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Extraction of spent hen proteins for adhesive application

by

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DEDICATION

I would like to dedicate this thesis to my beloved family. Achieving such goals could not have been possible if not for my loving parents. Throughout my tenor as a student here at University of Alberta, my father Manfu Wang and mother Yanjun Sun were always there for encouragement and constantly reminding me of the bigger picture in life. Without your endless love, I would never be at this stage.

ABSTRACT

Spent hen is a long existing issue of the poultry industry. The purpose of this study was to find alternative uses of spent hen proteins. Spent hen proteins were first extracted by pH-shifting process; the acid-aided extraction showed higher protein recovery than that of alkaline-aided extraction. Protein isolates underwent significant structural changes during freezing storage, especially for the acid-extracted proteins. Acid-extracted proteins had inferior structure stability and gelation ability than that of alkaline-extracted proteins, due to protein degradation during acid extraction and oxidation during freezing storage. Adding cryoprotectants (CP) however improved stability during storage. Adhesives prepared from acid extracted proteins without CP displayed lower viscosity, but higher adhesive strength than those of alkaline extracted proteins and proteins with CP addition during storage. Our study suggested acid-extracted proteins stored without CP addition exposed more reactive COO^- groups and formed new hydrogen bonds after curing, leading to improved adhesive performance.

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LIST OF ABBREVIATIONS

AAFC: Agriculture and Agri-Food Canada

ANS: 1-anilinonaphthalene-8-sulphonate

BSA: Bovine serum albumin

BSE: Bovine Spongiform Encephalopathy

CARB: California Air Resources Board

CP: Cryoprotectants

CUSTA: Canada-United States Free Trade Agreement

DNPH: 2, 4-dinitrophenylhydrazine

DOA: Death on arrival

DSC: Differential scanning calorimetry

DVB: Dynamic viscoelastic behaviour

EDTA: Ethylenediaminetetraacetic acid

FAO: Food and Agriculture Organization

FTIR: Fourier transform infrared spectroscopy

GLM: General linear models

Hb: hemoglobin

HCl: Hydrogen chloride

H_2O_2: Hydrogen peroxide

HSD: Honestly significant difference

Hz: Hertz

IPN: interpenetrating network

KBr: Potassium bromide

LVR: Linear viscoelastic region

MBM: meat and bone meal

MDI: methane-diisocyanate

MHC: Myosin heavy chains

MLC: Myosin light chains

MP: Myofibrillar protein

MPa: Megapascal

MP-SHo: myofibrillar protein surface hydrophobicity

MSM: Mechanical separated meat

MSPM: Mechanically separated poultry meat

MSTM: Mechanically separated turkey meat

MW: molecular weight

NaCl: Sodium chloride

NaOH: Sodium hydroxide

NMR: Nuclear magnetic resonance

O_3: Ozone

OSB: Oriented strand board

PEG: polyethylene glycol

PF: Phenol-formaldehyde

PUFA: Polyunsaturated fatty acids

SH-PI: Spent hen protein isolates

RFI: Relative fluorescence intensity

RIR: Rhode Island Red

R-SH: Reactive sulfhydryl

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM: Scanning electron microscope

SHM: Spent hen meal

SHo: Surface hydrophobicity

SP: Sarcoplasimic protein

SP-SHo: Sarcoplasmic protein surface hydrophobicity

S-S: sulphate-sulphate

TCA: Trichloroacetic acid

T-SH: Total sulfhydryl

UF: Urea-formaldehyde

WHO: World Health Organization

WL: White Leghom

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

The increasing global population and pressure on land and water resources indicates the importance of utilizing all available resources rather than simple disposal. The current disposal methods of spent hens (burial, composite and animal feed) are a growing problem due to increasing disposal cost and consequent environmental pollution for doing so (Black and Donald, 1992). Every year, approximate 22.5 million birds need to be removed from the egg industries in Canada (CEMA, 2006), at the egg producers' liability. Although the spent hens are partially sold as whole bird, cut-up parts (legs, breast and wings) and ground meat in the poultry meat market (Munira et al., 2006), the meat products made from spent hen birds are not able to compete with the broiler meat because of low yield, poor meat quality (Rouselle et al., 1984) and consumer preference (Babji and Gna, 1994), which make spent hen processing no longer profitable. Therefore, it is important to find a suitable way of utilizing spent hen carcasses in an economical beneficial and environmental secure manner.

On the way of seeking new possible approach to solve the spent hen problem, the concept of recoverying valuable protein components has attracted researchers' attention. There are two main methodologies has been developed regard in this aim. They are conventional surimi process and newly developed pH-shifting process. Although several studies has been conducted to test the organoleptic, sensory qualities and the functional properties of spent hen surimi (Nowsad et al., 2000a, b; Li, 2006), the new extracting method (pH-shifting process) has not yet been evaluated on spent hen meat. In this study, the efficiency of the pH-shifting extraction will be evaluated.

Nevertheless, the use of this spent hen protein isolate (SH-PI) is not limited to just for food applications since the proteins are also considered as a natural polymeric material for industrial applications (Chen, 2009). Using proteins for industry applications is not a newly developed research area. Since ancient time, animal proteins such as blood, casein and collagen has been used for making wood adhesive for the construction and painting purposes (Lambuth, 1994). However, they were replaced by the petroleum based adhesives rapidly after Industrial Revolution (Lambuth, 1994). Presently, the production of wood composites relies on the formaldehyde-based wood adhesives such as phenol-formaldehyde (PF) and urea-formaldehyde (UF). However, their dependence on exhaustible fossil fuels and the emission of carcinogenic formaldehyde emission prompt to develop an environmentally friendly adhesive from renewable natural resources (CARB, 2007; Huang and Sun, 2000). While the proteins recovered from underutilized agricultural by-products has been considered as an economically starting material. In the past two decays, researches on protein-based adhesives have been largely directed toward soy mill proteins. Based on their results, most soy protein-based adhesives suffered from relatively low bonding strength and poor water resistance due to their original native structure (Park, 1999). On the other hand, the protein structure was found to be altered during the pH-shifting extraction and the frozen storage (Kristinsson and Hultin, 2003, 2004; Decker et al., 1993; Xiong et al., 1993). In addition, the unique fibrous and globular structure of the muscle proteins may contribute to the formation of entanglements (physically entangled chains) or head-to-tail polymerization after curing. Thus a superior adhesive performance is expected from the muscle proteins.

1.2. Overview of spent hens

The poultry industry can be broadly divided into two categories: egg production layers, which spend most of their lives laying eggs and broilers, which live only a short period of time before slaughter for meat production. Laying hens are chickens raised for egg production; therefore, the majority of the nutrients they consume are used for this goal. The average live weight for commercial table egg layers is about 1.5 to 1.7 kg (Kondaiah and Panda, 1992). In addition, the hens' bones have become fragile by the time they complete their productive cycle as a result of demineralization for eggshell production (Kersey et al., 1997).

Every year millions of hens become "spent" at the conclusion of their laying cycle. They are referred to as "spent hens" because they are no longer at their peak production of number and quality of the eggs. Normally the production lifespan for hens is approximately one year. In that year, the average laying hen produces about 300-320 eggs, peaking at 24-26 weeks of age (AAFC, 2009). By 72 weeks of age, a flock's egg production rate starts to decline. In the United States, an industry practice called "forced moulting" is done by 75-80% of the layer industry for extending hens laying capacity into a second or third year (Bell, 1999). This process involves reducing feed and light for up to 18 days and attempts to mimic natural molting. Although the chicken's reproductive tract is rejuvenated during the molt, hens resume laying eggs at a reduced production level. Forced moulting is not a common practice for Canadian egg producers as it denies the food and light that the birds require. It is considered an unacceptable practice in Canada's Recommended Codes of Practice for the Care and Handling of Pullet, layers and spent fowl (CARC, 2003).

As a result, once the hens reach the end of their first laying cycle, they are culled and replaced by a younger and more productive flock. The culled spent hens are usually transported to the slaughtering house and sold for meat. However, the meat from the spent egg layers is categorized into the group of low value poultry meat due to the poor eating quality (tenderness, juiciness, color and flavor) and yield (Bilgili, 2000). With the increasing supply of broiler chicken, there is little demand for spent hens to produce cut-up poultry and mechanically separated poultry meat in the food processing plants (AFAC, 2003).

The limited usage of the spent hen meat leads to depreciation in the value of hens. Middleton (2000) reported that the on farm value of spent hens prior to disposal often approaches $0.00 because the transportation and labor cost for their removal from the farm lead to a net expense for the producer. Therefore, producers are interested in improved measures of flock disposal, especially methods that will increase income from the use of the spent layers.

1.2.1. Production status in Canada

In recent years, the production of laying hens increased with the rapid growth of global egg production. According to the Food and Agriculture Organization (FAO), the global laying hen flock was estimated at 4.73 billion birds in 2006 (FAO, 2010). Countries with the highest laying hen flocks were, in order, China, European Union, United States, Brazil, India, Indonesia and Mexico with 2.2 billion, 539.7, 344, 236, 215, 171 and 165 million, respectively (AAFC, 2007a). After the European Union with its 27 members, United States is considered to be the second largest egg production country and it exports

a considerable amount of spent hens to Canada due to the Canada-United States Free Trade Agreement (CUSTA). Approximately 26 million hens are expected to be trucked into Canada from the U.S each year (Clifton, 2000).

In Canada, there are approximately 1,100 table egg producers, with an average of 17,800 hens per farm (AAFC, 2007b). The data AAFC (2007b) conducted at 2007 illustrates that the number of laying hens per producer gradually increased to almost double from 1985 up to 2007. In 2007, 22.5 million female chicks that were housed for egg production (Table 1.1). Considering that the number of birds housed corresponds to the number of young pullets used to replace the old spent hens, a similar number must have been culled in the same period. With addition of the amount of spent fowls arriving from the United States, there is a significant supply of spent hens every year in Canada. The reduced demand for spent hen meat from the food industry coupled with the need for disposing of carcasses in an economically and environmentally secure manner puts a considerable economic burden on poultry producers. Both producers and researchers are making a unified effort to increase the market variability of spent fowl products from both food and non-food approaches.

Table 1.1. Eggs set and chicks hatched for egg production at feferally registered hatcheries, in thousands at 2006 and 2006. Adopted from (AAFC, 2007b).

Region	2006	2007
Eggs set and chicks hatched for egg production (males and females)	69,168	70,219
Chicks hatched for table egg production (males and females)	52,973	52,749
Hatching rate (%)	77	75
Placement of female egg production chicks	22,502	22,530
Ontario		
Eggs set and chicks hatched for egg production (males and females)	31,870	31,811
Chicks hatched for table egg production (males and females)	22,788	23,073
Hatching rate (%)	72	73
Placement of female egg production chicks	9,411	9,662
Quebec		
Eggs set and chicks hatched for egg production (males and females)	12,371	13,527
Chicks hatched for table egg production (males and females)	10,225	10,880
Hatching rate (%)	83	80
Placement of female egg production chicks	4,353	4,473

1.2.2. Composition and associated problems with spent hen meat

Information on the spent hen carcass components is necessary for considering the economic and quality of the processed products as well as the potential research directions for expanding their market. The main constituents of layer meat are water, protein, and fat. The accumulation of collagen leads to the tough texture associated with the spent hen meat. Studies showed that breed, gender, age and the feeding system influence animal growth rate, meat yield as well as the chemical composition (Kondaiah and Panda, 1992). Table 1.2 compares the chemical composition between broiler chicken and spent hens. Spent hens possess almost twice the collagen compared to broiler chickens, and the fat content is also significantly higher (7.15% VS 0.68%, respectively). Spent hen meat also has relative lower moisture, which may be related to its poor water holding capacity during processing.

In addition, weight is important in the marketing of live birds. The low yield of edible meat from spent hens is the primary reason that they are rejected by the processing plants. In general, the Rhode Island Red (RIR) is considered to be a heavy breed hen and the White Leghorn (WL) is classified as a light breed hen. Kondaiah and Panda (1987) reported that the deboned meat as a percentage of live weight was significantly higher in the case of RIR compare to the WL. Munira et al. (2006) compared the carcass quality of spent hens from five different breeds and concluded that the RIR carcass is superior in terms of quality (low abdominal fat) and quantity (higher shank weight), whereas the WL carcasses hold the lowest value.

Table 1.2 Chemical composition of raw broiler and spent hen meat.

Breed	Moisture (%)	Protein (%)	Total Collagen (mg/g muscle)	Fat (%)	Ash (%)
Broiler[1]	74.87±0.46	20.59±0.26	3.85±0.24	0.68±0.06	1.10±0.01
Spent hen[2]	67.46±3.13	24.36±0.26	6.47±0.20[3]	7.15±0.09	1.04±0.09

Source: [1] Adapted from Wattanachant et al. (2004)

[2] Adapted from Lee et al. (2003)

1.2.2.1. Heat-stable connective tissue

Meat toughness is considered to be the most important attribute in determining the eating quality of the meat and its market value (Lawrie, 1991). Despite usually only making up 2-10% of protein in muscle meat, collagen is a major contributor to the meat toughness (Gerrard and Grant, 2003). The structure of the muscle fiber bundles is maintained by three layers of connective tissue, which consist of the endomysium, enclosing each muscle fiber, perimysium, surrounding the bundles of these fibers and

epimysium, surrounding the whole muscle (Bailey and Light, 1989). The connective tissues are present throughout all types of muscle, thus the resulting background toughness is not easily corrected or avoided (Aberle et al., 2001). The major protein components of connective tissues are collagen and elastin, which are classified as insoluble stromal proteins. The structural integrity of collagen is less affected during post-mortem aging (Bailey and Light, 1989). There are several means for tenderizing meat chemically (marinated in salt or weak organics acid), physically (mechanical and high temperature moist-heating) or enzymatically (papain and ginger extract), which mainly focus on reducing the amount of detectable connective tissues without further degradation of the myofibrillar proteins (Chen et al., 2006). However, these methods are most effective when the animal is young. As the animals become mature and older, the collagen fibers become progressively stronger and more rigid, primarily due to the increased non-reducible cross-links between collagen molecules (Robins et al., 1973; Bailey and Light, 1989). This age-related heat-stable connective tissue has been associated with meat toughness (Bailey, 1998). In older animals such as spent hens, the molecular change in collagen is accompanied by maturation of edomysium and permysial thickness, which lead to decreased susceptibility to proteolytic enzymes and penetration of salt into the muscle. Thus, layer hens at approximately 60-72 weeks of age will produce tougher meat than broiler chickens, and the meat will be resistant to tenderization, which prevents its use in whole meat sales.

1.2.2.2. Fatty acid composition

The meat from spent hens is highly enriched with omega-3 fatty acids and has lower

cholesterol content, particularly in the breast muscle, both of which have health promoting benefits (Ajuyah et al., 1992). Several researchers evaluated the fatty acid profile in mechanically and manually separated meats of spent hens (Table 1.3). They found that both manually separated light and dark hen meat contained similar totals of unsaturated fatty acids, which were almost twice the amount of the saturated fatty acid concentration (Table 1.3). However, the higher fat content consisting of high amounts of polyunsaturated fatty acids (PUFA), which promotes faster oxidation in spent hen meat than broiler meat during processing and storage (Chueachuaychoo et al., 2011). The meat flavor would be changed because of oxidative breakdown of PUFA molecules, which produce abnormal volatile compounds during cooking and storage (Dinh, 2006). The problems associated with lipid oxidation have invoked industrial and public concerns since it leads to flavor deterioration, loss of nutritional value, protein functional property loss, biological spoilage, and environmental pollution (Frankel, 1984).

1.2.2.3. Bone fragmentations

Bone fractures in laying hens raise both welfare and economic concerns. At the end of the laying hens' production lives, the birds usually have very fragile skeletons because of the calcium demand for eggshell formation. Studies have shown that up to 30% of birds have freshly broken bones that occur during handling and transporting (Knowles, 1994); leading to public concern about animal welfare since these birds experience tremendous pain from the bone fractures. Bone fractures also cause an increased rate of birds' death on arrival (DOA) during transportation, causing economic losses to the processors. McCoy et al (2000) reported that 35% of caged spent hens (DOA) were caused by pathological fractures. In addition, Budgell and Silversides (2004) estimated that nearly 100% of birds had at least one bone fracture when they arrived at the processing plant, thus reducing the quality and usefulness of the meat due to bone residues in the final products.

1.3. Current major disposal methods for spent hens

Every year, egg producers have to find ways to dispose of their spent flocks in an environmentally safe, economically beneficial and animal welfare-friendly manner. Currently, the majority of the light spent fowl in Canada are slaughtered on farm by the producers (CEMA, 2006). The resulting carcasses are then buried, composted, or inspected and sold to rendering plants. In the following paragraphs, a detailed discussion about the steps and requirements involved in these disposal methods will be reviewed.

1.3.1. Burying

Burial in the landfill is considered to be the most convenient disposal method. A trench is dug somewhere on the farm and filled with carcasses. Then it is covered progressively with soil since the open ditch is a hazard and attracts predators. Weather is a factor that limits the use of trench methods because the soil becomes wet and frozen during winter time. Several variations have been made to improve the acceptability of burial disposal including constructed disposal pits, sanitary landfills and inverted feedbins. A properly designed burial pit has been used as a convenient, sanitary and practical method for handing poultry death at relatively low cost (Collins and Weaver, 1974.). The burial disposal method utilizes aerobic and anaerobic microorganisms to decompose organic materials. Mote and Estes (1982) reported that heating, agitating or cutting up the carcasses decreases the amount of time it takes for carcasses decomposition. However, an unpleasant odor produced by anaerobic decomposition could be spread in the area and decrease the air quality. Although the disposal pit is convenient and economical, it should be located above the ground water level and where the soil type is suitable for filtration of decomposed residues (Black and Donald, 1992). Burial is currently the main option supported by many countries for mass disposal of animal carcasses (DeRouchey et al., 2005). However, limitations of carcass burial are present in areas with shallow ground water and during winter months when soil may be frozen.

1.3.2. Composting

Composting of animal carcasses has become more popular in recent years due to increased costs associated with conventional animal rendering facilities and landfill limitations for the burial method. Composting is a neutral decomposition of organic

matter on carcasses by aerobic bacteria and fungi to produce a useful end-product that could be used as a soil amendment (Blake and Donald, 1992). The carcasses are sequentially arranged in the composting bins with layers of straw and manure in between, and each layer of carcasses is watered to keep it moist. Successful composting of organic matter could not occur unless the microorganisms are provided with the correct proportion of carbon, nitrogen, oxygen and moisture (DeRouchey et al., 2005). The carbon and ammonia nitrogen are supplied by the straw and carcasses, with the ideal C: N ratio falling between 25 to 35:1 (Murphy and Handwerker, 1988). An initial moisture content of 50% to 60% and an initial oxygen concentration > 10% are recommended (DeRouchey et al., 2005). The temperature is elevated in the bin due to the microbial activity, which helps to inactivate poultry-associated pathogenic microorganisms and other organisms present (worms and flies). The optimal temperature for thermophilic composting is between 43˚ to 66˚C, in which most pathogenic bacteria and parasites are killed and most viruses are inactivated (DeRouchey et al., 2005). A temperature higher than 70˚C will reduce the microbial population and lower than 40˚C will lead to inefficiently inactive pathogenic bacteria (Berge et al., 2009). Thus the temperature in the composting bins needs to be carefully monitored. With proper operation, Murphy (1988) indicated that composting poultry carcasses provides an economical and biological safe way of disposal. However, there are still potential biohazard concerns about its land application if the composting is done incorrectly or incompletely.

1.3.3. Rendering

Conventional rendering facilities offer an opportunity for converting spent hen

carcasses into a protein rich by-product meal that could be used for animal feed. The animal carcasses are ground and sterilized by heat under pressure. The heating process can effectively inactivate pathogenic agents (Norman, 2003). In addition, rendering is considered to be the most direct method of recycling nutrients from dead animal carcasses; however, the spread of pathogenic bacteria during transportation of carcasses to the rendering facility presents a substantial bio-safety problem (Blake and Donald, 1992). In order to deal with this threat, several methods have been used such as refrigeration, treatment with acid, or fermentation for preserving carcasses before and during transportation. Frozen methods can effectively inactivate microorganism activities. Acid treatment uses mineral or organic acids as preservatives, which kill pathogenic bacteria and do not affect the quality of the by-product meal (Lomax et al., 1991). Fermentation requires pre-ground carcasses and mixing with a measured carbohydrate source that allows lactic fermentation to proceed, which reduces the meat pH below 4.5 so that effectively inactivating the pathogenic bacteria associated with the carcasses (Murphy and Silbert, 1990; Dobbins, 1988). However, compared to the other disposal methods, additional transportation, pre-treatment (frozen, fermented and grounded) and machinery costs are the main drawbacks of the rendering method. In addition, the feeding trails of spent hen meal (SHM) to broiler chickens, laying hens and other mono-gastric animals showed the limitations of inclusion of SHM into animal feed formulas. Kersey and Waldroup (1998) found that SHM may only be effectively used at less than 10% - 15%. Beyond these levels, the amounts of digestible amino acids, body weight gains and carcass yields were reduced in the birds that received SHM. Douglas et al (1997) also found that digestibility of cystine was substantially lower than the other amino acids in

SHM. Bravo-Jimenez et al. (2009) observed the similar reductions in daily diet intake and weight gain of pigs when the amount of SHM increased in their diet. These findings suggested that animals might have difficulty digesting SHM and thereby using substantial levels of this feed ingredient is not recommended.

1.4. Problems associated with current disposal methods

1.4.1. Economic and product quality concerns

The modern Leghorn hen is a small-framed bird with a minimum of muscle mass. It was estimated that only about average 250 g of edible meat remains on their bodies, which yields a value to a processor that barely covers the cost of handling the birds (Kersey et al., 1997). In addition, the quality problems associated with the spent hen meat discussed in the previous section such as increased heat-stable collagen, greater susceptibility to lipid oxidation, and the broken bone residues, lead to a lower price when the producers try to sell the birds as raw material for food product processing. There are, however, a few more problems with using light fowl (WL) spent hens. The smaller size and low feather cover on the birds make them have difficulties in coping well with the stress and physical strain during transportation (Knowles, 1994). Thus the light fowl have relatively high numbers of dead on arrival (DOA) birds arriving at the processing facility. The on-farm slaughter police are used by producers to lighten the DOA loss and animal welfare issues that are associated with handling the spent hens; however, extra machinery and operation costs are required. For other disposal methods such as rendering and compositing, the initial cost for purchasing equipment, the labour cost for operation and the machinery cost for maintenance add up to a heavy economic burden that drives the

egg producer to seek a more profitable market for utilizing spent birds.

1.4.2. Environmental and biosafety concerns

Improper animal mortality disposals generate various environmental and health hazards. Land used for a burial site is limited because of the biological hazards created by decaying carcasses. An unpleasant odor is produced by the anaerobic breakdown of proteins, which can reduce the quality of life and decrease property values. The filtration and effluent of decomposed organic matter through the soil has the potential of leaching harmful nitrogen and sulfur compounds into ground water. This ground water contamination is considered to be a severe health problem since in most areas it is the source of drinking water for human and other livestock (Gingerich, 2003). Moreover, the burial method does not have a controlled system for inactivating pathogenic microorganisms, which may be capable of spreading diseases in soil as well as the plants, animals and humans that have contact with the contaminated soil (Kalbasi et al., 2005).

There are also potential pathogenic risks associated with composting of animal carcasses. Bacterial pathogens can survive outside the host organisms if composting temperatures are inadequate for their destruction (Mote et al., 1988). The potential re-growth of pathogenic organisms becomes a bio-safety concern of applying animal carcass compost as a land amendment. Regrowth of salmonellae and coliform bacteria in the composts that were incompletely stabilized has been reported by Mote et al. (1988).

A substantial percentage of spent hen meat is utilized by the rendering industry to produce SHM that may then be incorporated into animal feed. However, during the last two decades, animal infectious disease outbreaks such as Bovine Spongiform

Encephalopathy (BSE) have raised public concerns that infectious disease agents might be transmitted to the 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 the cage-raised hens are exposed to high levels of fecal dust and close contact with the surface of the eggshell where the salmonella contaminations are usually found (Baskerville et al., 1992; Gast and Beard, 1990). 51.8% of the poultry by-products being shipped to a feed mill were found to be positive for *Salmonella* (Snoeyenbos and Colleagues, 1967). It also has been reported that very low levels of *Salmonella* in feed can cause colonization in the intestinal tract of animals (Schleiffer, 1984). Recently, pet owners and farmers have moved toward choosing animal feeds that contain non-animal protein sources (e.g., soybean meal). As a result, the percentage of meat and bone meal that was accepted in the feed mills in the U.S has declined by approximately 50% during the period of 1999-2002 (Gamroth et al., 2006).

1.5. Extracting proteins from underutilized poultry meat

Increasing demands for processed poultry white meat have led to an oversupply of low value poultry resources including chicken dark meat, spent hens/broiler breeders meat, and mechanically separated poultry meat (MSPM). Extracting valuable protein components is considered a good value-added practice to better utilize these underused poultry resources.

1.5.1. Poultry surimi-like material

Surimi is made from myofribrillar proteins concentrated from mechanically deboned fish flesh by repeatedly washing with water, mixing with cryoprotectatnts, and then

freezing until used (Park, 2005). Due to the successful development of fish surimi process and an increasing market for surimi-based products, applying this surimi technology to other sources other than fish become an interesting area of study. The final product is then given a name called “suimi-like material” (Antonomanolaki et al., 1999; Nurkhoeriyati et al., 2010). Poultry (mainly chicken) meat is considered a good source for producing surimi-like material (Babji et al., 1995; Nowsad et al., 2000a, b; Jin, et al. 2007).

Spent hen meat as an underutilized poultry source that has also attracted some attention from the researchers. Nowsad et al. (2000b) reported that spent hen mince washed with 0.1% NaCl was less red in color and higher in collagen, gel strength, water-holding ability than unwashed mince. Babji and Gna (1994) found that the grinding and washing process of spent hen meat reduced sarcoplasmic proteins with increased myofibrillar proteins, which formed better gels for both broiler and spent hen surimi. A study done by Nowsad et al. (2000a) indicated that the gel strength and breaking strength were higher in spent hen surimi compared to broiler surimi under similar gelation conditions. Moreover, Li (2006) concluded that washed myofibrillar proteins from spent hen meat could be used to improve the functional properties of whole-muscle meat products. However, the efficiency of the surimi process was not reported in any of these studies. In addition, the gel quality was found to be markedly deteriorated in spent hen surimi after 8 weeks frozen storage even with the presence of cryoprotectants (Nowsand et al., 2000a). These results demonstrated the limitations of surimi processing for spent hen protein recovery, as revealed on protein yield and frozen stability. Hultin and Kelleher (2000)stated that traditional commercial surimi processing does not recover proteins effectively from

unconventional raw materials, in part because the material is rich in dark muscle containing high levels of PUFA and pro-oxidants (especially hemoglobin proteins). Moreover, the final products have poor gelation properties and also problems with lipid oxidation (Hultin and Kelleher, 2000). Therefore, there is a need to develop an alternative technique that has more efficient protein recovery for producing high quality muscle protein isolates from these unconventional raw materials, in particular for poultry meat by-products.

1.5.2. pH-shifting process

A new technology, pH-shifting method, was developed to address the problems associated with surimi process, especially for protein extraction from unconventional raw material. This process has several advantages as compare to surimi process including higher recovery yield, efficient removal of membrane lipids, acceptable protein functionality, and less water usage (Hultin and Kelleher, 1999, 2000; Yongsawatdigul and Park, 2004; Choi and Park, 2002; Hultin et al., 2005; Park and Lin, 2005). The process of pH shifting method is indicated in Figure 1.1. Unlike the conventional surimi process, the pH-shifting method utilizes the solubility of muscle proteins at different pH conditions. The protein structure is first unfolded at extreme acidic (pH 2-3) or alkaline (pH 11-12) conditions for solublization, which combine with the reduced slurry viscosity to achieve sufficient separation from insoluble components (skin, bones, connective tissue, neutral and membrane lipids) by high-speed centrifugation (Kristinsson et al., 2005; Hultin and Kelleher, 1999; Undeland et al., 2002). Then the soluble proteins are recovered by isoelectric precipitation where minimum protein solubility is achieved. This

insolublization of protein molecules is mainly due to lack of electrostatic repulsion, which allows refolding and aggregation of protein structure via hydrophobic interactions (Chang, Feng, and Hultin, 2001; Chang, Hultin, and Dagher, 2001; Feng and Hultin, 2001). Sarcoplasmic proteins are retained along with the myofibrillar proteins and are present in the final product of extraction (Hultin et al., 2005). The final protein isolates are usually readjusted to pH 6.2 and stored frozen until used. Many factors are important with regard to the quality of acid- or alkaline-aided protein isolates. The following sections discuss the details of protein denaturation induced by pH-shifting extraction and frozen storage, protein stabilizers (cryoprotectants) and the subsequent effectd on the products heat-induced gelling properties.

1.5.2.1. Protein denaturation

Denaturation of proteins occurs when the conformation and arrangement of polypeptide chains in the protein molecule are changed by intrinsic or extrinsic forces, which result in a different protein structure from its native state (Damodoran and Paraf, 1997). The protein structure can be partially unfolded or denatured by many factors such as pH variations, high salt concentration, or temperature fluctuations (Christen and Smith, 2000).

1.5.2.1.1. Protein denaturation by pH-shifting process

It has been demonstrated that the protein isolates from pH-shifting extraction were in their denaturated state. The conformation of the protein structure is largely altered by the unfolding/refolding process during the acidic or alkali solubilisation following the isoelectric precipitation. Kristinsson and Hultin (2003, 2004) concluded that the protein

structure is partially unfolded to different extenst depending on the extracting pH conditions (acid or alkaline). Here proteins retain secondary structure but lose most side-chain interactions in the distributed tertiary structure. While during refolding, the protein is able to partially recover its tertiary structure by non-specific interactions between exposed hydrophobic areas (Price, 2000). The denatured protein is not able to regain its native form likely being energetically trapped in partly folded intermediates (Kuznetsova et al., 1999; Kumar et al., 2000).

The pH-shifting extracted protein isolate is a complex system that contains both aggregated myofibrillar and sarcoplasmic proteins. Myofibrillar proteins make up to 60-70% of the muscle proteins and myosin is the most abundant protein in the myofibrils (Damodaran, 1997). The native myosin consists of a double-headed globular region and a left-handed twisted helical tail (Mccurdy et al., 1986). Sarcoplasmic proteins contribute 20-30% of total proteins and contain mainly globular proteinases involved in protein synthesis and degradation, fatty acid oxidation and glycolysis (Wang, 2006). Two studies has been conducted by Kristinsson and Hultin (2003, 2004) on the unfolding and refolding behaviors of both myosin and hemoglobin (Hb) during acid- and alkaline aided extraction. They found that cod myosin lost more helical structure and had more dissociation of light chains at acidic pH compared to at alkali pH. After refolding, myosin refolded from acidic solublization had more recovery of its tertiary structure (Kristinsson and Hultin, 2003), which may attribute to the higher recovery yield after acid-aided extraction. As for the Hb, the acidification created a more unfolded structure, subsequently resulting in a more misfolded Hb, shown to be more prooxidative in acid extracted protein isolates (Kristinsson and Hultin, 2004). One the other hand, proteolytic

hydrolysis also contributed to the degradation of isolated proteins from pH-shifting extractions. Cathepsins, heat-stable lysosomal proteinases, were found closely associated with myofibrillar proteins (Lanier, 2000) and were not removed by pH-shifting extractions. According to Undeland et al (2002) and Kim et al (2003), high proteinase activity was detected in acid-aided fish proteins that lead to extensive myofibrillar protein degradation. On the other hand, the low enzymatic activity found in alkaline-extracted protein, especially at pH 12.0, could be due to the complete removal and/or effective inactivation of cathepsin-type enzymes.

Based on these studies on the pH-shifting process, acidification causes more extensive protein denaturation and enzymatic degradation that leads to more misfolded protein states after refolding process.

1.5.2.1.2. Protein denaturation by freezing

Frozen storage is considered the most efficient and common way to preserve meat, meat products, and muscle protein isolates. However, the formation of ice crystals during frozen storage leads to cell disruption and destruction of muscle fibres (Sebranek, 1982), enhancing oxidative reactions in different meat components. Numerous studies have proved that the oxidatively-induced alterations in meat are responsible for protein denaturation (ie. polymerization or fragmentation, formation of carbonyl derivatives) and can lead to subsequent loss of solubility, gel-forming ability and water-binding capacity (Decker et al., 1993; Xiong et al., 1993; Kelleher et al. 1994). Lipid and protein oxidation are the two major forms of deterioration in frozen stored muscle meat.

Lipid oxidation is initiated by unsaturated fatty acids reacting with oxygen through a

free radical chain mechanism. Hydro-peroxides are formed that participate with many different decomposition pathways and lead to a large number of volatile compounds (Mottram, 1987). These breakdown products from oxidized lipids not only contribute to the meat rancidity and off-flavor problems, but also act as a catalyst for protein oxidation, leading to the formation of oxidized lipid-protein interactions. Khayat and Schwall (1983) reported that the formation of insoluble lipid-protein complexes via the binding of oxidised unsaturated lipids to proteins, lead to the toughened texture of the frozen stored seafood. Schilder (1993) also demonstrated that the degree of unsaturation of free fatty acid was positively correlated to the extent of polymerization and resulting isolubilization of oxidized chicken myobibrillar proteins. Moreover, Smith et al (1990) suggested that the deterioration of protein solubility may be caused by the presence of residual lipids in isolated myofibrils from chicken muscle.

The pH-shifting method provides a sufficient removal of PUFA (mainly phospholipids) from proteins during the acid or alkaline solubilisation step by high-speed centrifugation. It is considered an advantage of this process, especially for the frozen stability of isolated proteins. Hultin and Kelleher (2000) reported removal of 37 and 51% of phospholipids from chicken breast and thigh muscles, respectively, by acidic extraction. Undeland et al. (2002) reported 30-46% phospholipid removal from herring muscle by both acid- and alkaline-solubilization processes. A more recent study by Hrynets et al (2011a) on the recovery of functional proteins from mechanically separated turkey meat (MSTM) achieved an average 90.7% reduction of phospholipid by both acid- and alkaline aided extractions. In this situation, the lipid oxidation may not be significant during the frozen storage of pH-shifting extracted poultry protein isolates.

As for frozen-induced protein oxidations, recent studies show that muscle proteins, including sarcoplasmic, myofibrillar and stromal proteins, are all susceptible to oxidative damage caused not only by oxidizing lipids, but also metal ions and prooxidants in muscle, or generated during processing and storage (Xiong and Decker, 1995). These oxidative damages are demonstrated by changes in protein structure, protein polymerization or fragmentation, formation of amino acid derivatives and insoluble protein-lipid complexes (Xiong, 2000).

The most commonly measured products from protein oxidation are protein carbonyl derivatives. These compounds are formed via the decomposition of oxidized amino acid side chains, mainly from lysine, arginine, proline and threonine (Amici et al., 1989). In addition, the attack of reactive oxygen species on cysteine and methionine may also lead to the formation of disulphide cross-linkages among proteins or mixed disulphide conjugates with glutathione, since both contain susceptible sulfur atoms (Hu, 1994; Vogt, 1995). Decker et al. (1993) suggested that the aggregation could be produced by disulphide linkages from myosin and actin and result in higher molecular weight polymers. On the other hand, Davies et al. (1987) stated that the oxidative modification of protein structure can also involve direct fragmentation and provide denatured substrates for the intra or intercellular proteolysis. Soyer et al. (2009) demonstrated that the protein oxidation was significantly higher in the chicken leg meat than in the breast meat after freezing at -7˚C for 3 months, which was associated with an increase in carbonyl group content and a decrease in total sulphydryl groups. These results suggest that chicken dark (leg) meat is more susceptible to oxidative damages during frozen storage (Soyer et al., 2009). These studies indicate a general sensitivity of muscle

proteins to oxygen radicals during frozen storage.

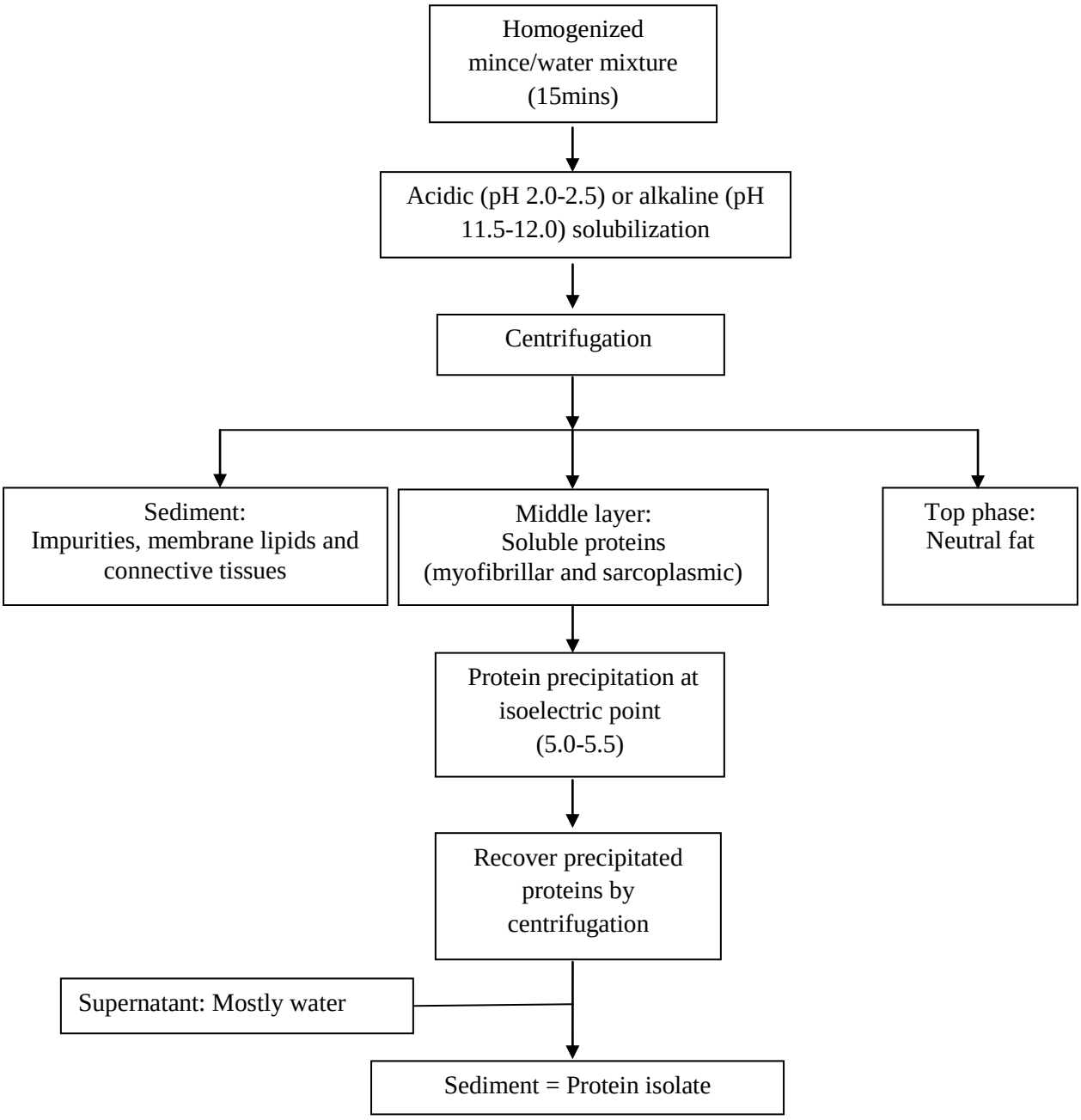

Figure 1.1 Schematic diagram of the acid- and alkaline- extraction process (Adopted from Kristinsson et al., 2005)

1.5.2.2. Protein stabilizers during frozen storage (cryoprotectants)

Unlike the unavoidable protein conformational changes during pH-shifting process, the frozen-induced protein denaturation can be inhibited by the addition of freezing stabilizers, usually referred to as cryoprotectants (CP). According to Park et al (1994), the first application of CP in preventing denaturation of fish protein during frozen storage dates back to 1959 and later became standard practice for modern surimi production. The most commonly used cryoprotective compounds are sucrose and sorbitol. These CP compounds could reduce the formation of large ice crystals and fixed water molecules around the preserved protein, which form a protective shield to stabilize the protein structure and prevent the frozen-induced protein aggregation (Arakawa and Timasheff, 1982). Polyphosphates are usually used to complement the cryoprotective effect of sugars due to their buffering and metal chelating effects in muscle proteins during freezing (Park and Lin, 2005).

The CP effect has been studied on the proteins isolated from surimi-like material (spent hens). Nowsad et al. (2000a,b) reported that CP had a beneficial effect on frozen stored spent hen surimi to protect the elasticity and cohesiveness of the gel. Ensoy et al. (2004) also reported that a CP mixture of 2% sucrose + 2% sorbital + 0.3% sodium pyrophosphate resulted in higher water-holding capacity of the frozen spent layer surimi.

The muscle protein isolate processed with the pH-shift method also needs additives to preserve them against denaturation under freezing. Thawornchinsombut and Park (2006) studied frozen stability of alkaline-aided extracted fish proteins and recommended using CP to prevent freeze-induced aggregation during frozen storage. Omana et al. (2010) reported that CP addition improved the elastic properties of the gel made from alkaline

extracted chicken proteins. No report has been conducted on CP effects on the proteins extracted by acid-aided process. In spite of the general higher protein yield with acid solubilization, the extensive protein denaturation during acid extraction and subsequentl pool gelling properties may reduce the industries interest to investigate further food applications from acid-extracted protein isolates.

1.5.2.3. Viscoelasticity of the muscle protein isolates extracted by pH-shifting process

Heat-induced gelation is the most frequently texted functionality of recovered muscle protein isolates. The dynamic rheological technique has been extensively used to determine the heat-induced gelation of muscle proteins (Hamann, 1988). It is expressed in the viscoelasticity measures of storage (G') and loss modulus (G''), which indicate elastic and viscous component of the gel, respectively. The gel forming ability during thermal processing is the primary interest of the industry in relation to the applicability of the extracted muscle proteins. The heating process unfolds protein structure which allows for hydrophobic and disulfide interactions at unfolded regions between protein molecules to form a continuous network structure with entrapment of water molecules (Visessanguan et al., 2000; Xiong, 1997). Upon cooling, the protein gel network is further stabilized and the reformation of hydrogen bonds leads to a more elastic gel (Hamann, 1988).

The pH-shifting process produces muscle protein isolates from fish with better gelation properties compared to the conventional surimi method. Yongsawatdigul and Park (2004) reported that the alkali process produced stronger gels from rockfish than did the

conventional surimi process. Kristinsson and Ingadottir (2006) also found that alkali-extracted fish proteins from tilapia formed better gels than the conventional surimi. A study was conducted by Kristinsson and Demir (2003) that compared the pH shift process and conventional washing on mackerel, catfish and mullet. They found that the best gels were produced by alkaline-extracted fish protein, followed by conventional surimi method and the acid-extracted obtained the worst gels. Choi and Park (2002) also reported that acid-extracted fish protein had lower gel forming ability than alkali-extracted samples. The lower gel quality is explained by greater proteolytic activity of lysosomal proteinases that are activated at low pH during acid-aided extraction (Choi and Park, 2002; Yongsawatidgul and Park, 2004). However, Hrynets et al. (2010b) reported that more viscoelastic gels were obtained by proteins extracted at pH 3.5 from MSTM compared to alkaline extraction. According to Lanier and Lee (1992), the protein source has a greater influence on the gelation characteristics based on the type of muscle (dark or light) that the proteins from which they are extracted. Different protein sources isolated under the same conditions have been observed with different functional properties, including gel strength, thermal stability and solubility (Oakenfull et al., 1997).

1.6. Utilization of proteins recovered from agriculture by-products as polymeric materials for non-food applications

As a consequence of considerable pubic and commercial interest in proteins isolated from agricultural by-products to be used for food applications, there is also a concurrent need to develop biodegradable polymeric material from these renewable resources. Developments of polymers from agricultural processing by-products are attracting

increased attention due to societal pressures for environmental responsibility and the recognition of limited petrochemical energy resources. Polymer by-product development will lead to a greater utilization of agricultural by-products and bring additional agricultural income.

In general, natural polymers with suitable structure and functional properties from agricultural sources are starch, protein, and cellulose (Chen, 2009). Proteins, in particular are differentiated positively from other biopolymers based on their good processability, advanced film-forming properties, strong adhesion to various substrates, and good surface-active properties. These functional properties are due to the protein molecule`s unique amphiphilic property. However, the native protein structure limits the availability of the reactive functional groups (ie. carboxylic acid, amino, sulphydryl and hydrophobic groups), which are mostly buried inside of the protein molecule. Thus, one advantage of using chemical extracted protein isolates for technical applications in contrast to food applications is that there are fewer restrictions with respect to protein denaturation (unfolding). In fact, for most applications, protein denaturation seems to become a requirement for some high quality products.

The studies on non-food applications of protein isolates from agricultural raw materials mostly adopt the concepts and tools developed in polymer science, which focuses on the relationships between molecular structure and functional properties (De Graaf et al., 2001). Three general approaches are used to elucidate the polymeric property of protein molecules: (1) observation of the modified protein structure at different scales (atomic and molecular); (2) correlation of the functional properties of the modified protein with

temperature, pressure, or incorporation of functional additives; (3) prediction of the macromolecular network formed after modifications base on the physical and macroscopic properties such as mechanical strength, surface morphological image and thermal stability of the modified protein structure.

Numerous vegetable and animal proteins such as wheat gluten (Irissin-Mangata et al., 2001; Largrain et al., 2005), corn zein (Lai and Padua,1997), soy proteins (Huang and Sun, 2000; Rhim et al., 2002), pea proteins (Gueguen et al., 1998; De Graaf, et al., 2001), collagen (Bigi et al., 2000), egg and milk proteins (Mauer et al., 2000) and fish myofibrillar proteins (Cuq et al., 1999) have been investigated as for their suitability in two major industrial applications: agricultural film for packaging and bio-based adhesive for plywood composition.

1.7. Wood adhesive industry

With the rapid increase in wood composite production worldwide, the total consumption of wood adhesives has greatly increased and adhesive industry has become one of the fastest growing industries in the world. Although the adhesive market had a significant decline 2008 due to a global economic crisis, it grew in the next few years with respect to both volume and dollar sales. The formulated adhesive world consumption researched 7.47 billion kilogram in 2009, which was worth US $ 20.6 billion market value (KNGInc, 2010). According to Global Industry Analysts report, the global formulated adhesive market is projected to reach 25.4 billion pounds by 2015. A modest 3% annual rate of growth is forecast for North American formulated adhesive volume trough by 2014 (KNGInc, 2010).

1.7.1. Adhesive mechanisms

Adhesion has been described as the state in which two surfaces are held together by chemical and/or physical forces (Sellers Jr., 1994). Sellers explained that the goal of wood adhesion is not simply to hold the material together, but to transfer mechanical loads and stresses away from the adhesive interface, thus the joined materials will not be easily separated. This required that the sum of adhesive forces (wood/adhesive molecular interactions), and cohesive forces (attractive forces between adhesive molecules) must be greater than the strength of the wood itself (Sellers Jr., 1994).

There are several theories have been proposed to explain the mechanisms of adhesion for wood bonding system, which are valid depending on the materials and conditions employed (Schultz and Nardin, 1994). Adsorption, mechanical interlock, covalent bonding, and interpenetrating networks (IPN) are the most frequently used principles to describe wood adhesion.

1.7.1.1. Adsorption theory

The adsorption theory is considered the fundamental mechanism of adhesion for wood (Shi and Gardner, 2001). This theory suggests that intermolecular forces (also known as secondary associations, and van der Waals forces), develop across the interface between adhesive and wood and are critical for the bonding strength (Comyn, 2005). Individually, these intermolecular interactions are weak, but summed collectively across the surfaces of the wood bondline, their strengths are more than adequate (Comyn, 2005). In order to achieve these forces, the adhesive must make intimate contact with the wood surface. This requires the adhesive to be liquid at some point during the bonding process to

effectively ‘wet’ the wood surface (Sellers Jr., 1994). The term ‘wetting’ refers to the contact of a liquid onto a solid surface, and the extent of wetting is measured by the contact angle. The contact angle represents a balance between the solid, liquid, and interfacial energies. Good ‘wetting’ is achieved when the contact angle is less than 90 degrees (Fig.1.2). This occurs when the solid has high surface energy and/or the liquid had low surface tension (Vick, 1999). When the liquid/solid attractive forces (adhesive forces) are greater than the liquid cohesive forces, the liquid will wet the surface. For wood bonding, favorable wetting is desirable because it promotes adhesive penetration and often prevents the entrapment of air onto the adhesive layer (Gardner et al., 1996; Saiki, 1984).

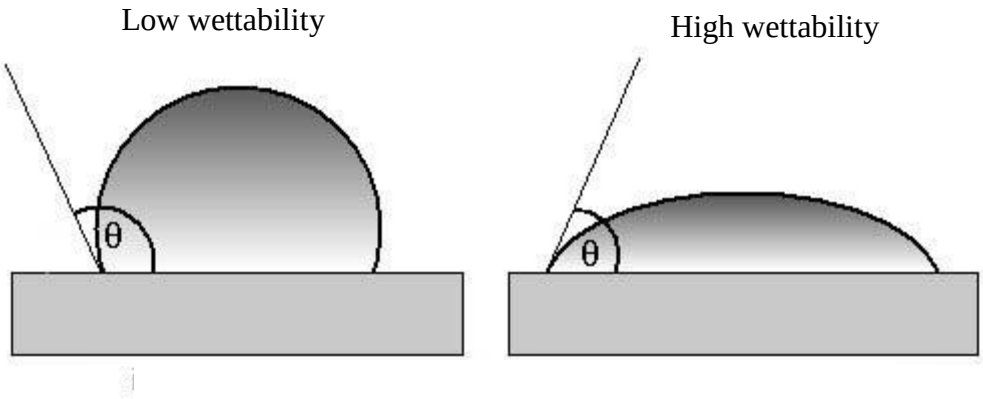

Figure 1.2. The contact angle of a water-borne adhesive with a wood surface is used as wettability index, for $\Theta < 90^{o}$ the liquid wet the wood surface. For $\Theta > 90^{o}$ the liquid does not wet the well.

1.7.1.2. Mechanical interlocking

Adhesive penetration is a critical concept in the mechanical interlocking theory. This theory suggests that upon solidification, penetrated adhesive molecules become anchored to the substrate resulting in stronger and tougher joints (Kamke and Lee, 2007; Vick, 1999). Wood surfaces are naturally porous and contain lumen with irregularities cut from machining prior to bonding. These provide a "tooth" like structure for the solidified adhesive to physically interlock with wood surface porous and lumen structure, on a scale of microns to millimeters, which improve adhesive strength. Penetration should be deep enough to transfer bond stresses away from the interface; however, excessive penetration may create a 'starved' bondline where too little adhesive remains at the interface (Kamke and Lee, 2007; Vick, 1999). Mechanical interlocking is considered necessary for good adhesion, but it is just one of the contributing factors.

1.7.1.3. Covalent bonding

Primary chemical bonds, or covalent bonds, between adhesive and wood may contribute to the wood adhesion mechanism. Covalent bonds are significantly stronger than secondary associations (Schultz and Nardin, 1994). Covalent bonds may not significantly increase the measured wood-bond strength (many non-covalent bonding adhesives are already stronger than wood itself), but they are desirable for improved durability against moisture (Pocius, 2002). There has been considerable speculation about the formation of covalent bonds among adhesive molecules or with wood surface molecules via the crosslinkage of various reactive functional groups available in both wood and polymer molecules. However, there is no direct evidence of covalent bonding at wood-adhesive joints (Sellers Jr., 1994; Vick, 1999). The recent NMR and FTIR

studies suggested a possibility of covalent bonding between wood and protein-based adhesive (Zhou and Frazier, 200; Lin et al., 2012).

1.7.1.4. Interpenetrating networks

For thermosetting adhesives, the molecular interpenetration network (IPN) via monomer diffusion and subsequent polymerization can explain the adhesive penetration on a molecular level (Marcinko et al. 2001). The IPN theory suggests that adhesives containing reactive monomers or very low molecular weight constituents that interpenetrate into the cell wall of wood molecules, which create a network between adhesive and wood due to both physical and chemical forces (Kamke and Lee, 2007). The adhesive mechanisms discussed in the previous section can penetrate the wood cell wall on the molecular level where lignin and hemicellulose are present. The adhesive molecule then can occupy the cell wall at the pore space and/or by creating an interpenetrating polymer network (IPN) within the wood molecules, thus blocking hydrophilic functional groups. The resin system bulking can reduce water uptake in the cell wall, resulting in better water resistance at the joints. This nano-scale adhesive principle is thought to contribute to the high bond strength and improve water durability (Kamke and Lee, 2007)

1.7.2. Factors affecting protein based wood adhesive performance

Although the protein polymers are widely recognized and used for producing formaldehyde-free wood adhesives, the fundamental nature of the adhesion of these materials remains poorly understood (Frihart, 2005). There are many factors that determine how a polymer adheres to the wood surface. When considering the adhesive

performance of protein polymers, the most critical properties are the adhesive viscosity, molecular weight distribution, and the exposure of functional groups on the surface of the protein structure. Processing parameters are also important, such as curing temperature, pressure, and total assemble/pressing time.

1.7.2.1. Viscosity

The viscosity of the adhesive plays an important role in its wood surface penetration behavior. There are three basic stages that must be satisfied by an adhesive before a proper bond can be formed: wood surface wetting, flow, and penetration (Nordqvist et al., 2010). These factors are largely dependent on the viscosity of the adhesive. Consequently, a lower adhesive viscosity is usually desired in practice because a smaller contact angle can be obtained during the assembly time. Low adhesive viscosity promotes greater interfacial strength due to its better penetration into the lumens and pores on the wood surface (Baldan, 2012). The differences in the appearance and the strength of wood bondlines are also related to the viscosity. The higher initial viscosity may lead to an impaired bonding strength due the inadequate surface wetting and penetration.

1.7.2.2. Molecular weight distribution

The particle size or molecular weight (MW) of the adhesive polymers has a close relationship with its viscosity. A deeper penetration was observed with most types of adhesives at low viscosity, while cell-wall penetration only occurred with an adhesive having a small MW component (Harada et al., 1968). High molecular weight adhesives occluded the pits or lumens on the surface of wood, which may inhibit wettability and degree of penetration. Tarkow et al. (1966) studied the critical MW of polyethylene

glycol (PEG) needed to permit penetration by the cell-wall of Sitka spruce. Their study identified a critical MW of 3 kDa for PEG at room temperature. The authors speculated that higher temperature would result in a larger critical MW due to increased mobility of the cell-wall polymers. Johnson and Kamke (1992) suggested that the deeper penetration caused by methane-diisocyanate (MDI) resin than PF resins was due to the low molecular weight of the resin. Kamke and Vanek (1994) suggested that low molecular weight of MDI resin would promote penetration into wood cell walls. They further hypothesized that an interpenetrating network (IPN) may be formed within the cell wall by this resin. In addition, Gollob et al. (1989) also reported that higher MW PF resin formulations tended to dry out and had little penetration at the wood bondline. Zheng (2002) attributed the reduction in penetration to an increased molecular weight, and subsequent increased viscosity. Some researchers further suggested that cell-wall penetration could be manipulated by controlling the molecular weight distribution of the formulated adhsives (which researchers).

1.7.2.3. Heat-pressure

In recent research, most protein-based adhesives are classified as thermosetting adhesives, meaning that the protein polymers in the adhesive only go through condensation at an elevated temperature. The normal processing temperature range is usually between 90ºC to 150ºC (Wang and Wu, 2012), which varies depending on the type of proteins and wood used for processing. Temperature influences wood penetration by affecting adhesive viscosity and cell wall permeability (Kamke and Lee, 2007). The wood adhesive initially decreases in viscosity with increase of temperature thus

promoting penetration. When the adhesive is held at a high temperature for a specific time, polymerization of the adhesive molecules initiates which increases in viscosity and eventually solidifies to create stable adhesive bonds. Wood, on the other hand, undergoes thermal expansion, the result of increased molecular motion with increasing temperature which also enhances adhesive penetration (Kamke and Lee, 2007). The temperature-induced increase in cell-wall penetration by adhesive polymers was reported by Tarkow et al. (1966) in their study of molecular size exclusion by the wood cell wall. Later, Sernek et al. (2002) noted that the rate of polymerization was much faster using heating and thus reduced the penetration time. The reactive functional groups from wood molecules as well as the protein molecules could react with each other or with compounds that have active hydrogen molecules such as water, alcohols, and amines. Heating was found to be useful on increasing the rate of these type reactions (Schmidt et al., 2005). In addition, it is theoretically possible for certain covalent bonds to form between hydroxyl groups in the wood (ie. cellulose) and the adhesive (unfolding protein molecules).

Johnson and Kamke (1994) reported increased adhesive penetration in wood by increasing pressure. White (1977) also found that increasing pressure up to 1000 kPa increased adhesive penetration into wood. Most research on protein-based adhesives used hot-pressing at the processing step for achieving better adhesive bonding properties to take advantage of both heat and pressure effects.

1.7.3. Concerns with petroleum-based (formaldehyde) adhesives

Nowadays, phenol-formaldehyde, urea-formaldehyde, melamine formaldehyde,

melamine urea formaldehyde resins are predominately used for the production of wood composite products (Rowell, 2005). However, concerns about the finite nature of petroleum resources and the environmental issues associated with carcinogenic formaldehyde emissions and the resins' non-degradable nature have raised concern by the government and consumers.

Petroleum is a non-renewable resource. An oil crisis will bring forward the dilemma of energy sustainability in the future (GFCP, 2008). One the other hand, an acceptable formaldehyde emission from wood composited products has been continuously reduced by government regulations due to an increased public awareness and consumer demands. In 2004, formaldehyde was re-classified as "carcinogenic to humans" by the World Health Organization (WHO). With this recognition, California Air Resources Board (CARB) has recently set strict limits on formaldehyde emissions for oriented strandboard production in consideration of both worker and consumer health (CARB, 2004). Therefore, the wood products industry has been forced to seek alternative materials satisfy both government and consumers. During the past decades, research on conversion proteins from renewable agricultural industry by-products to protein-based adhesive or intermediates applied in adhesive technology has been significantly intensified.

1.7.4. Animal protein based adhesive

The history of using protein-based adhesives as gluing materials can be traced back to ancient times. In early stages, research was focused on animal protein sources such as blood, milk proteins, fish skin extract and bovine serum albumin (Barone and Schmidt 2006). However, in the past two decays, the majority of research has been on plant

proteins, especially soy, due to their relatively lower price and abundant production (Barone and Schmidt, 2006). Even the soy protein-based adhesives suffer from poor water resistance and relative low adhesive strength. On the other hand, none of the animal protein sources are considered to be economically viable at a sufficiently low cost, large supply, and as consistent composition as soybean flour. Animal protein sources still have advantages over soy due to their special properties.

According to Salzberg (1962), casein glues were first manufactured in the nineteenth century, mostly used in wood industry. Although casein glues required long setting times to develop adhesion, the calcium caseinate formation gave better water resistance to these glues, while the animal gelatin glues remained sensitive to moisture (Lambuth, 1989). In most cases, casein glues are available as a powder and are mixed in water before use. The two main components, casein and an alkali, are involved; a third mixing chemical ingredient may also be added, such as copper chloride, to give a further improved durability by promoting crosslinking of casein molecules (Lambuth, 1989).

Nowadays, casein glues, are mostly replaced by synthetic glues in exterior woodworking, but there are still some casein applications in labeling adhesives, in the bottling industry, in interior woodworking, and in pressure sensitive systems (Salzverg, 1962).

Blood protein from beef or hogs has the best water resistance among all the commercial protein adhesives but has great inconsistency and a high spoilage rate (Paeker and Johnson, 1995; Lambuth 2003). Blood-based adhesives are made from spray-dried animal blood, which can be considered a disadvantage since drying is an expensive

process. As with the other protein adhesives, blood adhesive is formulated by re-dissolving the blood in water and dispersing it with an alkaline ingredient, such as caustic soda or hydrated lime (Selbo, 1975). Sometimes it can be incorporated into thermosetting resins (usually phenolic) to increase the adhesive's durability (Selbo, 1975).

Animal bone and hide glues are used in fine furniture manufacturing because they contain hydroxyproline compounds that form flexible bonds for good durability to indoor humidity changes (Pearson, 2003). They have many other uses but are being replaced by synthetic adhesives due to cost and the synthetics' superior water proofing property for exterior applications.

Compared with the petroleum formaldehyde-based adhesives, the major disadvantages of the protein-based adhesives is that they are subject to attack by microorganisms and much less resistant to moisture, which prevent them from being used for long term storage and exterior applications.

1.8. Hypothesis and research objectives

Spent hen meat is considered as an underutilized by-product from egg and poultry industries and also a good source for muscle protein extraction for specific applications. However, the extraction usually causes extensive protein denaturation during both process and storage steps, which may have negative influence on their suitability for food products preparation. Thus it is possible to investigate the value-added potential of using muscle protein isolates as a bio-polymeric material for industrial wood adhesive preparations, where denaturation is in fact required. In addition, the fibrous nature of the muscle protein (mainly myofibrils) may help to improve the adhesive properties such as

adhesion strength and water resistance.

1.8.1. Research objective

The objective of this thesis was to extract the protein components from spent hen meat by the pH-shifting process and explore value-added potentials for using these proteins in industrial applications, such as wood adhesive preparation, rather than for food or feed. In order to achieve this goal the following sub-objectives were defined.

(1) Determine the optimum extraction conditions (extracting pH, centrifugation speed etc) to achieve the highest protein recovery and content.

(2) Define the effect of extraction pH and frozen storage on the chemical and rheological properties of protein isolates obtained from spent hen meat.

(3) Investigate the adhesive properties of the chemically treated spent hen proteins as affected by acid- and alkaline-aided extraction and frozen storage.

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CHAPTER 2. CHEMICAL, RHEOLOGICAL AND SURFACE MORPHOLOGIC CHARACTERIZATION OF SH-PI EXTRACTED BY PH-SHIFT PROCESSING WITH OR WITHOUT THE PRESENCE OF CRYOPROTECTANTS[1]

2.1. INTRODUTION

Spent hens are egg-laying chickens considered to have outlived their productive lives, usually by about 60-72 weeks of age, which need to be replaced by younger and more productive flocks. In Canada, approximately 22 million spent laying hens need to be removed annually from production (AAFC, 2007).

In the poultry industry, processing of spent hens into meat has been practised as a way to dispose the hens and to maximize revenues. However, poor organoleptic and sensory qualities are the major problems of this meat. The general toughness, presence of bone fragments and the high aroma intensity, associated with spent hen meat limit its usage in processed food products (Rouselle et al., 1984). In addition, the genetic selection for smaller body weight results in low edible meat yield, which generates minimum profit for the processing plants, especially when the cost of labour and associated transportation is taken into account. Thus, an increasing number of birds are being composted and buried in landfills, which not only cause a loss of potential nutrients, but also concerns for the environment (Lyons & Vandepopuliere, 1996).

The newly developed protein extracting technology, pH-shifting process, has shown significant potential for producing functional protein isolates from poultry meat by-products (Hrynets et al., 2010a, 2010b). It could be an efficient approach to increase the market availability of spent birds from poultry and egg industries. During the pH-shifting extraction, protein structure is first unfolded at either low or high pH values and the

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soluble proteins are then collected after refolding and precipitation at their isoelectric point. The unfolding/refolding process, which varies depend on the acid or alkaline pH used during the extraction, results in different yield and physiochemical properties of the isolated proteins (Kristinsson et al., 2005).

The extracted protein isolates are usually kept frozen until use. Frozen storage can effectively reduce the microorganism activities but it also induces lipid and protein oxidations, which negatively affects functional properties such as protein solubility and water holding capacity (Kijowski & Richardson, 1996). The frozen-induced protein denaturation also reveals on the chemical properties changes of the isolated proteins, which specifically reflect on generation of carbonyl groups and decrease of sulfhydryl groups (Stadtman, 1990). On the other hand, gelation is one of the most important functional properties of the isolated protein and has often been impaired by the frozen storage (Thawornchinsombut & Park, 2006). The addition of cryoprotectants (CP) to the isolated muscle proteins were suggested to improve the gel quality by preventing cold-denaturation on myofibrillar proteins during frozen storage. During thermal processing, cryoprotected protein isolates formed a paste that, with increased water-holding capacity along with reduced viscosity, allowed for better gel strength and cohesiveness (Nopianti, Huda, & Ismail, 2010).

Several studies have been conducted on the heat-induced gelling properties of the spent hen surimi after frozen storage (Nowsad et al., 2000a, 2000b). To our knowledge, there are no reports about applying a pH-shifting process on spent hen meat and the physicochemical properties of the recovered proteins after frozen storage still remain

unclear. In addition, the CP effects on pH-shifting recovered proteins require further investigation. Although the effect of CP addition on improving the frozen stability and gelling property of the alkaline extracted muscle proteins has been reported in several studies (Thawornchinsombut and Park, 2006; Omana et al., 2010), there is limited data regarding the CP effects on the chemical, physical and functional properties of pH shifting recovered poultry proteins, especially for the proteins extracted by acid-aided processes that generally produced higher protein yield in fish protein extractions.

Therefore, the objectives of this study were to: (1) determine the efficiency of the pH-shifting method on protein recovery from underutilized residue spent hen meat from the carcasses; (2) investigate the chemical and the rheological properties of the isolated proteins as affected by the pH of extraction medium (acid and alkaline), the addition of CP, as well as the interaction of the two treatments; (3) understand the morphology and secondary structure changes of the protein isolates with and without CP.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Spent hen carcasses were obtained after removal of the cut-up parts (breast, legs and wings) from Lilydale Inc. (Edmonton, AB, Canada). The remaining meat on the frame was manually deboned and homogenized using a Kitchen-Aid Food processor (Model KFP 7500B, KitchenAid, St. Joseph, MI, USA) for 5 min. Spent hens mince (250 g) was filled into polyethylene bags and kept at - 20ºC until use. Before extraction, samples were thawed overnight at 4ºC. All the reagents and chemicals used in the study were of analytical grade.

2.2.2. Preparation of protein isolate by acid-aided and alkaline-aided extraction

The acid-aided and alkaline-aided protein extraction for spent hen mince was done according to the method as described by Hrynets et al. (2010a) with slight modifications. Based on the solubility profile of the raw spent hens' meat (Fig. 4), four levels of extraction pH were selected for acid and alkaline solubilisation: pH 2.0, 2.5 and pH 11.5, 12.0, respectively. Protein extractions were performed at 4ºC. For each extraction, 200 g of spent hens mince were homogenized with cold distilled water/ice mixture at a 1:5 ratio using a 900-Watt Food Processor (Wolfgang Puck WPMFP15, W.P. Appliances Inc., Hollywood, FL, USA) for 15 min. After homogenization, the meat slurry was set at 4ºC for 30 min. Then the pH of the homogenates was adjusted using 1 or 2 M HCl and NaOH to reach either acid solubilisation points or alkaline solubilisation points. After pH adjustment, samples were centrifuged at 15300×g for 20 min at 4ºC. Three layers: neutral lipids, soluble proteins and insoluble proteins (collagen) plus membrane lipids were obtained in the order from top to bottom. The middle layer of soluble proteins was subsequently recovered at pH 5.2. Protein precipitate was then collected by centrifugation (15300×g, 20 min, 4ºC). The precipitate was re-suspended in water/ice mixture (water/ice, 350 mL/350 g) by homogenization for 10 min and the homogenate pH was adjusted to 6.2, which is the average pH of the raw hen meat, and then collected via centrifugation at 15300×g.

2.2.3. Total protein content and recovery

The total protein content of both the spent hen meat and final protein isolates from different solubilisation pH values was estimated by a LECO (Nitrogen/Carbon) analyzer

(TruSpec®CN., MI., CA, USA.) . Spent hen meat and protein isolates were freeze dried (LABCONCO Inc., FreeZone12, MO., CA, USA) and grounded into fine power before analysis.

2.2.4. Cryoprotectants addition

After the extraction, protein moisture content was adjusted to 82%. Two levels of CP were added (with and without) to the extracted SH-PI. The CP is a blend of 5% sorbitol, 4% sucrose, 0.4% sodium bicarbonate, 0.3% tripolyphosphate and 0.03% sodium nitrite (Omana et al., 2010). The treated samples were kept in frozen conditions at -20ºC for 3 weeks.

2.2.5. Total (T-SH) and reactive (R-SH) sulfhydryl content

The T-SH and R-SH content were estimated as per the method of Kim, Park and Choi (2003). 25 mL of Tris-glycine buffer (pH 8.0) containing 5 mM of EDTA was added to 2.5 g of recovered proteins and homogenized for 1 min to obtain the protein extracts. The homogenate was filtered and in the case of R-SH, to 1 mL of filtrate, 4 mL of Tris-glycine buffer and 50 µL of Ellman's reagent were mixed together by vortex mixer (Fisher Scientific, On, Canada). In the case of T-SH, to 1 mL of the filtrate, 4 mL of 10M urea and 50µL of Ellman's reagent were added. The mixtures were kept for at 4ºC for 1 h with occasional stirring. The absorbance was measured at 412 nm using a spectrophotometer (V-530, Jasco Corporation, Tokyo, Japan). The sulfhydryl contents were calculated using the extinction coefficient of 13600 $M^{-1}cm^{-1}$ and presented as µmol/g of protein. The protein content of the filtrate was determined using the methodology of Gornall, Bardawill, & David, 1949.

2.2.6. Protein carbonyl content

The quantification of protein carbonyl content was performed as per the method described by Oliver et al. (1987) with modifications. The formation of protein hydrazones from the reaction between 2, 4-dinitrophenylhydrazine (DNPH) and protein carbonyls was determined by spectrophotometer (V-530, Jasco Corporation, Tokyo, Japan) at 365 nm. The total protein solution was extracted in 50 mM phosphate buffer containing 0.55 M potassium iodide from 2 g of sample. The protein solution was diluted in a concentration range of 0.7-1.0 mg/mL and mixed with 10% trichloroacetic acid (TCA; w/v) for precipitation. After centrifugation at 2000×g (4ºC for 10 min), the pellet was either mixed with 2 mL of 0.2% DNPH (w/v) in 2 M HCl, or 2 mL of HCl as blank. The solutions were incubated at 25ºC for 1 h with occasional stirring, then precipitated with 10% TCA and further centrifuged. The sediments obtained after this were washed 3 times with 1 mL of ethanol: ethyl acetate (1:1 v/v) solution and were precipitated with 10% TCA followed by centrifugation. The pellet was then dissolved in 2 mL of 6 M guanidine hydrochloride in 20 mM sodium phosphate buffer (pH 6.5). The absorbance was measure at 365 nm and 280 nm for determining protein hydrazones and protein content, respectively. The carbonyl content was standardized to protein concentration which is calculated from the absorbance at 280 nm in the HCl blanks. The bovine serum albumin (BSA) in guanidine hydrochloride was used for the standard curve. Carbonyl content was calculated and expressed as nmol carbonyl/mg of protein using an absorption coefficient of 22000 $M^{-1}cm^{-1}$.

2.2.7. Protein surface hydrophobicity (SHo)

The SHo of sarcoplasmic (SP) and myofibrillar (MP) proteins was determined according to the method described by Kim, Park, and Choi (2003) using 1-anilino-8-naphthalenesulfonate fluorescent probes (ANS; 8 mM in 0.1 M phosphate buffer, pH 7.4). To prepare SP, 2 g of sample was homogenised with 20 mL of 0.03 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 15300×g for 15 min at 4ºC. The supernatant was filtered through Whatman No.1 filter paper to obtain SP solution. The sediment was re-suspended in 50 mM phosphate buffer containing 0.55 M potassium iodide (pH 7.4) by homogenization and centrifuged at 15300×g for 15 min at 4ºC. Supernatant was filtered through Whatman No.1 filter paper to obtain MP solution. Protein concentration was determined using the method developed by Gornall, Bardawill, & David (1949). The protein solution was then serially diluted with the corresponding buffer to achieve a protein concentration ranging from 0.008% to 0.03% with a final volume of 4 mL. 20 µL of ANS solution was mixed with the sample solution and the relative fluorescence intensity (RFI) was measured at excitation wavelength of 355 nm and emission wavelength of 460 nm with a spectroflurometre (Thermo Electron Fluoroskan Ascent, Vantaa, Finland). The net RFI was calculated by subtracting the RFI of each sample measured without ANS from these with ANF. The initial slope of the fluorescence intensity against protein concentration was taken by linear regression analysis and used as an index of the protein SHo.

2.2.8. Fourier transforms infrared spectroscopy (FTIR)

The secondary structure of the treated proteins was estimated by a Nicolet Magna 750 FTIR spectrometer (Madison, WI, USA). The protein isolates with CP were purified by

dialysis in distilled water at 4ºC with magnetic stir with 3kDa molecular weight cut off dialysis tubing cellulose membrane (Sigma-Aldrich, St. Louis, MO, USA). The water was changed at 1, 2, 4, 8 and 12 h. The spent hen meat and protein isolates with different treatments were freeze dried and milled with potassium bromide (KBr) in a mortar to form a very fine powder and compressed into a thin sheet before analysis. The spectrum was conducted by accumulating 10 scans at a resolution of 8 cm^{-1}.

2.2.9. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated on precast 4–20% precise protein gels (Thermo Scientific., Rockford, IL) using a Mini-PROTEAN tetra cell attached to a PowerPack Basic electrophoresis apparatus (Bio-Rad Laboratories Inc., 1000 Alfred Nobel Drive, Hercules, CA, USA). 25 μg of each protein sample and standard protein marker from Bio-Rad (Bio-Rad Laboratories Inc., Hercules, CA, USA) were loaded and run at constant voltage of 200 V using Tris-HEPES-SDS as a running buffer. After staining and distaining, gels were scanned using an Alpha Innotech gel scanner (Alpha Innotech Corp., San Leandro, CA) with FluorChem SP software.

2.2.10. Scanning electron microscope (SEM)

The microstructures of the treated protein were observed under a Hitachi S-2500 scanning electron microscope (SEM, Nissei Sangyo America Ltd, CA, USA) as per method described by Wang and Wu (2012). The protein powder was evenly spread on the surface of a metal plate and coated with a thin layer of gold with a Gold Sputter Unit (Denton Vacuum, Moorestown, NJ, USA) before examination.

2.2.11. Protein solubility curves for fresh and isolated spent hen muscle proteins

The untreated and extracted spent hen protein solubility curves were conducted as described by Kim, Park, and Choi (2003) with slight modifications. For the untreated sample, 6 g of raw spent hen mince was mixed with 300 mL of deionized water in a homogenizer (Fisher Scientific, Power Gen 1000 S1, Scherte, Germany) at setting of 3 for 1 min. The homogenate (30 mL) was adjusted from pH 1.5 to 12.0 with 0.5 intervals, using 0.2 M and 1 M HCl or NaOH by a pH-meter (Denver Instrument, Ultra Basic, UP-10, Colorado, USA). The homogenate was centrifuged (Beckman Coulter Inc., Palo Alto, CA, USA) at 15300×g at 4ºC for 20 min. The protein concentration of the middle layer was measured by using the methodology of Gornall, Bradawill & David 1949 with bovine serum albumin as the standard. The protein solubility was expressed as mg/g of meat. The protein solubility curves for the acid- and alkaline-extracted protein isolates with and without CP addition were obtained followed the same procedure that described above.

2.2.12. Dynamic viscoelastic behaviour

The dynamic viscoelastic behaviour (DVB) of the treated protein isolates was determined as described in Hrynets et al. (2010b) using a Physica MCR Rheometer (Anton Paar GmbH, Virginia, USA) under oscillatory mode, equipped with a 2.5 cm parallel plate measuring geometry. The gap between the measuring geometry and plate was 1000 µm. Before testing, all samples were adjusted to equal moisture content. 4 g of defrosted protein isolate were mixed with 2.5% of sodium chloride (w/w) to a fine paste. The moisture content was standardized to 80% for all the samples. The paste was

subjected to DVB measurements in a temperature range of 4ºC to 80ºC (heating) and 80ºC to 4ºC (cooling). 2ºC/min heating and cooling rate was used under a controlled strain (0.5%) at a frequency of 1.0 Hz. The linear viscoelastic region (LVR) was determined by an amplitude sweep in a range of deformation from 0.1% to 10%. The storage modulus G', loss modulus G" and tan δ (G''/G') were presented as the result of the measurement.

2.2.13. Statistical analysis

The data for all chemical property assays were analysed as a 4 × 2 factorial ANOVA using the general linear models (GLM) procedure of SAS program (SAS version 9.0, SAS Institute, Cary, NC, USA, 2006). The model tested the main effects for extracting pH (acid: pH 2.0 and 2.5. alkaline: pH 11.5 and 12.0) and CP (with and without), as well as the interaction of the factors using residual errors. The entire experiment was replicated at least 3 times. Differences between group means were determined using HSD Tukey's adjustment and significance of difference was established at $p < 0.05$.

2.3. RESULTS AND DISCUSSION

2.3.1. Protein content and recovery

Protein recovery is an important factor to determine the efficiency of the extracting method for economic reasons. The protein recovery and content of the acid and alkaline extracted proteins from spent hen meat are listed in Table 2.1. The protein content for pH-driven protein isolates was in the range of 90-93% and no significant difference was found among four extracting pH values. However, the final protein recovery was higher

after acid-aided processes compared to the alkaline-aided processes. The protein recovery from the two acid extracts at pH 2.0 and 2.5 was 70.2% and 70.4%, and from the alkaline extractions at pH 11.0 and 12.0 was 60.8% and 62.1%, respectively. Nolsoe and Undeland (2009) summarized that the recovery of protein after acid and alkaline solubilisation is determined by three main factors: protein solubility at selected extraction pH values, size of the sediments after the solubilisation by centrifugation and the solubility at the chosen isoelectric point. The acidification slightly solubilized higher amounts of the total spent hen muscle proteins at pH 2.0 and 2.5 than did alkalization at pH 11.5 and 12.0 (Fig.2.4.-Native spent hen protein). It is also documented that acidification causes more muscle proteins to be denatured thus leading to more aggregation and precipitation at the isoelectric point compared to alkalization (Kristinsson et al., 2005). Another possibility could be that relatively larger sediment and floating layer were obtained during the alkaline process. Undeland, Kelleher, and Hultin (2002) proposed that the soluble proteins could be entrapped in the sediment and floating layer after first centrifugation and that the size of the two layers was larger in alkaline conditions compared to acid conditions. Hence, more proteins were collected at the isoelectric point after acid solubilisation as well as after pH readjustment to 6.2, resulting in higher final protein recovery.

Table 2.1. Protein content (%) and protein recovery (%) of the protein isolates[1] extracted from spent hen minced meat[2] (pH 2.0， 2.5, 11.5 and 12.0) at 15300×g (without cryoprotectants). Different letters indicate significant differences ($p \leq 0.05$).

Extraction pH	Protein recovery	Final protein content % (dry based)	Final protein content % (wet based)
pH 2.0	70.2±0.0^{a}	92.8±0.4^{ab}	16.7±0.5^{b}
pH 2.5	70.4±0.0^{a}	90.0±0.9^{ab}	16.2±0.2^{ab}
pH 11.5	60.8±0.0^{b}	93.1±1.4^{a}	16.8±0.2^{a}
pH 12.0	62.1±0.1^{b}	90.2±1.3^{ab}	16.2±0.1^{ab}

[1] Protein content and moisture of raw spent hen minced meat are 18.6% and 80%

[2] Moisture content of the isolated proteins were adjusted to 82%

2.3.2. Effect of pH and CP treatment on protein oxidation

2.3.2.1. T-SH and R-SH content

Sulfhydryl groups are considered to be the most reactive functional group in proteins, which can be easily oxidized to form disulfide bonds upon freezing or heating (Owusu-Ansah & Hultin, 1992; Feng & Hultin, 2001). In this study, the R-SH content was found to be the highest when proteins were extracted at pH 11.5, while no significant differences among the other three extracting pH values was observed (Table 2.2). However, it is important to point out that the T-SH groups were also different among the four pH treatments. In this case, the ratio of R-SH content to T-SH content for two alkaline (pH 11.5 and 12.0) treated samples were 71% and 70%, respectively, which were higher than the acidic treated samples at pH 2.0 (65%) and pH 2.5 (49%). The decline of R-SH/T-SH ratio implicates that more S-S crosslinks were formed in acid prepared protein isolates. In the model study of cod muscle, the acid treated hemoglobin (Hb) was

found to be more pro-oxidative than the alkaline treated Hb (Kristinsson and Hultin, 2004). By incorporating CP, a significant increase of both R-SH and T-SH content was observed (Table 2.2). The interactions of CP treatment with different extracting pH values were also significant for both R-SH and T-SH, especially in the acid extracted samples at pH 2. The data suggested that CP exerted their protection particularly when proteins were extracted at very low pH values by preventing S-S crosslinking thus reducing protein aggregation during frozen storage.

2.3.2.2. Protein carbonyl content

The carbonyl content was also measured to determine the oxidation level of the isolated proteins during frozen storage. The formation of carbonyl compounds is caused by reactive oxygen species attack to the side chains of protein that may then decompose to carbonyl derivatives. This conversion could ultimately result in a loss of protein structure integrity and increased susceptibility to proteolytic degradation (Stadtman, 1990). In this study, with absence of CP, carbonyl content increased significantly from 1.57 to 2.97 nmol/mg of protein after three weeks of frozen storage, indicating that freezing induced more protein oxidation (Table 2.2). A similar increase in carbonyl content has been reported during frozen storage of turkey breast meat (Chan, Omana, & Betti, 2011). The interaction of the two treatments (pH×CP) indicated that the CP effectively prevented the formation of carbonyl groups in pH-shift extracted protein isolates during frozen storage. Moreover, the acid extracted proteins were more oxidized by frozen storage without CP as revealed by the higher amounts of carbonyl content compared to the alkaline extractions. Stadtman (1990) and Xiong (2000) reported that

protein oxidations usually lead to the generation of carbonyl groups and the loss of sulfhydryl groups. The lower sulfhydryl group content in acid treated samples combined with the higher carbonyl content indicate that the acid prepared proteins are more susceptible to the oxidative deterioration during frozen storage. Xiong (2000) also reported that several amino acids such as histidine, methionine and lysine are more likely to be oxidized. The amount of lysine and histidine was found to be higher for the acid extracted proteins from MSTM compared to the alkaline extracted (Hyrnets et al., 2010a). The different amino acid composition might also have led to the preferential oxidative attack on the acid extracted proteins.

These results about sulfhydryl and carbonyl groups indicated that the oxidative damages on the isolated proteins during frozen storage could be alleviated by adding CP substances. Since extensive protein oxidations are commonly linked to decrease in muscle protein functionality, leading to decreasing solublity, weaker protein gels or less stable emulsions (Xiong, 2000), CP addition may improve the functional properties of the SH-PI.

2.3.3. Effect of pH and CP treatment on structural changes of spent hen proteins

2.3.3.1. Protein surface hydrophobicity

The hydrophobic interactions play a key role in stabilizing protein native structure, thus protein surface hydrophobicity is used to indicate any protein structure alterations during different processing and storage methods. Sompongse et al. (1996) also reported that frozen storage may induce the exposure of hydrophobic amino acids in the myosin molecules and lead to an increase in surface hydrophobicity of actomyosin.

Myofibrillar proteins SHo was significantly different depending on extraction pH values (Table 2.2). The highest value was obtained by proteins extracted at pH 12.0 (Ho = 270) and the lowest was at extracted pH 11.5 (Ho=161). Kristinsson and Hultin (2003) found that increased exposure of hydrophobic clusters is most pronounced for the protein refolded from alkaline condition. For protein extracted at pH 11.5, possibly more exposed hydrophobic pitches were re-buried inside the protein structure during the aggregation process and were less sensitive to the frozen induced exposure. By looking at the interaction pH×CP, addition of CP, in general, decreased MPSHo values with the exception of pH 12.0. The proteins extracted at pH 12.0 with CP showed the highest SHo value (Ho=300). This result implied that the CP combination used in this study may not effectively suppress the exposure of hydrophobic amino acids on proteins extracted in an extreme alkaline environment.

In the case of sarcoplasmic proteins, the SHo values of the all extracted proteins were significantly reduced by CP addition. Since the CP added protein isolates usually have better water retention upon frozen, the water soluble sarcoplasmic proteins structure might be better stabilized from the ice-induced disruptions, leading to reduced SHo values.

Table 2.2 Chemical properties of spent hens' protein extracted from acid-or alkali- aided solubilisation process as a function of 2 levels of cryoprotectants addition (with and without)[a]. Different letters indicate significant differences ($p \leq 0.05$)

		R-SH content (μmol/g protein)	T-SH content (μmol/g protein)	Carbonyl content (nmol/mg protein)	SP-SHo	MP-SHo
Extracted pH values (pH)						
pH 2.0		17±4^{b}	26±6^{c}	2.57±1.13^{a}	75±22^{d}	205±28^{b}
pH 2.5		17±4^{b}	35±6^{a}	2.37±0.92^{a}	86±44^{c}	190±13^{b}
pH 11.5		20±1^{a}	28±1^{b}	1.93±0.49^{b}	103±36^{b}	161±18^{c}
pH 12.0		17±1^{b}	24±2^{c}	2.21±0.71^{a,b}	120±50^{a}	270±37^{a}
Cryoprotectants (CP)						
+ CP		23±1^{a}	31±5^{a}	1.57±0.22^{b}	62±13^{b}	202±27
– CP		19±1^{b}	25±4^{b}	2.97±0.52^{a}	130±26^{a}	211±59
Interaction (pH×CP)						
pH 2.0	+ CP	21±2^{c}	31±1^{b}	1.56±0.18^{c}	56±11^{d}	180±6^{d}
	– CP	13±1^{e}	20±1^{e}	3.57±0.24^{a}	93±5^{c}	229±8^{b}
pH 2.5	+ CP	18±2^{d}	30±1^{c}	1.60±0.25^{c}	46±6^{d}	179±6^{d}
	– CP	16±3^{d}	40±1^{e,c,d}	3.14±0.19^{a}	126±3^{b}	200±4^{c}
pH 11.5	+ CP	20±2^{c,d}	27±2^{b,d}	1.52±0.19^{c}	70±3^{d}	148±8^{e}
	– CP	20±1^{c,d}	29±2^{b,c}	2.34±0.14^{b}	136±2^{b}	174±11^{d}
pH 12.0	+ CP	18±2^{a}	26±3^{a}	1.60±0.17^{c}	74±3^{cd}	300±17^{a}
	– CP	16±2^{b}	22±3^{b}	2.82±0.16^{b}	165±7^{a}	239±12^{b}
Source of Variation		*p-values*				
pH		<0.0001	<0.0001	0.0155	<0.0001	<0.0001
CP		<0.0001	<0.0001	<0.0001	<0.0001	0.0935
pH×CP		<0.0001	0.0001	0.0249	<0.0001	<0.0001

Means within column with no common superscript differ significantly ($p<0.05$).

[a] Results are presented as means ± standard deviations.

SP-SHo: sarcoplasmic protein surface hydrophobicity; MP-SHo: myofibrillar protein surface hydrophobicity

+CP: with cryoprotectants; –CP: without cryoprotectants.

Total n= 24

2.3.3.2. Fourier transforms infrared spectroscopy

FTIR technique is widely used for estimating protein secondary structure. Infrared absorptions around 1200 cm^{-1}, 1500 cm^{-1}, 1700 cm^{-1} and 3100-3300 cm^{-1} indicate amides III, II, I and amide B & A vibrational modes in protein structure, respectively (Goormaghtigh et al., 2006). These characteristic peaks had been observed in both the native and treated spent hen proteins (Fig. 2.1A). Amide I, A and B vibrational modes originate from C=O and N-H stretching, respectively, whereas amide II and III are attributed to C-N stretching coupled with N-H bending (Goormaghtigh et al., 2006).

The changes in the amide III vibrational range (1200-1360 cm^{-1}) of acid and alkaline extracted spent hen proteins without CP (Fig. 2.1B) suggested possible alterations in secondary structure after frozen storage. The bands in the region of 1269-1223 cm^{-1} normally assigned to β-sheet structure of proteins, while the α-helix absorption occurs near 1350-1330 cm^{-1} and 1300-1300 cm^{-1}(Goormaghtigh et al., 2006). FTIR spectra of the extracted proteins treated with CP resembled the peak pattern of the native spent hen proteins with slight decreases in the peak area of both β-sheets and α-helices. This small variation in peak area suggested that the protein structure was partially refolded after extraction. A peak shifting was observed in the sample with CP, from 1239.58 cm^{-1} to lower frequency (~1223.15 cm^{-1}). Based on the cryoprotective theory proposed by Matasumoto (1980), the additional –OH groups derived from CP may be responsible for hydrogen bonds formation with the active groups of the proteins shifting IR frequency to a lower value. In the case of extracted spent hen proteins without CP, significant decreases in the band intensity of the β-sheet structure at 1239.58 cm^{-1} combined with a

small increased peak area of α-helix at 1341.14 cm^{-1} were observed. These changes in the FTIR spectra indicated that the secondary structure of pH-shifting extracted spent hen proteins were greatly affected by frozen damage with the absence of CP. A decrease in β –sheets structure occurred to a greater extent in acid pH than in alkaline conditions compared to the untreated proteins. Yongsawatdigul and Hemung (2010) reported a similar β-sheet structure reduction in the threadfin bream sarcoplasmic proteins after pH-shifting treatments. They concluded that the secondary structure of isolated proteins might be less stable at acid pH than in alkaline conditions. The results of this study suggested that frozen storage increased the structural denaturation of the isolated spent hen proteins.

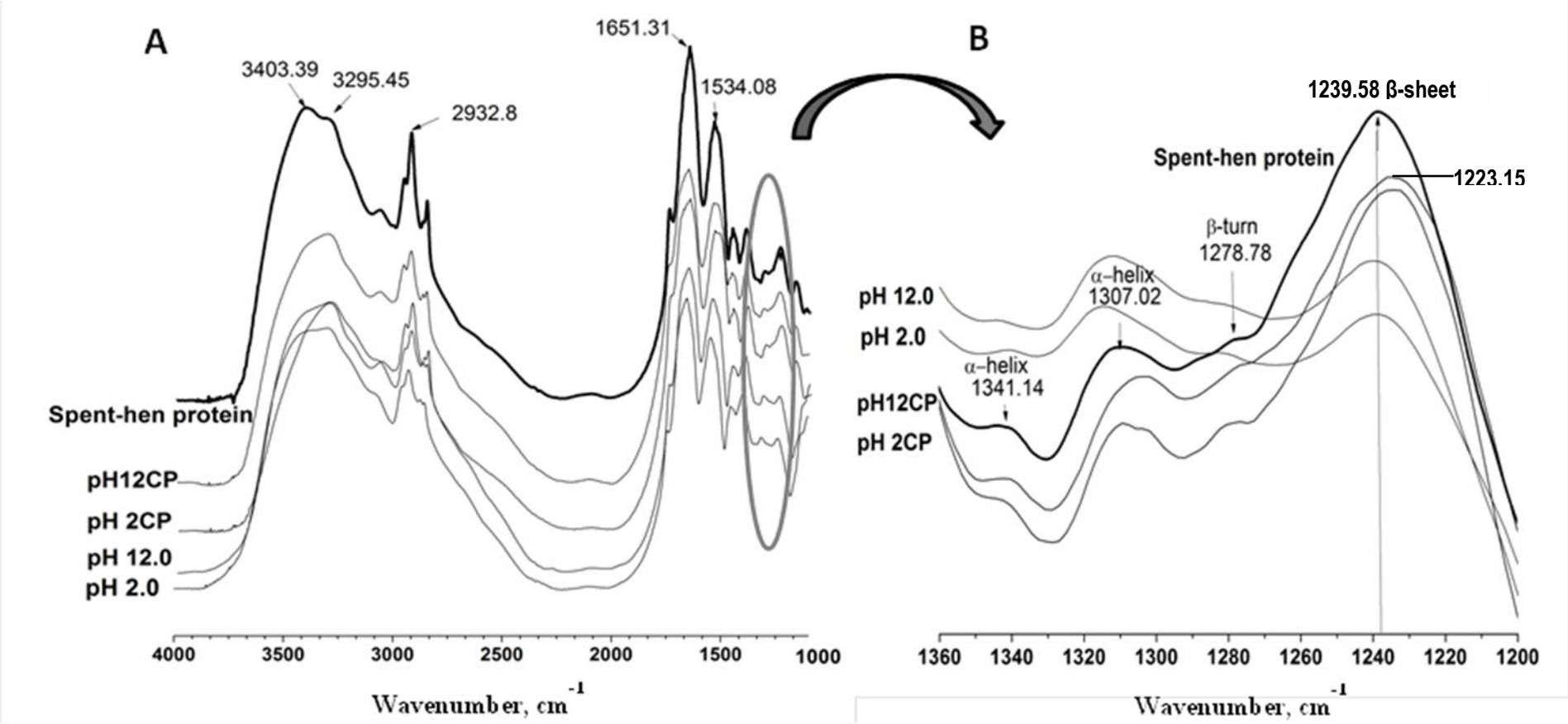

Figure 2.1. (A) FTIR full spectria of acid (pH 2.0) and alkaline (pH12.0) extracted SH-PI with and without cryoprotectants (CP) addition. (B) FTIR detailed spectra of acid (pH 2.0) and alkaline (pH12.0) extracted SH-PI with and without cryoprotectants addition.

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

SDS-PAGE profiles of proteins extracted at different pH values with/without CP addition were reported in Figure 2.2. The protein bands corresponding to myosin heavy chains (MHC), actin, and tropomyosin are located at molecular weight of 250, 45 and 37 kDa, respectively. Regardless of the CP addition, the electrophoretic profile of alkaline treated samples at pH 11.5 revealed a faint band at 250 kDa indicating the presence of MHC. Four comparative lower bands were observed between 90 kDa and 150 kDa, which could attribute to the products of myosin degradation. On the other hand, proteins prepared by acid extraction resulted in less bands compared to the alkaline treatments. The MHC fraction at 250 kDa almost completely disappeared, as well as some bands between molecular weights 40 and 150 kDa. Instead of showing more concentrated MHC and actin bands in the SDS-PAGE of pH-shifting extracted MSTM proteins (Hyrnets et al., 2010a), the extracted spent hen proteins after frozen storage revealed a loss of MHC and actin bands in their electrophoretic profiles. The absence of high molecular weight bands could be the result of proteolytic degradations, frozen-induced aggregation and/or polymerized MHC and actin by cross-linking during frozen storage of muscle proteins (Jiang, Hwang, & Chen, 1988). Cathepsins B, an acid activated lysosomal proteinase, were found to contribute more proteolytic hydrolysis during acid-aided protein extraction processes (Kim, Park, & Choi, 2003). The acid treated spent hen proteins were found to be more oxidized after frozen storage, which may lead to more proteolytic protein degradation. Oxidative modification can either induce generalized aggregation of proteins to higher molecular weight forms or a dispersed pattern of lower molecular weight protein fragmentations (Davies, 1987). In the present study, it appeared to be a

simultaneous occurrence of protein aggregation and autolysis in pH-driven extracted spent hen proteins for at least the first month of the frozen storage, especially for acid extracted proteins. A similar pattern of proteolytic degradation was observed in the extracted proteins with CP suggesting that the endogenous proteolytic enzymes might not be inhibited by cryoprotective substances.

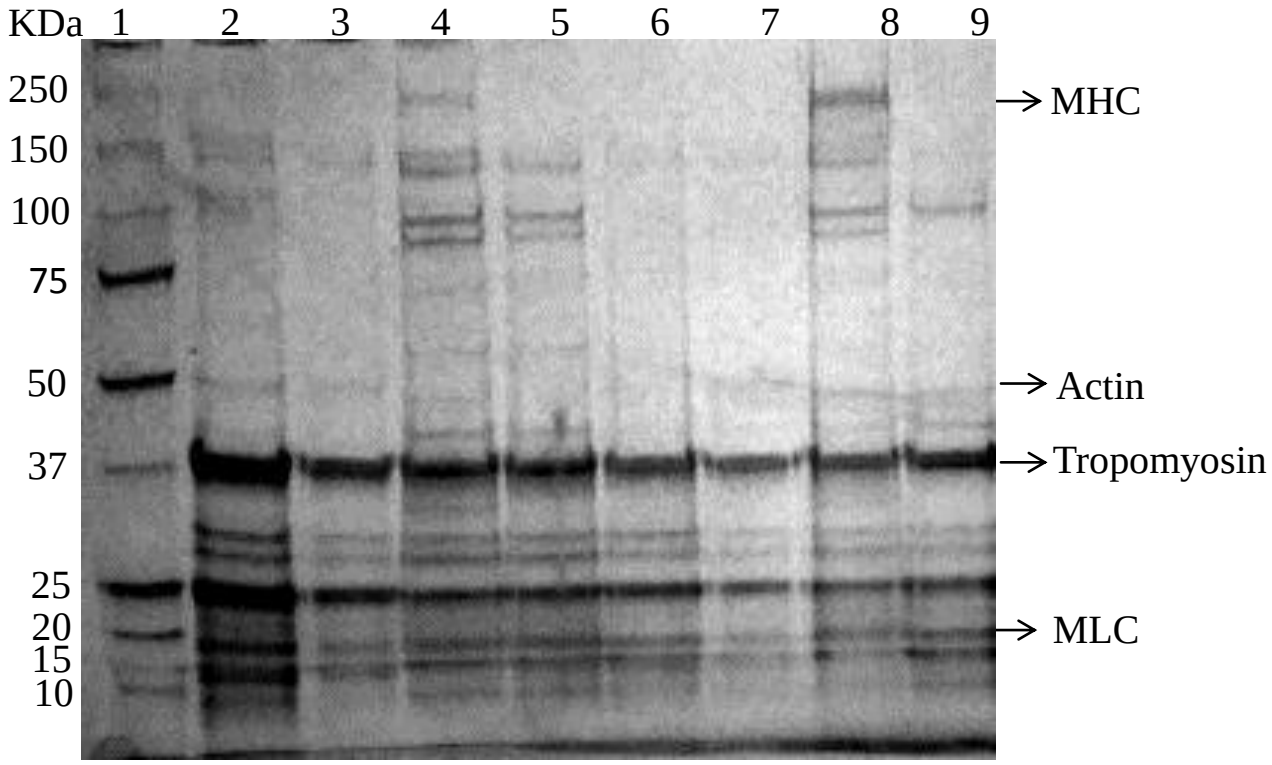

Figure 2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of different samples from acid and alkaline extraction processes with and without cryoprotectants. Lane1 is the moelecular weight standard. Lanes 2-5 refer to the proteins extracted at pH values of 2.0; 2.5; 11.5 and 12.0 without CP, respectively. Lanes 6-9 refer to the proteins extracted at pH values of 2.0; 2.5; 11.5 and 12.0 with CP, respectively. CP is stand for cryoprotectants

2.3.5. Surface morphology of the protein isolates

Scanning electron microscopy is commonly used to characterize the surface morphology for different biomaterials (Merrett et al, 2002). The SEM observations of acid-extracted, alkaline-extracted, acid-treated with CP and alkaline-treated with CP SH-

PI after 3 weeks of frozen storage were conducted at 1000× magnifications. The resulting micrographs were presented in Figure 2.3a, 2.3b, 2.3c and 2.3d, respectively. The acid and alkaline extracted proteins (Figure 2.3a & 2.3b) showed "molten globular"-like structures, which is the term that Kristinsson and Hultin (2003) used to describe the configuration of the myosin head after the pH-shifting extraction. No separate rod-like structure was observed, suggesting that the myosin rod parts might be aggregated along with the head portions during the refolding process and frozen storage. The particle size of the acid extracted protein isolates (Fig. 2.3a) was relatively smaller than the alkaline extracted ones (Fig. 2.3b) possibly due to the higher proteolytic activities found in acid treated proteins. Both protein isolates had a rough surface and sharp edges, which could be the result of frozen storage and freeze drying process. On the other hand, proteins treated with CP addition (Fig. 2.3c & 2.3d) showed a much larger particle size with a smoother surface and rounder edges. Large amounts of tiny particles were found in both graphs and yet had not been observed in the graphs without CP. This difference suggested that these tiny particles could be the CP substances blended with protein isolates after the extractions. The way that CP particles scattered on the surface and/or embedded within the protein structures suggested that a protective shield was formed to stabilize the structure of protein thus reducing the aggregation (i.e. S-S bridge formation) of individual protein molecules during frozen storage. Results reported in Table 2.2 about SH groups support this hypothesis.

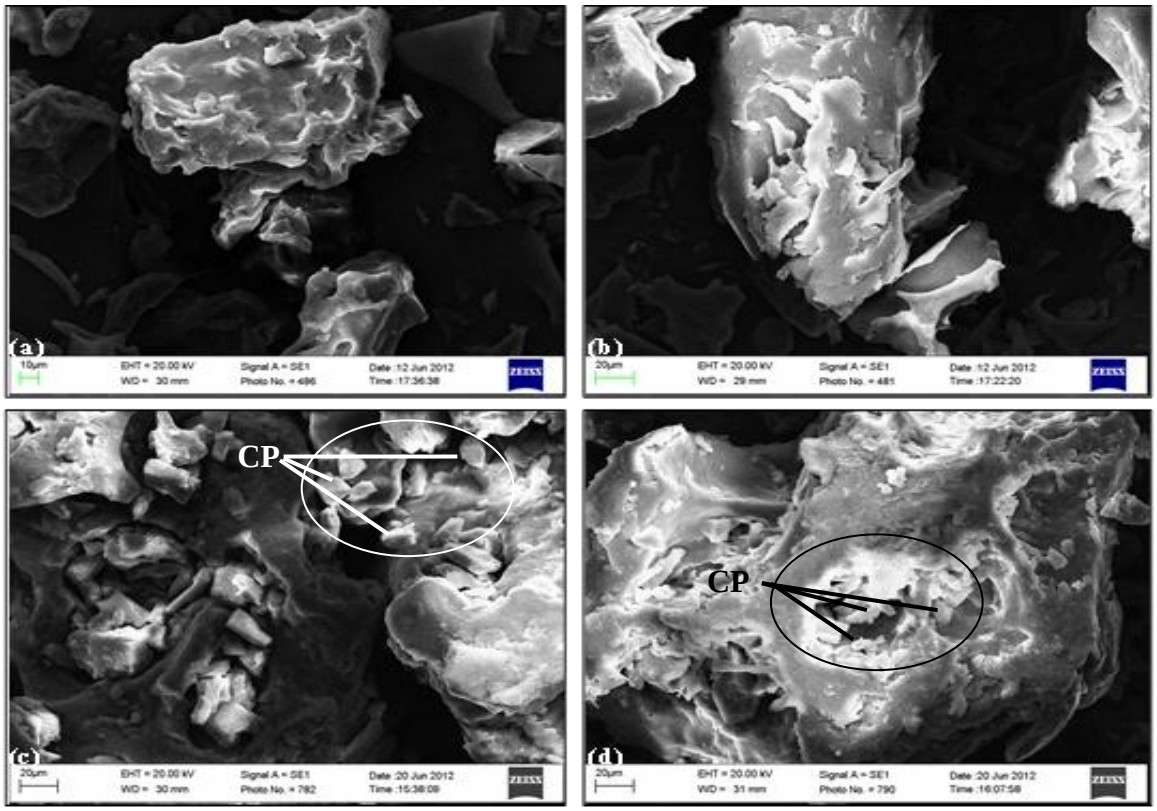

Figure 2.3. SEM micrographs of (a) SH-PI extracted at pH 2.0, (b) SH-PI extracted at pH 12.0, (c) SH-PI extracted at pH 2.0 with cryoprotectants, (d) SH-PI extracted at pH 12.0 with cryoprotectants. All the images were taking under 1000× magnifications.

2.3.6. Effect of pH and CP treatment on the functionalities of spent hen proteins

2.3.6.1. Solubility profile of the isolated proteins as a function of pH

The conformations of isolated muscle proteins are different from their native structures after the refolding process at the isoelectric point (Kristinsson & Hultin, 2003) and during frozen storage, which could influence their re-solubilisation ability. Therefore, solubility behaviour of modified spent hen muscle proteins with and without CP addition after freezing will be different from native spent hen proteins. The solubility curves of acid (pH 2.0) and alkaline (pH 12.0) extracted spent hen proteins with/without CP are presented in Figure 2.4 and Figure 2.5, respectively. The solubility curve of fresh spent

hen meat was used as a control for comparing the re-solubilisation level of the isolated proteins with different treatments. U-shaped curves were observed from both acid and alkaline treated proteins with and without CP. At the crests, the solubility values were increased almost two-fold for acid treated samples and three-fold for alkaline samples compared to the native proteins. This increase is much expected since the isolated proteins are more concentrated than the native proteins in meat and the electrostatic repulsion reached a maximum at extreme acid and alkaline pH points.

The solubility of both acid and alkaline extracted proteins without CP sharply dropped at pH 3.5 and remained lower than the control up to pH 11.0. The low solubility in this range indicated that the isolated proteins are more prone to aggregation than controls. According to the results presented in Table 2.2, isolated proteins without CP tended to form more S-S bonds and carbonyl groups, which could cause more protein aggregation and reduced solubility. Our results were also in agreement with the study of Thawornchinsombut and Park (2006) in which the authors found that frozen storage led to a decrease in solubility of the isolated fish proteins. For acid treated proteins, incorporation of CP slightly improved their solubility at the middle pH range (4.0-10.5) but did not exceed the control (Fig. 2.4). In the case of alkaline extractions, the CP added protein isolates obtained higher solubility than the control in the middle pH range, especially at pH 6.5 and 7.5 (Fig. 2.5). The alkaline-aided protein extraction seems to cause less denaturation than acid-aided one, which also concurs with the findings of Kristinsson et al. (2005). In addition, the acid extracted proteins has been found to be more oxidized than the alkaline extracted proteins during frozen storage, which could contribute to their lower solubility along the pH range (4.0-10.5). CP could prevent the

extracted protein structure from progressively denaturation and aggregation. Thus alkaline treated proteins showed higher solubility as a function of pH compared to the acid treated proteins and the control. The solubility curves from isolated proteins provided a better understanding on the conformational stability of the refolded proteins as affected by extracting pH and freezing. Moreover, the other functional properties of isolated proteins might be improved by knowing the re-solubilisation behaviour of protein isolates at different pH conditions.

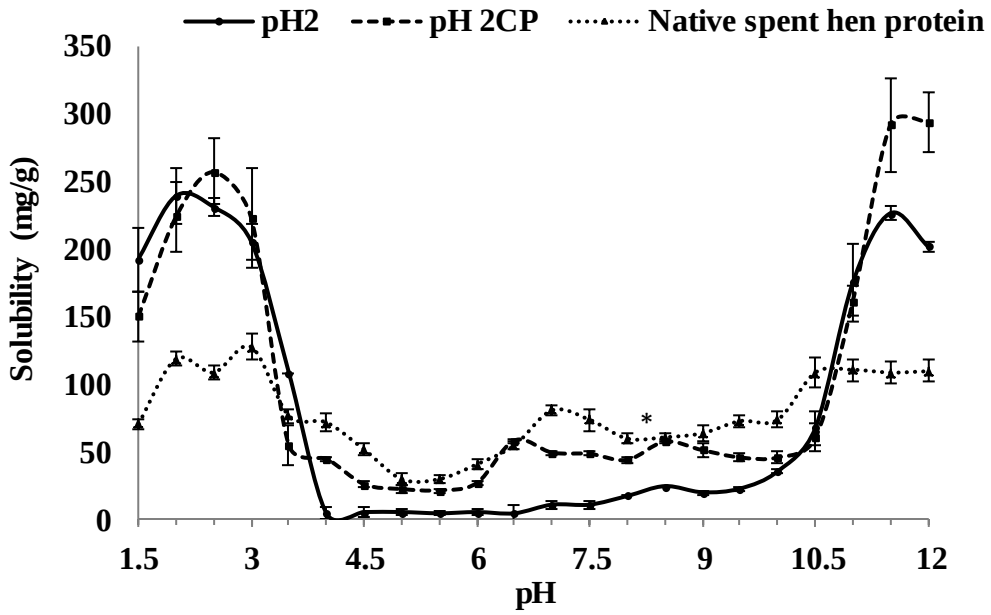

Figure 2.4. Solubility (mg/g) profile of native protein from spent hen meat and SH-PI by acid-aided (pH 2.0) extraction with and without cryoprotectants at pH values from 1.5 to 12.0. Results are presented as mean (n=4) ± standard deviation. *Cryoprotectants

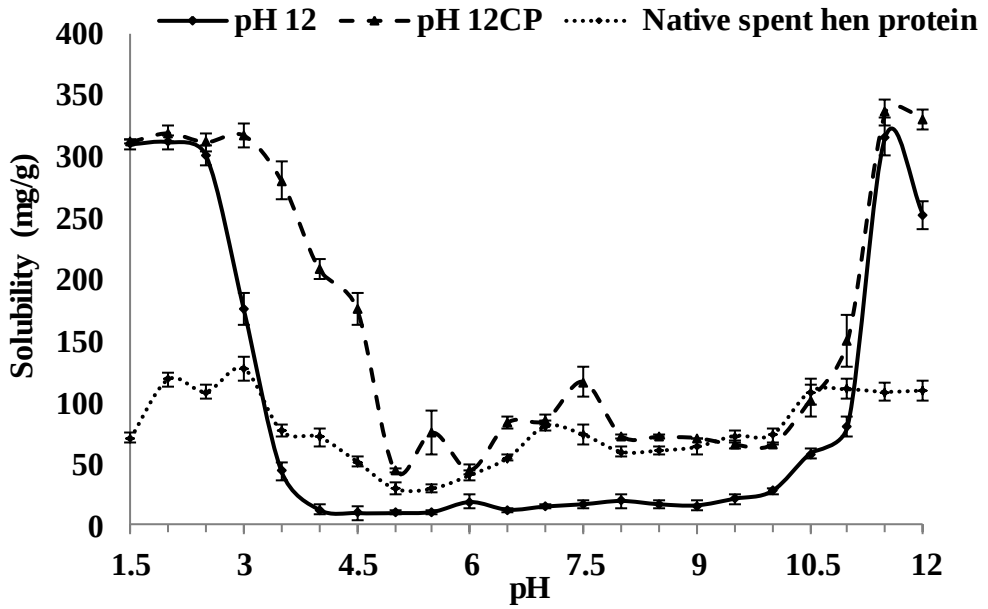

Figure 2.5. Solubility (mg/g) profile of native protein from spent hen meat and SH-PI by alkaline-aided (pH 12.0) extraction with and without cryoprotectants at pH values from 1.5 to 12.0. Results are presented as mean (n=4) ± standard deviation. *Cryoprotectants

3.6.1 Gelling properties of spent hen proteins

The dynamic rheological technique has been extensively used to determine the heat-induced gelation properties of myofibrillar proteins (Hamann, 1988). Muscle protein gel refers to a continuous network structure that involves various protein-protein interactions and that is capable of water entrapment. During heating, three steps are generally accepted to describe the protein gel formation: the initial denaturation of native proteins followed by the unfolding of the protein structure, then finally the aggregation of unfolded proteins to create new cross-links among protein molecules. Rheograms (heating) obtained for the proteins extracted at four pH values (2.0; 2.5; 11.5 and 12.0)

with and without CP are presented in Figure 6, respectively. The storage (G') and loss modulus (G'') measured the elastic and viscous component of the gel, respectively. Tan δ represents the energy lost as a result of viscous flow compared with the energy stored from elastic deformation in one deformation cycle (Sun & Arntfield, 2010). Usually a change in tan δ value indicates the start of gel formation and this change is often referred to as transition point of muscle proteins.

It is generally accepted that myofibrillar proteins are denatured during frozen storage of meat. Freeze-induced protein denaturation followed by aggregation during storage contributes to the considerable decrease in gel quality after thermal processing (Thawornchinsombut & Park, 2006). In this study, the SH-PI without CP after frozen storage (Fig. 2.6A) revealed continuing increases in G' and G" values at all four pH values in both heating and cooling processes. However, the tan δ values did not show a clear transition point, which is usually considered to be the indication of protein denaturation. Without a transition point, the general heat-induced gel formation principle could not be applied since denaturation is a pre-requisite for the formation of a viscoelastic protein gel. One explanation could be that without the cryoprotective effect, pH-driven extracted protein isolates were aggregated during frozen storage. Freeze denaturation induces S-S bridge formation in proteins. Generally the number of S-S in protein is related to the ability of proteins to survive heat denaturation (Vogt, Woel & Argos, 1997). In the present study, samples without CP was found to obtain lower RS/TS ratios compared to the samples with CP, which suggested that more S-S crosslinks were formed during the frozen storage. The extensive crosslinking probably resulted in a more aggregated structure, which was more resistant to heat denaturation. As a consequence,

the isolated proteins could not go through the proper thermal denaturation/aggregation steps, resulting in failure of gel formation. The huge increasing of G' values could represent a rubber-like elasticity due to the higher protein content compared to the samples with CP. Feng & Hultin (2001) reported that the extensive protein-protein interactions resulted in harder gel network but the protein-water binding was reduced, leading to local coagulants of aggregated proteins. For all the frozen SH-PI, a dry fibre-like film with no water entrapment was observed after cooling. This non-gel like appearance suggested that no fine protein gel network was formed by the frozen denatured spent hen proteins. Although the microstructure of the protein gel with and without CP was not evaluated in this study, it could be hypothesized that ice crystal growth during frozen storage would lead to major structural damages on the spent hen muscle proteins, which resulted in significant loss of gelling ability. Kijowski and Richardson (1996) observed a very coarse structure with no obvious fine network system from frozen chicken meat without CP under the scanning electron microscope.

In the present study, the CP were added in order to prevent the denaturation of SH-PI during frozen storage. During the heating phase (Fig. 2.6B), the G' showed a typical initial increase between 36 to 40 °C followed by a rapid increase to about 45°C then slowly increased up to 80°C. The initial increase at 36-40°C indicated the onset of gelation occurred where the myosin was unfolded (Sebranek, 2009). The rapid increase and then slowly increase in G' up to 80°C represented a more elastic gel network which was contributed by ordered aggregations among the unfolded protein molecules. This prolonged increasing range has not been shown in the rheograms of pH shift extracted chicken and turkey proteins in previous studies (Hrynets et al., 2010b; Omana et al.,

2010). The spent hen myofibrillar protein has been reported to contribute more rigidity and firmness in the mouth feeling (Yang & Froning, 1992). Moreover, Nowsad et al. (2000a) found that the spent hen protein surimi had higher gel strength and breaking strength compared to the broiler chicken surimi. The increased toughness of the spent hen protein might prolong the heat-induced gel formation process, as more energy and time was needed to break down the protein structure and then to reform an ordered protein-protein interactions. In addition, even the proteins were cryoprotected, freezing still could cause certain level of protein denaturation and aggregation, thus the protein structure become less prone unfolding when heated. Decreased G' values as further increased temperature above 60°C also did not observed except pH 2.0, suggesting the gel made from spent hen proteins was more stable at higher temperature compare the pH-shifting extracted chicken and turkey proteins (Hrynets et al., 2010b; Omana et al., 2010).

The highest G' value was obtained by proteins extracted at pH 12.0 at 179 kPa and then followed by pH 11.5 at 160 kPa, while the protein gels from acid extractions shown the lower G' values. The same order was observed in the increasing pattern of the loss modulus (G''). These results indicated that the alkaline treated proteins formed a more viscoelastic network. The interactions involved in the gel network formation are primarily hydrogen bonds, hydrophobic interactions, and disulphide bonds (Semejima et al. 1982). Since the alkaline extracted proteins had higher protein solubility at neutral pH range (6.0-7.5), more proteins are available for the gelation process. The higher protein surface hydrophobicity and reactive sulfhydryl groups from the alkaline treated proteins also could contribute more protein-protein interactions thus producing a stronger gel. In addition, the activation of cathepsin enzymes during the acid extraction also could be

responsible for the gel softening and the unstable gel network when temperature increased above 65ºC. Choi and Park (2002) found that a similar gel softening caused by cathepsin enzymes in acid extracted Pacific whiting fish protein isolates. Tan δ values indicated one major transition point at 50°C, 52.6°C and 57.2°C for acid extracted samples, extracted at pH 12.0 and at pH 11.5, respectively. For spent hen proteins, the transition point was shifted to a higher temperature compared to the protein isolated from other poultry meat, which reached its denaturation point at 47.3°C (Hrynets et al., 2010b; Omana et al., 2010). This migration corresponded to the prolongation of the gelation process found above, suggesting that the extracted spent hen proteins maintain their natural toughness and require higher temperature for structure unfolding. For the cooling profile, all samples showed an increase in G' and G'', which indicated a stronger gel formation with decreases in temperature (graph did not show). This increase is attributed to the formation of hydrogen bonds during cooling (Hamann, 1988).

Despite these small migrations on the temperature profile during the protein gelation process, the rheograms of the SH-PI with CP after frozen storage generally agreed with the viscoelastic behaviour of the fresh pH-shift extracted poultry proteins (Hrynets et al., 2010b). It suggested that the incorporation of CP could prevent the freeze damage toward the gel forming ability of the pH-shifted isolated poultry proteins, since without CP the SH-PI failed to form cohesive gel networks.

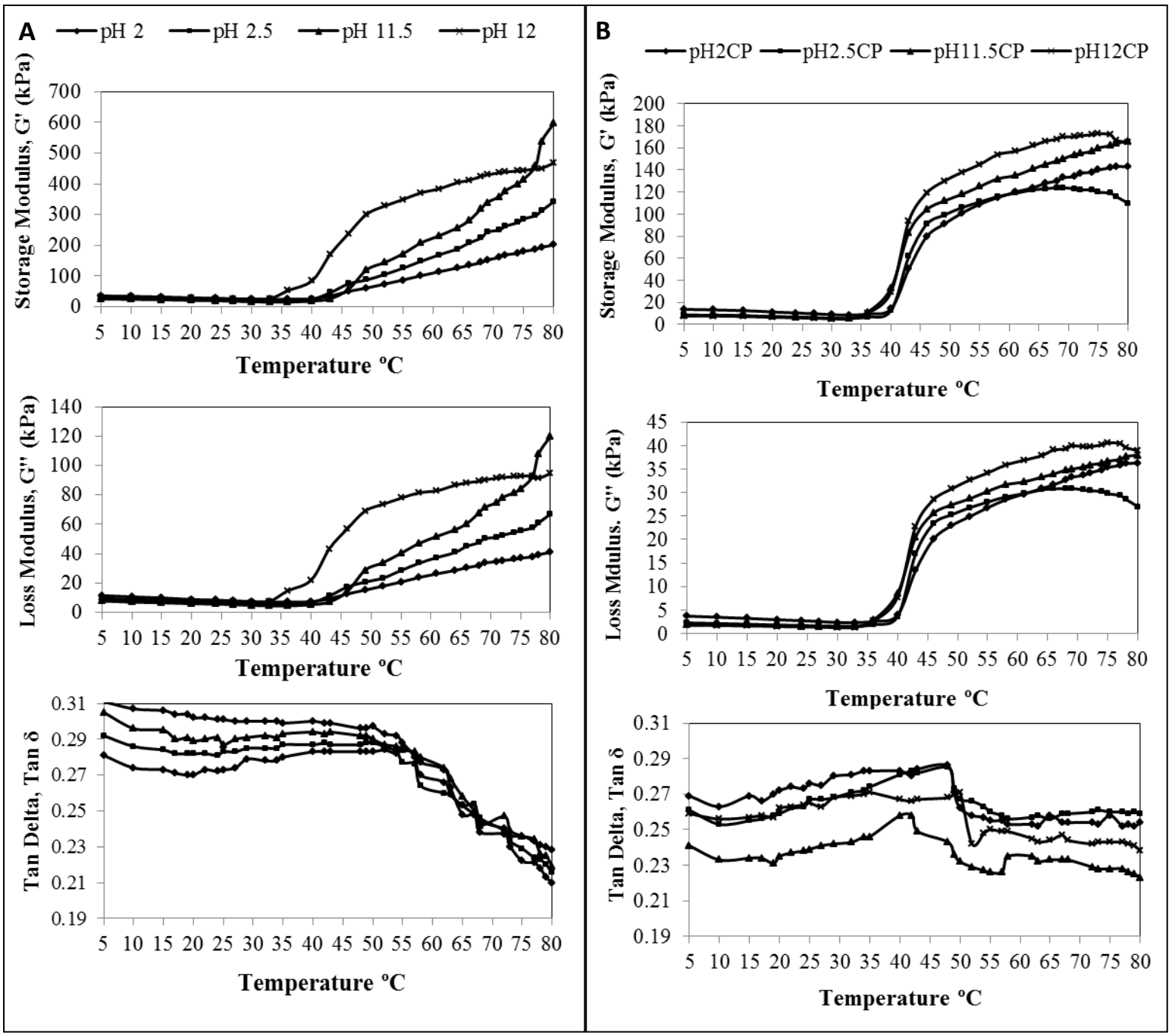

Figure 2.6. Changes in dynamic viscoelastic behavir of proteins recovered from spent hen meat at different extraction pH with and without cryoprotectants. The samples were prepared with 2.5% of NaCl addition. The rheograms show storage modulus (G'), loss modulus (G''), and tan delta (tan δ) development during heating from 4 to 80 ºC at 2ºC/min. *Cryoprotectants

2.4. CONCLUSION

This study demonstrated that pH-shifting method could be an efficient way to recover spent hen proteins. The data indicated that acid processing (pH 2.0 and 2.5) led to higher protein yields compared to the alkaline extraction. The FTIR spectra indicated that isolated proteins underwent significant structural and conformational changes after frozen storage, especially in the acid extracted samples. The presence of CP reduced protein oxidation, as revealed by the combination of higher reactive sulfhydryl groups and a significantly lower formation of carbonyl groups, which was found to be more effective on the acid extracted proteins. The SEM micrographs suggested that the cryoprotective substances were embedded around the protein structure to form a protective shield that reduced the formation of S-S bridges causing less aggregated protein structures. Thermal gelation and solubility of frozen SH-PI were strongly related to the presence of CP. With cryoprotection, a clear protein denaturation point was observed in all extracted samples; alkaline extracted protein isolates formed the highest viscoelastic gel and had the highest protein solubility around neutral pH (6.5-7.5). Without CP, the SH-PI were not able to form a fine gel network with effective water entrapment.

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CHAPTER 3. EFFECTS OF ACID- AND ALKAINE-AIDED EXTRACTIONS AND FREEZING ON THE ADHESIVE PROPERTIES OF RECOVERED SPENT HEN PROTEIN ISOLATES (SH-PI)

3.1. INTRODUCTION

Wood Adhesives were mainly prepared from casein, animal blood and gelatin as well as from some plant proteins before the introduction of synthetic adhesives (Lambuth, 1994). Petrochemical-based adhesives such as phenol-formaldehyde and urea-formaldehyde replaced protein adhesives had rapidly been adopted by the wood market due to their performance and affordable cost. They are now used heavily in the production of wood composite panels. However, the volatile formaldehyde emission in the preparation, curing process or during the application is known to be harmful to the environment and suspected to be carcinogenic according to the World Health Organization, which has been a subject of public concerns (CARB, 2007; Huang and Sun, 2000). Due to the increased awareness of being environmental responsible and realizing the finite nature of our petrochemical resources, developing natural-based formaldehyde-free adhesive has re-gained the interest of the researchers as well as the producers. The traditional protein-based adhesives suffered from relatively low bonding strength and poor water resistance due to their original native structure (Park, 1999). Based on the previous studies, protein denaturation using different denaturation agents such as alkali, urea, guanidine hydrochloride, and sodium dodecyl sulfate seems to be a prerequisite to achieve superior adhesive properties (Huang and Sun, 2000; Orliac et al., 2003). It has been established that partially unfolded protein molecules with a certain amount of

secondary structure is desirable for enhancing protein adhesion properties (Huang and Sun, 2000; Sun and Bian, 1999). During the denaturation (unfolding) process, internal hydrogen bonds are broken and more functional groups could be exposed and available for chemical bonding, thus to achieve desired properties.

During the past decade, researches on protein-based adhesives have been largely directed toward soy proteins (Huang and Sun, 2000; Sun and Bian, 1999; Nordqvist et al., 2010; Zhong et al., 2002). Spent hens, an underutilized meat by-product from egg industry, could be considered as another alternative protein source for adhesive preparation, since the general toughness of spent hens' meat and minimal edible meat recovery had made them less suitable for food applications (Rouselle et al., 1984). In addition, our previous study indicated that spent hen protein can be used for preparing adhesive (Wang and Wu, 2012).

Proteins are complex macromolecules and their native structure can be affected by processing conditions (Howell, 1996). Spent hen proteins were extracted using the pH-shifting method, which utilizes the pH-dependent solubility properties of muscle proteins to separate them from other components in the muscle (Mohan et al., 2007). The spent hen muscle protein structure was partially unfolded/refolded during either acid- or alkaline-aided extraction, leading to random aggregation of the protein peptide chains and the protein molecules are loosely associated with one another (Mohan et al., 2007; Kristinsson et al., 2005). After the unfolding/refolding process, more reactive groups such as carboxylic acid, sulfhydryl and hydrophobic groups were found to be exposed on the surface of protein structure (Mohan et al., 2007; Kristinsson and Hultin, 2004). On the other hand, the adhesive strength and water resistance of protein polymers are enhanced

by crosslinking, for which exposure of reactive groups is considered as a prerequisite. The extracted muscle protein isolate is a wet paste of concentrated myofibrillar and sarcoplasmic proteins with average of 80% moisture content. The unique fibrous structure of the myofibrillar proteins might contribute to the formation of entanglements (physically entangled chains) or head-to-tail polymerization thus to obtain a higher adhesive strength. Freezing is considered as the most efficient and economical way of preserving meat products as well as muscle protein isolates. Thus frozen SH-PI were used in this study inside of obtaining dry protein powder from energy intensive drying process. Moreover, protein denaturation also occurred during the freezing process due to the frozen-induced oxidative reactions (Estévez, 2011). In the present study, CP has been added to one group of samples, which were used as controls since addition of freezing stabilizers could inhibit frozen induced protein denaturation (Park, 1994).

To our knowledge, the effect of pH-shifting extractions and frozen induced oxidation on the adhesive properties of the isolated proteins has not yet been studied. Therefore, the objectives of this research were to [1] prepare an adhesive from frozen stored SH-PI which extracted by either acid or alkaline solubilisations; [2] compare the dry, wet and soaked bonding strength of the SH-PI based adhesives as a function of extracting pH and freezing effect; [3] investigate the thermal stability of the SH-PI before and after chemical modification; [4] analyzed the adhesive wood penetration and secondary structural alterations of the SH-PI based adhesives after curing process (heat-pressure).

3.2. MATERIALS AND METHODS

3.2.1. Materials

Spent hen carcasses were obtained after removal of the cut-up parts (breast, legs and wings) from Lilydale Inc. (Edmonton, AB, Canada). The remaining meat on the frame was manually deboned and homogenized using a Kitchen-Aid Food processor (Model KFP 7500B, KitchenAid, St. Joseph, MI, USA) for 5 min. Spent hens mince (250 g) was filled into polyethylene bags and kept at - 20ºC until use. Before extraction, samples were thawed overnight at 4ºC. All the reagents and chemicals used in the study were of analytical grade.

3.2.2. Protein extraction

Spent hen proteins were extracted either at pH 2.0 (acid-aided extraction) or at pH 12.0 (alkaline-aided extraction) based on the solubility profile of the raw spent hens as we previously established (Chapter 2). In brief, spent hens mince (200 g) were homogenized with cold distilled water/ice mixture at 1:5 ratio by a 900-Watt Food Processor (Wolfgang Puck WPMFP15, W.P. Appliances Inc., Hollywood, FL, USA) for 15 min. After homogenization, the meat slurry was set at 4ºC for 30 min. Then the pH of the homogenates was adjusted to pH 2.0 using 1M HCl or 12.0 using 1M NaOH to reach the required solubilisation pH points. After pH adjustment, samples were centrifuged at 15300×g for 20 min at 4ºC. Three layers: neutral lipids, soluble proteins and insoluble proteins (collagen) plus membrane lipids were obtained in the order from top to bottom. The middle layer of soluble proteins was recovered at pH 5.2, and then the protein precipitate was collected by centrifugation (15300×g, 20 min, 4ºC). The precipitate pH was readjusted to 6.2, which is the average pH of the raw hen meat.

3.2.3. Protein modifications

After the extraction, protein moisture content was adjusted to 82% and frozen stored at -20 ºC till use. Two levels of cryoprotectants (CP) were added (with and without) to the extracted SH-PI. The CP is a blend of 5% sorbitol, 4% sucrose, 0.4% sodium bicarbonate, 0.3% tripolyphosphate and 0.03% sodium nitrite (Omana et al., 2010). The SH-PI was thawed overnight in cooler at 4ºC before use. The thawed SH-PI was mixed with 3% sodium dodecyl sulfate (SDS) at a concentration of 5% (w/v) according to the method described in (Wang and Wu, 2012) with slightly modifications. The mixture was stirred at room temperature for 4 hr. The homogenized SH-PI slurry was used as protein adhesives for the adhesive strengths analysis.

3.2.4. Wood specimen preparation

The method described by Wang and Wu (2012) was used to prepare the wood specimens with slight modifications. Hard wood veneers (Brich) with a dimension of 0.7 mm × 20 mm × 120 mm (thickness, width, length, respectively) were used. The wood pieces were conditioned in a controlled chamber (ETS 5518, Glenside, PA, USA) with 50% humidity at 25ºC for a week prior to use. The modified SH-PI adhesive slurry (protein content: 0.05 ± 0.02 g mL^{-1}) was brushed evenly onto one end of each piece of wood veneer over an area of 5 mm × 20 mm. The amount of slurry on each piece was controlled, and variation was minimized by use of consistent brushing procedure. The two piece of SH-IP adhesive brushed wood veneer were allowed to rest at room temperature for 5 min. Then they were assembled by hand and hot pressed according the conditions determined by Wang and Wu (14) at 110 ºC, 3.5 MPa for 240 s (ABES II, Adhesive Evaluation Systems, Corvallis, OR, USA)

3.2.5. Adhesive strengths

The shear strength of adhesive is considered as the total force that required to apply for the separation of the two bonded surfaces and was measured using an Automated Bonding Evaluation System (ABES II, Adhesive Evaluation Systems, Corvallis, OR, USA). The dry adhesive strength was measured immediately after the hot pressing by pulling the assembled wood veneer specimen (ABES II, Adhesive Evaluation Systems, Corvallis, OR, USA). The wet and soaked strength was measured to determine the water resistance of the SH-PI adhesive. The hot press bonded wood specimens were soaked in tap water at room temperature for 48 h and the wet strength was tested immediately after soaking. To measure the soaked strength, the soaked samples were kept in a controlled chamber at 23ºC with 50% humidity for seven days prior to measurement.

3.2.6. Differential scanning calorimetry (DSC)

The thermal properties of the acid- and alkaline-aided extracted SH-PI with and without SDS modification was determined by differential scanning calorimetry (DSC) measurements. The DSC was performed under a continuous nitrogen purge on a heat flow differential scanning calorimeter (Perkin-Elmer, Norwalk, CT, USA). Samples were freeze dried and remoistured to 80% moisture content prior to analysis. The instrument heat flow and temperature were calibrated using pure indium. A large DSC pan was used and about 6 mg of sample was held at 25 °C for 1 min and then scanned from 25–100 °C at a heating rate of 1 °C/min.

3.2.7. Viscosity of the SH-PI based adhesives

The viscosity of the adhesive was determined using a controlled-stress Physica MCR 301 rheometer (Anton Paar GmbH, Virginia, USA) fitted with 2.5 cm parallel plate geometry. A gap of 1 mm between plates was used and the viscosity was measured at room temperature under a constant shear rate of 100/s.

3.2.8. Morphology of the bonding surface

The pulled bonding surfaces of wood specimens were observed under Hitachi S-2500 scanning electron mircroscopy (SEM, Nissei Sangyo America Ltd, CA, USA). A thin latey of gold was coated using a Gold Sputter Unit (Denton Vacuum, Moorestown, NJ, USA) on the pulled specimens before observation.

3.2.9. Effects of heat-pressure (curing) process on modified SH-PI structure

The secondary structure alterations of the acidic and alkaline extracted SH-PI adhesive before and after hot press were estimated by a Nicolet Magna 730 Fourier transforms infrared spectroscopy (FTIR) (Madison, WI, USA). The uncured and cured SH-PI adhesives were freeze dried and milled with potassium bromide (KBr) and compressed into a thin sheet before analysis. The spectrum was conducted by accumulating 1 scans at a resolution of 8 cm^{-1}.

3.2.10. Statistical analysis

The data for dry, wet and soaked adhesive strength were analyzed using the general linear models (GLM) procedure of SAS program (SAS version 9.0, SAS Institute, Cary, NC, USA, 2006). Each experiment was replicated at least 3 times. Differences between

group means were determined using HSD Tukey's adjustment and significance of difference was established at a 95% confidence interval ($P < 0.05$).

3.3. RESULTS AND DISCUSSION

3.3.1. Effect of protein extraction and freezing storage on the adhesive properties

Sodium dodecyl sulfate (SDS), a widely used anionic surfactant, was reported to improve adhesive bonding strength and water resistance (Sun and Bian, 1999). The protein molecules were unfolded by SDS molecules through interactions between the sulfate group and positively charged amino acid side-chains, as well as between the alkyl chain and hydrophobic side-chains (Yonath et al., 1977). The bonding performance was evaluated for the SH-PI adhesives in term of dry, wet and soaked bonding strengths (Fig.3.1). Adhesives prepared from the acid-aided extract (pH2.0+CP) SH-PI showed significant higher adhesive strength than that of the alkaline-aided extracted samples (pH12.0+CP). A study was conducted by Kristinsson and Hultin (2003) to elucidate the different unfolding/refolding mechanisms of muscle proteins at acid and alkaline pHs. On acid unfolding, the myosin rod was fully dissociated, while this dissociation was not observed at alkaline pH. Then on refolding process, the myosin globular head is less packed from acid solubilisation, resulting in less stable structure with more exposing hydrophobic groups. Therefore, with the same level of SDS modification, the acid extracted protein structure could be more unfolded than the alkaline extracted samples, which might subsequently lead to more entanglement among the unfolded protein molecules during curing (heat-pressure) process and reveal on a higher dry adhesive strength.

As for the freezing effect (without CP), the drying strengths were improved from both acid (pH2.0) and alkaline (pH12.0) extracted samples (Fig.3.1). With the combination of freezing denaturation, the acid extracted SH-PI adhesive obtained the highest dry strength value at 8.52 MPa. Studies have proved that the frozen induced oxidative reactions are responsible for protein denaturation, which could lead to a loss of protein structural integrity and functionality via protein polymerization (disulphate crosslinking) and fragmentation (proteolytice degradation) (Stadtman, 1990; Davies, 1987). Although these frozen induced protein structural alterations are unfavorable during food product processing, it seems to be desirable for the protein-based adhesive since oxidative reaction has been found to be useful on enhancing the bonding strength at curing process (Huang and Sun, 2000; Orliac et al., 2003; Zhong et al., 2002). In the present study, the data suggested that freezing storage has a positive effect on the adhesive strength of SDS modified SH-PI adhesive, while this effect seemed to be more effective combined with acid-aided extraction.

Both wet and soaked adhesive strengths are important properties that determine adhesive bond durability. During soaking, water molecules can penetrate into the glued area, interact with protein molecules, and weaken the interface between the proteins and the wood. In the present study, the soaked strength almost rebounded back to the level of dry strength, but wet strength was greatly decreased after 48 h of soaking for all samples (Fig.3.1).

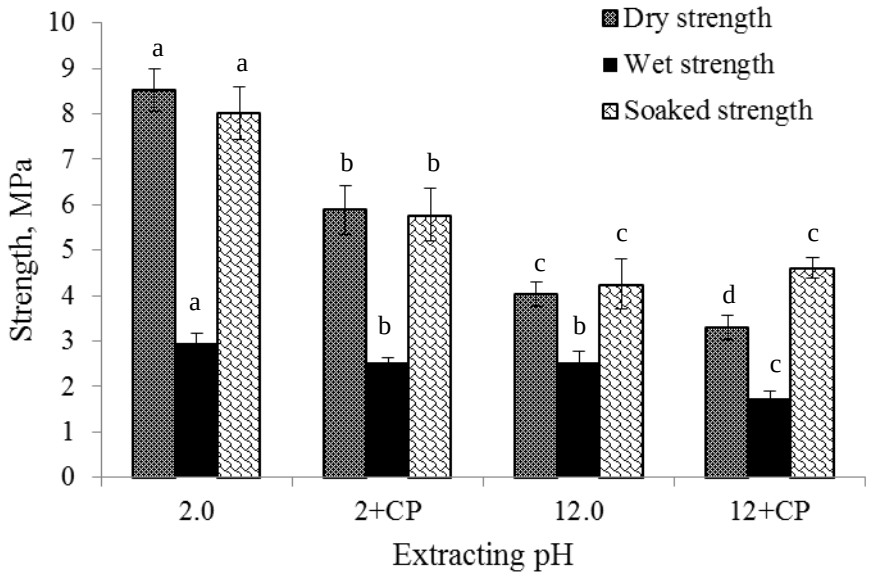

Figure 3.1. Dry, wet and soaked bonding strengths of wood composites bonded with adhesives that were prepared form proteins extracted at acid and alkaline pHs with and without cryoprotectants (CP) during freezing. Different letters indicate significant differences ($P \leq 0.05$).

3.3.2. The viscosities of SH-PI adhesives as a function of time and the corresponding bonding strengths

Adhesive penetration plays an important role in the adhesive bonding performance and participates in all of the potential adhesion mechanisms (Marra, 1992). According to the previous studies, the effectiveness of adhesive penetration is largely depended on the viscosity of the protein-based adhesive, which could be varied base on different modifications and the dry content of protein in the adhesive (Nordqvist et al., 2010). Higher dry protein content in the adhesive will lead to high viscosity and thereby decreasing its ability to properly wet, flow, and penetrate the wood surface. A high viscous adhesive is also more difficult to handle during preparation. In the present study,

the concentration of SH-PI is controlled at 5% (W/W, protein/water), before the viscosity became too high.

Table 3.1 indicates the viscosities of SH-PI adhesives from acid- and alkaline-aided extraction and the corresponding dry strengths at 1, 2, 4, 6 and 8 hr mixing time. The viscosity values of alkaline extracted SH-PI SDS suspension were higher than that of acid extracted SH-PI suspension; however, the corresponding adhesive dry strengths were significant lower from the alkaline-aided extract samples (Table 3.1). It was reported that the bonding strength of the modified soy adhesive with higher viscosity is much lower than the one with lower viscosity (Zhong et al., 2002). Adhesives with lower viscosity could improve the degree of adhesive penetration on the wood surface and reduce the possibility of developing voids along the bondline (Baldan, 2012). In addition, a low viscosity form of adhesive obtains superior flow characteristics, which increase the potential contact area between adhesive and wood elements, consequently leading to stronger adhesion strength. For some adhesive practice, a type of low molecular weight polymer was mixed with the adhesive to reduce the viscosity of the glue (Kamke and Lee, 2007). In this study, the acid-aided extraction might produce the protein isolates with suitable ratio of high to low molecular weight protein peptides thus to possess the proper viscosity which lead to superior wood penetration as well as the better bonding strength after curing. Mohan et al reported that the muscle proteins from acid-aided extraction resulted in the disappearance of some high molecular weight fraction (>160 kDa), while this disappearance was not observed from alkaline-aided extraction (Mohan et al., 2007). The authors suggested that it might be caused by the acid activated proteinases, which

dissociating the big protein molecule into low molecular weight fragments (Mohan et al., 2007).

Table 3.1.
Viscosity and dry bonding strength (DBS) of the SDS* modified SH-PI* adhesive from acid- and alkaline-aided extraction that tested at 1 hr, 2 hr, 4 hr, 6 hr and 8 hr mixing time.

Extraction	Acid		Alkaline	
	SDS*-pH2.0 SH-PI* adhesive		SDS*-pH12.0 SH-PI* adhesive	
Time	Viscosity (Pa.S)	DBS (MPa)	Viscosity (Pa.S)	DBS (MPa)
1 hr	0.75±0.2[b]	3.34±0.10[a]	2.23±0.21[a]	1.74±0.24[b]
2 hr	0.17±0.04[b]	6.80±0.58[a]	0.64±0.20[a]	2.00±0.39[b]
4 hr	0.16±0.04[b]	8.52±0.47[a]	0.32±0.04[a]	4.24±0.55[b]
6 hr	0.15±0.03	7.98±0.38[a]	0.22±0.05	4.52±0.21[b]
8 hr	0.15±0.04	7.55±0.11[a]	0.16±0.02	5.01±0.44[b]

*SDS: sodium dodecyl sulfate. *SH-PI: spent hen protein isolate. Different superscript letters indicate significant differences and no superscript letter indicate no significant differences ($P \leq 0.05$).

3.3.3. Surface morphology of the bond-lines between SH-PI adhesive and wood

The structural make-up of the wood bondline after curing will indicate the degree of adhesive penetrations and ultimately influence on the performance of the adhesive bonding strength (Kamke and Lee, 2007). The SEM observations of clean wood veneer surface, the pulled wood bondline with acidic and alkali extracted SH-PI adhesives after curing process were conducted at 1000×mignifications. The resulting micrographs were presented in Figure 3.2a, 3.2b and 3.2c, respectively. For the wood veneer surface, a clear image of lumen and porous structure was observed. The lumens on the wood surface provide a pathway for liquid form SH-PI adhesives to flow through, while the application of pressure facilitates the adhesive penetration into the porous cavities.

Even though the protein content in the joints is similar, the acid-aided extracted SH-PI adhesive covered more wood surface area (lumens and pores) than alkaline-aided extracted SH-PI adhesive (Fig.3.2b&c). The SEM images of pH2.0 SH-PI adhesive appeared to be more homogenized on the wood surface and tended to be present as a thin coating on the walls of the cell lumens. In contrast, the pH12.0 SH-PI adhesive tended to bulk the lumen and remain at the interface of the bondline. Zhong et al reported that the reduction in adhesive penetration related to increased molecular weight of protein polymers, and subsequent increased adhesive viscosity (Zhong et al. 2002). Therefore, this difference in degree of penetration could be the effect of adhesive viscosity since the high viscosity could inhibit flow of the adhesive on the wood surface. The lower viscosity of acidic extracted SH-PI adhesive combined with the higher bonding strength (Table 3.1) agreed with the better wood penetration from its SEM images (Fig. 3.2b).

Since the particle size directly affect the viscosity of the suspension (Kamke and Lee, 2007), one might conclude that large particle size might attribute to higher initial viscosity, which leading to an impaired bonding strength due the inadequate surface wetting and penetration.

The viscosity and SEM data suggested that protein molecules extracted in acid conditions with SDS modification produced peptide chains with suitable molecular weights, which could further enhance the adhesive bonding strength.

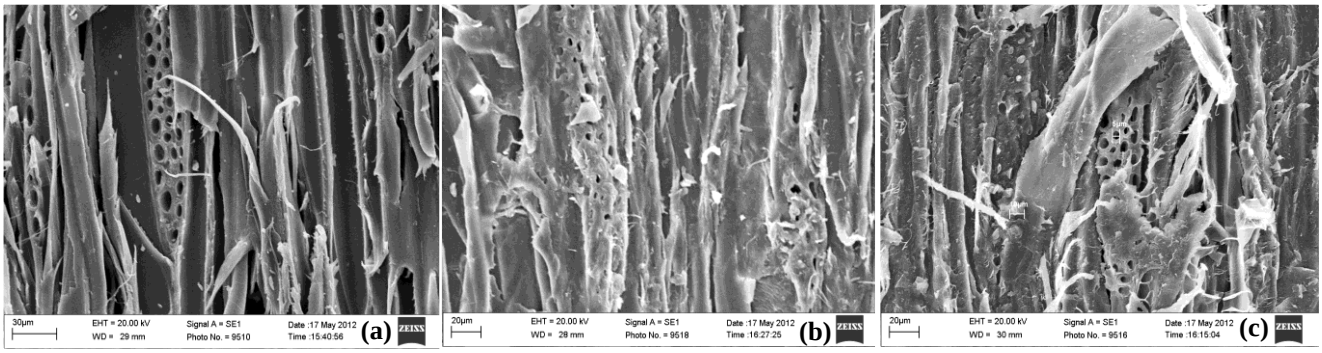

Figure 3.2. SEM of (a), (b) and (c) represent wood veneer surface, pulled wood sample after gluing with 3% SDS* modified acidic (pH2.0) extracted SH-PI* and pulled wood sample after gluing with 3% SDS modified alkaline (pH12.0) extracted SH-PI under 1000× magnification, respectively. *SDS: sodium dodecyl sulfate. *SH-PI: spent hen protein isolate.

3.3.4. Thermal stability of SH-PI before and after SDS-modification.

Considering the fact that SDS modified SH-PI adhesive were submitted to heat-pressure processes, the thermal denaturation points (temperature) of chemical extracted and SDS treated SH-PI were tested in order to determine the effect of the modifications on protein thermal properties. The typical transition temperatures for muscle proteins can range from 43 to 67ºC for myosin, 67 to 69ºC for sarcoplasmic proteins, and 71 to 83ºC for actin; the exact temperatures are subjected to pH and salt conditions (Findlay and Barbut, 1990).

In the present study, three endothermic transition temperature points were observed in SH-PI extracted at pH12.0 at 56.73ºC, 72.51ºC, 78.01ºC and 94.41ºC, which might corresponding to the unfolding points of myosin, sarcoplasmic and actin molecules, respectively (Fig. 3.3). These denaturation points shifted to a slightly higher temperature region as compare to the reported values (Findlay and Barbut, 1990). The formation of

the frozen induced protein oxidations could contribute to its resistance to thermal denaturation. Stadtman (1990) reported that the oxidative deterioration on protein molecules during frozen storage could cause increased S-S crosslinking formation (Stadtman, 1990), which will lead to a subsequently protein aggregation (Vogt et al., 1997). However, only one transition temperature at 96.83ºC was obtained by SH-PI extracted from acid pH, which is much higher than the highest denaturation temperature that reported for actin molecule (83ºC). Thus this denaturation point might not be temperature that is needed to unfold one type of muscle proteins but an accumulated energy that used to denature the refolded protein molecule mixture from acid pH. Since the muscle proteins extracted from acid pH were found to be more denatured (Kim et al., 2003), they may not able to maintain the individual form during the randomly unfolding process. Another explanation could be the proteolytic degradation of native proteins into fragments during the acid-aided extraction, thus the individual denaturation point for higher molecular weight myosin was not observed. Kim et al. (2003) has reported that the acid activated cathepsins B proteinase contributed more proteolytic hydrolysis during acid extraction process as revealed on disappearance of high molecular weight bands in SDS-PAGE, which suggested that the myosin molecules were degraded into lower molecular weight fragments. As for the sarcoplasmic proteins, Tadpitchayangkoon et al. (2010) found that it become less stable under extreme acidic condition due to pH induced denaturation, thus no endothermic transitions was observed. In addition, studies have found that the acid-aided extracted proteins are more prone to the frozen-induced oxidations (Kristinsson and Hultin, 2003). Davies (1986) concluded that the frozen-induced protein aggregations also could be random conglomerates of protein fragments

held together by non-covalent attractions such as hydrophobic and ionic bonds other than simple protein polymerizations to higher molecular weight.

The DSC data for SH-PI treated with SDS showed that decreased denaturation temperatures for both acid and alkaline extracted samples, which suggested that the heat capacity of the modified SH-IP proteins decreased (Fig.3.3). The decline of denaturation temperatures indicated the SH-PI/SDS interaction (electrostatic and hydrophobic) decreased the thermal stability of the protein by disrupting intermolecular bonds that maintain the protein conformation, lead to the greater the degree of protein unfolding (Huang and Sun, 2000; Schmidt et al., 2005). The SDS modification on the protein unfolding seemed to be more effective on the acid extracted SH-PI since its denaturation temperature was much lower (75.95ºC) than the alkali extracted SH-PI, which was denatured at 81.83ºC (Fig.3.3). The higher shear strength of SH-PI modified from acid-aided extraction (Fig.3.1) might have resulted from the adequate extent of unfolding that more reactive groups were exposed and became available for possible chemical crosslinking.

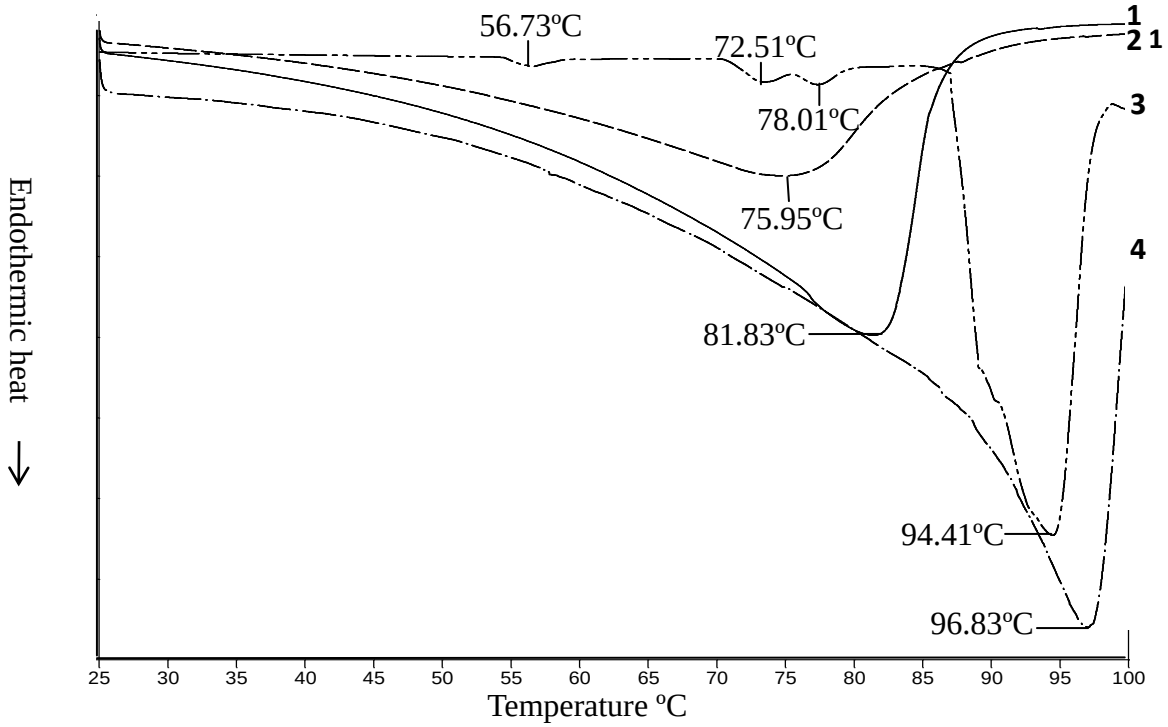

Figure 3.3. Differential scanning calorimetry (DSC) thermograms pH shifting extracted SP-PI and SDS modified SH-PI adhesives. 1: SDS-pH12.0 SH-PI. 2: SDS-pH2.0 SH-PI. 3: pH12.0 SH-PI. 4: pH2.0 SH-PI. The denaturation temperatures of each sample are marked beside peaks in each thermogram. SDS: sodium dodecyl sulfate. SH-PI: spent

3.3.5. Effect of the curing process on the structure changes of SDS modified SH-PI

The curing (heat-pressure) is essential for the adhesion process of the thermosetting adhesives, which indicated by transition of the adhesive from liquid to solid form (Pizzi, 1994). During thermosetting, the adhesive solidification occurs through chemical polymerization into cross-linked structures that resist to breakdown during pulling. The FTIR spectra of acidic and alkali extracted SH-PI adhesives were taken before and after the curing process (Fig.3.4) in order to illustrate the protein structure alterations caused by curing process. It appears that all samples tested have typical infrared absorption peaks at amide spectral bands I (C=O stretching vibration) at 1750-1651 cm^{-1} range,

amide spectral bands II (N-H in-plane bending vibration) at 1570-1520 cm^{-1} range and amide spectral bands III (C-N stretching vibration and N-H deformation vibration) at 1242-1230 cm^{-1} range (Nakanshi and Solomon, 1977; Chen et al., 2012). It indicates that the backbone structure of the SH-PI had not been changed during both pH-shifting process, SDS, and curing modifications.

A comparison of the cured and uncured samples FTIR spectra indicates that the curing process of acid and alkaline extracted SH-PI results in a significant decreasing of the absorption peak of functional group (C=O) at 1747.19 cm^{-1} combined with an more intensive absorption peak of functional groups (COO-) at 1395.14 cm^{-1}. This observation suggests the formation or exposure of more reactive carboxylic group (COO-) via the oxidative reaction promoted by heating process (Chen et al., 2012). Thus, crosslinking could be formed later when the SH-PI adhesive is cured with wood elements, which are rich in reactive hydroxyl groups. This type of interactions between adhesive and wood molecule is classified as adhesive force, which contributes to one of the two major forces that are most frequently used in the discussion of wood adhesion phenomenon. The other one is called cohesive force which refers to the interactions formed within the adhesive molecules. In the present study, the SDS modified SH-PI adhesives contain water as a carrier for better flow property, which will be evaporated or absorbed by the wood during the heating process so the adhesive could cure completely. A new absorption peak was observed at 1643.98 cm-1 in both cured samples as compare to the uncured samples. In addition, the peak at 1637.54 cm-1 in the uncured samples shifted to a lower frequency (1623.47 cm-1) in the cured SH-PI adhesive (Fig.3.4). This region was attributed to antiparallel and weakly hydrogen bonded β-sheets, which originated from N-H stretching

(Chen et al., 2012). The shift in this region might be caused by deprotonation during curing process. Chen et al. (2012) observed the similar pattern of decreasing in frequency of functional groups of N-H in soy protein-based adhesive after curing process. There is also a shift of absorption peak around 1519.90 cm^{-1} to higher frequency (1536.55 cm-1) in cured SH-PI adhesives, which could the result of re-formation of hydrogen bonding with the secondary amine. Lin et al. (2012) also reported a re-establishment of amide links and the conversion of the protonized products after curing of soy-based adhesive. These interactions among the protein molecules could contribute to the cohesive force of the SH-PI adhesive.

Base on the difference observed in the FTIP spectra of the cured and uncured samples, the curing process clearly caused protein secondary structural changes. Both adhesive and cohesive forces seemed to be reinforced by curing process. The peak shifting that observed at amide bands I and II region implicate the rearranging of hydrogen bonding due to the curing effect.

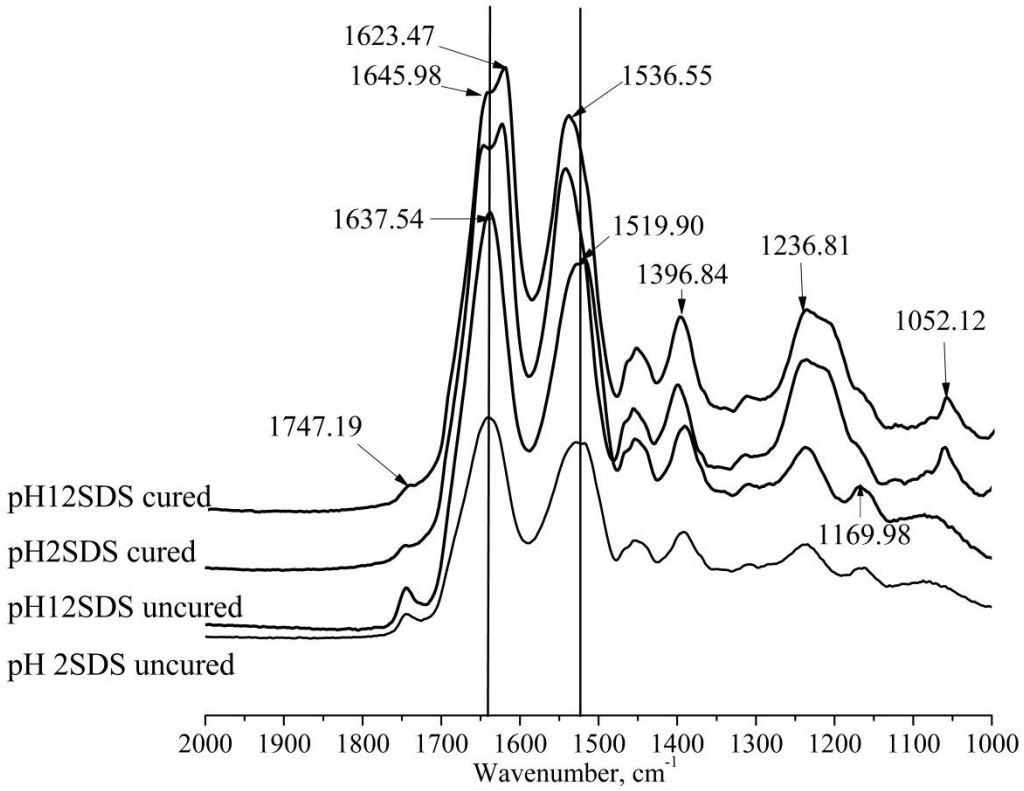

Figure 3.4. FTIR spectra of untreated spent hen protein as well as 3% SDS* modified acid (pH 2.0) and alkaline (pH12.0) extracted SH-PI* adhesive before and after curing (240 s, 110ºC & 3.5 MPa). *SDS: sodium dodecyl sulfate. *SH-PI: spent hen protein isolate.

3.4. Conclusion

According to this study, there is a clear difference in adhesive bond performance between acidic and alkali extracted SH-PI. The adhesive bonding strengths of acidic extracted SH-PI adhesive are superior and be further improved with the combination of freezing effect. The lower viscosity and better wood penetration showed in SEM for the acidic extracted SH-PI adhesive agreed with its advanced bonding strength and suggested that the acid and freezing treatment at extraction and storage stage might produce a more suitable SH-PI for preparing high performance wood adhesives. The DSC data indicated

the SDS modification was more effective on unfolding acidic extracted SH-PI structure. FTIR spectra indicated that the SDS modification did not cause obvious alterations on SH-PI secondary structure. Curing the SH-PI adhesives, on the other hand, resulted in the formation or exposure of reactive COO- group and the rearranged H-bonding in order to form corsslinkages with wood surface molecules or within the protein molecules thus the adhesive and cohesive strength might be both improved and the adhesive bonding performance would be subsequently enhanced.

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CHAPTER 4. MAIN FINDINGS OF THE PROJECT AND FUTURE WORK

4.1. Main findings of the project

As described in literature review, the main objective of this project is to extract the valuable proteins from the underutilized spent hen meat by pH-shifting process and explore the value-added potential of this chemical extracted SH-PI for both food and industrial applications (in this instance bio-based wood adhesive preparation). The first study was focused on evaluating the effect of the acid- and alkaline-aided extracting conditions and frozen-induced protein oxidation on the chemical and functional properties of the SH-PI, which are essential for their application in complex food matrix. In the second study, the chemical extracted frozen SH-PI was used to prepare wood adhesive via SDS and curing treatment. The adhesive properties were investigated and discussed as the effect of chemical extraction, frozen storage, SDS modification and curing process. Major findings are as follows:

- The acid-aided extraction provide higher protein recovery from the spent hen meat mince as compare to alkaline-aided extraction;
- For the food application, CP addition during frozen storage is necessary for stabilizing protein structure that further improves the gel-formation of the SH-PI;
- After the pH-shifting extraction, the acid treated SH-PI appeared to be more denatured and become more susceptible for frozen-induced oxidative reactions;
- For the adhesive preparation, the acid-aided extraction and the frozen-induced protein oxidations appeared to be beneficial as revealed better adhesive strength and wood penetration.

4.2. Future work and industrial implications

Although the possible application of adhesive prepared from chemical extracted SH-PI has been proved in this study, the material should ideally display the typical advantages as lower toxicity, biodegradability, lower price, ease of handing, abundance and renewable character (Chen, 2009; Huang and Sun, 2000; Rhim et al., 2002). Therefore, there are still two major problems that need to be considered for future studies on spent hen protein-based adhesive: the poor water resistance of the adhesive and the economic feasibility of the technology developed in this study.

One of the solutions for improving the low moisture resistance of the protein-based adhesives is to blend with synthetic resins. Breyer et al. (2005) have reported that blending the blood protein in the urea-formaldehyde resins resulted in an increase of adhesive binding strength and water resistance. Other kinds of blend also have been tested such as spray-dried animal blood mixed with soy flour and synthetic resins, which the blood proteins provided water resistance, while soy flour provided the granular consistency for machine applications (Breyer et al., 2005). This type of blending reduced the costs, as adhesives based only on protein source were very expensive (Kumar et al., 2002). Since we found that acid treatment and protein oxidation had a positive effect on the muscle protein adhesive properties, a designed acid and oxidative modification could be applied on SH-PI or even meat bone meal (Fig.4.1) in order to achieve the desired adhesive properties with simplified process.

In relation to the economic feasibility, the main drawback is the costly extraction process. The long term frozen storage also increases the budget for the production of SH-PI adhesive materials. A possible solution to overcome this issue is the use of a more

simplified process, which is showed in Figure 4.1. The starting material could be the dried mechanical separated spent hen meat or the meat and bone meal (MBM). Park et al (2000) has studied the possible use of protein concentrate from meat and bone meal for wood adhesive production. The authors pointed out the abundance of MBM. In the U.S., approximately 5.0×10^6 metric tons of MEM are produced annually and used domestically in animal feed production. In addition, MBM is rich in minerals especially the calcium ions, which was found to facilitate protein solubility (Park et al. 2000). It has been established that the degree of protein dispersion is a critical point for producing high performance protein-based adhesive (Kumar et al. 2002). Increased protein solubility could promote a more evenly dispersion of protein molecules during adhesive preparation. Hence, MBM could be directly treated with organic acid such as (HCl, citric acid and boric acid) and then oxidized by H_2O_2 or O_3, which was found to be useful on enhancing the adhesive properties (Chapter 3).

Feathers could also be incorporated in to the process to improve the adhesive binding properties and water resistance of the adhesive. Currently, approximately 2.25 billion kg of chicken feathers are produced annually in the U.S., which are mainly consumed by the feather meal industry for livestock feed production (Winandy et al., 2003). Over the past few years, researchers have tried to developed alternative usage of this large, cheap neutral fiber source in order to increase the value of this raw material, which is currently valued at $250/ton when sold for feather meal (Gentry et al., 2004). Birds' feathers generally contain 91% protein, which the majority is a type of fiber protein called keratin (Winandy et al., 2003). The feather keratin could be extracted by urea and reducing agents that the extracted material has a narrow distribution of molecular weight with

range of 10 kDa (Woodin, 1955). It has been reported that for achieving better adhesive strengths, smaller molecular weight (3 kDa-15 kDa) polymers are needed in the adhesive formula in order to enhance the adhesive wood penetration, which consequently lead to a higher adhesive strength (Kamke and Lee, 2007). In addition, the keratin fiber is considered hydrophobic, which make them more durable as compare to the isolated muscle protein. The keratin could also create extensive cross links when heated; it has been reported that the strength of this network is considered similar to nylon but with a density smaller than wood fibers (Winandy et la., 2003). Therefore, most of the studies were focused on mixing the keratin fiber with synthetic or protein-based resin to produce low density fiberboard panels. Barone and Schmidt (2005) reported that keratin feather fibers can be directly incorporated into the resin polymer by standard thermomechanical mixing technique, of which a strong adhesive bonding between keratin and polymer could be formed. Higher water resistance of the composite with incorporation of keratin fiber was also observed by Winandy et al. (2003). Thus, an improved adhesive strength and water resistance property can be expected when the keratin fiber is blended and treated with SH-PI adhesive. However, it requires further investigations on the proper ratio of keratin and SH-PI as well as which step it should be added during the adhesive preparation (Fig. 4.1).

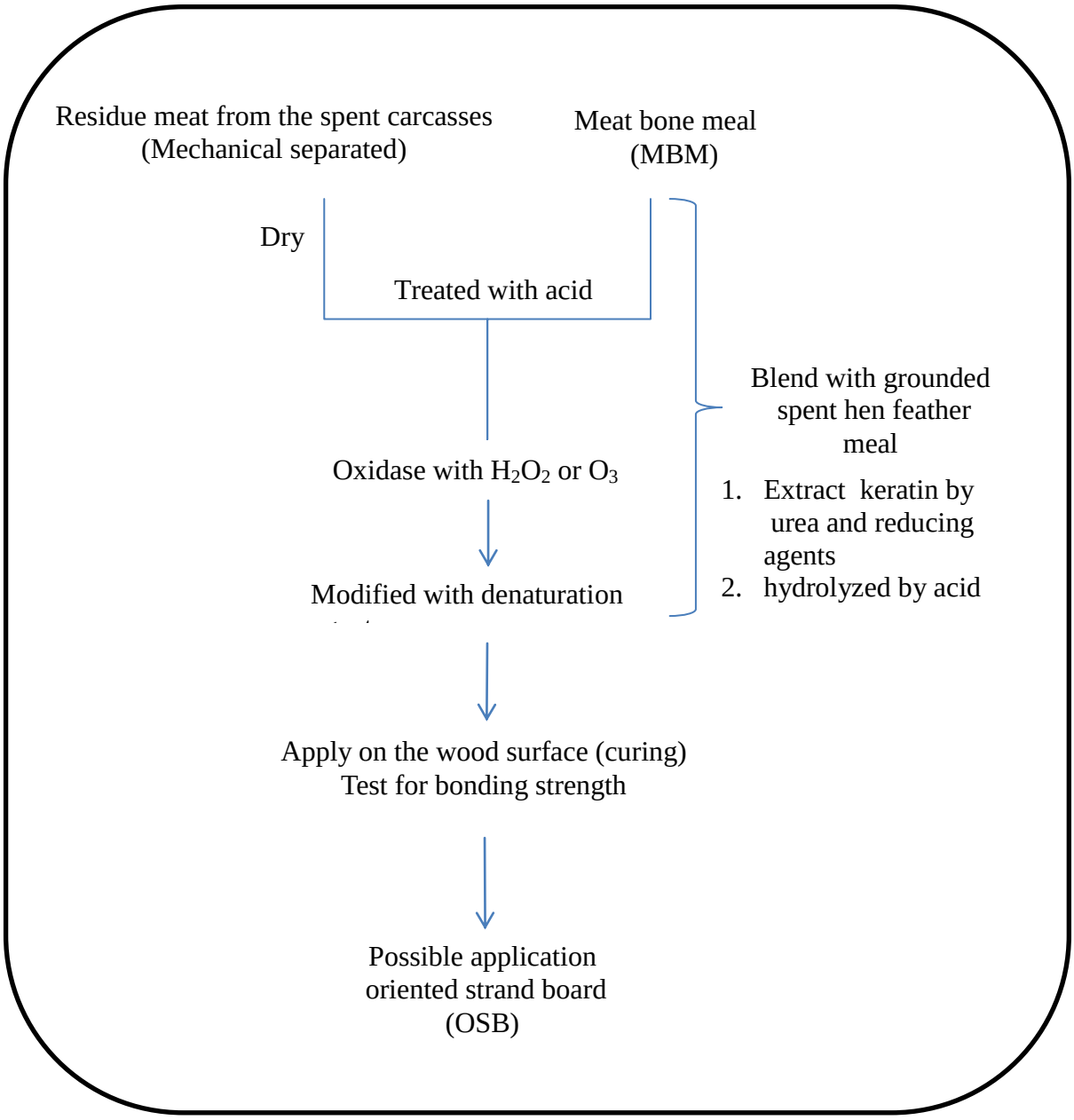

Figure 4.1. Schematic flow chart for the future studies on spent hen protein-based wood adhesive.

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