Proteins Derived Bionanocomposites from Poultry By-Product for Food Packaging Applications

by

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ABSTRACT

Spent hens, a poultry by-product, have little marketplace and their disposal methods are infeasible so to find alternative uses which are environmentally safe is prudent. In this study, proteins were extracted from spent hen by alkali aided extraction method with high recovery (74%) and purity (96%). For the preparation of proteins derived bionancomposite films, the types and ratio of different plasticizers (glycerol, sorbitol, ethylene glycol, poly(ethylene) glycol, butanediol), and chitosan as a cross linker were optimized. Glycerol was found a compatible plasticizer with 3% chitosan. Further, three nanoparticles (bentonite, glycidyl POSS and cellulose nanocrystals) with different concentration were used to evaluate the mechanical strength of the prepared bionancomposite films. The bentonite (5%) with 40% glycerol gave better results among all nanoparticles with mechanical strength of 11.37 MPa. While CNCs (5%) and glycidyl POSS (3%) provided the maximum mechanical strength of 6.86 MPa, and 6.47 MPa respectively. All protein derived bionancomposite films were characterized by the transmission electron microscopy (TEM), thermal gravimetric analysis (TGA), dynamic mechanical analysis (DMA), differential scanning calorimetry (DSC), X-ray diffraction (XRD) and attenuated total reflectance- fourier transform infrared spectroscopy (ATR- FTIR). The results show that a good intercalation and/or exfoliation of the protein biopolymers into clay interlayer galleries was observed leading to improved thermal, mechanical and barrier properties. These observations provided an important basis in the experimental design of high performance bionancomposite films for food packaging applications.

PREFACE

This thesis contains original work done by Muhammad Zubair and has been written according to the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research at the University of Alberta. The concept of this thesis originated from my supervisor Dr. Aman Ullah.

The thesis consisted of three chapters: Chapter 1 provides a general introduction on the context and the objectives of the thesis; Chapter 2 is a protein extraction from whole spent hen; Chapter 3 contains the work on preparation and characterization of the spent hen proteins derived bionanocomposites for food packaging applications.

Dr. Aman ullah and Dr. Jianping Wu greatly contributed to the experimental design, data interpretation, thesis preparation and edits. I was responsible for literature search relevant for the above studies, designing and performing laboratory experiments, data collection and analysis, and drafting the thesis.

DEDICATION

Dedicated to my beloved parents

ACKNOWLEGDEMENTS

There are many people who encouraged and supported me throughout my work, for which I am forever grateful to. First and foremost, I would like to express my sincere gratitude for my supervisor Dr. Aman Ullah for offering me this opportunity to pursue graduate study and for his devoted time and efforts throughout my degree. I am deeply grateful to my co-supervisor Dr. Jianping Wu for his invaluable motivation and intellectual support.

I am indebted to my dear parents, for their unconditional love, trust and encouragements throughout my life. I would also like to thank my brother and sisters. I am so blessed to have them always by my side. I will not be able to accomplish this without their love and support.

TABLE OF CONTENTS

ABSTRACT ii
PREFACE iii
DEDICATION iv
ACKNOWLEDGEMENTS v
LIST OF TABLES ix
LIST OF FIGURES x
CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW 1
1.1. Introduction 1
1.2. Biopolymers and their categories 3
1.3. Alternate source for food packaging production 5
1.4. Spent hen overview 6
1.4.1. Composition of spent hen meat 7
1.4.2. Muscle Proteins 7
1.4.3. Problems related to disposal of spent hens 9
1.4.4. Protein extraction from spent hens 10
1.5. Proteins derived bionanocomposites 13
1.5.1. Proteins 13
1.5.2. Main proteins used as a matrix 15
1.5.3. Main nanoparticles used as fillers in proteins derived bionanocomposites 20
1.5.4. Processing strategies for proteins derived bionanocomposites preparation 24
1.5.5. Common factors affecting proteins film formation 29
1.5.6. Film Properties 32
1.6. Potential application of spent hen proteins 37
1.7. Conclusion and research objectives 37
1.8. References 39

CHAPTER 2. PROTEIN EXTRACTION FROM WHOLE SPENT HEN 51
2.1. Introduction 51
2.2. Experimental section 54
2.2.1. Materials 54
2.2.2. Proximate analysis of raw material 54
2.2.3. Moisture content analysis of raw material 54
2.2.4. Determination of total fat contents 54
2.2.5. Determination of ash contents 55
2.2.6. Protein extraction from whole spent hen by salt aided method 55
2.2.7. Alkali aided extraction from whole spent hen 56
2.2.8. Preparation of proteins isolate at pH 11.5 57
2.2.9. Purity and recovery of isolated proteins 57
2.2.10. Collagen extraction 57
2.2.11. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) 58
2.2.12. Statistical analysis 58
2.3. Results and discussions 67
2.3.1. Water, protein, lipid and ash contents of raw material and protein isolates 67
2.3.2. Protein extraction by salt aided extraction 67
2.3.3. Protein Solubility 68
2.3.4. Protein purity and recovery 69
2.3.5. Lipid loss during alkali aided extraction 70
2.3.6. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS- PAGE) 70
2.3.6. Collagen extraction, recovery and purity 71
2.4. Conclusion 72
2.5. References 73
CHAPTER 3. SPENT HEN PROTEIN DERIVED BIONANOCOMPOSITES FOR FOOD PACKAGING APPLICATIONS 77
3.1. Introduction 77
3.2. Experimental section 79
3.2.1. Materials 79
3.2.2. Sample preparation 79

3.2.3. Film Preparation 80
33.2.4. Film Thickness 80
3.2.5. Thermal Property Measurement 80
3.2.6. Mechanical Property Measurement 80
3.2.7. X-Ray Diffraction (XRD) 81
3.2.8. Dynamic Mechanical Analysis (DMA) 81
3.2.9. Transmission Electron Microscopy (TEM) 81
3.2.10. ATR- FT-IR Analysis 81
3.2.11. Water vapor permeability (WVP) 82
3.3. Results and discussions 82
3.3.1. Structural analysis 82
3.3.2. Crystalline study of bionancomposite films 85
3.3.3. Effects on thermal properties 86
3.3.4. Thermal stability 88
3.3.5. Mechanical strength 90
3.3.6. Transmission electron microscopy analysis 93
3.3.7. Viscoelastic properties 95
3.3.7. Water vapor permeability (WVP) 97
3.4. Conclusion 98
3.5. References 100
BIBLIOGRAPHY 117

LIST OF TABLES

Table 1.1 Raw spent hen and broiler muscle meat chemical composition………………….8

Table 1.2 Mechanical properties of proteins derived bionanocomposites…………………34

Table 2.1 Proximate analysis of raw spent hen…………………………………………….67

Table 2.2 Protein purity and recovery by salt aided extraction……………………………68

Table 2.3 Protein purity and recovery by alkali aided extraction………………………….69

Table 2.4 Proximate analysis of spent hen feet…………………………………………….71

Table 3.1 Stress and strain of blend films without nanoparticles………………………….92

Table 3.2 Stress and strain of blend films with nanoparticles…………………………......92

Table 3.3 Water vapor permeability of films………………………………………………98

LIST OF FIGURES

Figure 1.1 Categories of biopolymer……………………………………………………………..5

Figure 1.2 Overview of the pH shift process..12

Figure 1.3 Schematic representation for proteins derived bionanocomposites by wet process....26

Figure 1.4 Schematic representation for proteins derived bionanocomposites by dry process....28

Figure 1.5 Schematic representation for proteins derived bionanocomposites by two- step process………………………………………………………………………………...............29

Figure 2.1 Schematic diagram of the sarcoplasmic, myofibrillar and stromal proteins extraction from whole spent hen ....……………………………………… ………………………59

Figure 2.2 Schematic diagram of the sarcoplasmic, myofibrillar, stromal and alkali soluble proteins extraction from whole spent hen……………………………………………….....…61

Figure 2.3 Schematic diagram of the sarcoplasmic, myofibrillar, stromal and alkali soluble proteins extraction from whole spent hen……………………………………….…………63

Figure 2.4 Schematic diagram of alkali aided proteins extraction from whole spent hen……...64

Figure 2.5 Schematic diagram of lipids analysis…………......…………………………………65

Figure 2.6 Schematic diagram of collagen extraction from chicken feet…………………….……66

Figure 2.7 SDS PAGE of alkali aided extracted proteins……………...………………………..71

Figure 3.1 ATR-FTIR spectra of (Control) Proteins with 40% glycerol, 3% chitosan (A) Proteins with 40% glycerol, 3% chitosan, 5% bentonite (B) Proteins with 40% glycerol, 3% chitosan, 3% POSS (C) Proteins with 40% glycerol, 3% chitosan, 5% CNCs……………………………….…...83

Figure 3.2 Amide I region of 2nd derivative spectra of (Control) Proteins with 40% glycerol, 3% chitosan (A) Proteins with 40% glycerol, 3% chitosan, 5% bentonite (B) Proteins with 40% glycerol, 3% chitosan, 3% POSS (C) Proteins with 40% glycerol, 3% chitosan, 5% CNCs……….………………………………………………………………………………84

Figure 3.3 X-ray diffractograms of (Control) Proteins with 40% glycerol, 3% chitosan (A) Proteins with 40% glycerol, 3% chitosan, 5% bentonite (B) Proteins with 40% glycerol, 3% chitosan, 3% POSS (C) Proteins with 40% glycerol, 3% chitosan, 5% CNCs………………….86

Figure 3.4 DSC heat flow signals of spectra of (Control) Proteins with 40% glycerol, 3% chitosan (A) Proteins with 40% glycerol, 3% chitosan, 5% bentonite (B) Proteins with 40% glycerol, 3% chitosan, 3% POSS (C) Proteins with 40% glycerol, 3% chitosan, 5% CNCs............…………87

Figure 3.5 TGA and DTGA curves of spectra of (Control) Proteins with 40% glycerol, 3% chitosan (A) Proteins with 40% glycerol, 3% chitosan, 5% bentonite (B) Proteins with 40% glycerol, 3% chitosan, 3% POSS (C) Proteins with 40% glycerol, 3% chitosan, 5% CNCs…………………………………………………………......……………………...89

Figure 3.6 Stress-Strain curves of spectra of (Control) Proteins with 40% glycerol, 3% chitosan (A) Proteins with 40% glycerol, 3% chitosan, 5% bentonite (B) Proteins with 40% glycerol, 3% chitosan, 3% POSS (C) Proteins with 40% glycerol, 3% chitosan, 5% CNCs……….....…………………………………………………………………………...91

Figure 3.7 TEM images of (I) Proteins with 40% glycerol, 3% chitosan (II) & (III) Proteins with 40% glycerol, 3% chitosan and 5% clay (IV & V) Proteins with 40% glycerol, 3% chitosan, 5% CNCs (VI & VII) Proteins with 40% glycerol, 3% chitosan, 3% POSS…………………………………………………………………......……………...94

Figure 3.8 DMA thermograms E´(I) and tan delta (II) of (Control) Proteins with 40% glycerol, 3% chitosan (A) Proteins with 40% glycerol, 3% chitosan, 5% bentonite (B) Proteins with 40% glycerol, 3% chitosan, 3% POSS (C) Proteins with 40% glycerol, 3% chitosan, 5% CNCs………………………………......………………………………………………..…..96

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Currently, global annual production of plastics is more than 300 million tons with an annual growth rate of about 5%. Crude oil resources are the main source of plastics production, which makes the plastic industry dependent on those resources. The excessive use of petroleum resources creates plastic pollution that has become a major threat to the environment (Siracusa, Rocculi, Romani, & Dalla Rosa, 2008). The largest application for plastic is packaging (Mihindukulasuriya & Lim, 2014), which is the third biggest industry in the world and represents 2% of GDP in the developed world. Worldwide, there was a 31% increase in total municipal waste between 1988-2005 and packaging was a major (37%) contributor (Tang, Kumar, Alavi, & Sandeep, 2012). Food packaging accounts for most packaging (Lagaron & Lopez-Rubio, 2011), contributing almost two-thirds of packaging waste (Tang *et al*., 2012). The extensive use of petroleum resources to produce polymers leads to concerns about environmental security, economic stability, and sustainability. Excessive reliance on petroleum resources can be eased by increasing bioplastics production using sustainable and renewable resources (Reddy *et al*., 2013).

Presently, both academia and industry has focused their attention on the development of bio-based polymers and innovative processes to develop biomaterials which can be used as food packaging to decrease widespread dependence on fossil fuels and move the industry to a renewable material basis. The use of biopolymers for the development of food packaging is driven by biodegradability, renewability and a low carbon footprint (Reddy, Vivekanandhan, Misra, Bhatia, & Mohanty, 2013).

Biopolymers, due to their biodegradable nature, are one of the favorite choices to be exploited and developed into environmentally benign food packaging materials (Tang *et al*., 2012). Bio-

based food packaging produced from biopolymers can be composted and leave behind organic by-products such as carbon dioxide and water (Othman, 2014).

Unfortunately, the use of biopolymers as food packaging materials has downsides such as poor mechanical, thermal, and barrier properties, unlike conventional material produced from petroleum resources. Thus, many researchers are trying to enhance the properties of biopolymers. Studies have recognized bionanocomposites as a promising material to improve mechanical and barrier properties of biopolymers. A bionancomposite is a multiphase material with two or more constituents, a biopolymer, a continuous phase or matrix, and a nanofiller (<100 nm), a discontinuous nano dimensional phase. The nano-sized fillers play a central role, acting as a reinforcement to increase the mechanical and barrier properties of the matrix. The matrix (biopolymer) tension is transferred to the nanofillers through the boundary between them (Arfat, Benjakul, Prodpran, Sumpavapol, & Songtipya, 2014; H. M. C. de Azeredo, Mattoso, & McHugh, 2011; Kanmani & Rhim, 2014; Trovatti *et al*., 2012). Integrating nanofillers such as silicate, clay, zinc oxide (ZnO), silver, copper, gold and titanium dioxide (TiO_2) with biopolymers may enhance their mechanical, thermal and barrier properties. The optimal addition of different nanoparticles also helps to improve other functions in food packaging, such as antimicrobial activity, bio-sensing, and oxygen scavenging ability (H. M. De Azeredo, 2009; H. M. C. de Azeredo *et al*., 2011).

Bionanocomposites can be used as an active food packaging whereby the food package interacts with food in some ways by releasing beneficial compounds such as antioxidant or antimicrobial agents, or by eliminating unfavorable elements such as water vapor and oxygen. Bionanocomposites can also be smart food packaging, perceiving properties of the packaged food such as microbial contamination or the expiry date. Bionanocomposites use a mechanism to

register and convey information about the quality or safety of the food. The nano-reinforcement of biopolymers into bionancomposite materials is vital for food packaging to mitigate the environmental issue and improve physical properties of the biopolymers. Many studies are being carried out to make not only new monomers from biological resources by industrial biotechnology or chemical processes (Reddy *et al*., 2013) which can be polymerized into biopolymers but also efforts are underway to improve the functional properties of food packaging materials (H. M. C. de Azeredo *et al*., 2011).

1.2. Biopolymers and their categories

Biopolymers or biodegradable plastics are materials in which at least one step in the degradation process occurs when naturally occurring organisms leave behind organic products such as H_2O and CO_2 (Sorrentino, Gorrasi, & Vittoria, 2007). Under suitable conditions of temperature, moisture, and oxygen availability, biodegradation leads to breaking down of the plastics in a way which is safe for the environment. Biopolymers are considered an alternative to petro-based plastics since they are abundant in nature, biodegradable and sustainable (Chandra & Rustgi, 1998). The natural biopolymers such as starch, cellulose, chitosan, and agar, which are derived from carbohydrates, are the most common type of biopolymers used for food packaging applications (Othman, 2014).

Current technology has led to the formation of synthetic biopoloymers like polylactic acid (PLA), polycaprolactone (PCL), polyglycolic acid (PGA), polyvinyl alcohol (PVA), and polybutylene succinate PBS (Rhim, Park, & Ha, 2013). The synthetic biopolymers have the potential to create a renewable and sustainable industry. Additionally, biopolymers properties such as durability, flexibility, high gloss, clarity, and tensile strength can be enhanced (Othman, 2014).

Biopolymers can be broadly divided into different classes based on the origin of the raw materials and their manufacturing processes as shown in Fig. 1.1:

(a) Natural biopolymers such as plant carbohydrates including starch, cellulose, chitosan, alginate, agar, and carrageenan; and animal or plant origin proteins including soy protein, corn zein, wheat gluten, gelatin, collagen, whey protein, and casein;

(b) Synthetic biodegradable polymers, which are petroleum or biomass derived such as poly(l-lactide) (PLA) derived from biomass, and poly (glycolic acid) (PGA), poly (€-caprolactone) (PCL), poly (butylene succinate) (PBS), and poly (vinyl alcohol) (PVA);

(c) Third type of biopolymers are obtained by microbial fermentation like polyesters, such as poly (hydroxyalkanoates) (PHAs) including poly(β-hydroxybutyrate) (PHB), poly (3 hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and microbial polysaccharides, such as pullulan and curdlan (Bordes, Pollet, & Avérous, 2009; Clarinval & Halleux, 2005).

The industrial use of biopolymers as food packaging material is limited, showing relatively poor mechanical and barrier properties compared to petroleum-based food packaging (Tang *et al*., 2012). The interconnected issues related to biopolymers are their performance, processing, and cost. Performance and processing are common problems to all biodegradable materials regardless of their origin. Their applications have been limited due to a number of issues, in particular brittleness, high gas and water permeability. (Pandey, Reddy, Kumar, & Singh, 2005; Saltelli, Chan, & Scott, 2000)).

However, weighing the benefits of biopolymers for food packaging applications, particularly to meet concerns for a sustainable and secure environment, many researchers are focused on improving the properties of biopolymers as a food packaging materials (Othman, 2014).

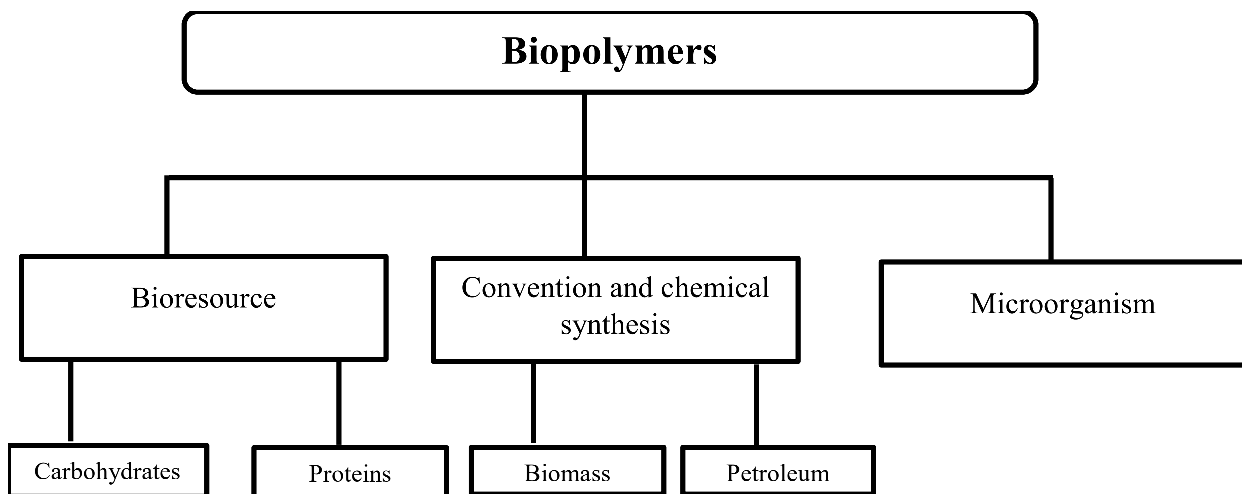

Figure 1.1 Categories of biopolymers (adapted from Rhim *et al*., 2013)

1.3. Alternate source for food packaging production

The ever-increasing world population and scarcity of water resources and land, drive researchers to devise ways to better utilize all available resources instead of simply disposing of things we no longer need. Similar to many industries, the poultry industry is also facing the challenge to produce high quality products that meet consumer needs, increase profit, and satisfy environmental regulations and laws (Freeman, Poore, Middleton, & Ferket, 2009). One area of increasing interest to packaging manufactures is the production of bioplastic for food packaging from renewable and sustainable waste resources.

According to the Canadian Egg Marketing Agency, in Canada, every year more than 32 million birds are removed from the egg industries at egg producer's liability (CEMA, 2006). Spent hens are old laying hens, largely considered a by-product of egg and hatching egg production. The egg production cycle of spent hens is usually for one year. They are referred to as "spent hens" because they are no longer at their peak production of egg number and quality. The average laying hen produces about 300-320 eggs per annum and is at the peak of egg production at 24-26 weeks (A.

F. A. C. AAFC, 2009). Some of the spent hens are sold as cut-up parts (legs, breast and wings), whole bird, and ground meat (Munira *et al*., 2006). The spent hen has only 166 g of white meat (Freeman *et al*., 2009) which makes spent-hen processing no longer economically profitable. Further, their muscles become tough due to the formation of a high amount of collagen as they get old. The toughness reduces its usage in meat products and this decreases economic value (Sams, 1997). So, the meat products made from a spent hen are not able to compete with broiler meat due to poor quality and low yield.

The current disposal methods for spent hens which include burying, compositing, and incineration are often environmentally and economically infeasible. Finding alternatives to disposal that reduce pollution and are profitable is challenging for the poultry industry (Freeman *et al*., 2009).

The concept of recovering valuable protein components from spent hens and converting these proteins into food packaging material is one way to resolve the environmental and economic aspects of the spent hens' issue. Although several studies have been done on spent hens solely for food applications, other industrial applications like food packaging have not been explored yet. Turning the spent hen carcasses into biodegradable food packaging benefits the farmers, the environment, and the economy in general.

1.4. Spent hen overview

In Canada in 2015, there were 2690 regulated chicken producers; 531 registered turkey producers, 244 broilers hatching egg producers, and 1021 egg producers. The egg market is divided into two parts: table eggs and processed eggs. In 2015, the table egg market accounted for 73% of the Canadian market while the processed egg market contributed 27%. Canadian egg farms can range

from a few hundred to more than 400,000 hens with an average Canadian flock size of 20,811 hens.

The poultry industry can be classified into two main categories: egg production layers and broilers. Egg production layers (who eventually become spent hens) spend most of their lives laying eggs and become available (usually at 85-100 weeks) for use in further processed products as they complete their egg laying cycle (Nowsad, Kanoh, & Niwa, 2000). The commercial table egg layers have an average live weight of about 1.5 to 1.7 kg (Kondaiah & Panda, 1992); broilers or broiler chickens raised solely for meat live only a short period (five to seven weeks) before being slaughtered for meat production.

1.4.1. Composition of spent hen meat

It is important to know the components of a spent hen carcass to expand the market value for food and nonfood applications. The major constituents of spent hen meat are water, fat, proteins, and ash as shown in table 1.1. The muscle proteins composition of spent hens is discussed below

1.4.2. Muscle Proteins

The spent hen muscle proteins are myofibrillar proteins, sarcoplasmic proteins and stromal proteins. Myofibrillar proteins, also called salt soluble proteins account for 50-60% of the total muscle proteins and are soluble at a higher salt concentration. Myofibrillar proteins are made up of different proteins: myosin (55%), actin, troponin and tropomyosin (40-45%); and desmin, synemin, α-actinin, nebulin and other structural proteins (1-5%). Myosin (M.W 540,000 daltons) is a long rod-shaped protein composed of six subunits including four light chains and two heavy chains. The heavy chains are comprised of the myosin head; a movement occurs when the myosin heads connect with actin. Myosin is the most important protein among myofibrillar protein with

highly charged amino acids and forms a thick filament (Yates, Greaser, & Huxley, 1983). Thin filaments contain three main myofibrillar proteins; actin, tropomyosin and troponin. Actin (M.W 42,000 daltons) consists of two chains which are twisted together and help to make up the thin filament. Tropomyosin, another protein made of thin filaments, surrounds the actin molecule. It comprises 5% of the myofibrillar proteins and is rod-shaped. Troponin exists in globular form and is the third type of thin filament protein.

Sarcoplasmic proteins, generally low molecular weight proteins, are in the sarcoplasm (cellular fluid) of the myofiber. These make up 30% of the total muscle proteins and have strong binding ability. Sarcoplasmic proteins are myoglobin, hemoglobin, cytochromes, glycolytic enzymes and creatine kinase. Their isoelectric point (pI) is generally between 6-7 and they are considered to be water soluble proteins (Scopes, 1970).

Stromal proteins contribute 10-20% of total muscle protein contents, have isoelectric point of 7.2. These proteins are unique because they have non-charged non-polar units of glycine (33%) and hydroxyproline (10%) amino acids. Stromal proteins provide strength and protection to the muscle tissues. The composition and abundance of stromal proteins greatly affect the quality of meat. The major protein of this group is collagen, which is the most abundant protein (20-25% of total body proteins) in an animal's body (Strasburg, Xiong, & Chiang, 2008). Other important stromal proteins are elastin and mitochondrial proteins (Barbut, 2002).

Table 1.1 Raw spent hen and broiler muscle meat chemical composition

Breed	Moisture (%)	Protein (%)	Fats (%)	Total collagen (mg/g muscle)	Soluble collagen (mg/g muscle)	Ash (%)
Spent hen	67.64±3.13	24.36±0.26	7.15±0.09	6.47±0.20	29.36±0.87	1.04 ±0.09
Broiler	74.84 ±0.46	20.59±0.26	0.68 ±0.06	3.85 ±0.24	19.14±0.48	1.10 ±0.01

Source: Adapted from Wattanachant, Benjakul & Leward, 2004

1.4.3. Problems related to disposal of spent hens

There are many concerns related to disposal of spent hens. These issues both environmentally and economically are not secure and feasible. Every year millions of birds have to be disposed of by the poultry industry. This presents waste management issues that create environmental and health concerns. Burying of carcasses leaves in-ground residue for years, which can contaminate the ground water by producing harmful nitrogen and sulfur compounds from the decomposed organic matter and affect humans and livestock. Land used for a burial site is limited because of the biological hazards created by decaying carcasses. An offensive odor is produced near these places which reduces the quality of human and animal life near those sites. Transportation to specific disposal sites is an additional cost. Moreover, the burial method has no controlled system for inactivating pathogenic microorganisms. Thus it creates a risk of spreading diseases to animals and humans that have contact with the contaminated soil (Kalbasi, Mukhtar, Hawkins, & Auvermann, 2005)

Incineration may offer a biologically safe and suitable way to dispose of large number of birds on farms. But operational costs, turnaround time and disposal of ash create air quality issues that need to be addressed.

There are also potential pathogenic risks related to composting animal carcasses. For successful composting, workers should have good knowledge and skill to execute the process efficiently. Temperature plays a main role in composing, the bacterial pathogens can survive outside the host organisms if the temperature is not high enough to promote biodegradation (C. R. MOTE & ESTES, 1982) posing a health risk. The re-growth of pathogenic organisms becomes a bio-security concern of applying animal carcass compost as a land amendment. Mote *et al.*

reported the regrowth of salmonellae and coliform bacteria during composting that were incompletely stabilized (C. Mote, Emerton, Allison, Dowlen, & Oliver, 1988).

A significant percentage of spent hen meat is used by the rendering industry to make spent hen meal that could be incorporated into animal feed. During the last two decades, animal infectious disease outbreaks, such as Bovine Spongiform Encephalopathy have raised public concern that infectious disease agents might be transmitted to farm animals through animal protein-based feed (Koller *et al*., 2005). The spent hen carcasses are more likely contaminated with salmonellae and coliform bacteria because cage-raised hens are exposed to high levels of fecal dust and have close contact with the surface of eggshells, which is where salmonella contaminations are generally found (Baskerville *et al*., 1992; Gast & Beard, 1990). More than half (51.8%) of the poultry by-products being shipped to a feed mill were found to be positive for Salmonella (Snoeyenbos, Carlson, McKie, & Smyser, 1967). It also has been reported that very low levels of Salmonella in feed can cause a salmonella colonization in the intestinal tract of animals (Berge & Wierup, 2012). Nowadays, pet owners and farmers prefer non-animal protein sources for animal feeds, e.g., soybean meal. Consequently, the percentage of meat and bone meal that was accepted in US feed mills has declined by approximately 50% from 1999-2002 (Gamroth, Downing, & French, 2006)

1.4.4. Protein extraction from spent hens

Proteins are natural biopolymers that can be used for food and non-food applications. Spent hen meat composition clearly shows a big portion of valuable protein which can be extracted and further used for industrial processing. Proteins extraction from spent hen with low cost is the demand for further processing. Many methods have been used to extract protein but the pH shift method is among the more commonly used.

1.4.4.1. The pH-shift method

The pH-shift protein isolation method is also called acid/alkaline solubilization, the precipitation method, or the isoelectric solubilization/precipitation method (H. O. Hultin & Kelleher, 1999; Nolsøe & Undeland, 2009; Undeland, Kelleher, & Hultin, 2002). Proteins solubility is changed in water at different pH values. The solubilizaition process works on this principle. Generally, this method is performed in steps in a laboratory, as summarized in Fig. 1.2 In the pH-shift method, the sample is mixed with water that provides a medium for proteins solubilization. The proteins are homogenized to increase the surface interactions between the proteins and water. (H. O. Hultin & Kelleher, 1999). The insoluble myofibrillar proteins are held together in aggregates at physiological pH. Both sarcoplasmic and myofibril proteins interact with water as they have polar amino acids. Most muscle proteins are negatively charged at physiological pH. By lowering the pH, the proteins first become neutral and then positively charged when the amino group is protonated. Similarly, the proteins become more negatively charged by the addition of a base and the subsequent deprotonation.

The interaction between protein and water is increased due to the overall increased protein charge. Likewise, individual proteins start to separate from each other because of the charged amino acids on the proteins, which repel each other. In this manner they attract more water and partly unfold, becoming more soluble (Step 2, solubilization) (Kristinsson & Hultin, 2003). On the way towards solubilization, the protein aggregates swell via the water attraction and the viscosity of the solution is considerably increased. Attracting enough water, most of the muscle proteins become completely soluble and the viscosity is lowered again (Undeland, Kelleher, Hultin, McClements, & Thongraung, 2003). Under these conditions, the proteins can be separated, using centrifugation, from insoluble material such as bones, some proteins and neutral lipids (H. O.

Hultin & Kelleher, 1999, 2000). After centrifugation, three layers can be observed (Step 3, separation): a sediment in the bottom containing insoluble material (e.g. bones, debris, and membrane lipids), a middle layer of solubilized proteins, and an upper layer of fat contents. Sometimes, a fourth gelatinous layer forms on top of the bottom sediment. This is thought to be a mix of solubilized and non-solubilized proteins (H. Hultin, Kristinsson, Lanier, & Park, 2005). The dissolved proteins that remain in solution are then adjusted to a pH close to the pI of the proteins, generally between pH 5 and 6 (step 4, precipitation) (Stefansson & Hultin, 1994). During this pH adjustment, proteins start to refold, though not necessarily to their native conformation. At the pI, there is a minimum of interaction between water and proteins and proteins are precipitated down, called protein isolate, collected from the solution via a second centrifugation (Step 5, isolation).

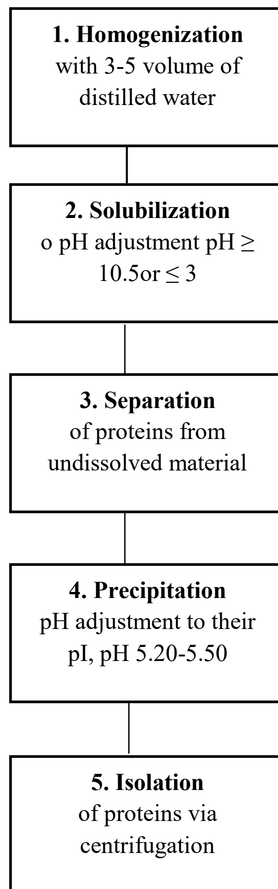

Figure 1.2 Overview of the pH shift process (adapted from Nolsoe & Undeland., 2009)

The shifting process has advantages over the traditional method like surimi production. The protein yield is higher due to more recovery of sarcoplasmic proteins. The removal of lipids, connective tissues and other impurities is more efficient due to the use high speed centrifugation during this process (H. Hultin *et al*., 2005; H. O. Hultin & Kelleher, 2000). The method can be applied to complex samples (e.g. whole carcass or by- product) (Gehring, Gigliotti, Moritz, Tou, & Jaczynski, 2011). The shifting process can be performed on an industrial scale as previously reported. (McCURDY, Jelen, Fedec, & Wood, 1986).

1.5. Proteins derived bionanocomposites

1.5.1. Proteins

Amongst renewable resources, proteins are one of the agricultural products, being used as a raw material to produce bio-based plastics. Proteins are the hetero-biopolymers which have various network structures due to different amino acid chains which provide many chemical functional groups as compared to other bio-based macromolecules like carbohydrates. Apart from this, proteins are highly available, biodegradable, and not eco-toxic, which makes them suitable for developing bioplastics (Angellier-Coussy *et al*., 2013).

Proteins consist of 20 different amino acids, are characterized by different structures that allow various interactions leading to bondings of different energy. The functionalities of protein-based materials are determined primarily by the composition of the amino acids constituting their primary sequence and more precisely on the chemical reactivity of the amino acid side chains: i.e., whether they are hydrophilic or hydrophobic, acidic or basic, and aromatic or aliphatic. Depending on the chemical specificity of the amino acids, different kinds of interactions can be established leading to intra- and inter-polypeptidic chain linkages. Among these linkages, noncovalent bond

interactions are the most representative as they play a major role in stabilizing protein's three-dimensional structure.

Amino acids are usually classified in groups depending on their ability to establish interactions via either covalent linkages (amino acids involved in disulfide bonds, 330–380 kJ/mol), hydrophobic interactions (between apolar amino acids, 4–12 kJ/mol), ionic interactions (implying ionized polar amino acids, 42–84 kJ/mol), or hydrogen bonds (involving nonionized polar amino acids, 8–40 kJ/mol. Noncovalent bonds impart stability to the structures generated by their collective action although they are weak chemical forces ranging from 4 to 30 kJ/mol (Garrett & Grisham, 1996). Van der Waals forces (involving both polar and apolar nonionised amino acids, 0.4–4 kJ/mol) are also implicated but their effect on protein structure is insignificant. In addition to noncovalent interactions, covalent disulfide bonds (formed by the oxidation of two thiol groups) play a vital role in the stability of the proteins containing cysteine residues (Angellier-Coussy *et al*., 2013). The functional properties of the final materials will depend on the influence of these different kinds of interactions that are closely associated with the characteristics of the protein raw material that can also be enhanced by the conditions used to process and store the protein-based materials. Moreover, this heterogeneous system provides many opportunities for cross-linking or chemical grafting.

Protein-based films have become a research focus because of their better film-forming properties, low cost, and biodegradable nature. A limitation to their wide use is their intrinsic reactivity and lower inertia when compared with conventional petrochemical-based plastics. Due to their hydrophilic nature, they are very sensitive to microbial spoilage (Robertson, 2009).

1.5.2. Main proteins used as a matrix

Protein films have been developed from wheat gluten, soy protein, gelatin, corn zein, casein, and whey proteins.

1.5.2.1. Gluten based nanocomposites

Wheat gluten protein is an enriched protein complex, comprising a mixture of gliadins and glutenin. The molecular weight of wheat gluten protein ranges from 15,000 to 85,000 kDa (Gennadios, 2002; Pallos, Robertson, Pavlath, & Orts, 2006). Wheat gluten is an interesting raw material that can be used for food packaging due to its unique viscoelastic and film-forming properties.

Wheat gluten-based films have properties that make them attractive for food-quality preservation: strength and flexibility, CO_2/O_2 permeability in dry conditions, and good grease and aroma barriers. In addition, they are heat-sealed and translucent. Glutenins have disulfide-linked polymeric chains whereas gliadins are mostly monomeric single-chain polypeptides. Wheat gluten is a viable substitute for synthetic plastics in food packaging due to its high barrier properties and hydrophobicity. Wheat gluten proteins can undergo disulfide interchange upon heating, leading to the formation of a covalent three-dimensional macromolecular network (Tang *et al*., 2012). One major problem related to gluten processing for food packaging material is lack of basic understanding of gluten's tertiary and quaternary structures (Petersen *et al*., 1999). So far, very few studies have been done on wheat gluten-based bionanocomposites. Two types of nanoclay were used: Cloisite Na+ (MMT) and Cloisite 10A (quaternary ammonium modified MMT) to synthesize bionancomposite formation from wheat gluten (Olabarrieta, Gällstedt, Ispizua, Sarasua, & Hedenqvist, 2006). The films were cast using ethanol/water solutions from pH 4 to 11. It was revealed that film was almost completely exfoliated at pH 11, the most brittle, the strongest and

stiffest when MMT was used. Additionally, it showed the highest reduction in water vapor permeability (Tang *et al*., 2012).

1.5.2.2. Gelatin based nanocomposites

Gelatin, found mainly in animal skins and bones, is prepared by chemical or thermal hydrolysis of collagen chains. Commercially, it is produced by using two processes; type B gelatin is obtained by an alkali followed by a solubilization at neutral pH (60–90°C) whereas type A gelatin comprises an acid extraction. Gelatin has a large number of glycine, proline and 4-hydroxyproline residues. Gelatin is a heterogeneous mixture (containing between 300 and 4,000 amino acids) of single or multi-stranded polypeptides, each with extended left-handed proline helix conformations (Ray & Bousmina, 2005).

Gelatin was one of the first food proteins to be used as raw material not only for food applications but also in the pharmaceutical and photographic film industries due to its availability, low production cost and excellent film-forming ability. Gelatin has been successfully used to form films by casting that is transparent, flexible, water-resistant, and impermeable to oxygen (Cuq, Gontard, & Guilbert, 1998).

Zheng *et al*. synthesized gelatin/MMT nanocomposites for the first time with the expectation that resulted materials properties would be improved by using nanoparticles. In their findings, the tensile strength and Young's modulus of gelatin-based nanocomposites were improved remarkably, which changed with clay (MMT) contents and the pH of the gelatin matrix (J. P. Zheng, Li, Ma, & Yao, 2002). However, gelatin poor mechanical properties limit its use as a food packaging material. Many techniques such as vapor crosslinking, orientation methods, and use of fillers such as hydroxylapatite and tricalcium phosphate have been developed to reinforce

gelatin-based films but the films are still not strong enough, particularly in the wet form (Tang *et al*., 2012).

1.5.2.3. Soy protein-based nanocomposites

Soy proteins have mainly polar amino acids. The major components of soy proteins are *β*-conglycinin (35%), and glycinin (52%). Bionanocomposites made from soya proteins isolates (SPI) have good biodegradability but poor flexibility (Mo, Sun, & Wang, 1999). Various chemical treatments and plasticizers have been used to overcome the brittleness and water resistance of SPI-based films. Glycerol, ethylene glycol, and propylene glycol have been used and found to be better plasticizers than 1,3-propanediol for soy proteins-based films. By the addition of suitable nanoparticles with an SPI/plasticizer mixture, flexible films have been obtained (Tang *et al*., 2012). Yu *et al*. established an effective method to exfoliate MMT lamellae in water using ultrasonics (L. Yu, Dean, & Li, 2006). He prepared MMT-reinforced soy protein films plasticized by a mixture of glycerol and water. Chen and Zhang synthesized highly exfoliated and intercalated SPI/MMT nanocomposites by using a solution intercalation method in a neutral aqueous medium and studied the correlation between the microstructure and mechanical properties (Chen & Zhang, 2006). The results showed that the heterogeneous distribution of the surface positive charges provided a net negative charge on soy globulins to anchor the negative-charged MMT interlayers (the distance between two MMT layers). Electrostatic attraction and hydrogen bonding on the interfaces of soy protein and MMT led to a good dispersion of MMT layers in the protein matrix. When the MMT content was less than 12% (w/w), a highly exfoliated structure was formed due to the better dispersion of MMT in a protein matrix. The intercalated structure was predominant when the MMT content was more than 12%. The results also showed that better dispersion of MMT layers in the protein matrix and a strong interaction between SPI and MMT enhanced the mechanical

strength and thermal stability of SPI/MMT nanocomposites. Yu *et al*. prepared soy protein nanocomposite sheets using rectorite (REC) with compression molding method. REC is a 1:1 layered silicate which is composed of a regular stacking of mica-like layers and MMT-like layers. Interlayer spacing in REC can be approximately 2.4 nm as compared to 1.2 nm of MMT. Therefore, REC is likely to allow better penetration of protein molecules between interlayers (J. Yu, Cui, Wei, & Huang, 2007). The results showed an exfoliated nanocomposite structure until the REC content reached 12%, after which intercalated structures were formed. This is consistent with the mechanical properties results, with the TS of the nanocomposite sheets being maximum (12.92 MPa) at a REC content of 12%. The percentage of elongation at the break (%E) of nanocomposite sheets lowered sharply as the REC content increased (Tang *et al*., 2012).

1.5.2.4. Corn zein

Corn zein is found in the endosperm of corn. It is a group of alcohol soluble (prolamins) proteins with a molecular weight of 18 to 45 kDa. Corn zein is soluble in 60% - 70% ethanol. It is produced commercially by extraction with aqueous alcohol followed by drying to a granular powder. Zein derived films can be prepared by casting method. Glycerol is a commonly used plasticizer to reduce the brittleness of zein films. However, glycerol tends to aggregate because of the weak interaction between protein and glycerol molecules. An aggregation of glycerol leads to loss of flexibility in the film. A mixture of glycerol and polyethylene glycol (PEG) has been used to reduce the aggregation rate in zein films. The tensile strength of zein films is similar to that of wheat gluten films, while the water vapor permeability (WVP) of zein films is lower than or similar to those of other protein films but higher than that of low density polyethylene (LDPE). Lipids have been used to increase the WVP of zein films. Since zein has helical conformation, its oxygen

permeability is higher than that of highly cross-linked wheat gluten (Hernandez-Izquierdo & Krochta, 2008; Padua, Wang, & Gennadios, 2002).

1.4.2.5. Milk proteins

Biodegradable films can be obtained from total milk proteins or components of milk proteins. Milk proteins are classified into two types; casein and whey protein (Tang *et al*., 2012). Casein contributes 80% of total milk protein, consists of three main components, α, β, and γ, with molecular weights ranging from 19kDa to 25 kDa. Casein forms colloidal micelles in milk. These micelles are stabilized by calcium phosphate bridging. Casein precipitates when milk is acidified to its isoelectric point (pH = 4.6). Caseinate-based biodegradable films can be obtained by casting process. Water vapor permeability of caseinate-based films is comparable to the permeability of wheat gluten and soy protein films.

Whey proteins, which comprise 20% of total milk proteins, contain a mixture of proteins such as α-lactalbumin (M.W 14 kDa), β-lactoglobulin (M.W 18 kDa), bovine serum albumin, and immunoglobulins. β- lactoglobulin comprises approximately 57% of total whey protein. It remains soluble in milk serum after casein is coagulated during cheese or casein production. It has two disulfide groups, one free sulfhydryl group, and hydrophobic groups located in the interior. α-lactalbumin contains about 20% of total whey protein and has four disulfide bonds.

The formation of whey protein films required heat denaturation in aqueous solutions. Denaturation involves breaking existing disulfide bonds. When the bonds break, new intermolecular disulfide and hydrophobic bonds are formed (Tang *et al*., 2012). The WVP of whey protein films is affected by moisture, nature, and the concentration of plasticizer. Whey protein isolate (WPI) based films have poorer WVP than soy protein, caseinate, corn zein, and wheat gluten films (Hernandez-Izquierdo & Krochta, 2008; Krochta & Mulder-Johnston, 1997).

1.5.3. Main nanoparticles used as fillers in proteins derived bionanocomposites

Layered silicates are the nanoscale particles most commonly used as reinforcing agents in protein-based matrices. Carbon nanotubes, polysaccharide (cellulose, chitin and starch nanocrystals), silica, and TiO_2 nanoparticles are used in a less extent (Angellier-Coussy *et al.*, 2013). Nanoscale additives can be categorized into two types: inorganic and organic nanostructured materials. The inorganic nanostructured materials include silica, TiO_2, and carbon nanotubes. Organic nanostructured materials include cellulose, chitosan/chitin, and starch nanocrystals.

1.5.3.1. TiO_2

TiO_2, also known as titanium (IV) oxide with chemical formula TiO_2, occurs in nature in the form of the well-known minerals rutile, anatase, and brookite. Titanium dioxide nanoparticles are relatively inexpensive to produce (Anwar, Kassim, Lim, Zakarya, & Huang, 2010) and have the potential to act against all kind of microbes (Cerrada *et al.*, 2008). It has unique physical and chemical properties such as a high refractive index, opacity, brightness and excellent optical transmittance in the visible range. The key purpose of TiO_2 in food packaging is to protect food from the oxidizing effects of ultraviolet (UV) radiations (Ghanbarzadeh, Oleyaei, & Almasi, 2015).

Oxygen and ethylene scavenger activities of TiO_2 have been extensively investigated. Nanocrystalline titania acts as O_2 scavenger in food packaging under UV radiation to maintain low level of O_2. The photocatalytic effect of TiO_2 has been found to act as a catalyst to promote the biodegradation of used organic packaging materials. TiO_2 nanoparticles have been used as fillers in whey protein-based matrices and found a significant increase in the mechanical strength of films due to the electrostatic attraction between the negatively charged group of WPI and positively charged Ti^+ (Y. Li *et al.*, 2011; Zhou, Wang, & Gunasekaran, 2009).

1.5.3.2. Layered silicates

Smectites, and particularly montmorillonites (MMT), are the layered silicates mostly used to improve the properties of protein-based materials. These have got much attention from the packaging industry due to their large availability, low cost, and ability to impart a significant increase in mechanical strength of reinforced materials (H. M. De Azeredo, 2009; Sorrentino *et al*., 2007). These layered silicates belong to the phyllosilicate family and display a perfect crystalline structure consisting of two-dimensional layers where a central octahedral sheet of alumina or magnesia is linked with two external silica tetrahedrons (Alexandre & Dubois, 2000). The layered silicates give high barrier properties against carbon dioxide, ultraviolet radiations, oxygen, moisture and volatile compounds. Perhaps these are the most important properties that nanocomposite-based food packaging can offer. The problem with the use of such nanofillers is proper dispersion within a polymeric matrix due to their tendency to form agglomerated tactoids (Angellier-Coussy *et al*., 2013). To modulate the mechanical and barrier properties of protein-based materials, a well-exfoliated or even intercalated nanocomposite structure is required. One significant characteristic of clays is that the space between layers comprises hydrated cations such as Na^+ or K^+ that can experience an exchange reaction with organic cations such as alkylammoniums. Thus, organically modified layered silicates can be purposely prepared to impart functionality and compatibility with polymers (Ghanbarzadeh *et al*., 2015).

Several studies have showed improvement in the mechanical strength of silica/whey protein-based films, making it feasible to use. The optical and tensile properties of WPI-based films influenced by blending with organically modified MMT and Cloisite 30B. Studies have found that addition of 5% nanoclay significantly improved the water vapor barrier properties of

films. The wheat gluten film's water sensitivity was reduced by the presence of MMT (Rungsinee Sothornvit, Hong, An, & Rhim, 2010; Rungsinee Sothornvit, Rhim, & Hong, 2009).

1.5.3.3. Silica

Nanosilicon dioxide is a type of amorphous powder with a molecular structure having three-dimensional network. It is a permitted food additive and used in food packaging. It has been reported that nanosilica improved the mechanical or barrier properties of several polymer matrices. In the field of bionanocomposites materials, silica (SiO_2) is mostly used in colloidal suspensions of amorphous spherical nanoparticles whose properties and structure depend directly on synthetic process. The surface of silica nanoparticles is naturally hydrophilic and can easily be chemically modified to create new functionalities or enhance the nanoparticles' compatibility with a hydrophobic polymer matrix. Up to now, colloidal suspensions of silica nanoparticles have only been studied as fillers in a soy protein-based matrix (Ai, Zheng, Wei, & Huang, 2007).

1.5.3.4. Carbon nanotubes

Carbon nanotubes (CNTs) are cylindrical tubes formed by rolled-up graphene sheets with a very high aspect ratio. Carbon nanotubes are of two types depending on their structure: single-walled carbon nanotubes (SWCNT), which consist of a single sheet of graphene, a cylinder with a diameter of 1 nm and a length of up to several centimeters; multiwalled carbon nanotubes (MWCNT), consist of an array of cylinders formed concentrically and have outer diameters of about 30 nm and lengths of 1–100 μm. Until now, SWCNTs have not been used in protein-based matrices because of dispersion difficulty within a biopolymer matrix. These carbon nanotubes tend to aggregate into large bundles owing to their high flexibility and surface energy (Ai *et al*., 2007). CNTs have some prominent characteristics including high strength, good thermal and electrical conductivity and other functional properties like flame retardation, crystallization behavior and

antibacterial behavior. Up till now, the reinforcing effect of MWCNT has been evaluated in gelatin (P. Li, Ping Zheng, Lu Ma, & De Yao, 2003) and SPI matrices (H. Zheng, Ai, Wei, Huang, & Chang, 2007). Researchers have observed that SPI nanocomposites containing 0.25 wt. % of MWCNT have improved mechanical and water resistance properties. These improvements were attributed mainly to the interactions at the interface of the SPI chain and the MWNT walls.

1.5.3.5. Cellulose

Cellulose nanofibers can be obtained from acid hydrolysis of cellulose fibrils or microcrystalline cellulose. Cellulose has many advantages as it is widely available (the most abundant polysaccharide in the world), low in cost, biodegradable, low density and high mechanical strength. These properties make cellulose attractive for producing low-cost, high strength and lightweight composites. Fundamentally, two types of nanoreinforcement can be obtained from cellulose: microfibrils and whiskers (Podsiadlo *et al*., 2005). Cellulose nanocrystals have low hydrophilicity compared to most biopolymer based matrixes. Thus, the total solubility of water molecules in composites is decreased. Meanwhile, strong hydrogen bonding between the matrix and cellulose decreased the OH group in the matrix. The less permeable filler with better dispersion in the matrix gave better mechanical strength (H. M. De Azeredo, 2009; H. M. C. de Azeredo *et al*., 2011).

1.5.3.6. Starch nanocrystals

Starch nanocrystals are obtained from mild acid hydrolysis of starch below its gelatinization temperature. The amorphous regions are hydrolyzed and allow the separation of crystalline lamella which is more resistant to hydrolysis. The reinforcing effect of the starch nanocrystals can be attributed to filler/matrix interactions due to the formation of hydrogen bonding. The starch presence also slows the recrystallization of the matrix during aging in the

humid atmosphere (Lin, Huang, Chang, Anderson, & Yu, 2011). Up till now, only pea starch nanocrystals have been studied (Zheng *et al*., 2009) as reinforcing agents in an SPI-based matrix. Authors found that SPI films containing 2 wt.% starch nanocrystals showed an increase in ultimate tensile strength. Starch nanocrystals have a highly reactive surface covered with hydroxyl groups, which provides the possibility of chemical modification to create new specific functions and thus expand the starch nanocrystals applications.

1.5.3.7. Chitin/Chitosan

Chitin whiskers can be obtained from acid hydrolysis of chitin. It is the second most abundant polysaccharide in the world after cellulose (Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010). The most important derivative of chitin is chitosan, which is obtained by partially deacetylating chitin under basic hydrolysis or by enzymatic hydrolysis in the presence of chitin deacetylase (Ghanbarzadeh *et al*., 2015). Both chitin and chitosan are biodegradable, nontoxic and biocompatible polymers. Lu *et al*. (2004) reported that the addition of chitin whiskers in SPI greatly improved chitin's tensile strength and elastic modulus as well as its water resistance (Lu, Weng, & Zhang, 2004).

1.5.4. Processing strategies for proteins derived bionanocomposites preparation

For the formation of a new three- dimensional network of proteins, proteins derived bionanocomposites are first unfolded and then realigned. Later this structure is stabilized by new inter and intramolecular interactions. Formation of a macromolecular network from proteins involves three steps:

Step I: Stabilizing polymer chains in the native state by breaking of low energy intermolecular bonds.

Step II: Shaping (arrangement and the orientation) polymer chains.

Step III: Removing the disruptive agent and forming a new three-dimensional network stabilized by new bonds and interactions.

Proteins-based films often required plasticizers to allow their thermo-processability and improve material flexibility due to proteins high glass transition temperature which is usually above their temperature of degradation. Water is considered as a natural plasticizer for proteins-based films. The other widely used plasticizers are polyols (glycerol, sorbitol), mono, di- oligosaccharides and fatty acids (Angellier-Coussy *et al*., 2013). Regardless of the nature of the nanoparticle used to form protein-based nanocomposites, three processing methods are commonly used (Fig. 1.3, 1.4, 1.5).

1.5.4.1. Wet process (Dispersion of nanoparticles from solution)

The wet process is also called solution casting and is suitable for most of the proteins (Fig 1.3). This method is based on the proteins dispersion or solubilization in a solvent such as ethanol, water and, occasionally acetone. Whether a protein can be dispersed in solution depends on the amino acid sequence, the nature of the disruptive agents, solution's pH and its ionic strength (Guillaume, Pinte, Gontard, & Gastaldi, 2010). In aqueous media, proteins generally expose their polar amino acids at the interfaces with the solvents whereas the hydrophobic portions move inside the structure to avoid solvent contact.

In the case of layered silicates, it is challenging to intercalate protein chains within the galleries (Cho, Ullsten, Gällstedt, & Hedenqvist, 2007). For this reason, clay nanoparticles are exfoliated into single layers when using solvents in which the proteins are easily dispersed. Proteins then adsorb onto the delaminated surfaces of the clay and when the solvent is evaporated, the sheets reassemble, sandwiching the proteins. It is possible to change the pH of the film-forming solution to positively enhance the protein net charge in favor of electrostatic interactions with the

negatively charged clay surfaces. This makes it easier for the proteins to penetrate in the clay galleries.

To date, the preferred method for making protein-based nanocomposites has been to disperse nanoparticles in solution. The reasons for this are:

(a) The method does not require plasticizers.

(b) Processing is relatively easy.

(c) The method makes it possible to obtain well dispersed nanocomposite structures.

In some solution intercalation cases, solvent is evaporated by freeze-drying, leading to good dispersion of nanoparticle. The resulted dried powder is shaped using a dry process. The freeze drying process is generally used for cellulose, chitin, starch nanofillers, which are found in aqueous colloidal suspension forms (Alexandre & Dubois, 2000; H. Zheng, Ai, Chang, Huang, & Dufresne, 2009; H. Zheng et al., 2007)

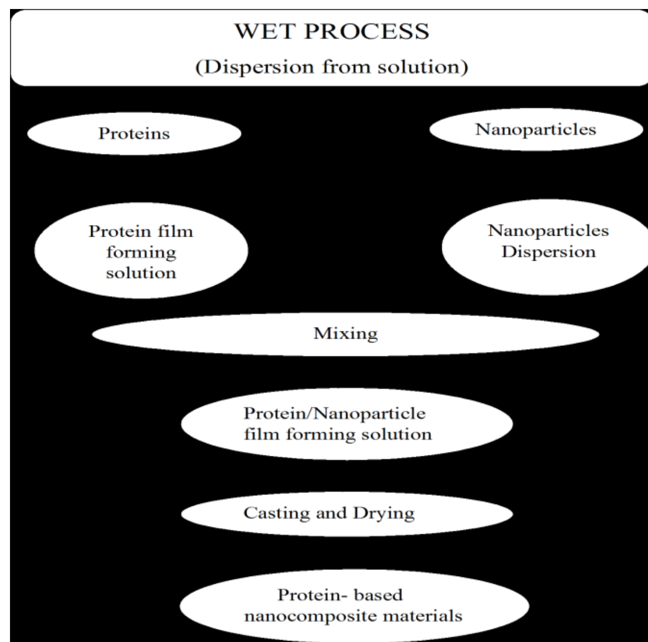

Figure 1.3 Schematic representation for proteins derived bionanocomposites by wet process (adapted from Angellier- Coussy *et al.*, 2013)

1.5.4.2. Dry process

Dry processes are based on the thermoplastic properties of proteins including extrusion and thermomolding. Under shearing and heating, the protein matrix in the molten state is mixed with nanoparticles (Fig 1.4).

The proteins are heated above their softening point to form a homogeneous protein melt, an essential requirement for dry processes. The covalent and non-covalent bonds between protein molecules can be disrupted by the addition of disrupting agents such as urea, sodium sulfite and sodium dodecyl sulfate along with plasticizers. Though most proteins are suitable for the extrusion process, to date only soy protein isolates and wheat gluten have been converted to bionancomposite materials using a dry process (Angellier-Coussy *et al.*, 2013). In case of clay/protein-based material, polypeptide chains can go into the galleries and form either an intercalated or exfoliated structure when the clay surface is sufficiently compatible with the proteins. Studies showed that the entropic and enthalpic factors control the outcome of polymer intercalation. Exfoliation or phase separation depends on the favorable interactions between polymer and clay surfaces to compensate the entropy loss due to the polymer confinement and settlement between the clay layers. The temperature difference between the polymer glass transition temperature and processing temperature is very important because it determines polymer mobility which in turn plays a major role in intercalation kinetics (Vaia, Jandt, Kramer, & Giannelis, 1996). Since it requires no solvent, melt intercalation is considered a promising and environmentally benign alternative to polymer intercalation from solution. Additionally, dry processes are compatible with current industrial plastic processing (Ojijo & Ray, 2013).

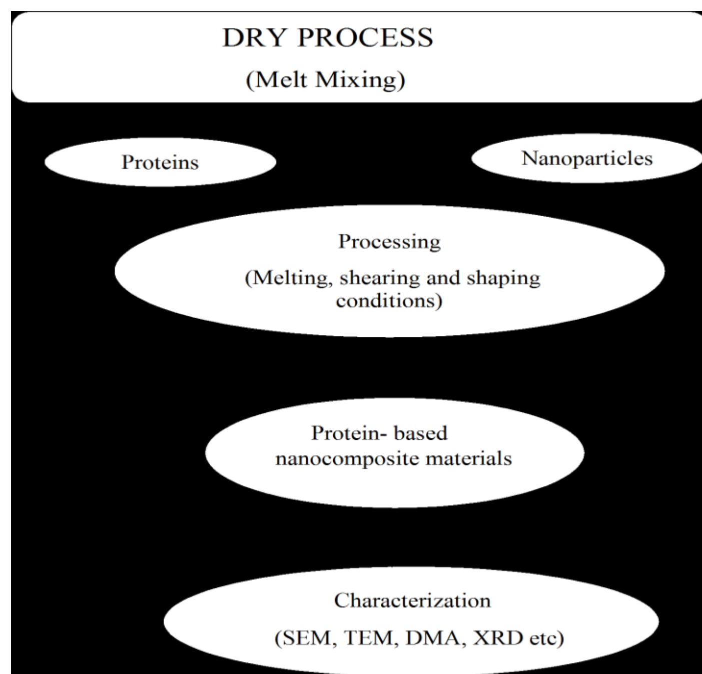

Figure 1.4 Schematic representation for proteins derived bionanocomposites by dry process (adapted from Angellier- Coussy *et al.*, 2013)

1.5.4.3. Two-step process

The two-process step consists of solution casting and melt extrusion (Fig 1.5) Chang *et al.,* and Chen *et al.,* described the process in two steps:

1. Nanofiller is dispersed in distilled water. These solutions are then added to protein isolate emulsions with constant mixing.
2. The mixture is blended with plasticizer.

This method successfully disperses the filler in the protein matrix. The resultant material is strong due to hydrogen bonding interactions on the interfaces of the protein and nanofiller. Such interactions are beneficial to the intercalation and delamination of the MMT layers in the protein matrixes (Chang *et al*., 2009; Chen & Zhang, 2006). Thus, the mechanical strength and thermal stability of the plastics is significantly enhanced because of the fine dispersion of the nanoparticle layers and the strong electrostatic attractions on the interfaces (Lin *et al*., 2011).

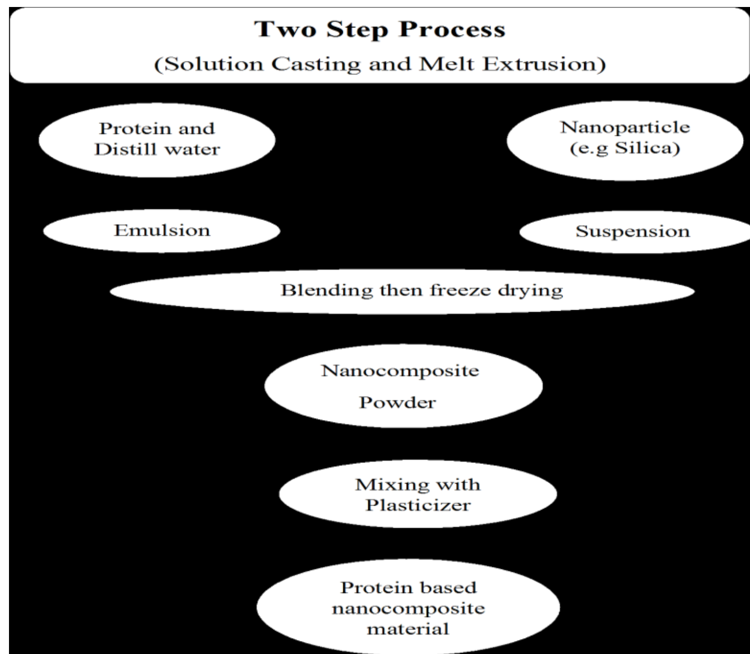

Figure 1.5 Schematic representation for proteins derived bionanocomposites by two- step process (adapted from Angellier- Coussy *et el.*, 2013)

1.5.5. Common factors affecting proteins film formation

Several factors come into play during making of protein-basd bionancomposite films. These factors greatly influence the films functional properties. For the last two decades, research has been focused on these parameters to achieve good protein film properties. As proteins and processing methods have their own parameters, optimizing various combinations of the parameters is quite challenging to commercialize the protein-based films.

1.5.5.1. Plasticizers

Plasticizer plays a vital role in making a good film. Proteins derived films are brittle and stiff because of interactions among molecular chains. These interactions are hydrogen bonding, electrostatic forces and hydrophobic interactions. Plasticizers modify the protein three-dimensional structure by entering between the protein chains, physiochemically associating with the protein chains and increasing free volumes and chain mobility (Gennadios, 2002). Adding

plasticizer to protein biopolymers reduces the chain interactions, lowers its glass transition temperature and improves the film flexibility (Swain, Biswal, Nanda, & Nayak, 2004). At the same time, the film permeability and elongation increase and the mechanical strength decrease.

Water, glycerol, propylene glycol, sorbitol, sucrose, polyethylene glycol, fatty acids, lipids and monoglycerides are commonly used plasticizers for protein-based films (Selling, Sessa, & Palmquist, 2004; Q. Wang, Crofts, & Padua, 2003). The most frequently used is glycerol (3%-50% w/w) as it is hydrophilic. Glycerol also affects the moisture content by attracting water from surrounding which influences the properties of the films (Gennadios, Brandenburg, Weller, & Testin, 1993). Several studies have looked at how different types and amounts of plasticizers affect the molecular reactions of protein-based films. It has been reported that increasing concentrations of glycerol (from 0% to 33% (w/w) enhance the extensibility of wheat gluten film but reduce elasticity and water vapor barrier properties (Nathalie Gontard, Guilbert, & CUQ, 1993). Pommet *et al.,* found that increased plasticizer (octanoic acid) resulted in protein aggregations including sulfhydryl/disulfide interchanging (Marion Pommet, Andreas Redl, Stéphane Guilbert, & Marie-Hélène Morel, 2005). Another study showed that adding hydrophobic plasticizers can extensively enhance the moisture barrier properties of soy protein-based films (Rhim *et al*., 2013).

1.5.5.2. Temperature

Protein molecules are very sensitive to temperature changes. The structural mechanism of proteins can be controlled by the temperature used for films formation. During heating, protein molecules transform from a glassy to a rubbery state. Meanwhile, the molecules become disordered and the mobility of the chains increases. Heating promotes protein network cross-linking through the interactions of disulfide and hydrophobic bonds (Hernandez-Izquierdo & Krochta, 2008; Sabato *et al.,* 2001). On the other hand, too high temperature can induce protein

degradation, therefore weakening the film's protein network structures. Differential scanning calorimetry (DSC) is extensively used to characterize the thermal transitions of proteins. The glass transition temperature (T_g), melting, aggregation, crystallization, thermal denaturation, and protein degradation can be studied using DSC. For example, after thermal denaturation, soy protein film exhibited a single T_g instead of the two characteristic T_g values (Jinwen Zhang, Mungara, & Jane, 2001). Fitzsimons reported the existence of an exothermic peak that shows the slow formation of aggregation in a thermal gelation of whey proteins (Fitzsimons, Mulvihill, & Morris, 2007).

1.5.5.3. pH

pH plays an important role in protein films prepared from water soluble materials such as whey protein isolate and soy protein isolate, because the solubility of these proteins is isoelectric point (pI) dependent. During the dissolution of proteins, the interactions between the solute macromolecules are neutralized by combining with the solvent (Banker, 1966). The charge groups repel each other and produce spacing between the polymer chains when the functional groups on a linear polymer become ionized during dissolution. The greater the degree of dissolution creates more charges on the chain and greater unfolding of the polymer chain. As the charges on the polymer chains increase, the interactions between the polymer chains and the solvent increase. The proteins are soluble at extreme low or high pH, away from their isoelectric point. Gennadios studied the effect of pH on soy protein isolate film and observed that highly acidic pH or alkaline conditions hindered the formation of soy protein isolate film (Gennadios *et al*., 1993). Kinsella and Philip reported that films formed near the isoelectric point of major proteins are stronger (Wittaya, 2012).

1.5.5.4. Other additives

In some cases, cross-linking agents or enzymes are also employed in a film forming process to improve moisture resistance, cohesion, mechanical strength, and barrier properties. The most commonly used enzymes for this purpose are transglutaminases and peroxidases (Motoki, Aso, Seguro, & Nio, 1987). For example, the egg protein network treated by transglutaminases showed highly reduced water vapor permeability (WVP) (Lim, Mine, & Tung, 1998). Chemical crosslinking reagents such as formaldehyde have been added to wheat gluten films to increase covalent binding between protein molecules, leading to higher tensile property and lower elasticity (Micard, Belamri, Morel, & Guilbert, 2000). However, adding covalent cross-linking agents to food-grade films is still extremely dubious.

1.5.6. Film Properties

Mechanical properties and water vapor permeability are the most important properties for food packaging films which are discussed below

1.5.6.1. Mechanical properties

Mechanical properties are essential for food packaging materials as they protect food under stress during transportation, handling and storage. Tensile strength (TS), elongation (Ɛ) and elastic modulus are the important mechanical properties of food packaging films. It is very critical to maintain structural integrity and improve mechanical handling of the food packaging films (Rhim & Lee, 2004). Tensile strength (TS) shows the maximum stress tolerated by the films before fracture and is usually expressed as MPa. TS indicates ability of film to maintain physical integrity that is essential to obtain good barrier properties of the food packaging materials. Elongation (Ɛ), is an extent to which film can stretch before breaking and expressed in percentage of film original length (Krochta, 2002). Basically, it represents the flexibility and extensibility of the films. The

mechanical properties of materials depend on the extent of inter and intra- molecular interactions. Proteins derived nanocomposite films mechanical performance mainly results from covalent bonds or other high energy bonds. Studies suggested that specific protein nanoparticle interactions and nanofiller dispersion play a major role in the mechanical properties.

Layered silicates have been mostly used as a reinforcing agent in proteins derived films. Most studies are reported on unmodified clays, particularly montmorillonite (MMT). Both MMT and protein are hydrophilic in nature, help to give an enhanced nanodispersion, leading to the improved mechanical properties (Angellier-Coussy *et al*., 2013). Cellulose whiskers or chitin, starch nanocrystals, carbon nanotubes and silica have also been used to study the effect on mechanical properties of proteins derived films (Grossman, Nwabunma, Dufresne, Thomas, & Pothan, 2013). Table 1.2 shows some of the proteins derived bionanocomposites films mechanical properties.

Table 1.2 Mechanical properties of proteins derived bionanocomposites

System	Sample	E (MPa)	TS (MPa)	Ɛ (%)
Protein/TiO_2	Whey (33 wt% glycerol)	-	6	95
	0.1 wt% TiO_2	-	10	95
	0.25 wt% TiO_2	-	10.2	98
	0.5 wt%TiO_2	-	9.2	96
	1 wt% TiO_2	-	8.4	75
	2 wt% TiO_2	-	6.9	27
	Whey (50 wt% glycerol)	31 ± 3	1.7 ± 0.1	55±1
	0.5 wt% TiO_2	66 ± 3	2.4 ± 0.1	54±1
	1 wt% TiO_2	63 ± 2	2.2 ± 3.1	40±1
	2 wt% TiO_2	44 ± 3	1.9 ± 0.2	15±1
	4 wt% TiO_2	39± 4	1.8 ±0.1	12±1
Protein/ Silica	SPI (30 wt% glycerol)	174	6.8	108
	4 wt% SiO_2	310	11.3	133
	8 wt% SiO_2	195	7.9	175
	12 wt% SiO_2	295	9.9	59
	16 wt% SiO_2	275	9.6	37
	20 wt% SiO_2	240	9.3	27
	24 wt% SiO_2	115	9.2	11
Protein/Carbon nanotubes	SPI (30 wt% glycerol)	88	6.9	171
	0.25 wt% MWCNT	261	11.6	193
	0.5 wt% MWCNT	252	10.7	153
	0.75 wt% MWCNT	248	9.1	148
	1 wt% MWCNT	101	6.2	158
	2 wt% MWCNT	118	7.8	160
	3 wt% MWCNT	177	9.8	165

Protein/Chitin or Cellulose Whiskers	SPI (30 wt% glycerol)	26	3.3	205
	5 wt% chitin whiskers	32	3.9	134
	10 wt% chitin whiskers	34	5	86
	15 wt% chitin whiskers	45	5.7	82
	20 wt% chitin whiskers	158	8.4	29
	30 wt% chitin whiskers	106	6.3	25
	SPI (30 wt% glycerol)	44	5.8	188
	5 wt% cellulose whiskers	51	6.3	133
	10 wt% cellulose whiskers	62	6.9	115
	15 wt% cellulose whiskers	74	7.6	95
	20 wt% cellulose whiskers	90	8.1	61
	30 wt% cellulose whiskers	133	8.1	36
Protein/Starch nanocrystals	SPI (30 wt% glycerol)	108	6.8	171
	1 wt% starch nanocrystals	259	9.5	60
	2 wt% starch nanocrystals	313	10.2	51
	4 wt% starch nanocrystals	270	8.8	34
	8 wt% starch nanocrystals	248	8.5	68
	16 wt% starch nanocrystals	205	6.1	12

Source: Adapted from (Angellier-Coussy *et al.*, 2013)

1.5.6.2 Water vapor permeability

Another important feature of food packaging material is the barrier properties such as, moisture, CO_2 and O_2. Preservation and safety of foods are the key features of food packaging. Shelf life of packaged food is associated with the degradation reactions. Food packaging has role in transferring moisture vapors which participate in degradation of food. Moisture barrier property plays crucial part for the texture (softness, firmness and crispness) preservation and microbial spoilage or pathogen species (Grossman *et al*., 2013). The main challenge to make protein-based films for food packaging is to optimize the conditions (temperature and pressure), and proteins and plasticizer concentration to attain the required water vapor transmission rate. Protein's amino acid composition and their distribution determines the barrier properties (CUQ, AYMARD, CUQ, & GUILBERT, 1995). If the protein structural arrangement allows some of the groups free, the film may have possibility to interact with other molecules in the environment. In general, hydrophilic groups in proteins favor moisture transportation rather than other gases (CO_2 and O_2) permeability. The permeability of proteins derived bionancomposite films can be controlled by number of factors, such as, nature of protein, plasticizer and nanoparticle (polar or non-polar groups), testing conditions and film thickness. Studies suggested that proteins derived films have excellent gas barrier properties in absence of moisture particularly, against oxygen. Baker *et al* reported that wheat gluten based films have 800 times lower oxygen permeability than low density polyethylene (Bakker & Eckroth, 1986). On the other hand, proteins derived films have relatively high water vapor permeability. The water vapor permeability can be lowered by using hydrophobic proteins and heat them to increase cross linking by compression molding (R Sothornvit, Olsen, McHugh, & Krochta, 2003).

1.6. Potential application of spent hen proteins

Myofibrillar and sarcoplasmic proteins are mostly extracted using alkali-aided extraction. These alkali-aided extracted proteins have been studied for food applications, but not yet explored for food packaging. Alkali-extracted proteins have good cohesive properties. Apart from that, only myofibrillar proteins have been studied as edible films for food packaging (CUQ *et al*., 1995). Additionally, these proteins are known to be hydrophilic (Omana, Xu, Moayedi, & Betti, 2010) as aspartic acid and glutamic acid are the major amino acids of these isolated proteins. The hydrophilic nature of these proteins may make spent hen proteins a good candidate to mix with hydrophilic nanoparticles to give them good mechanical strength.

1.7. Conclusion and research objectives

Based on the literature review, spent hen meat has the potential to be converted into proteins derived bionancomposite films. However, research in this area is very limited. The goal of this research is to extract proteins from spent hen meat and exploring the value-added potential of spent hen proteins as food packaging films.

The specific objectives are:

(1) To optimize conditions for protein extraction from spent hen carcasses to achieve the highest recovery and purity.

(2) To form films from spent hen proteins by compression molding technique using different plasticizers such as glycerol and sorbitol.

(3) To optimize the concentration for plasticizers and study which one works best for these proteins.

(4) To add different nanoparticles to modulate the proteins film mechanical strength.

(5) To investigate the addition of different percentages of nanoclay (bentonite), nanocrystalline cellulose and glycidyl POSS nanoparticles and their effect on the proteins films mechanical strength.

(6) To study the proteins film structure changes during processing (ATR-FTIR), thermal stability (TGA, DSC), viscoelastic properties (DMS), crystallinity (XRD), and WVP to determine the effects of nanoreinforcement on material properties.

The core piece of this work is to find an alternative way for the disposal of spent hen carcasses and produce a method to develop spent hen protein-based food packaging material for industrial applications. The success of this work can benefit local farmers in regards of the spent hen disposal issue and can help industries to make food packaging from renewable, sustainable, and biodegradable materials.

1.8. References

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CHAPTER 2. PROTEIN EXTRACTION FROM WHOLE SPENT HEN

2.1. Introduction

Besides the challenges of mortality and litter disposal, the poultry industry is also facing an economical barrier because the cost of removing spent hen often exceeds their value for meat. Since spent hens have low market value as there is only 166 g of white meat per spent hen and their disposal (burial, composting or incineration) is often infeasible and these methods raise environmental concerns as well (Aho, 1999; Freeman et al., 2009). Additionally, age makes spent hen muscles very tough due to the formation of heat stable collagen (Nakamura, Sekoguchi, & Sato, 1975). Therefore, finding alternatives to disposal that are environmentally safe and secure is prudent and a great challenge for the poultry industry.

Spent hens are egg laying hens that stop laying eggs usually by 85-100 weeks of age. After that, these birds become available for further processing (Nowsad et al., 2000). In Canada, more than 32 million birds are removed at the end of their laying cycle and need to be replaced by younger and more productive flocks (AAFC, 2006). The average live weight of the egg layers is about 1.5-1.7 kg (Kondaiah & Panda, 1992).

One possible way to enhance the utilization of spent hen is to isolate valuable components. Since spent hens contain 17% of proteins on wet basis therefore this is a huge sustainable bioresource which can be extracted and further used for industrial applications. Several methods have been developed for the protein extraction like mechanical separation and surimi processing. Low processing yields and usage of excessively large volumes of water are limiting factors of these approaches (Hrynets, Omana, Xu, & Betti, 2011). The recently developed method for protein extraction is alkali aided extraction, also known as acid/base extraction. This technique is based on the principal of pH dependent solubility. The process comprises initial solubilization of proteins

at high or low pH value at which the protein structure is unfolded, followed by refolding/precipitation at the isoelectric point (Kristinsson, Theodore, Demir, & Ingadottir, 2005). Under acidic and basic conditions, proteins carry a net positive and net negative charge respectively which is an important characteristic for obtaining high solubility accompanied by low viscosity of the initial homogenate (Hrynets *et al*., 2011).

The protein recovery during alkali aided method is chiefly determined by three major factors: the solubility of the proteins at extreme acidic/basic conditions, the size of the sediment formed during the centrifugation, and the solubility of the proteins at the pH selected for precipitation. Ideally, the acid or alkali driven solubilization should be high while the other two factors should be low (Nolsøe & Undeland, 2009). Usually, high protein recovery is obtained by pH-shift processing compared to the conventional surimi method which is associated with the retention of sarcoplasmic proteins (Nolsøe & Undeland, 2009). The extracted proteins are usually kept frozen until use since frozen storage can efficiently decrease the microbial activities. Apart from this, storage at high temperature also induces protein and lipid oxidation that negatively affects functional properties like protein solubility and water holding capacity (Kijowski & Richardson, 1996).

In addition to protein extraction from spent hen, collagen was extracted from spent hen feet. Collagen is the most abundant protein in mammals and the major protein constituent of connective tissues, bones, skin, cartilage and tendon. It constitutes about 23-30% of the total body proteins (Huda, Seow, Normawati, & Aisyah, 2013). Mammals, poultry and fish are major sources of collagen. Poultry feet have collagen in abundance which can be extracted and used to produce collagen based films (Pachence, 1992). Collagen having molecular weight of 300 kDa is of three types, type I, type II and type III. Type I making up of 90% of the total collagen in organisms, has

numerous advantages as it is widely available, sustainable and renewable resource, biocompatible, and degradable (Yang & Shu, 2014). It has wide applications in various fields such as packaging, cab be used as an edible film and for encapsulation (Rodziewicz-Motowidło *et al*., 2008; Sadowska, Kołodziejska, & Niecikowska, 2003). According to Statistics Canada, 1067 million chickens were produced in 2015. From this number, we can imagine how many chicken feet are produced every year. The collagen can be extracted using a number of common methods such as salting out, acidic, enzymatic and alkaline method. The acidic method was used during the collagen extraction from chicken feet since this method gives a much higher fraction of collagen when compared to other processes. Hydrolysis is also faster during this process when compared with enzymatic and salting out methods (Yang & Shu, 2014).

During the study for protein extraction, other than alkali aided extraction method, salts of KCl and NaCl with different ionic strength of phosphate buffers of varying pH have been used to extract proteins. Data from these methods is presented very briefly since protein purity and recovery was very low from these experiments.

The objectives of this study were as follows: (1) to extract protein from whole spent hen (including skin, bones, breast, leg and gizzard) with high protein recovery and purity; (2) to determine the effect of pH on the protein recovery and purity from whole spent hen; (3) to extract collagen protein from chicken feet with high recovery and purity.

2.2. Experimental section

2.2.1. Materials

The whole spent hen (including skin, bones, breast, leg and gizzard) was received from a local market with the help of Alberta Hatching Egg Producers (AHEP) and feet were obtained from a local supplier (Edmonton, AB, Canada). The whole spent birds were ground at Agri-Food Discovery Place (AFDP). The birds were first ground using a meat grinder with a 5mm plate and then passed through a Stephan Emulsion Mill with a 1.3mm disk. The ground meat was divided into different samples. Each sample was filled in polyethylene bags and kept at -20 °C until use. Before extraction, sample was thawed overnight at 4 °C. HCl, NaOH, Na_2HPO_4, KH_2PO_4, NaH_2PO_4, NaCl and KCl were purchased from Sigma Aldrich and used as received. Precast gel (4-20%) ready gel (Bio- Rad Laboratories Inc; Hercules, CA) was used to separate protein by SDS-PAGE.

2.2.2. Proximate analysis of raw material

The moisture, proteins purity, total fats and ash contents for raw spent hen and spent hen feet were determined as follows

2.2.3. Moisture content analysis of raw material

Moisture contents of raw spent hen, protein isolates and feet material were determined according to AOAC method (William Horwitz, 2005)

$$\% \text{ Moisture contents (w/w)} = \frac{\text{Wet weight- Dry weight x 100}}{\text{Wet weight}}$$

2.2.4. Determination of total fat contents

Lipid extraction of raw spent hen, its feet and protein isolates was done by Folch method, shown in diagram 2.5 (Pérez-Palacios, Ruiz, Martín, Muriel, & Antequera, 2008). It was

performed using the original extraction ratio of 20 parts chloroform: methanol (2:1, v/v) to 1 part of sample. 5 grams of sample was mixed with 100 ml of chloroform: methanol (2:1, v/v). The mixture was homogenized, centrifuged (13,000 x g) for 10 minutes and filtered. Further, 5 ml of distilled water was poured into the filtrate and the resulting mixture was shaken vigorously. The final biphasic system was separated by centrifugation (13,000 x g) for 10 mins. The upper aqueous phase was removed. The lower phase (chloroform) was filtered through anhydrous sodium sulphate and collected. Lipid contents were then gravimetrically determined after chloroform was evaporated with a rotary evaporator under vacuum.

2.2.5. Determination of ash contents

Ash contents of raw spent hen material, feet and protein isolates were determined by AOAC (William Horwitz, 2005). Sample was dried and ground before ash contents determination. The sample was kept in muffle furnace at 550 °C overnight. The ash content is calculated from the loss in weight that occurs during incineration which is as follows

$$\% \text{ Ash} = \frac{\text{Weight of ash x 100}}{\text{Weight of sample}}$$

2.2.6. Protein extraction from whole spent hen by salt aided method

The proteins were extracted using modified method developed by Wang *et al.,* as shown in diagram 2.1 (H. Wang, Zhang, Cao, Zhang, & Chen, 2013). This method was used with modifications as presented in diagrams 2.2 and 2.3. Whole spent hen protein sample was mixed with phosphate buffer (15.6 mmol/L. Na_2HPO_4, 3.5 mmol/L, KH_2PO_4) of pH 7.5 (w:v 1/6) and homogenized the mixture for 30 mins. The resulting homogenate was centrifuged (1000 X g/ min) for 10 mins. The supernatant (I) was collected and residue (I) was re-extracted with 1 L of the same phosphate buffer, homogenized, and centrifuged again as described above. After removal of supernatant (II) from the residue (II), both supernatant (I & II) were combined and acetone was

slowly added to precipitate down the sarcoplasmic proteins. The precipitated material was centrifuged, and precipitates were washed with deionize water several times to get sarcoplasmic proteins. Ice-cold phosphate buffer (0.05 mol/L. NaH_2PO_4, 0.05 mol/L, Na_2HPO_4 containing 1.1 mol/L KCl) at pH 7.5 was added to residue (II) by 1:10 ratio. The mixture was homogenized for half an hour followed by centrifugation at 1000×g for 10 mins. The resulting residue III was collected and suspended in 5-fold volume of the same ice cold phosphate buffer followed by homogenization and centrifugation. After 2 cycles of homogenization, the resulting residue IV was washed with distilled water and used as the stromal protein fraction. Supernatant III and IV were combined and dialyzed against deionized water (dialysis membrane of molecule cutoff =3,500 Da was used). The water was changed after every 2 h initially and after 12 h, it was changed every 8 h during dialysis. The obtained pellet was myofibrillar protein.

Further, the extraction was also carried out with buffer of pH 8, in the same sequence as the process shown in diagram 2.2. In addition to that, the residue IV was subjected to alkali extraction (Shahidi & Synowiecki, 1996). The NaOH was added to residue IV with the ratio of 1:4. The resulting mixture was homogenized and centrifuged. The alkali soluble proteins in supernatant were precipitated by using HCl at 5.5 pH value and the residue had stromal proteins.

Furthermore, the procedure was also modified by using NaCl salt solution (1.2 M) and adjusted pH of the mixture at 6.5 to extract myofibrillar proteins as shown in diagram 2.3 (Munasinghe & Sakai, 2004). The rest of procedure was same as mentioned in above process.

2.2.7. Alkali aided extraction from whole spent hen

McCurdy method was used for protein extraction with some modifications (McCURDY et al., 1986) as shown in diagram 2.4. The whole spent hen sample was mixed with ice cold distilled water (1:4) by using homogenizer for 15 mins. The pH of the resulting mixture was raised to 12

by using 2.0 M NaOH under constant stirring. Further the slurry was centrifuged (13,000 x g) at 4 °C for 10 minutes using refrigerated centrifuge. Thus, three layers were formed, an upper layer of fat, middle layer of soluble proteins and a bottom insoluble layer. The middle layer was separated carefully and pH was adjusted to 5.75 by using 4.0 M HCl to precipitate the proteins. The precipitated proteins were centrifuged (13,000 x g) at 4 °C for 10 minutes.

2.2.8. Preparation of proteins isolate at pH 11.5

The extraction process was carried out in the same sequence as described earlier with some difference at the solubilization step. Here the proteins were initially solubilized at pH of 11.5.

2.2.9. Purity and recovery of isolated proteins

The total protein contents of both the raw spent hen meat, chicken feet and purity of protein isolates were determined by a LECO (Nitrogen/Carbon) analyzer (TruSpec®CN., MI., CA, USA.). Spent hen meat and protein isolates were freeze dried (using LABCONCO Inc., FreeZone12, MO., CA, USA) and ground into fine power before analysis. The recovery was measured using the reported method (Omana et al., 2010). It was expressed as difference in total content of isolates and raw material.

$$\text{Protein recovery (\%)} = \frac{\text{Protein content of isolate (g) x 100}}{\text{Protein content of raw material (g)}}$$

2.2.10. Collagen extraction

The spent hen feet were ground finely and kept at -20 °C before use. For the collagen extraction, the ground frozen feet were thawed first at 4 °C overnight. The acid method was used developed by Liu *et al* with some modifications as shown in diagram 2.6 (Liu, Lin, & Chen, 2001). The feet sample was mixed with distilled water (1:5) and homogenized it for 1 hour. After that, 5% lactic acid was added, the mixture pH was adjusted to 2.75 and kept at 4 °C for 72 hours. At the end of soaking, the mixture was homogenized followed by filtration to remove bone residue.

The solution was neutralized by using 2M NaOH to precipitate the collagen. This neutral solution was centrifuged (13000 x g) at 4 °C for 15 mins. The supernatant was discarded and precipitated collagen was freeze dried to remove the moisture.

2.2.11. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE)

SDS- PAGE of alkali aided extracted proteins was done according to the method described by Laemmli (Laemmli, 1970). Precast (4-20%) ready gel (Bio- Rad Laboratories Inc; Hercules, CA) was used to separate proteins in a Mini- PROTEAN tetra cell attached to a Powerpack Basic electrophoresis apparatus. For each sample, 20 µl was loaded and ran at a constant voltage of 150 V. After staining and destaining, gels were scanned uing an Alpha Innotech gel scanner (Alpha Innotech Corp, San Leandro, CA) with FluorChem SP software. Standard protein marker from Bio-Rad (Bio-Rad Laboratories Inc., Hercules, CA, USA) was loaded into a separate well.

2.2.12. Statistical analysis

Each experiment was done in at least triplicate and their mean values were used for statistical analysis. IBM SPSS statistics software (Version 23 SPSS Inc, USA) were used to analyze the data of each experiment.

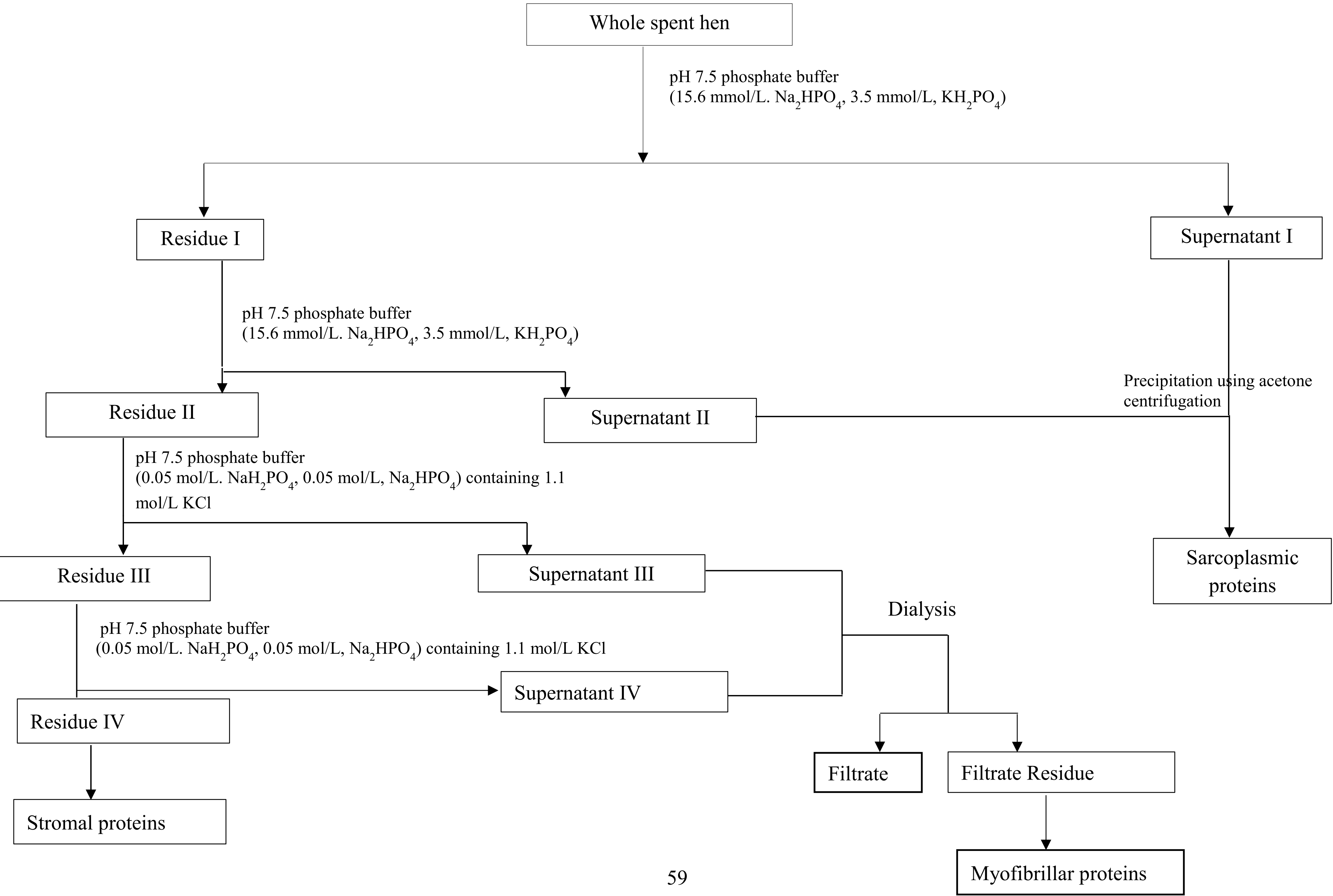
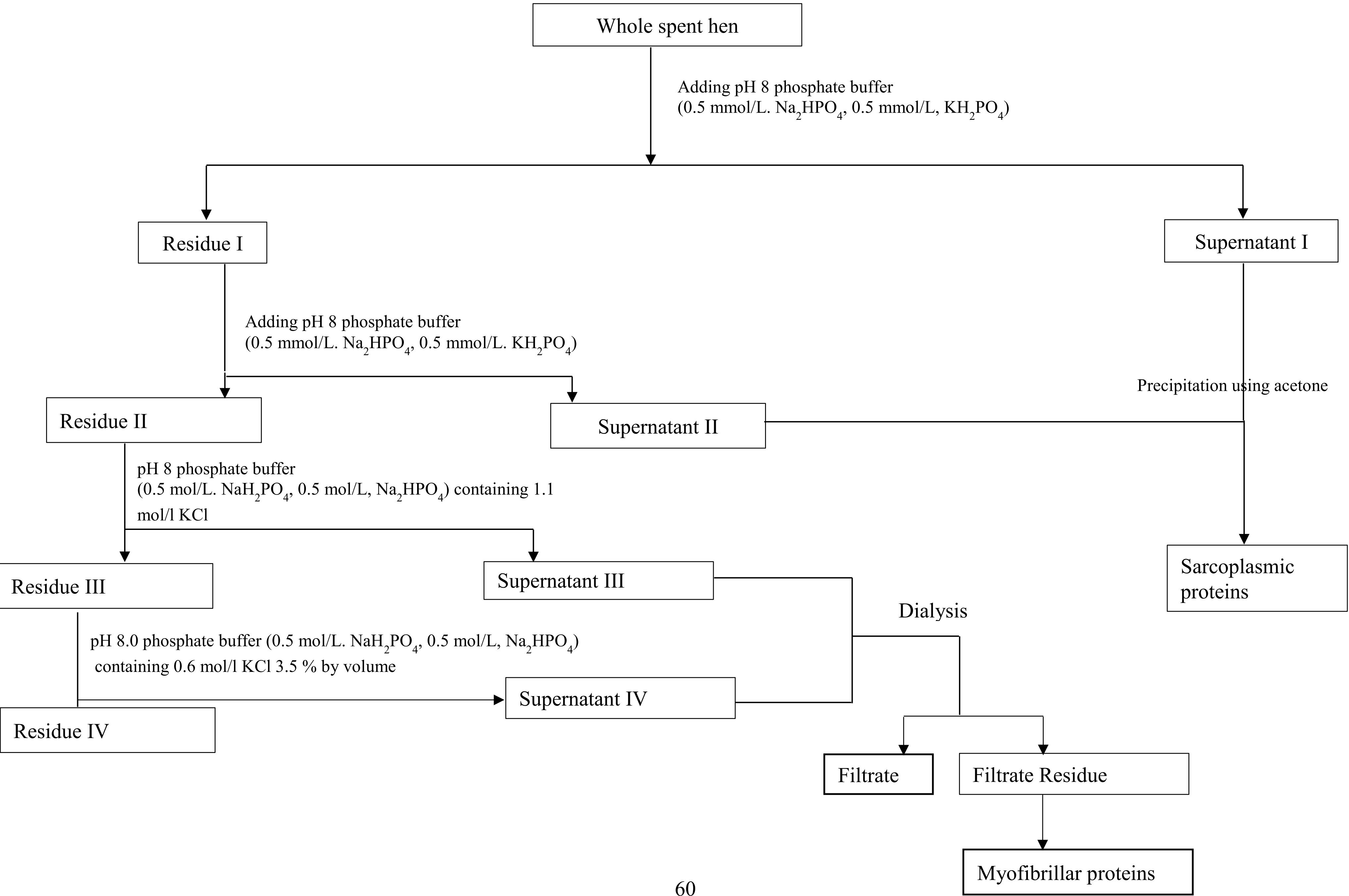


Figure 2. 1 Schematic diagram of the sarcoplasmic, myofibrillar and stromal proteins extraction from whole spent hen



Whole spent hen
Adding pH 8 phosphate buffer
(0.5 mmol/L. Na_2HPO_4, 0.5 mmol/L, KH_2PO_4)
Residue I
Supernatant I
Adding pH 8 phosphate buffer
(0.5 mmol/L. Na_2HPO_4, 0.5 mmol/L. KH_2PO_4)
Precipitation using acetone
Residue II
Supernatant II
pH 8 phosphate buffer
(0.5 mol/L. NaH_2PO_4, 0.5 mol/L, Na_2HPO_4) containing 1.1 mol/l KCl
Residue III
Supernatant III
Sarcoplasmic proteins
Dialysis
pH 8.0 phosphate buffer (0.5 mol/L. NaH_2PO_4, 0.5 mol/L, Na_2HPO_4)
containing 0.6 mol/l KCl 3.5 % by volume
Supernatant IV
Residue IV
Filtrate
Filtrate Residue
Myofibrillar proteins

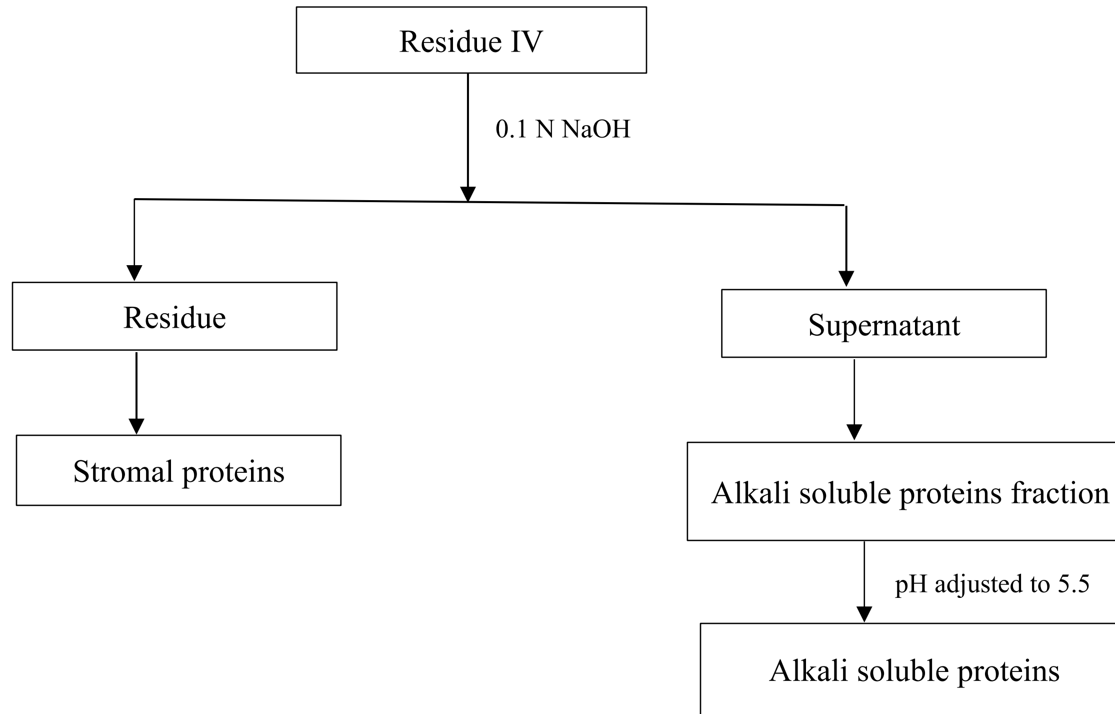
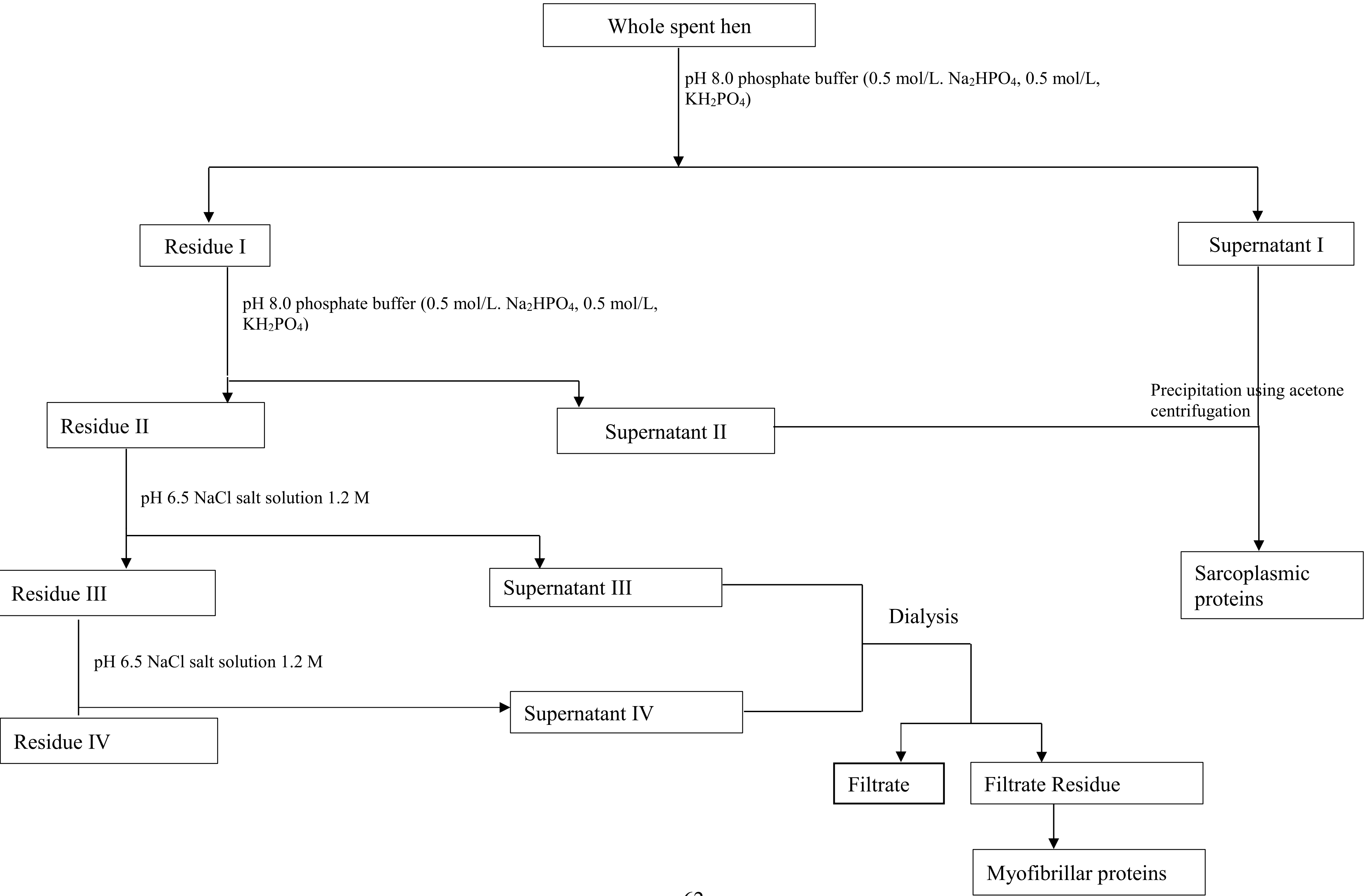


Figure 2. 2 Schematic diagram of the sarcoplasmic, myofibrillar, stromal and alkali soluble proteins extraction from whole spent hen


Whole spent hen
pH 8.0 phosphate buffer (0.5 mol/L. Na_2HPO_4, 0.5 mol/L, KH_2PO_4)
Residue I
Supernatant I
pH 8.0 phosphate buffer (0.5 mol/L. Na_2HPO_4, 0.5 mol/L, KH_2PO_4)
Precipitation using acetone centrifugation
Residue II
Supernatant II
pH 6.5 NaCl salt solution 1.2 M
Residue III
Supernatant III
Sarcoplasmic proteins
Dialysis
pH 6.5 NaCl salt solution 1.2 M
Supernatant IV
Residue IV
Filtrate
Filtrate Residue
Myofibrillar proteins

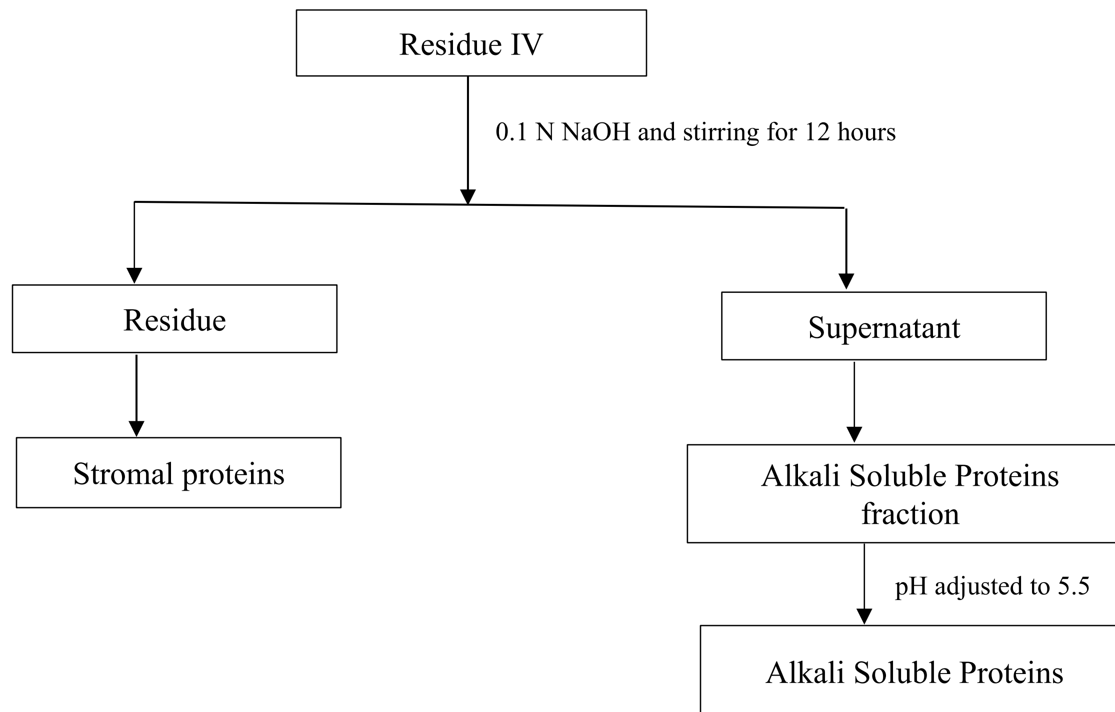


Figure 2. 3 Schematic diagram of the sarcoplasmic, myofibrillar, stromal and alkali soluble proteins extraction from whole spent hen

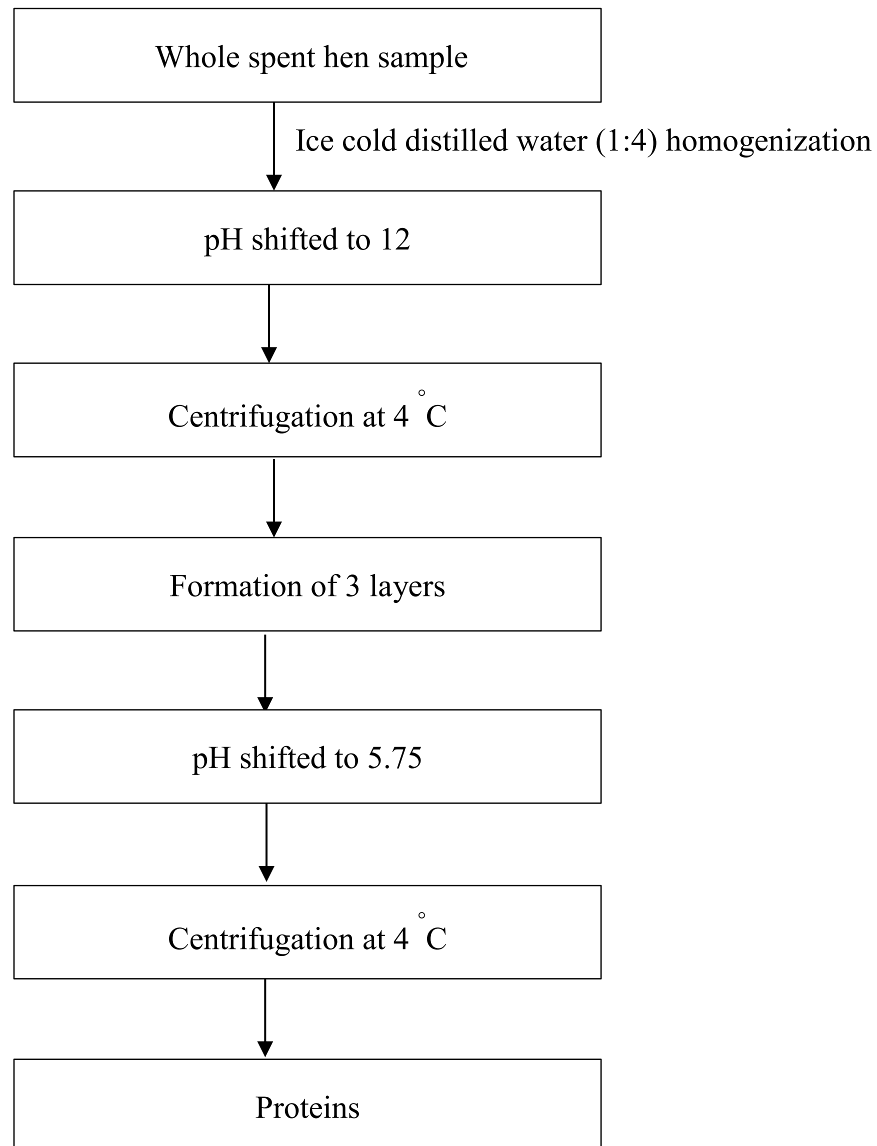


Figure 2. 4 Schematic diagram of alkali aided proteins extraction from whole spent hen

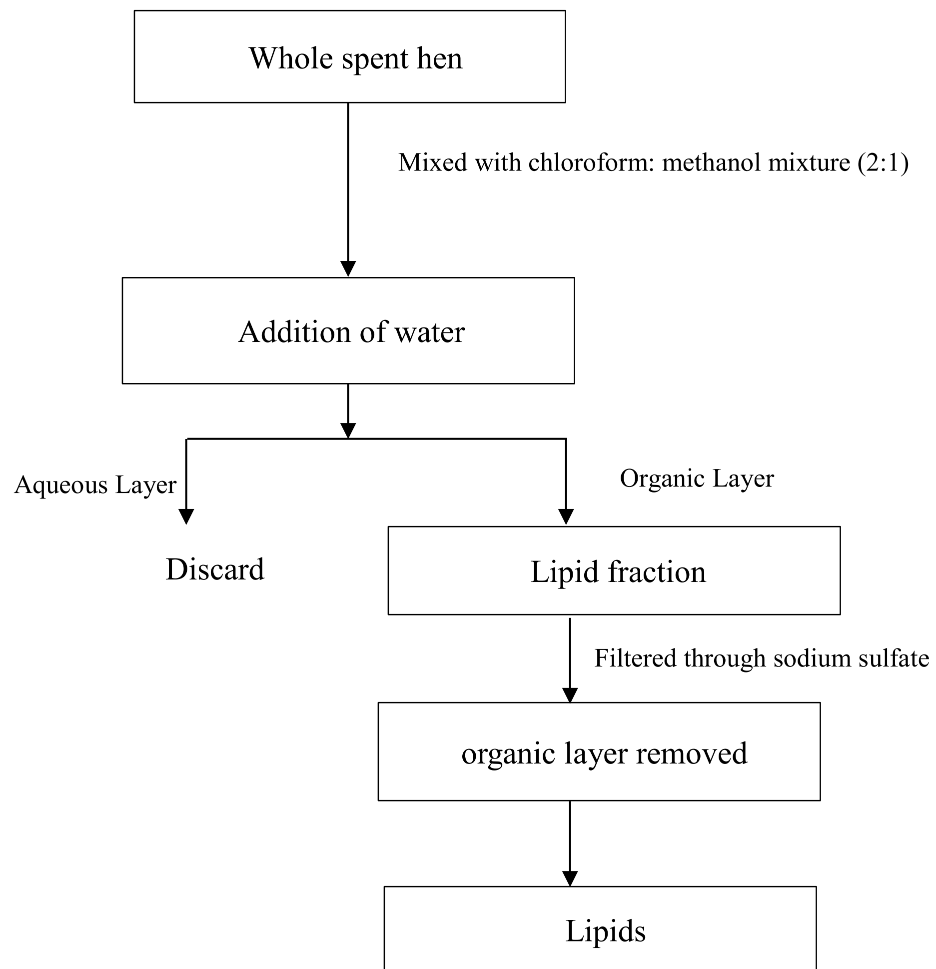


Figure 2.5 Schematic diagram of lipids analysis

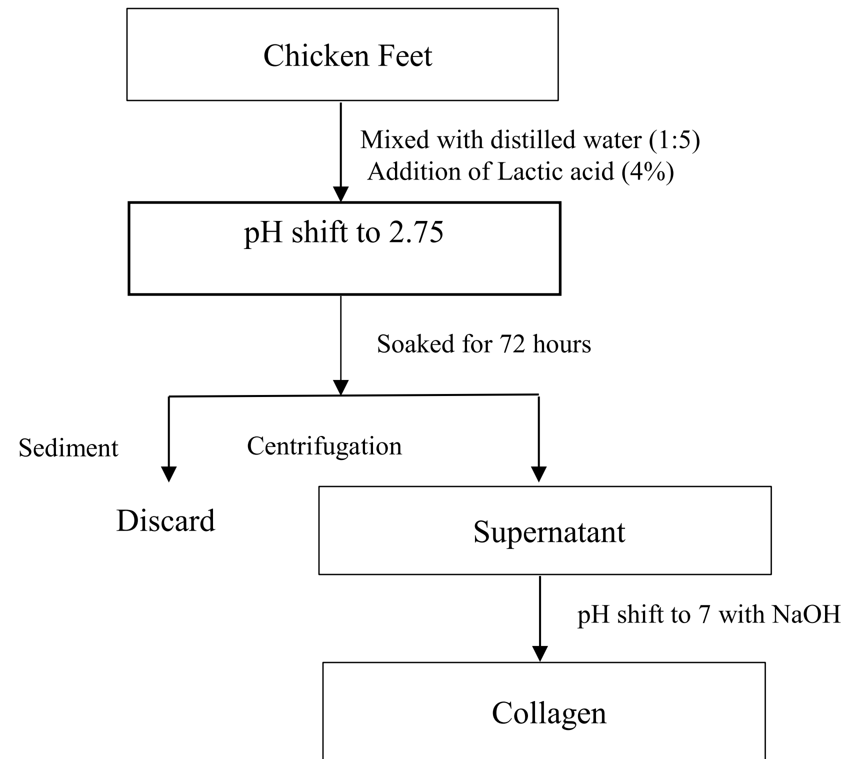


Figure 2.6 Schematic diagram of collagen extraction from chicken feet

2.3. Results and discussions

2.3.1. Water, protein, lipid and ash contents of raw material and protein isolates

The moisture content of the spent hen was 67%. While the protein and lipid contents of the raw spent hen sample were 17% and 14% respectively on a wet basis (table 2.1). The water content (85%) of the protein isolates was significantly higher than that of the spent hen raw material but the solubilization pH either 11.5 or 12 did not affect much. The ash content was investigated in both raw material and protein isolates. It was slightly lower in the raw material as compared to protein isolates (2.06% Vs 2.40%) because during protein precipitation there is salt formation that contributed to ash content.

Table 2.1 Proximate analysis of raw spent hen

Quality Parameter	% (Wet basis)
Moisture	67.72±2.68
Protein	17.05±0.04
Fat	14.07±0.02
Ash	2.06±0.03

Results are presented as means ± standard deviations

2.3.2. Protein extraction by salt aided extraction

Protein recovery and purity by using different buffer solutions of varying ionic strength was lower than the previously extraction of proteins from fish. The protein recovery was only 27% and proteins purity were as follows

Table 2.2 Protein purity by salt aided extraction

Protein class	Protein purity (%)
Sarcoplasmic	3.39±0.09s
Myofibrillar	36.47±0.03
Stromal	36.33±0.01
Alkali Soluble	91.84±0.21

Results are presented as means ± standard deviation

2.3.3. Protein Solubility

Literature suggest that the poultry meat proteins are more soluble at pH value of 11.5 (Hrynets *et al*., 2011). That is why pH 11.5 and 12.00 were selected for solubilization of the proteins. The reason for using the pH-shifting method on whole spent hen is the solubility of proteins is maximum at low and high pH values. The high solubility is necessary to separate proteins efficiently from the undesired meat constituents such as connective tissues, lipids and impurities. On the other hand, low solubility is required to precipitate the soluble proteins at their isoelectric point. In this study, the lowest solubility or highest precipitation was at pH 5.75 which is the isoelectric point of most proteins in the spent hens. Although this value is different from previous studies (pH5.50) for the precipitation of muscle proteins (Hrynets *et al*., 2011). This may be because the starting material is quite different from those studies, here whole spent hen was used to isolate proteins. The negative and positive charges are equal at the isoelectric point. Thus, association among protein molecules is strong due to the ionic interactions. Consequently, precipitation occurs since protein-solvent interactions are replaced by the protein-protein interactions. Solubility of proteins is increased with alkalization since proteins become positively

charged and provide more binding sites to water and increased protein- water interactions (Hamm, 1996).

2.3.4. Protein purity and recovery

Protein recovery is of prime importance to determine the developed method efficacy related to cost. The method of pH shift has been extensively used for fish muscles, chicken residue and mechanically separated turkey meat. However, in this study we have used this method with slight modification for the whole spent hen to extract the proteins. Alkali aided extraction method is better than the acid aided extraction due to the fact that there is less denaturation of proteins during the former process (Omana *et al*., 2010).

Table 2.3 Protein purity and recovery by alkali aided extraction

Extraction Ph	Protein purity (%)	Recovery (%)
11.5	77.97±0.10	64.46±0.45
12.0	96.0±0.03	74.02±0.04

Results are presented as means ± standard deviations

The solubility of proteins at higher pH (12) and precipitation at 5.75 lead to higher protein recovery compared to precipitation at pH 5.50. The protein recovery with this method was 74 % which is quite higher compared to the same method used for protein extraction from turkey and fish (Kristinsson *et al*., 2005). The protein contents of the recovered proteins was 96% which is higher than the previous alkali aided extraction methods reported by Kristinsson *et al* and Omana *et al* (Kristinsson *et al*., 2005; Omana *et al*., 2010). Optimal pH for protein solubility was 12 which is consistent with Omana result but higher purity as achieved compared to his method probably because we used whole spent hen. The subsequent precipitation was done at pH 5.75.

The recovery at pH 11.5 is less as shown in table 2.3. The hydrophobicity is increased at higher pH which has positive effect on protein recovery. This can be explained on myofibrillar protein aggregation at their isoelectric point, these proteins aggregate and precipitate, giving rise to hydrophobic interactions. Therefore, the protein becomes unfolded and, as a result, exposure of hydrophobic amino acids may lead to enhanced aggregation and precipitation at the isoelectric point (Ingadottir, 2004; Taskaya, Chen, Beamer, Tou, & Jaczynski, 2009)..

2.3.5. Lipid loss during alkali aided extraction

The total fat content of the raw spent hen sample used for protein extraction was 14% on the wet basis of the starting material. Lipid contents at pH value 11.5 and 12 were 44% and 45% respectively. The lipid loss was less as compared to previous studies done on fish (Nolsøe & Undeland, 2009). Less amount of water was used during protein extraction, a ratio of 1:5 is usually suggested to enhanced lipid removal, particularly polar phospholipids (Omana *et al*., 2010).

2.3.6. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS- PAGE)

SDS is extensively used to resolve the proteins in complex mixtures. Proteins are separated on the basis of their molecular weights (Laemmli, 1970). Four major bands were identified at 250 kDa, 100 kDa, 45 kDa and 37 kDa. The first band 250 kDa corresponds to myosin heavy chain (MHC), the band at 100 kDa relates to c-proteins, while bands at 45 kDa and 37 kDa are associated with actin and tropomyosin respectively. Two bands were observed between 100 kDa and 200 kDa, which could be attributed to the myosin degradation as previously reported during alkali aided extraction (Omana *et al*., 2010).

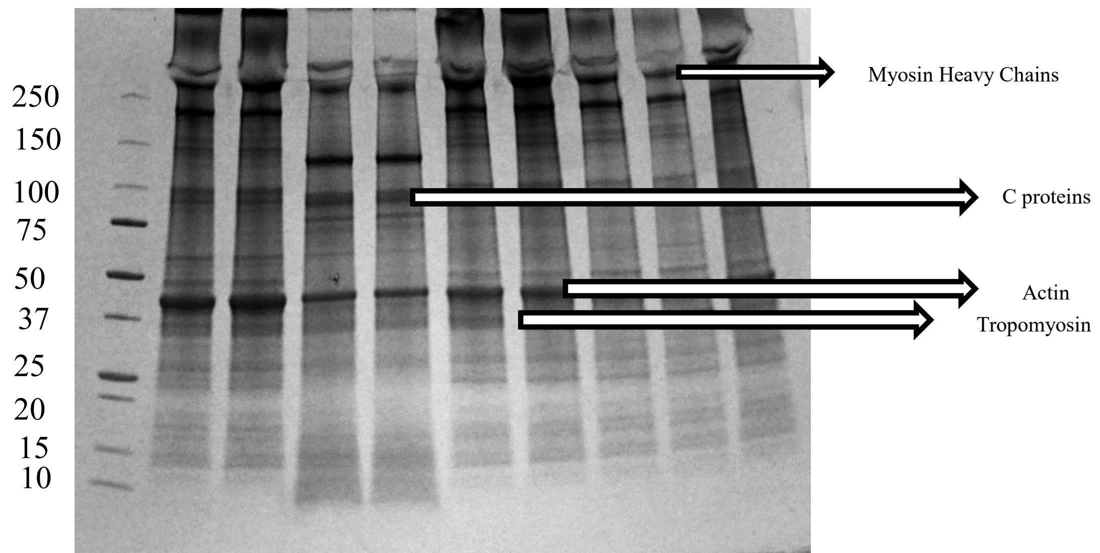


Figure 2.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of alkali aided extracted proteins

2.3.6. Collagen extraction, recovery and purity

Table 2.4 shows spent hen feet proximate analysis; the feet contain 63% moisture, 19% crude proteins, 11% crude fat and 6% ash contents. The data were similar to the results obtained by Li *et al* (2001). The crude collagen purity was 70% while its recovery was 35%, which is higher than the previous study done by Li *et al* (2001). Since the method, we used for collagen extraction was slightly different. The higher ash contents can be due to high level of bone residue.

Table 2. 4 Proximate analysis of spent hen feet material

Quality Parameter	% (Wet basis)
Moisture	63.18±1.50
Crude protein	19.7±0.04
Fat	11.80±0.04
Ash	6.02±0.20

Results are presented as means ± standard deviations

2.4. Conclusion

In summary, the protein recovery from whole spent hen was 74% while the protein purity in the final isolate was 96%. The protein recovery is higher as compare to the previous studies done. The SDS-PAGE showed that, the protein isolated contains four different kinds of proteins including actin, myosin heavy chain, tropomyosin and c-proteins. The protein recovery and purity is higher at pH value of 12 as compared to 11.5 since protein solubilization is greater at higher pH values. The lipid loss was the same whether extraction was done at a pH value of 11.5 or 12 because the same volume of water was used during extraction at different pH values. The protein recovery and purity with other method used for protein extraction was significantly lower than pH shift method. Hence, current studies suggested that the pH shift process is a better extraction method for protein isolation from whole spent hen. The amount of collagen extraction from spent hens' feet was 35% with protein contents of 70%. It was found that collagen extraction gave a better recovery by dissolving feet at pH 2.75 for 72 hours.

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CHAPTER 3. SPENT HEN PROTEIN DERIVED BIONANOCOMPOSITES FOR FOOD PACKAGING APPLICATIONS

3.1. Introduction

For the last two decades, there is growing attention in the production of synthetic polymers films to protect food. With the increasing population and pressure on petroleum resources and the environment, use of renewable and sustainable resources to produce biodegradable films that can enhance food quality and reduce waste disposal concerns are being explored (Krochta, 2002). Presently, proteins have got much interest for the production of food packaging with great potential to replace petro-based polymer films. Proteins are abundantly available, biodegradable and non-ecotoxic, which make them favorable choice for developing bioplastics (Angellier-Coussy *et al*., 2013)

Proteins generally exhibit poor mechanical properties, limited processability due to fragility and brittleness during thermoformation, which restrict their potential for food packaging application. Plasticizers provide the mobility to proteins in order to overcome these problems (Vieira, da Silva, dos Santos, & Beppu, 2011). Water, glycerol, propylene glycol, sorbitol, sucrose, polyethylene glycol, fatty acids, lipids and monoglycerides have been mostly used to improve proteins film forming capability (Selling *et al*., 2004; Q. Wang *et al*., 2003). Due to their hydrophilic nature, they are very sensitive to microbial spoilage and water. Therefore, substantial amount of plasticizer is needed to enhance thermos-processibility and film flexibility. (Grossman *et al*., 2013)

Recent investigations found that heat-curing of proteins produces stronger films with better mechanical strength and barrier properties. Both plasticizer and heating have major roles in the thermal processing of protein derived films. Upon heating proteins are unfolded leading to the

development of new interactions like hydrogen bonding and hydrophobic interactions to reinforce the proteins networks (Angellier-Coussy *et al*., 2013). The plasticizer addition is often vital to protein films to enhance film extensibility and flexibility by decreasing the interactions between polymer chains whereas increasing intermolecular spacing (Y. Wang, Cao, & Zhang, 2006).

Moreover, the most recently used technology to enhance mechanical strength and barrier properties of proteins is the use of nanofillers. The addition of nanoparticle in protein biopolymers leads to outstanding physical properties since these have large surface area to volume ratio which helps to strengthen interfacial adhesion between nanoparticles and protein molecules (Azeredo *et al*., 2009; H. M. C. de Azeredo *et al*., 2011; Zhao, Qiu, Yan, & Yang, 2011). Layered silicates are the most commonly used nanoscale particles as reinforcing agents in protein-based matrices. Carbon nanotubes, polysaccharide like cellulose, chitin, starch nanocrystals, silica, and TiO_2 nanoparticles are used in a lesser extent (Angellier-Coussy *et al*., 2013).

In this study, the proteins extracted from spent birds were plasticized, nanoreinforced and thermally processed. The plasticizers like glycerol, poly (ethylene glycol), ethylene glycol, 1,2-butanediol and sorbitol were used. Each plasticizer with 10%, 20%, 30%, 40%, and 50% (w/w) were used. However, no cohesive blends were obtained using ethylene glycol, 1,2- butanediol and sorbitol, showing that these plasticizers were ineffective to obtain suitable material for further study. Glycerol was selected for detailed study and materials were prepared using compression molding using various concentrations of cross-linker (chitosan) and nanoparticles. The moisture content (20%) with varying concentration of plasticizers was optimized for film formation. Three nanoparticles (bentonite, cellulose nanocrystals and glycidyl POSS) were studied with 1%, 3% and 5% concentration. The cross-linker was added with 1%, 3% and 5%. The addition of nanoclay (bentonite) gave better mechanical strength as compare to cellulose nanocrystals and glycidyl

POSS. The thermal, mechanical, water vapor permeability and crystallinity of the prepared films were investigated to see the effect of cross-linker, nanoparticles and plasticizers on the film overall properties.

3.2. Experimental section

3.2.1. Materials

The proteins were extracted from spent birds as described in chapter 2. The Chitosan, Glutaraldehyde, Glycerol, 1,2 Butanediol, Sorbitol, Ethylene Glycol, Polyethylene glycol, Cellulose nanocrystal (CNC), Bentonite, Glycidyl POSS, HCl, NaOH were purchased from Sigma Aldrich and used as received.

3.2.2. Sample preparation

The Blends of spent hen proteins with different concentrations of glycerol were prepared in a laboratory blender. After that, these blends were sealed in plastic bags and kept at room temperature overnight so that glycerol could sufficiently incorporate in to the spent hen proteins. The films were prepared by compression molding and plasticizer concentration was optimized. The blends with 40 and 50% glycerol displayed better properties compared to other concentrations. These two plasticizer concentrations were selected and blends were prepared using different concentrations of crosslinker chitosan (1, 3 & 5%) to optimize the crosslinker. Similarly, these blends were also put in plastic blends and placed overnight at room temperature. Blends with 3% chitosan displayed better properties compared to other concentrations of chitosan.Three samples for each nanoparticle were prepared by varying concentration 1%, 3% and 5% in each blend (40%, 50% glycerol and 3% chitosan). These mixtures were first blended in a laboratory blender followed by constant stirring for 30 mins. Blends were sealed in plastic bags and kept at room temperature

for 72 hours to give maximum time to nanoparticles to interact with the proteins. In all above-mentioned blends, moisture was kept constant which was 20%.

3.2.3. Film Preparation

Films of plasticized material was prepared by compressing molding the resin for 10 mins at 120 °C and 3500 psi pressure using a carver press.

33.2.4. Film Thickness

Digital caliper (digi-max caliper, sigma-aldrich,USA) was used to measure thickness and width of films at three different places and valued were averaged. The average film thickness was used for the determination of mechanical properties, dynamics mechanical properties and water vapor permeability.

3.2.5. Thermal Property Measurement

The degradation patterns of whole spent hen protein powder and derived films were studied using TGA Q50 (TA Instrument, USA) under a continuous nitrogen flow between 25-600 °C at a heating rate of 10 °C/min.

DSC was performed by calorimetric apparatus (2920 Modulated DSC, TA Instrument, USA) in a continuous flowing nitrogen atmosphere. The instrument was calibrated for heat flow and temperature using a sample of pure indium. All samples were scanned at a heating rate of 5 °C per minute in a temperature range of 25-300 °C.

3.2.6. Mechanical Property Measurement

Tensile properties (tensile strength, tensile modulus and break elongation) of the films (in triplicate) were measured at room temperature using universal testing machine (autograph AGS-X shimadzu, Canada) using standard procedure described in ASTM D822. Each specimen

dimensions were 9 mm X 50 mm. A 50 N load cell with a crosshead speed of 0.90 mm/sec was used. Before testing, samples were equilibrated at 25 $^{\circ}$C and 65 RH for 48 hours.

3.2.7. X-Ray Diffraction (XRD)

X- Ray diffraction analysis was done using a XRD (Rigaku Ultima IV unit) with Co radiation operated at 38 kV and 38 mA. The samples were scanned from 5° to 90° (2Θ) in a continuous scanning mode at rate of 2°/min with a 0.02 step size.

3.2.8. Dynamic Mechanical Analysis (DMA)

The viscoelastic properties of films were measured by using DMA Q800 (TA Instrument). The films were conditioned for 48 hours in a desiccator at 50% RH. The analysis was carried out in a tensile mode at an oscillatory frequency of 1 Hz with an applied deformation of 0.2% during heating with a temperature scan between -90 to 200 $^{\circ}$C with a heating rate 2 $^{\circ}$C /mins

3.2.9. Transmission Electron Microscopy (TEM)

CM20 FEG TEM/STEM (Philips) instrument was use to take TEM images of film operated at 80 kV. The films were fixed before analysis. The film was cut into a very tiny piece. Put into a 2.5% glutaraldehyde. 2% paraformaldehyde and 0.1M phosphate buffer (pH 7.2-7.4). Film stored in fixative until processing. After that, it was dehydrated through a graded ethanol series (20 mins) for each film sample solution. Then infiltration with spurr resin was done (1:1 ratio for 2 hours). Sample was embedded in flat molds with fresh spurr resin followed by curing at 70 $^{\circ}$C in oven overnight. Film sample was sectioned of 80 nm thickness and picked up on copper grid.

3.2.10. ATR- FT-IR Analysis

FT-IR using ATR technology (Bruker Optics, Esslingen, Germany) equipped with a single bounce diamond ATR crystal was used to determine functional group changes of the powdered protein and derived films. The analysis for each sample was performed in the wavelength range

between 410-4000 cm^{-1}. All sample spectra were collected at 16 scans at a resolution of 4 cm^{-1} and averaged using OPUS software version 6.5 provided by Bruker. A background spectrum of the clean ATR crystal was collected before applying and collecting the sample spectrum. Spectral examination, measurements and processing were done using nicolet omnic software.

3.2.11. Water vapor permeability (WVP)

Water vapor permeability was carried out based on the method described by ASTM standard method E96-95 with some modifications. The film samples were sealed on a permeation cell with exposed area 3.14×10^{-3} consisting of anhydrous calcium chloride (0% RH). The cell was kept in desiccator with sodium chloride saturated solution (75% RH). The cell weight was observed after every 24 hours for 144 hours.

3.3. Results and discussions

3.3.1. Structural analysis

FTIR was used to understand the structural changes in the generated films compared to neat protein biopolymer as shown in figure 3.1. All the films show characteristics bands of peptide bonds which have been assigned as Amide A, Amide I, Amide II and Amide III (Aluigi *et al.*, 2007; Idris *et al.*, 2013; Xu, Cai, Xu, & Yang, 2014). The transmission band at 3274 cm^{-1} is the stretching vibrations of O-H and N-H which is recognized as Amide A, the band at 2919 cm^{-1} is related to the symmetrical CH_3 stretching vibration (Edwards, Hunt, & Sibley, 1998). The band at 1623 cm^{-1} is assigned to the C=O stretching (Amide I) while band at 1536 cm^{-1} come from N-H bending and C-H stretching is the amide II. The amide III with a weak band at 1231 cm^{-1} is combination of C-N stretching and N-H in plane bending along with influence from C-C stretching and C=O bending vibration.(Jing Zhang *et al.*, 2013). The subtle changes in vibrational bands give evidence about the formation of some new interactions due to processing.

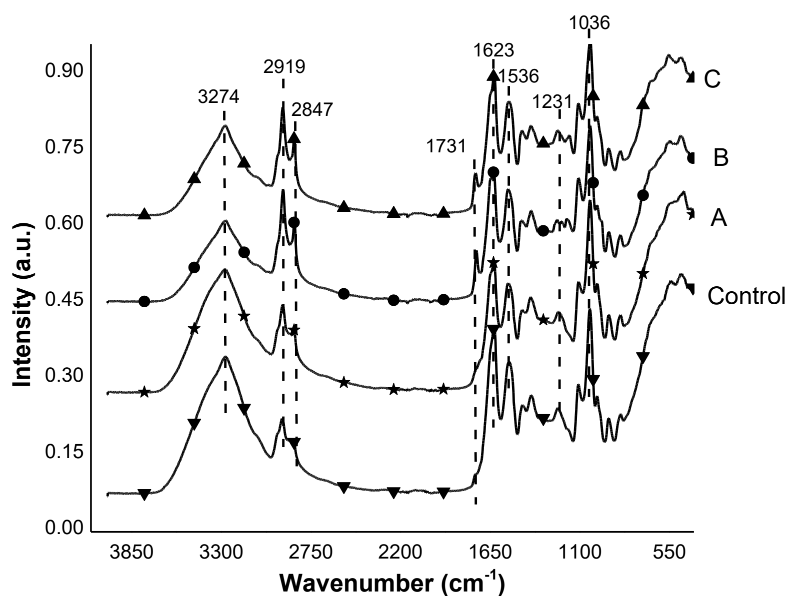


Figure 3.1 ATR-FTIR spectra of **(Control)** Proteins with 40% glycerol, 3% chitosan **(A)** Proteins with 40% glycerol, 3% chitosan, 5% bentonite **(B)** Proteins with 40% glycerol, 3% chitosan, 3% POSS **(C)** Proteins with 40% glycerol, 3% chitosan, 5% CNCs

From the literature review, the peaks at 3274 cm^{-1} (Amide A) related to α-helix of proteins structure and is mainly due to hydrogen bonded N-H and O-H stretching vibrations, the range of 1536-1515 cm^{-1} (Amide II) is attributed to the β-sheet structure, the peaks between1664 cm^{-1} 1650 cm^{-1} (Amide I) is the combination of α-helix and β-sheet (Martinez-Hernandez, Velasco-Santos, De Icaza, & Castano, 2005; Senoz & Wool, 2010). Thus, all the derived films possess two micro-structures of α-helix and β-sheet.

ATR-FTIR spectra of films B and C with glycidyl POSS and Cellulose nanocrystals (CNCs) respectively have two bands at 2847 cm^{-1} and 1731 cm^{-1} with more intensity as compare to control and A, are related to carbonyl C-H and C-O stretching respectively. The more is carbonyl group involved in hydrogen bonding, lesser the intensity expressed in the spectra. It means that sample A is more involved in hydrogen bonding in this region, as a result its intensity is reduced

which clearly showed that clay has better interactions with the proteins as compare to POSS and CNCs (Lewis, McElhaney, Pohle, & Mantsch, 1994).

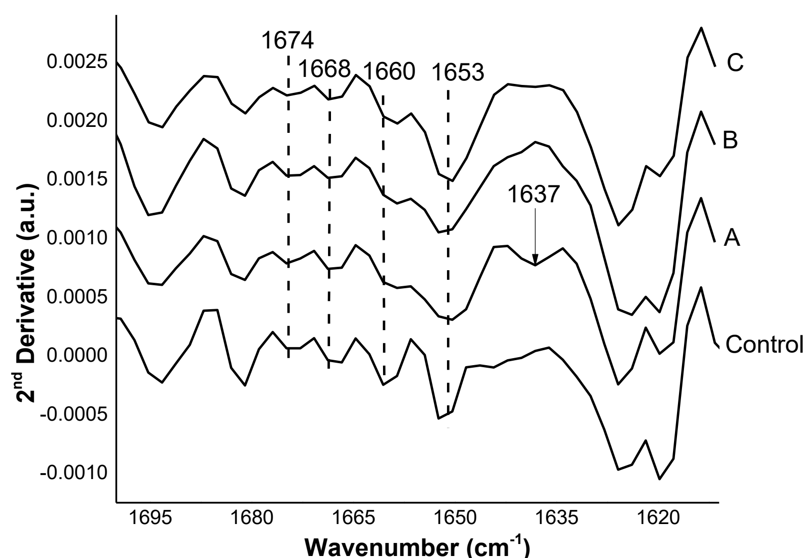


Figure 3.2 Amide I region of 2nd derivative spectra of **(Control)** Proteins with 40% glycerol, 3% chitosan **(A)** Proteins with 40% glycerol, 3% chitosan, 5% bentonite **(B)** Proteins with 40% glycerol, 3% chitosan, 3% POSS **(C)** Proteins with 40% glycerol, 3% chitosan, 5% CNCs

Second derivative of proteins derived films is shown in figure 3.2. For clarity, the spectra are showed on an offset scale. Second derivative analysis revealed the absorption band as negative bands in the second derivative spectrum. Apart from that it allowed the direct separation of amide I band into its components. The main component bands exhibited at 1653 cm^{-1}, 1660 cm^{-1} in all films can be assigned to α- helices and 3_{10} helices respectively (Ullah & Wu, 2013). The other peak which is at 1674 cm^{-1} can be assigned to antiparallel β- sheets/aggregated strands (Jackson & Mantsch, 1995). The significant difference can be observed in case of A, one extra band is observed at 1637 cm^{-1}, 40% glycerol with 5% clay may promote the formation of higher number of β- sheets and make more interaction with the proteins. The peak at 1625 cm^{-1} indicates the

presence of strong intermolecular hydrogen bonding between the protein and clay due to the better dispersion which is confirmed by the sharp peak at 3274 cm^{-1} figure 3.1 (Ullah & Wu, 2013).

3.3.2. Crystalline study of bionancomposite films

To determine the crystal structure, X-ray diffraction (XRD) is an important technique. XRD patterns of clay (bentonite) and derived films are shown in figure 3.3. All samples have broad peaks at about 19° which corresponds to β-sheet structure while the peak at 17.8° relates to the diffraction pattern of α-helix (Ma, Qiao, Hou, & Yang, 2016). Though, these two peaks usually are not to be clearly assigned due to the overlapping signals, resulting the broad single peaks at 19° (Khosa & Ullah, 2014; Popescu & Augustin, 1999). In this perspective, all the derived films present two structures of α-helix and β-sheet which is consistent with the results from ATR FT-IR. XRD diffractograms of nanoclay derived films shows great reduction or complete disappearance of crystallinity peaks as compare to pure clay. The peaks assigned to nanoclay at 2θ of 6.01°, 19.81°and 28.63° is completely vanished in case of A. On the other hand, the peak at 19.81° in case of F is just reduced. This demonstrated homogeneous dispersion of nanoclay (bentonite) in the protein chains thorough exfoliation/ intercalation. The similar phenomenon is observed in TEM analysis. In addition to that, two new crystallinity peak can be observed in D at 2θ of 31.66° and 45.47°.

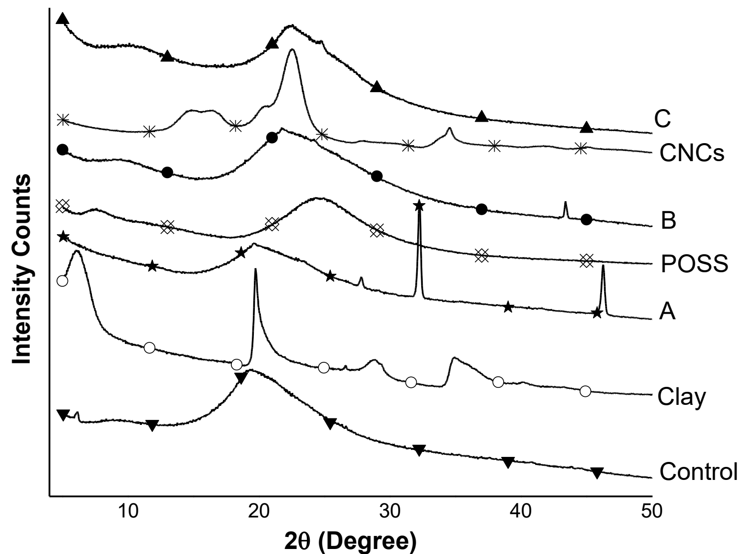


Figure 3.3 X-ray diffractograms of **(Control)** Proteins with 40% glycerol, 3% chitosan **(A)** Proteins with 40% glycerol, 3% chitosan, 5% bentonite **(B)** Proteins with 40% glycerol, 3% chitosan, 3% POSS **(C)** Proteins with 40% glycerol, 3% chitosan, 5% CNCs

From the films B and C diffractogram, it clearly shows that there is clear shift in the crystallinity peaks of pure POSS and pure CNCs. The peak intensity indicates the content of crystal structure (Ma *et al*., 2016). From the above discussion, it shows that both films with and without clay possess two kinds of crystal structure of α-helix and β-sheet. In case of glycidyl POSS and cellulose nanocrystals, there is nothing noticeable, only a shift in the β- sheet structure peaks.

3.3.3. Effects on thermal properties

DSC measurement of pure proteins and their derived bionancomposite films were done by heating the sample from 25 to 300 $^{\circ}$C as represented in the figure 3.4. Normally, the two peaks can be identified with glycerol based films: a glycerol-rich region where glycerol is loosely attached to proteins and a protein rich region where glycerol has greater interactions with proteins as found in literature (Chen & Zhang, 2005). Here these two peaks are not clear due to the overlapping of these regions.

The first broad peak in all derived films related to the loss of water which is attached to polar groups of the proteins via hydrogen bonding.(Ullah, Vasanthan, Bressler, Elias, & Wu, 2011) The figure shows a relatively sharp peak assigned to the loss of water molecules at lower temperature (116.52 °C, 139.13 °C and 153.21°C) for control, B and C respectively which are with 40% glycerol. A broad smaller peak in case of A with nanoclay at higher temperatures of 163.55°C was observed due to the presence of more interactions which were developed by the addition of 40% glycerol and 5% nanoclay that contributed towards highest tensile strength of this blend among all.

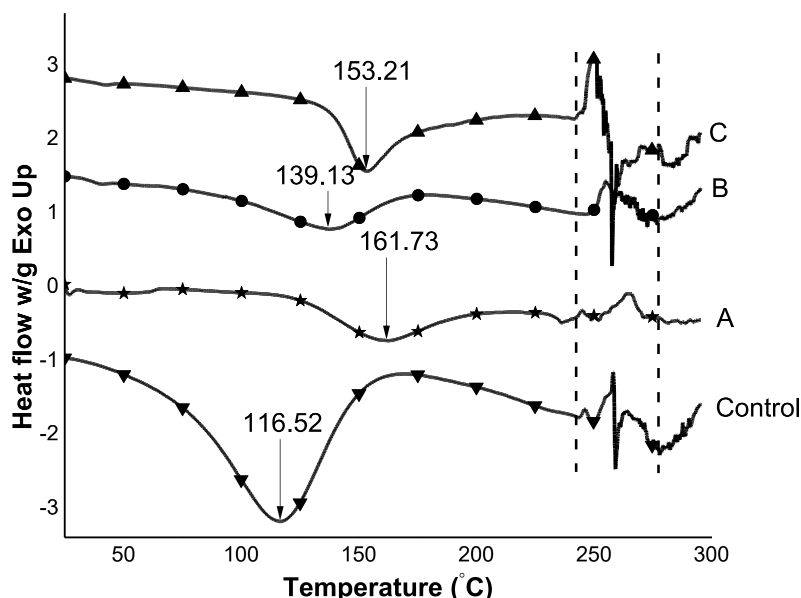


Figure 3.4 DSC heat flow signals of **(Control)** Proteins with 40% glycerol, 3% chitosan **(A)** Proteins with 40% glycerol, 3% chitosan, 5% bentonite **(B)** Proteins with 40% glycerol, 3% chitosan, 3% POSS **(C)** Proteins with 40% glycerol, 3% chitosan, 5% CNCs

The peaks around 270 °C attributed to crystalline melting or the glass transition for the spent hen proteins and is the breakdown of greater interaction of proteins with the glycerol. The better clay dispersion with the polar groups of protein decreased the number of polar groups

available for moisture uptake through hydrogen bonding. A sharp transition in the melting range of clay based films was observed in which could be ascribed to the presence of nanoclay in the protein biopolymer galleries. In addition, some of the hydrophilic groups of clay interact with hydrophilic groups of protein, which was originally present in the interior of protein film. This behavior in fact is due to better interaction which makes loss of internal moisture difficult and therefore a broad and delayed peak is observed. As a comparison of clay based films with glycidyl POSS and CNCs nanocrystals derived films, the major difference in the glass transition or crystalline melting region of the proteins is around 250 $^{\circ}$C

3.3.4. Thermal stability

The TG and DTG curves of pure proteins and derived films are presented in figure 3.5. The weight loss in all films were observed 1-2 % before 100 $^{\circ}$C which is due to the evaporation of water including free water and bound water (Ma *et al*., 2016). Up to temperature 188, all four blends are thermally stable, followed by a sharp loss between 188 to 425 $^{\circ}$C. The weight loss in case of A (5% nanoclay and 40% glycerol) is the least among all derived films due to the better dispersion of nanoclay in the protein matrix as evident from the TEM image. Further it is confirmed from the tensile strength of this blend which has highest strength among all derived films. From DTG graph, it clearly depicts all the blends reach a maximum decomposition between 310-327 $^{\circ}$C.

The TG and DTG curve represent that the delay in onset of loss temperature in the plasticizer loss zone (140- 250 $^{\circ}$C) in case of A, is increased due to the stronger interactions between clay and proteins. Similarly, loss between 188-217, in case of A, is at higher temperature as compared to other three blends. This is consistent with tensile strength and DSC data. Furthermore, at temperature 425 $^{\circ}$C, there is loss up to 72% which is related to the denaturation of

helix structure, skeleton decomposition and destruction of peptide chain/bridge linkages (Martinez-Hernandez *et al*., 2005).

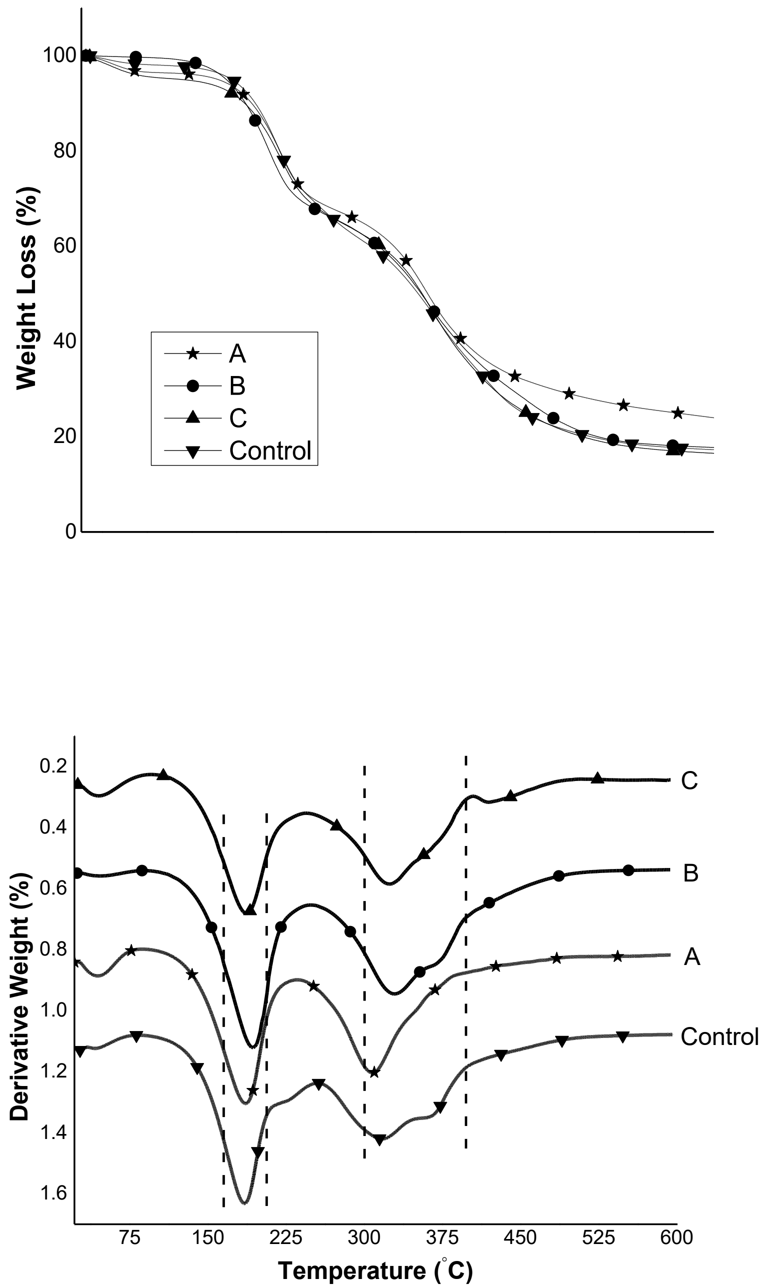


Figure 3.5 TGA and DTGA curves of **(Control)** Proteins with 40% glycerol, 3% chitosan **(A)** Proteins with 40% glycerol, 3% chitosan, 5% bentonite **(B)** Proteins with 40% glycerol, 3% chitosan, 3% POSS **(C)** Proteins with 40% glycerol, 3% chitosan, 5% CNCs

From the TGA graph, peaks B and C which are related to the glycidyl POSS and CNCs derived films show that there is more weight loss in these nanoparticles (16-17%) as compare to bentonite is 22%. The less degradation in the latter is related to the better interaction of clay with the proteins.

3.3.5. Mechanical strength

Stress- strain curve is commonly used to study polymer properties of bionancomposite derived films as it directly affects the applications of polymers. The properties typically examined are tensile strength and percent elongation at break. Glassy materials have tensile strength above 30 MPa and almost no elongation. While materials having tensile strength more than 5MPa and break elongation more than 100% are considered as rubbery materials. Thermoplastic material which lying in the glass transition zone have properties between them (Marion Pommet, Andréas Redl, Stéphane Guilbert, & Marie-Hélène Morel, 2005).

The tensile tests were performed to assess the effect of cross linking agent (chitosan) and nanoparticles on the mechanical properties of the bionancomposite films. Tensile properties of the resulting films are given in table 3.1 (without nanoparticle) and table 3.2 (with nanoparticle). The results revealed an increment in mechanical properties by the addition of 3% chitosan. In addition to that, by adding 5% bentonite, a remarkable enhancement in mechanical strength is observed (11.37 MPa). Proteins derived film with 50% glycerol and 3% chitosan has maximum break elongation which is 1590. This is because of the plasticizer usually increase the flexibility and extensibility of the material. While its interactions at a molecular level increase tensile strength and stiffness.

The properties of polymers can be modified by using cross-linker like chitosan. The amino groups of chitosan can bind to the proteins across protein chain leading to crosslinking which can alter the properties of films. The addition of chitosan improves the mechanical strength. Its film-forming ability is recognized on the basis of the H-bonds network formation in the solid state which may contribute towards improved tensile properties (Younes & Rinaudo, 2015).

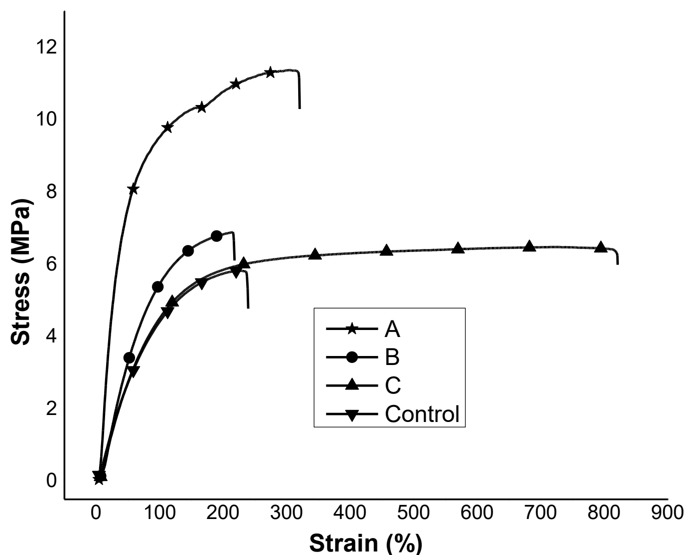


Figure 3.6 Stress-strain curves of **(Control)** Proteins with 40% glycerol, 3% chitosan **(A)** Proteins with 40% glycerol, 3% chitosan, 5% bentonite **(B)** Proteins with 40% glycerol, 3% chitosan, 3% POSS **(C)** Proteins with 40% glycerol, 3% chitosan, 5% CNCs

Furthermore, the addition of bentonite increase the tensile property of films in case of 40% glycerol and 5 % clay along with 3% chitosan which is also confirmed from the TEM images. As in this case, the dispersion is better. The well dispersed nanoparticles enhance the strength as reported by Jose *et al* (Jose, George, Maria, Wilson, & Thomas, 2014).

Table 3.1 Stress and Strain of the blend films without nanoparticle

Glycerol (%)	Chitosan (%)	Stress (MPa)	Elongation at break (%)
40	----	2.43	474
50	----	2.65	474
40	3	5.81	239
50	3	4.57	1590

The stress-strain curve of blends with each nanoparticle having highest mechanical strength is shown in figure 3.6. The tensile strength of CNCs and glycidyl POSS derived films is less than the nanoclay based films. The maximum tensile strength obtained by using cellulose nanocrystals is 6.47 MPa and 6.86 MPa with glycidyl POSS. From the table, it clearly shows that 40% glycerol gave better results with 5% bentonite or CNCs. While glycerol concentration does not have effect on the mechanical strength of glycidyl POSS based films as the strength in both cases is same i.e. 6.86 MPa.

Table 3.2 Stress and strain of the blend films with nanoparticle

Nanoparticle Type	Particle (%)	Glycerol (%)	Stress (MPa)	Elongation at break (%)
Bentonite	5	40	11.37	322
	5	50	7.33	853
Glycidyl POSS	3	40	6.86	211
	3	50	6.86	116
Cellulose nanocrystals	5	40	6.47	821
	5	50	5.67	827

3.3.6. Transmission electron microscopy analysis

TEM of spent hen protein derived film were done to study how nanoparticles were dispersed within the protein matrix. The image I shows the TEM image of sample without nanoparticle. While other images are with nanoparticles. If we look at the in images I, there is no indication of nanoparticle presence in this blend. While other images show presence of nanoparticles in the proteins based films. TEM images (II and III) are clay based films, mixed intercalation and exfoliation dispersion as stacks of silicate layers are uniformly dispersed in the polymer matrix. This distribution can be attributed to insertion of proteins chains into the galleries of bentonite which resulted high exfoliation/intercalation of silicate layers. The mechanical strength results also support these TEM images II and III. There is clear aggregation can be seen in case of CNCs derived films (TEM images IV and V). Darks spots are visible due to the presence of high electron density of Si atoms in the POSS molecule (Arshad, Kaur, & Ullah, 2016) which is clearly seen in TEM images VI and VII. TEM images of POSS and CNCs derived films show that the distribution of these nanoparticles is not good as compare to bentonite that may contributed to low mechanical strength of the resulted films.

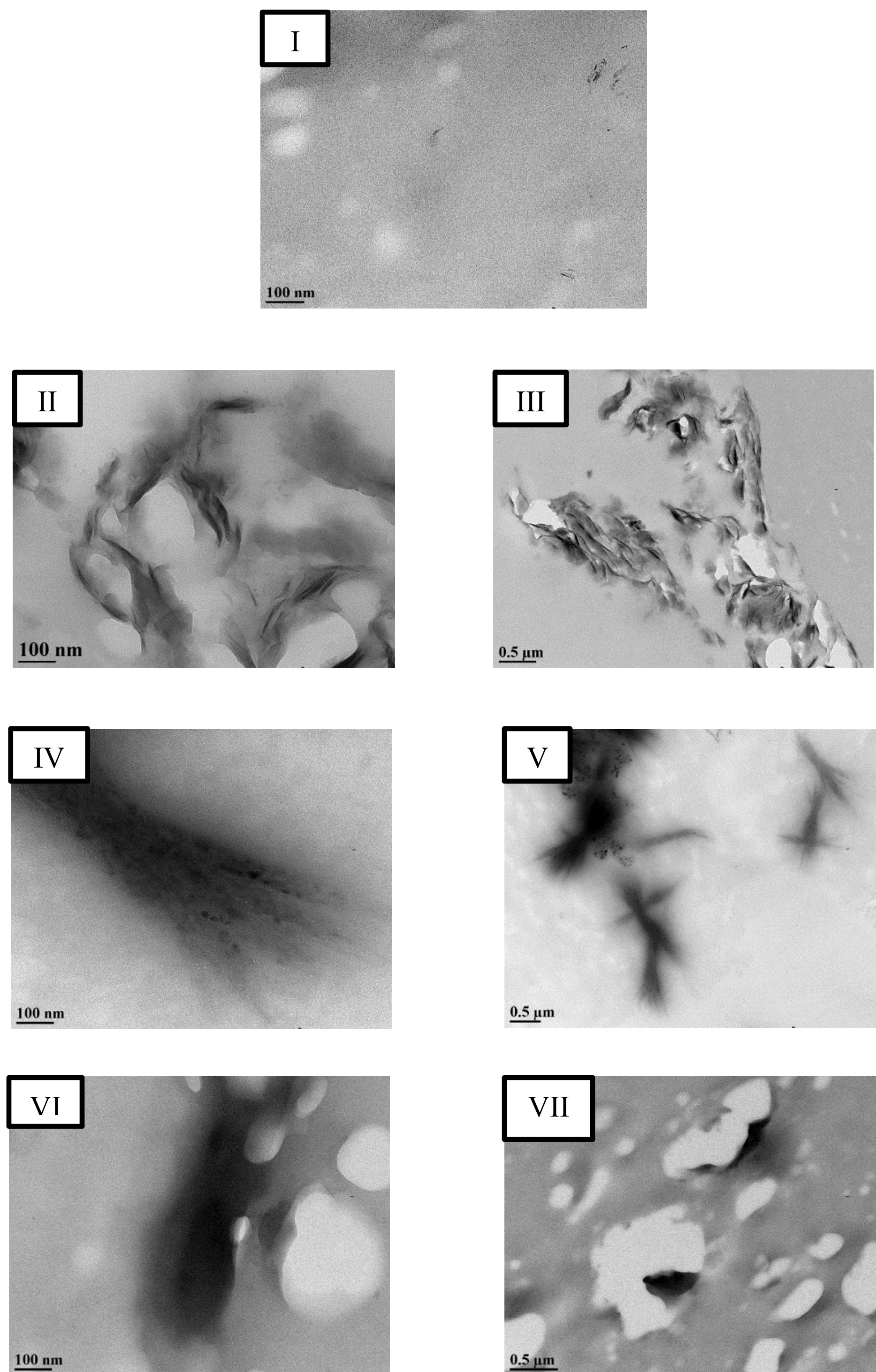
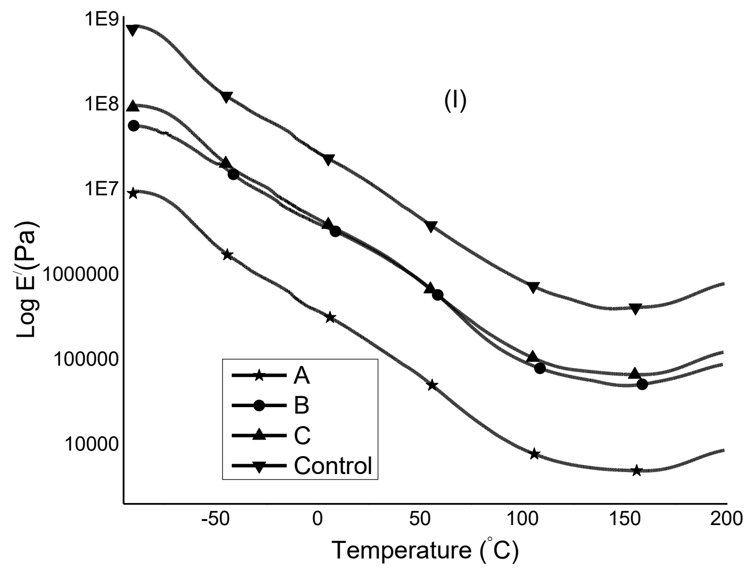


Figure 3.7 TEM images of **(I)** Proteins with 40% glycerol, 3% chitosan **(II) & (III)** Proteins with 40% glycerol, 3% chitosan and 5% clay **(IV & V)** Proteins with 40% glycerol, 3% chitosan, 5% CNCs **(VI & VII)** Proteins with 40% glycerol, 3% chitosan, 3% POSS

3.3.7. Viscoelastic properties

Proteins experience several thermal transitions on heating or cooling as they are semi-crystalline polymers. DMA is a technique which is used to measure the viscoelastic properties of the polymers with changing temperature. Thermal transitions are generally related to the chain mobility. The most important transition among them is the glass transition (T_g) which is associated to the onset of major chain motions (Bengoechea, Arrachid, Guerrero, Hill, & Mitchell, 2007). The single narrow transition for all blends represent a good compatibility of glycerol with spent hen proteins. The low molecular weight of glycerol enables it to incorporate itself into the polymer chain and lubricate it easily. Thus, the protein-glycerol interaction is developed at the expense of protein-protein interaction. This is in agreement with the free volume theory of the plasticization (Ullah *et al*., 2011).

The tan delta curve shows the maximum for all blends between 73 $^{\circ}$C and 85 $^{\circ}$C. From the tan delta curves, it can be observed that there is increase in the tan delta peak size in case of A (40% glycerol, 3% Chitosan, 5% nanoclay) as compare to control, B (POSS based film) and C (CNCs derived film). As size of tan delta revealed the volume of fraction of the material experiencing transition, therefore higher change in tan delta peak, it can be suggested that A plasticized material undergoes glass transition phenomenon to greater extent. This shows that interactions involved between proteins and glycerol is quite homogeneous in this case.



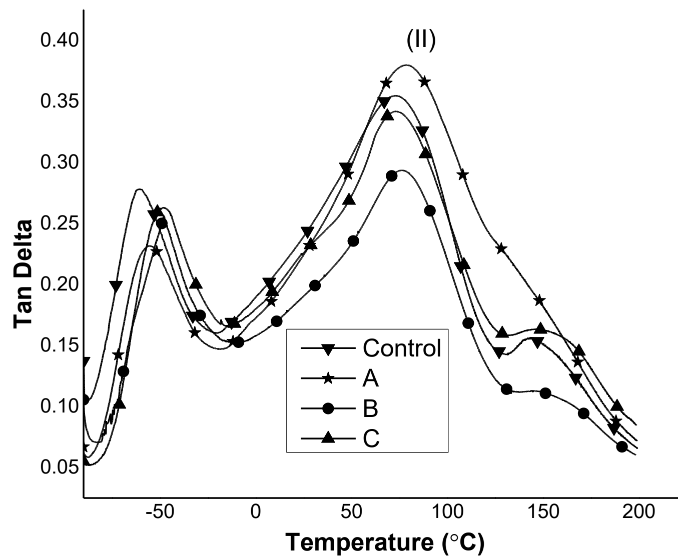


Figure 3.8 DMA thermograms E´(I) and tan delta (II) of **(Control)** Proteins with 40% glycerol, 3% chitosan **(A)** Proteins with 40% glycerol, 3% chitosan, 5% bentonite **(B)** Proteins with 40% glycerol, 3% chitosan, 3% POSS **(C)** Proteins with 40% glycerol, 3% chitosan, 5% CNCs

According to theory of the plasticization, a decrease in the rubbery modulus determine the compatibility of plasticizer with the matrix. E´ depends on the density of the polymer crosslinks

above its glass transition. It is predicted that lower the density of polymer/polymer crosslinks the higher the decrease in rubbery modulus (N Gontard & Ring, 1996) Similar effect is observed here, by the addition of chitosan and glycerol contribute towards the lowering of rubbery modulus especially with clay.

The tan delta of glycidyl POSS and CNCs derived films, a clear difference can be seen in as compare to clay based films. The maxima of tan delta are at lower temperature as compared to clay derived films. In addition to that size of the peaks are smaller which indicates that small volume fraction of the material undergoes thermal transitions. It may be contributed to weak interactions between filler and protein matrix that become the cause of non-homogeneous mixing of these nanoparticles within the protein layers.

3.3.7. Water vapor permeability (WVP)

Water vapor permeability and water vapor transmission rate of all films is show in table 3.2. The WVP ranges from 2.18-2.36 g. mm/ m^2. d. kPa. These film are less permeable compared to those prepared from similar kinds of proteins like myofibrillar proteins from turkey and fish.(CUQ *et al*., 1995) The films based on 40% glycerol with 3% chitosan and 5% clay is least permeable as this is consistent with TEM, mechanical strength and TGA results. From the results, it can be inferred that by increasing glycerol content, the water vapor permeability is increased. Since glycerol is hydrophilic in nature that may increase the WVP. Similar results have been reported previously (da Rocha, Loiko, Gautério, Tondo, & Prentice, 2013). The addition of chitosan decreases the water vapor permeability as interaction developed between chitosan molecule and proteins which reduces the number of sites to interact with the water molecules. Nanofiller addition further lowered the water vapor permeability because of the protein interactions with the filler.

Table 3.3 Water vapor permeability of films

Sample code	Water vapor transmission rate ($g/m^2.d$)	Water Vapor Permeability ($g. mm/ m^2. d. kPa$)
Control	0.29	2.25
A	0.28	2.18
B	0.30	2.36
C	0.28	2.18

WVP of **(Control)** Proteins with 40% glycerol, 3% chitosan **(A)** Proteins with 40% glycerol, 3% chitosan, 5% bentonite **(B)** Proteins with 40% glycerol, 3% chitosan, 3% POSS **(C)** Proteins with 40% glycerol,3% chitosan, 5% CNCs

3.4. Conclusion

We successfully extracted proteins from poultry by-product (whole spent hens and feet) with high recovery and purity. The first attempt to introduce chitosan into the glycerol plasticized spent hen proteins with enhanced mechanical strength was developed in this study. Furthermore, bentonite was added to the chitosan glycerol plasticized protein blend. The chitosan was disturbed well within protein matrix, helped nanofiller distribution and crosslinking of protein chanis. Thus, the mechanical strength is significantly enhanced. Nanoclay derived films have highest mechanical strength among all used nanofillers leading to lower water vapor permeability. The other nanoparticles such as POSS and CNC were poorly dispersed and therefore did not substantially improve the properties of films. The above results suggested that proteins from spent birds can be successfully extracted using simple and industrially scalable alkali aided extraction method and extracted proteins can be used to develop protein based bionanocomposites films with improved mechanical and barrier properties. The developed bionanocomposites have great future potential to be used as a food packaging materials.

Film Images

Film obtained using glycerol

Film obtained using chitosan

Film obtained using ethylene glycol

Film obtained using nanoclay

3.5. References

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