Conversion of Avian Collagen to Gelatin and Cryoprotective Peptides

by

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Abstract

Gelatin - a gel-forming, multifunctional biopolymer obtained from the processing of collagen - has long been used in the food industry. Gelatin hydrolysates obtained by enzymatic hydrolysis have various bioactivities. Although porcine and bovine sources are principally used for commercial gelatin production, the demand for gelatin derived from alternative sources is increasing mainly due to the religious, ethical, sustainability, as well as health and safety reasons. Therefore, in this research underutilized poultry by-products were developed as a potential source for gelatin and collagen production. In the first study, gelatins were extracted from chicken and turkey heads with optimized pre-treatment and extraction methods at 50 °C and 60 °C, and their physico-chemical properties (amino acid composition, molecular weight, microstructure, rheological behavior, Bloom strength) and functionalities (solubility, emulsifying and foaming properties) were examined. All the obtained poultry gelatins exhibited an excellent Bloom greater than 200 g, and the gelatin achieved from turkey heads at 50 °C showed superior gel attributes with a Bloom of 368±8 g. Except for source of collagen, the manufacturing method mostly affects both the physico-chemical and functional properties of gelatin. Traditional gelatin production involves a time and energy intensive pre-treatment step, with the enormous consumption of water and chemical reagents. Therefore, in the second study, a new, efficient and environmental-friendly pre-treatment approach was developed for gelatin extraction from mechanically separated turkey meat (MSTM) using an isoelectric solubilisation/precipitation process (ISP). The resulting gel had an improved quality (bloom strength 353.2 g) compared to the gel made by the conventional manufacturing procedures. The first part of the research demonstrated the possibility of using poultry by-products as an alternative gelatin source; here the features of these poultry gelatins were

comparable to those from porcine or bovine sources. However, the lower yields of this process may represent a limitation in commercializing these poultry gelatins.

To maximize the value of gelatin or collagen obtained from poultry by-products, collagen produced from chicken skin was hydrolyzed by trypsin, and then the cryoprotective effect of these collagen hydrolysates was evaluated in different food systems with two studies. Firstly, the ice-growth inhibition activities of collagen peptides were determined in a sucrose model system as compared to natural antifreeze proteins (AFPs). The ice crystal dimension in the model systems was evaluated under a microscope after thermal cycles, and the amino acid sequences of several ice-controlling peptides were identified. The addition of certain collagen peptides at 1 mg/mL effectively reduced approximately 70% of ice crystal growth in the sucrose systems, in a similar manner as do AFPs. In the second study, the cryoprotective effect of collagen hydrolysates against freeze-denaturation of actomyosin systems was investigated in relation to ice-controlling activity. Smaller ice crystals induced by collagen hydrolysates may decrease protein unfolding, oxidation and functionality loss during frozen storage. Overall, this thesis provides a research foundation for producing high quality gelatin from poultry sources. It demonstrates the potential of how chicken collagen peptides can be a substitute for sweet commercial cryoprotectants like sucrose or sorbitol used in frozen foods.

Preface

This thesis is an original work of Lihui Du. It is presented in manuscript format and consists of seven chapters.

Chapter 1 introduction describes the research background, hypothesis and objectives of this work.

Chapter 2 is the literature review and focuses on collagen and gelatin, common cryoprotectants and mechanisms, antifreeze proteins and the potential for collagen hydrolysate to be used as a novel cryoproetctant.

Chapter 3 to chapter 6 constitute the main content of the thesis.

Chapter 3 was published as Du, L., Khiari, Z., Pietrasik, Z. and Betti, M. (2013). Physicochemical and functional properties of gelatins extracted from turkey and chicken heads. *Poultry Science*, 92, pp.2463–2474.

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Chapter 6 was published as Du, L. and Betti, M. (2016). Chicken collagen hydrolysate cryoprotection of natural actomyosin: Mechanism studies during freeze-thaw cycles and simulated digestion. *Food Chemistry*. 15 (211), 791-802.

Chapter 7 is the conclusion chapter and briefly summarizes the thesis, and discusses implications of the results and suggests future research in this field.

I was responsible for the experimental design, data collection and analyses as well as the manuscript composition. Dr. Z. Khiari assisted with experimental design and contributed to manuscript edits of the chapter 3 and 4. Dr. Z. Pietrasik assisted with the proofreading of the chapter 3, and L. Keplová took part in the data collection of chapter 4. Dr. M. Betti was the supervisory author and was directly involved with concept formation and manuscript composition.

Dedicated to

my father Yingji Du, my mother Yuanting Li,

my grandmother Meixiao Luo

and my husband Yu Qian,

In the memory of

my beloved grandfather Xuliang Li.

Whose love has always been encouraging me to go forward.

致

我的父母和家人

是你们给了我坚持的勇气和力量

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Abbreviations

a* – redness/greenness
ACE – angiotensin I converting enzyme
AFGPs – antifreeze glycoproteins
AFPs – antifreeze proteins
ANOVA - analysis of variance
b* – yellowness/blueness
BSA – bovine serum albumin
BSE – bovine spongiform encephalopathy
CB – gelatin extracted from collagen biomass
CCH – chicken collagen hydrolysates
CD – circular dichroism
CHS1 – chicken head gelatin extracted at 50 °C
CHS2 – chicken head gelatin extracted at 60 °C
CL – cooking loss
D [4, 3] – mean droplet size
DVB – dynamic viscoelastic behavior
EM – expressible moisture
ER – endoplasmic reticulum
F1-F5 – five fractions obtained from SEC
FACIT – fibril associated collagens with interrupted triple helices
FE – foaming expansion
FS – foaming stability
G' – elastic modulus
G'' – viscous modulus
HPLC – high performance liquid chromatography
ISP – isoelectric solubilisation/precipitation process, pH-shifting
L* – lightness
LC-MS/MS – liquid chromatography tandem mass spectrometry
MACIT – membrane associated collagens with interrupted triple helices
MS – mass spectrometry
MSPM – mechanically separated poultry meat
MSTM – mechanically separated turkey meat
MSTMS1 – MSTM gelatin extracted at 50 °C
MSTMS2 – MSTM gelatin extracted at 60 °C
NAM – natural actomyosin
PPI – poultry protein isolate
R-SH – reactive sulfhydryl content
S-S – disulfide bond
S1-S5 – five synthesized collagen peptides
sbwAFP – AFP from the spruce budworm
SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC – size-exclusion chromatography
SEM – scanning electron microscopy
SU – sucrose
Surface Ho – surface hydrophobicity
SUSO – sucrose and sorbitol blend (1:1)
T-SH – total sulfhydryl content
THS1 – turkey head gelatin extracted at 50 °C
THS2 – turkey head gelatin extracted at 60 °C
vCJD – variant Creutzfeldt-Jakob disease
δ - phase angle
Δ D [4, 3] – change of the droplet size

Chapter 1- General introduction and objectives

Gelatin is a multifunctional protein biopolymer derived from collagen, the principal constituent of animal skin, bone, and connective tissue (Karim and Bhat, 2009). Gelatin has long been used in the food industry, as a gelling, thickening and film-forming agent, as well as an emulsifier and a stabilizer (Schrieber and Gareis, 2007). Although the majority of commercial gelatins come from porcine and bovine sources, their utilization is either restricted or limited due to cultural, religious and safety issues (Gómez-Guillén et al., 2007). Interest in fish gelatin arose after an outbreak of bovine spongiform encephalopathy (BSE) (Eysturskarð et al., 2009). However, an insufficient availability of raw material, the potential presence of allergens, and inferior rheological properties impede both production and usage of fish gelatin (Karim and Bhat, 2009). Underutilized poultry by-products is another suitable source for gelatin production to meet the increasing and changing demands, but needs to be investigated.

Besides the source of collagen to make gelatin, the method of manufacture greatly affects its physico-chemical and functional properties (Montero and Gomez-Guillen, 2000; Norziah et al., 2009). The conventional process of gelatin production involves acid or alkali pre-treatment, thermal extraction and then purification (Karim and Bhat, 2009); this is usually time and energy intensive, particularly in the pretreatment step. At a pilot project in our lab, we recovered the functional myofibrillar proteins from mechanically separated turkey meat (MSTM) using an isoelectric solubilization/precipitation process (ISP). Here, a substantial amount of collagenous biomass was isolated as a waste material after the centrifugation steps (Hrynets et al., 2011; Omana et al., 2010). This collagen biomass was deemed to be a suitable for gelatin production. Furthermore, this made it possible to use ISP as a pretreatment replacing the traditional steps

involved in gelatin manufacturing, since it also recovers functional myofibrillar proteins at the same time.

As a versatile ingredient, new applications for collagen or gelatin are continuously being discovered by researchers. For instance, enzymatic hydrolysis of gelatin can produce biologically active peptides exhibiting anti-oxidative, anti-microbial and angiotensin I converting enzyme (ACE) inhibitory effects (Kim et al., 2001; Gómez-Guillén et al., 2010; Herregods et al., 2011). Recently, novel cryoprotective properties of gelatin hydrolysates have drawn researchers' interest (Wang and Damodaran, 2009). Alternative food cryoprotectants are in demand; common commercial cryoprotectants, like sucrose and sorbitol, are too sweet and antifreeze proteins/peptides obtained from nature exist in limited quantities. Research on gelatin's cryoprotective ability is still very limited to date. No previous studies have been published on the cryoprotective peptides from poultry collagenous sources, and furthermore, no clear mechanism associated with the cryoprotective effect of collagen or gelatin peptides has been elucidated.

Therefore, during my four-year PhD program, first I was dedicated to confirm the possibility of producing high quality gelatin from underutilized poultry by-products, and also to develop an efficient pretreatment method for gelatin extraction. These studies aimed to prove that also poultry by-products represent a valuable and sustainable resource for manufacture of gelatin. After that, I was also interested to verify that collagen derived from avian by-products could be exploited to generate cryoprotective (i.e. ice-growth inhibition) peptides. I was eager to know what are the mechanisms conferring their anti-freeze effect in food systems. The latter part of my research focused on this novel activity of chicken collagen hydrolysate, not only to maximize the value of poultry by-products, but also to give benefit to both food industries and consumers alike.

The studies conducted in this PhD thesis try to address the following questions:

1. Can poultry by-products be used as a source of high functional gelatin?

Chapter 3 "*Physicochemical and functional properties of gelatins extracted from turkey and chicken heads*" is a study that aimed to valorize poultry by-products by extracting and evaluating the functional properties of gelatin. Poultry heads is a by-product not fit for human consumption, mainly consists of skin, comb, wattle, cartilages and bones. However, due to its collagen content, this by-product remains an appreciable source for gelatin extraction (Rivera et al., 2000).

The specific objectives of the study 1 were:

a) To optimize a method for gelatin preparation from minced chicken and turkey heads;

b) To evaluate and compare the physico-chemical and functional properties of gelatins obtained from chicken heads and turkey heads.

2. Can the isoelectric solubilization/precipitation process (ISP) be used as a pretreatment to extract gelatin from MSTM?

Chapter 4, "*Preparation and characterization of gelatin from collagen biomass obtained through a pH-shifting process of mechanically separated turkey meat*" aimed to valorize the collagen biomass obtained during ISP of mechanically separated turkey meat. During the ISP of MSTM, the main products sarcoplasmic and myofibrillar proteins are efficiently isolated. Meanwhile, significant amounts of fat and insoluble collagenous biomass are also produced and

discarded. The collagen biomass, has high purity and may represent a valuable source for gelatin preparation.

The specific objectives of the study 2 were:

a) To develop a new process of gelatin preparation from MSTM through the ISP;

b) To evaluate the gelatin obtained from the newly developed process and compare this to the gelatin extracted by the conventional process.

3. Does chicken collagen contain amino acid sequences that possess ability to control ice crystal size during freezing?

Chapter 5 "*Identification and evaluation of cryoprotectvive peptides from avian collagen: ice growth inhibition activity compared to type I antifreeze proteins in sucrose model systems*" was aimed at identifying sequences of amino acids within the collagen molecule that possess cryprotection capacity and to compare their activity with winter flounder antifreeze proteins. Gelatin hydrolysates produced from bovine sources have been reported to show a cryoprotective effect by controlling ice crystal size (ice growth inhibition) in an ice cream mix (Wang and Damodaran, 2009). This may relate to the special amino acid sequence in the collagen, and chicken collagen may also contain those encrypted cryoprotective sequences.

The specific objectives of the study 3 were:

a) To evaluate the ice inhibition property of the chicken collagen hydrolysate in a sucrose model system;

b) To identify the sequence and secondary structure of the ice-controlling peptides;

c) To compare the ice growth inhibition of identified collagen peptides with that of natural antifreeze proteins.

4. Does chicken collagen hydrolysate play a cryoprotective role on protein through controlling ice growth and further protect proteins from freeze-denaturation?

Chapter 6 "*Collagen hydrolysate cryoprotection of natural actomyosin: mechanism studies during freeze-thaw cycles and simulated digestion*" aimed to elucidate the cryoprotection mechanism of collagen peptides on a wet myofibrillar protein concentrate (i.e. surimi). During frozen storage, freeze-denaturation plays the dominant role in the functional quality changes of surimi (Matsumoto, 1980). This process includes dehydration, oxidation, aggregation and cross-linking of myofibrillar proteins. It is of interest to investigate the cryoprotective effect of chicken collagen peptides in preventing freeze-protein denaturation in this type of product and to link this property with its ice-growth inhibition.

The specific objectives of the study 4 were:

a) To determine the ice-controlling effect of chicken collagen hydrolysate in a natural actomyosin model system;

b) To evaluate the cryoprotective effect of chicken collagen hydrolysate against protein unfolding, oxidation and crosslinking of natural actomyosin;

c) To determine the cryoprotective effect of chicken collagen hydrolysate on the functional properties and digestibility of natural actomyosin;

d) To elucidate a possible mechanism of the cryoprotective effect of chicken collagen hydrolysate on natural actomyosin.

Chapter 2 – Literature review

2.1. Collagen and gelatin from alternative sources

2.1.1. Collagen, collagen biosynthesis and structure

2.1.1.1. Collagen classification and biosynthesis

The collagen family is a major member of the extracellular matrix contributing to the scaffolding. It is the most abundant protein constituting about 30% of total animal protein and is found in skin, bone, tendons, ligament and connective tissues (Shon et al., 2011). In general, collagens function as a building material to provide strength and resilience for maintaining the stability and structural integrity of tissues, and they also play an important role in the early stages of organogenesis as well as in the regulation of cell behaviour (Prockop and Kivirikko, 1995).

Based on the polypeptide composition, 28 distinct collagen types have been identified. Also the collagen family can be classified into different subfamilies according to their supramolecular assembly; for example, fibrillar collagens, basement membrane collagens, and so on as listed in Table 2.1 (Engel and Bächinger, 2005). Among all the current variants of collagen, type I, II, III and V are the most prevalent and they are all fibrillar-forming collagens. Table 2.2 shows backbone information on chain composition, molecular and supramolecular assembly, as well as tissue distribution and pathology of the I, II, III and V collagen types (Engel, 2005). Type I collagen is the most abundant (around 80% of total collagen) and a well-studied collagen. It is distributed in all connective tissues, but exists dominantly in bone, tendons, skin, ligaments and the cornea. It is essential for the tensile strength of bone, and 80% of Type II collagen is found in cartilages, usually associated with proteoglycans and acting to cushion joints. Type III collagen is

Table 2.1. Collagen families and their respective collagen types. The table is reproduced from Engel and Bächinger (2005).

Collagen Family	Collagen Type
Fibril forming collagens	I, II, III, V, XI, XXIV, XXVII
Basement membrane collagens	IV
FACIT collagens	IX, XII, XIV, XVI,XIX, XX, XXI
Short chain collagens	VIII, X
Anchoring fibrils	VII
Multiplexins	XV, XVIII
MACIT collagens	XIII, XVII, XXIII, XXV
Collagen type VI	VI

Table 2.2. The details of the most abundant collagen types. The table is reproduced from Engel (2005).

Type	Chain composition	Molecules	Subfamily	Tissue distribution (selection)	Pathology
I	α1(I), α2(I)	$[\alpha1(I)]_2$ α2(I); $[\alpha1(I)]_3$	Fibrillar	Widespread: skin, bone, tendon, ligament, cornea	Osteogenesis imperfect I–IV, Arthrochalatic type of Ehlers-Danlos syndrome (VIIA, VIIB), Classical type of Ehlers-Danlos syndrome
II	α1(II)	$[\alpha1(II)]_3$	Fibrillar	Cartilage, vitreous	Achondrogenesis II, Hypochondrogenesis, Spondyloepiphyseal dysplasia congenita, Kniest dysplasia, Late onset Spondyloepiphyseal dysplasia, Stickler dysplasia
III	α1(III)	$[\alpha1(III)]_3$	Fibrillar	Skin, vessel, intestine, uterus	Vascular type of Ehlers-Danlos syndrome, Hypermobile type of Ehlers-Danlos syndrome (rare), familiar aortic aneurysm
V	α1(V), α2(V), α3(V), α4(V) (rat)	$[\alpha1(V)]_2\alpha2(V)$; $[\alpha1(V)]_3$; α1(V)α2(V) α3(V)	Fibrillar	Widespread: Bone, skin, cornea, placenta, Schwann cell (rat)	Classical type of Ehlers-Danlos syndrome

widely spread in collagen I-containing tissues except bone (Gelse, Pöschl, and Aigner, 2003), and its content depends on the age of animals, with skins from young animals containing up to 50% while the content is approximately 5 to 10% less in older animals (Schrieber and Gareis, 2007). Type V collagen typically forms heterofibrils in the presence of type I collagen in corneal stroma, which is very important for the regulation of corneal fibril diameter (Birk et al., 1988).

Fibrillar collagen biosynthesis is a multistep process involving both intracellular and extracellular steps (Figure 2.1). It follows the normal pathway for any secreted protein. Firstly, the preprocollagen is translated from ribosome-bound mRNA in a fibroblast and the signal sequence imports the polypeptide chain into the rough endoplasmic reticulum (ER). After the removal of the signal peptide by a signal peptidase, the precursor of collagen, procollagen α-chain is formed (Gelse, Pöschl, and Aigner, 2003). In the ER, the procollagen chain undergoes a series of post-translational modifications resulting in the assembly of procollagen molecules. These reactions include modification of proline residues to hydroxyprolines, modification of lysines to hydroxylysines, N- and O-linked glycosylation, trimerization, disulphide bonding, prolyl cis–trans isomerization and folding of the triple helix (Hulmes, 2008). The procollagen molecules are then translocated to the Golgi apparatus where they are packaged into secretory vesicles before being released to the extracellular matrix (Gelse, Pöschl, and Aigner, 2003). The later processes of biosynthesis in the extracellular space forms mature collagen molecules, where the N-terminal and C-terminal propeptides are removed from procollagen molecules by the procollagen peptidases, resulting in the release of triple-helical collagen molecules, and the assembly of collagen fibrils. The molecular arrangement into collagen fibrils is stabilized by the formation of intermolecular

covalent crosslinks through the action of lysyl oxidase, which contributes to the mechanical resilience of final collagen fibrils (Gelse, Pöschl, and Aigner, 2003; Lodish, 2000).

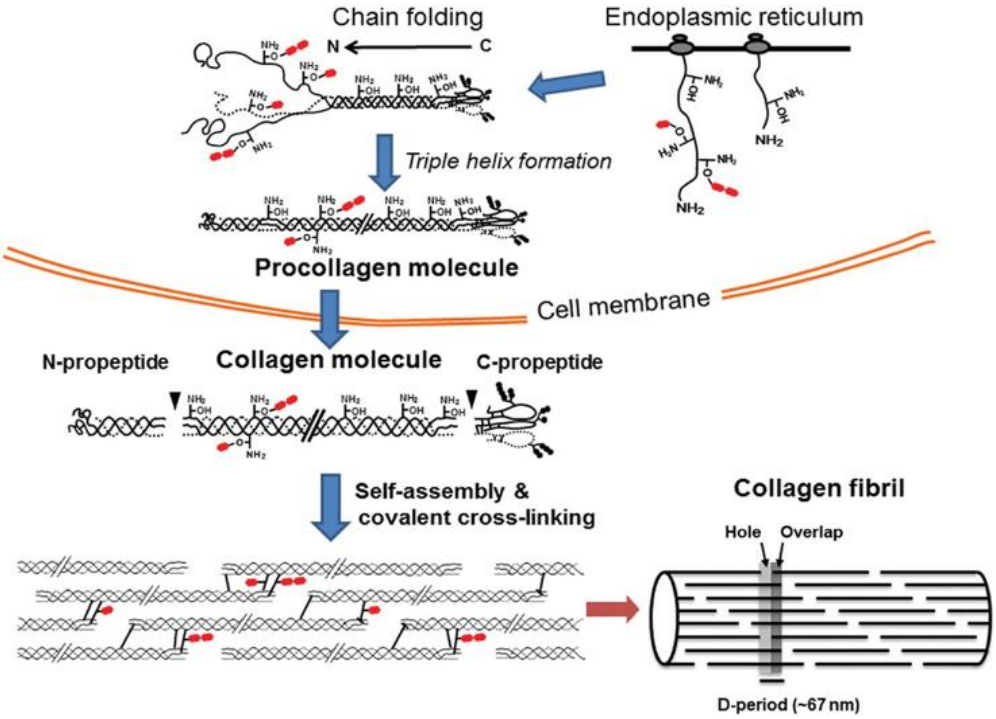


Figure 2.1. Major events during collagen biosynthesis. The graph is from Yamauchi and Sricholpech (2012), with permission of the Portland Press.

2.1.1.2. Collagen structure

Despite the many kinds of collagen, each molecule is consistently composed of a combination of three α-chains from at least 46 different polypeptide chains, and these three chains always coil together to form a right-handed helix (Shoulders and Raines, 2009; Figure 2.2). For instance, type I collagen consists of two α1(I) chains and one α2(I) chain, while type II collagen has three same α1(II) chains (Table 2.2). This fundamental collagen triple helix unit is approximately 300 nm in length with a diameter of around 1.5 nm and has a molecular weight about 300 kDa (Lodish, 2000). The repeating amino acid motif Gly-X-Y contained in each

collagen polypeptide chain of over 1000 amino acid residues is recognized as the hallmark of collagen, in which X and Y can be any amino acids but predominantly are proline and hydroxyproline (Ogawa, 2003). These characteristic triplets are crucial for stabilization of the collagen structure. The hydrogen atoms on the recurring glycine residues are oriented towards the center of the collagen molecule, which form intra-molecule hydrogen bonds between N-H groups of glycine residues and C=O groups of the other amino acid residues (mainly proline and hydroxyproline) on adjacent chains. In this way, the three strands are densely packed together. Moreover, the fixed angle of C-N peptidyl-proline or peptidyl-hydroxyproline enables the polypeptide chains to twist and fold into a three-stranded helix (Lodish et al., 2000). During collagen fibril assembly, inter-molecular covalent cross-links are of particular importance (Figure 2.2). In most cases, the cross-linking happens between lysine and lysine, hydroxylysine and hydroxylysine, or lysine and hydroxylysine residues in the telopeptide regions, during which the lysine or hydroxylysine residues are converted to aldehydes with the aid of lysine oxidase, and then spontaneously condense with other aldehydes or unreacted lysine and hydroxylysine residues to develop crosslinks (Hulmes, 2008). In mature collagen fibrils, crosslinks can occur through glycation as well (Avery and Bailey, 2008). A typical collagen fibril in a tendon has a diameter between 50 to 500 nm. Multiple fibrils are further bundled to form collagen fibers of about 50-300 μm in diameter (Figure 2.2), via inter- and intra-fibrillar cross-links and by interacting with the proteoglycans in the extracellular matrix (Fratzl, 2008). In food industry, collagen film has been applied in the co-extrusion process as a decent alternative to natural casings and sausage guts which have relatively high cost (Barbut, 2010). The impact of various acids and pH values, different types of salts, and co-gelling proteins have been demonstrated to affect collagen gel

properties that might improve the film quality of the co-extruded collagen casings (Oechsle et al., 2014; Oechsle et al., 2015a; Oechsle et al., 2015b).

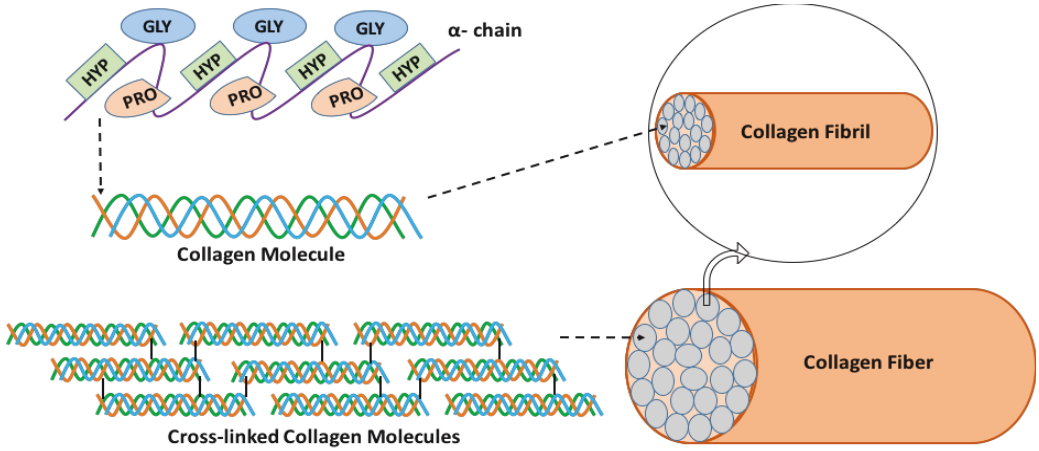


Figure 2.2. Collagen molecule structure to a collagen fiber.

2.1.2. Conversion of collagen to gelatin

2.1.2.1. Gelatin market

Gelatin is the partially denatured form of collagen. Different from insoluble collagen, gelatin can be solubilized in warm water, which makes gelatin a better material in the food industry. Another important feature of gelatin is its ability to form a thermally reversible gel, providing even more applications (Haug and Draget, 2011). Beyond its original applications, the ongoing expansion of the gelatin market is due to this non-fat, odourless protein filling growing consumer demand for sources of healthy, low fat and high protein. According to the report form Grand View Research, Inc., the global gelatin market is expected to reach 485.6 kilotonnes by 2020 from 373.3 kilotonnes in 2013, growing at a CAGR (compound annual growth rate) of 3.85% from 2014 to 2020. In terms of revenue, the market was valued at USD 2,012.5 million in 2013 and is anticipated to achieve USD 3,177.0 million by 2020, with a CAGR of 6.72%. Second to the largest gelatin

market in Europe (40%), North America possessed almost 25% of the global gelatin market in 2013, with 91.3 kilotonnes valued at USD 501.1 million, and this growth is expected to continue. Here the food and beverage segment represents the largest application of gelatin in North America.

2.1.2.2. Gelatin production

The nature and amount of collagen in different tissues and different kinds of animals are diverse. Due to the heterogeneity of the different types of gelatin of interest, the preferences and techniques vary with manufacturers regarding selection of raw materials, chemical reagents used in processing, the extent of treatment, and the subsequent processes during manufacturing. However, over time the principle of gelatin preparation has remained virtually unchanged (Djagny, Wang and Xu, 2001).

Gelatin is a biopolymer produced by extraction and hydrolysis of collagen. Type I collagen is the main type used for gelatin manufacture, which contains no cysteine and is ubiquitous in all connective tissues. Trace cysteine found in some gelatin is mainly due to the presence of type III collagen in the raw material used for the extraction (Cole, 2000). Gelatin production can be divided into three stages: pre-treatment of raw materials, gelatin extraction, and final purification (Karim and Bhat, 2009). Initially to prepare the collagen for gelatin extraction, the raw materials are chemically pretreated to remove non-collagenous proteins, fat, and impurities, followed by acid or alkali –aided swelling to partially cleave the cross-links in the native insoluble collagen. In the extraction step, a period of heating (usually above 45°C) results in the breaking of intramolecular hydrogen bonds and a portion of covalent bonds; this further dissociates the triple helix structure of collagen molecules and transforms fibrous collagen into randomly coiled gelatin (Figure 2.3) (Gómez-Guillén et al., 2011). The degree of conversion of collagen into gelatin is associated with

the severity of both pretreatment and extraction process, which depends on pH, temperature, and the length of extraction (Johnston-Banks, 1990). The gelatin obtained is subsequently purified and clarified by filtration, deionization, concentration and lastly, sterilization (Schrieber and Gareis, 2007).

Gelatin can be classified into two types, depending on the pre-treatment procedure with either acid or alkaline. Type A gelatin is obtained under acid pretreatment conditions and exhibits an isoelectric point at pH 8–9, while type B gelatin is derived from alkaline pretreatment with an isoelectric point at pH 4–5 (Hanani, 2015). Employing acid or alkaline in pretreatment relies on the degree of cross-linking in raw materials (Gómez-Guillén et al., 2011). Normally acid is used for the immature collagens such as pig skin or fish skin, while alkaline works for the mature collagens with more complicated crosslinks such as bovine hide (Montero et al., 1990 and Hanani, 2015).

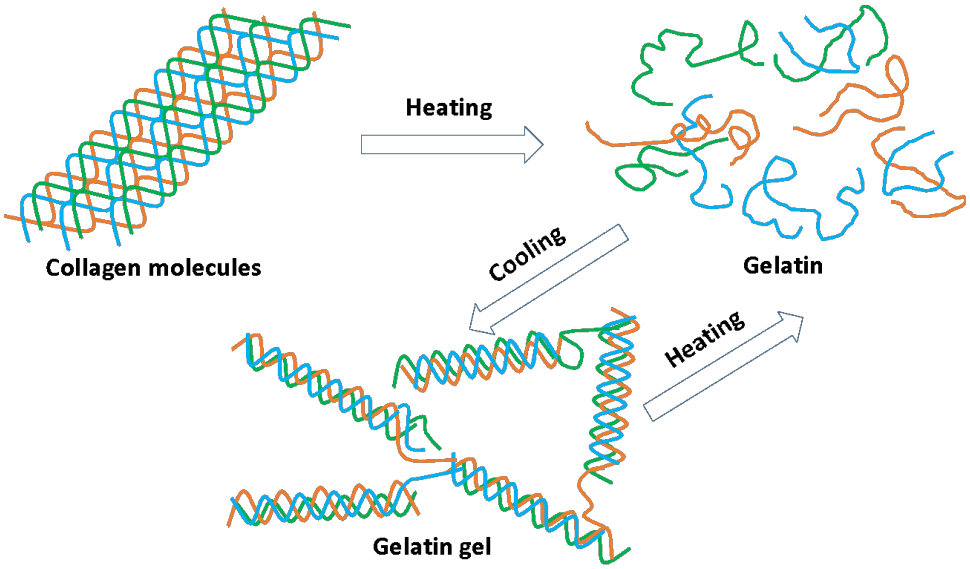


Figure 2.3. Conversion of collagen to gelatin by heating, and the formation of gelatin gel by cooling.

Gelatin is a mixture of different polypeptide chains of collagen, frequently presenting a typical band pattern of type I collagen in terms of molecular weight distribution, with a characteristic α1/α2 chain ratio of around 2 and the presence of β (two covalently linked α-chains) and γ (three α-chains) components (Stainsby, 1987). The properties of gelatin are dependent on various factors, such as the raw materials, the collagen type, and the production processes. For example, gelatin extracted from fish sources or extracted with more intense conditions, such as an extended extraction time or higher extraction temperature, will result in lower molecular weight fragments, which influences gelatin's functionalities (Johnston-Banks, 1990). Coming from collagen, gelatin contains large amounts of glycine (around 26%), proline (around 15%) and hydroxyproline (around 14%), and it lacks tryptophan and is deficient in cysteine, methionine, tyrosine and histidine (Eastoe, 1955). However, variations in amino acid composition are found among the gelatins prepared from different sources, ages, and different manufacturing processes, which also play an important role in the repertoire of gelatin properties (Hanani, 2015).

2.1.2.3. Gelatin properties and applications

The most important physicochemical property of gelatin is gelation, or the property to form a gel. Gelatin forms a gel during cooling and transforms to a liquid during heating (Figure 2.3). The mechanisms and the interactions leading to the formation of the gelatin gels are not well elucidated, but most studies agree that it is governed by the partial reformation of the three-dimensional network that formerly existed in the parent collagen. During cooling, the random-coiled polypeptide chains of gelatin orientate to certain configurations and subsequently produce junction zones by the partial formation of ordered triple helices as in collagen (Renard, van de Velde and Visschers, 2006; Saha and Bhattacharya, 2010; Ziegler and Foegeding, 1990). The

development of this three-dimensional network during cooling is capable of immobilizing the liquid and converts a viscous gelatin liquid into an elastic solid or gel (Djagny, Wang and Xu, 2001). Researchers have suggested that hydrogen bonds, electrostatic and hydrophobic interactions are involved in the formation of gelatin gel architecture (Bello and Vinograd, 1958; Ferry,1948; Schrieber and Gareis, 2007). The quality of gelatin is usually measured by gel strength or "Bloom" strength, named after Oscar T. Bloom who invented a bloom gelometer to measure the stiffness of gelatin in 1925. The Bloom values of commercial gelatins are within the range of 50 to 300 g, and those with higher Bloom indicate a stronger gel with higher melting and gelling points (Schrieber and Gareis, 2007). As mentioned previously, the properties of gelatin are affected by animal origins, method of processing, and the molecular weight and amino acid composition of gelatin molecules. The high molecular weight chains/aggregates and content of imino acids (proline and hydroxyproline) are important for forming strong gelatin gels (Benjakul, Kittiphattanabawon and Regenstein, 2012). In the food industry, based on the type of final product desired, gelatins with different Bloom strength are selected to achieve desired functional qualities and textures. Except for gelling and viscoelastic properties, other important functionalities include solubility, foaming ability, emulsification and film-forming ability (Gómez-Guillén et al., 2011).

Due to its versatility, gelatin has long been used in food, pharmaceutical, photographic and cosmetic applications. Since early 19th century, chilled and dried gelatin films have been utilized in pharmaceutical applications to produce hard capsules or soft capsules to encapsulate medicines and make them no longer taste bitter. Later on, gelatin dressings, surgical sponges and plasma expanders were developed. Gelatin was also used for coating tablets and making microcapsules to protect bioactive substances from light and oxygen (Wood, 1977). In the same period, gelatin was

used in photographic materials. In terms of cosmetics nowadays, gelatin is used widely in lipsticks, hair and skin care products (Schrieber and Gareis, 2007).

Currently, gelatin is broadly recognized as a food ingredient and its food applications represent the largest segment of the gelatin market. The prevalence of edible gelatin can be dated back to 1845, when the first gelatin dessert was made by Peter Cooper in the United States. In the 20th century, gelatin is used as an important ingredient in marshmallows, gummy bears and yogurts, all of which are extremely popular (Schrieber and Gareis, 2007). Pure, dry commercial gelatin is generally tasteless, odourless, and is a non-fat protein. In the food industry, gelatin is used extensively as a foaming agent and stabilizer, clarifier, gelling agent, and thickener, in products like desserts, baked products, dairy, meat products, and also in drinks containing tannins (Hsieh and Ofori, 2011). Gelatin can react with tannins to form precipitates that clarify the drinks. In the manufacture of marshmallows, gelatin is used as foaming agent to produce a stable foam system imparting a light and airy texture. Gelatin is commonly added as a stabilizer and texture enhancer in bakery products, such as cakes and breads, as well as in icing and dairy products. During cheese production, casein usually loses its emulsifying properties after fermentation, however the use of gelatin compensates for this loss and prevents the creaming of milk fat. Moreover, gelatin plays important roles in the meat industry by efficiently improving the water holding capacity, texture and taste of meat products (Hsieh and Ofori, 2011) in products such as meat jellies, sausages and meat loaves. The major applications of gelatin in foods are summarized in Table 2.3.

Table 2.3. Major gelatin applications in foods. The table is reproduced from Hanani (2015).

Product category	Function	Food products
Meat	Binding and glazing agent, casings, coatings	Canned meats, jellied meats, sausages, meat
Bakery and confectionary	Stabilizer, binding and gelling agent, emulsifier, elasticity and chewability improver, coating	Icing, glazes, creaming fillings, marshmallows, toppings, mousses, chiffons, gummy bears, gelatin and wine gums, toffees, chewing gums, compressed tablets, nougats, licorice, chocolate, and sugar-filling products
Dairy	Stabilizer, emulsifier, texture improver, water binder, increasing viscosity, fat replacement	Yogurts, creams, buttermilks, margarines, cheeses
Desserts	Stabilizer, gelling agent, coating	Pudding, custard, ice creams, milk ices, syrups, gravies, fruits
Wines and juices	Clarifying agent	Wines, beers, fruit juices, vinegar

2.1.3. Alternative gelatin sources

The raw materials for gelatin production have traditionally been skins or hides, bones, tendons and cartilages from pig and bovine sources. Approximately 80% of the edible gelatin in Europe comes from pigskin and 15% is from cattle hide split. The remaining 5% is produced from pig and cattle bones and fish (GME, 2016). In North America, pig skin is the major source for edible gelatin production, while bovine hide is the least preferred (Hsieh and Ofori, 2011). According to the report of Grand View Research, pig skin was the most used raw material for global gelatin production accounting for 40.7% in 2013, followed by bones (29.5%) and bovine hide (28.4%).

With the increasing demand for gelatin, some concerns and issues associated with the gelatin origins were made apparent. These concerns are mainly related to religious sentiments, the

transmission of pathogenic vectors from animals as well as sustainablility concerns (Karim and Bhat, 2009). For example, Muslims, Jews and Hindus do not consume pork-related and/or cow-related products due to religious reasons. The outbreak of bovine spongiform encephalopathy (BSE) also raised concerns about the use of any bovine-derived food ingredient, since foods contaminated with the BSE prion may be responsible for variant Creutzfeldt-Jakob disease (vCJD) (Hsieh and Ofori, 2011). In addition, the animal rights movement has made the public aware of issues concerning animals in the past twenty years, and more people act by the tenets of veganism. Promoting healthy diets and seeking "ethical" substitutes for animal-sourced ingredients become popular in vegans. Therefore, research that identifies and develops alternatives to mammal-derived gelatin are continuing to gain interest. From a sustainable point of view, the interest in economical and environmental friendly management of industrial by-products and wastes in meat industries provides a good concept for gelatin production.

2.1.3.1. Fish sources

Fish gelatin is considered to be a suitable alternative having been studied intensively and is readily available in the market. Surprisingly, waste from fish processing after filleting can account for as much as 75% of the total catch weight (Shahidi, 1994). About 30% of such waste consists of skin and bone with a large amount of collagen that can be utilized to produce fish gelatin (Gómez-Guillén et al. 2002). Until now, gelatin has been extracted from various cold-water (e.g., cod, hake, salmon and Alaska pollock) and warm-water fish (e.g., tuna, tilapia, catfish, shark and Nile perch) under different conditions (Karim and Bhat, 2009; Benjakul, Kittiphattanabawon and Regenstein, 2012). However, gelatins extracted from these sources, especially cold-water fish, have lower gel strength and make less stable gels than those derived from pig or cattle. This is

mainly attributed to their differences in molecular weight distributions and imino acid contents (Gómez-Guillén et al., 2011). This inferior rheological property of fish gelatin is a major limitation for its applications. To improve its rheological properties, research has been conducted on modifications of fish gelatin with other substances, such as salts, glycerol, glutaraldehyde, sugars, and enzymes (Alfaro et al., 2014). The insufficient availability of a particular fish type, inconsistent gelatin quality due to the variable composition of the sources, and allergy risks are other issues that undermine using fish gelatin. In addition, the unpleasant odour and its high price due to trends in relative low yields are continuing challenges for fish gelatin manufacturers (Karim and Bhat, 2009).

2.1.3.2. Poultry sources

Poultry sources like chicken skin, feet, meat residues and bone have also been developed as gelatin sources, but the literature is still limited. In 2001, Liu, Lin and Chen (2001) and Lim, Oh and Kim (2001) extracted gelatin from chicken feet and evaluated the effect of processing conditions on gelatin characteristics. The gel strength and texture profile of chicken feet gelatin were determined by Almeida and Lannes in 2013, who demonstrated that chicken gelatin had a high Bloom value and great texture profile. Rafieian, Keramat and Kadivar (2011) and Rammaya, Voon and Babji (2012) confirmed that deboned chicken meat residue can be used as a raw material for gelatin extraction and optimized the extraction conditions. Another very comprehensive study with poultry gelatin obtained from chicken skin was reported by Sarbon, Badii and Howell (2013), showing superior Bloom strength and rheological properties of chicken skin gelatin compared to bovine hide gelatin. A high imino acid content in chicken skin gelatin is important for the gelling process and gel strength (Nik Aisyah et al., 2014). Gelatins from other poultry sources: duck and

other birds are under investigation as well (Huda et al., 2013; Lee et al., 2012). Currently no poultry gelatin is commercially available in the market, which may due to the greater price of poultry sources and lower availability of poultry materials compared to mammalian gelatin (Nik Aisyah et al., 2014). However, these recent findings proved that poultry gelatin has good potential as an alternative due to its excellent gel quality. More poultry by-products should be investigated, such as low value mechanically separated meat; for example, chicken and turkey heads could also be considered as raw materials for gelatin production to meet the increasing demands.

2.1.3.3. Insects

In certain countries such as Sudan, insects are also regarded as an additional source of gelatin. The most well-known ones are melon bug (*Aspongopus viduatus*) and sorghum bug (*Agonoscelis pubescens*), which were extracted to obtain oil for cooking and medical purposes, as well as for producing gelatin. The FTIR spectra of insect gelatin is similar to that of commercial gelatin (Mariod and Adam, 2013) and it contains all essential amino acids; however, the quantity of insect protein produced is limited (Hanani, 2015).

2.1.3.4. Yeasts

Gelatin produced from yeast systems has also been described by microbiologists and it always required a co-expression of animal prolyl 4-hydroxylases (Duan et al., 2011; Nokelainen et al., 2001; Olsen et al., 2001; Toman et al., 2000; Vaughan et al., 1998; Vuorela et al., 1997). However, Bruin et al. (2002) found that a methylotrophic yeast *Hansenula polymorpha* unexpectedly had the endogenous capacity for prolyl 4-hydroxylation and consequently was able to produce gelatin without the need for co-expression. All these findings proved the possibility of yeast to be an alternative source for gelatin preparation. Besides, the advantage of this

environmental friendly technique is to produce non-animal derived and quality-controllable gelatin. However, the scale-up production and the acceptability for consumers are still the concerns.

2.2. Novel cryoprotectants in food production

2.2.1. Common cryoprotetcants and cryoprotection mechanisms

2.2.1.1. Deterioration of foods during freezing and frozen storage

Freezing is one of the most common means of food preservation in the industrialized world. Refrigeration has been used to effectively extend the shelf-life of food since Paleolithic and Neolithic times, when people used ice and snow to cool food and inhibit spoilage. Developments in freezing and frozen foods technology represent a technological revolution in the later half of the twentieth century. Even today, there is a growing need for frozen processed foods in consumers' lifestyles, with most modern households having access to a freezer to store and use ready-to-cook foods conveniently. At the end of the twentieth century, the frozen food market increased at approximately 10% per year, with about 25% of refrigerated food being frozen (Evans, 2008). The long history of freezing preservation of foods has demonstrated that it effectively lowers the activity of microorganisms and enzymes. Ice crystallization during freezing reduces the amount of liquid water which further inhibits bacterial growth and retards the deterioration of food products (Sun, 2012).

However, ice formation during freezing is often associated with freezing damage of food products, either directly through mechanical effects created by ice crystals or indirectly by concentrating solutes in the unfrozen phase (Zaritzky, 2012). Slow freezing rates, improper storage temperatures, temperature fluctuations generating freeze-thaw cycles during frozen storage, and

deficient food packaging can all catalyze recrystallization and the formation of large ice crystals, which usually increases the degree of freezing related damage, resulting in the loss of food quality (James, 2008; Reid, 1997).

In the case of food tissues, both extracellular and intracellular ice crystals are created when freezing commences, causing cell dehydration, osmotic stress and the breakage of cell membranes, particularly at low freezing rates that favor large extracellular ice formation (Nesvadba, 2008). Freezing damage involves multiple and interrelated physical and chemical changes in food systems. In general, except for the physical changes mentioned above, cell volume expansion (water convert to ice), dislocation of water (osmotic stress) and mechanical damage by ice, other physical modifications of food systems also occur. These include cracking during the freezing process, moisture migration where moisture irreversibly moves to the internal package surface, freezer burn where the food surface is desiccated due to the inadequate packaging, and ice recrystallization to increase the ice crystal size during frozen storage (Zaritzky, 2012).

In terms of chemical changes, freezing and frozen storage can affect the chemical reactions and chemical characteristics of food products, downgrading the food quality. The major chemical changes that proceed during freezing and frozen storage are enzymatic reactions, protein denaturation and oxidation, deterioration of lipids and loss of vitamins (Zaritzky, 2000). Storage at low temperatures can decrease the activity of enzymes but does not inactivate them, therefore some hydrolytic enzymes, lipolytic enzymes and proteases remain active during frozen storage. The presence of ice crystals can disrupt the frozen tissues and release these enzymes and chemical substances to influence food properties, causing for instance lipid oxidation, rancidity, and the destruction of pigments (Zaritzky, 2012). Freeze-induced damage to protein is mainly associated

with ice formation and recrystallization, dehydration, exposure to concentrated solutes and oxidation, all of which may cause protein denaturation, protein aggregation, protein oxidation and loss of protein functionalities (Gonçalves, Nielsen and Jessen, 2012). Furthermore, the loss or degradation of water-soluble vitamins in foods is another detrimental consequence of freezing. Temperature fluctuations, including repeated freeze-thaw cycles during frozen food transportation and storage, play a major role in the changes in food quality (Singh, 2000).

Freezing damage exists in various food systems. For example, the formation of large ice crystals in ice cream is undesired since it results in an undesirable coarse texture; solid foods from living tissues such as meats, fruits and vegetables with delicate cell structures are prone to be damaged by ice crystals; freeze-induced lipid oxidation, protein denaturation and related functionality losses are commonly observed in frozen fish, meats, poultry and their products, egg products and dough (Potter and Hotchkiss, 1995; Zaritzky, 2000).

2.2.1.2. Conventional cryoprotectants used in foods

Cryoprotectants are usually added prior to freezing in food formulation in order to mitigate deleterious changes during the freezing process and frozen storage, since maintaining a proper and constant temperature during food frozen storage is not always feasible. The incorporation of cryoprotective compounds ensures the stability and extends the shelf-life of food products during frozen storage and thawing. Regarding foods with intact cell structures such as vegetables, fruits and meats, retaining their cellular structure and controlling water movement are the major concerns. Hence cryoprotective efforts mainly focus on applying novel technologies in both freezing methods and packaging of these types of foods (Silva, Gonçalves and Brandão, 2008). On the other hand, for formulated foods, cryoprotectants are always mixed with the food materials themselves

to preserve important components and features (MacDonald and Lanier, 1997). For instance, cryoprotectants help stabilize proteins in meat and fish products (e.g. meatballs and surimi), maintain the smooth texture of frozen desserts, and preserve the rheological properties and sensory attributes of frozen bakery products.

The most common cryoprotectants used in frozen food products are carbohydrates and sugar alcohols, especially sucrose and sorbitol, due to their low cost and high efficiency. Of particular interest is their application on Surimi products. Surimi is a wet concentrate of myofibrillar proteins, which is obtained by washing away the fat, sarcoplasmic proteins and connective tissues from the raw animal flesh, and importantly, followed by the addition of cryoprotectants to maintain the protein functionalities during frozen storage (MacDonald and Lanier, 1994; Zhou et al., 2006). Sych et al. (1990) investigated the cryoprotective effects of different materials on cod-surimi proteins during frozen storage, and found that sorbitol, glucose, sucrose and sucrose/sorbitol at a level of 8% (w/w) showed the best protein stability. Phosphate has been reported to minimize the negative impact of frozen storage on protein, and it is usually added in combination with sugars/polyol in meat systems. Rather than being treated with phosphate or sugar/polyol alone, better stabilization of mullet myofibrils during frozen storage treated with a combination of sugar/polyol and phosphate was observed by Park and Lanier (1987). Nowsad et al. (2000) also demonstrated that the combination of 8% sucrose/sorbitol (1:1) and 0.2% Na-tripolyphosphate had a beneficial effect on frozen stored spent hen meat mince, with improved protein functionalities. Commercially, a blend of 4% sucrose, 4% sorbitol and 0.3% phosphate (typically sodium tripolyphosphates or hexametaphosphates) is used as cryoprotectant in manufacturing of fish, meat or poultry mince products (Jaczynski et al., 2012).

A commercial cryoprotective blend can effectively alleviate the influences of freezing or frozen storage, however it imparts an unfavourable sweet taste to final products, especially meat products. For this reason, other types of low sweetness carbohydrate-based cryoprotectants have been explored. Lactitol, Litesse®, Palatinit®, polydextrose®, trehalose, and some polysaccharides, such as maltodextrin and chitosan have revealed a cryoprotective potential in food products (Auh et al., 1999; Carvajal, MacDonald and Lanier, 1999; Dey and Dora, 2011; Herrera and Mackie, 2004; Miura Takayanagi and Nishimura, 1992; Park, Lanier and Green, 1988; Sych et al., 1990; Zhou et al., 2006). Additionally, a group of hydrocolloid ingredients have been used in ice cream formulations as stabilizers, to create a smoother texture and retard ice crystal growth in ice cream during frozen storage. The functionality of several hydrocolloids (carboxylmethylcellulose, guar gum, sodium alginate, xanthan gum and κ-carrageenan) related to the rheological, physical and sensory characteristics of ice cream was studied by Soukoulis, Chandrinos, and Tzia (2008). They concluded that the addition of hydrocolloids improved the mouth feel, flavor perception and stability of frozen ice cream. The cryoprotective effect of hydrocolloids has been mainly attributed to the increased viscosity of food systems that limits water mobility, thus controlling the growth of ice crystals. However, the effectiveness of stabilizers in ice cream also relies on the particular formulation ingredients, most critically water and sugar contents (Goff, 2012).

The cryoprotective property of amino acids was also studied in protein systems; it was hypothesized that active amino acids would contain as least two of the following functional groups: -COOH, $-NH_2$, $-SO_3H$, -OH, -SH, =O, or an imino group. (Noguchi and Matsumoto, 1975). Later, Jiang et al. (1987) substantiated part of the findings of Noguchia and Matsumoto (1975), but also suggested that the cryoprotective capacity of certain amino acid is highly pH dependent. A

protective effect was noticed when the isoelectric point of the particular amino acids was lower than the pH of the protein solution.

2.2.1.3. Mechanisms of cryoprotection in frozen foods

Different mechanisms of the common cryoprotective substances have been proposed over the years. The three well accepted theories include 1): preferential exclusion of cryoprotectants from the surface of proteins; 2) increasing glass transition temperature; 3) freezing point depression.

As early as 1980, Matsumoto proposed a possible mechanism for cryoprotection, in which cryoprotectants were considered to interact with protein directly through hydrogen or ionic bonds. The cryoprotectant coated protein can then hardly take part in reactions with other protein side chains, thus stabilizing the protein structure and retarding protein unfolding and aggregation in frozen conditions.

Afterwards, the preferential solute exclusion theory evolved. Arakawa and Timasheff (1982) showed that the addition of sugars (lactose and glucose) in protein solution resulted in an unfavorable free-energy change (increased surface tension of water), which led to the exclusion of the sugar from the protein surface, leaving the proteins in a more hydrated state and therefore protected during the freezing process. Carpenter and Crowe (1988) tested 28 different compounds including sugars, polyols, amino acids, and salts, to protect lactate dehydrogenase from freeze-thawing damage. This study provided more evidence consistent with the preferential exclusion hypothesis, and also suggested that steric hindrance effects may explain why certain large-size molecules are preferentially excluded from contact with proteins, where water can form hydrogen bonds to almost any region on a protein's surface without geometric constraint. The phenomenon

of preferential solute exclusion has been explained by the amphiphilic nature of proteins (Park, 1995). He illustrated that the hydrophobic fractions on the protein surface impede the formation of hydrogen bonds between the protein surface and some hydrophilic compounds, such as sugars. Therefore, sugars are excluded from the protein surface and the protein is then surrounded by a water shell, maintaining its native conformation and protected from freeze-denaturation.

Low molecular weight cryoprotectants protect proteins by altering the thermodynamics of the native protein state, whereas high molecular weight polymers increase the glass transition temperature to accelerate the conversion of a food system to the glass state during freezing (Levine and Slade, 1988ab). When a solution transitions into a glass state, the mobility of molecules and all the reactions in the system slow down greatly. This "cryostabilization" can effectively minimize freeze-induced damage of food systems by immobilizing water in the glass structure and significantly retard the deterioration processes (Park, 1995). The relationship between the solute concentration and the glass transition temperature was illustrated by Roos and Karel (1991) as shown in Figure 2.4.

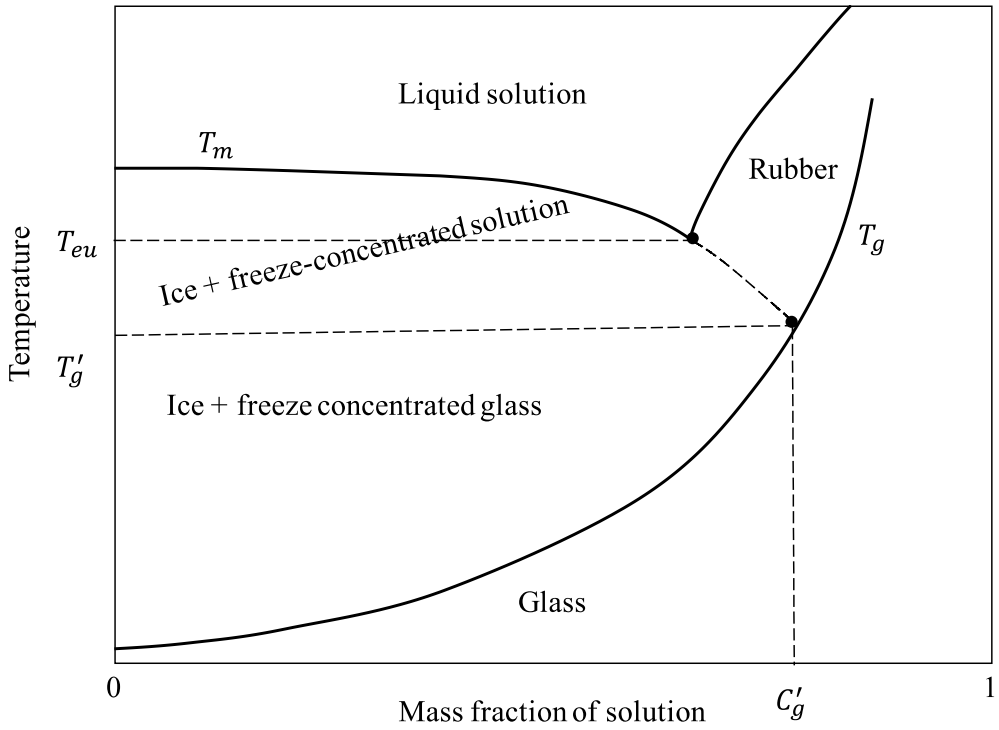


Figure 2.4. Schematic phase diagram of a solute-water binary system. Tg represents the transition into the glass phase. The figure is reproduced from Zaritzky (2012).

Another important mechanism of common cryoprotectants is freezing point depression; a colligative property which solely depends on the concentration of the solute added, especially small molecular weight compounds, such as sugars, salts etc. The degree of depression is directly proportional to the molar concentration of solutes (Zaritzky, 2012). The presence of solute lowers the effective number of solvent molecules that can produce a phase transition from liquid to solid. Even at low temperatures, there still exists some water molecules not presenting as "ice"; not in the solid phase. With increasing solute concentration, the freezing point is depressed leaving a portion of the water unfrozen in the system, thus minimizing to some extent the damage induced by ice formation (Jaczynski et al., 2012).

Nowadays, the main challenge in food cryoprotection is to develop cryoprotectants with less sweetness than the standard sugars used, yet still have minimal negative impact on the food's textural, rheological and sensory characteristics.

2.2.2. Antifreeze proteins

In nature, "cryoprotectants" exist in a wide range of living organisms, such as Antarctic fish, insects, plants and fungi thriving under subzero environments that most organisms could not tolerate. These antifreeze proteins (AFPs) synthesized within these organisms, also called thermal hysteresis proteins, play a key role in protecting biological tissues from damage during harsh winters or in cold conditions, mainly by depressing the freezing point in a non-colligative manner and inhibiting the growth of ice (Griffith and Ewart, 1995).

AFPs were first discovered in the blood of Antarctic fish in the early 1970s. The temperature of Arctic and Antarctic ocean water is around −1.9 °C, but only 40–50% of the freezing point depression in the Antarctic fish blood can be accounted for the colligative effect of electrolytes such as sodium chloride (Duman and DeVries, 1975). Small proteins were found to be responsible for the remainder of this depression. Across all species studied, these AFPs depress the freezing point of body fluids by thermal hysteresis, a non-colligative process that does not affect the melting point at the same time (Wathen and Jia, 2005).

Shortly after the discovery of AFPs in fish, proteins with this ice-growth inhibitive activity were discovered in other biological systems such as animals, insects, plants and microorganisms (Cheng, 1998).

2.2.2.1. Antifreeze proteins in various sources

1) Fish

Table 2.4. General characteristics and structures of fish AFPs.

Characteristic	AFGP	Type I AFP	Type II AFP	Type III AFP	Type IV AFP
Mass (kDa)	2.6-33	3.3-4.5	11-24	6.5	12
Primary structure	(AAT) repeat	A rich	Disulfide stabilized	General	Alanine rich or glutamine/glutamate rich
Amino acid bias	>60% A; >30% T	A ~60%	Cysteine rich 9%	General	Glutamine/glutamate rich (26%)
Secondary structure	Extended 3-fold helix	α-Helix	Mixed coil	β-sandwich	α-Helix
Tertiary structure	Extended	Single α-helix	Globular, C-type lectin fold	Globular	Helical bundle
Heterogeneity	Polymer 1–8	Repetitive/ nonrepetitive	Ca dependent/independent	Isoforms, pIs 6–10 dimer	NA
Ice binding	Close to 10–10	20-21/2-1-10	11–21/?	Close to 10-10	NA
Homologues or antecedents	Trypsinogen gene	NA	Ca-dependent lectins and Li thostathine	NA	Lipoprotein domain
Natural source	Antarctic noothenioid; northern cods	Right-eyed flounders (winter flounder, two yellow tail flounder); Sculpins (Shorthorn Grubby)	Sea raven, smelt, herring	Ocean pout, wolfish, eel pout	Longhorn sculpin
Expression or synthesis	Chemical synthesis or natural source	Recombinant and natural source	Recombinant and natural source	Recombinant and natural source	Natural source

AFPs have been classified into two main groups: non-glycosylated antifreeze proteins and antifreeze glycoproteins (AFGPs) (Hew and Yang, 1992). AFGPs constitute the largest fraction of protein in the blood serum of Antarctic notothenioids and Arctic cod, consisting of repeating units of $(Ala\text{-}Ala\text{-}Thr)_n$ with a connection to a disaccharide moiety (Galβ1-3GalNAcα1-) at the Thr

residue. Their molecular mass ranges from 2.6 kDa to 33 kDa (Harding, Anderberg and Haymet, 2003). Aside from AFGPs, the other AFPs from fish have been classified into 4 types (type I to type IV) in the order of their discovery, with type I AFP being the most extensively studied. Several researchers have compared and reviewed the properties of AFPs among different types of fish (Cheng, 1998; Davies and Sykes, 1997; Davies et al., 1999; Ewart et al., 1999; Fletcher et al., 1998; Yeh and Feeney, 1996). The characteristics of different AFP types are presented in Table 2.4 (Davies and Sykes, 1997; Venketesh and Dayananda, 2008).

According to Fletcher et al. (1998) and Ewart et al. (1999), the thermal hysteresis or freezing point depression effect of fish AFPs is between 0.6 ºC and 1.5 ºC. This is less compared to the AFPs obtained from other species, such as insects. All the types of fish AFPs appear to function by binding to ice crystals and inhibiting the growth of ice in a certain direction (Davies and Hew, 1990). Wilson et al. (2002) attributed this moderate thermal hysteresis to the fact that the fish AFPs can bind to only one plane on the ice crystal, although at higher concentrations they may be able to bind to additional faces. However, another study from Marshall et al. (2004) uncovered a new AFP from winter flounder which showed equivalent hyperactivity to insect AFPs, providing a better explanation for the freezing resistance of polar fish.

2) Plants

AFPs have been found in many cold acclimated plants. Plant AFPs possess a low thermal hysteresis effect with only 0.2 ºC to 0.4 ºC depression, however they have an extraordinary effect on inhibition of ice recrystallization (Venketesh and Dayananda, 2008). Griffiths and her colleagues (1992) were the first to report the ice-inhibition activity of extracts obtained from plants, and since then, many studies have been carried out on this phenomenon. AFPs have been found in

different parts of plants, including seeds, stems, crowns, barks, branches, leaves, flowers, etc. (Griffith and Ewart, 1995; Atící and Nalbantoğlu, 2003; Griffith and Yaish, 2004). The common names of plants showing this activity are listed in Table 2.5.

Griffith and Ewart (1995) and Sidebotton et al. (2000) presented the outstanding inhibitive activity of winter rye AFPs on ice recrystallization; winter rye AFPs in solutions can exert the inhibition effect at as low concentration as 25 μg/L. Regand and Goff (2005, 2006) reported that sucrose solutions containing more than 0.05% of total protein from winter wheat grass extracts significantly reduced the ice growth under frozen conditions, and the presence of 0.13% winter wheat grass protein in the solutions diminished ice growth as much as 74%.

Table 2.5. Cold-acclimated plants containing AFPs. The table is reproduced from Hassas-Roudsari and Goff (2012).

Common Names					
Aarrow leaf plantain	Buttercup	Daylily	Heath rush	Potato	Water sedge
Antarctic hairgrass	Butterfly bush	Douglas fir	Heather	Prickly burr	Weeping forsythia
Bamboo	Cabbage	Dutchman's breeches	Jacob's ladder	Shasta fern	White oak
Barley	Carrot	Eastern cottonwood	Kale	Sheep sorrel	Winter & spring canola
Beech	Chickweed	Elder	Maple	Spurge	Winter & spring rye
Bittersweet nightshade	Chickweed	Evergreen legume	Oat	Timothy	Winter & spring wheat
Bluegrass	Crack willow	Fescue	Peach	Tussac grass	Winter cress
Brown rush	Creeping bentgrass	Garlic mustard	Perennial ryegrass	Violet	Wood aster
Brussel's sprout	Dandelion	Geranium	Periwinkle	Virginia waterleaf	Wood sorrel

This excellent ice-inhibition activity of plant AFPs has been linked with their unique structures that have multiple and hydrophilic ice-binding domains. Some plants secret these AFPs into the apoplast and their accumulation here can effectively inhibit the growth of extracellular ice and attenuate the cell damage in freezing conditions. Additionally, AFPs from winter rye and some other plants are homologous to phathogenesis-related proteins which provide protection against psychrophilic pathogens (Griffith and Yaish, 2004). The molecular weight of plant AFPs vary in species, ranging from 12 kDa to 67 kDa based (Hassas-Roudsari and Goff, 2012)

3) Insects

Insect AFPs are considerably more active than those from fish and plants. Their maximum activity is 3–4 fold greater than those from fish and they are 10–100 fold more effective at micromolar concentrations (Graether et al., 2000). Around 50 species of insects (Duman et al., 2004) and many terrestrial arthropods (Tursman and Duman, 1995; Sjursen and Somme, 2000; Duman et al., 2004) have been found to contain these hyperactive AFPs in their hemolymph. While representative species from several families of insects are known to produce AFPs, the majority are beetles (Duman et al., 2004).

Granham et al., (1997) purified an AFP from *Tenebrio Molitor* (yellow mealworm), which is a threonine and cysteine rich protein with a molecular mass of about 8.4 kDa. It showed a 5.5 ºC thermal hysteresis at a concentration of only 1 mg/mL. The ice-binding properties of AFP from spruce budworm（*Choristoneura fumiferana*） was reported by Graether et al. (2000), and the structure of this 9 kDa AFP was characterized as a combination of β-helix and β-sheets. The β-helix and β-sheet structure, as well as the repetitive amino acid motif (Thr-X-Thr) found in insect AFPs, are believed to be important for the interaction with the ice surface, contributing to the

hyperactivity of these particular AFPs (Graether et al., 2000; Graham et al., 1997; Hakim et al., 2013; Leinala, Davies and Jia, 2002; Li et al., 1998; Strom, Liu and Jia, 2005). In a recent study from Graham and Davies (2005), the AFP from winter-active snow fleas was found to have a distinct structure composed of repeating Gly-X-X (glycine-rich) units, with a molecular mass ranging from 6.5 to 15.7 kDa. These snow fleas AFPs have a 5.8 ºC depression of freezing point from melting point. For some insects that can survive at very low temperatures (-40 ºC to -70 ºC), the AFPs in their bodies were considered functionally differently from the AFPs in most insects, since the several degrees of thermal hysteresis is insufficient for them to stay alive under those extreme conditions. The ice-growth suppression activity likely plays a major role in protecting their cells from ice damage, however, the particular functions of these AFPs are still unclear (Duman et al., 2004).

4) Bacteria and fungus

Within the last two decades, AFPs have been found in a variety of bacteria from cold habitats (Lorv, Rose and Glick, 2014). The AFPs from these bacteria commonly possess weak thermal hysteresis values compared to animals, but with good ice recrystallization inhibition activities (Duman and Olsen, 1993; Sun et al., 1995; Yamashita et al., 2002). Consequently, studies focusing on bacterial antifreeze activity mainly measure ice recrystallization inhibition and ice morphology shaping. *Pseudomonas putida* GR12-2 is originally isolated from the Canadian Arctic, it is capable of proliferating at low temperatures like 5 ºC, and surviving at freezing temperatures as low as -20 ºC to -50 ºC without the assistance of cryoprotectants. AFPs from this bacterium showed a low thermal hysteresis of 0.11 ºC, and the ice crystals were shaped into hexagonal bipyramids. Gilbert et al. (2004) isolated 19 bacteria strains that produce AFPs from Antarctic

lakes, and demonstrated they all showed a significant recrystallization inhibition activity. In the bacterium *Marinomonas primoryensis*, a highly active AFP with a thermal activity of 2 ºC at 0.1 mg/mL has been observed similar to insects and fishes, pointing out that the thermal hysteresis strategy does also exist in bacteria (Gilbert et al., 2005). Moreover, to thrive under subzero temperatures, bacteria (particular Gram-negative bacteria) were also reported to produce ice-nucleation proteins. These proteins can significantly reduce supercooling and catalyze ice nucleation, thus leading to shortening of freezing time (Wang and Sun, 2012).

Fungal AFPs have been discovered in snow molds; these fungi have pathogenic activities against dormant plants under snow cover. Snow molds include two major fungal taxa of ascomycetes and basidiomycetes, and one pseudofungal taxon of oomycetes. Among them, AFPs were only identified in the basidiomycetes *Coprinus psychromorbidus* and *Typhula ishikariensis* (Xiao et al., 2009). Their thermal hysteresis and ice modification activities have been reported in either their cellular extracts or the isolated AFPs (Duman, J. and Olsen, 1993; Venketesh and Dayananda, 2008). Recently, Kondo et al. (2012) reported the crystal structure of a 223-residue secreted AFP from the snow *Typhula ishikariensis*, whose main structural element is an irregular β-helix with six loops of 18 or more residues that lies alongside an α-helix. β-helices have been involved in AFPs from different origins and seem ideally structured to bind to several planes of ice.

2.2.2.2. Proposed mechanisms

As mentioned previously, AFPs come from different organisms, and their structure and amino acid compositions are diverse. However, they present the same two unique functions. Firstly, they are able to depress the freezing point of water in the presence of ice crystals in a non-

colligative manner (thermal hysteresis), and secondly they can inhibit ice crystal growth. Although the specific mechanism behind this has not been completely elucidated, the "adsorption-inhibition mechanism" is generally recognized as the mechanism by which these AFPs work. Here, they have the ability to bind to ice and restrict the ice growth at that point, therefore smaller ice crystals grow with a curvature on the ice surface between the bound AFPs instead of growing freely (Figure 2.5) (Barret, 2001; Venketesh and Dayananda, 2008; Yeh and Feeney, 1996). The concept is exemplified in Figure 2.5. After Raymond and DeVries (1977) first pointed out this theory, a possible explanation for the thermal hysteresis was provided by the Gibbs– Thomson model by Yeh and Feeney (1996).

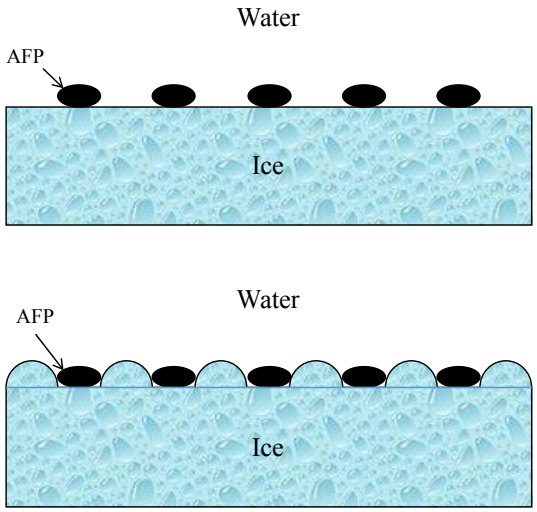


Figure 2.5. Model of the adsorption-inhibition mechanism, adapted from Barret (2001).

The lowering of freezing temperature (ΔT) due to the presence of finitely sized ice crystals is given by:

$$\Delta T = \frac{2\Omega\gamma T_0}{\rho_{min}\Delta H_0}$$

where Ω is the molal volume of ice, γ the isotropic surface energy, T_0 is the transition temperature of pure water, ρ the ice crystal radius and ΔH_0 the latent heat of fusion. Once the mixture freezes, ρ approaches ∞ and ΔT becomes zero. Hence, the equation describes a freezing hysteresis that is directly proportional to the interfacial energy and inversely proportional to the radius of the ice crystal. Therefore, the modified ice front that results from the adsorption-inhibition mechanism will experience a depressed freezing temperature. The AFPs may be adsorbed on the ice crystal surface via some types of interaction at the ice-water interface (Chapsky and Rubinsky, 1997; Grandum et al., 1999). Wen and Laursen (1993) proposed a two-step adsorption mechanism; at low AFP concentrations reversible hydrogen-bonding between AFPs and ice exists; once the concentration exceeds a certain point, the AFP molecules on the surface begin to interact and pack together resulting in a cooperative intermolecular interaction. However, Davies et al. (2002) suggested that the van der Waals and hydrophobic effects are more important than hydrogen bonding during AFPs adsorption.

The structure of AFPs has a major influence on their antifreeze activity, since it is responsible for the affinity and specificity of ice-binding. The binding models of some typical AFPs have been studied extensively (Baardsnes et al., 1999, Chen and Jia, 1999; Davies et al., 2002; Hakim et al., 2013; Jia et al., 1996; Kuiper et al., 2015; Leinala, Davies and Jia, 2002; Patel and Graether, 2010; Sicheri and Yang, 1995; Strom, Liu and Jia, 2005). The isomer (HPLC-6) of type I AFPs from winter flounder is an alanine-rich single α-helix with 37 amino acids, that contains three 11 amino acid repeats (Thr-X_2-Asx-X_7), where X is generally Ala (DeVries and Lin,1977; Davies, Roach and Hew) (Figure 2.6a). The regularly spaced Thr, Asx and Leu residues lie on one flat face of the helix and have been thought to form hydrogen bonds with oxygen atoms

on the prism plane and/or pyramidal plane of ice. This is important since water molecules have the same periodicity on the ice surface. In particular, the proposed distance (4.5 Å) between the threonine hydroxyl and carboxyl groups of aspartate matches to the spacing (4.5 Å) between the oxygen atoms of water molecules on prism planes of a hexagonal ice crystal, so that the AFPs can be aligned on the ice face and interact with water molecules (DeVries, 1984; knight et al., 1991). Later, Baardsnes et al. (1999) proposed another ice-binding site - Thr-Ala-Ala of the HPLC-6 isomer, suggesting that steric interaction and the hydrophobic effect are the determining energetic factors in binding between AFPs and ice. The ice morphology with the addition of type I AFPs is modeled in Figure 2.6b.

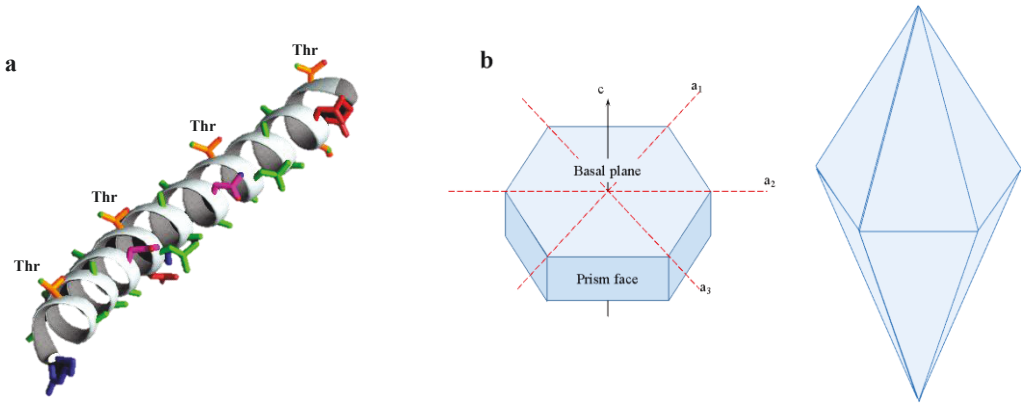


Figure 2.6. a: Model structure of the type I AFP from winter flounder (Sicheri and Yang, 1995). The model is adapted from http://pout.cwru.edu/~frank/index.html with permission. b: The left one is a common hexagonal ice crystal, and the right one is the modified ice crystal by the type I AFPs during growth. The AFPs are adsorbed to the prism/pyramidal faces of ice crystal and shape the ice into a bipyramidal configuration.

The highly active AFP from the spruce budworm, *Choristoneura fumiferana*, (sbwAFP) provides an excellent model for antifreeze simulations (Graether et al., 2000; Kuiper et al., 2015; Nada and Furukawa, 2011; Nutt and Smith, 2008). It has a β-helix structure with a triangular cross-section forming three parallel β-sheet faces and a hydrophobic core (Figure 2.7). The regular array

of repeating Thr-X-Thr motifs may be the ice binding face, in which the spacing between the threonines in the neighboring Thr-X-Thr motifs ideally match to the repeating dimensions of 4.5 Å along the a-axis in both prism and basal planes of ice (Figure 2.7b). At right angles to this repeating dimension, the distance between threonines in the TXT motifs makes an ideal match to the 7.35 Å spacing on the prism plane (Figure 2.7a), and a fairly close match to the 7.8 Å spacing on the basal plane (Figure 2.7c) (Graether et al., 2000). The binding ability of sbwAFP to both of the prism and basal planes may be the key to its exceptional activity (Drori et al., 2014). Pertaya et al. (2008) directly visualized the adhesion of fluorescently tagged sbwAFP on basal planes of ice crystals with fluorescence microscopy. They found that the sbwAFP accumulated at the six prism plane corners and the two basal planes of hexagonal ice crystals. While bipyramidal ice crystals are found in type I AFP solutions, a hexagonal plate shape of ice results with the sbwAFP.

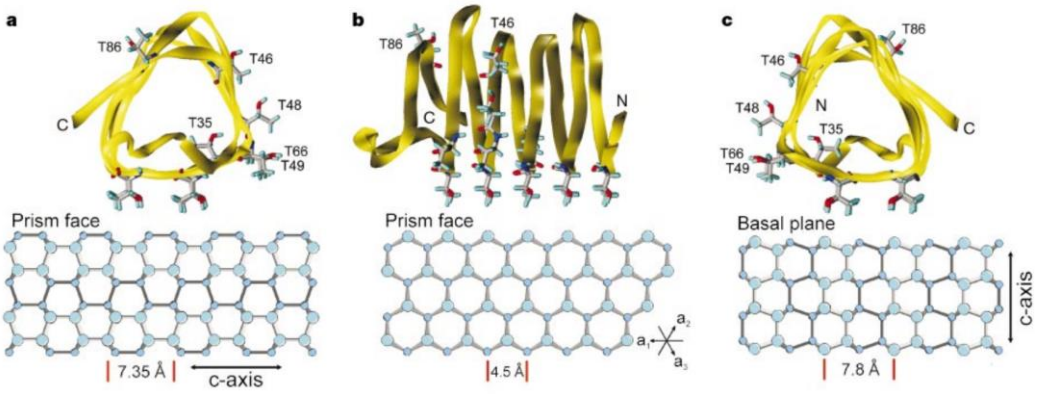


Figure 2.7. The ice-binding model of sbwAFP on different ice faces. Model source is from Graether et al. (2000), with permission of the Nature Publishing Group.

Another reasonable mechanism of AFP is the ice nucleation theory. From the functional point of view, prevention of ice nucleation minimizes uncontrolled ice crystal growth. However, evidence to support this theory is still not sufficient (Barret, 2001). In 2003, the first study on the

effect of AFPs on ice nucleation was investigated using a newly developed technique called the "double oil layer micro-sized ice crystallization technique" by Du, Liu and Hew (2003). Here they found type III AFP can inhibit the ice nucleation process by adsorbing onto both the surface of ice nuclei and that of dust particles.

2.2.2.3. Applications of AFPs in food

The applications of AFPs are promising for frozen food products. AFPs are able to inhibit recrystallization of food systems during frozen storage, transportation and thawing, thus preserving food texture and quality (Venketesh and Dayananda, 2008). Since AFPs are located in extracellular spaces in freezing tolerant organisms, this implies that these proteins can be added to foods by physical means such as mixing, injection, soaking or vacuum-infiltration (Griffith and Ewart, 1995). However, so far the literature on the use of AFPs in food applications is still limited; most of the studies were conducted in only few selected food systems, such as frozen dairy or ice cream products, meat, fish and frozen dough products (Table 2.6) (Ustun and Turhan, 2015).

Table 2.6. Previously studied food applications of AFPs. Table is reproduced from Ustun and Turhan (2015).

Source of AFP	Product used	References
Fish	Ice cream	Feeney and Yeh, 1998
Fish (type III AFP)	Ice cream	Clarke et al. 2003
Antarctic cod and the winter flounder	Frozen and chilled meat	Payne et al. 1994
Antarctic cod	Frozen meat	Payne and Young, 1995
Tilapia hybrids (type III AFP)	Frozen and chilled fish	Boonsupthip and Lee, 2003
Polar fish grubby sculpin	Frozen dough	Panadero et al. 2005
Winter wheat grass	Ice cream	Regand and Goff, 2006
Carrot	Frozen dough	Zhang et al. 2007; 2008
Winter wheat	Frozen dough	Xu et al. 2009
Carrot	Frozen noodles	Ding et al. 2014

The limitations of AFPs in commercial food applications are attributed to several factors. Firstly, is the cost of production and purification of AFPs from natural sources. Currently, the isolation and purification of AFPs involves complicated and time-consuming processes with low yields, and thus is not favorable for commercial development (Ustun and Turhan, 2015). The chemical synthesis of analogous compounds would theoretically be much cheaper and commercially viable. The direct use of concentrated extract containing AFPs has been considered; however, it is also problematic because naturally occurring enzymes in the extract could be detrimental to the food preservation. Applying an enzyme inactivation to the extract would likely impact the structure and activity of AFPs as well (Kontogiorgos et al. 2007). Today, both the AFGP and the AFPs from winter flounder are available commercially, but the cost is about $500/g - still only suitable for research purposes. The safety of consuming AFPs as food additives in the long-term is another concern. The toxicity of AFPs in certain specialized cells (e.g. human oocytes) and the possibility for causing allergic reactions need to be further considered and investigated (Tomczak and Crowe, 2002; Venketesh and Dayananda, 2008).

2.2.3. Collagen hydrolysate as a novel cryoprotectant

Due to the undesirable sweetness of the common cryoprotectants, such as sucrose and sorbitol, used in food systems, especially in meat, and the limitations related to using natural antifreeze proteins, there is a demand for the investigation of new alternative food cryoprotectants. The preferred cryoprotectant for food systems should have the ability to inhibit ice formation, come from a natural source, be non-toxic, and have minimal sweetness.

Protein hydrolysates produced by various enzymes have many bioactivities. Collagen being the most abundant protein that exists in animals, the bioactivities of its hydrolysates have

been explored broadly the in last decades. Collagen/gelatin hydrolysates from different sources have been shown to have antimicrobial activities, antioxidant properties, antihypertensive effects, and skin enhancive effects (Gómez-Guillén et al., 2011; Han et al., 2015; Inoue, Sugihara and Wang, 2016; Zhang et al., 2010).

Normally, collagen hydrolysates have been produced from pig skin or bovine hide (Jia, Zhou, Lu, Chen, Li and Zheng, 2009). However, outbreaks of mad cow disease and the banning of collagen from pig skin and bone in some regions for religious reasons, have made it necessary to find new marine or poultry sources that are safer and healthier for consumers (Gómez-Guillén et al., 2011). Therefore, more studies have focused on the discarded collagenous materials from poultry and fish processing. These materials are valuable sources of hydrolysates or peptides with many bioactive properties (Cheng, Liu, Wan, Lin and Sakata, 2008; Cheng, Wan, Liu, Chen, Lin and Sakata, 2009; Nam, You and Kim, 2008; Saiga et al., 2008). These collagenous materials may include skins, tunics, bones, fins and scales.

In 2007, a novel property of collagen hydrolysate was noticed by Damodaran (2007). The study demonstrated that gelatin hydrolysate derived from bovine collagen can act as a cryoprotectant by inhibiting the growth of ice in ice cream. He hypothesized that the unique glycine-rich, repetitive amino acid sequence in the collagen molecule (Gly-X-Y) has a surprising similarity with the hyperactive AFP found in the snow flea. This collagen hydrolysate confers the ability to control ice growth with the same adsorption-inhibition mechanism proposed for AFPs found in natural sources. Despite the different structures of AFPs, they all possess specific conformations which are capable of aligning and adapting to ice surfaces. The molecular modeling of the gelatin peptides binding to ice in this study demonstrated that the hydrolyzed gelatin peptides

with the repeating units may form an oxygen triad plane with oxygen-oxygen distances similar to those found in ice nuclei (4.5 Å), thus binding to the prism face of ice nuclei and inhibiting ice growth, possibly via hydrogen bonding. Kittiphattanabawon et al. (2012) also discovered a cryoprotective effect of gelatin hydrolysate prepared from blacktip shark skin that can reduce surimi protein denaturation during repeated freeze-thawing. In 2015, Nikoo, Benjakul and Xu demonstrated that the addition of gelatin hydrolysate in fish mince was able to inhibit the displacement of bound water surrounding proteins by freezable water, stabilizing the water associated with myofibrils during the freeze-thaw process. Several other studies have been performed on the hyperthermia protective effect that collagen peptides from fish or pig skin can have on different bacteria, and one particular peptide (GAIGPAGPLGP) with this effect was identified (Wang et al., 2011; Wang et al., 2014; Wang et al., 2015). Moreover, Cao et al. (2015) purified a hyperactive ice-binding peptide (GLLGPLGPRGLL) from pig skin which showed a thermal hysteresis of 5.28 °C.

Despite the literature on this topic still being scarce to date, the potential of collagen hydrolysate as a novel cryoprotectant is remarkable. Since collagen represents a "natural" compound and has been widely used as a nutritional supplement or in cosmetic products, acceptance as a food additive by consumers should be good. In addition, since collagen hydrolysates have a low sweetness and the supply is not a problem, it would be a suitable candidate as a commercial cryoprotectant. However, there are some issues that need to be further investigated which have not been emphasized yet in the literature. The mechanism of collagen cryoprotective peptides is still unclear and the sequence identification of the active peptides needs to be investigated further. Collagen peptides can improve the texture of ice cream through the inhibition of ice crystals; however, does this activity take an effect in protein systems to protect the protein

from freeze-denaturation as well, or does it act only as an antioxidant or as a regular cryoprotectant? If the mechanism is similar to AFPs, a comparison between the activity of AFPs and collagen hydrolysate is still lacking. Cryoprotective collagen hydrolysates have been found in pig, bovine and fish sources, whereas poultry collagens have not been investigated which may represent a viable source. Last but not least, the sensory attributes of foods with the addition of collagen hydrolysate is still missing, and reveal a critical area of research for their use commercially as cryoprotectants.

Chapter 3 - Physico-chemical and functional properties of gelatins extracted from turkey and chicken heads

3.1. Introduction

According to the Food and Agriculture Organization (FAO), global poultry meat production and consumption are growing steadily with a projected annual growth of 3.6% (FAO, 2012). In 2010, around 78 million tonnes of poultry meat were produced worldwide (FAO, 2012). It is estimated that around 22 to 30% of the global poultry production is considered to be by-products (Haines, 2004) such as head, feet, viscera, blood and feathers (Zhu et al., 2010). In developed countries, animal by-products are rendered into animal feed or composted for other agricultural purposes (Bolan et al., 2010), while in many developing countries, burial in landfills is a common disposal method (Williams, 2010). The disposal of poultry by-products in landfills is not only costly but also risky for human and environmental contamination (Williams, 2010).

Poultry by-products, such as low value mechanically separated meat and bone, have the potential to be used for food ingredients including protein isolates (Hrynets et al., 2011) and chondroitin sulfate glycosaminoglycans (Nakano et al., 2010). Although not consumed by humans, poultry head (skin, comb, wattle, cartilages, and bones) is an appropriate source for gelatin production due to its high collagen content (Rivera et al., 2000).

Collagen is a fibrous insoluble protein representing about 30% of total animal protein and is most commonly found in skin, bone, tendons, ligament and connective tissues (Shon et al., 2011). Collagen has a characteristic triple helical structure which collapses and forms gelatin upon heating (Mile et al., 2000). The source of collagen and the method of manufacture greatly affect the

physico-chemical and functional properties of the gelatin (Montero and Gómez-Guillén, 2000; Norziah et al., 2009). The process of gelatin production involves pre-treatment, extraction and purification (Karim and Bhat, 2009). The purpose of pre-treatment with acid or alkali is to remove non-collagenous proteins and other impurities in addition to cleaving the collagen cross-links.

In industry, gelatin has been widely used for food, cosmetic, nutraceutical, and pharmaceutical purposes (Schrieber and Gareis, 2007). Although commercial gelatins are mainly produced from pig skin, bovine bone and hide (Montero and Gómez-Guillén, 2000), their utilization is either restricted or limited due to cultural, religious and safety reasons (Gómez-Guillén et al., 2007). Interest in fish gelatin rose after the outbreak of bovine spongiform encephalopathy (BSE) (Eysturskard et al., 2009). However, the insufficient availability of raw material, the potential presence of allergens and the inferior rheological properties limit both production and usage of the fish gelatin (Karim and Bhat, 2009). Hence, under-utilized poultry by-products may be a suitable source for gelatin production to meet the increasing demand.

In this study, gelatin was prepared from chicken and turkey heads and their composition and functional properties were evaluated.

3.2. Materials and methods

3.2.1. Materials

Two different raw materials (ground broiler chicken heads and turkey heads) were compared in this study. The ground poultry heads were kindly provided by Rossdown Natural Foods & Rossdown Farms (Abbotsford, BC, Canada). Upon arrival to the Food Protein Chemistry Laboratory in a frozen state, the ground material was divided into three main batches and stored at -20 °C until use in less than three months. All the chemicals were analytical grade.

3.2.2. Gelatin extraction process

Gelatin was extracted from chicken and turkey heads according to the method of Gelatin Manufacturers of Europe (GME, 2000) with some modifications.

Ground chicken and turkey heads (~1 kg) were separately mixed with distilled water at a ratio of 1:4 (w/v) and stirred for 15 min at 4 °C, then filtered through a 1 mm pore size sieve (Fisherbrand U.S. Standard Brass Test, Thermo Fisher Scientific Inc., Newington, NH, USA). The clean sample was treated with 15 mM $NaHCO_3$ solution at a ratio of 1:4 (w/v) while stirring for 1 h at 4 °C and then centrifuged at 10,000 × g for 10 min at 4 °C using an Avanti J-E high-performance centrifuge (Beckman Coulter Inc., Palto Alto, CA, USA). This step was repeated 3 more times until no fat was observed in the supernatant.

The clean defatted sample was then treated with 0.1 M NaOH at a ratio of 1:10 (w/v) for 6 h at 4 °C and the alkaline solution was changed every 2 h. Alkali-treated samples were washed with distilled water and mixed with 0.05 M acetic acid solution at a ratio of 1:10 (w/v). After stirring for 18 h at 4 °C, acid-treated samples were washed with distilled water to remove the acid.

Gelatin from the pre-treated poultry heads was extracted in two stages at two different temperatures (Figure 3.1). Sample was mixed with distilled water at a ratio of 1:10 (w/v) and the pH was adjusted to 7.0 using 1 M NaOH. In the first stage (S1), gelatin was extracted at 50 °C for 18 h under continuous stirring in a jacketed glass beaker (Chemglass Life Sciences, Vineland, NJ, USA) connected to a Haake S7 heated bath circulator (Thermo Fisher Scientific, Newington, NH, USA). Soluble gelatin was separated from the insoluble material by filtration through a 1 mm pore size sieve (Fisherbrand U.S. Standard Brass Test, Thermo Fisher Scientific Inc., Newington, NH, USA). Gelatin in the insoluble material was then extracted at 60 °C for 6 h at the second stage (S2).

Extracted gelatins were separately filtered using Whatman No.4 filter papers and deionised using Rexyn I-300 (H-OH) beads (Acros, NJ, USA) until reaching a conductivity value lower than 50 µSiemens/cm (Oakton Acorn CON6, Vernon Hills, IL, USA). Gelatin was then evaporated under vacuum at 50 °C using a Heidolph Rotavapor (Hei-VAP Collegiate WB/G3) fitted with a Heidolph HB digital heating bath and Brinkmann auto-purge vacuum system (Heidolph Brinkmann LLC., Schwabach, Germany). Concentrated gelatins were dried by a freeze dryer (Labconco Corporation, Kansas City, MO, USA). As a result, four groups of gelatin were produced: chicken head gelatin extracted at 50 °C (CHS1) and 60 °C (CHS2), and turkey head gelatin extracted at 50 °C (THS1) and 60 °C (THS2).

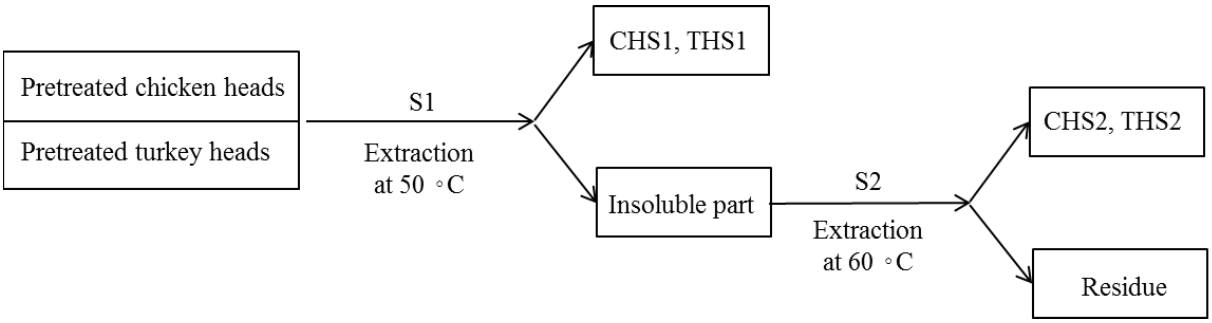


Figure 3.1. Chart of the gelatin extraction steps.

3.2.3. Physico-chemical properties of extracted gelatins

3.2.3.1. Proximate analysis

The method of the Association of Official Analytical Chemists (AOAC, 2000) was used to measure moisture (950.46), ash (920.153), and fat (960.39) contents of raw materials and extracted gelatins. Protein content was determined by a TruSpec CN carbon/ nitrogen determinator (Leco Corp., St. Joseph, MI, USA) using nitrogen conversion factors of 6.25 for chicken and turkey heads and 5.4 for gelatins (Eastoe and Eastoe, 1952).

3.2.3.2. Determination of hydroxyproline (Hyp) content

Hyp is a very abundant amino acid in collagenous material and the determination of its content gives an estimation of the collagen level. Hyp contents of raw materials were measured according to the method of Edwards and O'Brien (1980). The collagen content in the raw material was estimated by multiplying the Hyp content by 7.14, that reflects the fact that collagen contains about 14% hydroxyproline.

3.2.3.3. Extraction yield

Gelatin extraction yield was calculated on a dry basis as follows:

$$Yield(\%) = \frac{Dry\ weight\ of\ extracted\ gelatin\ (g)}{Dry\ weight\ of\ collagen\ in\ the\ raw\ material\ (g)} \times 100$$

3.2.3.4. Color measurement

The color of 6.67% (w/v) gelatin gels was evaluated by a Minolta CR-400 colorimeter (KonicaMinolta Sensing Americas, Inc., Ramsey, NJ, USA). The colorimeter, which has an aperture of 8 mm, was calibrated using a CR-A43 white calibration plate. The color parameters were determined using L*, a* and b* values (indicating lightness, redness/greenness and yellowness/blueness, respectively).

3.2.3.5. Amino acid composition

A known quantity of gelatin was subjected to hydrolysis with 6 M HCl containing 0.1% phenol for 1 h at 160 °C in a vacuum-sealed hydrolysis vial. Hydrolysates were labeled with AccQ-Tag Ultra Derivatization Kit (Waters, Milford, MA) according to the manufacturer protocol. Labeled amino acids were then analyzed by a HPLC system (Agilent 1200 Series) using AccQ-Tag C_{18} column (3.9 x 150 mm, Waters) at an absorbance of 254 nm for detection. Norleucine

(Sigma-Aldrich, Inc., Edmonton, Canada) was added as an internal standard. Results were presented as residues per 100 residues. In order to have a general overview of the amino acid composition of the gelatins, only two samples of each gelatin were randomly evaluated. The amino acid analysis was conducted at the Institute for Biomolecular Design (IBD, University of Alberta, Edmonton, Canada).

3.2.3.6. SDS-polyacrylamide gel electrophoresis

Protein patterns of gelatins were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Khiari et al. (2013) with a slight modification. Gelatin solutions (5 mg/mL protein content) were diluted 1:1 (v/v) with sample buffer containing β-mercaptoethanol then heated to 90 °C for 5 min. Ten microliters of samples (2.5 mg/mL) and molecular weight markers (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were loaded on a precast 4–20% ready gel (Bio-Rad Laboratories Inc., Hercules, CA, USA). The samples were run at a constant voltage of 120 V on a PowerPack Basic electrophoresis apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA). The gel was then stained using Coomassie Brilliant Blue R250 and destained using a mixture of distilled water, methanol, and acetic acid in a ratio of 50/40/10 (v/v/v). Gel was scanned using Image Scanner (Amersham Biosciences, Uppsala, Sweden).

3.2.4. Rheological and textural properties of extracted gelatins

3.2.4.1. Determination of dynamic viscoelastic behavior (DVB)

The dynamic viscoelastic behavior of 6.67% (w/v) gelatin samples was determined using a Physica MCR 301 rheometer (Anton Paar GmbH, Ashland, VA, USA) according to the method described by Binsi et al. (2009) with some modifications. A preliminary amplitude sweep test was

first performed to determine the viscoelastic range. The visocoelastic properties of gelatin samples were measured under oscillation using a 2.5 cm parallel plate geometry with a gap of 0.5 mm between plates. The analysis was run under a constant strain of 5%, a constant frequency of 10 s^{-1} and the temperature changed from 5 to 45 °C and back to 5 °C with a heating/cooling rate of 0.5 °C/min. The evolution of the elastic modulus (G'), the viscous modulus (G'') and the phase angle (δ) was plotted as a function of temperature.

3.2.4.2. Bloom strength

The bloom strength of chicken and turkey head gelatins was determined following the British Standard 757:1975 (BSI, 1975) with a slight modification. Gelatin solutions (6.67%, w/v) were prepared with distilled water at 50 °C and set into bloom jars (150 mL, 85mm height, 59 mm inside diameter). Gelatin gels were then kept in a refrigerated chamber at 7- 8 °C for 18 h. The bloom strength was determined by a TA-XT2 Texture Analyzer (Stable Micro System Ltd., Surrey, UK) equipped with a 1.27 cm diameter flat-faced cylindrical Teflon probe and using a load cell of 5 kN. The analysis was run at a constant speed of 0.5 mm/s and the bloom strength was calculated as the maximum force (g) registered at a penetration depth of 4 mm.

3.2.4.3. Determination of gel microstructure by Cryo-Scanning Electron Microscopy (Cryo-SEM)

The microstructures of chicken and turkey head gelatin gels were evaluated in cryo-mode according to the method of Stuart and Panitch (2009) with some modifications. Gels at a concentration of 6.67 % (w/v) were first cut in cubes (with a thickness of 2-3 mm) and mounted on the sample holder by bonding adhesive. Gelatin cubes were frozen at -207 °C by Slush Nitrogen and then fractured, and warmed at -40 °C, 10^{-5} Torr vacuum for 30 min to sublime the water.

Finally, the fractured samples were sputtered with gold coating and visualized with a scanning electron microscope (JEOL JSM-6301, Tokyo, Japan) at an acceleration voltage of 5 kV.

3.2.5. Functional properties of extracted gelatins

3.2.5.1. Determination of solubility

The effect of pH on gelatin solubility was determined according to the method described by Khiari et al. (2013) with some modifications. Gelatin samples were first dissolved in distilled water to a final concentration of 2% (w/v) then 5 mL of each gelatin solution was transferred into a glass test tube. The pH was then adjusted from 2.0 to 12.0 with either 1M HCl or 1M NaOH and the final volume of each solution was made up to 10 mL by adding distilled water having the same pH as the analyzed sample. All samples were centrifuged at 9,000 × g for 10 min at room temperature. The protein content of the sample before and after centrifugation was estimated by the Biuret assay (Robinson and Hodgen, 1940) using bovine serum albumin as a protein standard. Gelatin solubility was expressed as the ratio of protein content in the supernatant with respect to protein content in the initial sample.

3.2.5.2. Determination of emulsifying properties

Emulsions of oil-gelatin were prepared based on the method of Pearce and Kinsella (1978) with some modifications. Gelatin was first dissolved in distilled water at different concentrations (0.5, 1, 2 and 3% (w/v)) then 8 mL of each gelatin solution was mixed with 2 mL of commercial corn oil (Mazola, ACH Food Companies, Inc., USA). The mixture was homogenized at 20,000 rpm for 1 min at room temperature. An aliquot of the emulsion was diluted 100-fold with 0.1% (w/v) SDS solution then analyzed by a multi-angle static light scattering device (Mastersizer 2000S, Malvern Instruments, U.K). The emulsifying properties of the poultry gelatins were estimated

through the particle size distribution of the oil droplets in the emulsion. The volume mean diameter (D [4, 3]) value was used to indicate the mean droplet size and was calculated with the following formula:

$$D[4,3] = \sum n_i d_i^4 / \sum n_i d_i^3$$

where, n_i is the number of particles with diameter d_i.

The emulsifying stability of the emulsion was estimated after storing the samples for 1 week at 4 °C and expressed by Δ D [4, 3] (change of the droplet size during one week).

3.2.5.3. Determination of foaming properties

Measurements for foaming expansion (FE) and foaming stability (FS) of gelatins were carried out according to the method reported by Shahidi et al. (1995) with some modifications. Gelatin solutions, at different concentrations (0.5, 1, 2 and 3% (w/v)), were first prepared in distilled water then 10 mL from each sample were transferred into 25 mL graduated glass cylinders. Foam was generated at room temperature by homogenizing each gelatin solution at 13,000 rpm for 1 min using a Power Gen 1000 S1 homogenizer (Fisher Scientific, Schwerte, Germany). The volumes of gelatin solutions at 0 and 60 min after homogenization were recorded. The FE and FS were calculated according to the following equations.

$$FE(\%) = (V_T / V_0) \times 100$$

$$FS(\%) = (V_t / V_0) \times 100$$

Where V_T represents the total volume after homogenization at 0 min; V_0 stands for the initial volume of the gelatin solution (10 mL) and V_t indicates the volume of foam that remained after 60 min.

3.3. Statistical analysis

The whole extraction process was replicated three times. Except for the amino acid determination, each analysis/assay was conducted in triplicate. One-way ANOVA was conducted to evaluate the effect of the different gelatin groups (CHS1, CHS2, THS1 and THS2) on the dependent variables considered in this study using the SPSS statistic software (Version 20.0, SPSS Inc., Chicago, Il, USA). For emulsion and foaming properties, two-way ANOVA was conducted using gelatin groups and concentrations as fixed effects. Comparison among means was evaluated by performing studentized range test (Tukey HSD Test) at 5% significance level.

3.4. Results and discussion

3.4.1. Characterization of raw materials: chemical composition

Chicken and turkey heads had moisture as the major component with contents of 78.3 and 82.9%, respectively. The ash content was relatively high for both poultry heads (4.6 and 5.3% for chicken and turkey, respectively) probably due to the presence of minerals in head bones. Turkey heads had significantly lower protein and fat contents (8.7 and 5.7%, respectively) compared to chicken heads (10.6 and 9.4 %, respectively) which might be due to variation between the two species. The amount of Hyp in raw material represents the maximum possible quantity of gelatin that can be extracted. No significant difference of Hyp content (on a dry weight basis) was observed between chicken and turkey heads (2.2 and 2.3%, respectively), which represented about 15% collagen content.

3.4.2. Physico-chemical properties of extracted gelatins

3.4.2.1. Gelatin extraction yield

Gelatin extraction yield on a dry basis was expressed as the amount of extracted gelatin with respect to total amount of collagen in the raw material (Table 3.1). Turkey heads produced significantly higher gelatin (62.8 %; S1 + S2) than chicken heads (52.3 %; S1 + S2), with more gelatin extracted in S1 for turkey (38.0%) and chicken (31.2%) heads than those heads (24.8 and 21.1%, respectively) in S2. There are several factors that may affect the extraction yield such as the extraction time, acid used in pre-treatment, washing step and collagen content in raw materials (Muyonga et al., 2004; Cheow et al., 2007).

Table 3.1. Proximate composition and recovery yields of gelatins extracted from poultry heads.

Gelatin	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)	Yield (%)	Bloom strength (g)
CHS1	9.7 ± 0.1^{a}	0.05 ± 0.00^{ab}	88.7 ± 0.5^{d}	0.5 ± 0.1^{a}	31.2 ± 1.0^{b}	248 ± 5^{c}
CHS2	9.0 ± 0.1^{b}	0.03 ± 0.01^{c}	90.1 ± 0.0^{c}	0.6 ± 0.0^{a}	21.1 ± 0.6^{d}	200 ± 3^{d}
THS1	8.6 ± 0.0^{c}	0.06 ± 0.00^{a}	90.3 ± 0.0^{b}	0.2 ± 0.0^{b}	38.0 ± 1.0^{a}	368 ± 8^{a}
THS2	9.1 ± 0.1^{b}	0.03 ± 0.00^{bc}	90.9 ± 0.1^{a}	0.3 ± 0.0^{b}	24.8 ± 0.6^{c}	332 ± 6^{b}
P - values	< 0.001	= 0.003	< 0.001	< 0.001	< 0.001	< 0.001

Values were given as mean ± standard deviation (n=3). Different letters in the same column indicate significant ($P < 0.05$) differences between the means.

3.4.2.2. Chemical composition

The proximate analysis and yields of extracted gelatin are presented in Table 3.1. The moisture content of all gelatins, regardless of the raw material and the extraction step, ranged from 8.6 to 9.7%. Ash was detected in trace quantities (0.03 to 0.06%) and fat was found to be less than 1%. It has been reported that ash content lower than 0.5% suggests high quality gelatins (See et

al., 2010). Moreover, the low moisture, ash and fat contents may indicate that the pre-treatment, the demineralization and the drying steps were effective in removing fat, soluble salts and water.

For all extracted gelatins, crude protein was the major component and ranged from 88.7 to 90.9%. Regardless of the extraction stage, the protein content of turkey head gelatin was higher ($P < 0.05$) than that of chicken heads and the same transition of high protein content was found in the gelatin of S2 over the gelatin of S1. A similar result was observed by Rammaya1 et al. (2012) who observed an increase of protein content for gelatin extracted from mechanically deboned chicken meat at higher temperature. According to Fonkwe and Singh (1997), high temperature increases the rate of decomposition of collagen and subsequently leads to formation of more gelatinous proteins.

3.4.2.3. Color of gelatin

Appearance of gelatin is of commercial importance since it is associated with consumer acceptance. Color of all extracted gelatins was expressed in terms of L*, a*, and b* (Table 3.2).

There were significant differences in color properties among the samples. In general, gelatin from chicken heads showed higher values for lightness (L*) and yellowness (b*) but lower a* values in comparison with turkey head gelatins. The color of gelatins depends not only on the raw material used but also on the extraction conditions. For instance, gelatins from the same raw materials extracted in S2 showed higher ($P < 0.05$) lightness values compared with gelatins extracted in S1. The lower L* values of gelatins, obtained in S1, might be associated with the longer extraction time. A prolonged extraction period would increase the degradation of gelatin and would favor the Maillard reaction leading to a decrease of L* values (Ahmad and Benjakul,

2011). Although the color parameters may lower the appearance attributes, they do not affect the functional properties of the gelatins (Ockerman and Hansen, 2000).

Table 3.2. Color of gelatins extracted from poultry heads**.**

Gelatin	L*	a*	b*
CHS1	75.1±0.3^{b}	-1.7±0.0^{d}	0.5±0.2^{a}
CHS2	78.5±0.2^{a}	-1.6±0.0^{c}	-0.5±0.1^{b}
THS1	57.7±0.2^{d}	-0.7±0.0^{a}	-1.3±0.1^{c}
THS2	62.0±0.7^{c}	-1.1±0.0^{b}	-0.4±0.1^{b}
P - values	< 0.001	< 0.001	< 0.001

Values were given as mean ± standard deviation (n=3). Different letters in the same column indicate significant ($P < 0.05$) differences between the means.

3.4.2.4. SDS-polyacrylamide gel electrophoresis

The SDS-PAGE profiles of the gelatins from poultry heads (Figure 3.2) were similar and showed the protein band characteristics of type I gelatin, containing three major bands for two α-chains (α_1 upper and α_2 lower) and a dimer of their cross-linked chains (referred to as β-chain). For both species, α_1 and α_2 chains had an estimated molecular weight of around 134 and 120 KDa, respectively.

It appears that turkey head gelatins had an increased number of high molecular weight aggregates and also more bands in the lower molecular weight range than chicken head gelatins. Besides, it seems that gelatin degradation occurs as the preparation temperature increased from 50 °C to 60 °C where more fractions of low molecular weight were observed. According to Muyonga et al. (2004), elevated temperature promotes the cleavage of gelatin chains leading to the formation of lower molecular weight components.

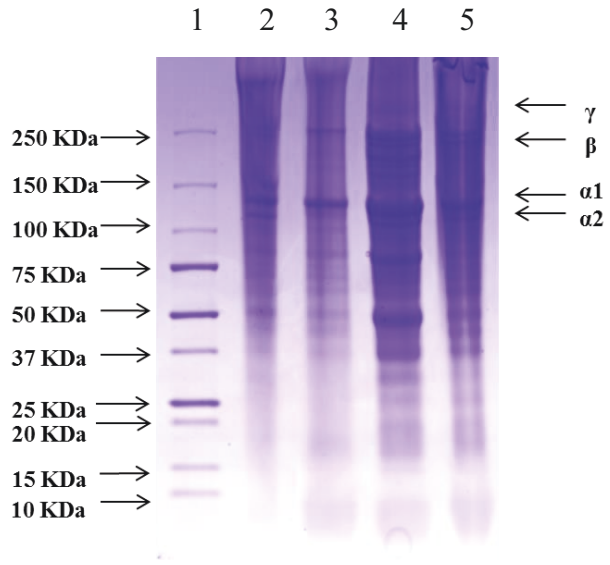


Figure 3.2. SDS-PAGE patterns of gelatins extracted from poultry heads. Lane 1: Molecular weight marker. Lane 2: CHS1. Lane 3: CHS2. Lane 4: THS1. Lane 5: THS2.

3.4.2.5. Amino acid composition

The amino acid composition of chicken and turkey head gelatins, expressed as residues per 100 residues, is presented in Table 3.3. All extracted gelatins had similar amino acid profile with glycine, proline, hydroxyproline and alanine being the major amino acids. Glycine constituted approximately 32.5% of the total amino acid content. Proline and hydroxyproline together accounted for about 22.6%. It has been reported that proline and hydroxyproline play a major role in re-stabilizing the triple helix of heat denatured collagen (i.e. gelatin), which is based on hydrogen bonding through hydroxyproline hydroxyl groups (Giménez et al., 2005). All poultry head gelatins had a very low content of tyrosine and were free of histidine. In this study, tryptophan and cysteine were not detected which may indicate that the extracted gelatins were type I. However, it is possible that during the amino acid determination, tryptophan and cysteine are completely lost by acid hydrolysis (Lourenço et al., 2002).

Table 3.3. Amino acid composition (residues/100 residues) of gelatins extracted from poultry heads.

Amino acid	CHS1	CHS2	THS1	THS2
Asx[1]	3.2	3.1	3.3	3.5
Glx[2]	8.3	8.9	8.0	7.1
His	ND[3]	ND	ND	ND
Arg	5.6	5.5	5.7	5.4
Thr	1.9	1.9	1.8	1.8
Ala	12.0	12.0	12.1	12.1
Ser	1.9	2.0	2.0	2.5
Cys	ND	ND	ND	ND
Tyr	0.5	0.4	0.4	0.5
Gly	32.2	32.5	32.2	33.1
Pro	12.3	12.2	12.0	12.2
Hyp	10.0	9.9	11.2	10.4
Val	2.8	2.5	2.4	2.3
Met	0.8	0.8	0.7	0.8
Lys	3.1	3.2	3.0	3.0
Ile	1.2	1.1	1.2	1.3
Leu	2.6	2.4	2.4	2.4
Phe	1.6	1.6	1.6	1.6
Trp	ND	ND	ND	ND
Total	100	100	100	100
Imino acids (%)	22.3	22.1	23.2	22.6

Values were given as an average of two replicates per treatments (n=2). 1: The sum of aspartic acid and asparagine. 2: The sum of glutamic acid and glutamine. 3: not detected.

3.4.3. Rheological and textural properties of extracted gelatins

3.4.3.1. Dynamic viscoelastic behavior (DVB) of gelatins

Figure 3.3 shows the evolution of the storage (G') modulus (A, B), the loss modulus (G'') (C, D) as well as the phase angle (δ) (E, F) as functions of temperature. In the cooling process, the elastic (G') and the viscous (G'') moduli of all gelatins increased rapidly from 27 °C to 5 °C (Figure 3.3, A, C). A sharp decrease of phase angle (Figure 3.3, E) was also observed in this region indicating that the gelatin solutions started to form a gel. During the heating process, the elastic (G') and viscous (G'') moduli gradually decreased from 10 °C (Figure 3.3, B, D). A sharp decrease

of both moduli took place between 17 °C and 35 °C as a result of the transition from gel to solution. The gelling temperatures of these gelatins varied between from 26.2 °C to 28.2 °C (Figure 3.3, A, C, E) while the melting temperatures ranged around 33.7 °C to 34.2 °C (Figure 3.3, B, D, F).

Turkey head gelatins had higher values for parameters describing viscoelastic properties (mainly the elastic modulus *G'*) than chicken head gelatins and, regardless of the species, gelatins extracted in S1 showed better gelling ability than those extracted in S2.

The higher viscoelastic properties of turkey head gelatin could be due to the difference in the molecular weight distribution (Figure 3.2). Turkey head gelatins had larger amounts of high molecular weight chains compared to chicken head gelatins which may partially recreate the native collagen structure (Stainsby, 1987).

3.4.3.2. Bloom strength of gelatins

Bloom strength of gelatin is one of the most important parameters to evaluate the grade and physical quality of a gelatin (Schrieber and Gareis, 2007). Significant differences were observed among the bloom strength of chicken and turkey head gelatins (Table 3.1). THS1 exhibited the highest ($P < 0.05$) bloom strength (368 g) followed by THS2 (333 g), both of which were higher ($P < 0.05$) than those of CHS2 (200 g) and CHS1 (248 g). The bloom values of extracted gelatins were similar and in the same range of those from commercial bovine and porcine gelatins (Karim and Bhat, 2009). The higher ($P < 0.05$) bloom strength observed with turkey head gelatins may be associated with the higher molecular weight distribution (Figure 3.2).

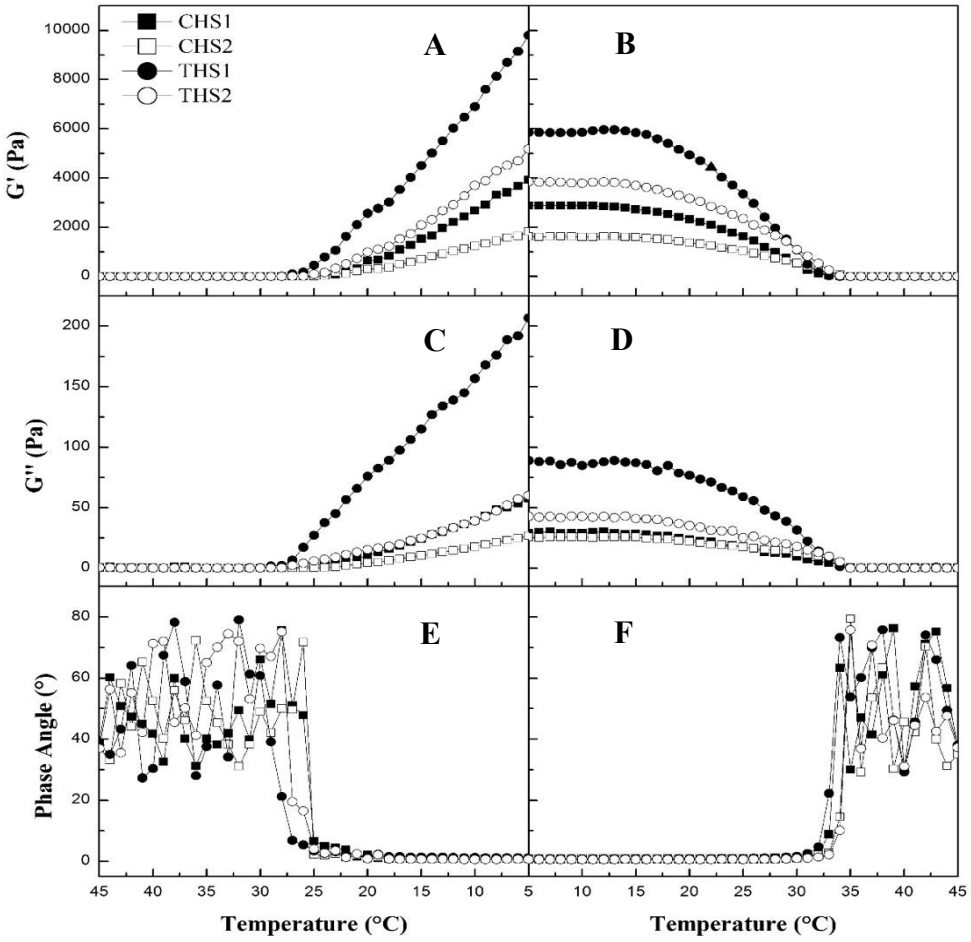


Figure 3.3. Evolution of viscoelastic properties of poultry head gelatins upon cooling (45 – 5 °C) and heating (5 – 45 °C) A: Evolution of elastic properties (*G′*) of gelatins upon cooling. B: Evolution of elastic properties (*G′*) of gelatins upon heating. C: Evolution of viscous properties (*G″*) of gelatins upon cooling. D: Evolution of viscous properties (*G″*) of gelatins upon heating. E: Change of phase angle (δ) of gelatins upon cooling. F: Change of phase angle (δ) of gelatins upon heating.

3.4.3.3. Gel microstructure

The microstructure of chicken and turkey head gelatin gels was investigated by Cryo-SEM at 5,000 × magnification (Figure 3.4). Turkey head gelatins had higher numbers of cross-linked small pores and denser networks compared to chicken head gelatins which presented obviously large voids. This result may reveal better gel properties of turkey head gelatins compared to

chicken head gelatins. According to Ahmad et al., (2011), larger numbers of interconnected small voids and denser crosslinks are indications for stronger gel structure.

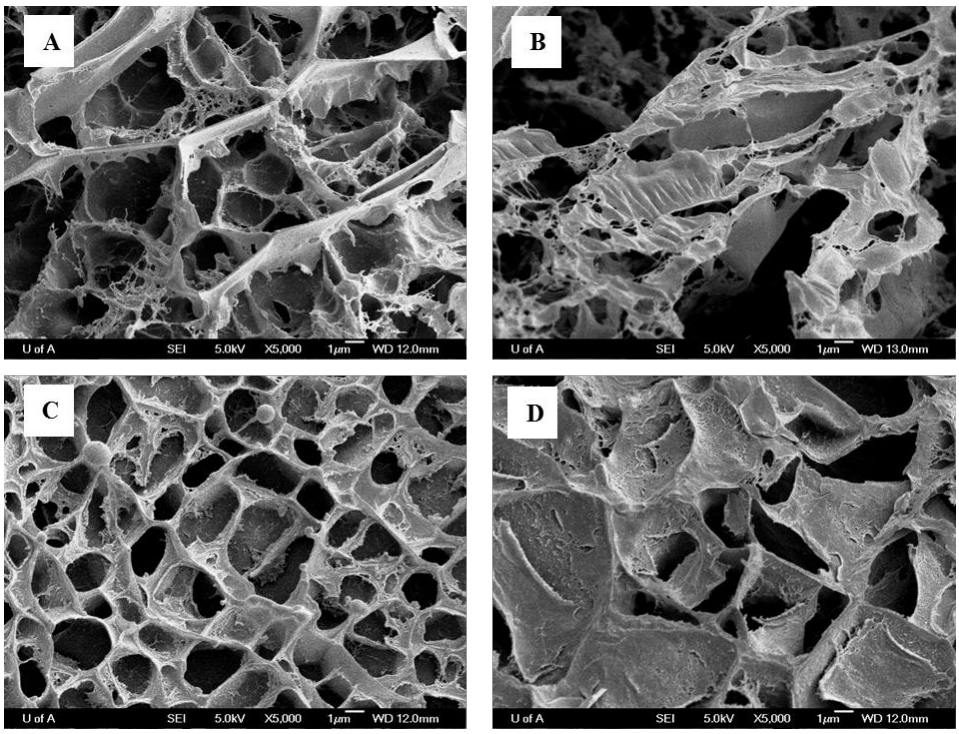


Figure 3.4. Cryo-Scanning Electron Microscopy (× 5,000) of gelatin gels from poultry heads. A: CHS1. B: CHS2. C: THS1. D: THS2.

For both turkey and chicken heads, gelatins extracted in S1 showed more uniform and smaller pores as well as tighter structures than gelatins obtained in S2. This may indicate that the gel matrix formed at 50 °C was stronger than that formed at 60 °C.

The Cryo-SEM results were in accordance with the bloom strength and DVB analyses, in which gelatins obtained from turkey material and extracted in S1 showed better rheological and textural properties, which was supported by the presence of larger amounts of high molecular weight chains in their protein patterns (Figure 3.2).

3.4.4. Functional properties of extracted gelatins

3.4.4.1. Solubility

Gelatin solubility was measured in the pH range of 2 to 12 and the result is shown in Figure 3.5. The solubility curves of gelatins extracted from the same source showed a similar trend, regardless of the extraction stage. The highest solubility percentages for CHS1 and CHS2 were observed at pH 4 and 10. Turkey head gelatins (THS1 and THS2), on the other hand, showed the highest solubility percentages at pH 2 and 12. The lowest solubility percentage for all gelatins, regardless of the source and the extraction conditions, was obtained at pH 8, corresponding to the isoelectric point.

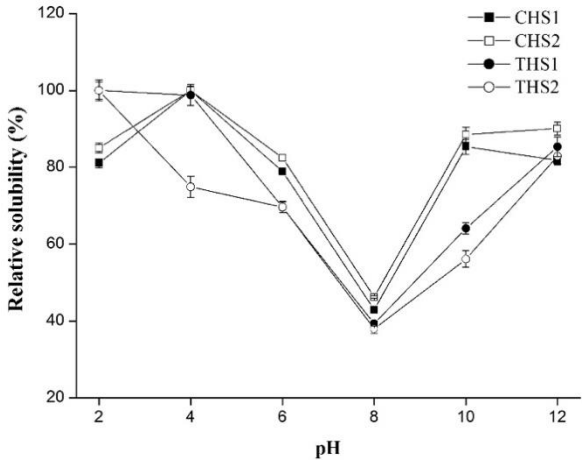


Figure 3.5. Solubility of gelatins extracted from poultry heads in the pH range of 2-12 (n=3).

It has been reported that the isoelectric point of gelatin varies between pH 5 and 9, depending on the alkaline or acidic pre-treatment (Schrieber and Gareis, 2007). In this study, the higher isoelectric point of turkey and chicken head gelatins was mainly due to the use of acetic acid to cleave the collagen cross-links. It is known that the use of acid in the preparation of gelatin (type A gelatin) does not affect the amino acid composition of native collagen while the use of

alkali (type B gelatin) results in deamidation of glutamine and asparagine to glutamic and aspartic acids, respectively and leads to a shift of isoelectric point toward lower pH values.

3.4.4.2. Emulsifying properties of gelatin

Emulsifying capacity and stability of gelatin samples were evaluated by the average mean droplet diameter (D [4, 3]) using a laser diffraction technique (Table 3.4). It was reported that the smaller the particle size of the droplets and the lower the change in size during a period of time indicate better emulsifying properties (Dickinson and Lopez, 2001).

The emulsifying capacity of extracted gelatins was significantly affected by both the concentration and gelatin group. In addition, a significant interaction was found between these two main effects. In general, the increase of gelatin concentration resulted in an increment of the D [4, 3] (Table 3.4)**,** indicating a negative relationship between the protein concentration and the emulsifying capacity. According to *Cheftel* et al. (1985), the protein concentration plays a major role in the emulsification process, and at low concentration, the diffusion and adsorption of protein at the oil-water interface is favorable. Regarding the gelatin groups, turkey head gelatins showed better emulsifying capacity compared to chicken head gelatins as proven by the smaller D [4, 3]. The presence of low molecular weight peptides in turkey head gelatins (Figure 3.2) may have improved their emulsifying capacity by facilitating the diffusion at the surface of oil droplets (Surh et al., 2006). A statistically significant interaction effect (gelatin × concentration) was found for D [4, 3] values. In general, the negative effect of increasing gelatin concentration on emulsifying capacity was more evident in THS1 and THS2 rather than CHS1 and CHS2. Moreover, the emulsifying capacity of CHS2 was more influenced by the gelatin concentration compared to

CHS1. However, the gelatin group showed a stronger effect at 0.5% (w/v) with turkey head gelatins having the least D [4, 3] ($P < 0.05$) values.

After one-week storage at 4°C, the droplet size increased for all gelatins. The interaction effect of gelatin group and concentration on the emulsifying stability of gelatins was also found to be significant (Table 3.4). In contrast to the emulsifying capacity and for all gelatin groups, increasing the gelatin concentrations lead to a decrease of Δ D [4, 3] values ($P < 0.05$). This result indicated better emulsifying stability at higher gelatin concentrations. CHS2 was strongly influenced by the concentration level and showed the greatest Δ D [4, 3] at 0.5% ($P < 0.05$). According to Sajjadi (2007), higher viscosities of dispersions are usually obtained at higher protein concentrations. As a consequence, a better emulsion stability is obtained probably due to reduction of the coalescence rate at high viscosity. At 1% (w/v), the gelatin group effect was dominant. THS1 and THS2 showed significantly lower Δ D [4, 3] compared to CHS1 and CHS2, revealing a higher emulsifying stability.

Table 3.4. Emulsifying properties of gelatins extracted from poultry heads.

		D [4, 3][1] (μm) t = 0	Δ D [4, 3][2] (μm) t = 7 days
Gelatin			
CHS1		10.0±1.2^{b}	2.9±1.8^{a}
CHS2		13.4±4.1^{a}	3.1±2.5^{a}
THS1		8.0±2.5^{c}	1.5±1.3^{b}
THS2		8.5±2.3^{c}	1.4±1.3^{b}
Concentration (%)			
0.5		7.0±2.4^{d}	4.4±1.7^{a}
1		8.9±1.4^{c}	2.6±1.5^{b}
2		11.0±2.3^{b}	1.3±0.7^{c}
3		13.0±3.7^{a}	0.5±0.2^{d}
Interaction (Gelatin × Concentration)			
CHS1	0.5	9.1±0.4^{def}	4.3±0.3^{b}
	1	9.3±0.2^{def}	4.5±0.4^{b}
	2	9.8±0.6^{cde}	2.2±0.4^{cd}
	3	11.7±0.5^{c}	0.5±0.2^{e}
CHS2	0.5	9.0±0.4^{def}	6.8±0.4^{a}
	1	10.8±0.5^{cd}	3.4±0.2^{bc}
	2	14.8±1.1^{b}	1.5±0.3^{de}
	3	19.0±0.4^{a}	0.7±0.2^{de}
THS1	0.5	4.3±0.1^{h}	3.5±0.1^{bc}
	1	7.5±0.1^{fg}	1.2±0.1^{de}
	2	9.7±0.1^{cde}	0.7±0.4^{de}
	3	10.5±0.6^{cd}	0.4±0.2^{e}
THS2	0.5	5.5±0.3^{gh}	3.1±1.8^{bc}
	1	7.9±0.1^{ef}	1.1±0.2^{de}
	2	9.7±0.2^{cde}	0.8±0.1^{de}
	3	10.9±0.4^{cd}	0.5±0.2^{e}
Sources of variation		*P*-values	
Gelatin		< 0.001	< 0.001
Concentration		< 0.001	< 0.001
Gelatin × Concentration		< 0.001	< 0.001

Values were given as mean ± standard deviation. For interaction part, n=3. Different letters in the same column of each effect indicate significant ($P < 0.05$) differences between the means. 1: Mean droplet size of emulsion immediately after preparation. 2: The change of mean droplet size of emulsion after one week.

Table 3.5. Foaming properties of gelatins extracted from different poultry heads.

		FE[1] (%)	FS[2] (%)
Gelatin			
CHS1		120±15^{d}	105±2^{c}
CHS2		125±16^{c}	104±2^{c}
THS1		133±13^{b}	107±2^{a}
THS2		136±12^{a}	106±2^{b}
Concentration (%)			
0.5		114±6^{d}	104±2^{d}
1		121±9^{c}	105±1^{c}
2		131±9^{b}	106±2^{b}
3		148±4^{a}	108±2^{a}
Interaction (Gelatin × Concentration)			
CHS1	0.5	107±3^{h}	103±1^{fg}
	1	110±1^{h}	105±1^{cde}
	2	121±0^{f}	104±1^{ef}
	3	142±1^{b}	107±1^{bc}
CHS2	0.5	110±1^{h}	101±1^{g}
	1	115±2^{g}	104±0^{def}
	2	125±1^{e}	106±0^{cde}
	3	149±1^{a}	106±0^{bcd}
THS1	0.5	116±0^{g}	105±0^{def}
	1	129±1^{d}	106±0^{cde}
	2	138±0^{c}	108±0^{b}
	3	150±2^{a}	110±1^{a}
THS2	0.5	122±0^{ef}	105±0^{def}
	1	130±0^{d}	105±0^{cde}
	2	140±0^{bc}	108±1^{b}
	3	151±1^{a}	108±1^{b}
Sources of variation		*P*-values	
Gelatin		< 0.001	< 0.001
Concentration		< 0.001	< 0.001
Gelatin × Concentration		< 0.001	< 0.001

Values were given as mean ± standard deviation. For interaction part, n=3. Different letters in the same column of each effect indicate significant ($P < 0.05$) differences between the means. 1: Foaming expansion. 2: Foaming stability.

3.4.4.3. Foaming properties of gelatin

The foaming expansion and foaming stability of gelatin samples at different concentrations are presented in Table 3.5. A significant interaction effect of gelatin group and concentration was observed for both FE and FS. Unlike the emulsifying capacity, increasing the concentration of gelatins significantly increased the FE. The higher FE at higher protein concentration may be due to the higher diffusion rate at the interface (Carrera and RodrÍguez Patino, 2005). On the other hand, turkey head gelatins had significantly higher FE than chicken head gelatins. This might be due to their larger amount of high molecular weight components that usually form stronger foams (Khiari et al., 2013). The interaction effect of gelatin and concentration was also significant for all gelatin groups, especially in CHS2.

The FS showed similar trends to the FE (i.e. higher FS observed at higher concentrations and turkey head gelatins having higher FS than to chicken head gelatins). According to Zayas (1997), increasing the protein concentration usually produces denser and stable foams probably due to the larger thickness of interfacial films.

3.5. Conclusion

Gelatin was extracted from two under-utilized poultry sources (chicken and turkey heads) in two stages at different temperatures (50 and 60 °C). The extraction conditions and the raw material significantly affected the properties and quality of the final product. In general, gelatins from the same materials extracted in S1 were better than those extracted in S2, in terms of textural and viscoelastic properties. Comparing both poultry sources, turkey head derived gelatins showed better rheological, textural and functional properties than chicken head gelatins. Hence, turkey head gelatins can be considered as a new alternative to mammalian gelatins due to their excellent

functional properties. However, their low extraction yields may limit their industrial feasibility. Future research needs to explore other poultry by-products to implement large-scale commercial gelatin production.

Chapter 4 - Preparation and characterization of gelatin from collagen biomass obtained through a pH-shifting process of mechanically separated turkey meat

4.1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), the meat sector is growing rapidly as a result of the rising demand for poultry meat and its derivatives (FAO, 2012). The latest statistic estimated the global poultry meat production to be around 102 million tons per year (FAOSTAT, 2013). Along with this large production, significant quantities of by-products and wastes are generated. In an attempt to recover the meat adhering to the frames and bones and to add value to these by-products, a mechanical deboning process was developed. This process is capable of recovering up to 70% of the meat, usually referred to as mechanically separated poultry meat (MSPM) (Froning and McKee, 2001). MSPM is considered to be a low-cost meat product and has been widely incorporated into a variety of meat product formulations, such as sausages, nuggets and patties (Froning and McKee, 2001). However, the relatively high fat, bone and connective tissue content in MSPM limits its industrial usage (Liang and Hultin, 2003).

Several studies were undertaken to investigate the recovery of valuable protein from MSPM (Hrynets et al., 2010; Hrynets et al., 2011). Functional proteins from MSPM have been successfully isolated by either the surimi or the pH-shifting processes. Due to high water consumption and the elevated level of organic matter in the waste water associated with the surimi process, the pH-shifting process became a popular method for protein isolation from MSPM. In

this technology, sarcoplasmic and myofibrillar proteins are first solubilized at acidic or alkaline conditions then precipitated at their isoelectric point. Protein isolates are the main products of the pH-shifting process. However, significant amounts of fat and insoluble biomass (mainly consisting of collagen) are also produced and discarded. The collagen biomass, which is obtained as a by-product during the pH-shifting operation, may represent a valuable source for gelatin preparation. Gelatin is a multifunctional ingredient that has long been used in the food industry, as a gelling, thickening, and film-forming agent as well as an emulsifier and stabilizer (Schrieber and Gareis, 2007). The source of collagen and the manufacturing process significantly affect the physicochemical and functional properties of gelatin (Montero and Gómez-Guillén, 2000). The traditional method of gelatin production involves the pretreatment of raw material followed by extraction and purification steps (Karim and Bhat, 2009). Acid and/or alkali are usually used in the pretreatment steps to remove impurities and cleave the collagen crosslinks, then gelatin is a produced by a partial thermal denaturation of collagen.

The majority of commercial gelatins come from porcine and bovine sources. However, due to some cultural, religious or safety reasons (Gómez-Guillén et al*.,* 2007), mammalian gelatins may not meet the growing demand for halal and kosher products (Arnesen and Gildberg, 2007). Poultry by-product contains a high proportion of collagen making it a potential alternative source for gelatin extraction (Nakano et al., 2012). Recent studies showed that gelatin can be recovered from chicken skins (Sarbon et al., 2013), chicken and turkey heads (Du et al., 2013) as well as mechanically deboned chicken and turkey residues (Fonkwe and Singh, 1997; Rafieian et al., 2013). The preparation of gelatin from MSPM has not yet been investigated. Therefore, the main objective of this research was to explore the possibility of gelatin preparation from MSTM using

two different extraction approaches: The conventional manufacturing method and a newly developed process. In the conventional method, MSTM was chemically pretreated to remove non-collagenous proteins, fat and impurities, followed by an acid-aided swelling step and gelatin was finally obtained in a series of batch extractions. In the newly developed approach, gelatin was produced after the recovery of a collagen biomass from MSTM, through a pH shifting process, followed by an acid solubilization and a thermal denaturation. The advantage of the latter method would be the recovery, along with collagen, of other important functional proteins (for example actin and myosin in the form of protein isolate) making the process more profitable. Physicochemical and functional properties of the extracted gelatins were also studied.

4.2. Materials and methods

4.2.1. Materials

For the conventional extraction process, three separate batches of frozen MSTM were purchased from Lilydale (Edmonton, AB, Canada) and thawed overnight at 4 °C prior to usage. Collagen biomass, a by-product obtained during the preparation of poultry protein isolate (PPI) through the pH-shifting technology (Figure 4.1) and used in the newly developed process, was collected from three separate batches of 20 kg MSTM. The extraction and separation of PPI and collagen biomass were performed in the pilot plant at Food Processing Development Centre (Leduc, Alberta) according to the acid-aided pH-shifting process as described by Hrynets et al. (2011). Collagen biomass was stored at -20 °C in polyethylene bags until use. All the chemicals and reagents used in this study were of analytical grade.

4.2.2. Gelatin preparation

Gelatin from MSTM was obtained from two different extraction methods.

Conventional Thermal Process. This process was based on chemical elimination of non-collagenous proteins, fat and impurities followed by thermal solubilization of collagen (Du et al., 2013). One kilogram of MSTM was washed with distilled water at a ratio of 1:5 (w/v) for 15 min followed by centrifugation at 8,000 × g for 10 min at 4 °C using an Avanti J-E High-performance centrifuge (Beckman Coulter Inc., Palto Alto, CA, USA). This step resulted in removing soluble impurities, and in order to dispose of non-collagenous proteins and fat, the sample was successively treated with NaCl, NaOH and butanol (BuOH). Briefly, the water washed sample was first mixed with 0.5 M NaCl solution at a ratio of 1:5 (w/v) under stirring at 4 °C with regular changes every 15 min for a total period of 45 min. The residue obtained after centrifugation at 20,000 × g for 10 min at 4 °C was treated with 0.25 M NaOH at a ratio of 1:10 (w/v) for 6 h with regular changes every 2 h. After centrifugation, the residue was treated with 2.5% BuOH at a ratio of 1:10 (w/v) for 24 hours with three changes. The clean, defatted collagen material was recovered after centrifugation and was subjected to a swelling step using 0.05 M acetic acid at a ratio of 1:10 (w/v) for 4 h under stirring. The acid-treated sample was washed twice with distilled water for 10 min each time. Gelatin from MSTM was produced in a series of batch extractions at 50 °C and 60 °C as described in the section 3.2.2.

Newly Developed Process. This process was based on recovering collagen biomass from MSTM using the pH-shifting technology as developed by Hrynets et al. (2011) (Figure 4.1), followed by solubilization of collagen and thermal transformation. Collagen biomass was mixed with distilled water at a ratio of 1:5 (w/v) and stirred for 15 min followed by centrifugation at 8,000 × g for 10 min at 4 °C. This step was repeated 3 more times to remove fat. Collagen was solubilized in 0.5 M acetic acid at a ratio of 1:100 (w/v) containing pepsin (MP Biomedicals, LLC, Solon, OH)

at a ratio 1:20 (w/w) for 18h at 4 °C. Soluble collagen was recovered by centrifugation at 8,000 × g for 10 min at 4 °C and precipitated at pH 8 using 1M NaOH. The precipitated collagen was collected by centrifugation and washed 3 times with water (1:5, w/v) in order to remove the salt (sodium acetate). Purified collagen was finally obtained after centrifugation at 20,000 × g for 10 min at 4 °C.

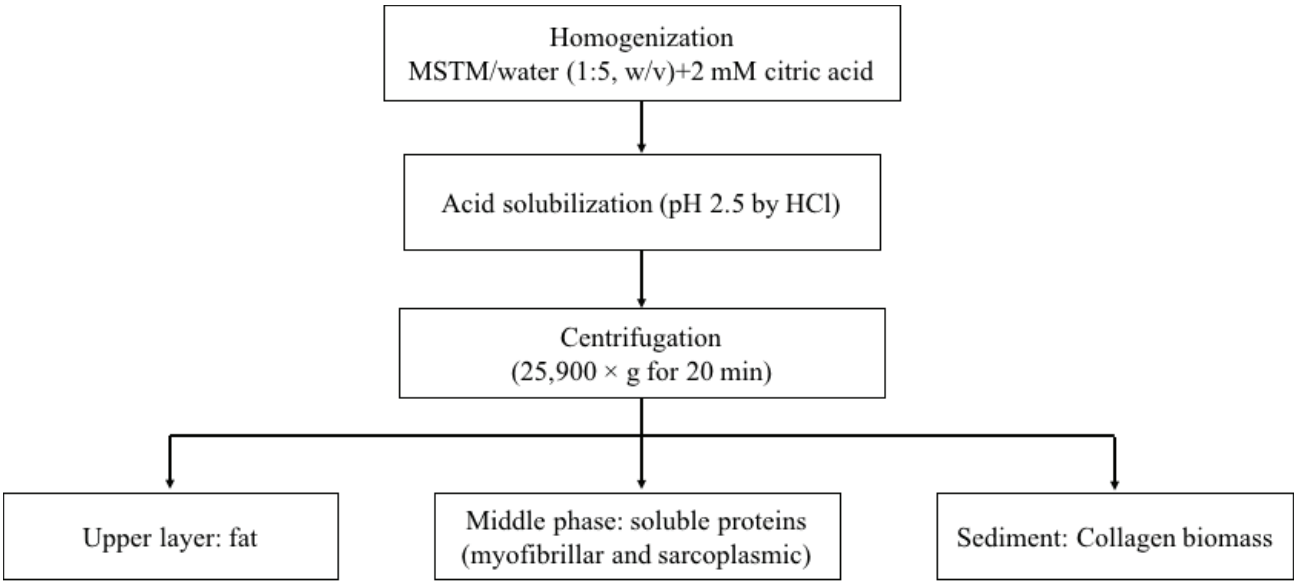


Figure 4.1. Preparation of collagen biomass by pH-shifting technology.

Extracted collagen was homogenized with distilled water at a ratio of 1:3 (w/v) and gelatin was produced after thermally treating the sample at 60 °C for 6 h. The soluble gelatin was recovered by centrifugation at 25 °C and then freeze-dried.

In total, three gelatin groups were obtained: gelatin extracted in the first stage from MSTM (MSTMS1), gelatin extracted in the second stage from MSTM (MSTMS2) and gelatin extracted from collagen biomass (CB).

4.2.3. Characterization of extracted gelatins

All the analyses were the same as described in section 3.2.3.

4.2.4. Rheological and textural properties of extracted gelatins

The dynamic viscoelastic behavior (DVB), Bloom strength and gel microstructure were determined by the same methods as in section 3.2.4.

4.2.5. Functional properties of extracted gelatins

The methodology for evaluating foaming properties and emulsifying properties of gelatin was according to the section 3.2.5.

4.3. Statistical analysis

The entire experiment was replicated three times and each assay was replicated at least twice. Data was presented as mean ± SD (standard deviation). Analysis of variance (ANOVA, one-way and two-way) was used for mean comparison at 0.05 significance level by performing a studentized range test (Tukey HSD Test). All statistical analyses were done using SPSS statistic software (Version 20.0, SPSS Inc., Chicago, IL, USA).

4.4. Results and discussion

4.4.1. Characterization of raw materials

The chemical compositions of MSTM and collagen biomass are shown in Table 4.1. Moisture and fat were the major components of the two raw materials, followed by protein and ash. MSTM had significantly higher protein (16.1%), fat (21.4%) and ash (3.7%) contents than collagen biomass (3.4%, 6.1% and 0.2%, respectively). However, significantly higher moisture content (90.1%) was observed for the collagen biomass compared to the MSTM (59.3%). The

higher moisture in collagen biomass may have been due to the greater water absorption during the pH-shifting process.

On a dry weight basis, collagen biomass had significantly higher Hyp content (5.7%) compared to MSTM (1.5%). The collagen content in the collagen biomass (41%) was ~4 times higher than that from MSTM (11.0%) which could be due to the accumulation of collagen during the pH-shifting process.

Materials	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Hydroxyproline (%, dry weight basis)	Collagen (%, dry weight basis)
MSTM	59.3±2.5^{b}	3.7±0.8^{a}	16.1±0.8^{a}	21.4±0.8^{a}	1.5±0.1^{b}	11.0±0.4^{b}
Collagen biomass	90.1±0.9^{a}	0.2±0.0^{b}	3.4±0.0^{b}	6.1±0.2^{b}	5.7±0.5^{a}	40.9±3.8^{a}
P- values	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Table 4.1. Proximate analysis of MSTM and collagen biomass.

Values were given as mean ± standard deviation (n=3). Different letters in the same column indicate significant ($P < 0.05$) differences between the means.

4.4.2. Characterization of extracted gelatins

4.4.2.1. Gelatin extraction yield

The temperature and the process duration play a major role in converting collagen to gelatin. It was reported that increasing the temperature improves the extraction yield (Rafieian et al., 2013). However, the application of severe temperatures and long processing time result in lowering both the visual and the functional properties of gelatin (Schrieber and Gareis, 2007). Since the collagen in the newly developed process has been already solubilized and precipitated, it was only heated to 60 ºC for 6 h in order to efficiently maximize its conversion into gelatin, without negatively affecting its properties.

Table 4.2. Proximate analysis and recovery yields of gelatins extracted from MSTM and collagen biomass.

Gelatin	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Yield (%)
MSTMS1	11.2±0.3[a]	0.1±0.0[b]	85.2±0.3[b]	1.8±0.0[a]	4.1±0.5[b]
MSTMS2	10.1±0.3[b]	0.1±0.0[b]	88.5±0.8[a]	1.5±0.0[b]	2.2±0.0[b]
CB	11.6±0.4[a]	1.9±0.0[a]	86.1±0.1[b]	0.4±0.1[c]	13.5±1.5[a]
P - values	= 0.003	< 0.001	< 0.001	< 0.001	< 0.001

Values were given as mean ± standard deviation (n=3). Different letters in the same column indicate significant ($P < 0.05$) differences between the means.

Gelatin extraction yields (on a dry basis) from both MSTM and collagen biomass were expressed as the weight of extracted gelatin (g) with respect to the total mass of collagen in the raw materials (g) (Table 4.2). The amount of gelatin extracted from the collagen biomass (13.5%) was significantly higher than that obtained from MSTM (6.3%, S1+S2). This might not only be due to the higher Hyp content in collagen biomass (Table 4.1) but also due to the usage of pepsin during the collagen solubilisation process. Pepsin is known to cleave crosslinks at the telopeptide region in collagen fibers resulting in a higher extraction yield (Nalinanon et al., 2008).

However, it is also important to mention that the extraction yield of the newly developed process would be 5.4±0.6% if calculated based on the collagen content found in the initial MSTM. This yield was not significantly different from the total extraction yield (MSTMS1+MSTMS2) obtained through the traditional process. Despite of the same yield obtained from these two different methods, the newly developed method was more efficient than the conventional method because of the reduced processing time (67 h and 28 h respectively).

4.4.2.2. Chemical composition

The proximate analysis of the extracted gelatins is presented in Table 4.2. In this study, all extracted gelatins were high in protein (85.2 - 88.5%) and low in ash (0.1 - 1.9%) and fat (0.4 - 1.8%).

The moisture content of all gelatins varied from 10.1 % to 11.6 %. This moisture range is considered to be microbiologically safe since higher water content may lead to microbial growth (Schrieber and Gareis, 2007). According to GME (2000), gelatin should be fat-free and the recommended level of ash should be less than 2%. The higher ($P < 0.05$) ash content in CB (1.9%) compared to MSTMS1 and MSTMS2 may be due to the usage of NaOH during the precipitation of collagen which resulted in salt formation. It seems that the newly developed process is more efficient than the traditional method in removing fat as proven by the lower amount of fat in CB (0.4%). With regard to the protein content, MSTMS2 contained significantly more protein (88.5%) than MSTMS1 (85.2%) and CB (86.1%). The higher protein content of MSTMS2 compared to MSTMS1 was in agreement with the result reported by Rammaya et al. (2012) who observed an increase in protein content at higher extraction temperature for gelatin extracted from mechanically deboned chicken meat residue.

4.4.2.3. Color of gelatin

The colors of the extracted gelatin gels were evaluated by the L*, a* and b* parameters (Table 4.3). CB showed lower ($P < 0.05$) lightness, greenness and yellowness compared to MSTMS1 and MSTMS2. The low L* value for CB (36.4), which reflected a darker gelatin color, could be due to the result of Maillard reaction between the amino acids in gelatin and residual sugars. In fact, in the newly developed method, high pH and temperature were used to precipitate

collagen and convert it to gelatin, respectively. These conditions (elevated pH values and temperature) are known to accelerate the Maillard reaction (Rich and Foegeding, 2000) resulting in lower lightness value.

Table 4.3. Color and bloom strength of gelatins extracted from MSTM and collagen biomass.

Gelatin	L*	a*	b*	Bloom strength (g)
MSTMS1	80.9±1.9[a]	-2.1±0.1[b]	4.3±0.5[b]	338 ±11[b]
MSTMS2	80.0±0.8[a]	-2.2±0.1[c]	7.2±0.2[a]	309±8[c]
CB	36.4±0.7[b]	-1.8±0.1[a]	1.0±0.2[c]	353±5[a]
P - values	< 0.001	< 0.001	< 0.001	< 0.001

Values were given as mean ± standard deviation (n=3). Different letters in the same column indicate significant ($P < 0.05$) differences between the means.

Gelatin gels, obtained from MSTM, had similar lightness values (80.9 and 80.0 for MSTMS1 and MSTMS2, respectively). However, significantly higher greenness (-2.2) and yellowness (7.2) were observed with MSTMS2. This result was in agreement with that reported by Nagarajan et al. (2012) who observed that the increase in color parameters is correlated with the increase in temperature, as higher temperatures would raise the number of free amino groups capable of inducing the non-enzymatic browning reaction.

4.4.2.4. Protein patterns of gelatins

The molecular weight distribution of gelatins produced from the two processes was estimated by the SDS-PAGE and is shown in Figure 2. CB comprised of three major bands; two α-chains (α_1 upper and α_2 lower) and one β-chain (Figure 4.2, Lane 1). The α-chains and β-chain were also observed in MSTMS1 along with some low molecular weight bands (Figure 4.2, Lane 2). However, MSTMS2 had different protein patterns than both MSTMS1 and CB. All the three α_1, α_2 and β bands were totally hydrolyzed into lower molecular weight components (Figure 4.2, Lane 3) probably due to the effect of higher temperature and the long extraction time.

The presence of high molecular weight protein bands in CB is an indication that the newly developed process resulted in less degradation of collagen molecules compared to the traditional method. Although CB and MSTMS2 were extracted at the same temperature (i.e. 60 °C), the greater protein degradation observed in MSTMS2, may be associated with the longer treatment time rather than the temperature.

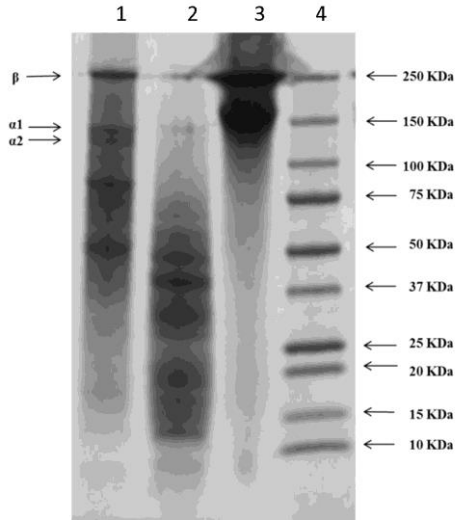


Figure 4.2. The SDS-PAGE patterns of gelatin extracted from MSTM and collagen biomass. Lane 1: CB; lane 2: MSTMS1; lane 3: MSTMS2; lane 4: molecular weight markers.

4.4.2.5. Amino acid composition of gelatins

The amino acid composition of gelatins, presented as number of amino acid residues per 100 residues, is shown in Table 4.4. Gly, Pro, Ala and Hyp were the most abundant amino acids in all gelatins. The three amino acids Gly, Pro and Hyp accounted for more than 53% of the total amino acid content. All gelatins, regardless of the extraction process, had very low amounts of His and Tyr, while Trp and Cys were not detected. The absence of Trp and Cys may suggest that all extracted gelatins were from type I collagen.

The imino acids (Hyp and Pro) are believed to play a major role in stabilization of the collagen triple helix structure (Fernandez-Diaz et al., 2001). In this study, no significant differences among the imino acid contents were observed for all the extracted gelatins. However, MSTMS2 had significantly higher amounts of hydrophobic amino acids (Ala, Val, Met, Ile, Leu, Phe) compared to both MSTMS1 and CB. According to Khiari et al. (2013), differences among the hydrophobic amino acid contents may affect the functional properties of gelatins.

Table 4.4. Amino acid composition (residues/100 residues) of gelatins extracted from MSTM and collagen biomass.

Amino acid	MSTMS1	MSTMS2	CB	*P* - values
Asx[1]	4.3±0.7	3.9±0.4	4.7±0.9	0.401
Glx[2]	8.0±0.4	8.0±0.5	7.4±0.5	0.271
His	0.0±0.0	0.0±0.0	0.3±0.5	0.422
Arg	5.6±0.2	5.7±0.3	5.5±0.4	0.754
Thr	1.6±0.2	1.7±0.2	1.6±0.4	0.961
Ala	12.7±0.2	12.8±0.7	12.1±0.5	0.258
Ser	1.7±0.6	1.8±0.6	2.2±0.7	0.645
Cys	ND[3]	ND	ND	--
Tyr	1.0±0.6	0.4±0.1	0.8±0.2	0.212
Gly	31.8±0.6	31.5±0.5	31.5±1.0	0.868
Pro	12.6±0.2	12.3±0.4	12.5±0.9	0.802
Hyp	9.5±0.4	9.8±0.7	9.6±1.4	0.893
Val	2.0±0.2	2.5±0.6	2.3±0.3	0.270
Met	0.8±0.1	0.8±0.1	0.7±0.1	0.171
Lys	2.7±0.0	2.9±0.1	3.0±0.2	0.072
Ile	1.3±0.1	1.4±0.1	1.3±0.1	0.422
Leu	2.8±0.1	3.0±0.1	2.8±0.2	0.190
Phe	1.6±0.1	1.6±0.1	1.5±0.1	0.317
Trp	ND	ND	ND	--
Total	100	100	100	--
Imino acids (Pro + Hyp)	22.1±0.3	22.1±0.6	22.1±0.8	1.0
Hydrophobic amino acids	21.1±0.2[b]	22.1±0.4[a]	20.7±0.3[b]	0.003

Values were given as mean ± standard deviation (n=3). Different letters in the same row indicate significant ($P < 0.05$) differences between the means. 1: The sum of aspartic acid and asparagine. 2: The sum of glutamic acid and glutamine. 3: Not detected.

4.4.3. Rheological and textural properties of extracted gelatins

4.4.3.1. Dynamic viscoelastic behavior (DVB) of gelatins

The DVB of gelatins was investigated under a temperature sweep test in the range of 5 – 45 °C (Figure 4.3). During the cooling step, both the elastic (*G'*) and the viscous (*G"*) moduli of all gelatins (Figure 4.3, A and C) increased rapidly from 25 °C to 5 °C. The sharp decrease of the phase angle at 25 °C (Figure 4.3, E) indicated the transition of gelatins from solution to gel. During the heating step, the elastic (*G'*) and viscous (*G"*) moduli gradually decreased from around 8 °C and a marked decrease of both elasticity and viscosity was observed between 15 °C and 33°C, which represented the gelatin melting zone. The gelling temperatures of these gelatins varied between 27.0 °C to 27.9 °C while the melting temperatures ranged from 31.4°C to 34.5 °C.

The CB showed better viscoelastic properties in terms of higher gelling and melting temperatures as well as elastic and viscous moduli compared to gelatin extracted from MSTM. This might be associated with the molecular weight distribution of gelatins. Gelatin obtained from the new process had a large amount of higher molecular weight chains (α and β chains) while those obtained from the traditional process were more prone to thermal degradation. The presence of high molecular weight components in CB might have resulted in partial renaturation of the collagen triple helices and therefore enhanced its viscoelastic properties (Gómez-Guillen et al., 2002).

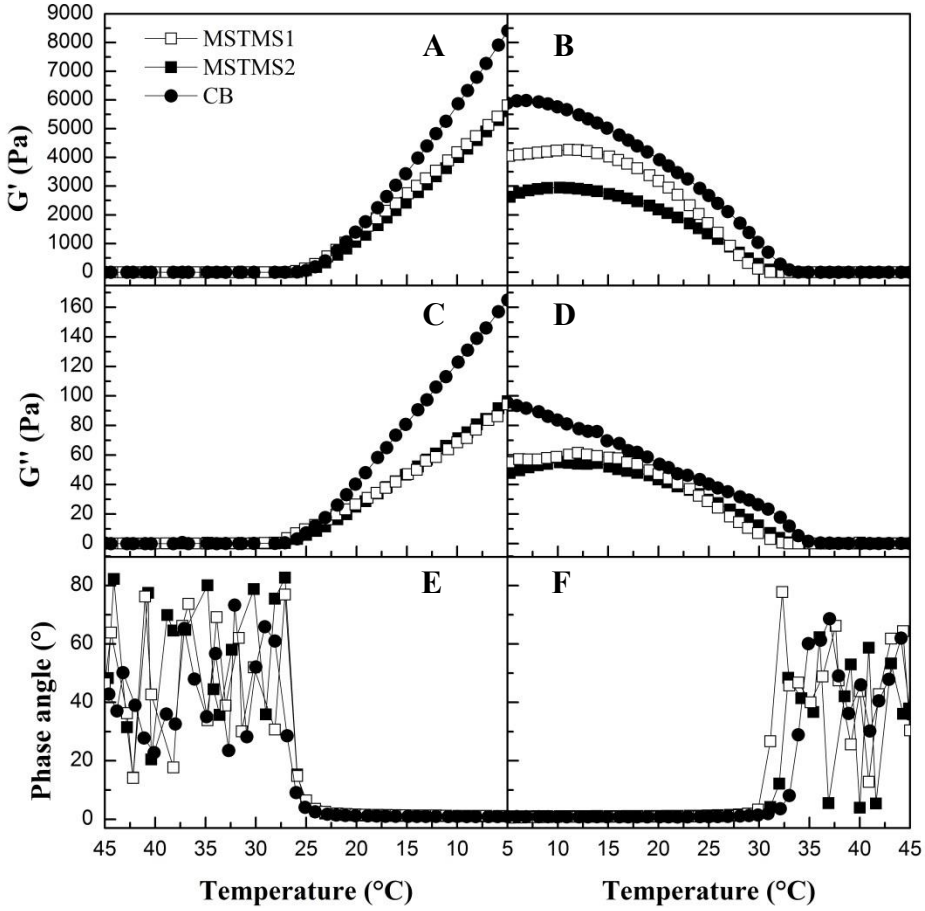


Figure 4.3. Evolution of viscoelastic properties of MSTMS1, MSTMS2 and CB upon cooling (45–5°C) and heating (5–45°C). A: Evolution of elastic properties (*G*') of gelatins upon cooling. B: Evolution of elastic properties (*G*') of gelatins upon heating. C: Evolution of viscous properties (G") of gelatins upon cooling. D: Evolution of viscous properties (*G*") of gelatins upon heating. E: Change of phase angle (δ) of gelatins upon cooling. F: Change of phase angle (δ) of gelatins upon heating.

4.4.3.2. Bloom strength of gelatins

Bloom strength is the most important quality associated with the price and industrial application of gelatins. Significant differences were observed among the gel strengths of the different extracted gelatins (Table 4.3). CB exhibited the highest ($P < 0.05$) bloom strength (353 g) followed by MSTMS1 (338 g) and MSTMS2 (309 g). The molecular weight distribution and the amino acid composition of gelatins are the main factors affecting the bloom strength. High

molecular weight gelatins can effectively form strong gels compared to low molecular weight ones (Nagarajan et al., 2012). Since all gelatins had similar imino acid contents (Table 4.4) then the differences among the gel strengths may be due to the molecular weight distribution (Figure 4.2). As discussed earlier, CB contained large amounts of high molecular weight components compared to MSTMS1 and MSTMS2 which might have imparted greater bloom strength. On the other hand, the partially thermally degraded MSTMS2 had significant amounts of low molecular weight fractions compared to MSTMS1 and as a consequence exhibited lower gel strength.

The bloom strength of all gelatin gels, regardless of the extraction process, varied from 309 to 353 g which corresponded to high bloom gelatin range and was comparable to the strength of porcine and bovine gelatins (Schrieber and Gareis, 2007).

4.4.3.3. Gel microstructure

The internal microstructure of the cryo-fractured gelatin gels was determined using a scanning electron microscope (SEM). The gelatin gel micrographs, obtained at 500 × magnifications, are shown in Figure 4.4. Gelatins extracted from MSTM through the conventional process (MSTMS1 and MSTMS2) had similar internal microscopic structures characterized by well-organized protein networks with evenly distributed pores (Figure 4.4 A and B, respectively). However, gelatin extracted from collagen biomass had highly porous microstructure (Figure 4.4 C). Compared to MSTMS1 and MSTMS2, CB gels contained greater number of small-size pores which is an indication of a rigid gel (Wangtueai and Noomhorm, 2009). Both the bloom strength analysis and the scanning electron microscopy were in accordance with this finding.

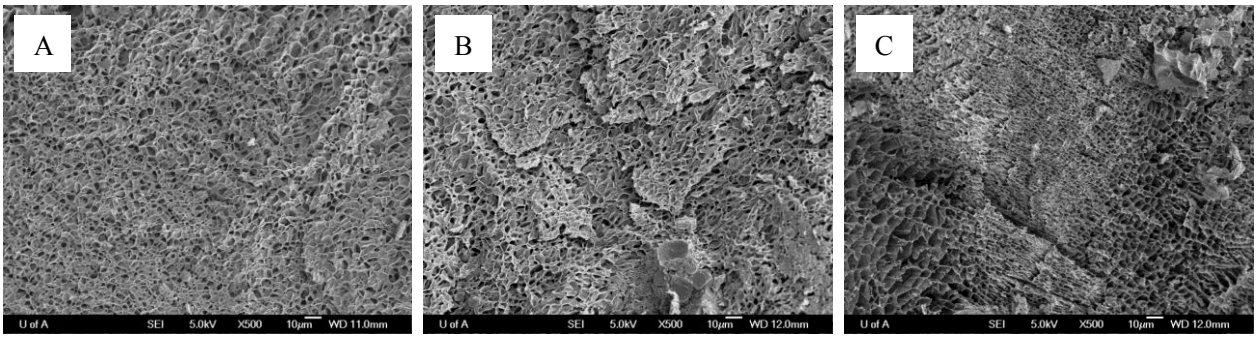


Figure 4.4. SEM (×500) of gelatin gels obtained from MSTM and collagen biomass. A: MSTMS1. B: MSTMS2. C: CB.

4.4.4. Functional properties of gelatin

4.4.4.1. Foaming properties of gelatin

The FE and FS of extracted gelatin are shown in Table 4.5. The interactive effect of gelatin groups and different levels of gelatin concentrations on the foaming properties of gelatins was significant. It was observed that, regardless of the extraction method, both the FE and FS significantly increased with the increment of gelatin concentration (from 0.5% to 3%). On the other hand, the concentration effect was less pronounced on the gelatins obtained from CB. In general, higher protein concentration leads to higher amounts of proteins migrating to the surface of air bubbles. As a consequence, a rapid foam formation with a denser stable film at the air/water interface is observed (Zayas, 1997).

At the same gelatin concentration, MSTMS2 showed greater ($P < 0.05$) FE compared to MSTMS1 and CB. This may due to the presence of low molecular weight chains (Figure 4.2, Lane 3) and larger numbers of hydrophobic groups (Table 4.4), which might have resulted in an efficient protein adsorption at the air/water interface (Khiari et al., 2013). FS can be influenced by various factors, such as the surface tension, viscosity and the electrical repulsion (Ahmad and Benjakul, 2011). MSTMS2 exhibited the highest ($P < 0.05$) FS (especially after 60 min) at all gelatin

concentrations which was probably due to its higher ($P < 0.05$) amount of hydrophobic amino acid residues (Ala, Val, Ile, Leu, Met, Phe) compared to the other two gelatins (Table 4.4). The positive effect of protein hydrophobicity on the foam properties has been previously demonstrated by Townsend and Nakai (1983).

Table 4.5. Foaming properties of gelatins extracted from MSTM and collagen biomass.

		FE[1] (%)	FS[2] (%)	
			30 min	60 min
Gelatin				
MSTMS1		126±12[b]	122±12[b]	117±11[c]
MSTMS2		134±10[a]	131±11[a]	129±10[a]
CB		126±5[b]	122±6[b]	118±7[b]
Concentration (%)				
0.5		117±5[d]	112±5[d]	108±6[d]
1		125±4[c]	121±4[c]	118±5[c]
2		135±5[b]	130±8[b]	127±9[b]
3		139±6[a]	136±5[a]	131±5[a]
Interaction (Gelatin × Concentration)				
MSTMS1	0.5	110±0[h]	106±1[g]	101±1[g]
	1	120±1[g]	119±1[d]	115±0[e]
	2	135±1[c]	125±1[c]	121±1[d]
	3	140±0[b]	139±1[a]	130±0[b]
MSTMS2	0.5	121±1[g]	116±1[ef]	115±0[e]
	1	131±1[de]	126±1[c]	125±0[c]
	2	141±1[b]	141±1[a]	139±1[a]
	3	145±0[a]	140±0[a]	138±0[a]
CB	0.5	119±1[g]	115±1[f]	109±1[f]
	1	125±1[f]	119±1[de]	115±0[e]
	2	129±1[e]	126±1[c]	122±1[d]
	3	132±1[cd]	129±0[b]	127±1[c]
Sources of variation			P-values	
Gelatin		< 0.001	< 0.001	< 0.001
Concentration		< 0.001	< 0.001	< 0.001
Gelatin × Concentration		< 0.001	< 0.001	< 0.001

Values were given as mean ± standard deviation. For interaction part, n=3. Different letters in the same column indicate significant ($P < 0.05$) differences between the means. 1: Foaming expansion. 2: Foaming stability.

4.4.4.2. Emulsifying properties of gelatin

The emulsion activity and stability of gelatin samples from different processes were evaluated by the mean droplet size (D [4, 3]) of emulsions (Table 4.6). A smaller emulsion droplet size and smaller change in the droplet size (Δ D [4, 3]) during storage indicate better emulsion activity and stability, respectively.

There was no significant interaction of gelatin groups and different concentrations on the emulsion activity of gelatins. With respect to the concentration effect, a significant improvement in the emulsion activity, as indicated by the reduction of D [4, 3] value, was noticed with the decrease in the gelatin concentration (from 3% to 0.5%). This might be due to the fact that the diffusion and adsorption of protein at the oil-water interface is favorable at lower protein concentrations (Cheftel et al., 1985).

Regarding the gelatin group's effect, the average droplet size of emulsions prepared with MSTMS1 and MSTMS2 were smaller ($P < 0.05$) than that prepared with CB. This was probably due to differences in molecular weight distributions (Figure 4.2) and hydrophobic amino acid contents (Table 4.4). The smaller size peptide chains present in gelatins extracted from MSTMS2 may have enhanced the emulsion capacity by facilitating the diffusion at the surface of oil droplets (Surh et al., 2006). In addition, the significantly larger amounts of hydrophobic amino acid residues in MSTMS2 may have improved the emulsion activity by lowering the interfacial tension through binding to the oil fraction at the oil-water interface (Kato and Nakai, 1980)

The stability of emulsions was evaluated by the Δ D [4, 3] after one week of storage (Table 4.6). The interaction effect of gelatin groups and concentrations on the emulsion stability of gelatins was significant. Contrary to emulsion activity, increasing gelatin concentration resulted in

better emulsion stability for these three gelatin groups. Higher protein concentrations lead to higher viscosities of dispersions and subsequently reduce the coalescence rate and improve the emulsion stability (Sajjadi, 2007).

Table 4.6. Emulsifying properties of gelatins extracted from MSTM and collagen biomass.

		D [4, 3][1] (μm)	Δ D [4, 3][2] (μm)
Gelatin			
MSTMS1		8.5±0.4^{b}	3.0±3.3^{a}
MSTMS2		7.4±0.5^{c}	2.5±2.4^{b}
CB		9.6±0.6^{a}	2.1±1.9^{b}
Concentration (%)			
0.5		7.9±0.9^{d}	6.4±1.7^{a}
1		8.4±1.0^{c}	2.4±0.5^{b}
2		8.7±1.0^{b}	1.0±0.4^{c}
3		9.1±1.0^{a}	0.3±0.2^{d}
Interaction (Gelatin × Concentration)			
MSTMS1	0.5	8.1±0.0	8.3±0.3^{a}
	1	8.4±0.1	2.5±0.3^{de}
	2	8.7±0.2	0.8±0.1^{fgh}
	3	9.0±0.2	0.3±0.1^{gh}
MSTMS2	0.5	6.7±0.2	6.2±1.3^{b}
	1	7.3±0.2	1.9±0.2^{def}
	2	7.6±0.0	1.4±0.3^{efg}
	3	8.0±0.0	0.5±0.2^{gh}
CB	0.5	8.9±0.2	4.7±0.4^{c}
	1	9.5±0.1	2.9±0.1^{d}
	2	9.9±0.0	0.8±0.1^{fgh}
	3	10.3±0.0	0.2±0.0^{h}
Sources of variation		*P* - values	
Gelatin		< 0.001	< 0.001
Concentration		< 0.001	< 0.001
Gelatin × Concentration		= 0.102	< 0.001

Values were given as mean ± standard deviation. For interaction part, n=3. Different letters in the same column within the same effect indicate significant ($P < 0.05$) differences between the means. 1: Mean droplet size of emulsion immediately after homogenization. 2: The change of mean droplet size of emulsion after one-week storage.

Gelatin groups strongly affected ($P < 0.05$) the emulsion stability. No significant differences were observed among the emulsion stabilities of all gelatins at protein concentrations

of 1, 2 or 3% (w/v). However, at 0.5% gelatin concentration, CB showed the smallest Δ D [4, 3] value (4.7) compared to MSTMS1 and MSTMS2 (8.3 and 6.2, respectively) indicating better emulsion stability.

4.5. Conclusion

Gelatins were extracted from MSTM following two different methods. One was a traditional two-stage batch thermal extraction and the other was a new process based on recovering gelatin from a collagen biomass obtained through the pH-shifting technology. Both processes significantly affected the properties and quality of the final products. Gelatin prepared by the newly developed process had better rheological and textural characteristics but darker color compared to those produced through the traditional thermal way. Results from this study showed that high quality gelatin can be extracted from MSTM using an alternative process to the traditional thermal extraction method. Despite the darker color of gelatin, the newly developed process seems to be industrially more advantageous compared to the traditional process in terms of recovery of valuable meat proteins (protein isolate and collagen biomass) as well as time saving and reduction of water and chemical reagent consumption.

Chapter 5 - Identification and evaluation of cryoprotective peptides from chicken collagen: ice growth inhibition activity compared to type I antifreeze proteins in sucrose model systems

5.1. Introduction

In nature, antifreeze proteins (AFPs) exist in a wide range of living organisms, such as antarctic fish, insects, plants, bacteria and fungi, all thriving in a subzero environment. AFPs were first discovered in the blood of Antarctic fish in the early 1970s (Duman and DeVries, 1975). These antifreeze substances are synthesized in their bodies and are believed to play a role in protecting biological tissues from freeze damage during harsh winters or in cold surroundings, through non-colligatively depressing the freezing point without affecting melting point (thermal hysteresis) and inhibiting the ice crystal growth in their body fluids (Griffith and Ewart, 1995). The related mechanism is called "adsorption-inhibition", in which AFPs bind to the ice surface inhibiting its growth (Barret, 2001). Specific amino acid sequences contained in the AFPs and subsequent protein conformations are responsible for this special activity (Davies and Sykes, 1997). In food, freezing and frozen storage are important to preserve many foods, however, food quality can be impaired under freezing temperatures (Matsumoto, 1980). Ice formation is often associated with freezing damage of food products, either directly through mechanical effects created by ice crystals, or indirectly by concentrating the solute in the unfrozen phase (Zaritzky, 2012). Therefore, it is important to control and minimize the abuse associated with the formation of the larger ice crystals. These are mainly attributed to prolonged frozen storage, temperature fluctuation and repeated freeze-thawing cycles during food storage and transportation (James, 2008). In general,

most of the deleterious effects of freezing on foods involve dehydration, ice recrystallization, protein denaturation, lipid oxidation and vitamin loss. These changes can further induce deteriorations of food flavor, texture, and functional properties (Zaritzky, 2011). To alleviate these undesirable modifications and extend the shelf-life, the addition of cryoprotectants in frozen food is necessary.

The most common cryoprotectants used in the food industry have been low molecular weight sugars and polyols. These cryoprotectants retard the freezing damage of foods mainly though 1): solute-exclusion in which the cryoprotectants are preferentially excluded from the protein surface, due to the increased solution surface tension with the addition of cryoprotectants, and thus hydrating proteins to stabilize their native structure (Carpenter and Crowe, 1988); 2) colligatively depressing the freezing point of food systems. Sucrose and sorbitol are popular cryoprotective additives because of their availability, low cost and effectiveness (Portmann and Birch, 1996). Despite these excellent features, their strong sweetness and high calorie content are not acceptable for diabetics and dietarians. Also, the sweetness in particular food applications, such as in meat products (i.e. surimi products), is not favorable to consumers (Nopianti et al., 2012). Additionally, even though the high sugar content in frozen desserts is essential for creating fine textures with tiny ice crystals, these molecules do not effectively prevent ice recrystallization during the temperature fluctuations that may occur during frozen storage, and thus negatively affect the textural characteristics (Goff, 2012). Carbohydrate-based cryoprotectants with reduced sweetness have been explored by researchers, but there are still issues related to the Maillard reaction, viscosity or the effectiveness (Portmann and Birch, 1996). As a consequence, there is growing demand to develop alternative food cryoprotectants. The antifreeze proteins from natural

sources are an option, but unfortunately their application has been restricted by limited availability (Ustun and Turhan, 2015).

The enzymatic hydrolysis of proteins can produce various biologically active peptides. Collagen is the most abundant component of animal protein. Collagen/gelatin hydrolysate bioactivities have been explored extensively in last decades, revealing antimicrobial activity, antioxidant properties, antihypertensive and skin enhancing effects (Gómez-Guillén et al., 2011; Han et al., 2015; Inoue, Sugihara and Wang, 2016; Zhang et al., 2010). Recently, the potential cryoprotective properties of gelatin hydrolysates have drawn researchers' interests; the ice-growth inhibition effect of gelatin peptides may be a consequence of the unique tri-peptide repetitions (Gly-X-Y) found in the collagen molecule, similar to the structure (Gly-X-X) existing in the hyperactive AFP from snow fleas (*Hypogastrura nivicola*) (Damodaran, 2007). To date, several more studies have provided more information about the cryoprotective properties of gelatin hydrolysates (Cao et al., 2015; Kittiphattanabawon et al., 2012; Limpisophon et al., 2014; Nikoo et al., 2015, Wang et al., 2011; Wang et al., 2014; Wang et al., 2015). However, studies on the structure and amino acid sequence of the ice-inhibitive collagen peptides are still very scarce, and a direct comparison of the activity between collagen peptides and AFPs from nature has not been provided yet. Furthermore, the mechanism on how the active collagen peptides inhibit ice growth is still unclear.

Chicken skin is one of the by-products of poultry industries and is an underutilized material, accounting for 4% weight of a chicken (Feddern et al., 2010). Chicken skin is mainly used as animal feed or as a source of fat; clearly, the value of its collagen has not been fully explored (Cliché et al., 2003, Sarbon et al., 2013). Therefore, it would be of interest to confirm that avian

collagen can be used to generate ice-controlling peptides. In this study, the inhibitory effect of trypsin digested chicken collagen hydrolysates on ice crystal growth was evaluated in sucrose model systems after seven thermal cycles under frozen conditions. The amino acid sequences were identified and the molecular structures of several ice growth inhibitory peptides were elucidated. Moreover, the activity and structure of identified collagen peptides were compared to the type I AFPs isolated from winter flounder (*Pseudopleuronectes americanus*), since it is the most well-studied AFP reference.

5.2. Materials and methodology

5.2.1. Materials

Broiler chicken skin (sacrificed at 42 days) was provided by the Poultry Research Center of University of Alberta. Upon arrival to the Food Protein Chemistry Laboratory, skin pieces were packaged into polyethylene bags and stored at - 20 ºC until used in less than 2 months. All solvents used in liquid chromatography were HPLC grade and the buffers were prepared with filtration (0.22 µm). Type I antifreeze proteins (AFPs) from winter flounder with a purity > 90% was purchased from A/F Protein Inc., U.S. and the synthesized peptides (purity > 98%) were purchased from Biomatik Corporation, Canada. All the chemicals were analytical grade.

5.2.2. Experimental design

Three batches of collagen hydrolysates were prepared from chicken skin and pooled. In this study, the inhibitory effect of the obtained collagen hydrolysates on ice crystal growth was evaluated in sucrose model systems under a microscope, and the amino acid sequences of the peptides showing this ice-controlling activity were elucidated. Five of the identified collagen

peptides were synthesized, and their ice growth inhibition effects and secondary structures were examined and compared to the fish type I AFPs.

In the first part of the study, the ice growth inhibition activity of the whole collagen hydrolysates at different concentrations (0%, 4% and 8%, w/v) was evaluated in sucrose solutions after seven thermal cycles between -16 ºC and -12 ºC. The total solid content (collagen hydrolysate content + sucrose content) of the models was 27% (w/v) and sucrose solutions at 19 and 23 % (w/v) were also used as controls. Therefore, a total of 5 treatments were subjected to three independent thermal cycles, resulting in a total of 15 glass slides (3 glass slides × 5 sucrose model systems) which were analyzed for their ice dimension. After proving its positive action, the collagen hydrolysate was fractionated by size-exclusion chromatography (SEC), and the fractions revealing the greatest ice growth inhibition activity were selected and subjected to sequence identification. At this stage, a total of 15 glass slides (3 glass slides × 5 fractions) were analyzed for ice crystal size, each containing 8% (w/v) of the fraction and 19% (w/v) sucrose. In the second part of this study, five identified peptides were synthesized and tested in a 23% (w/v) sucrose solution. The reduction of ice crystal dimensions induced by these selected peptides was compared to that of fish Type I AFP; bovine serum albumin (BSA) was used as a negative control. In this last experimental phase, 105 glass slides (3 glass slides × 5 peptides × 7 concentrations) were tested for the synthesized peptides, and 27 glass slides (3 glass × 1 AFP × 9 concentrations) were tested for the fish AFP. Lastly, to elucidate the influence of structure on this ice-controlling effect, the secondary structures of both synthesized peptides and AFP were detected and compared.

5.2.3. Chicken skin preparation

Frozen chicken skins were thawed in a cold room (4 °C) overnight before preparation. The skins were cut into small pieces (1 cm^2) and washed with excessive distilled water to remove a portion of impurities.

5.2.4. Collagen extraction

Acid soluble collagen was produced from the prepared chicken skin according to Singh et al. (2011) with modifications. This method was based on chemical elimination of non-collagenous proteins, fat and impurities followed by acid solubilization of collagen and subsequent collagen precipitation. All processes were conducted at 4 °C with continuous stirring.

To remove non-collagenous impurities, the prepared skin was first soaked in 0.1 M NaOH at a ratio of 1:10 (w/v) for 6 h and the alkaline solution was changed every 2 h. The alkaline treated skin was washed by deionized water until reaching to a neutral pH, and then defatted by 10% (v/v) butyl alcohol at a ratio of 1:10 (w/v) for 24 h with regular changes every 8 h. After the removal of alcohol, the defatted skin was homogenized with distilled water using a homogenizer (Waring Commercial, Torrington, CT, USA) for 1 min, followed by solubilizing in 0.5 M acetic acid at a ratio of 1:10 (w/v) for 42 h. The mixture was then subjected to centrifugation (Avanti J-E, Beckman Coulter Inc., Palto Alto, CA, USA) at 20,000 × g for 1 hour at 4 °C, and the supernatant was collected afterwards. The acid soluble collagen was recovered from the supernatant by centrifugation after adding NaCl to a final concentration of 2.0 M.

The precipitated collagen was mixed with three volumes of distilled water and again centrifuged at 20,000 × g for 10 min at 4 °C. The supernatant was discarded and this step was repeated four times to reduce the amount of salt and acid. The resultant collagen slurry was further

purified by dialyzing (dialysis tubing, MWCO 12.4 kDa, Sigma-Aldrich) against distilled water with changes every 12 h for a total period of 48 h. After purification, the collagen was lyophilized (Labconco Corporation, Kansas City, MO, USA).

5.2.5. Collagen hydrolysate preparation

The collagen extracted from chicken skin was hydrolyzed by trypsin (porcine pancreas, Sigma-Aldrich) (Trauger et al., 2002; Zhang et al., 2009), which is considered to provide advantages for peptide sequence identification. For this purpose, one gram of freeze-dried collagen was mixed with 80 mL of 50 mM NH_4HCO_3 (pH 8.0), followed by heating at 80 °C for 10 min in a water bath (Isotemp 2320, Fisher Scientific, Marietta, OH, USA) to inactivate the endogenous enzymes. After cooling to 30 °C, 1 mL of freshly prepared trypsin solution (50mg/mL in 50 mM NH_4HCO_3 solution, pH 8.0) was added into the collagen suspension and the mixture was incubated at 37 °C under agitation for 18 h. Trypsin was inactivated by heating the solution to 80 °C for 10 min after digestion. The hydrolyzed collagen solution was then filtered by using Whatman No. 1 filter papers and desalted by dialyzing (MWCO 100-500 Da) against distilled water. Three grams of collagen was digested in total and pooled together before lyophilization. The produced chicken collagen hydrolysates (CCH) were stored at −20 °C for later use.

5.2.6. Collagen hydrolysate addition in sucrose model systems

Different concentrations (0%, 4% and 8%, w/v) of the obtained CCH were mixed into sucrose solutions containing different levels of sucrose (27%, 23% and 19%, w/v), respectively, to compose a total solid content of 27% (w/v) in the systems (Wang and Damodaran, 2009). In order to prepare the model systems, sucrose and CCH were first dissolved in distilled water at greater concentrations than required, and then mixed the certain amounts to achieve the final

concentrations. The 19%, 23% and 27% (w/v) of sucrose solutions without CCH addition were used as controls.

5.2.7. Ice growth inhibition determination

The size of ice crystals in model systems was evaluated by a microscope after thermal cycles according to Damodaran (2007) with slight modifications. In this analysis, 2 μL of sample was injected on a microscope glass slide and covered by a glass coverslip with a press to allow the sample to evenly spread. The glass slide was then placed in a temperature-controlled stage (Linkam LTS 350, Linkam Scientific Instruments, Tadworth, UK) that was equipped on a polarized light microscope (Leica DMRX, Leica Microsystems Inc., Concord, ON, Canada). The slide was quickly frozen to -25 °C with a freezing rate of 20 °C/min and held for 5 min to set the frozen state. To simulate the thermal cycles in a household freezer, the stage was slowly warmed to -16 °C at 2 °C/min and then cycled between -16 °C to -12 °C at the rate of cycle/2 min. Images at 10 × magnifications of ice crystals in each sample was recorded after seven thermal cycles using the camera integrated in the microscope (the sucrose controls were captured at -25 °C before cycles as well), and the average dimension of ice crystals was analyzed with the Image J program (Particle size analysis SOP). Three observations for each sample were conducted and analyzed. The image was first converted to an 8-bit type and the scale bar was calibrated by pixel units. After subtracting the background, the threshold process was performed to segment the ice particles. Area measurement of the ice crystals was then analyzed from size 0 to infinity with included holes and excluded on edge settings. The average area of the ice crystals was then obtained for each observation.

5.2.8. Size fractionation of collagen hydrolysate

In order to identify peptides with the ability to prevent ice growth, CCH was fractionated using size-exclusion chromatography (SEC) on a Superdex peptides 10/300 GL column (GE Healthcare Life Sciences, Canada) connected to an Agilent 1100 high performance liquid chromatography (HPLC) system (Agilent Technologies, US). To observe the size distribution of CCH, 50 μL of CCH (10 mg/mL in HPLC water) was loaded on the column and separated by an isocratic elution at a flow rate of 0.45 mL/min with 50 mM sodium phosphate buffer (containing 50 mM NaCl, pH=7.0). The detection of the peptides was performed at a wavelength of 214 nm. As the outcome of this fractionation, five fractions (F1 to F5) were collected from each CCH injection and pooled. The purity of the fractions was monitored by examination of the amino acid profiles. The obtained fractions were desalted by dialysis membrane (MWCO 100-500 Da) for 72 h before dehydration, and the ice-controlling activity of lyophilized fractions was evaluated. To measure the ice inhibition activity of the fractions, 8% (w/v) of each fraction was added into 19% (w/v) of sucrose solution to reach a final solid content of 27% (w/v), and the ice crystal dimension was analyzed as described in the section 5.2.7.

5.2.9. Sequence identification by LC-MS/MS

Collagen peptides in the fraction showing the best ice growth inhibition were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Nanoflow HPLC (Easy-nLC II, Thermo Scientific) coupled to a LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific, Bremen, Germany) was used to resolve and analyze the fraction. Peptide mixtures were injected onto the column at a flow rate of 3000 nL/min and resolved at 500 nL/min using a 130 min linear gradient from 0 to 45% v/v aqueous acetonitrile in 0.2% v/v formic acid. The mass spectrometer

was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 60000 and m/z range of 100-2000. The multiply charged precursor ions were sequentially fragmented by using collision induced dissociation, and spectra of their fragments were recorded in the linear ion trap. Data was processed using Proteome Discoverer 1.4 (Thermo Scientific) and the Uniprot *Gallus gallus* collagen database was searched using SEQUEST (Thermo Scientific). Search parameters included a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.8 Da. Peptides were searched by trypsin cleavage with carbamidomethyl cysteine as a static modification and hydroxyproline, oxidized methionine and deamidated glutamine and asparagine as dynamic modifications. The analysis was conducted by the Alberta Proteomics and Mass Spectrometry Facility in the University of Alberta.

5.2.10. Ice growth inhibition of the synthesized collagen peptides

Five collagen peptides (S1 to S5) were selected among the identified peptides with the highest abundance (%). The selected collagen peptides were synthesized and their ice-controlling effect was determined in a model system containing 23% (w/v) of sucrose solution (Regand and Goff, 2005). The incorporation of BSA in the model system was used as a reference and the type I AFPs from winter flounder was employed as a comparison. The concentration ranges of the synthesized peptides (0.1 to 1 mg/mL) and AFP (0.01 to 0.5 mg/mL) applied in this analysis were determined according to preliminary tests, and the change of solid content was negligible. The analysis was conducted as described in section 5.2.7.

5.2.11. Secondary structure identification

Far-UV Circular Dichroism (CD) was utilized to identify the secondary conformations of the synthesized peptides and the fish AFP, as described by Greenfield (2006). The samples were dissolved in 0.1 M phosphate buffer (pH 7.4) with a concentration of 0.5 mg/mL and filled in 1 mm quartz cuvettes. The spectra were recorded as averages of five scans at 4 °C in the wavelength range from 190 to 250 nm, with a resolution of 2 nm on a OLIS DSM 17 UV-Vis-NIR CD spectrophotometer (Bogart, Georgia, USA). The reference (0.1 M phosphate buffer) spectrum was automatically subtracted from each sample spectrum, after which the data were smoothed and converted to mean residue ellipticity (Θ) units ($deg.cm^2.dmol^{-1}$). The analysis was operated by the Analytical and Instrumentation Laboratory in the University of Alberta.

5.3. Statistical Analysis

All the treatments and ice-growth inhibition analyses were carried out in triplicate. Data were subjected to the one-way analysis of variance (ANOVA) using SPSS statistical software (version 20.0, SPSS Inc., Chicago, USA), and comparison among means was assessed by conducting a studentized range test (Turkey HSD Test) at the 0.05 significance level. The curve fitting was performed by a non-linear fitting using GraphPad Prism software (version 4.0, San Diego, CA, USA).

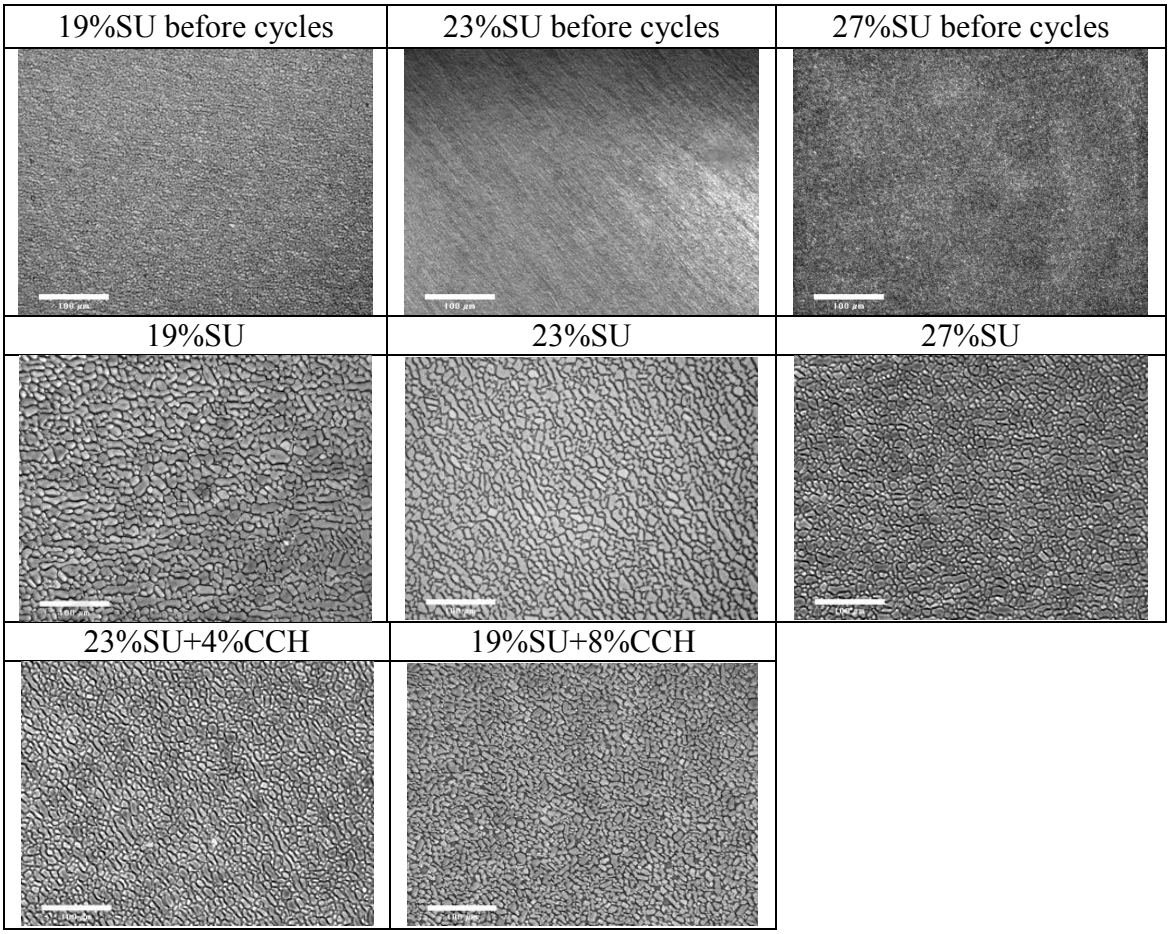
5.4. Results and discussion

5.4.1. Ice growth inhibition of collagen hydrolysate

The ice crystal growth in the control treatments containing 19%, 23% and 27% (w/v) of sucrose were observed under the microscope before and after seven thermal cycles (between -16 °C and 12 °C). The captured ice crystal images are shown in the Figure 5.1A. Before thermal

cycles, ice crystals observed at -25 °C presented very fine and tiny structures, with an average dimension smaller than 6.8 μm^2 in all of the sucrose solutions. However, after experiencing the temperature fluctuations during the thermal cycles, the mean size of the ice crystals significantly increased to 84.5 ±4.3, 64.9±1.5 and 42.8±3.4 (μm^2) in the 19%, 23% and 27% (w/v) sucrose systems, respectively, and clear differences ($P < 0.05$) among the three controls were noted (Figure 5.1B). This sharp increase of the ice crystal dimensions after thermal cycles is mainly due to ice recrystallization. Ice recrystallization is significantly accelerated as a result of temperature fluctuations. As the temperature rises, the smaller ice crystals melt, while during the recooling phase the resulting moisture preferentially diffuses toward to the surface of larger ice crystals rather than on the nascent forming new ice nuclei (Pham and Mawson, 1997). Therefore, as a consequence of this phenomenon, the amount of ice crystals normally decreases while the ice crystal size increases. Flores and Goff (1999) also showed that the increase in size of ice crystals occurred with temperature cycles. However, despite the ice crystal size enlarging significantly after the thermal cycles, the treatment with 27 % sucrose showed the least increase; this negative relationship between ice dimension and sucrose concentration is likely associated with the "antifreeze" function of sucrose. The addition of small MW molecules like sucrose has been known to depress the freezing point of solutions in a colligative manner, for which the reduction of freezing point is directly proportional to the molality of the solute. Thus, greater sucrose concentrations result in a greater amount of unfrozen water, capable of attenuating the formation of large ice crystals (Hartel, 1996).

A.



B.

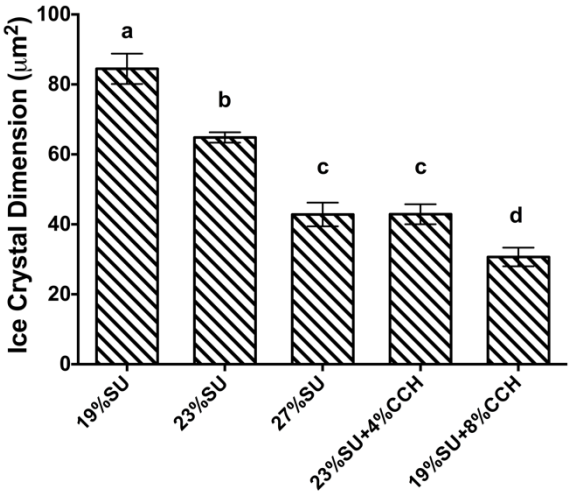


Figure 5.1. Ice crystal images (A) and average ice crystal size (B) of the sucrose model solutions (SU) with or without addition of collagen hydrolysates (CCH). Results are means ± standard deviations (n=3) in B.

Since the solute content in solution plays a role in determining the ice crystal size, 4% and 8 % (w/v) of CCH were mixed in the sucrose solutions with 23% and 19% (w/v) sucrose, respectively, to retain a constant solid content in the model systems. The ice crystal pictures of the systems after thermal cycles are presented in Figure 5.1A, while their respective average ice crystal dimension is shown in Figure 5.1B. Compared to the 23% sucrose control treatment, the incorporation of 4% CCH reduced ($P < 0.05$) the mean ice crystal size from 64.9±1.5 to 42.9±2.9 (μm^2). This value was not different from that obtained in the control solution containing 27% sucrose, which has the same solid content as the 23% sucrose + 4% CCH treatment. This proves that the presence of CCH can inhibit the ice crystal growth, and that the replacement of 4% sucrose with an equivalent amount of CCH does not significantly change the ice crystal size. However, when the CCH concentration increased to 8%, the ice crystal dimension was significantly decreased compared to the 27% sucrose solution (Figure 5.1B). This implies that the addition of 8% CCH is more active than an equivalent amount of sucrose in controlling ice crystal size. Therefore, CCH likely contains peptides that, on top of working in a colligative way as sucrose does, may also act as an ice-controlling agent.

5.4.2. Ice growth inhibition of collected fractions and peptide sequence identification

In order to understand and isolate the collagen peptides responsible for the ice inhibition activity of the CCH, the hydrolysate was separated into fractions using size-exclusion chromatography (Figure 5.2). A total of five fractions (F1 to F5) were collected and their ice-controlling effects are reported in Figure 5.3A and 5.3B. The treatment with 8% F1 in 19% sucrose solution exhibited the largest ($P < 0.05$) ice crystals among all the treatments, with an average ice size of 80.3±5.8 μm^2. Among the other fractions, the only one showing an appreciable effect was F3, which reach the level of 40.2 ±2.8 (μm^2) similar to the one obtained for the 27% sucrose solution.

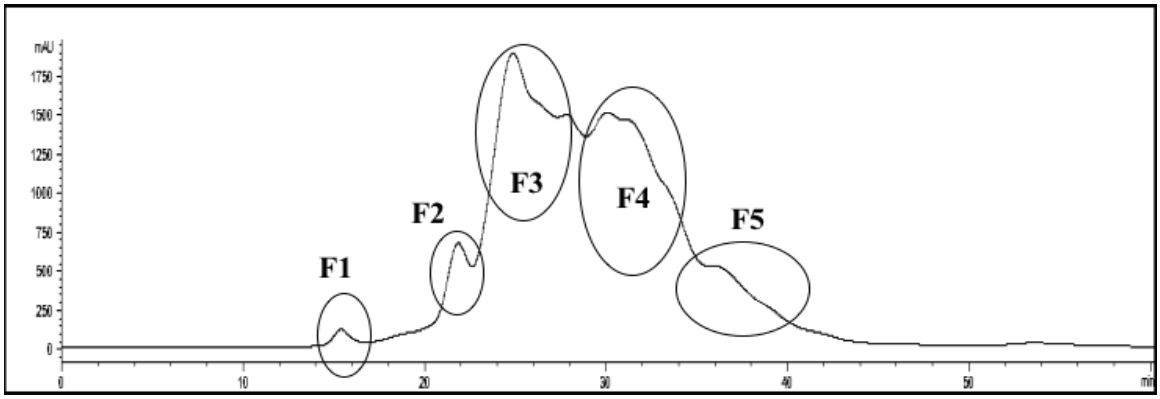
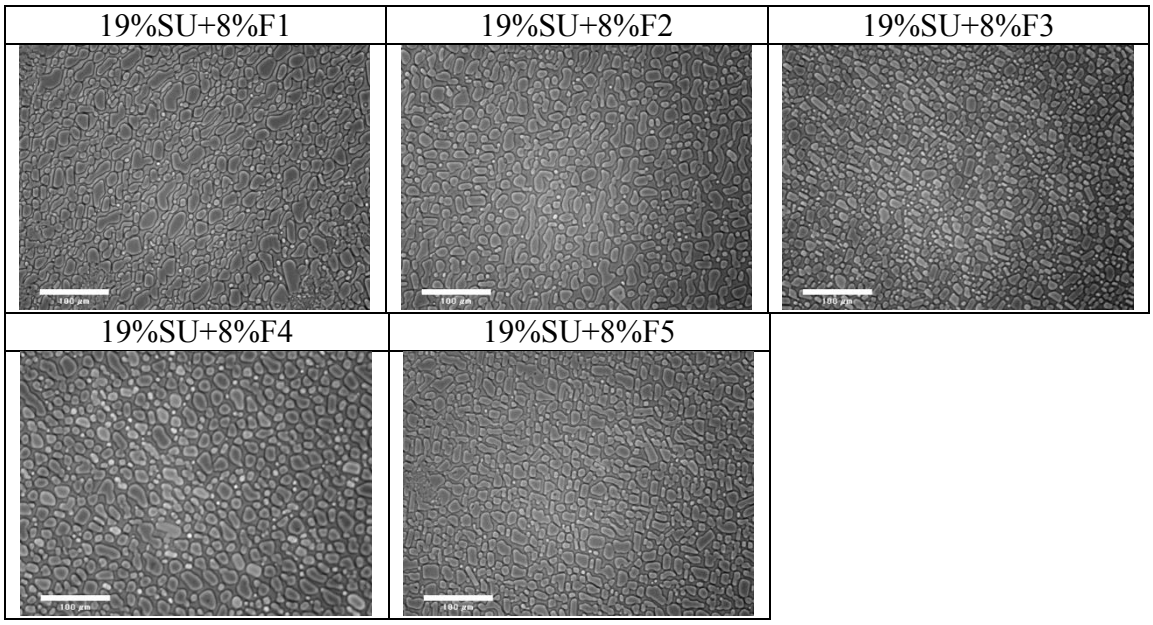


Figure 5.2. Fractionation of the collagen hydrolysates (CCH) by size exclusion chromatography.

The champion fraction F3 was therefore selected and subjected to LC-MS/MS for sequence identification. In this study, the protease trypsin was used to hydrolyze collagen. Since trypsin cleaves the C-terminal to positively charged arginine or lysine, the resulting peptides are in a desired mass/charge range in MS and can be efficiently fragmented in the tandem MS. This leads to a better signal intensity (ionization efficiency in the other words) and as a sequence more informative y-ion series. In addition, trypsin has high cleavage specificity and hydrolysis efficiency. The LC chromatograph of F3 is shown in Figure 5.4A, in which the majority of peptides

A.



B.

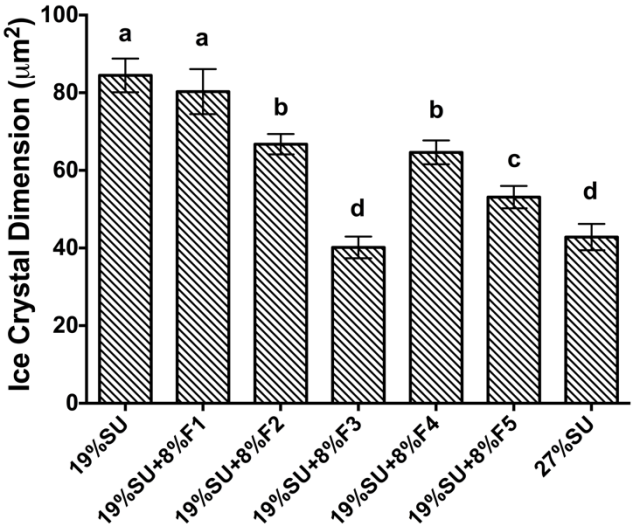


Figure 5.3. Ice crystal images (A) and average ice crystal size (B) of the 19% (w/v) sucrose model solution (SU) with the addition of 8% (w/v) of each fraction. Results are means ± standard deviations (n=3) in B.

was eluted from 10 to 60 min and the peptide MS spectrum within that retention time is presented in Figure 5.4B. These charged peptides were subsequently fragmented and distinguished through matching to the MS/MS fragment spectra in the database. Among the peaks observed in the MS spectrum, several tryptic collagen peptides were identified, but only the major peaks with the greatest abundance are labelled (Figure 5.4B) and listed (Table 5.1). Here it is noticed that the molecular weight of F3 belongs to a range from about 1500 Da to 2700 Da. The molecular weight of bioactive protein or peptides has been linked to their activities; different molecular weights of AFPs and collagen hydrolysates have been reported to exhibit the inhibitory properties on ice crystal growth (Damodaran, 2007; Davies and Sykes, 1997; Wang and Damodaran, 2009). The top five identified peptides are reported in Table 5.1 as S1 to S5, and their fragmentation spectra are documented in the Appendix A. The amino acid sequences of the peptides are consistent with the repetitive triplets (-Gly-X-Y-) occurring in the native collagen structure, where X and Y have a good probability to be proline or hydroxyproline, respectively. This characteristic glycine-rich tripeptide unit of collagen peptides, surprisingly similar to the hyperactive AFP found in snow fleas (Graham and Davies, 2005; Lin et al., 2007), is considered to be the main factor responsible for the ice inhibition. This effect for natural AFPs is proposed to occur with an analogue mechanism otherwise known as the adsorption-inhibition mechanism. The adsorption-inhibition mechanism depicts that AFPs have the specific conformations to bind to ice and then create micro-curvatures at the ice front between the bound AFPs; this makes it thermodynamically unfavorable for spontaneous ice growth to occur (Barret, 2001; Knight and Wierzbicki, 2001; Venketesh and Dayananda, 2008; Yeh and Feeney, 1996). The hydrolyzed gelatin peptides with the repeating elements may form an oxygen triad plane with oxygen-oxygen distances similar to those found in

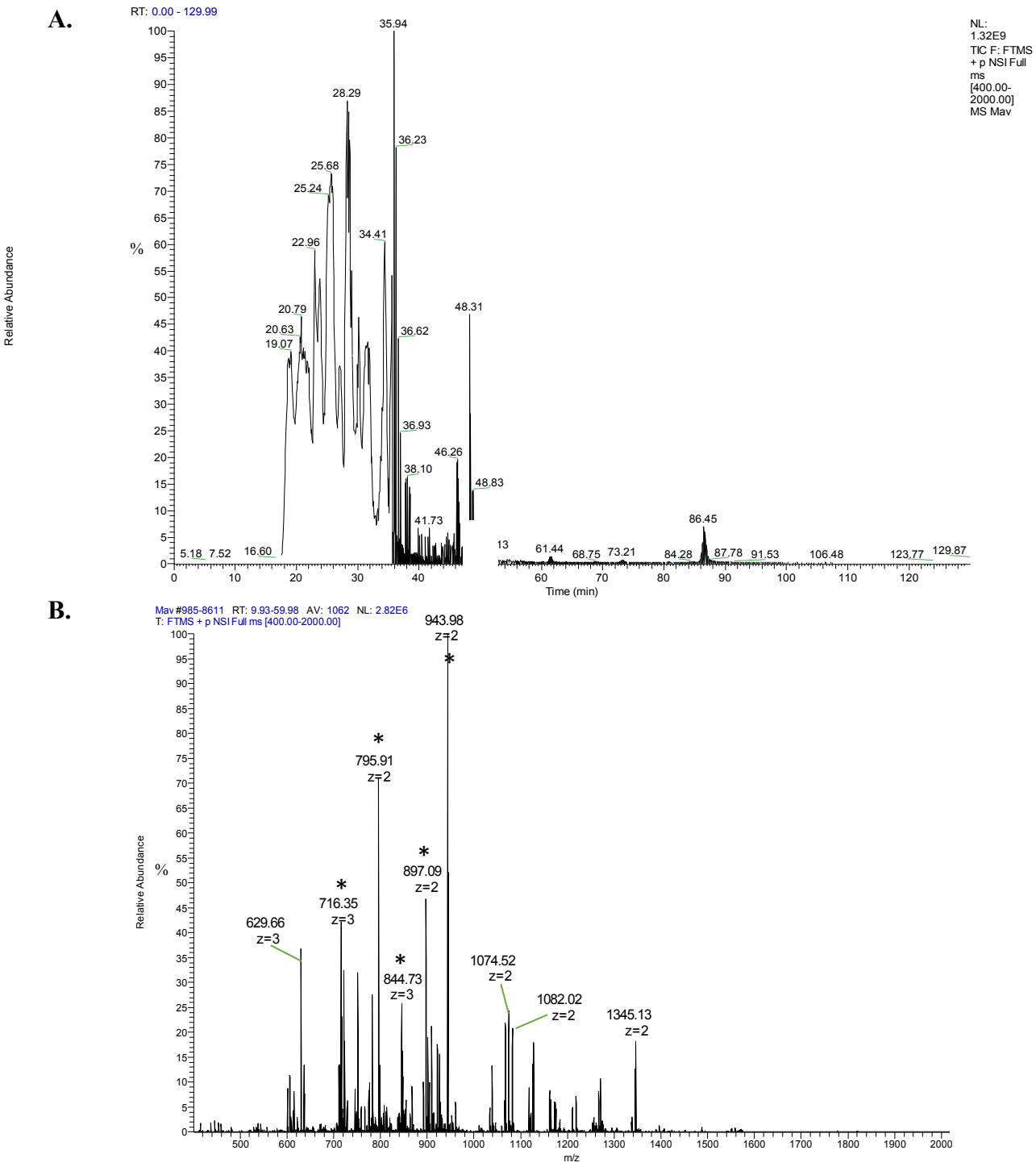


Figure 5.4. The liquid chromatograph (A) and MS spectrum (B) of the fraction 3 obtained in the LC-MS/MS analysis.

Table 5.1. The identified sequences of the most abundant peptides in fraction 3. The five most intensive peptides are labeled as S1 to S5.

m/z	Sequence		MH^+ (Da)
943.98 (z=2) and 629.66 (z=3)	VGPHypGPSGNIGLHypGPHypGHypAGK	(S1)	1886.96
795.91 (z=2)	GLTGPIGHypPGPAGAHypGDK	(S2)	1590.82
897.09 (z=3)	GFSGIQGPPGPHypGSHypGEQGPSGASGPAGPR	(S3)	2689.27
716.35 (z=3)	GSPGADGPIGAHypGTHypGPQGIAGQR	(S4)	2147.05
844.73 (z=3)	GNDGAHypGAAGPHypGPTGPAGPHypGFHypGAAGAK	(S5)	2532.18
1082.02 (z=2)	SHypGADGPIGAHypGTHypGPQGIAGQR		2163.05
1074.52 (z=2)	GSHypGADGPIGAHypGTPGPQGIAGER		2148.04
1345.13 (z=2)	GFSGLQGPHypGPHypGAHypGEQGPSGASGPAGPR		2689.26

ice nuclei (4.5 Å), thus binding to the ice surface possibly via hydrogen bonding, and further inhibiting ice growth (Damodaran, 2007). The considerable content of glycine is believed to contribute to a low steric barrier for ice binding and participate in hydrogen bonding interactions (Lin et al., 2007). In addition, proline has a cryoprotective effect on cells and proteins (Pemberton et al., 2012; Withers and King, 1979), which may also be associated with the ice-controlling property of collagen peptides that naturally contain abundant proline. To verify the ice-binding hypothesis on the tryptic chicken collagen peptides, the five major peptides of F3 were synthesized in a pure form (mentioned as S1 to S5, Table 5.1), and subjected to the thermal freezing cycles.

5.4.3. Ice growth inhibition of synthesized peptides vs. type I AFPs

The type I AFPs from winter flounder have been fully characterized as alanine-rich, α-helical proteins with a molecular weight of 3300 Da to 4500 Da (Davies and Sykes, 1997). For comparison, an ice inhibition test on natural type I fish AFPs was conducted under the same

conditions as with the synthesized peptides, and BSA was used as a negative control. In this analysis, the sucrose content was kept as 23% (w/v), and the addition of synthesized peptides or AFP in the sucrose system was less than 0.1%, in order to minimize the change of total solids and avoid a differential colligative effect.

The curve reported in Figure 5.5A illustrates the mathematical relation between AFP concentration and the ice crystal size, which is fitted into an exponential decay model with an R^2 of 0.97. As the curve shows, the AFP started to control the ice growth at a very low concentration of 0.01 mg/mL (corresponding ice image is shown in Figure 5.6A), while a prominent decline from 63.2±2.4 to 21.2±4.3 μm^2 was observed when the AFP concentration reached to 0.1 mg/mL (image shown in Figure 5.6A). A plateau was gradually attained over 0.1 mg/mL and displayed an ice dimension of 4.5±0.3 μm^2 with 0.5 mg/mL AFP (image shown in Figure 5.6A), equal to an almost 100% ice inhibition. These results show the excellent efficacy of fish AFPs to control ice growth in a sucrose model system and are in line with the findings of Hagiwara et al. (2011). The type I AFPs have a helix periodicity of three turns every 11 residues reflected in a recurring sequence of ($ThrX_{10}$), where X is frequently alanine (Hobbs et al., 2011; Sicheri and Yang, 1995). The hydrophobic alanine-rich face in the type I AFPs is likely important for ice-binding and inhibition (Patel and Graether, 2010).

With regard to the five collagen peptides (Figure 5.5B), four of them (S2, S3, S4, S5) possessed an ice growth inhibition effect within the concentration range from 0 to1 mg/mL, decreasing the ice size from 64.9±1.5 μm^2 to less than 30 μm^2 (images shown in Figure 5.6B), and 5 mg/mL of the peptides (not shown) were applied in preliminary tests to ensure the plateau has been reached at 1 mg/mL. Compared to AFPs, the active peptides had smaller molecular weights,

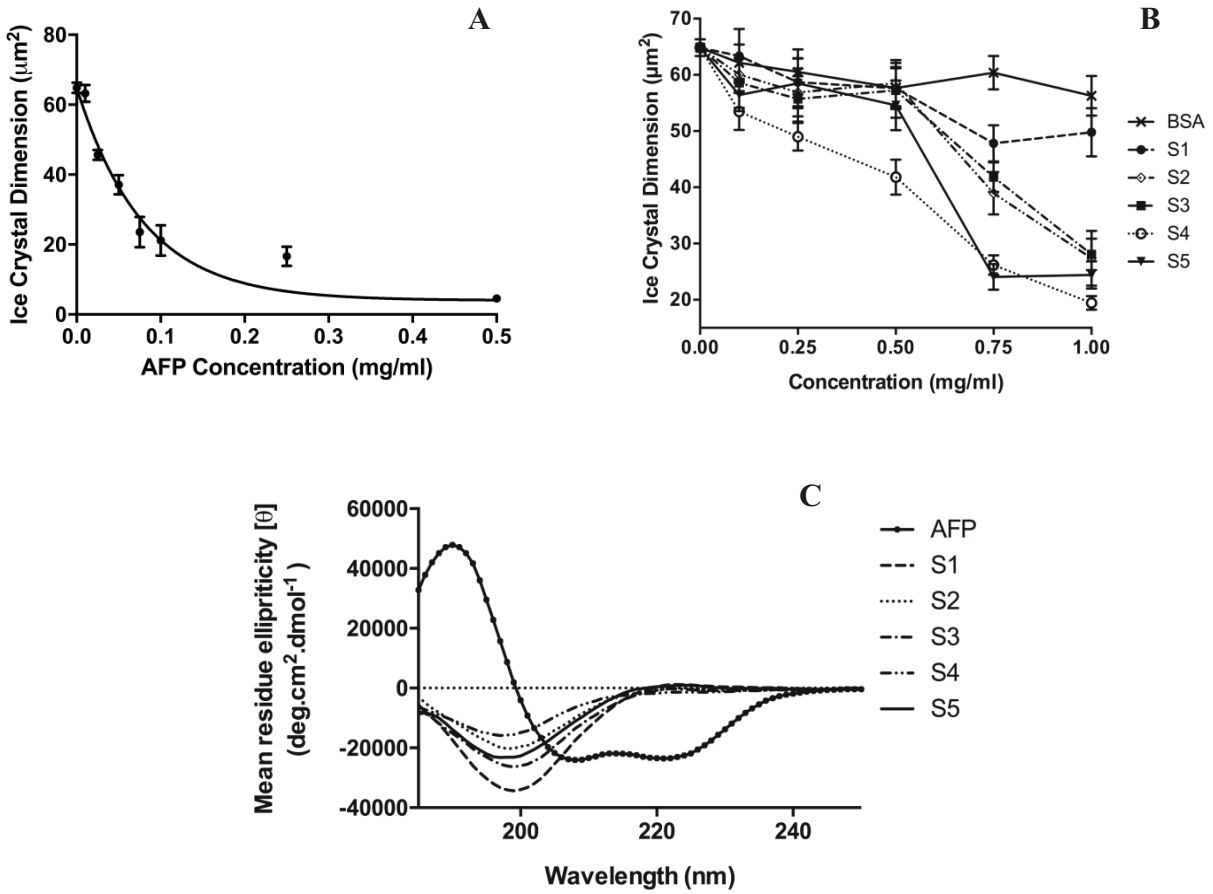
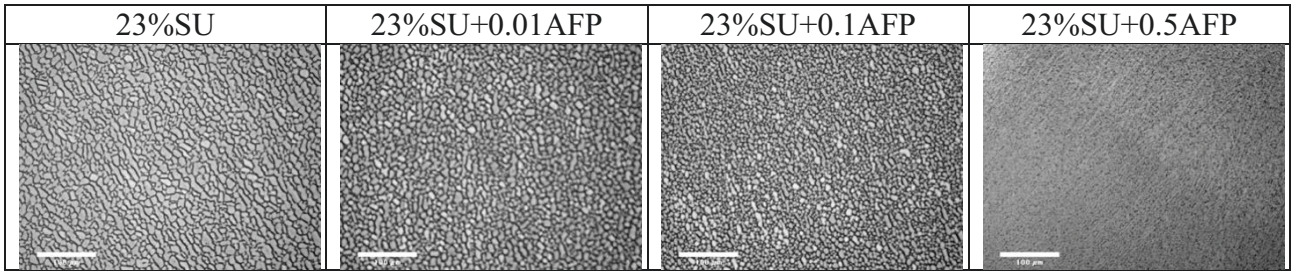


Figure 5.5. Average ice crystal sizes of 23% (w/v) sucrose model systems with the addition of different concentrations of fish AFPs (A, fitted in a model of $Y=60.86*\exp^{(-12.59*X)}+4$, $R^2=0.9693$) or synthesized (S1-S5) collagen peptides (B). The far-UV CD spectra of the AFPs and synthesized collagen peptides (C). Results are means ± standard deviations (n=3) in A and B.

from 1500 Da to 2700 Da, and this is in agreement with the findings from Wang and Damodaran (2009). Among the four peptides, S4 and S5 showed the best potential. The S4 presented the greatest activity with a final ice dimension of 19.4±1.2 μm^2 at 1 mg/mL, accounting for about a 70% reduction in ice-growth. The S5 was able to rapidly reduce the ice size to a similar level of S4 at a concentration from 0.5 to 0.75 mg/mL (images shown in Figure 5.6B). Similar to the AFPs, the greater appearance of alanine in the primary structure of S5 may result in hydrophobic regions that promote the binding to the ice crystals. According to the peptide alignment results shown in Appendix B, the sequences of the S4 and S5 have great similarities (query coverage ranging from 66% to 100%) with some amino acids sequences found in the two AFPs of snow flea (Q38PT6 and D7PBP2 in Uniprot database). Interestingly, the GADG amino acid sequence in S4 and the two GAAG sequence components existing in S5 are found exactly to match with specific amino acid sequences in those two snow flea AFPs (Figure 5.7). Moreover, these two sequences are also very similar to the short peptides GPAG and GGAG which have been proposed to have ice-inhibition activity (Kim et al., 2009).

Taken together these results imply that the synthesized peptides can inhibit the ice-growth in a similar non-colligative manner as with AFP. They all showed a notable decrease in the dimension of ice crystals in the range of 0.1-1.0 mg/mL. However, the AFP possessed a much greater effectiveness and efficiency than the S4 and S5, working at a 10-fold lower concentration. These differences may mainly relate to their structure details as discussed in the following paragraph.

A.



B.

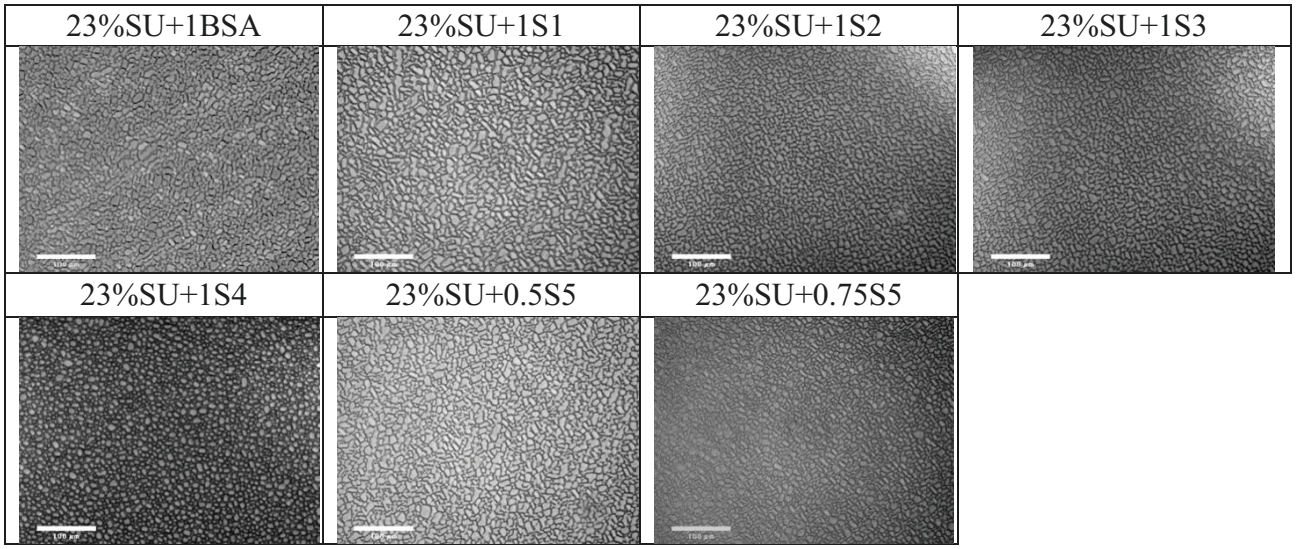


Figure 5.6. Selected ice crystal images of 23% (w/v) sucrose model systems (SU) with the addition of different concentrations (mg/mL) of fish AFPs (A) or synthesized (S1-S5) collagen peptides (B).

5.4.4. Evaluation of secondary structures of AFP and identified collagen peptides

Besides the differences existing in the primary amino acid sequence between the collagen peptides and the AFP, secondary structural differences may also explain the different ice inhibition activities. Far-UV CD (190 to 250 nm) was employed for the evaluation of the secondary structure of the fish AFPs and the five identified peptides. In a typical CD spectrum, an α-helical structure has negative bands at 222 nm and 208 nm and a positive band at 193 nm. The well-defined anti-

parallel β-sheets have negative bands at 218 nm and positive bands at 195 nm, while the disordered structures have very low ellipticity above 210 nm and negative bands near 195 nm (Greenfield, 2006). The CD spectra of the treatments are shown in Figure 5.5C. The AFP clearly showed a classic curve of α-helical structure, as documented in the literature. Different from native collagen, the spectra of collagen peptides had a more disordered conformation rather than a defined α-helix structure, but did correspond to the characteristic spectrum of denatured collagen with a negative peak around 200 nm and no positive peak above 200 nm (Lopes et al., 2014). Therefore, the greater regularity of the AFP structure may be the key reason for its better ice-controlling activity, which is associated with specific ice-binding sites. Based on the spectra, the S4 revealed the lowest negative ellipticity near 200 nm, indicating a less unordered configuration and corresponding to its appreciable ice inhibition activity, while the S1 showed the greatest degree of disorder. These results seem to indicate that a lower level of unordered structures in the peptides plays a role on its activity.

6.5 kDa snow flea antifreeze protein

```
        10          20          30          40          50
MAQMKFILVA FLVVLAVSWA NACKGADGAH GVNGCPGTAG AAGSVGGPGC
        60          70          80          90         100
DGGHGGNGGN GNPGCAGGVG GAGGASGGTG VGGRGGKGGS GTPKGADGAP

GAP
```

15.7 kDa snow flea antifreeze protein

```
        10          20          30          40          50
MLPSRIIGVL LFCCIGGIIS CKAPNGADGR SNGEAGGAGT AGANGCDGGD
        60          70          80          90         100
GGNGFPGTGS AAGGAGGIGG VGGAGAKGGN GGKGGIGASS ESAAGGAGGA
       110         120         130         140         150
GGAAGAGSSG PGGQGGEGGK GGKANGVTGT GGAGGIGGVG GAGSGGQPGG
       160         170         180         190         200
NGGNAGLGGD SVTAGGAGGT GGAGGAGTPG GRGGNGANGG SGHGNPGGRG
       210         220
LPGAAGLPVG GGGAGGIGGG GGA
```

Figure 5.7. Match of the sequence components of S4 (GADG) and S5 (GAAG) in the sequences of the two antifreeze proteins from snow flea. The appearances of the two sequences from S4 and S5 are in bold and underlined.

5.5. Conclusion

In this study, several ice-controlling peptides were identified and compared with the well-studied fish type I AFPs. The results demonstrate that specific collagen peptides like GSPGADGPIGAHypGTHypGPQGIAGQR (2147.1Da) and GNDGAHypGAAGPHypGPTGPAGPHypGFHypGAAGAK (2532.2 Da) can reduce ice crystal growth up to about 70% in a 23% (w/v) sucrose system when used at a concentration 1 mg/mL. This appreciable activity may be attributed to a good adsorption on ice surface as similar to the AFPs, therefore controlling the ice expansion during the recrystallization process. These findings show that specific collagen peptides are responsible for the cryprotection capacity of the collagen hydrolysate obtained from chicken skin. Despite the ice growth inhibition capacity of these

collagen peptides being not as efficient as type I AFPs, they nonetheless have good cryoprotective activity. Unlike the limited availability and impractical cost of fish AFPs, the relative abundant access to underutilized chicken skin collagen make collagen hydrolysates a promising alternative antifreeze protein to explore further.

Chapter 6 - Chicken collagen hydrolysate cryoprotection of natural actomyosin: mechanism studies during freeze-thaw cycles and simulated digestion

6.1. Introduction

Freezing is a common technique to preserve meat products and extend the shelf-life. However, due to the ice formation and temperature fluctuations during frozen storage, meat products undergo a series of physical and chemical changes, including dehydration, ice recrystallization, protein denaturation, lipid oxidation, and vitamin loss. These changes further cause the deterioration of flavour, texture, and functional properties of meat products (Zaritzky, 2011).

Freeze-induced protein denaturation occurs when proteins suffer dehydration and salt concentration during ice formation and recrystallization. This process consequently leads to protein unfolding, aggregation and oxidation (Zaritzky, 2011). Muscle proteins are particularly susceptible to freeze-denaturation and the functionality losses of meat proteins resulting from freeze-denaturation are commonly found in frozen fish and poultry products (Xiong, 1997). The addition of cryoprotectants to frozen meat products is important in order to alleviate the undesirable impact of frozen storage, and so they can retain their functional properties, such as protein solubility, water holding capacity and gel-forming ability.

The mechanism by which cryoprotectants exert their protective function in food depends on their chemistry and the specificity of the food system. The most commonly used commercial cryoprotectants in meat products are low molecular weight sugars and polyols, such as sucrose and

sorbitol. These compounds take effect mainly through three mechanisms: i) depressing the freezing point of water in a colligative manner; ii) coating the protein molecules by forming ionic bonds or hydrogen bonds with protein functional groups (Matsumoto, 1980); iii) through the solute-exclusion theory where sugars increase the surface tension of aqueous solution of protein, resulting in sugars being preferentially excluded from the protein surface, and thus hydrating proteins to stabilize their native structures (Carpenter and Crowe, 1988). However, the addition of these cryoprotectants imparts a sweet taste and high calorie value to the food, sometimes deemed unfavorable by certain populations, especially in meat products. Therefore, alternative cryoprotectants with reduced sweetness have been an interest to food scientists. Recently, Damodaran (2007) demonstrated that gelatin hydrolysate derived from bovine collagen could also act as a cryoprotectant by inhibiting the growth of ice in ice cream. He hypothesized that the unique repetitive amino acids sequence in the collagen molecule (Gly-X-Y) played a role in controlling ice growth with a mechanism similar to the one proposed for antifreeze proteins (AFPs) found in fishes, insects, or plants living in subzero temperatures. AFPs possess specific conformations which are capable of aligning and adapting to ice surfaces. The AFP ice-inhibition activity is explained by an "adsorption-inhibition mechanism" in which the AFPs bind onto the growing surfaces of ice crystals limiting their expansion (Raymond and DeVries, 1977). The growth of ice crystals can destroy the hydration sphere surrounding a protein leading to protein unfolding and hydrophobic interactions, considered to be the primary cause of freeze-denaturation in fish protein (Hanafusa, 1973). Kittiphattanabawon et al. (2012) also discovered the cryoprotective effect of gelatin hydrolysate prepared from blacktip shark skin to reduce surimi protein denaturation during repeated freeze-thawing. They indicated that the suppression of protein

freeze-denaturation by adding gelatin hydrolysate may be related to more unfrozen water molecules surrounding proteins. Furthermore, some collagen peptides may also exhibit antioxidant activity and protect proteins against oxidation (Wang et al., 2013). To date, several more studies have been published to provide further information about the cryoprotective nature of collagen-based hydrolysate (Cao et al., 2016; Limpisophon et al., 2015; Wang et al., 2014; Wang et al., 2015) and several cryoprotective sequences from collagen have been identified. However, research on this topic is still scarce.

Previously, our research group has shown that poultry by-products can serve as an alternative source for high quality collagen and gelatin production (Du et al., 2013ab). Further investigation of these cryoprotective peptides following enzymatic hydrolysis is warranted, since there is no scientific literature regarding poultry by-products as a source of functional additives against freeze-denaturation of myofibrillar proteins.

In this study, collagen hydrolysate was prepared from chicken skin which is one of the most underutilized poultry by-products. Its cryoprotective effect against protein freeze-denaturation was evaluated on the natural actin-myosin complex (actomyosin, NAM) obtained from chicken breast muscle. This comprehensive study was aimed to elucidate the mechanism of chicken collagen hydrolysate (CCH) in retarding protein freeze-denaturation, starting from the inhibitive effect of CCH on ice growth, followed by analyzing its cryoprotective action on NAM conformation and oxidation after frozen storage. Finally, the influence of these modifications on protein gel formation and *in vitro* digestibility was also investigated as functional tests relevant to the food industry.

6.2. *Materials and methods*

6.2.1. Materials

Broiler chicken skin (sacrificed at 42 days) was kindly provided by Poultry Research Centre of University of Alberta (Edmonton, AB, Canada). Upon arrival to the Food Protein Chemistry Laboratory, the skin pieces were packaged into polyethylene bags and stored at - 20 ºC until use in less than 2 months. The chicken breasts used for NAM extraction were purchased from a local supermarket and stored at 4 ºC for fewer than three days. All chemicals were of analytical grade.

6.2.2. Experimental design

Collagen was extracted from broiler chicken skin, and the collagen hydrolysate was obtained after trypsin hydrolysis. In the first part of this study, the effects of CCH on ice crystal dimensions in a NAM model system were conducted. Three batches of CCH were produced from chicken skin, freeze-dried and then pooled for later experiments. Subsequently, CCH at 4%, 8%, and 12% of total protein content (w/w) were uniformly mixed with NAM extracted from *Pectoralis major* muscle of broiler chickens, and subjected to freeze-thaw cycles as reported in section 6.2.7. Two NAM controls without addition of CCH were prepared; one had a NAM concentration of 3 mg/mL, while the other had a NAM concentration of 3.4 mg/mL equivalent to the treatment containing NAM + 12% CCH. A treatment similar to a commercial blend consisting of 8% (w/w) sucrose/sorbitol (sucrose:sorbitol = 1:1) mixture (SUSO) in NAM was also performed and used as a positive control. The experiment consisted of a total of 6 treatments which were applied to three independent NAM batches. The material in each tube was analyzed after freeze-thaw cycles, as described in section 6.2.7.

In the second part of the study, the focus was to investigate the influence of CCH on protein denaturation and protein oxidation during the freeze-thaw cycles, and how these chemical changes affect the NAM functionalities (solubility, water holding capacity, and gelation) as well as NAM microstructure before and after thermal processing. Before the analyses, CCH and SUSO were removed using a washing procedure. The treatments were the same as discussed in the first part of the study, but without the second NAM control. A fresh NAM without freeze-thaw was employed as an extra positive control in this part. Finally, the last part of the study was dedicated to understanding how the treatments affected the digestibility of both raw and cooked NAM in a simulated gastrointestinal digestion (section 6. 2.13). In this case, a 2 x 6 factorial design was used testing the main effects of "cooking" (raw or cooked NAM) and cryprotectant treatments (CCH 0%, CCH 4%, CCH 8%, CCH 12%, 8% SUSO and untreated fresh NAM).

6.2.3. Chicken skin preparation

6.2.4. Collagen extraction

6.2.5. Collagen hydrolysate preparation

For the sections 6.2.3 to 6.2.5, please refer to sections 5.2.3 to 5.2.5.

6.2.6. Natural actomyosin (NAM) extraction

Natural actomyosin (NAM) was extracted from the chicken breast using 0.6 M KCl according to Yarnpakdee et al. (2009). The protein content of NAM was determined by the Biuret procedure (Torten and Whitaker, 1964) with bovine serum albumin (BSA) as a standard. All the obtained NAM pellets were pooled, with a final protein concentration of 38 mg/mL. The extracted protein was kept on ice and subjected to treatments or analyses within 12 h.

6.2.7. Effect of CCH on ice-growth in NAM model system

For this analysis, NAM solution was constituted by 3 mg/mL of protein in 0.6 M KCl solution with the presence of various concentrations (4%, 8% and 12%, w/w) of CCH or 8% (w/w) of SUSO, respectively. Two NAM controls were prepared: control 1 was a 3 mg/mL NAM solution and control 2 was 3.4 mg/mL NAM solution which corresponds to the same solid content as in the NAM+12%CCH treatment. The ice-growth inhibitive activity of CCH was evaluated according to Damodaran (2007) with slight modifications, as described in the section 5.2.7. In this case, the slide was quickly frozen to -20 °C and the image of ice crystals was captured after 7 thermal cycles.

6.2.8. Freeze-thawing of isolated NAM

The extracted NAM pellet with 38 mg/mL was treated with 0%, 4%, 8%, and 12% (w/w) CCH and 8% (w/w) SUSO, respectively. All samples were subjected to 7 freeze-thaw cycles with 18 h freezing at -20 °C and 6 h thawing at 4 °C for each cycle (Cheung, Liceaga and Li-Chan, 2009). A fresh extracted NAM without any addition of cryoprotectants was used as a second positive control for comparison with the CCH treatments.

6.2.9. Microstructure evaluation of NAM paste with scanning electron microscope (SEM)

The microstructures of freeze-thawed NAM samples (38mg/mL) were randomly chosen from treatments with 0%, 8% (w/w) CCH and SUSO, respectively, and were observed using SEM after freeze-drying. The fractured samples were sputtered with gold coating and visualized with a SEM (JEOL JSM-6301, Tokyo, Japan) at different magnifications, ranging from 1000 × to 19000 × magnification, using equipment at the Scanning Electron Microscope Lab of the Earth Sciences Department.

6.2.10. Evaluation of cryoprotective effect of CCH on NAM unfolding and oxidation

Prior to all analyses, including protein solubility, 3 g of each freeze-thawed or fresh NAM sample was vigorously agitated with 30 mL of chilled distilled water using a spatula, followed by a centrifugation at 8000 × g for 10 min at 2 °C. The supernatant was discarded, and this step was repeated twice to remove the CCH or SUSO which could interfere with the analyses and affect the results (Kittiphattanabawon et al., 2012).

6.2.10.1. Surface hydrophobicity

The surface hydrophobicity of fresh and treated NAM was measured according to the method described by Omana et al. (2010b), using 8 mM 1-anilinonaphthalene-8-sulfonic acid (ANS) in 0.1 M phosphate buffer (pH 7.0) as a fluorescent probe. The NAM solution was prepared with sonication (1 min, Careche and Li-Chan, 1997) in 0.01 M phosphate buffer (pH 6.0, containing 0.6 M NaCl) and diluted with the same buffer to 0.125, 0.25, 0.5 and 1 mg/ml for the test.

6.2.10.2. Total and reactive sulfhydryl contents

One gram of NAM pellet was dissolved in 10 mL tris–glycine buffer (pH 8.0) containing 5 mM EDTA with intermittent vortex for 20 min. The total and reactive sulfhydryl (-SH) contents of NAM samples were estimated per the method described by Chan, Omana and Betti (2011a) using Ellman's reagent. The protein content of NAM solution was determined by the Biuret procedure.

6.2.10.3. Protein carbonyl content

The protein carbonyl content of the NAM extracts was quantified according to the spectrophotometric method of Wehr and Levine (2013) by derivatizing proteins with 2,4 dinitrophenylhydrazine (DNPH).

6.2.11. Cryoprotective effect of CCH on NAM functionalities

6.2.11.1. Protein solubility

The protein solubility of NAM samples was determined according to the procedure of Chan, Omana and Betti (2011b) with modifications. The washed NAM sample was homogenized with 10 mL 0.05 M phosphate buffer containing 0.6 M KCl (pH 7.0) for 2 min and the homogenate was then filtered through Whatman No.1 filter paper. The protein content of the filtrate was assessed using the Biuret method and was presented as the protein solubility (mg/mL).

6.2.11.2. Cooking loss

As reported in section 6. 2.10, for all the following functionality tests, the CCH or SUSO were removed from each sample and the protein concentration in each sample was adjusted to 38 mg/mL. Cooking loss of the fresh NAM and freeze-thawed NAM was determined after cooking at 75 °C in a water bath for 10 min. Prior to heating, 2.5% (w/w) of solid NaCl was mixed into 2 g of NAM paste to aid gel formation. After cooking, the sealed eppendorf tubes were immediately cooled down by tap water and samples were removed and drained on tissue papers. The weight of the sample before (NAM+salt) and after cooking was recorded, and cooking loss was calculated as follows:

$$\text{Cooking loss (\%)} = [(W_1-W_2)/W_1] \times 100,$$

where W_1 and W_2 is the weight before and after cooking, respectively.

6.2.11.3. Expressible moisture

Expressible moisture of cooked NAM samples was measured using a texture profile analyzer (TA-XT Express, Stable micro systems, Ltd., Surrey, England) according to the method of Omana et al. (2010a) with slight modifications. One gram of cooked protein gel was placed between four pre-weighed Whatman No.1 filter papers and compressed with a target force of 1000 g for 30s under adhesive test mode. The protein gel residue on the filter papers was removed using a spatula. The weight difference of the filter paper before and after the test was expressed as a percentage of the original cooked gel weight, and was reported as the expressible moisture.

6.2.11.4. Gel-forming property

The gelation property of NAM samples was evaluated by their dynamic viscoelastic behaviors (DVB) during heating using a Physica MCR Rheometer (Anton Paar GmbH, Virginia, USA). The analysis was carried out according to the method of Omana et al. (2010a) with modifications. During the preliminary analyses, the linear viscoelastic region (LVR) was determined by an amplitude sweep in a range of deformation from 0.1% to 10%. Two grams of NAM was mixed thoroughly with 2.5% (w/w) NaCl and loaded on the Peltier plate of the rheometer. The gel-forming property of NAM was monitored under oscillatory mode with a 2.5 cm parallel plate measuring geometry from 4 ºC to 80 ºC at a rate of 2 ºC/min. The gap between the measuring geometry and the Peltier plate was set at 1.0 cm. The constant strain and frequency used in the measurements were 0.5% and 1.0 Hz, respectively. The changes of storage modulus (elasticity, G') and loss modulus (viscosity, G'') of NAM were plotted during the measurements. The ratio of these two values (tan δ, G''/G') was also recorded.

6.2.12. Microstructure of NAM gels

The cooked NAM was prepared as described in the section 6.2.11.2. The prepared samples were sent for fixation in the Oncology Lab of the Cross Cancer Institute (University of Alberta, Canada) before imaging. NAM gels were fixed using paraformaldehyde, glutaraldehyde and osmium tetroxide, and then dehydrated by ethanol, according to a standard SEM sample fixation protocol (Beckman.illinois.edu). The gels were completed dried in a critical point dryer (Bal-Tec CPD 030, Leica Microsystems, Germany). Fractured samples were then mounted on the holder and coated with gold. The microstructure of the NAM gels was visualized under a field emission SEM (Zeiss Sigma FESEM, Germany) in the nanoFAB Lab of the University of Alberta, and the images magnified and captured at 1,000×, 5,000× and 10,000×.

6.2.13. In vitro gastrointestinal digestion of NAM

In order to understand the effect of CCH on digestibility of NAM after frozen storage, an *in vitro* gastrointestinal digestion was performed on both raw and the cooked NAM according to the method described by Cinq-Mars et al. (2008) with slight modifications. The raw or cooked NAM containing 75 mg of protein was homogenized with 10 mL distilled water and the pH was adjusted to 2.0 with 6 M HCl. The dissolved pepsin (40 mg/mL, pH 2.0) was then added into the NAM sample (E/S 1:25 w/w) and the mixture was incubated under agitation for 1 h at 37 ºC. After adjusting the pH of pepsin-digested sample to 5.3 with 0.9 M $NaHCO_3$ and further to pH 7.5 with 6 M NaOH, the prepared pancreatin solution (40mg/mL, pH 7.5) was immediately added (E/S 1:25 w/w). The mixture was then incubated under agitation for 2 h at 37 °C, and the digestion process was terminated by submerging in 80 °C for 15 min. After digestion, the solution was centrifuged at 10,000 x g for 10 min and the supernatant was used for analysis.

6.2.13.1. Digestibility

The peptide content of each collected supernatant was determined using o-Phthaldialdehyde (OPA) per the method of Church et al. (1983), with L-leucine as a standard. The peptide yield of digested protein was expressed as digestibility (%) and calculated with the following equation:

$$\text{Digestibility (\%)} = [(C_2\text{-}C_1)/C] \times 100,$$

where C1 and C2 are the peptide contents before and after digestion, respectively.

And C is the initial protein content of the NAM before digestion.

6.2.13.2. Size-exclusion chromatography (SEC)

To elucidate the differences of molecular weight profiles among the digested NAM samples, the obtained supernatant was pooled from the replications and analyzed using SEC as described in 5.2.8. Two standard reference mixtures composed of Cytochrome c (12,384 Da), Aprotinin (6,512 Da), insulin chain b oxidized (3,496 Da), Angiotensin (1046.2 Da), Met-enkephalin (573.7 Da) and Val-Tyr-Val (379.5 Da) were injected and separated under the same conditions. A standard curve was obtained by plotting the ratio of peak elution volume (Ve) to the void volume (V_0) against the logarithm of MW (Khiari, Ndagijimana and Betti, 2014). The results are shown in Appendix D.

6.3. Statistical analysis

Except for the microstructure determination, each assay was performed in triplicate or more. Data were analyzed using one-way and two-way analysis of variance (ANOVA), and comparison among means was assessed by conducting a studentized range test (Tukey HSD Test) at the 0.05 significance level. The Pearson's correlation analysis between the NAM chemical properties

(surface hydrophobicity, sulfhydryl and carbonyl contents) and functionalities (solubility, cooking loss and expressible moisture) was also performed. All statistical analyses were done using SPSS statistical software (version 20.0, SPSS Inc., Chicago, USA).

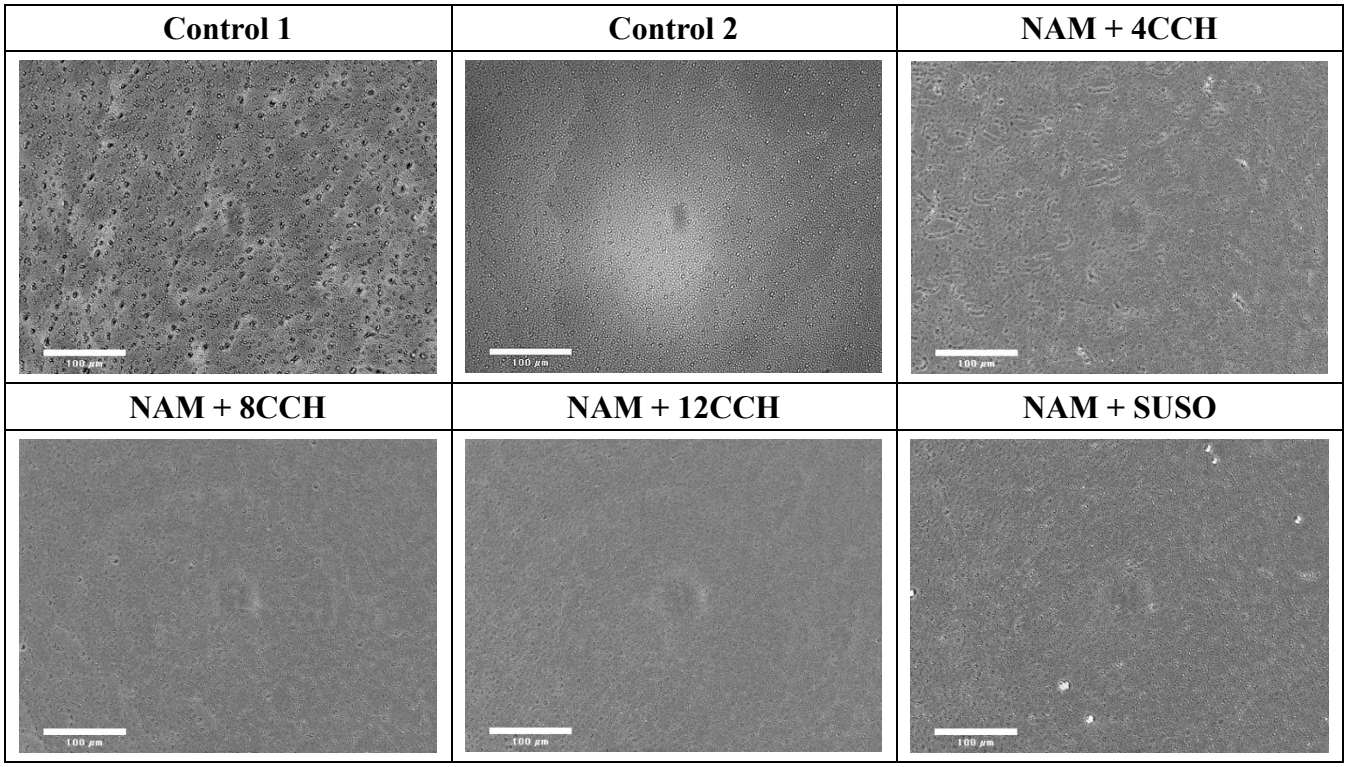
6.4. Results and discussion

6.4.1. Effect of CCH on ice-growth in NAM model systems

Damodaran (2007) reported that the ice crystal growth inhibition from bovine collagen peptides likely follows a mechanism similar to that of AFPs. The repeated amino acid motif, Gly-X-Y, which is very similar to the repetitive sequences found in AFPs from snow fleas, was thus conceived as the key in controlling ice crystal structure and dimension. This tripeptide repeating sequence in collagen hydrolysate may adopt a flat face with oxygen atoms geometrically aligned with the distance between two oxygen molecules in ice nuclei; this facilitates hydrogen bonding and thus shapes the ice crystals (Damodaran, 2007). Smaller ice crystals decrease the mechanical damage inflicted to a protein by better preserving the hydration layer surrounding the native protein, thereby preventing its unfolding and aggregation.

Ice crystal dimensions of control and treated NAM are reported in Figure 6.1. Figure 6.1A shows images of ice crystal growth in NAM model systems in the presence of different levels of CCH or SUSO after 7 freeze-thaw cycles, and Figure 6.1B shows the average size of ice crystals calculated from the image program. The NAM solution in the absence of CCH (control 1) had the largest ($P < 0.05$) ice crystals with an average size of 6.1±0.3 μm^2, and it was not significantly different from the ice size observed in control 2. This indicates that the change of protein content in this range (3.0 to 3.4 mg/mL) does not influence the ice growth in the system. However, the addition of CCH at 8 and 12 % in NAM solution, which had a total protein content lower or

A.



B.

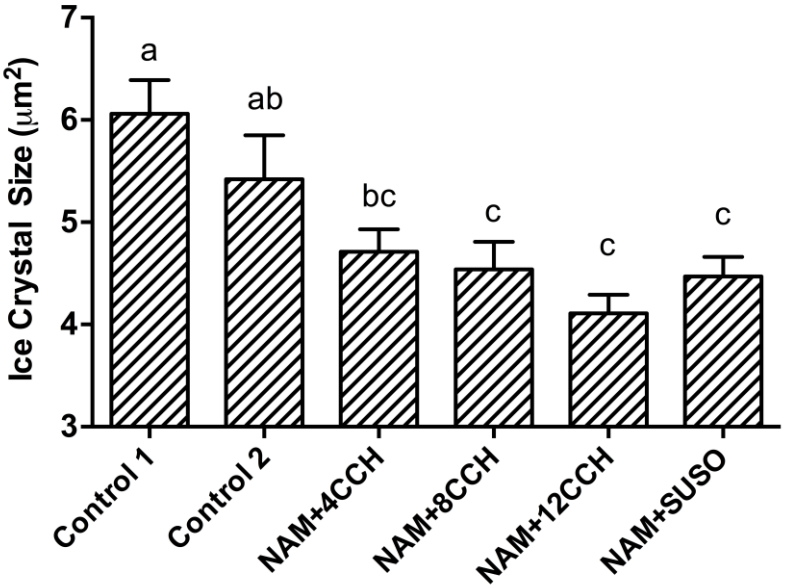


Figure 6.1. Ice crystal growth in NAM model solutions with CCH or SUSO treatments. A: Ice crystal images of the NAM model systems captured under a microscope after 7 thermal cycles. B: Average ice crystal dimensions (n=3) of the NAM model systems.

equivalent to control 2, significantly reduced the ice crystal sizes to a level similar to the SUSO treatment (4.5±0.2 μm^2). This suggests that the presence of CCH in the NAM model system can inhibit the ice crystal growth or ice recrystallization effectively during the freeze-thaw cycles, and its effect was at the same level as commercial cryoprotectants, sorbitol and sucrose. These results are similar to the ones obtained by Damodaran (2007) in ice cream model system.

6.4.2. Microstructure of NAM pastes after freeze-thaw cycles

Ice formation can dramatically change the microstructure of proteins. For instance, freeze-denaturation can alter the texture of protein-based food, a phenomenon in which newly aggregated and cross-linked protein structures are formed in relation to the growth of ice crystals. In some applications, the "freeze-induced texturization" has also been proposed to develop new protein fibers with a very organized sheet protein structure stabilized by disulfide bonds (Consolacion and Jelen, 1986). Therefore, in order to gain complementary proof about the relationship existing between ice crystal dimensions and protein microstructure, selected freeze-thawed samples (NAM + 0% CCH, NAM + 8% CCH and NAM + SUSO) were viewed under the SEM after being freeze-dried. We hypothesized that smaller ice crystals as a consequence of the cryprotectant addition would minimize the changes on the NAM microstructure.

SEM images are shown in Figure 6.2A. At 1000 × magnification, and important differences were noticed in relation to the treatments. More numerous and larger micropores – seen as black void spaces -were observed in NAM treatments without the addition of 8 % CCH or SUSO. These large micropores were likely developed after ice sublimation and were related to the dimension of the ice crystals. Furthermore, in the sole NAM sample, "dented" protein sheets structures – seen as the white regions - were more evident and numerous compared to NAM treated with SUSO or

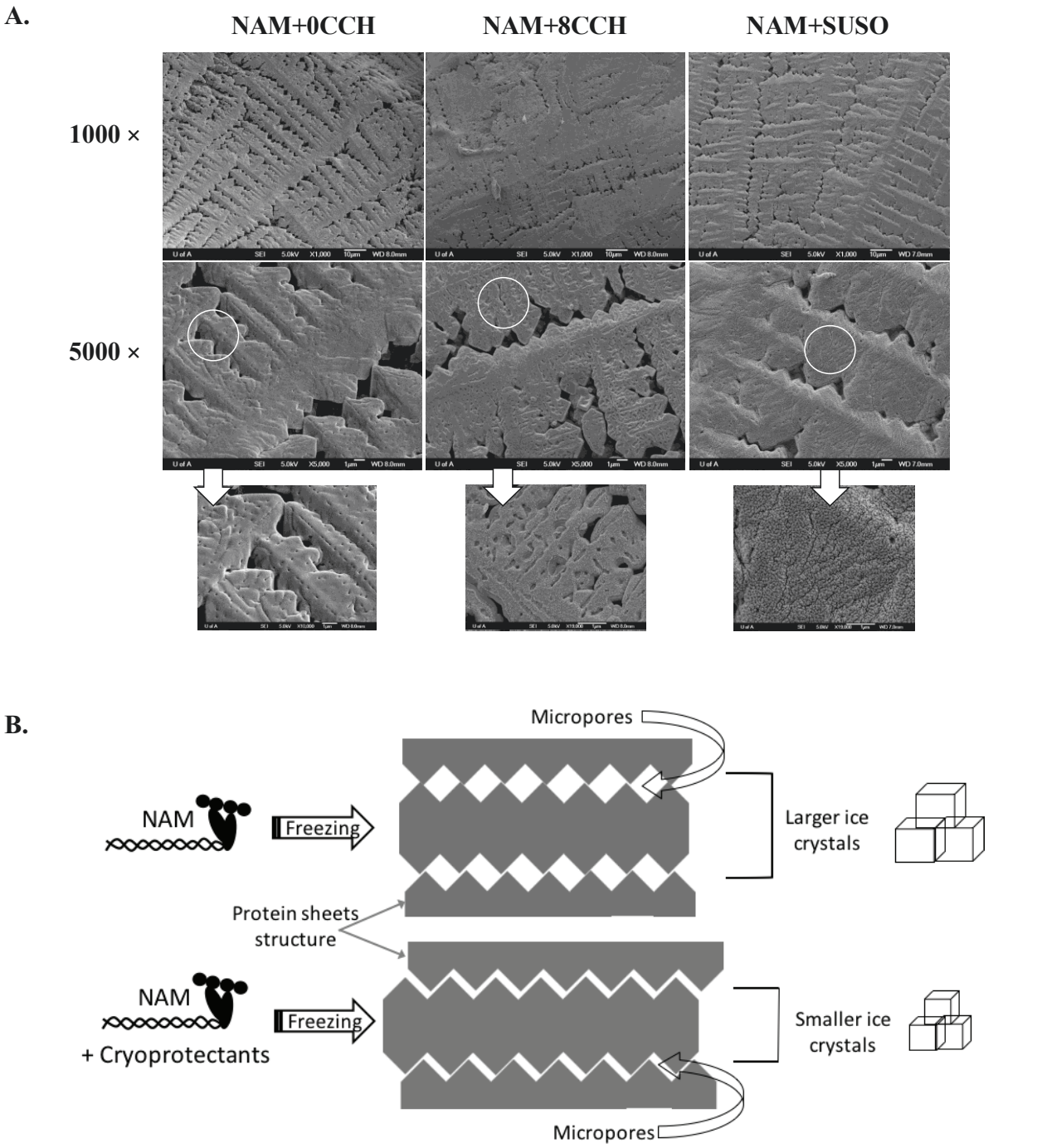


Figure 6.2. Scanning electron microscopy images (at 1000 ×, 5000 ×, 10,000 × and 19,000 × magnifications) of freeze-thawed NAM with CCH or SUSO treatments after freeze-drying (A), and the possible schematic illustration of the effect of cryprotectants (CCH and SUSO) on ice growth in NAM structures during freeze-thaw cycles (B).

CCH, where more homogeneous surfaces with less micropores were noted. At 5000 × magnification, the characteristic "dented" structure was even clearer. By increasing the magnification to 10,000 × and 19,000 ×, other morphogenetic differences were detected on the protein sheets' surfaces. For instance, in the NAM without cryoprotectants, the surface morphology was a flat sheet characterized by very few scattered "microcavities", whereas in the treatment with 8 % CCH, there were stretched and larger micro cavities which resemble "microcraters". At a magnification of 19,000 ×, NAM with SUSO presented a white surface covered with amorphous sucrose/sorbitol microparticles, with no microcavities observed. It is apparent that the addition of CCH or SUSO provided more homogenous microstructures in which the dented fibrous structures were minimized. These results are important, since a strong "fiberization", or texturization, effect of the ice crystals on NAM would negatively affect the subsequent heat-induced gelation, where proteins would withstand the unfolding process during heating (see paragraph 6.4.4.2 and 6.4.4.3). A possible schematic illustration of the mechanism is presented in Figure 6.2B. After freezing, smaller micropores were observed in the protein structure of the NAM treated with CCH or SUSO compared to the untreated NAM, indicating that smaller ice crystals were formed in the protein structure with the presence of CCH or SUSO during freezing, which is consistent with the findings reported in section 6.4.1.

6.4.3. The effect of CCH on NAM unfolding and oxidation

6.4.3.1. Surface hydrophobicity

The alteration of protein surface hydrophobicity is highly correlated with protein conformational and functional changes, which usually implies the exposure of hydrophobic amino

acids to the protein surface. Variations of surface hydrophobicity of NAM treated with different levels of CCH or SUSO are shown in Table 6.1.

Significant differences on surface hydrophobicity among the freeze-thawed NAM samples were detected. The NAM with 0% CCH showed the greatest ($P < 0.05$) surface hydrophobicity, not significantly different from the NAM treated with 4% and 8% CCH. However, the surface hydrophobicity of NAM with 12 % CCH was the same as the surface hydrophobicity found in fresh NAM and in the treatment with SUSO. This indicates that addition of 12% CCH or 8% of SUSO was able to reduce protein structural changes after a period of frozen storage. Kittiphattanabawon et al. (2012) also observed that the incorporation of gelatin hydrolysate lowered the surface hydrophobicity of fish NAM. A possible explanation to this phenomenon refers to the inhibitive effect of collagen hydrolysate on ice crystal growth. As highlighted previously, smaller ice crystals might have retarded the damage of the hydrate shell surrounding the native proteins during freezing, leading to less protein unfolding. Another possible explanation may be due to hydrophilic interactions between collagen peptides and proteins keeping the NAM in a folded state and limiting the exposure of hydrophobic amino acids to the protein surface (Kittiphattanabawon et al., 2012).

6.4.3.2. Total and reactive sulfhydryl contents

Sulfhydryl groups are one of the most reactive functional groups in myofibrillar proteins since they are involved in protein cross-linking upon the heat-induced gelation. During cooking of meat, free sulfhydryl groups (-SH) on myosin molecules can be oxidized to form disulfide bridges (S-S), inducing the formation of a tridimensional elastic gel network capable of retaining water and lipid droplets (Smyth, Smith and O'Neill, 1998). During cooking the formation of S-S bridges

are at the expense of free -SH groups, and the reduction of free -SH due to oxidative conditions (i.e. freezing) prior to cooking may have detrimental effects on the gelation process. This is because the cross-linked (S-S) myofibrillar proteins will resist the unfolding process that is an important step to form elastic gels (Wang, Wu and Betti, 2013). It is thus necessary to minimize the oxidation of –SH groups to S-S bridges prior to cooking.

The total and reactive sulfhydryl contents of the NAM in relation to the treatments are shown in Table 6.1. Fresh NAM had the greatest ($P < 0.05$) total sulfhydryl group content with the amount of 50.2±1.9 μmol/g protein, while the untreated NAM after freeze-thaw cycles had the fewest ($P < 0.05$), with 29.9±1.2 μmol/g protein. The 40% reduction of the total sulfhydryl content in NAM after freeze–thaw suggests the occurrence of oxidation during frozen storage. A similar trend was noted for the reactive sulfhydryl contents as well. Except for the oxidation of -SH groups, protein aggregation may also lead to less reactive -SH groups detected on the surface of frozen NAM than in fresh NAM, since they would be buried in the aggregated protein clusters (Benjakul et al., 2003). However, a significant increase of total and reactive -SH contents was observed in freeze-thawed NAM with the addition of CCH (especially 8% or 12%) or SUSO compared to the untreated NAM. This phenomenon may be due to the antioxidant capacity of certain collagen peptides that could minimize protein oxidation (Wang et al., 2013). Nikoo, Benjakul and Xu (2015) also reported that gelatin hydrolysate can retard the loss of sulfhydryl groups in unwashed fish mince induced by freeze–thawing. The sulfhydryl content results were generally in accordance with the surface hydrophobicity results, and implied that the addition of CCH induced less protein unfolding, and consequently less sulfhydryl groups were exposed to the protein surface to be oxidized. Significant negative correlations between sulfhydryl groups—both total and reactive—

and surface hydrophobicity (r = −0.73 and r = −0.70, respectively) were found (Appendix C). This suggests that the collagen hydrolysate can protect against freeze-induced denaturation by reducing the NAM unfolding (i.e. through reducing the ice crystal size) and reducing amino acid oxidation.

6.4.3.3. Carbonyl group content

Another method to directly assess the degree of protein oxidation is the determination of carbonyl group content (Table 6.1). Carbonyl compounds are formed by the preferential attack of reactive oxygen species on the side chain of amino acid residues, which causes loss of protein structural integrity (Chan et al., 2011a). The freeze-thaw processes often give rise to protein oxidation of myofibrillar proteins, and further impair protein functionalities. In agreement with the results reported in section 6.4.3.2, the carbonyl content of untreated NAM increased significantly from 1.5±0.2 to 2.4±0.1 nmol/mg of protein after freeze–thaw cycles. These were similar values seen in a study using spent hen proteins (Wang, Wu and Betti, 2013). The addition of 8% or 12% CCH or SUSO in frozen NAM significantly lowered the amount of carbonyls, similar to the results reported by Nikoo et al. (2015). However, there was no difference between the NAM treated with 4% CCH and the untreated NAM after the freeze–thaw process. The results suggest that the freeze-oxidation of NAM proteins could be effectively reduced by adding 8% or 12% CCH. The reasons behind this phenomenon are likely due to a dual mechanism involving both the antioxidant capacity of CCH against protein oxidation and its ability to prevent NAM unfolding. As highlighted in the previous section, an increase in protein unfolding would induce more protein oxidation. Specifically, the amino acids susceptible to oxidative attack (i.e. cysteine and methionine) would be exposed to a pro-oxidant solute environment as a consequence of the freeze-induced dehydration (Utrera, Parra and Estévez, 2014). This is corroborated by the significant

Table 6.1. Chemical and functional properties of fresh or freeze-thawed NAM with CCH or SUSO treatments.

	Surface Ho	T –SH (μmol/g protein)	R–SH (μmol/g protein)	Carbonyls (nmol/mg protein)	Solubility (mg/mL)	CL (%)	EM (%)
Fresh NAM	364±21^{b}	50.2±1.9^{a}	41.0±2.1^{a}	1.5±0.2^{c}	8.4±0.4^{a}	40.4±0.9^{d}	28.6±0.9^{d}
NAM+0CCH	469±23^{a}	29.9±1.2^{d}	20.7±1.0^{d}	2.4±0.1^{a}	1.3±0.1^{e}	60.7±2.4^{a}	47.2±1.7^{a}
NAM+4CCH	427±33^{ab}	32.3±1.3^{cd}	23.9±1.0^{c}	2.1±0.1^{ab}	2.5±0.3^{d}	51.5±1.7^{b}	40.8±1.5^{b}
NAM+8CCH	412±20^{ab}	35.9±0.4^{b}	27.8±0.5^{b}	1.9±0.1^{bc}	5.3±0.4^{c}	45.7±1.3^{c}	35.2±1.2^{c}
NAM+12CCH	393±12^{b}	34.9±0.7^{bc}	28.2±1.0^{b}	1.8±0.2^{bc}	7.0±0.2^{b}	47.2±1.3^{c}	33.0±1.5^{c}
NAM+SUSO	394±38^{b}	34.5±0.6^{bc}	26.9±0.8^{bc}	1.7±0.2^{c}	6.5±0.3^{b}	46.3±1.0^{c}	34.4±1.0^{c}
P-values	=0.006	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Values are given as mean ± standard deviation (n=3). Different letters in the same column indicate significant ($P < 0.05$) differences between the means. Surface Ho: Surface hydrophobicity; T-SH: Total sulfhydryl content; R-SH: Reactive sulfhydryl content; CL: Cooking loss; EM: Expressible moisture.

positive correlation (r = 0.77) between the carbonyl content and surface hydrophobicity results. Since severe protein oxidation often can negatively affect protein functionalities, it was of interest to further investigate whether the changes brought about by the addition of CCH would have an impact on protein functionality.

6.4.4. The effect of CCH on NAM functionalities

6.4.4.1. Solubility

The ability of proteins to be dispersed in water, also known as "protein solubility", is not only an important functional property per se, but also a fundamental factor that influences other functional properties including gelation, emulsion activity and foaming. In general, protein solubility can be used as an indirect indicator of protein denaturation and oxidation, where increased protein denaturation and oxidation are generally associated with a decrease in solubility with subsequent negative effect on heat-induced gelation (Xiong, 1997). The solubility of the different NAM samples in salt buffer is shown in Table 6.1. The fresh NAM, with a value of 8.4 ± 0.4 mg/mL, was the most soluble in saline buffer. On the other hand, all the NAM samples after freeze-thaw cycles displayed significantly lower solubility, ranging from 1.3 ± 0.1 to 7.0 ± 0.2 mg/mL. In general, increasing the CCH concentration significantly improved the solubility, and the treatment with 12% CCH was comparable to the solubility of the positive control (SUSO).

Overall, samples with higher surface hydrophobicity, fewer sulfhydryl groups and increased carbonyl groups (Table 6.1) displayed lower solubility (Table 6.1). This was substantiated by the significant correlations among solubility and surface hydrophobicity (r = −0.79), reactive and total -SH contents (r = 0.82 and 0.73, respectively), and carbonylation level (r = −0.84) (Appendix C). In summary, these results indicate that the treatments with smaller ice

crystal sizes resulted in less protein unfolding with subsequently less amino acid exposure to the concentrated, oxidative environment resulting from the freeze-induced dehydration. As a consequence, NAM was less aggregated/cross-linked and thus more soluble.

6.4.4.2. Cooking loss and expressible moisture

Water holding capacity is an important functionality of meat proteins, which determines the yield of products and the meat quality. In practice, the water holding capacity of meat products is often estimated by cooking loss and expressible moisture, which are at the same time related to the sensory attributes and consumer acceptability (Zhuang and Savage, 2013). The effect of different treatments on cooking loss and expressible moisture are shown in Table 6.1. A similar trend for both analyses was observed, where fresh and frozen NAM with 0% CCH had the smallest and the largest values ($P < 0.05$), respectively. After 7 freeze–thaw cycles, a significant decline of the cooking loss and expressible moisture was noticed in all of the NAM containing CCH or SUSO compared to the NAM with 0% CCH, and there were no significant differences among the treatments of 8% CCH, 12% CCH and SUSO. Greater cooking yields may indicate that the NAM samples treated with CCH or SUSO formed better gel networks after cooking which were able to retain more water, also confirmed by the results reported in paragraph 6.4.4.3. Since expressible moisture also provides an insight into the juiciness of the NAM gels, the addition of CCH or SUSO may enhance the juiciness of meat products. Conformational changes driven by protein unfolding and oxidation can unbalance protein intramolecular and protein–water interactions, leading to the loss of water retention ability (Utrera et al., 2014). This study reemphasizes that samples with low water holding capacity have high levels of protein unfolding and protein oxidation (see Pearson correlation in Appendix C). A proposed mechanism on how gelatin hydrolysate exerts its positive

effect on water holding capacity was elucidated by Nikoo et al. (2015) while studying the effect of freeze–thaw cycles on unwashed fish mince by using low field NMR spectroscopy. These researchers demonstrated that the displacement of water from the intra- to the inter-myofibrillar compartment (free water) was minimized in the presence of hydrolyzed gelatin. As a consequence, this would leave more water within the myofibrils with positive effects on water holding capacity. Moreover, the large amount of hydroxyl groups of SUSO, and hydrophilic groups exiting in CCH, may have played a role in stabilizing the water surrounding the proteins, thus preserving the native protein structures during freeze-thaw cycles.

6.4.4.3. Gel-forming ability

Dynamic viscoelastic behavior of the proteins during thermal denaturation is a widely used method to assess the gelation properties of myofibrillar proteins (Chan et al., 2011b). The changes in storage modulus (*G*'), loss modulus (*G*") and tan δ during heating from 4 to 80 °C at a rate of 2 °C/min are shown in Figure 6.3. A two-step gelation was found for all NAM samples (Figure 6.3A and 6.3B), where a sol–gel transition occurred in the range of 45 - 55 ˚C (Figure 6.3C) as shown by tan δ and the correspondent increase in storage modulus (*G'*). Xiong and Blanchard (1994) also reported the formation of an elastic matrix of myofibrillar proteins at a temperature range from 45 to 55 ˚C. This transition, which corresponds to the first step of gelation, is mainly associated with unfolding of myosin's globular heads, to expose hydrophobic amino acids residues, followed by an aggregation via hydrophobic interactions to form a first elastic protein network (Zayas, 1997). Furthermore, the temperature increments from 50 to 55 ˚C resulted in the dissociation of the actin-myosin complex and the denaturation of the myosin tail, causing a slight decrease of *G*' (Benjakul et al., 2001). The second gelation step started at 55 ºC at the end of the

sol-gel transition, and finished at 80 ºC, where the maximum *G*' values were obtained. In this step an irreversible three-dimensional gel matrix was developed due to the formation of intermolecular covalent disulfide bonds (Ding et al., 2012). This step generated large molecules through protein polymerization and an increase of the elasticity and hardness of the gels. Interestingly, the *G*' values in correspondence to the sol–gel transition were the lowest in the frozen NAM without addition of CCH and SUSO (Figure 6.3A). This may be attributed to the extensive protein aggregation/cross-linking during the freeze-thaw cycles as a consequence of the protein conformational changes, such as unfolding and exposure of hydrophobic amino acids, and oxidation through disulfide bond formation. As previously discussed, the extensive formation of S-S bridges prior to thermal process would lead to heat stable protein structures (fibrous and "dented" protein sheets, see section 6.4.2) that could resist unfolding during the temperature sweep with subsequent negative effects on NAM gelation. In this treatment, the lowest *G*' value was also observed at the end of the gelation process at 80 ºC. Increased *G*' values in both steps were observed in the NAM treated with CCH or SUSO, which indicated that more native proteins were preserved under the cryoprotective treatments and superior gel networks with higher elasticity were arisen after heating. Among the CCH treatments, 8% and 12% exhibited better effect than the 4% CCH.

The changes of viscosity (*G*") (Figure 6.3B) were generally consistent with the *G*', with two-step changes in the same temperature ranges. However, all the *G*" values were much lower than the *G*' values, suggesting a more elastic nature of formed gels. Unlike the *G*', the *G*" showed smaller values in the second step than the values in the first major peak, which further imply that a more elastic and stronger gel was obtained at a higher temperature, while a looser gel structure with relative higher viscosity was formed at the first stage. The order of the G" values from

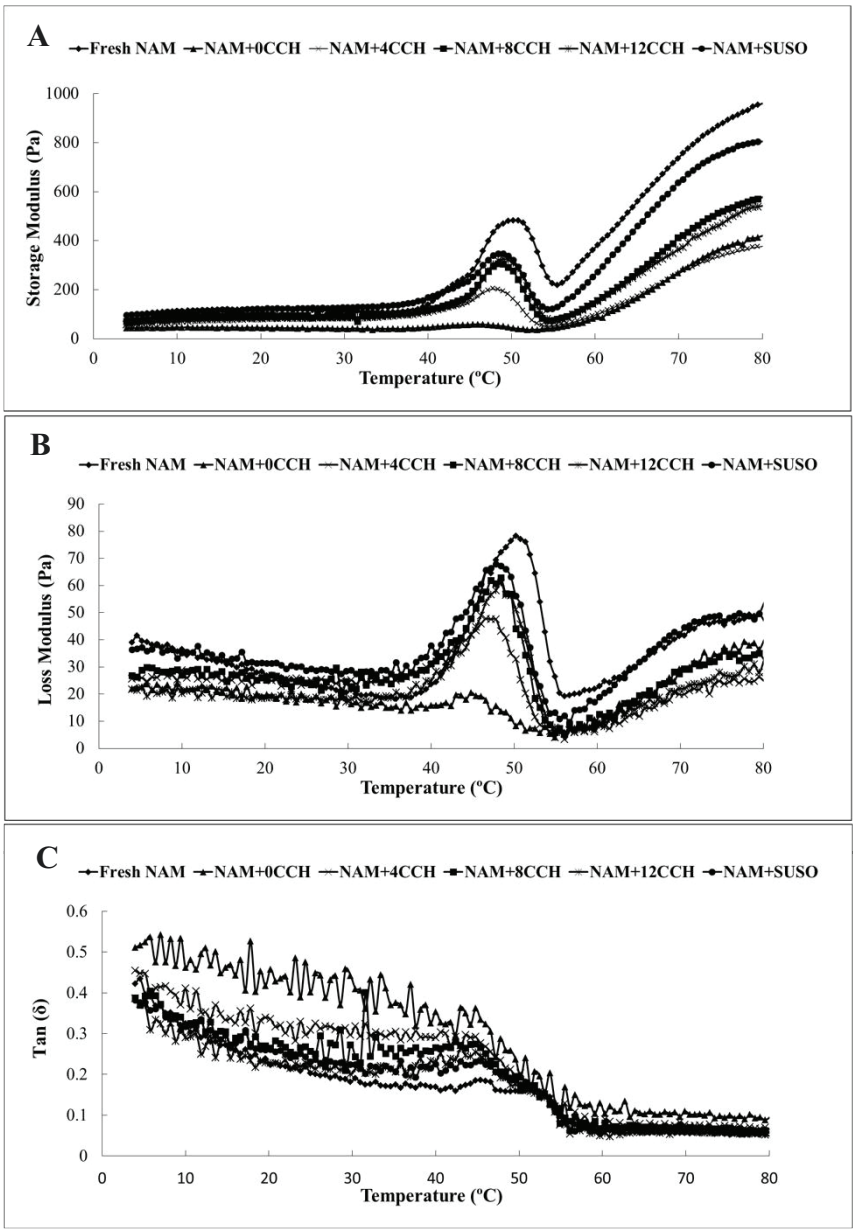


Figure 6.3. Changes in dynamic viscoelastic behavior (DVB) of fresh or freeze-thawed NAM with CCH or SUSO treatments. The developments of storage modulus G' (A), loss modulus G'' (B), and change of tan δ (C) upon heating from 4 to 80˚C at 2 ˚C min^{-1} are shown.

different samples were similar to the order of *G'* values, with the largest in fresh NAM and SUSO treatment, followed by the NAM treated with CCH and non-treated NAM. In summary, the addition of CCH at the level of 8% or 12%, as well as SUSO at the level of 8%, were beneficial in terms of preserving the ability of NAM to develop a functional elastic gel.

6.4.5. Microstructure of NAM gels

Microstructural visualization of cooked gels under electronic microscope helps to elucidate the type of protein–protein and protein–solvent interactions. For instance, a string-like gel network can be obtained mainly through protein-water interactions, while an aggregate (coagulum)-type gel network is obtained when protein-protein interactions are dominant in the system. To visualize the microstructure of the NAM gels after cooking, four NAM samples (the fresh NAM, the freeze-thawed NAM, and the NAM treated with 8% CCH or SUSO) were selected and observed under SEM at different magnifications (Figure 6.4). In general, all the NAM gels had a coagulum-type network in which the number and dimension of the pores changed in relation to the treatments. In this experiment, fresh NAM formed a uniform porous gel with fairly smooth surface and a fine, cage-like network (Figure 6.4) with a significant ability to retain water, while the treatment without CCH or SUSO revealed a gel network with protein clusters having fewer pores and reduced capacity to retain water (Section 6.4.4.2). This may be attributed to the failure of protein unfolding during heating, since NAM had been already in an aggregated and cross-linked form after freeze-thaw cycles, as confirmed by the microstructure of frozen NAM (Figure 6.2A), the sulfhydryl group contents (Table 6.1), and the rheology results (Figure 6.3). Regarding Figure 6.4, the addition of CCH and SUSO did not result in the formation of protein clusters compared to NAM

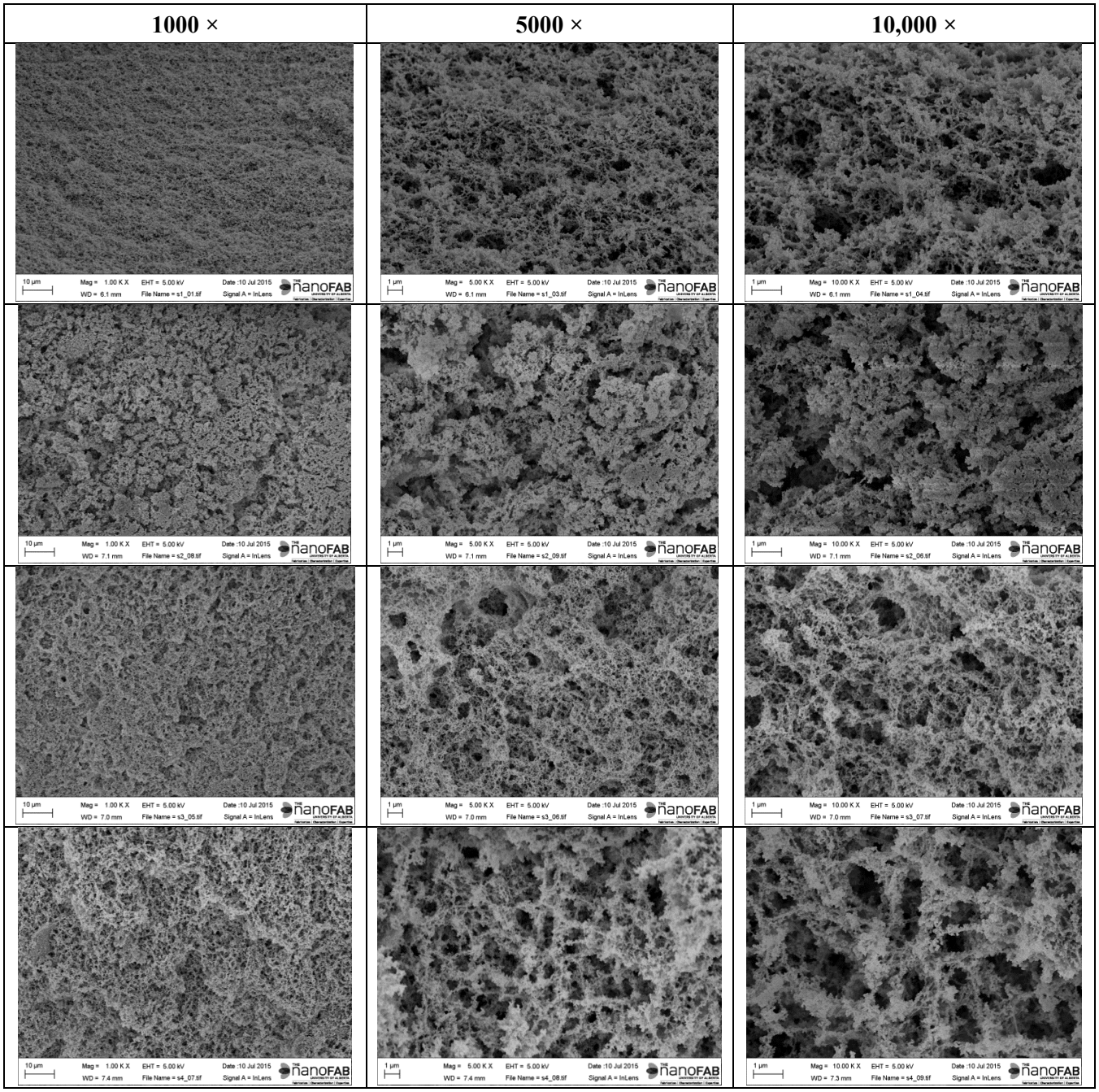


Figure 6.4. Scanning electron microscopy images (at 1000 ×, 5000 × and 10,000 × magnifications) of the cooked gels formed in fresh or freeze-thawed NAM with CCH or SUSO treatments. From top to bottom: Fresh NAM, NAM+0CCH, NAM+8CCH and NAM+SUSO.

alone, but instead created a protein network similar to the one observed in fresh NAM. The main noticeable differences were however related to the dimension and number of the pores appearing in the treatments. The treatments with CCH or SUSO resulted in the formation of larger pores as compared to the fresh NAM sample. Stevenson, Liu and Lanier (2012) found a positive correlation between the pore size of salted muscle mince gels and the cooking loss, suggesting that smaller pore sizes caused lower cooking loss. This was also demonstrated by our results: the treatment with smallest pores in fresh NAM corresponded to the greatest water holding capacity (section 6.4.4.2).

6.4.6. In vitro gastrointestinal digestion of NAM

6.4.6.1. Digestibility

In the previous sections it has been demonstrated that CCH can reduce ice crystal size and prevent unfolding and oxidation of NAM with subsequent positive effects on protein-water interactions and gel forming ability, resulting in an elastic protein matrix. It was therefore of interest to understand whether different elasticity of NAM gels affects their digestion in a simulated gastrointestinal system. The influence of CCH on frozen protein digestibility was estimated in both raw and cooked frozen NAM by measuring peptides released from the original protein (Table 6.2). The main effect of treatments ($P < 0.001$) indicate that the addition of SUSO or CCH at large concentrations (8% and 12%, respectively) increased the digestibility of frozen NAM, and that the fresh NAM was more digestible than the frozen ones. This confirmed that elastic and less oxidized NAM gels were more digestible in this study.

There was no significant effect ($P = 0.58$) of cooking on digestibility. However, by looking at the interaction "cooking × treatments" ($P = 0.002$), more insights can be achieved. The

significant effect of interaction originates from the effect of "cooking" on fresh NAM and frozen NAM without cryprotectants, where cooked fresh NAM was less ($P < 0.05$) digestible than uncooked fresh NAM. This is in agreement with the study of Kaur et al. (2014) who reported a significantly lower digestibility of cooked beef, and indicated that the intermolecular cross-links and aggregates formed during heating may reduce protein susceptibility to enzymatic proteolysis. From these interaction results, it is also evident that once NAM has been subjected to freezing, its digestibility was independent from the cooking effect. Hence, the presence of cryoprotectants (CCH or SUSO) improved the digestibility of frozen NAM no matter if it was cooked or not, indicating the positive effects of the cryoprotectants during the freeze–thaw cycles. Since protein conformation greatly influences the accessibility of the bonds susceptible to proteolytic enzymes, the lower levels of aggregation and oxidation found in CCH or SUSO treatments (section 6.4.3) could be the reason for their better digestibility. In addition, the protein clusters observed in the untreated NAM gel may mask the sites available for enzymatic digestion and therefore decrease digestibility. In summary, the cryprotectants not only improved the functionality of myofibrillar proteins, but also played an important role on NAM digestibility. The intensity of size-exclusion chromatographs of digested NAM also demonstrated similar results obtained in this section, and it showed that the molecular weight distribution of NAM digests was from 108 to 5118 Da with no considerable changes among all the samples (Appendix D).

Table 6.2. *In vitro* digestibility of fresh or freeze-thawed NAM in raw or cooked state with CCH or SUSO treatments.

		Digestibility (%)
Cooking		
Raw		38.1±5.5[a]
Cooked		38.4±4.2[a]
Treatments		
Fresh NAM		45.6±3.5[a]
NAM+0CCH		32.9±2.2[d]
NAM+4CCH		34.5±1.8[d]
NAM+8CCH		38.3±3.5[bc]
NAM+12CCH		37.5±2.1[c]
NAM+SUSO		40.7±2.0[b]
Interaction (Cooking × Treatments)		
Raw	Fresh NAM	48.1±1.9[a]
	NAM+0CCH	32.7±1.9[f]
	NAM+4CCH	33.9±1.6[ef]
	NAM+8CCH	36.7±2.5[def]
	NAM+12CCH	37.4±2.8[cde]
	NAM+SUSO	39.9±2.0[bcd]
Cooked	Fresh NAM	43.1±2.8[b]
	NAM+0CCH	33.2±2.6[ef]
	NAM+4CCH	35.1±1.8[ef]
	NAM+8CCH	39.9±3.8[bcd]
	NAM+12CCH	37.6±1.1[cde]
	NAM+SUSO	41.6±1.6[bc]
Sources of variation		*P*-values
Cooking		=0.58
Treatments		<0.001
Cooking × Treatments		=0.002

Values are given as mean ± standard deviation. For interaction part, n=3. Different letters in the same column indicate significant ($P < 0.05$) differences between the means.

6.5. Conclusion

This study elucidates the cryoprotective mechanism of the collagen hydrolysates against freeze-denaturation of actomyosin. We show that the ice-growth inhibition properties of 8% and 12% of collagen peptides are crucial in controlling the unfolding of actomyosin, and consequently minimizing the exposure of the oxidizable amino acids to a concentrated solute environment. This, in turn, effectively reduces aggregation (lower value of surface hydrophobicity and higher protein solubility) and oxidation (increased sulfhydryl group content and less carbonyl content) of actomyosin molecules during frozen storage, with subsequent positive effects on functionalities comparable to the commercial blend of SUSO. The incorporation of collagen hydrolysates greatly enhanced the water holding capacity and gelling property of frozen actomyosin. An elastic, cross-linked gel microstructure was detected in the treatment with collagen peptides, while the untreated frozen actomyosin was unable to form an interconnected gel network. Furthermore, the elastic gels obtained due to the addition of collagen peptides were found to be more prone to the gastro-intestinal digestion. Hence, the collagen hydrolysate produced from chicken skin could be considered as a potential non-sweet cryoprotectant alternative for meat products. Since collagen hydrolysate has been commercialized as a health supplement for anti-aging, anti-oxidant, and anti-hypertensive functions, providing a double benefit would be another advantage of utilizing collagen hydrolysates as a frozen food preservative.

Chapter 7-Conclusions, implications and future research

This doctoral research started with the aim of finding a more valuable application for the vast quantity by-products underutilized in the poultry meat processing industry. It ended up being more about understanding the mechanism of how collagen peptides protect myofibrillar proteins against freezing damage. Most of these by-products produced contain a large amount of connective tissue, rich in collagen. This molecule can be used and converted into many different valuable products for applications in both food and pharmaceutical industries. For instance, historically the immense collagen biomass from the pig and bovine industries is recovered and has been largely used in the production of mammalian gelatin. However, due to health and safety issues (i.e. Bovine spongiform encephalopathy) associated with bovine gelatin, and cultural issues associated with the consumption of pig gelatin, new sources of collagen for gelatin production are needed to satisfy consumer demand. Avian gelatin may therefore represent a valuable alternative to mammalian ones. In this research, I concentrated efforts to confirm the possibility of producing high quality gelatin from underutilized poultry by-products, and to develop an efficient and sustainable pre-treatment for gelatin extraction. After this, I became interested in the bioconversion of collagen to bioactive peptides, particularly regarding cryoprotective properties. Collagen peptides have shown several interesting activities including anti-aging, anti-oxidant, and anti-hypertensive. Recently bovine and fish gelatin peptides have been shown to possess an ice-controlling ability and subsequent cryoprotective capacity, properties that may be used for the protection of food against freezing damage. These capacities were postulated to be a consequence of the repetitive amino acid sequence in collagen. No specific sequence in this regard was known at the time my research started. Therefore, the cryoprotective activity of collagen hydrolysates derived from avian by-

products were investigated in two different food models; a protein system and a sugar system. Amino acid sequence identification was investigated and possible mechanisms for cryprotection were proposed. Reported below, a summary of the knowledge generated from this doctoral thesis.

1. **Identification of a novel raw material suitable for gelatin production:**

High quality gelatins were produced from chicken heads, turkey heads and mechanically separated turkey meat (MSTM) in these studies, which suggest that poultry and poultry by-products can serve as an alternative source for making gelatin.

2. **Isoelectric solubilisation and precipitation process as a possible pre-treatment for poultry gelatin production:**

The isoelectric solubilization/precipitation process (ISP) used to isolate sarcoplasmic and myofibrillar proteins from MSTM created a considerable amount of collagenous biomass as a by-product. The gelatin could be extracted from this by-product efficiently and had excellent properties.

3. **Novel applications of poultry collagen hydrolysate:**

The novel cryoprotective activity of poultry derived collagen hydrolysates was demonstrated in this research. This broadens the potential applications of poultry collagen/gelatin, and also provides a possible value-added solution to compensate for the relatively low yields of poultry gelatins. The cost of producing collagen/gelatin hydrolysate is about two times higher than producing gelatin, but it is sold ten times more expensive than gelatin.

4. **Sequence identification, structural characterization and ice growth inhibition properties of identified collagen peptides:**

It was confirmed that certain chicken collagen peptides can non-colligatively inhibit ice crystal growth in a sucrose model system with a similar behaviour as natural antifreeze proteins (AFPs). The amino acid sequence and structural identification of ice-controlling peptides, as well as a comparison with natural AFPs helped to understand the mechanism. To the best of my knowledge, this is the first research on ice growth inhibition of chicken collagen peptides.

5. Cryoprotection mechanism of collagen peptides against freeze-induced denaturation of isolated actomyosin

The mechanism of the cryoprotective effect of chicken collagen peptides in a protein system was linked to collagen peptides' ice inhibition activity. Proteins showed less denaturation, oxidation and functionality loss with the addition of peptides. The potential of using chicken collagen hydrolysates to serve as a new cryoprotectant substituting for sweet cryoprotectants, such as sucrose or sorbitol, in frozen foods is appreciable.

The results obtained here have significant implications on poultry by-product utilization and sustainability of the industry. According to the widely-cited report by the United Nations Food and Agriculture Organization (FAO) in 2006, about 18% of annual worldwide greenhouse gas (GHG) emissions is attributed to the livestock sector, and a report from Worldwatch (2009) showed how livestock and their by-products actually account for 51% of annual worldwide GHG emissions. GHG emissions is a major cause of the greenhouse effect associated with environmental issues. Among different livestock, the production of poultry represents the least water consumption and the best feed conversion, and the most efficient use of land compared to pork, beef or goat (beef production is the most resource-costly (Globalagriculture.org, 2016). Therefore, poultry

represents a more sustainable source of animal protein and by-products compared to pigs and cattle. Also, the excellent quality of poultry gelatin obtained from poultry by-products (chapter 3 and chapter 4) supports its use in gelatin production. Global poultry meat production is currently about 110 million tonnes per year (FAO, 2015); this statistic represents significant quantities of by-products and wastes generated. However, if these by-products were better utilized in gelatin/collagen hydrolysate production, they would be valued more than USD 5.5/kg or USD 60/kg for collagen hydrolysate. As high functional gelatins, the poultry gelatin can be used as not only a desirable food ingredient, but also a decent material to produce capsules, sponges and films. These improvements would be anticipated to be significant from both environmental and economical points of view.

These results of this doctoral work show promise for the application of chicken collagen peptides as non-sweet, versatile food cryoprotectants; whether used in frozen desserts to maintain a fine texture or in protein-based frozen foods to reduce the deterioration of quality as occurs with common cryoprotectants. Collagen hydrolysate has been already commercialized as a health supplement for anti-aging, anti-oxidant, and anti-hypertensive benefits. Hence, if collagen hydrolysates could also be utilized as a food preservative added to frozen foods, it would provide a double benefit. Multi-functional food ingredients such as collagen hydrolysates represent a growing trend in food applications.

In terms of future research related to this work, the effect of chicken collagen peptides on the thermal hysteresis level (non-colligative freezing point depression) is expected to be similar to that exerted by the AFPs, and needs investigation. Despite the fact that ice growth inhibition of

collagen peptides was not as efficient as AFPs, they may have similar or better thermal hysteresis activity. A hyperactive collagen ice-binding peptide GLLGPLGPRGLL exhibiting a thermal hysteresis as high as 5.28 ºC has been reported (Cao et al., 2016). The cryoprotective properties of collagen peptides could also be evaluated in other food and cell systems, and more thorough studies elucidating the antifreeze mechanism should be of interest, such as the crystallography of collagen peptides, and analysis of ice-binding sites and ice crystal morphology. Furthermore, in order to utilize collagen peptides in practical food applications, the sensory attributes of the final products need to be considered.

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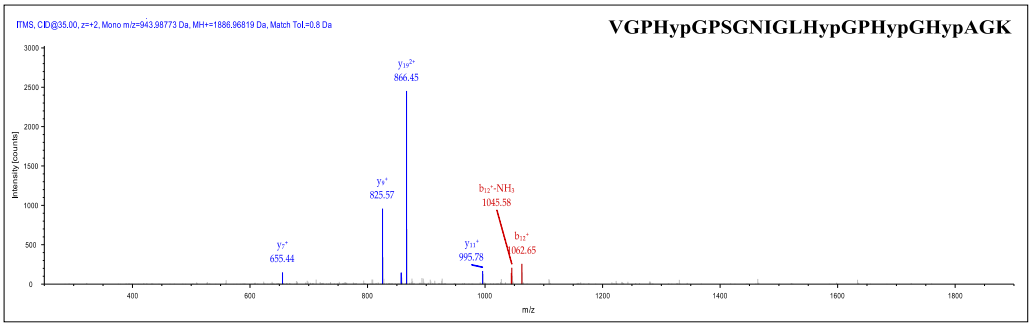
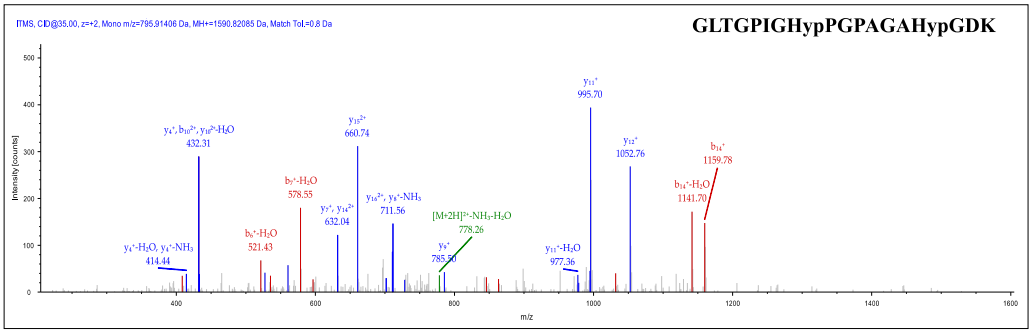
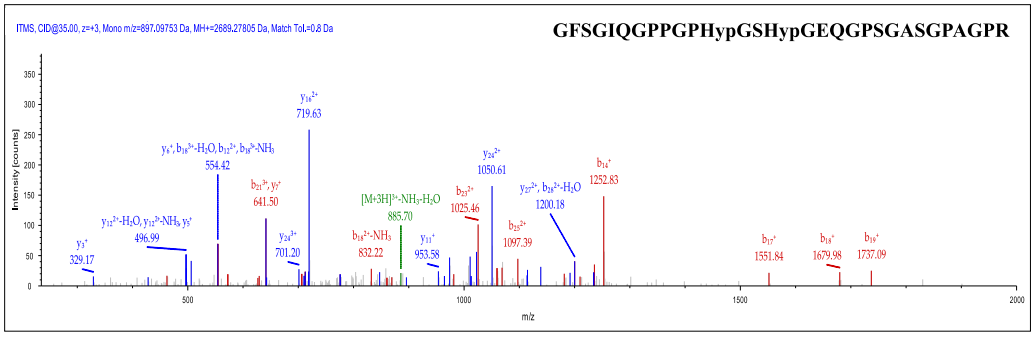
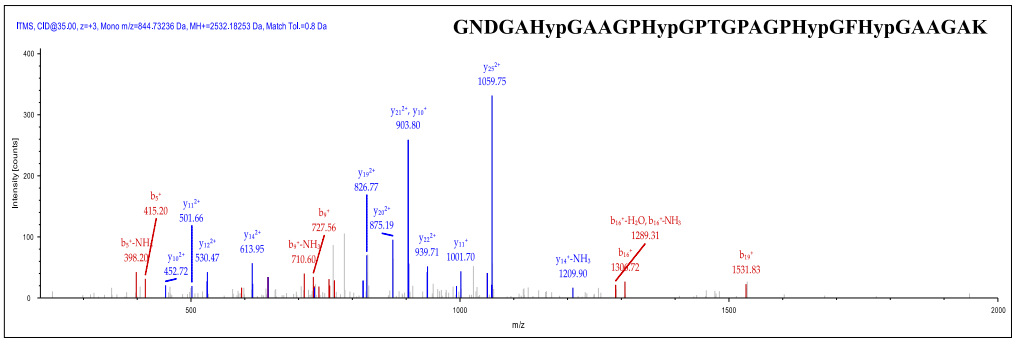
Appendix A.

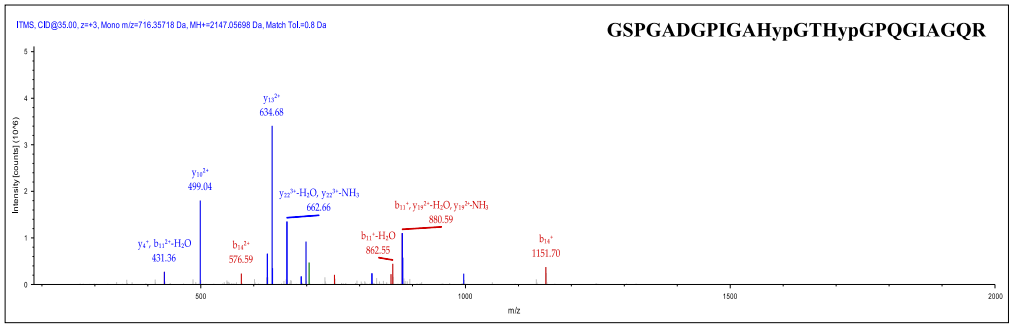
The fragment spectra of the five most abundant peptides in the identified list (Table 5.1), obtained by the LC-MS/MS analysis.





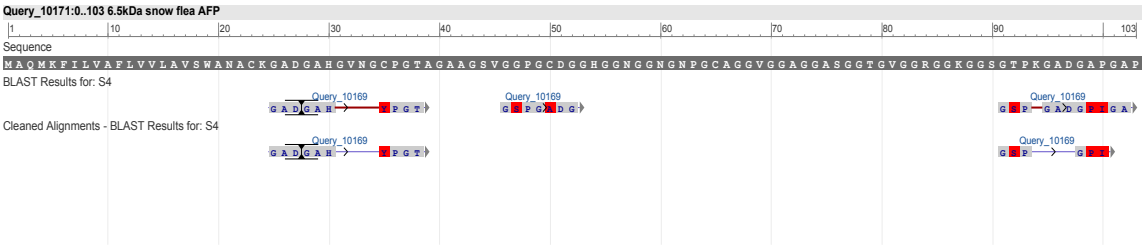



ITMS, CID@35.00, z=+3, Mono m/z=844.73236 Da, MH+=2532.18253 Da, Match Tol.=0.8 Da
GNDGAHypGAAGPHypGPTGPAGPHypGFHypGAAGAK
Intensity [counts]
m/z

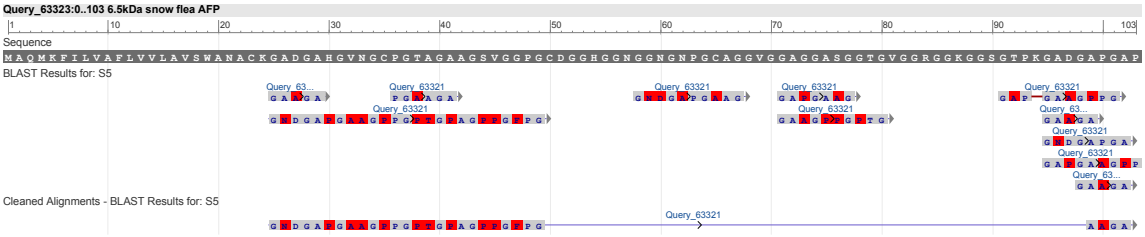

ITMS, CID@35.00, z=+3, Mono m/z=716.35718 Da, MH+=2147.05698 Da, Match Tol.=0.8 Da
GSPGADGPIGAHypGTHypGPQGIAGQR
Intensity [counts] (*10^6)
m/z

Appendix B.

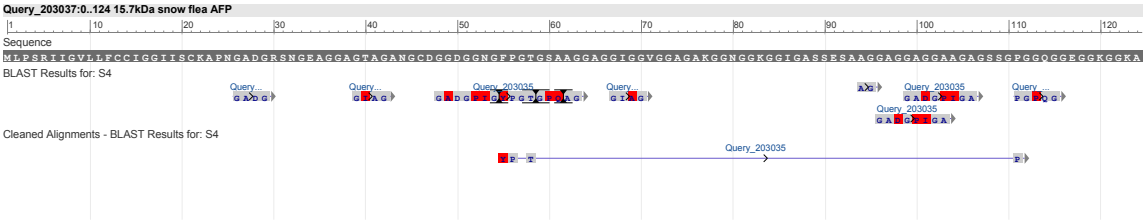
Sequence alignments of S4, S5 with two snow flea antifreeze proteins by BLAST. The proline showed at 12 and 15 positions in S4, and 6, 12, 21 and 24 positions in S5 are hydroxyproline

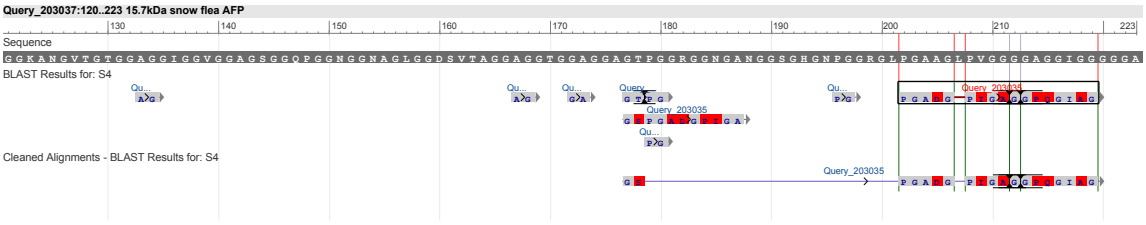


Query coverage 66%

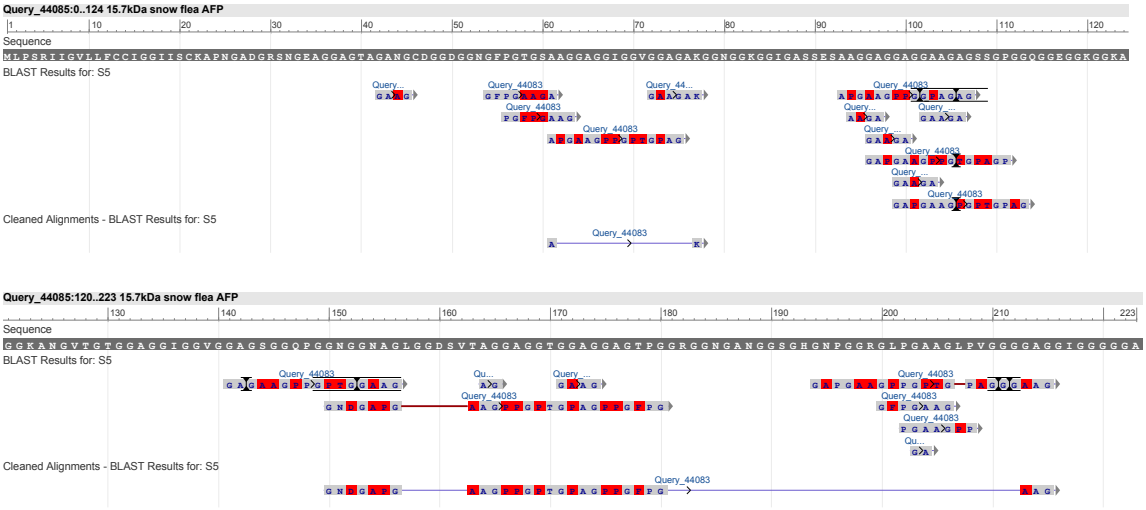


Query coverage 96%





Query coverage 83%



Query coverage 100%

Appendix C.

Pearson's Correlation Coefficient among the NAM chemical and functional properties.

	Surface Ho	Total -SH	Reactive -SH	Carbonyls	Solubility	CL	EM
Surface Ho[1]	1	-.73**	-.70**	.77***	-.79***	.80***	.83***
Total –SH[2]	-.73**	1	.93***	-.72**	.73**	-.76***	-.77***
Reactive –SH[3]	-.70**	.93***	1	-.77***	.82***	-.83***	-.84***
Carbonyls[4]	.77***	-.72**	-.77***	1	-.84***	.88***	.88***
Solubility	-.79***	.73**	.82***	-.84***	1	-.90***	-.96***
CL[5]	.80***	-.76***	-.83***	.88***	-.90***	1	.97***
EM[6]	.83***	-.77***	-.84***	.88***	-.96***	.97***	1

. Correlation is significant at the 0.01 level (P< 0.01); *. Correlation is significant at the 0.001 level (P< 0.001).

1: Surface hydrophobicity. 2: Total sulfhydryl content. 3: Reactive sulfhydryl content. 4: Carbonyl content

5: Cooking loss. 6: Expressible moisture

Appendix D.

Size-exclusion chromatographs of fresh or freeze-thawed NAM with CCH or SUSO treatments after digestion (detected at 214 nm). The chromatographs show the digests of raw NAM samples (A), the digests of cooked NAM samples (B), and the two standard mixtures (C).