\) log reduction up to 8 hours and then produced a significant by \((p<0.05)\) increased inhibition \((3.7\) log\) between 8-hour and 24-hour incubation.

Table 6.7: Log reduction of *Salmonella typhimurium* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2 Hour</th>
<th>4 Hour</th>
<th>8 Hour</th>
<th>24 Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>6.97 ± 0.06</td>
<td>6.91 ± 0.05</td>
<td>6.83 ± 0.04</td>
<td>6.76 ± 0.10</td>
</tr>
<tr>
<td>Acetate film</td>
<td>0.86 ± 0.14(c)</td>
<td>0.91 ± 0.15(c)</td>
<td>0.96 ± 0.05(c)</td>
<td>1.05 ± 0.11(c)</td>
</tr>
<tr>
<td>Formate film</td>
<td>2.71 ± 0.09(b)</td>
<td>2.86 ± 0.10(b)</td>
<td>2.87 ± 0.07(b)</td>
<td>3.7 ± 0.29(b)</td>
</tr>
<tr>
<td>Citrate film</td>
<td>3.49 ± 0.24(a)</td>
<td>3.85 ± 0.37(a)</td>
<td>&gt; 4.83(a)</td>
<td>&gt; 4.76(a)</td>
</tr>
</tbody>
</table>

*All analyses were based on three separate experiments with each mean ± standard deviation being an average of three determinations. Means within each row followed by the same letters are not significantly different \((P> 0.05)\).

†Log survivors of *Salmonella typhimurium* inoculated at 6.98 ± 0.06 (mean ± standard error) CFU/mL without any film was used as control.

Effects of edible films on *Salmonella typhimurium* have been reported earlier. Natrajan and Sheldon (2000) demonstrated a 4.3 log reduction of *Salmonella typhimurium* on inoculated broiler skin exposed to nisin coated polyvinyl chloride film. Sheldon and others (1996) reported a 4.23 log reduction of *Salmonella typhimurium* in pads treated with nisin formulations. Sheldon (2001) applied nisin formulations to *Salmonella typhimurium* inoculated on tray pads and demonstrated 3.1 log reduction. Compared to these published data, reduction of *Salmonella typhimurium* in the present study by more than 4.7 log by chitosan citrate film and 3.7 log by chitosan formate film is outstanding.
Effect of crawfish chitosan films against *Shigella sonnei* is shown in Table 6.8. As with *Listeria monocytogenes*, and *Staphylococcus aureus*, chitosan acetate films produced minimal inhibition against *Shigella sonnei*. The chitosan formate films accounted for about 1 log reduction at 4 hours of incubation and 2.6 log reduction at 24 hours. The citrate films showed the highest antibacterial activity against *Shigella sonnei* with more than 5 log reduction at 8 hours of incubation (Table 6.9).

Table 6.8: Recovery of *Shigella sonnei* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log CFU/mL*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 Hour</td>
</tr>
<tr>
<td>Control†</td>
<td>7.04 ± 0.08a</td>
</tr>
<tr>
<td>Acetate film</td>
<td>6.88 ± 0.09a</td>
</tr>
<tr>
<td>Formate film</td>
<td>6.71 ± 0.14a</td>
</tr>
<tr>
<td>Citrate film</td>
<td>4.94 ± 0.36b</td>
</tr>
</tbody>
</table>

*All analyses were based on three separate experiments with each mean ± standard deviation being an average of three determinations. Means within each vertical column followed by the same letters are not significantly different (P> 0.05).
†An inoculum of *Shigella sonnei* at 7.01 ± 0.06 (mean ± standard error) CFU/mL without any film was used as control.
ND = Not detected.

### 6.4 CONCLUSIONS

This study confirms that crawfish chitosan can be used to develop antimicrobial edible films effective against both Gram positive and Gram negative food pathogenic bacteria. Chitosan acetate films showed poor inhibitory effect against *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Shigella sonnei*. Although chitosan acetate films
outweighed other films in terms of their mechanical properties, they demonstrated minimal antibacterial effects similar to bacteriostatic effects with negligible bacterial reduction over a period of 24 hours.

Table 6.9: Log reduction of *Shigella sonnei* survivors from crawfish chitosan film with different organic acids incubated at 37°C for 24 hours.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2 Hour</th>
<th>4 Hour</th>
<th>8 Hour</th>
<th>24 Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>7.04 ± 0.08</td>
<td>7.08 ± 0.06</td>
<td>7.10 ± 0.04</td>
<td>7.13 ± 0.04</td>
</tr>
<tr>
<td>Acetate film</td>
<td>0.16 ± 0.09b</td>
<td>0.24 ± 0.09c</td>
<td>0.20 ± 0.11c</td>
<td>0.13 ± 0.07c</td>
</tr>
<tr>
<td>Formate film</td>
<td>0.33 ± 0.14b</td>
<td>1.04 ± 0.10b</td>
<td>1.21 ± 0.06b</td>
<td>2.59 ± 0.22b</td>
</tr>
<tr>
<td>Citrate film</td>
<td>2.11 ± 0.36a</td>
<td>3.78 ± 0.43a</td>
<td>&gt; 5.10a</td>
<td>&gt; 5.13a</td>
</tr>
</tbody>
</table>

*All analyses were based on three separate experiments with each mean ± standard deviation being an average of three determinations. Means within each row followed by the same letters are not significantly different (P> 0.05).†Log survivors of *Shigella sonnei* inoculated at 6.98 ± 0.06 (mean ± standard error) CFU/mL without any film was used as control.

Chitosan formate films were effective against *Staphylococcus aureus*, *Salmonella typhimurium*, and *Shigella sonnei*, causing more than 5, 3.7 and 2.5 log reduction at 24 hour incubation, respectively. Chitosan formate films produced poor inhibitory effect against *Listeria monocytogenes* with less than 1 log reduction at 24-hour incubation. Based on antibacterial and packaging properties, chitosan formate films can be used as antibacterial packaging to control *Staphylococcus aureus, Salmonella typhimurium, and Shigella sonnei*, except *Listeria monocytogenes*.

Chitosan citrate films were highly effective against *Listeria monocytogenes*, *Staphylococcus aureus, Salmonella typhimurium*, and *Shigella sonnei*. The effect of chitosan
citrate films against *Listeria monocytogenes* and *Staphylococcus aureus* was prominent with more than 5 log reduction within 4 hours of incubation. Furthermore, chitosan citrate films indicated its potential antibacterial effect against *Bacillus cereus* and *Vibrio vulnificus* as indicated by the zone inhibition tests. This study indicates the possibility of formulating an antibacterial edible film, especially crawfish chitosan citrate film, active against a broad spectrum of bacteria.

6.5 REFERENCES


CHAPTER 7

CONCLUSIONS
Development of antimicrobial films from biopolymers has been a challenge. Many critical factors such as film-forming ability, mechanical strength, gas and water barrier properties, storage stability, and antimicrobial properties have to be optimized in order to successfully develop an antimicrobial film.

The film-forming ability of crawfish chitosans varied with different film casting organic acids. Acetic, formic, and citric acids could be used as a film casting solvent to produce flexible and transparent films, resembling plastic films. Malic, lactic, and ascorbic acids were not recommended due to the undesirable hydrophilic nature or brittleness of the film produced.

Among many factors, the swelling and solubility of chitosan films in water were very critical in selecting film formulations for packaging requirements. Chitosan citrate films, which completely solubilized in water, may be utilized for edible film formation but not for packaging applications. Films formed with DMCA acetate and DPMCA formate, which had the lowest swelling can be selected for packaging applications.

Film casting solvents and chitosan types significantly influenced the sorption behavior of crawfish chitosan films. On average, chitosan formate films adsorbed more moisture than chitosan acetate or citrate films. While acetate and formate films maintained their flexibility, the citrate films exhibited brittleness at the lower water activity levels. The sorption behavior of crawfish chitosan films could be best predicted by the GAB model ($R^2 = 0.97-0.98$). Analysis of sorption isotherms indicated that clustering of water molecules took place when water activity exceeded 0.57.

The water vapor permeability of crawfish chitosan films was significantly influenced by the chitosan types and film casting solvents used to form the films. The chitosan citrate films
showed the lowest WVP, followed by chitosan acetate films. The highest WVP was obtained for DPMCA chitosan formate films.

The type of chitosan and acid used to form the films significantly influenced the tensile properties of the films. While acetic and formic acids formed hard and tough films with crawfish chitosans, citric acid formed weak and soft films that are unsuitable for packaging application. The mechanical properties of crawfish chitosan films found to be superior to many biopolymers and comparable to synthetic polymers.

The crawfish chitosans can be used to develop antimicrobial edible films effective against both Gram positive and Gram negative food pathogenic bacteria, especially when citric acid was used as a film casting solvent. Chitosan acetate (DMCA) films showed poor inhibitory effect against *Listeria monocytogenes, Staphylococcus aureus, Salmonella typhimurium,* and *Shigella sonnei.* Chitosan formate (DPMCA) films were effective against *Staphylococcus aureus,* *Salmonella typhimurium,* and *Shigella sonnei,* causing more than 5, 3.7 and 2.5 log reduction at 24 hour, respectively.

Chitosan citrate films were effective against *Listeria monocytogenes, Staphylococcus aureus, Salmonella typhimurium,* and *Shigella sonnei,* and produced 4.4, 5.6, 4.7, and 5.1 log reduction in 24 hours, respectively. The effect of chitosan citrate films against *Listeria monocytogenes* and *Staphylococcus aureus* was prominent with more than 5 log reduction observed within 4 hours of incubation. Furthermore, chitosan citrate films indicated its potential antibacterial effect against *Bacillus cereus* and *Vibrio vulnificus* as indicated by the zone inhibition tests.

This study indicated the possibility of formulating antibacterial edible films active against a broad spectrum of bacteria using selected crawfish chitosans and formic and citric acid as film
casting solvents. Crawfish formate films, with desirable mechanical properties, would be recommended for the formation of antimicrobial packaging film while the citrate films with their complete solubility were more suitable for antimicrobial edible film formation.
VITA

The author is native to Hatton, a small town in the hill county of Sri Lanka. He received his primary and secondary education from the Highlands College, Hatton, Sri Lanka, and entered University of Peradeniya in 1990. He received his Bachelor of Science degree in agricultural science while concentrating in food engineering in 1994, and his Master of Science degree in post harvest technology of fruits and vegetables in 1997 both from the University of Peradeniya, Peradeniya, Sri Lanka.

The author served as a lecturer in food science and technology at the Sabaragamuwa University of Sri Lanka from 1996 to 2000. He joined the Department of Food Science, Louisiana State University, in August 2000, to pursue his doctoral studies. He received a Doctor of Philosophy degree in food science with areas of concentration in food engineering and food microbiology.

The author is a son of Mr. and Mrs. Annamalay Kandasamy and the brother of Puwaneswary, Manohar, Shivakumar, and Bharathy. He is also the husband of Radha Subburathinam.