Development of Edible Films from Gelatin Extracted from Argentine Shortfin Squid (*Illex Argentinus*) with the use of an Enzyme (Pepsin) Aided Process

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This thesis is dedicated to my mother and father

ABSTRACT

Gelatin, a protein biopolymer, can be easily derived from marine biomass. Studies have been carried out on different marine gelatin sources. However, there is still limited research on films formed using squid gelatin. For this study, gelatin was extracted from shortfin squid (*Illex argentinus*) using an enzyme-aided method, specifically pepsin, under different reaction conditions; enzyme concentrations of 25, 15 and 5U of pepsin/g of dehydrated squid, heat extraction temperatures of 45 °C, 55 °C and 65 °C and extraction durations of 12, 18 and 24 hours. These parameters were observed to have a direct effect on not only the quality and yield of gelatin but as well as the edible films formed and their respective properties. Optimal conditions for each parameter were determined and used to extract subsequent gelatin for the formation of edible films. Results indicated that the optimal enzyme concentration, 25 U/g, produced the highest yield of gelatin at 6.59% ± 0.7221. Through the BCA assay, the highest protein concentration was determined to be of 671.5 µg/ml ± 0.0045. The lowest extraction temperature, 45 °C in conjunction with the lowest extraction duration, 12 hours, was determined to be the shrinkage temperature at which the triple helix arrangement of the polypeptide subunits in the collagen molecule will collapse due to the non-covalent and covalent inter- and intra- molecular bonds breaking, converting into gelatin. However, the lack of fragments of beta and alpha chains indicated the extraction of low-quality gelatin. Film forming solutions of 4% and 8% gelatin-glycerol films were characterized to have a tensile strength of 0.229 ± 0.0037 N/mm^2 and 0.939N/mm^2 ± 0.0104 respectively. The elongation at break remained relatively stable at 27.2% ± 0.1979 and 22.3% ± 0.0824 respectively. The water solubility of both films was calculated to be 100%. The opacity of the films indicated that both films were effective in blocking both UV and visible light at opacities higher than 90% showing effectiveness in blocking lipid oxidation. The present study indicated that it is possible to extract gelatin and despite it being low quality, edible films with adequate properties were formed. Further studies are required in order to explore optimization for gelatin extraction as well as film properties.

RESUMÉ

Gélatine, un biopolymère de protéine peut être facilement dérivé la biomasse marine. Études ont été menées sur des sources différentes de gélatine marine, cependant, il n'y a encore peu de recherches sur les films formés à l'aide de la gélatine de calmar. Pour cette étude, la gélatine a été extraite des taupes de calmar (*Illex argentinus*) en utilisant une méthode enzymatique assistée par plus précisément de la pepsine, dans des conditions de réaction différente; concentrations de l'enzyme de 25, 15 et 5 U de pepsine/g de calmar déshydratés, températures d'extraction de chaleur de 45 °C, 55 °C et 65 °C et extraction des durées de 12, 18 et 24 heures. Ces paramètres ont été observés à avoir un effet direct non seulement sur la qualité et le rendement de la gélatine, mais ainsi sur les films comestibles formés et leurs propriétés respectives. Des conditions optimales pour chaque paramètre ont été déterminées et utilisées afin d'extraire la gélatine subséquente pour la formation de films comestibles. Les résultats indiquent que la concentration de l'enzyme optimale, 25 U/g, produit le rendement le plus élevé de la gélatine à 6,59 % ± 0,7221. En effectuant un dosage de la BCA, la plus forte concentration de protéines a été déterminée de 671.5ug/ml ± 0,004 5. La température d'extraction la plus basse de 45 °C, en conjonction avec la durée d'extraction la plus basse de 12 heures, était déterminée comme étant la température de rétrécissement au cours de laquelle l'arrangement de la triple hélice des sous-unités polypeptidiques dans la molécule de collagène s'effondrera en raison de la rupture non covalente, mais aussi covalente des liaisons inter et intramoléculaires résultant de la conversion de la gélatine. Cependant, l'absence de fragments de bêta et les chaînes alpha indiquent l'extraction de la gélatine de mauvaise qualité. Les solutions filmogènes de films de gélatine-glycérol à 4% et 8% ont été caractérisées par une TS de 0,229 ±0,003 7 N/mm^2 et 0.939N/mm^2 ± 0,010 4 respectivement. L'agrile du FRÊNE est demeuré relativement stable à ± 0,082 4 de ± 0,197 9 et de 22, 3 % et 27,2 % respectivement. Solubilité dans l'eau de ces deux films a été évaluée à 100 %. L'opacité des films a indiqué que les deux films ont été efficaces pour bloquer les UV et la lumière visible à opacité supérieure à 90 %, ce qui indique l'efficacité dans le blocage de l'oxydation des lipides. La présente étude a indiqué qu'il est possible d'extraire de la gélatine et bien qu'il soit de mauvaise qualité, des films comestibles avec les propriétés adéquates ont été formés. D'autres études sont nécessaires afin d'explorer l'optimisation pour l'extraction de la gélatine, mais aussi les propriétés du film.

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CHAPTER 1:

1 GENERAL INTRODUCTION

In recent years there has been more attention generated towards the manufacturing of edible and biodegradable films. The increased usage of synthetic packaging films has led towards serious ecological problems (Flieger, Kantorová, Prell, Řezanka, & Votruba, 2003). Although complete disuse and replacement of these synthetic non-biodegradable packaging may not be entirely possible, the application of edible and biodegradable films in the food industry would have a major positive impact on this issue. Currently, polyethylene terephthalate (PET), polyvinylchloride (PVC), polyethylene (PE), Polypropylene (PP), polystyrene (PS) and polyamide (PA) are the predominantly used petrochemical based plastics in the industry (Siracusa, Rocculi, Romani, & Rosa, 2008). Although advantageous in numerous aspects, these materials are not recyclable or biodegradable. Hence there exists a need to develop packaging films and coatings that are both economically friendly and biodegradable.

Biodegradable plastics are of growing interest especially as edible films and coatings, as the type of chemical bonds in the material will determine in how much time the microbes will biodegrade the material (Guilbert, Gontard, & Gorris, 1996). These synthetic polymers that are biodegradable can be divided into three categories; polymers directly extracted/removed from biomass, polymers produced by classical chemical synthesis using renewable bio-based monomers and polymers produced by microorganisms or genetically modified bacteria (Shalini & Singh, 2009). For this study, the focus is on developing such biodegradable plastic packaging from the first category under which the protein gelatin is of particular interest and is known for being commonly used due to its film-forming properties.

Gelatin is one of the most abundantly used collagen derivatives in the food industry. In fact, its applications are not limited to that and can also be seen in the nutraceuticals, pharmaceutical, and photography industries. Its applications are very broad and diverse due to the numerous advantageous properties it presents. Due to its clarity, bland flavor, stability, texture properties and emulsifying characteristics, gelatin can easily be incorporated into a food matrix (Herpandi, Huda,

& Adzitey, 2011). Common applications can be seen especially in confectionery and dairy products, for specific desired effects (Herpandi et al., 2011). Gelatin is obtained from the insoluble protein, collagen, through controlled hydrolysis (Bourtoom, 2008). It is composed of a unique sequence of amino acids; it contains considerably high contents of glycine, proline, and hydroxyproline. Gelatin also contains both single and double unfolded hydrophilic chains which attribute to its high film forming properties (Nur Hanani, Roos, & Kerry, 2014). Gelatin solutions are capable of forming thermo-reversible gels upon cooling due to its chains undergoing conformational disorder-order transition which allows it to recover the collagen triple-helix structure (Bourtoom, 2008). With the addition of plasticizers, gelatin films can be formed and be used for encapsulation of low moisture or oil phase food ingredients. They can also be used to form coatings on meats for protection from oxygen, moisture and oil transport (Jorge et al., 2014).

The global demand for gelatin has increased over the years, rendering gelatin a product of a rapidly growing market. In 2003, the annual world output for gelatin reached 278 300 tons and since then has only increased to 326 000 tons (Karim & Bhat, 2009). However, the more common sources are still of pig-skin origin, bovine hides and bones. Unfortunately, other sources only contribute a little more than 1% even though data has shown an increase in the percentage of gelatin being used from other sources (Karim & Bhat, 2009).

With greater attention geared towards gelatin extraction from marine species, numerous studies have successfully extracted gelatin from salmon fish, big-eye snapper, and sea urchin. These studies have shown that generally, the functional properties of gelatin differ mainly due to the amino acid content and molecular weight distribution (Jorge et al., 2014). It was also noted that the melting temperature of gelatin extracted from warm-blooded fish is generally higher than that of gelatin extracted from cold-water fish (Gilsenan & Ross-Murphy, 2000). Thus indicating that warm-water fish gelatin is preferred for the development of films compared to cold-water fish. Although limited research exists on this topic, a study by B. Giminez et al. (2009) studied the physico-chemical and film forming properties of cold-water giant squid gelatin and reported that properties of the films could easily be enhanced (Giménez, Gómez-Estaca, Alemán, Gómez-Guillén, & Montero, 2009).

Compared to other fish species, a special feature of squid collagen is its high degree of cross-linking due to the high amount of hydroxyproline (Gómez-Guillén et al., 2002). Furthermore, both the tunic and mantle of the squid contain high amounts of collagenous residues that could yield high amounts of gelatin. This difference from other sources of gelatin (mainly mammalian sources) makes it important to note how film-forming abilities will differ when using squid gelatin. For this reason, the source of gelatin being studied will be from cold-water species shortfin (*Illex argentinus*) squid.

1.1 RESEARCH OBJECTIVES:

Objective 1: The objective is to study the effect of the reaction conditions, (extraction temperature, and extraction time and enzyme concentration) on the gelatin yield. The gelatin will be analyzed by determining the gelatin yield, protein concentration, molecular weight distribution and the hydroxyproline content. This will aid in the selection of the best gelatin sample to be used for the formation of edible films.

Objective 2: From the gelatin extracted from objective 1, edible films will be formed and then characterized by studying its tensile strength, elongation at break, water solubility, water uptake and opacity.

Overall, the study aims to obtain a high-quality gelatin with exceptional film forming properties to produce biodegradable films. The successful development of such edible films can have a massive positive impact on plastic waste, and for this reason, it is important to continue research to further study alternatives. These films can also have positive impacts on the preservation of food by protecting them from light and oxygen. Gelatin extracted from marine sources have been observed to provide good film forming properties, however, there is still much room for improvement and optimization.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Biodegradable Films

Food products, whether they be in their raw materials or processed form, require protection to increase their preservation. The purpose of food packaging is focused on the conservation and protection of all food types especially from oxidative and microbial spoilage and for an extended shelf life. However, the increased usage of plastic packaging plastics has led towards larger environmental issues increasing the need for alternatives.

To date, petrochemical based plastics have been used as the conventional packaging materials, as they are available in high quantities at a lower cost and provide excellent packaging functions such as good tensile and tear strength, good barrier properties to oxygen and aroma compounds and heat sealability (Tharanathan, 2003). The disadvantages of using such material for packaging are their low water vapor transmission rate and more importantly they are completely non-biodegradable (Tharanathan, 2003).

The concept of biodegradability refers to the use of raw materials derived from either replenishable agricultural feedstocks or the wastes from the marine food processing industry, for the production of a packaging material (Flieger et al., 2003). The usage of these raw materials provides the additional advantages of capitalizing on the conservation of natural resources as well as the packaging material disintegrating and compositing into potential fertilizers and soil conditioner (Tharanathan, 2003).

2.1.1 Prerequisites of Packaging Films

Packaging films are required to meet certain standards to fulfill their roles. There exist seven main requirements to be met, of which some are product specific and may not apply to for some commodities (Kader, Zagory, Kerbel, & Wang, 1989).

1. Good food packaging films need to provide a slow but controlled respiration of the product through reduced oxygen absorption.
2. They need to provide a selective barrier to gasses and water vapor.
3. The package must provide a modified atmosphere depending on the internal gas composition. This allows for a more direct regulation of the ripening process and increases the shelf life of the commodity.
4. For the confectionery industry, packaging needs to lessen the migration of lipids.
5. The packaging needs to maintain its structural integrity and improve mechanical handling.
6. It needs to serve as a vehicle for the incorporation of food additives such as flavors, colors, antioxidants and antimicrobial agents.
7. Most importantly, the packaging needs to either completely prevent or reduce microbial spoilage during extended storage.

The most commonly used packaging films; polyethylene, polyvinylidene, polyester, polyamide and cellophane currently provide all of the prerequisites mentioned above (Tharanathan, 2003). However, these demands can also be easily met with bio-based polymers (biodegradable material).

2.2 What are Bio-Based Polymers?

Bio-based polymers are defined as materials that are derived from renewable sources and are recognized as biodegradable according to the standards that are outlined by the EU Standardization Committee (Shalini & Singh, 2009). Bio-based polymers generally have more diverse chemistry and architecture in relation to their side chains (Shalini & Singh, 2009). This makes it more possible to modify the final properties of the packaging. These polymers are divided into three main categories depending on their origin and production method (Petersen et al., 1999).

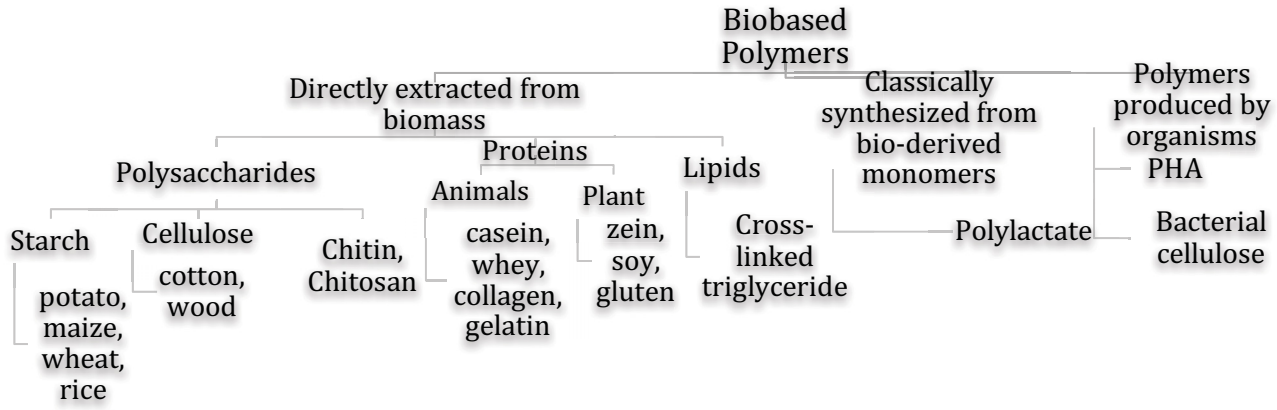

Figure 2-1. Schematic presentation of bio-based polymers, adapted from Shalini and Singh (2006).

2.2.1 Category 1

Category 1 includes polymers that are directly derived from biomass. These are most commonly polysaccharides and proteins derived from marine and agricultural products (Shalini & Singh, 2009). These polymers tend to be hydrophilic and crystalline by nature causing issues during its processing and functionality especially when used as packaging for moist products. However, when used as packaging, these polymers exhibit excellent gas barriers properties (Weber, 2000). Three main polysaccharides of interest are most commonly used; starch, cellulose, and chitin. Proteins commonly utilized for the development of films include gelatin, corn zein, wheat gluten and soy protein (Shalini & Singh, 2009).

2.2.2 Category 2

Category 2 includes polymers that are produced by classical chemical synthesis from bio-based monomers. The use of chemical synthesis to make these polymers allows a wider range of possible bio-polyesters especially from agricultural resources such as corn or wheat, or agricultural waste products such as whey or green juice (Shalini & Singh, 2009). The polymeric material of most interest from category 2 is polylactic acid. This material has the highest potential to be utilized on

a large scale in the industry as a renewing packaging material (Weber, 2000).The polylactic acid produced films have that the advantage of good water vapor barriers and low gas transmittance.

2.2.3 Category 3

Category 3 contains polymers that are produced directly by natural or genetically modified organisms. These biopolymers are mostly used in industry because they are more biodegradable and biocompatible. The most common ones include polyhydroxyalkanoates (PHA's), of which hydroxybutyrate (PHB) is the most utilized (Shalini & Singh, 2009). The films produced by PHA's have very low water vapor permeability which allows them to be readily used as coatings over cheese (Shalini & Singh, 2009).

The focus of the study is to develop biodegradable plastic packaging, specifically edible films, using a marine source. This falls under the first category pertaining to protein and polysaccharide biopolymers. Gelatin extracted from marine sources is of high interest, as it is known for its exceptional film forming properties.

2.3 Edible Films

Edible films are defined as a thin layer of material that act as barriers towards moisture, oxygen, and solute movement in the food matrix. These films can coat foods as free-standing films or as a continuous layer between food components (Bourtoom, 2008). This form of packaging utilizes polymeric materials such as polysaccharides, proteins, and lipids. The main advantage of using edible films in the food industry over traditional synthetic films is due to their ability to be consumed; there is no need for package disposal. Also, due to the advantageous biodegradable aspect, the disposal of the film will not harm the environment. It would rather easily become a part of the environment without any harmful effects.

The usage of edible polymers for film formation holds much potential in today's world. With an increasing number of possible edible ingredients that can be used, there exist endless possibilities for optimization of the film properties. This increases the application of these films for more practical products such as fruits, nuts, and other fresh produce (Gennadios, Hanna, & Kurth, 1997).

Edible films can be tailored to prevent deteriorative inter-component moisture and solute migration in foods and can also be carriers for antimicrobial and antioxidant agents (Bourtoom, 2008).

2.3.1 Film Formations

In the development of an edible film, it is important to contain at least one ingredient that is a natural polymer capable of forming a cohesive and continuous structural matrix (Bourtoom, 2008). Cohesion forces such as covalent bonds, ionic bonds, and hydrogen bonds are responsible for the functional properties of edible films and coatings. The cohesive strength is dependent on the biopolymer structure and chemistry and film forming procedures, film forming parameters and the concentrations of plasticizers (Janjarasskul & Krochta, 2010). In general, when forming edible films using solid fats, waxes or resins can be melted and solidified (Janjarasskul & Krochta, 2010). The bio-polymers are dissolved in a solvent such as water, alcohol or a mixture of other solvents (Bourtoom, 2008). During this process, it is common to add plasticizers, antimicrobial agents, colors or flavors. In order to facilitate the dispersion, heating of the solutions and adjustment of their pH is often carried out, depending on the polymer being used (Bourtoom, 2008).This allows for the productions of a smoother and more consistent film. The solution is then cast and dried at the desired temperature and relative humidity for a specific duration of time. This will result in the production of a free-standing film. Aside from forming free-standing films, edible films can be applied directly on foods through dipping, spraying, brushing and panning.

2.3.2 Film Additives

Film additives are additional materials incorporated for the enhancement of structural, mechanical and handling properties. They can also be added to provide active functions to the films(Janjarasskul & Krochta, 2010). Typical additives include emulsifiers, antimicrobials, antioxidants, and plasticizers.

Emulsifiers are surface-active compounds that have both polar and nonpolar characteristics. The addition of emulsifiers is essential for the formation and stabilization of lipid particles emulsion films (Janjarasskul & Krochta, 2010). They are also added to obtain sufficient surface wettability, which will result in proper surface coverage and adhesion to the coated surface (Krochta, 2002). Typical emulsifiers include lecithin, polysorbate, sorbitan monooelate.

Antimicrobials are commonly added to edible packaging to control the growth of microorganisms. Conventional antimicrobials used in edible packaging include chitosan to be used against yeast and molds, essential oil extracts from plants such as cinnamon, onion, clove and garlic that contain phenolic compounds have a broad range antimicrobial effects (Janjarasskul & Krochta, 2010). Bacteriocins that are produced from bacteria and contain protein that also has different antimicrobial functions (Janjarasskul & Krochta, 2010).

Antioxidants are added to either prolong the start or slow down the rate of oxidation reactions. Depending on the mechanism of actions there can either be primary or secondary antioxidants used. Primary antioxidants also known as chain-breaking are free radical acceptors that will delay the propagation step of autoxidation (Eça, Sartori, & Menegalli, 2014). Examples of primary antioxidants are butylated hydroxytoluene and tocopherols that are the most commonly used (Janjarasskul & Krochta, 2010). Secondary antioxidants also known as preventative antioxidants work to slow down oxidation through different means such as chelation of pro-oxidant metals, decomposing hydroperoxides as well as promoting the antioxidant activity of primary antioxidants (Eça et al., 2014). Examples include citric acid, ascorbic acid and tartaric acid (Janjarasskul & Krochta, 2010).

Plasticizers are incorporated into the polymeric network and compete for chain to chain hydrogen bonding with the polymer chains (Sothornvit & Krochta, 2005).This will increase the overall film forming ability and the properties. They are small molecular weight hydrophilic agents typically mono-, di- or oligosaccharides such as fructose and glucose, polyols such as glycerol and sorbitol, lipids and their derivatives (Vieira, da Silva, dos Santos, & Beppu, 2011). The type of plasticizer used depends typically on the compatibility, efficiency, permanence, and economics (Janjarasskul & Krochta, 2010).

2.3.3 Protein Films: Characteristics and Properties

Proteins are described as a random copolymer of amino acids that contain side chains that can be easily modified by chemical means (Shalini & Singh, 2009). This allows for a broad range of tailoring to obtain desired properties of protein films as a packaging material. In recent years, proteins have gained high importance as having the most potential as edible packaging materials (Janjarasskul & Krochta, 2010). In general, proteins can either exist as fibrous proteins or globular

proteins. Fibrous proteins are water insoluble and are the primary structure materials of many animal tissues. These proteins are associated with each other through hydrogen bonds that form fibers in parallel structures. Globular proteins are soluble in water as well as aqueous solutions of acids, bases or salts. Their structures are more complicated spheres that are held together through hydrogen, ionic, hydrophobic and covalent disulfide bonds (Sunde & Blake, 1998). The chemical and physical properties of these proteins depend on the type of amino acid residue and their placement on the protein polymer chain (Bourtoom, 2008). This placement will determine how they will interact with other components in a film formation.

Protein films are formed once the protein is dispersed and dissolved in a solvent, which then evaporates. The solvents generally used for protein film compositions are limited to water, ethanol or a mixture of both (Kester & Fennema, 1986). However, these proteins are required to form a more extended structure for protein films. This is achieved by denaturing the protein which can be carried out through different means such as heat, acid or base (Bourtoom, 2008). An increased extension of these proteins will allow the chains to bond with each other once again through hydrogen, ionic, hydrophobic and covalent bonding (Bourtoom, 2008). A protein's ability to form cohesive films depends on the chain-to-chain interactions that in return depend on the degree of chain extension and most importantly, the nature and sequence of amino acid residues. Protein film matrices are formed depending on the protein, treatment, and fabrication conditions that produce modified film forming properties (Janjarasskul & Krochta, 2010). If along the polymer chains there is uniform distribution between polar, hydrophobic and thiol groups, this will increase the interactions. This will form films that are stronger and less permeable to gasses, vapors, and liquids but less flexible in nature (Kester & Fennema, 1986). However, these films are not flexible in nature.

Protein polymers that contain groups that can easily form hydrogen or ionic bonds will form films that have an excellent permeability to oxygen. However, these films have a higher affinity for moisture. This is why, protein films are expected to have higher oxygen barriers at lower humidity (Wu, Weller, Hamouz, Cuppett, & Schnepf, 2002). They are easily influenced by the relative humidity due to their hydrophilic nature. The most popular protein polymers that have been used

to form edible films are casein, whey protein, corn zein, wheat gluten, soy protein, mung bean protein, peanut protein and of course gelatin (Bourtoom, 2008).

2.3.3.1 Gelatin Films

Collagen is widely found in nature as the major constituent of skin, bones and connective tissues. On a molecular level, collagen is a right-handed helical rod composed of three parallel-intertwined alpha chains (Haug, Draget, & Smidsrød, 2004). One turn in collagen's super-helix is made up of three amino acid residues in the sequence of Gly-X-Y where X and Y are often proline and hydroxyproline (Asghar & Henrickson, 1982). The presence of proline and hydroxyproline is what allows the alpha chain to form a left-handed helix and stabilizes the secondary structure of the single helix (Haug et al., 2004). Gelatin is an insoluble fibrous protein that is obtained from collagen by controlled hydrolysis. It is composed of a unique sequence of amino acids and is high in glycine, proline, and hydroxyproline (Bourtoom, 2008). Its structure consists of a mixture of single and double unfolded hydrophilic chains. Gelatin is capable of forming thermo-reversible gels upon cooling. These gels are formed by cross-linking between amino and carboxyl components of amino acid residue side groups. These films are composed of approximately 20-30% gelatin, 10-30% plasticizer, such as glycerol or sorbitol, and 40-70% water (Bourtoom, 2008). Gelatin aqueous solutions at 40 degrees will be in the sol state and during the gelation process its chains will undergo a conformational disorder-order transition (Ross-Murphy, 1997). This will recover the triple helix structure found in collagen when extraction temperature is lowered below the coil-helix transition temperature, forming an active film (Haug et al., 2004). These films can be used to encapsulate low moisture or oil phase food ingredients and pharmaceuticals. As other protein films, gelatin films provide protection against oxygen and light. This feature makes gelatin films possible to be used as coatings on meats to reduce oxygen, moisture and oil transport (Jorge et al., 2014).

2.4 What is Gelatin?

Gelatin is a water-soluble protein, which is derived from collagen. It is a colorless and tasteless gel, which is commonly used as a gelling agent for food preparation (Arvanitoyannis, 2002). Gelatin is extracted from collagen through partial thermal denaturation. This is a process in which the non-covalent bonds will be disrupted resulting in the gelling ability of gelatin; however, it can be partially reversible (Karim & Bhat, 2009).

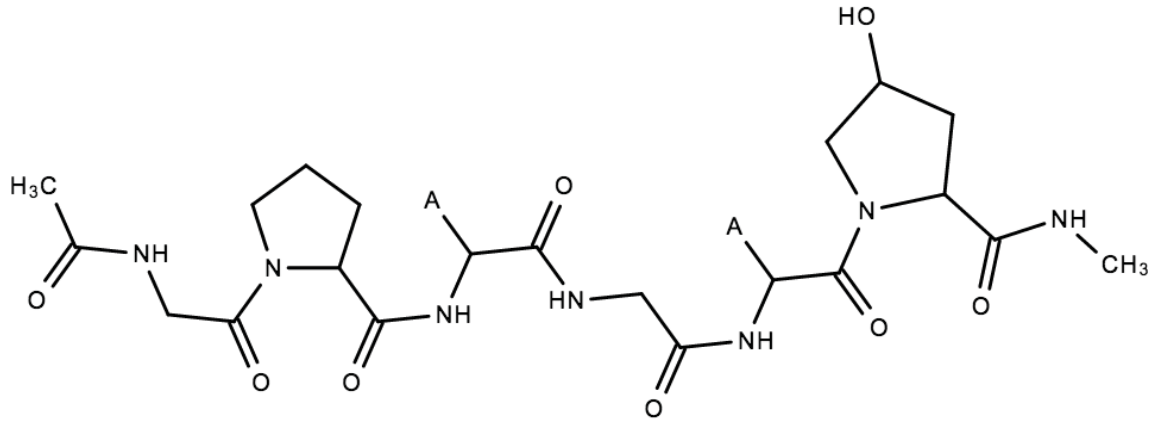

Figure 2-2. Molecular structure of gelatin.

Usually, gelatin is extracted from mammals such as pig's skin and cow skins and bones. However, there is safety hazard linked to gelatin extracted from bovine sources due to the outbreaks of bovine spongiform encephalopathy, commonly known as mad cow disease. Furthermore, religious constrictions in the Islamic and Judaism religion do not permit the consumption of gelatin extracted from non-halal or non-kosher sources. (Yang et al., 2007) For these reasons, there is a higher demand for fish gelatin. This has created a shift in the production of gelatin from bovine sources towards marine sources, increasing its production from 1.5% (Karim & Bhat, 2009).

2.4.1 Fish Gelatin

From fish, gelatin can be extracted from the skin, bone and the scales of different seafood, especially from their by-products. Fish skin, considered to be one of the major by-products from fish processing, is known to cause waste and pollution. However, it can be a valuable source of

gelatin as the skin contains the largest amount of collagen (Karim & Bhat, 2009). According to a study carried out in Japan, it was reported that amount of collagen which was present in fish skins, which were considered waste, contains approximately 50% collagen on a dry basis (Nagai & Suzuki, 2000). This creates an abundance of gelatin from an alternative source that can be easily produced at a low cost. However, fish gelatin does exhibit lower functional properties such as a lower gel strength and stability, when compared to gelatin that is extracted from other sources (Karim & Bhat, 2009).This limits its application in the food industry and has led towards the development of methods that could potentially improve its properties and make it more applicable in all fields.

Production of fish gelatin is not a new concept and has been present since the 1960's (Karim & Bhat, 2009). The method of extraction used for it was mostly acid based and was applied mainly in industrial applications. The main difference between gelatin that is extracted from fish and mammals is all a result of the different molecular weight distribution and amino acid content; particularly the content of imino acids; proline and hydroxyproline (Asghar & Henrickson, 1982). However, if extracted under the correct conditions, some fish gelatin sources may exhibit properties similar to those of mammalian gelatin. For example, gelatin that is derived from the skin of brown-banded shark had the ability to be a gel that was set at room temperature (Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2010). This means that the sharkskin gelatin most likely contains similar amino acids, especially the imino acids, as mammalian gelatins.

2.4.2 Properties of Fish Gelatin

Regarding application in food, it is important to understand in details the gel strength, viscosity, gelling and melting point properties of the extracted gelatin (Karim & Bhat, 2009). These properties are affected by factors such as average molecular weight, molecular weight distribution, the concentration of the gelatin solution, gel maturation time, gel maturation temperature, pH and finally the salt content (Kołodziejska, Kaczorowski, Piotrowska, & Sadowska, 2004). There are numerous tests, which can be carried out to study the rheological properties, emulsifying, foaming, film forming and sensory properties of extracted fish gelatin (Karim & Bhat, 2009). It is noted that the source and the types of collagen will affect the properties of the extracted gelatin.

2.4.2.1 Chemical and Structural Properties

When comparing the amino acid compositions in collagen present in fish skin to that found in mammalian collagens, the hydroxyproline and proline contents were found to be much lower in fish, and the levels of serine and threonine were found to be much higher (Balian & Bowes, 1977). The low levels of imino acids are the reason why gelatin that is extracted from fish denatures at a lower temperature. Furthermore, it was noted that warm-water fish gelatins contain higher levels of imino acid content when compared to cold-water fish gelatins (Gilsenan & Ross-Murphy, 2000). Mammalian gelatins contain 30% proline and hydroxyproline contents; warm water fish gelatin contains 22-25% whereas cold-water fish gelatin contains 17% (Muyonga, Cole, & Duodu, 2004a).

A study carried out in 2006 demonstrated data, which supported the percentages mentioned above. The study depicted that cold-water fish gelatins had significantly lower levels of hydroxyproline, proline, valine, and leucine residues when compared to mammalian gelatins (Avena-Bustillos et al., 2006). In contrast, cold-water gelatin contained significantly higher levels of glycine, serine, threonine, aspartic acid, methionine and histidine residues (Avena-Bustillos et al., 2006). Furthermore, when comparing the remaining levels of amino acids, it was observed that cold-water fish and mammalian gelatins contain the same percentages of alanine, glutamic acid, cysteine, isoleucine, tyrosine, phenylalanine, homocysteine, hydroxylysine, lysine and arginine residues (Avena-Bustillos et al. 2006). The lower levels of proline and hydroxyproline are what provide the fish gelatin with its low gel ability and melting temperature (Karim & Bhat, 2009). When studying the gelling ability of different compounds, it is important to look at the stability of the super-helix structure of the gelatin. This structure is stabilized by steric restrictions which are imposed by both pyrrolidine rings of the imino acids as well as the hydrogen bonds which are formed between amino acid residues (Harrington & Rao, 1970). Hence why the low levels of imino acids found in fish gelatin result in a low, stable gel.

As mentioned previously, the functional properties of gelatin are most influenced by the molecular weights, structures, and composition. When gelatin is being produced, there is a conversion of the extracted collagen. This conversion will yield molecules of different mass because of the breakage of inter-chain covalent crosslinks and the unfavorable breakage of some of the intra-chain peptide

linkages (Zhou, Mulvaney, & Regenstein, 2006). This means that at the end of the process, the gelatin that is extracted will have a lower molecular weight than the original collagen. It also results in a mixture of components that contain molecular weights ranging in size from 80-250 kDa (Imeson, 1997).

When looking in further details at the collagen structures present in fish and mammals, it was noted that β-chain and γ-chain aggregations were present in both. However, higher levels of β-chain and γ-chain present in fish gelatin will cause unfavourable effects on the functional properties; it will lower the viscosity, melting and setting points and will increase the setting time (Karim & Bhat, 2009). All of these effects were observed in pollock and salmon gelatins (Muyonga et al., 2004a).

2.4.2.2 Rheological Properties

Gelatin contains features that categorize it as a thermo-reversible gel. This is due to the fact that the bonding energy is feeble inside fish gelatin (Karim & Bhat, 2009). The main physical properties of gelatin gels are gel strength and gel melting points, which are both dependent on molecular weight. It is also reliant on the complex interactions that occur due to the amino acid composition and the ratio between α/β chains in the gelatin (S. M. Cho et al., 2004).The gel strength is directly correlated with the α-chain content in gelatin. The higher the α-chains are, the greater the strength of the gel. However, if there is a high ratio of peptides with molecular weights that are either higher or lower than a number of α-chains, this will decrease the gel strength (S. M. Cho et al., 2004).

One of the unique properties of gelatin is its ability to produce thermally reversible gels. This means that fish gelatin gels will begin to start melting at a temperature below that of the average human body. This is often referred to as its "melt-in-the-mouth" property (Karim & Bhat, 2009). For porcine and bovine gelatin, the gelling and melting points tend to range from 20 to 25 degrees Celsius and 28 to 31 degrees Celsius respectively. In comparison, fish gelatin range from 8 to 25 degrees Celsius and 11 to 28 degrees Celsius respectively (Arnesen & Gildberg, 2007). Furthermore, when comparing the melting points between cold-water fish and warm-water fish, it was determined that cold-water fish have significantly lower melting points due to the low levels of imino acid contents and proline hydroxylation (Giménez et al., 2009).

These rheological properties are a function of temperature and the concentration of gelatin, which is present in different species. These properties are developed during the transformation of collagen into gelatin. The process involves the disintegration of the helical structures into random coils (Hill, Ledward, & Mitchell, 1998). When they are cooled down the random coils will transition into helix's however, during this time they are attempting to reform back to their original structure (Karim & Bhat, 2009). This structure will be responsible for the strength and integrity of the gelatin gel.

2.4.2.3 Emulsifying and Foaming Properties

Gelatin has the ability to be used as a foaming, emulsifying and wetting agent not only in food but also in the pharmaceutical, medical and technical application industry. This is due to its surface-active properties. Studies have proven that gelatin has an active-surface due to the hydrophobic areas on its peptide chains, making it a good candidate to form oil-in-water emulsions (Karim and Bhat 2009). However, when comparing gelatin to other emulsifying agents such as globular proteins and Arabic gum, it is considered to have weaker surface-active substances (Karim and Bhat 2009). Therefore, there is a slight modification carried out in order to prevent gelatin from producing large droplets during homogenization. Instead attaching non-polar side groups to its peptides hydrophobically modifies it to prevent droplets (Toledano and Magdassi 1998). An alternate method is to use it alongside anionic surfactants (Toledano and Magdassi 1998).

Interestingly enough, it was noted that even with these modifications, there was still a small presence of large droplets in the emulsions even if there was a high protein concentration (Surh, Decker, and McClements 2006). When comparing the amount of droplets produced between high molecular weight fish gelatin and low molecular weight fish gelatin emulsions, there was an inverse relationship noted. The higher the molecular weight, the fewer droplets formed and the less destabilization of the emulsion (Surh, Decker, and McClements 2006). It was later concluded that this effect was attributed to the thickness of an adsorbed gelatin membrane increasing with a higher molecular weight. Furthermore, it was concluded that emulsions produced from both low and high molecular weight fish gelatins were fairly stable even when subjected to the following conditions: high salt concentrations of 250 mM sodium Chloride, thermal treatments of 30 and 90 degrees Celsius for 30 minutes and different pH values ranging from 3-8 (Surh, Decker, and McClements 2006).

In general, there are few studies conducted on the emulsifying and foaming properties of fish gelatin. Studies that have been carried out to indicate that fish gelatin emulsions have moderate stability when used for creaming. In 2006, J. Surh conducted a study which concluded that the physical stability of oil in water emulsions is influenced by the molecular weight of the fish gelatin as well as the pH, salt content and thermal processing which is carried out during the extraction (Surh, Decker, and McClements 2006).

2.4.2.4 Film Forming Properties

From the numerous studies that have been carried out to exhibit the production of films from fish gelatin, it can be concluded that all fish gelatin express excellent film-forming properties. However, there are still some differences between marine and mammalian gelatin.

In general, gelatin films that are formed from warm-water fish species depicted stress and elongation break that was similar to that of bovine bone gelatin (Muyonga, Cole, & Duodu, 2004b). However, when comparing water vapor permeability, it was discovered that fish gelatin exhibited a lower value (Gómez-Guillén, Ihl, Bifani, Silva, & Montero, 2007). This can be explained by looking at the amino acid composition. As mentioned before, fish gelatin expresses a much higher hydrophobicity due to its lower content of proline and hydroxyproline. Typically, with a higher content, this would mean that there would be more hydroxyl groups available to form hydrogen bonds (Avena-Bustillos et al., 2006).

The same was true for fish gelatin extracted from cold-water fish; they showed significantly lower water vapor permeability due to as mentioned above, the increased hydrophobicity. For this reason, it was concluded that utilizing lower molecular weight fish gelatin would have a more desired effect for film forming (Karim & Bhat, 2009).

2.4.2.5 Sensory Properties

A study carried out in 2000, (Choi & Regenstein, 2000) compared the physicochemical properties of pork and fish gelatin and the effect that the melting point would have on the sensory characteristics of gels.

A quantitative descriptive analysis was carried out to determine the effect of the melting point on the different sensory characteristics. The results indicated that flavored fish gelatin used to produce dessert gel products contained a less undesirable off-flavor and off-odors. On the other hand, when those same gel products were made with pork gelatin there was a more desirable release of flavor and aroma. The main difference between the two sources of gelatin was the melting point (Choi & Regenstein, 2000). Despite the Bloom values being equivalent, the lower melting temperature of the fish gelatin aided in releasing the fruit aroma, flavor and sweetness(Choi & Regenstein, 2000). However, the mouthfeel provided from the pork gelatin was more desirable due to the slow melting temperature.

2.5 Gelatin Production

The process of producing gelatin is carried out in three main steps: pre-treatment, extraction, and drying. As mentioned previously, it is important to be aware of the fact that the conditions used during extractions are directly related to the yield of gelatin and its properties. Therefore, different conditions exist for pre-treatment, extraction and drying depending on what source the gelatin is being extracted (Karim & Bhat, 2009).

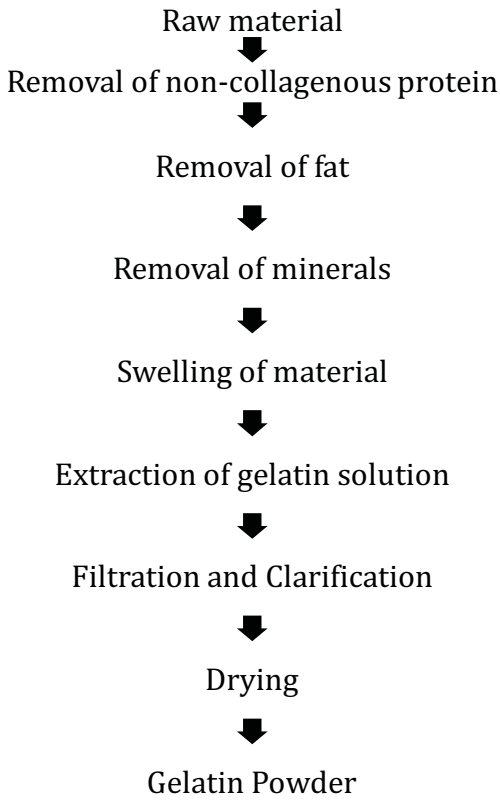

Figure 2-3: Overview of steps involved for gelatin extraction, adapted from Nollet et al. (2012).

2.5.1 Pretreatment

Pre-treatment is an important step as its purpose is to increase the purity of the gelatin that is being extracted (Regenstein, Zhou, & Shahidi, 2007). The pre-treatment step involves the removal of non-collagenous materials such as proteins, fat, and minerals to increase the yield of gelatin. After the removal process, swelling of these pretreated materials is carried out.

2.5.1.1 Removal of Non-Collagenous Protein

For the removal of non-collagenous protein, an alkaline solution is utilized. They are known for being capable of removing high amounts of non-collagenous materials by breaking down inter-chain cross-links (Regenstein et al., 2007). Alkaline solutions are also capable of inactivating proteases that have the potential of breaking down collagen (Regenstein et al., 2007). This inactivation allows them to prevent the loss of gelatin earlier on in the process.

When preparing for the removal of non-collagenous protein, the type of alkali being used does not make any significant difference in the process. However, the concentration utilized is important, as studies have shown that higher times and concentrations during extraction led towards lower yields of gelatin. This is due to the alkaline solution attacking the telopeptide region of the collagen molecule, which causes some collagen to be solubilized (Yoshimura, Terashima, Hozan, & Shirai, 2000). Depending on which type of fish the gelatin is being extracted, the temperature, duration, and concentration of alkali vary.

2.5.1.2 Removal of Fat

In general, any fish sources of gelatin that have high amounts of fat are known to cause adverse effects on film forming gelatin. The fats present the risk of forming soaps during the pre-treatment and will contaminate the final yield of gelatin (Nollet et al., 2012). Interestingly enough, despite this potential risk, most fish gelatin extractions do not require the removal of fat especially if the fat content is very low. For higher levels of fat, a defatting step is carried out using hot water (Muyonga et al., 2004b)

2.5.1.3 Removal of Minerals

The removal of minerals is carried out to facilitate the extraction of the gelatin from bones or scales. Fresh bone is most commonly treated using hydrochloric acid solution (Nollet et al., 2012). This solution is utilized mostly for inorganic compounds since the bone is composed majorly of that, this means that it will carry out the removal efficiently. Although on average, the acid concentration that is used will range between 2 to 6 percent HCl, it can change depending on which fish is being used. Furthermore, the temperature and demineralization time can also vary depending on the source of gelatin. Acid phosphates can also be used to dissolve calcium phosphate (Nollet et al., 2012)

Furthermore, it is important that two things need to be avoided during demineralization. The first is the acid hydrolysis of proteins and the second is using high temperatures during the process. This can be prevented by using lower temperatures as high temperatures favor the hydrolysis of the protein resulting in a lower yield of gelatin (Muyonga et al., 2004b).

2.5.2 Swelling of Pretreated Raw Materials

Once the removal of the non-collagenous has been completed, the next step is swelling. Swelling is a major step as it enhances the efficiency of extraction by favoring protein unfolding. The unfolding is a result of disturbing the non-covalent bonding, making the extraction of collagen possible (Nollet et al., 2012). There are different acidic and alkaline methods used to cause the swelling of the raw materials. Acidic process yield type A gelatin whereas alkaline methods yield type B gelatin (Nollet et al., 2012).

2.5.2.1 Acid Process

The purpose of this process is to convert the collagen into a form that is more suitable for extraction. Acid hydrolysis is a milder treatment, which is more commonly used on animals, such as pigs, that have been slaughtered at a younger age (Foegeding, Lanier, & Hultin, 1996). Although this method is used on animals, it also works on fish gelatin. This process results in stripping organic substances and releasing free α-chains for the extraction step to be carried out. The acid process involves using a wide variety of acids such as sulfuric and hydrochloric acid (Johnston-Banks, 1990).

However, sometimes the acid pre-treatment can partially inactivate endogenous proteases. This inactivation will cause enzymatic breakage of the intra-chain peptide bonds in collagen (Peng & Regenstein, 2005). This jeopardizes the yield of gelatin that is extracted at the end of the process. It can be avoided with the simple addition of pepsin and a protease inhibitor. This was proved with a study, which was conducted in 2008. The purpose of the research was to compare swelling methods using a pepsin-aided process and protease inhibitor and the conventional acidic process (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008). Results indicated that the pepsin-aided process used on a big-eye snapper fish, not only had a higher yield but also expressed higher bloom strength. In comparison, the conventional method actually depicted levels of degradation to the gelatin (Nalinanon et al., 2008).

2.5.2.2 Alkaline Process

The alkaline process yields type B gelatins, and it also used on both animals and fish sources. This process involves the deamidation and the degradation through alkali hydrolysis, and it will result in a lowered chain length, which will affect the quality of the gelatin (Foegeding et al., 1996).

Generally, lime is used for this hydrolysis because it is a milder method compared to the acidic process and will not cause any significant damage. Concentrations of up to 3% are used in combination with low levels of calcium chloride or even caustic soda (Johnston-Banks, 1990). However, the process does have a longer duration of at least eight weeks especially with the addition of caustic soda, which required a pre-treatment period of 10 to 14 days (Johnston-Banks, 1990) .

2.5.2.3 Acid Process in Conjunction with Pepsin

Traditionally, between the acidic and alkaline process, the latter is popular as it has the ability to extract a high yield of gelatin from high degree of cross-linking. That being said, the conditions are still considered harsh and present the risk of developing random hydrolysis of peptide bonds and the decomposition of some amino acids (Slade & Levine, 1987). This will cause the resulting gelatin to have differing molecular weight distribution, which means varying quality standards.

As mentioned before, the quality of gelatin is dependent on the molecular weight and the length of collagen chains. The higher the molecular weight and the length are, the higher the quality of the extracted gelatin. Depending on the raw material, the process of swelling is selected. Acidic processes are generally used to obtain high quality gelatins, as it will destroy less amino acid (Slade & Levine, 1987). The more cross-linkages broken, the higher the yield of the gelatin and if the majority of them are acid-labile collagen crosslinks, the acidic method should be the chosen one. However, higher numbers of cross-links can also result in an overall lower yield. To solve this issue, proteases can be added in order to aid in the solubilizing of the collagen and cleavage of the crosslinks (Galea, Dalrymple, Kuypers, & Blakeley, 2000). To test this theory, the 2008 study carried out by Nalinanon et al. showed that gelatin yield from big eye snapper skin that was treated with pepsin was two-fold higher than that treated without it (Nalinanon et al., 2008). This means that the addition of such proteases needs to be considered during pre-treatment.

2.5.3 Extraction of Gelatin

The extraction of gelatin can only be carried out after the collagen has converted into gelatin. The process of conversion occurs on a molecular level. The first step is to apply enough heat that the collagen fibrils will shrink to less than one-third of their original length. This occurs at the critical temperature, which is known as the shrinkage temperature. At the shrinkage temperature, the fibers

are disassembled, and the triple helix arrangement of the polypeptide subunits in the collagen molecule will collapse (Foegeding et al., 1996). The collapse is due to the many non-covalent bonds as well as covalent inter and intra-molecular bonds breaking. The heat will disrupt most of the hydrogen bonds that are responsible for stabilizing the collagen structure (Foegeding et al., 1996).

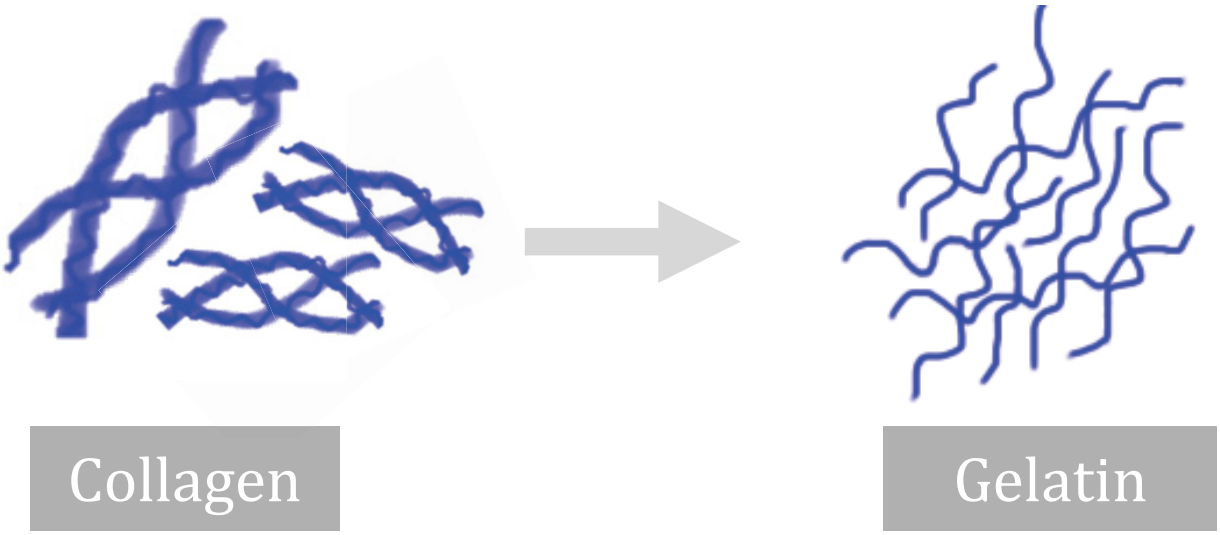

Figure 2-4. Visual representation of collagen converting into gelatin, adapted from Nollet et al. (2012)..

This will result in the helical collagen structure converting into an amorphous state known as gelatin (Nip et al. 2008). From the different methods of extraction that are used for the various sources of fish, the extracted gelatin has a molecular weight which varies from 15 to 400kDa (Nollet et al., 2012).

The primary goal of extraction is to be able to extract the highest quality gelatin with the max yield and the desirable physical properties. Therefore, the factors need to be adjusted according to which property is most desired. The adjustment of pH and other parameters can result in shorter extraction time yet still provide high-quality gelatin with high bloom strength (Johnston-Banks, 1990).

2.5.4 Drying

The last step involved in the production of gelatin is drying. After the extraction, the gelatin is filtered to remove any insoluble matter such as fat, collagen fibers, and any other residues. To carry out the filtration, activated carbon or diatomaceous earth is used commonly to make the gelatin solution clear (Johnston-Banks, 1990). Once this is complete the next steps are to evaporate, sterilize and finally dry. Since the pre-treatment and extraction steps can be very time-consuming it is important that these measures be carried out as efficiently and as quickly as possible. Methods of drying include freeze drying, hot-air drying, and spray drying. Of these three, freeze-dried gelatin is known for expressing the highest gel strength and foam formation; however, its foam stability was the lowest. Spray-dried gelatin expressed the highest emulsion capacities (Nollet et al., 2012)

2.6 Squid Gelatin Extraction

To date, the extraction of gelatin from squid, for the purpose of making edible films, has been limited to the study of Gomez-Guillen in 2002 (Gómez-Guillén et al., 2002) and then once again by B. Giminez et al. in 2009 (Giménez et al., 2009). Prior studies focus more on the use of squid gelatin for hydrolysis to obtain gelatin peptides that exhibit antioxidant properties (Lin & Li, 2006). Furthermore, the studies carried out on squid gelatin were using the species of Dosidicus *Gigas* more commonly known as Giant Squid and *Loligo Formasona.*

There have been many studies conducted on the extraction of gelatin from marine sources. The extraction methods include both enzymatic aided and non-enzymatic methods. Gomez-Guillen et al. (2002) utilized a non-enzymatic extraction method on giant squid (Gómez-Guillén et al., 2002). This method used a mild acidic procedure using 0.05M acetic acid for the swelling step followed by an overnight extraction (12 hours) at a high temperature of 80 degrees C. However; these conditions provided a low gelatin yield. Furthermore, low alpha chain yields were also reported which consequently led towards the weak gelling ability of the gelatin (Giménez et al., 2009).

A study conducted in 2006 by Lin and Li carried out the extraction of gelatin using a different treatment, to obtain gelatin peptides with antioxidant properties from jumbo flying squid skin (Lin & Li, 2006). The swelling step was performed using 0.02% H2SO4 instead of acetic acid.

Subsequently, the gelatin extraction was carried out overnight (12 hours) at a temperature of 45 degrees C (Lin & Li, 2006). This study reported a high gelatin yield of 10.9% however, there was no characterization of the gelatin carried out. (Lin & Li, 2006)

The study conducted out by Gomez-Guillen et al. in 2009 aimed to characterize the gelatin extracted and the respective films formed. The extraction was carried out using an enzyme-aided process using pepsin in conjunction with acetic acid (Giménez et al., 2009). This method of extraction is a modification of a method that was initially carried out by Nalinanon, Benjakil et al. in 2006 on the fish, big eye snapper. Briefly, the method involves pre-treating the fish skins in NaOH followed by the swelling of the skins using acetic acid in conjunction with pepsin. The gelatin is then extracted at 60 degrees for 18 hours (Nalinanon et al., 2008). Using a modified version of this method, Gomez-Guillen et al. obtained extraction yields of 12% and 4% on wet basis for G1 and G2 extracts respectfully (Giménez et al., 2009).

There exist in literature usage of enzyme-aided processes of gelatin extraction from fish species. Research in this area shows that a proper digestion of the fibrous collagen with proteases can be carried out to increase the yield of gelatin. Furthermore, it is important that for highly cross-linked collagen as is the case for squid gelatin, it is necessary to increase the yield using proteases (Nalinanon et al., 2008). However, there is a possibility of high collagen hydrolysis to occur which would damage the gelling ability. The enzyme pepsin has been used as was the case in the study carried out by Nalinanon, Benjakil et al. in 2006. The pepsin being utilized in this study was extracted from the stomach of the fish (big eye snapper) from which the gelatin extracted. The enzyme is known to cleave peptides in the telopeptide region of native collagen. The study shows that with the addition of proteases, the yield increased. The highest obtained yield was 40.3% with the addition of pepsin added at 15 U/g of treated skin in the presence of inhibitor SBTI. The presence of inhibitor SBTI was added to the solution during extraction to ensure that the beta sheets and alpha 1 & 2 chains were not completely degraded (Nalinanon et al., 2008).

With the success of the previous study, Giminez et al. used a modified version of the procedure for gelatin extraction. The pepsin enzyme was from porcine stomach mucosa with an activity of 662 U/mg solid from Sigma-Aldrich. The study involved characterization of two types of gelatin;

the initial gelatin extraction (G1) was followed by a subsequent gelatin extraction (G2) using the same pieces of squid that were used for the initial extraction. The molecular weight distribution between the two samples had noticeable differences. G1 had more clearly defined alpha chains than G2 .(Giménez et al., 2009) Overall, the SDS-polyacrylamide gel electrophoresis showed that higher molecular weight components and possible fragments of β components could be hardly detected. When studying the puncture force and breaking deformation values, between the films formed from G1 and G2, G1 was more resistant. Both films exhibited high water solubility (over 90%) which is due to the lack of alpha chains polymers obtained; in general mammalian gelatin is reported to have a lower water solubility value due to high amount of hydroxylysine residues (Giménez et al., 2009). The films also expressed lower water vapor permeability due to the predominance of low molecular weight components which created a denser protein matrix that had lower volume. Regarding color, squid gelatin films exhibited a lower yellow component compared to fish gelatin derived from other marine sources. Despite appearing quite transparent, the opacity values indicated that the gelatin films were far more opaque compared to films formed from tuna (Giménez et al., 2009). The data shows that under the extracted conditions, polymers of alpha chains and high molecular weight aggregates could not be efficiently extracted. However, gelatin could still be used to form homogenous, flexible and easily handled films that have the most potential in the food industry as film coatings.

2.7 Conclusion

In conclusion, the extraction of gelatin from other sources is important and vital for further advancements in this field of study. The development of biodegradable edible films can contribute towards immense reduction of environmental pollution. Their role as active and functioning barriers against oxidation and moisture can increase its applications especially in the food industry. The development of such films from marine biomass would only have a stronger and more positive impact on the environment and the issues surrounding it. Studies have shown that squid is an invaluable source of gelatin and can be used for the development of biodegradable. The properties of the gelatin extracted will in turn determine how strong and resilient the films they can form will be. These properties are directly related to the structure and amino acid content of the gelatin being extracted hence why the conditions of extraction are very important. The pretreatment method, the

type of acids and alkalines being used along with their concentrations will all have an effect on the collagen that is being treated for the conversion of gelatin. The extraction temperatures and durations will also determine whether the gelatin extracted will be of high or low quality. Studying the extraction method and the effect that these parameters will have on the properties of the gelatin extracted will aid in determining what films can be formed and for what applications they can be used.

CHAPTER 3:

3 MATERIALS AND METHODS

3.1 Materials Required:

3.1.1 Biological Specimen:

Illex Argentinus Squid was purchased from OCN Import fish market, Montreal. The skins were stored at -20°C until used for extraction.

3.1.2 Enzyme:

Pepsin from porcine stomach mucosa (an activity of 427 U/mg solid) was purchased from Sigma-Aldrich, Steinheim, Germany.

3.1.3 Reagents:

Acetic acid, hydrochloric acid, isopropanol, ammonium persulfate, glycerol was obtained from Fisher Scientific, New Jersey, USA. BCA Assay Kit is purchased from Thermo Scientific, Rockford, USA. Activated charcoal, hydroxyproline, mercaptoethanol, Tetramethylethylenediamine (TEMED), sodium hydroxide, Ehrlich's reagent was purchased from Sigma-Aldrich, Steinheim, Germany. Chloramine-T was acquired from Acrose Organics, New Jersey, USA. SDS sample buffer laemmli was purchased from Biorad, Ontario, Canada. Whatman #4 and #1 paper was purchased from Whatman Limited, Buckinghamshire, England.

3.1.4 Machinery:

Isotemp Vacuum Oven Model 281A and Isotemp hot air oven were purchase from Fisher Scientific, New Jersey, USA. Shaking Water Bath 25 was purchased from Thermo Fisher Scientific, Massachusetts, USA. Mini protean electrophoresis set was purchased from Biorad, Ontario, Canada. Science Teaching Incubator was acquired from Labline, California, USA. Instron Universal Machine model 4500 was purchased from Instron Corporation, Canton, MA. Freeze dryer Lyph Lock 18 was purchased from Labconco, Missouri, USA. DU 800 spectrophotometer was purchased from Beckman Coulter, California, USA. Micrometer Model 030025 was purchased from Marathon watch company Ltd., Richmond Hill, Ontario.

3.2 Sample Preparation

The *Illex argentinus* squid was obtained frozen and was stored in a freezer at temperature of -20°C for four months before being used. The squid was thawed and cleaned by hand when gelatin extraction was ready to be carried out. The squid's offals were removed and discarded with only the fins and mantles being used for gelatin extraction. These parts were cut into small pieces of 1x1cm and then dehydrated for 12 hours at 45°C using a Fisher Scientific Isotemp Vacuum Oven Model 281A (New Jersey, USA).

3.3 Gelatin Extraction Method

The extraction method carried out was that conducted out by B. Giménez et al. (2009) using giant squid (Dosidicus Gigas) which is a modified version of the protocol used by S. Nalinanon et al. (2008) on bigeye snapper fish. The dehydrated samples were then pretreated in 0.025M NaOH (ratio of 1:10 w/v) for 2 hours under continued gentle stirring with the NaOH solution changed every hour. After pre-treatment, the squid samples were then swelled in 0.2M acetic acid (ratio of 1:10 w/v) in conjunction with pepsin (concentrations of 5U/g of squid, 15U/g of squid and 25U/g of squid) from porcine stomach mucosa, for 48 hours at 4°C. Squid samples were then filtered using Whatman #4 paper and washed with distilled water (ratio of 1:100 w/v) before gelatin extraction is carried out in distilled water (ratio 1:10 w/v) at 45°C, 55°C and 65°C for 12, 18 and 24 hours in a water bath. Solutions were then filtered using Whatman #4 paper are then dried using a Labconco freeze dryer (Missouri, USA).

3.3.1 Gelatin Yield

A rough estimation of the gelatin yield is calculated using the following formula:

$$\%\ yield\ = \frac{weight\ of\ freeze-dried\ gelatin}{weight\ of\ dehydrated\ squid\ sample} \times 100$$

3.3.1.1 Hydroxyproline Content

Hydroxyproline content of gelatin was determined according to the method of S. Nalinanon et al. (2008) with a slight modification. The samples were hydrolyzed with 6M HCl in glass tubes at a temperature of 110°C for 12 hours in a Fisher Scientific Isotemp hot air oven (New Jersey, USA). The hydrolysate was clarified with activated charcoal and filtered through Whatman #4 filter paper. The filtrate is then neutralized using 10 M NaOH, 1M NaOH, and 0.1M NaOH to a pH between 6.0 and 6.5. 0.1mL of the neutralized sample is then transferred into a test tube, and 0.2mL of isopropanol is added. This solution is then mixed and 0.1mL of an oxidant solution, a mixture of 7%(w/v) chloramine T and acetate/citrate buffer, pH 6, at a ratio of 1:4(v/v), was added and stirred. 1.3mL of Ehrlich's reagent solution is added along with isopropanol at a ratio of 3:13(v/v). This mixture is then heated for 25 minutes at 60 °C in a Thermo Fisher Scientific Water Bath (Massachusetts, USA). The samples are then removed and cooled down using running water for 2-3 minutes. The samples are then diluted to 5 ml with isopropanol and the absorbance was then measured against water a wavelength of 558 nm using a Beckman Coulter DU 800 spectrophotometer (California, USA). The standard hydroxyproline solutions for the standard curve were prepared at concentrations ranging from 10 to 60 ppm. The content of hydroxyproline was calculated and expressed as mg/g of sample.

3.3.2 BCA Analysis

The protein content was determined using the Pierce BCA Protein Assay Kit. The BCA standard curve was prepared by diluting the Albumin (BSA) stock solution to have a ranging concentration from 0 µg/mL to 2000 µg/mL of BSA concentration. The BCA working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent. The unknown samples were prepared by dissolving 0.001 g of gelatin in 1mL of distilled water. 0.1mL of each standard and each unknown sample was then mixed with 2.0mL of the WR and mixed well. The test tubes were then covered and incubated a temperature of 37°C for 30 minutes in a water bath. After 30 minutes, the tubes were cooled to room temperature. The absorbance of the samples and the standards were then measured within 10 minutes at an absorbance of 562nm against water using the Beckman Coulter DU 800 spectrophotometer (California, USA). The standard curve as prepared by plotting the average blank-corrected 562nm measurement for each BCA standard vs. its concentration in µg/mL.

3.3.3 SDS Electrophoresis PAGE - Molecular Weight Distribution

To analyze the molecular weight distribution, the gelatin samples were prepared by dissolving 0.01g in distilled water. 100 µL of this solution was then added to a loading buffer comprised of 10% SDS, 5% mercaptoethanol and 0.002% bromophenol blue. The protein samples were then boiled for 5 minutes at a temperature of 100 °C. They were then analyzed by SDS-PAGE using a 5% stacking gel and a 10% resolving gel using a Mini Protein Electrophoresis Set from Biorad, (Ontario, Canada). The loading volume was 20 µL in all lines. The protein bands were stained with Coomassie Brilliant Blue R250. A low molecular weight Biorad SDS marker kit (Ontario, Canada) consisting of phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.O kDa), carbonic anhydrase (30.0kDa), trypsin inhibitor (20.1kDa) and α-lactalbumin (14.4 kDa) was used to determine molecular weight of protein samples.

3.4 Film Formation

Edible films were formed using the method by B. Giménez et al. (2009) which prepared them through casting. The gelatin filmogenic solutions were prepared by dissolving the freeze-dried gelatin in distilled water at 4% w/v at 45 °C for 20 minutes. The plasticizer glycerol was added to the heated solution at a ratio of 0.30g/g gelatin. Amounts of 10 mL of the filmogenic solutions were poured into plexiglass plates (55mm X 16mm) and dried at room temperature for 48 hours. Before being characterized, films were conditioned over a saturated solution of NaBr in 50 ± 5 % RH for 40 hours at 23-25°C.

3.4.1 Mechanical Properties - Elongation, Tensile Strength & Thickness

Elongation and tensile strength were determined according to the ASTM Standard 638-10(Shi & Dumont, 2014). Film samples were cut into dog-bone shapes and conditioned for 40 hours at 23-25°C in 50 ± 5 % RH. Tests were carried out using an Instron Universal Testing Machine (Model 4500, Instron Corporation, Canton, MA) at a crosshead rate of 50 mm/min. Three replicates of each film were tested. Film thickness was measured using a laboratory micrometer (030025, Marathon watch company Ltd., Richmond Hill, Ontario) at six random positions. Tensile Strength and Elongation were calculated using the following formulas:

$$Tensile\ Strength\ (TS) = \frac{(Maximum\ Load\ (N))}{Initial\ Cross-Sectional\ Area\ of\ Sample\ (Thickness\ x\ 5\ mm)}$$

$$Elongation\ at\ Break\ (EAB) = \frac{Displacement\ at\ Maximum\ Load\ (mm)}{Original\ Gage\ Length\ (30mm)}$$

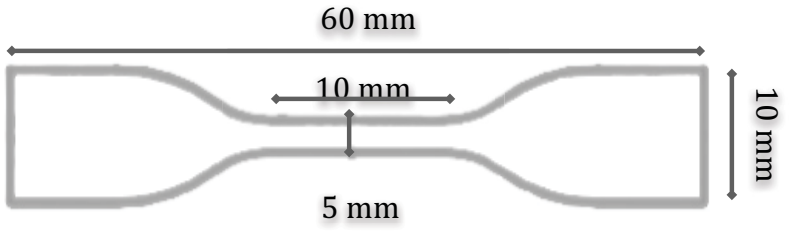

Figure 3-1. Dimension of the dogbone shape that films were cut into for analysis.

3.4.2 Solubility of Film

The water solubility of the film was determined according to the procedure of Jeya et al. (2012) (Jeya Shakila, Jeevithan, Varatharajakumar, Jeyasekaran, & Sukumar, 2012). The films were cut into a size of 4x4cm with three replicates. The films were weighed and then placed in a beaker with 15 mL distilled water. It was stirred gently at 22 °C for 15 hours. The solution was then filtered using Whatman #1 filter paper. The residue collected on the filter paper was then dried in a Fisher Scientific Instron hot air oven (New Jersey, USA) at 105 °C for 24 hours. The solubility of the film was then calculated according to the following equation:

$$Solubility = \frac{Initial\ weight\ of\ the\ sample - Weight\ of\ the\ undissolved\ desiccated\ Film\ Residue}{Initial\ weight\ of\ the\ sample}$$

3.4.3 Water Uptake of Film

Films were cut into a size of once again 4x4cm with three replicates each. The films were weighed before and then immersed in water at room temperature. The films were removed from water and weighed at 5 min, 10 min, 15 min, 20 min, 25 min and finally 30 minutes. The finally weights were recorded and the water uptake was expressed in percentage.

3.4.4 Opacity of Film

The opacity of the film was determined using the method of Limpisophon et al. (2009) (Limpisophon, Tanaka, Weng, Abe, & Osako, 2009). Films were cut into sizes of 12 x 34 mm, with at least three replicates. They were placed in a glass cuvette and exposed to the both visible and UV wavelengths of 200, 280, 350, 400, 500, 600, 800 nm using a Beckman Coulter DU 800 spectrophotometer (California, USA) to determine the % Transmittance. The opacity of the film was calculated using the following equation:

$$Opacity\ (\%) = 100\% - (\%\ Transmittance\ at\ each\ wavelength)$$

CHAPTER 4:

4 RESULTS AND DISCUSSION

4.1 Extraction of Squid Gelatin Using Pepsin Aided Method

For the extraction of gelatin from *Illex Argentinus* squid (shortfin), there were three different parameters that were studied during the process. These parameters were the effect of the enzyme concentration on the gelatin yield, the effect of the extraction temperature on the gelatin yield and the effect of the extraction duration on the gelatin yield. This part of the study falls under the first objective which is to extract gelatin under different conditions in order to determine which sample would be best suited for film formation. The studies which were being used as a reference for gelatin extraction of squid, Giminez B et al.(2009) and Nalinanon et al. (2008) used an extraction temperature and duration of 45°C and 12 hours respectively (Arnesen & Gildberg, 2007; Giménez et al., 2009; Nalinanon et al., 2008). Nalinanon et al. (2008) reported that the enzyme concentration that gave the highest yield of gelatin was 15 U/g of material. For this reason, these values were considered in the range of values being studied.

The first parameter being studied was the enzyme concentration; there were three different concentrations used during the swelling step, 5 U/g of dehydrated squid, 15 U/g of dehydrated squid and 25 U/g of dehydrated squid. The extraction temperature and extraction duration was kept stable at 45°C and 12 hours respectively. This was the first parameter studied because the concentration which aided in extracting a higher quality gelatin would then be used for the subsequent gelatin extractions.

The second parameter being considered was the extraction temperature; again there were three different temperatures being used during the heat extraction, 45°C, 55°C and 65°C. The extraction duration was kept stable at 12 hours, and the best enzyme concentration was also kept as the standard concentration used. The gelatin that was extracted which exhibited the highest quality would then be used as the standard temperature during the study of the third parameter.

The third parameter being studied was the extraction duration, where three durations, 12, 18 and 24 hours were used during the heat extraction. For these samples, again the best enzyme concentration and extraction duration were used as the stable values in order to see the isolated effect of extraction duration on the yield of gelatin.

4.1.1 Parameter 1 - Effect of Enzyme Concentration (5U/g, 15U/g, and 25U/g)

4.1.1.1 Effect of Enzyme Concentration on Gelatin Yield

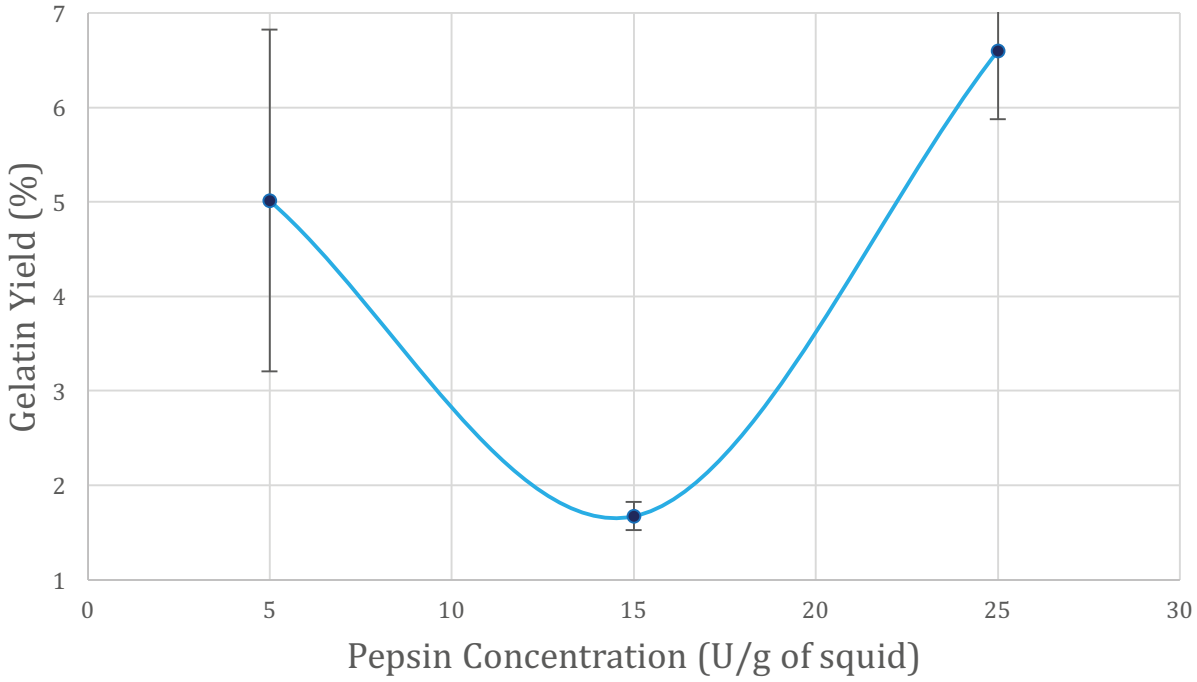

*Figure 4-1. Plot of gelatin yield (%) vs. pepsin concentration (U/g of squid) to see the effect of enzyme concentration on yield. *Reaction Conditions: 12-hour extraction at 45 ℃.*

When comparing the gelatin yield obtained from the different enzyme concentrations utilized, there is no direct relationship observed as can be seen from Figure 4-1. It is noted that the highest enzyme concentration of 25U/g provides the highest average yield of gelatin at 6.60%. The lowest

concentration of enzyme, 5U/g rendered a gelatin yield of 5.02%. The lowest yield of gelatin, 1.67% is observed when an enzyme concentration of 15U/g is used.

The data obtained is contradicting to the findings of a study carried out in 2008 by Nalinanon et al. According to the method employed by Nalinanon et al. (2008) the results indicated that in general, the yield of gelatin extracted with the aid of big-eye snapper pepsin (BSP) was approximately twofold higher than gelatin that was extracted without the use of BSP (Nalinanon et al., 2008). The results of this study also depicted that with the increase of BSP levels used, a range of 0 -15 U/g, the yield of gelatin also increased linearly (Nalinanon et al., 2008). However, Figure 4-1 depicts that there is no linear relationship depicted between the gelatin yield and the pepsin concentration that is used during the swelling process. The purpose of using an enzyme-aided method within the acidic swelling step was to observe an increase in the yield of gelatin extracted overall.

With the addition of pepsin, acid-labile collagen cross-links are disrupted without causing any peptide bond hydrolysis or amino acid degradation; allowing gelatin to be obtained efficiently (Nalinanon et al., 2008; Slade & Levine, 1987). The inter- and intra- molecular covalent cross-links of collagen that create the telopeptide region is a site that pepsin can effectively hydrolyze (Drake, Davison, Bump, & Schmitt, 1966). It is capable of solubilizing collagen during the acid-swelling process and in result can create a looser structure that can ease the extraction of gelatin (Nalinanon et al., 2008). In order for there to be an improvement in the yield, there must be a proper digestion of the fibrous collagen with proteases when extracting from highly cross-linked collagen (Peterson & Yates, 1977). For this study, since squid is known for its high cross-linking, it is possible that there was not an appropriate digestion period and for this reason, the enzyme pepsin was not capable of loosening the structure to allow better gelatin extraction.

Furthermore, it was visually observed that the gelatin samples obtained under the different enzyme conditions looked dramatically different from each other. The 5U/g freeze-dried samples were powders and had less resemblance to the most conventional gelatins that have been previously extracted from marine sources. The powder-like texture gave rise to suspicions that this sample was not entirely gelatin and was in fact contaminated with other minerals and sediments such as

salt. Both the 15U/g and 25U/g samples were closer in visual resemblance to each other and had textures most resembling conventional extracted gelatin. In order to further confirm these suspicions, the protein content of the samples was tested using a BCA Assay.

4.1.1.2 Protein Content Determination with BCA Assay

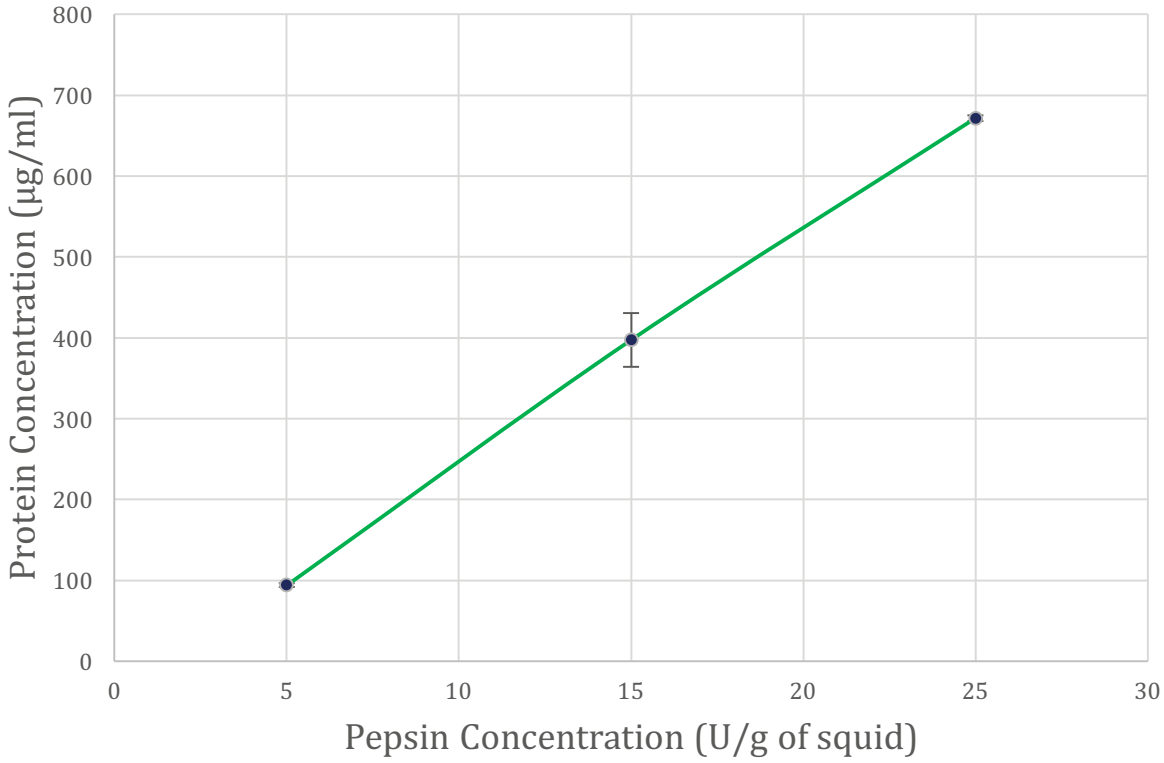

*Figure 4-2. Plot of protein concentration (µg/mL) vs. pepsin concentration (U/g of squid) *Reaction Conditions: 12-hour extraction at 45℃.*

The BCA Assay is a method that carries out the quantification of the total protein content through a colorimetric detection. This process involves the reduction of Cu^{+2} into Cu^{+1} by protein while in an alkaline medium; this is also known as the biuret reaction (Brown, Jarvis, & Hyland, 1989). The method is highly sensitive and selective with its colorimetric detection of the cuprous cation when using a reagent which contains bicinchoninic acid (Smith et al., 1985). The purple color change which is observed is a result of the chelation of two molecules of BCA with one cuprous

ion forming a water-soluble complex. This complex will exhibit a strong absorbance at 562nm that is relatively linear over increasing protein concentrations. In more details, the color formation with BCA depends on the macromolecular structure of protein, the number of peptide bonds and most importantly, the presence of four specific amino acids; cysteine, cystine, tryptophan and tyrosine (Wiechelman, Braun, & Fitzpatrick, 1988).

Figure 4-2 depicts a linear relationship between the protein concentration in the gelatin samples and their respected pepsin concentration. The gelatin sample extracted with the lowest pepsin concentration also has the lowest protein concentration at a value of 94µg/mL. The gelatin sample extracted with a pepsin concentration of 15U/g has a protein concentration of 397.25µg/mL. The highest pepsin concentration used during extraction had the highest protein concentration of 671.5µg/mL. By looking at the protein concentration, it was possible to confirm that the samples extracted were not pure gelatin and that there was some contamination involved. Particularly in the first sample, the presence of salts was evident. Most likely, the salts were formed when the NaOH solution was used to inactivate the enzyme pepsin in the acetic acid solution. Before the gelatin extraction, the samples are washed with distilled water to remove the enzyme and other sediments. It is possible that the washing step was not efficient in removing the contaminants and were, therefore, present in the freeze-dried samples.

The lack of protein and the presence of salt in the samples can also give insight to the gelatin yield curve depicted in Figure 4-1. The gelatin yield was determined using a basic equation that takes the overall freeze-dried weight into account as well. With the increasing protein concentrations, it can be hypothesized that there is a high presence of salt. The weight of this salt was also taken into account when calculating the yield therefore; Figure 4-1 is not an accurate representation of the gelatin yield.

After determining the protein concentration, the molecular weight distribution was observed in order to see if the gelatin samples showed presence of the beta and alpha bands needed to form films. The molecular weight distribution also indicates what proteins are present in the sample.

4.1.1.3 SDS Bands - Molecular Weight Distribution

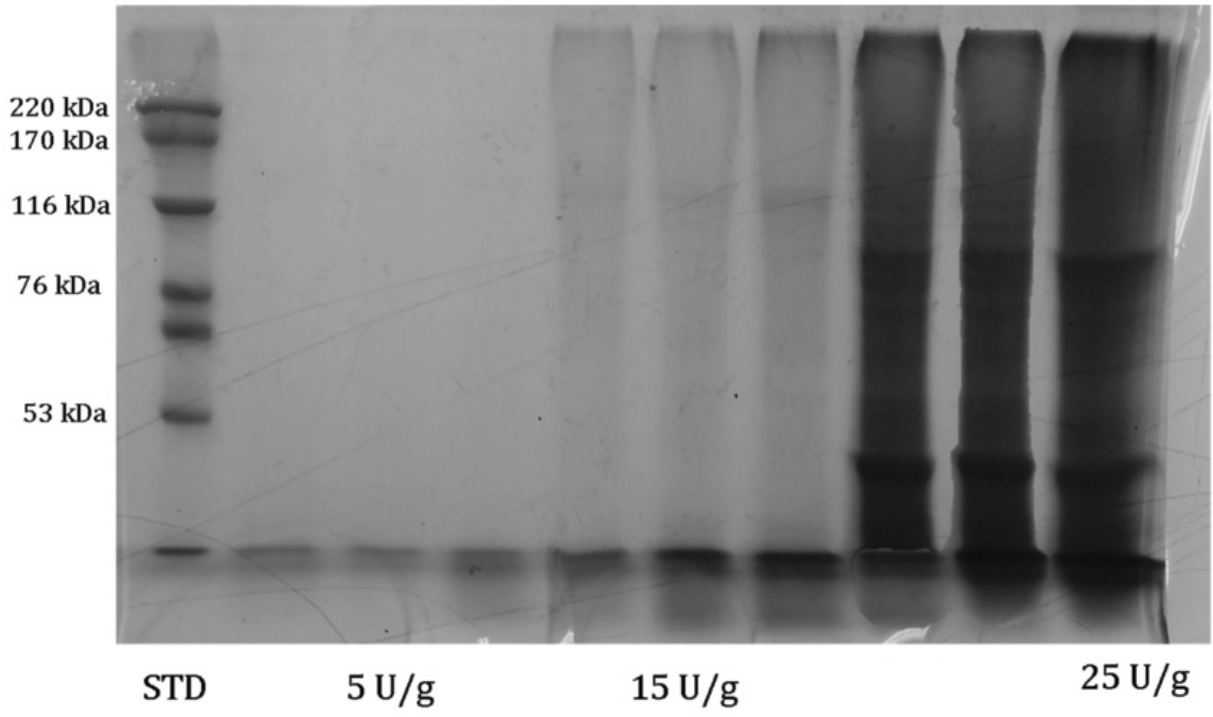

Figure 4-3. Electrophoretic analysis (SDS-PAGE) of gelatin preparations (triplicates of each sample with varying extraction durations were used) in the presence of 2-β Mercaptoethanol.

Figure 4-3 depicts the molecular weight distribution of the extracted gelatin sample. In Figure 4-3 a high molecular weight standard was used. From the bands that appeared for the different samples it can be noted that there is no presence of high molecular weight bands. Components which have higher molecular weights of alpha chains, fragments of beta components are hardly detected. For the gelatin samples that did have bands show up, they are of a lower molecular weight. As mentioned before, presence of lower molecular weight bands suggests that poor films will be formed as low molecular weight gelatin tends to form glue-like sticky films rather than plastic like films that are made from high molecular weight. When comparing the three enzyme concentrations, Figure 4-3 clearly shows that the lowest concentration, 5U/g, has no protein bands confirming that there was no gelatin extracted; the powder-like material that was extracted was most likely entirely salts that had been freeze-dried during the process and is not fit for forming films. Gelatin samples extracted using an enzyme concentration of 15U/g has a very faint band at

116kDa indicating a faint presence of the protein ß-galactosidase (Garfin, 1990). However, lack of other protein bands indicates that this sample is poor to be used for film formations. The gelatin samples with the highest enzyme concentration of 25U/g had three main lower molecular weight protein bands present. The more prominent bands are the 97kDa and the 47kDa, indicating the presence of the proteins Phosphorylase b and Ovalbumin. Upon close examination, the protein band of Serum Albumin at 66kDa can also be seen to be present. The presence of these three lower molecular weight protein bands indicates that although there is protein present, the overall gelatin extracted is of lower quality and will most likely form poor films.

Overall, it can be determined that the enzyme concentration did not necessarily increase the yield as was seen in previous studies. The lack of linear relationship depicted between the two variables as well as the visual observations of the samples brought upon the conclusions that not all extracted samples were gelatin and this was confirmed by both the BCA Assay and the SDS electrophoresis. Samples were contaminated with salt that was not efficiently washed off the skins and in return contributed to the yield calculations. Both analyses indicated that with the increasing enzyme concentration, the protein concentration increased with the presence of more protein bands. Furthermore, polymers of alpha chains and high molecular weight fragments were not efficiently extracted under the conditions used for this study. According to other studies, this can possibly be because of the pepsin having a selective effect on the telopeptide region of collagen and only cleaving the alpha chains. From the three different samples collected using the different enzyme concentrations, it was concluded that the concentration of 25U/g used gave extracted gelatin with the highest protein concentration and the most protein bands. For this reason, this enzyme concentration was then used for all gelatin extraction to be carried out.

4.1.2 Parameter 2 - Effect of Extraction Temperature (45℃, 55℃ and 65℃)

4.1.2.1 Effect of Extraction Temperature on Gelatin Yield

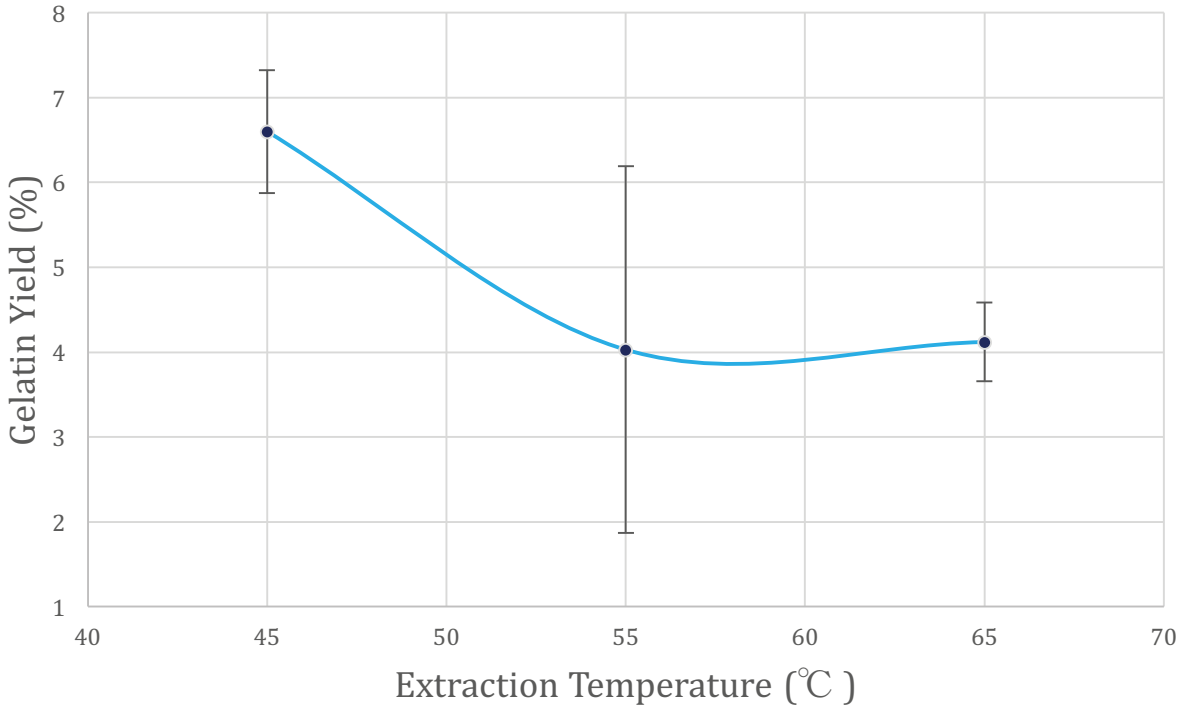

*Figure 4-4. Plot of gelatin yield (%) vs. extraction temperature (℃) to see the effect of extraction temperature on yield. *Reaction Conditions: 12-hour extraction using 25 U of pepsin/g of squid.*

The extraction temperature plays an important role as it can alter both the yield and the properties of the gelatin being extracted (Kołodziejska, Skierka, Sadowska, Kołodziejski, & Niecikowska, 2008). This parameter can have a direct effect on the molecular weight range of the fragments forming gelatin. The temperature at which the hydrolysis occurs allows for amide bonds to undergo extensive hydrolysis thus generating fragments varying from 16 to 150 kDa (Asghar & Henrickson, 1982; A. Bailey, 1985). Compared to mammalian skins, fish skins are more suitable as a source of gelatin due to its capability of being extracted at relatively moderate temperatures (below 50℃) but with higher yields (Giménez, Gómez-Guillén, & Montero, 2005; Gómez-Guillén et al., 2002; Kołodziejska et al., 2004).

When studying the effect that the extraction temperature would have on the gelatin yield, Figure 4-4 depicts a more direct relationship between the two variables; as the extraction temperature increases, there is an overall decline observed in the gelatin yield. At a temperature of 45℃, the highest gelatin yield is obtained at a percentage of 6.60%. As the temperature increases to 55℃, there is a decline in the yield to 4.03%. Between the temperature of 55℃ and 65℃, the yield stays relatively the same around 4.0%. A study conducted in 2007 looked at the effect of both extraction time and temperature on the yield of gelatin. The study extracted gelatin from different fish offals and varying temperatures and times, and its results indicated that overall, the yield increased with an increase in both temperature and time (Kołodziejska et al., 2008). However, it also indicated that overall, an extraction temperature of 45℃ and extraction time between 15 and 60 minutes were the most optimal conditions for gelatin extraction (Kołodziejska et al., 2008). From the data obtained for squid gelatin, the results are agreeable with the study; between the three extraction temperatures, 45℃ appears to be the most optimal.

The overall decrease in the yield of gelatin could possibly be attributed to the higher temperatures which is favoring protein unfolding and an overall loss of protein at temperatures higher than 45℃ (Dill et al., 1995). The loss of protein was also observed through the BCA Assay. However, it is also possible that at a temperature of 55℃, there is just simply lower gelatin extracted compared to 45℃. Furthermore, when visually observing the gelatin samples, it was noted that gelatin that was extracted at 55℃ and 65℃ was less resembling to that extracted at 45℃. The sample extracted at 45℃ resembled conventional gelatin the most while the other samples appeared not to be pure in nature. Furthermore, it was noted that since the yield calculation is based on a general formula, the contaminants present in the gelatin samples are altering results.

4.1.2.2 Protein Content Determination with BCA Assay

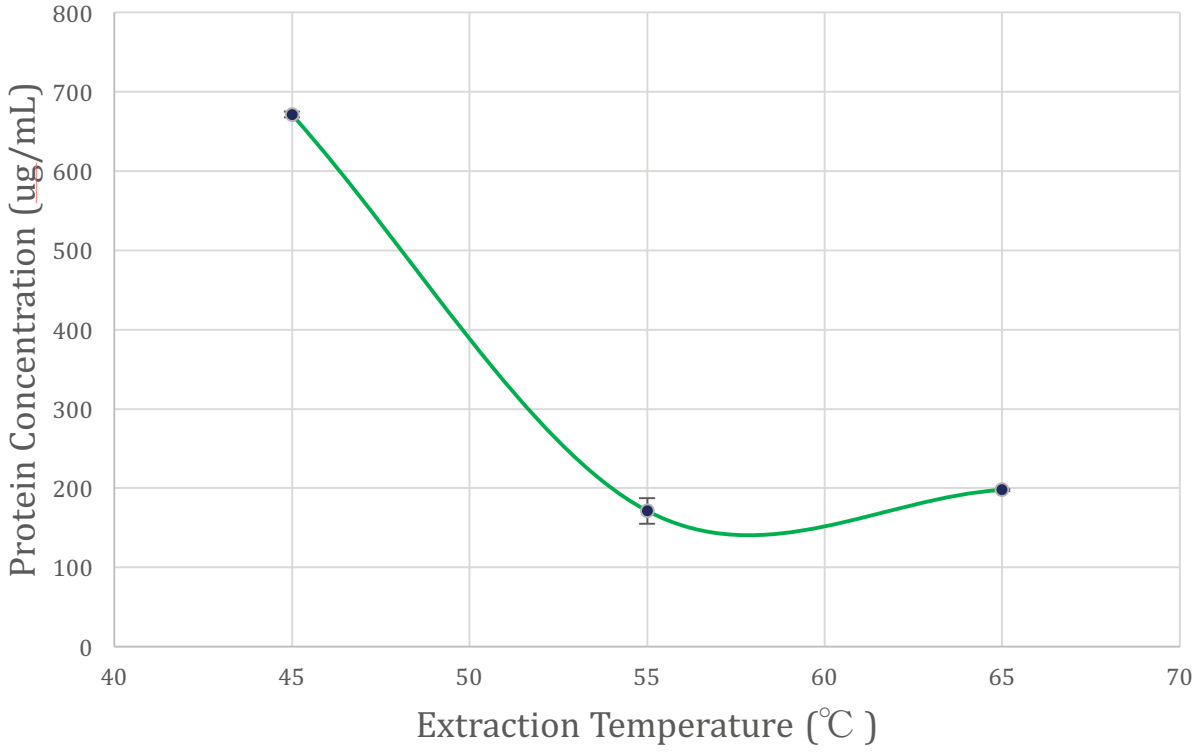

*Figure 4-5. Plot of protein concentration (µg/mL) vs. extraction temperature (℃) *Reaction Conditions: 12-hour extraction using 25 U of pepsin/g of squid.*

When looking at the relationship between the protein concentration and the extraction temperature, Figure 4-5 shows that there is an overall decline much like Figure 4-4. The protein concentration decreases from 671.5µg/mL to 171.4µg/mL. This is a pretty large decrease between a temperature increase of 10 degrees. Between the temperatures of 55℃ and 65℃, the protein concentration stays relatively the same with a slight increase to 197.76µg/mL. The decline in protein concentrations indicates that there was an overall loss of protein as temperature was increased. It is possible that for the extracted gelatin samples, the temperatures of 55℃ and 65℃ were favoring protein unfolding.

However, it is also possible that there was less gelatin extracted at 55℃ and 65℃ which can account for the lower protein concentration. At those high temperatures, it is possible that the solubility of collagen was disrupted and overall did not allow its conversion into a more amorphous state. It has been reported that squid collagen is less soluble compared to other fish species (S.-Y. Cho & Kim, 1996). Low collagen solubility has also been reported for other invertebrates even after heat-denaturing at 90℃ (Mizuta, Yoshinaka, Sato, & Sakaguchi, 1995). The visual observations indicated that the gelatin samples extracted using 55℃ and 65℃, had presence of more salt compared to the gelatin sample of 45℃. In order to confirm presence of salt as well as to see what protein bands were present in the gelatin samples, the molecular weight distribution was determined by once again carrying out an SDS electrophoresis.

4.1.2.3 SDS Bands - Molecular Weight Distribution

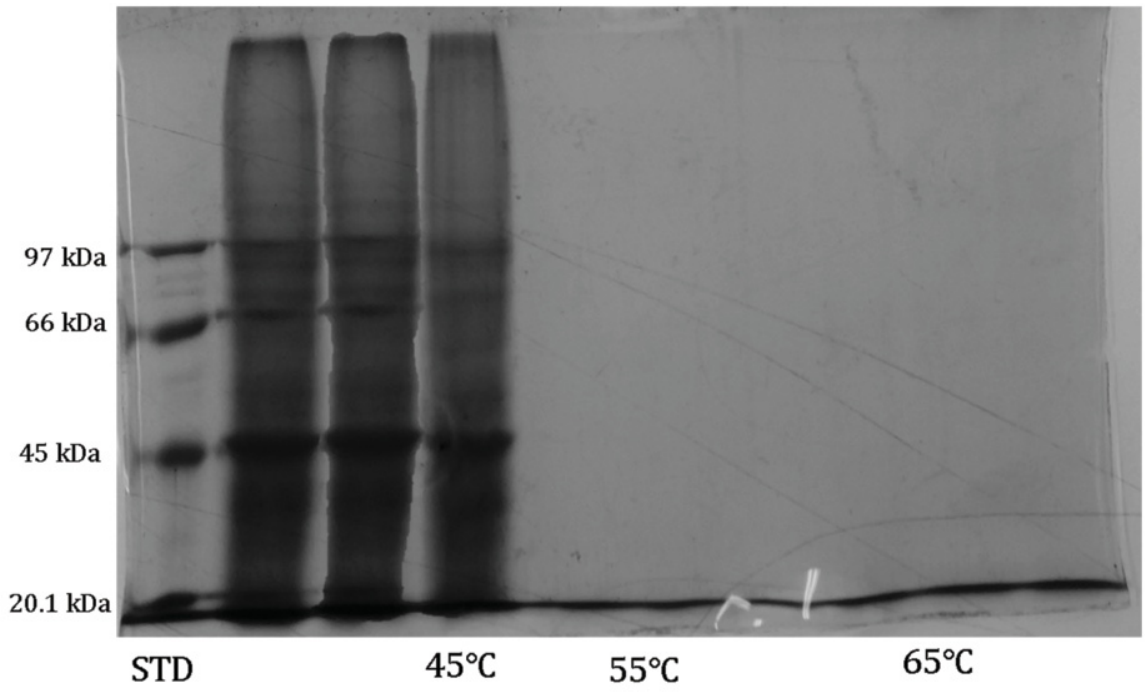

Figure 4-6. Electrophoretic analysis (SDS-PAGE) of gelatin preparations (triplicates of each sample with varying extraction durations were used) in the presence of 2-β Mercaptoethanol.

First most, since the electrophoresis carried out on the previous extractions indicated that there was presence of lower molecular weight protein bands present in gelatin samples, a lower molecular weight standard was used for the following samples for electrophoresis.

As can be seen from Figure 4-6, there are no protein bands that were found to be present in both extracted samples of 55℃ and 65℃. Due to the high temperatures and the low protein concentrations in the samples, it was determined that these two temperatures presented conditions much too harsh for gelatin extraction and therefore no gelatin was extracted. Temperatures were either too high for extraction and had instead caused more protein breakdown during the extraction and resulted in there being contamination within the samples once again. For this reason, when freeze-dried, the samples contain mostly salt as the broken down protein was not capable of

forming gelatin fibrils (Eastoe, 1957). The presence of salts indicates, as mentioned before the inefficient washing of the skins before heat extraction. However, it was noted that at a temperature of 45℃, the gelatin samples once again contain three main protein bands at 97kDa, 66kDa and 45kDa indicating presence of Phosphorylase b, Serum Albumin, and Ovalbumin. Figure 4-6 confirms that the gelatin extracted using a temperature of 45℃ is the best sample to use for development of edible films.

Overall, it was concluded that the following conditions, despite the presence of the enzyme pepsin, were not suitable once again for extracting gelatin with higher molecular weight alpha and beta fragments. The decrease in protein concentration and gelatin yield suggest two possibilities; the extraction temperatures were either too high and in result, favored protein unfolding or the higher temperatures affected the solubility of collagen limiting its conversion into the amorphous state of gelatin (Eastoe, 1957). The rate of collagen conversion into gelatin depends on the processing parameters; therefore, it is hypothesized that the higher temperatures could have had an effect on that. The study carried out by Kolodziekska et al. (2008) that observed the effect of extracting time and temperature on the yield of gelatin from different fish offal indicated that with the increase of extraction temperature the yield of gelatin had also increased (Kołodziejska et al., 2008). Furthermore, except for one fish sample, their study indicated that the solubility of collagen was not affected up till a temperature of 70℃. When comparing this to the results obtained in this investigation, conclusions are contradictory. However, since 45℃ was determined to be the most efficient for gelatin extraction, this temperature was used for all following extractions.

4.1.3 Parameter 3 - Effect of Extraction Duration

4.1.3.1 Effect of Extraction Time on Gelatin Yield

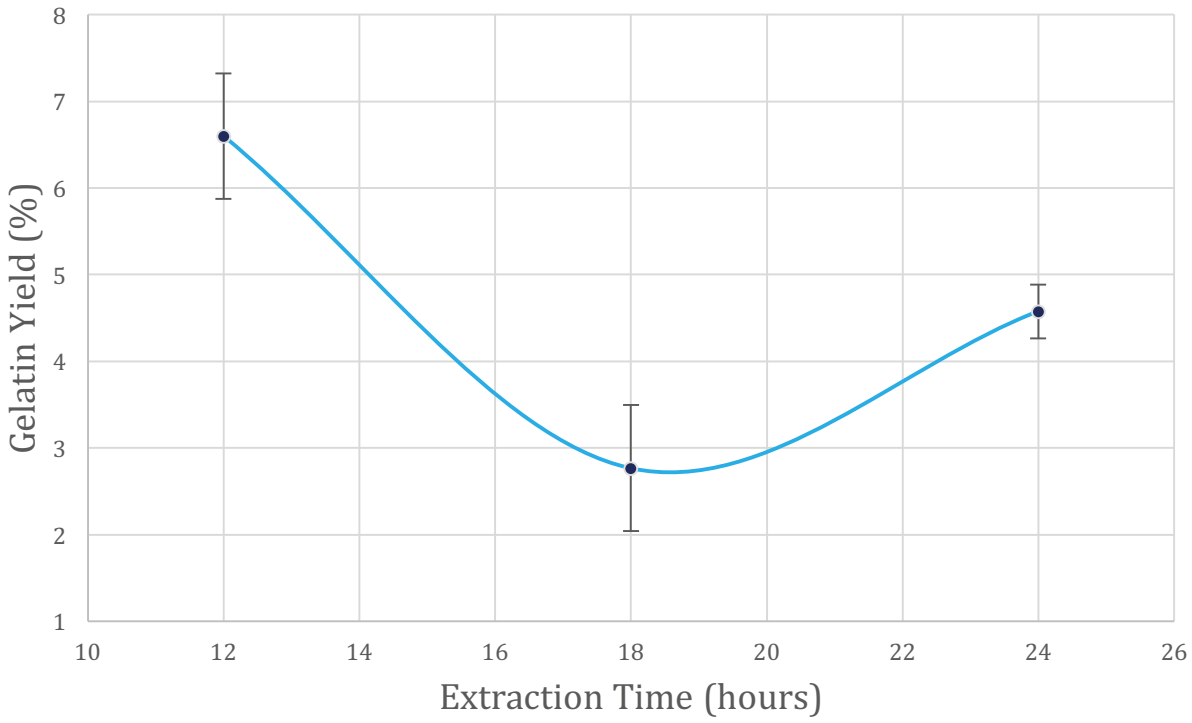

*Figure 4-7. Plot of gelatin yield (%) vs. extraction duration (℃) to observe effects of extraction duration on gelatin yield. *Reaction Conditions: 12-hour extraction using 25 U of pepsin/g of squid .*

The third parameter being studied looked at the effect of the extraction duration on the yield of extracted gelatin. Figure 4-7 indicates that overall, the yield decreases, however, there is a slight increase at the last duration. At an extraction duration of 12 hours the highest yield is obtained at 6.56%; as the extraction duration is increased by 6 hours, the yield decreases to 2.77% and then increases to 4.58% after 24 hours. Overall, the decrease in the yield can be attributed towards the prolonged exposure to heat which can also favour protein unfolding, therefore, resulting in an overall loss. The slight increase in the yield towards the end, however, does not necessarily mean that the gelatin content increased. Once again, Figure 4-7 is not a concrete depiction of the gelatin

yield due to the fact that a general equation is being used in order to get a general idea of the gelatin yield obtained. The equation takes into consideration the weight of all freeze-dried substances which can include other sediments and salts.

A study carried out in 2007 by Kolodziejska et al. showed that overall, with increased extraction duration, from 15 to 120 minutes, the yield of gelatin from different fish offal increased (Kołodziejska et al., 2004). Despite the fact that some gelatin samples indicated higher yield values compared to others, overall there is an increase observed. However, the results obtained from our study, show no such pattern. Furthermore, the extraction durations used for our study are significantly different and much longer as most studies carried out on squid used longer durations (Arnesen & Gildberg, 2007; Giménez et al., 2009; Gómez-Guillén et al., 2002; Lin & Li, 2006; Mendis, Rajapakse, Byun, & Kim, 2005).

When visually observing the samples, it was noted that samples extracted at 18 and 24 hours had a more powder-like appearance compared to the 12 hours. The samples seemed to be contaminated with other sediments, most likely salts that were obtained during the freeze-drying. Once again, the presence of salt can be attributed towards the inefficient washing of the skins before heat extraction. To confirm this, the protein concentration was determined through a BCA analysis.

4.1.3.2 Protein Content Determination with BCA Assay

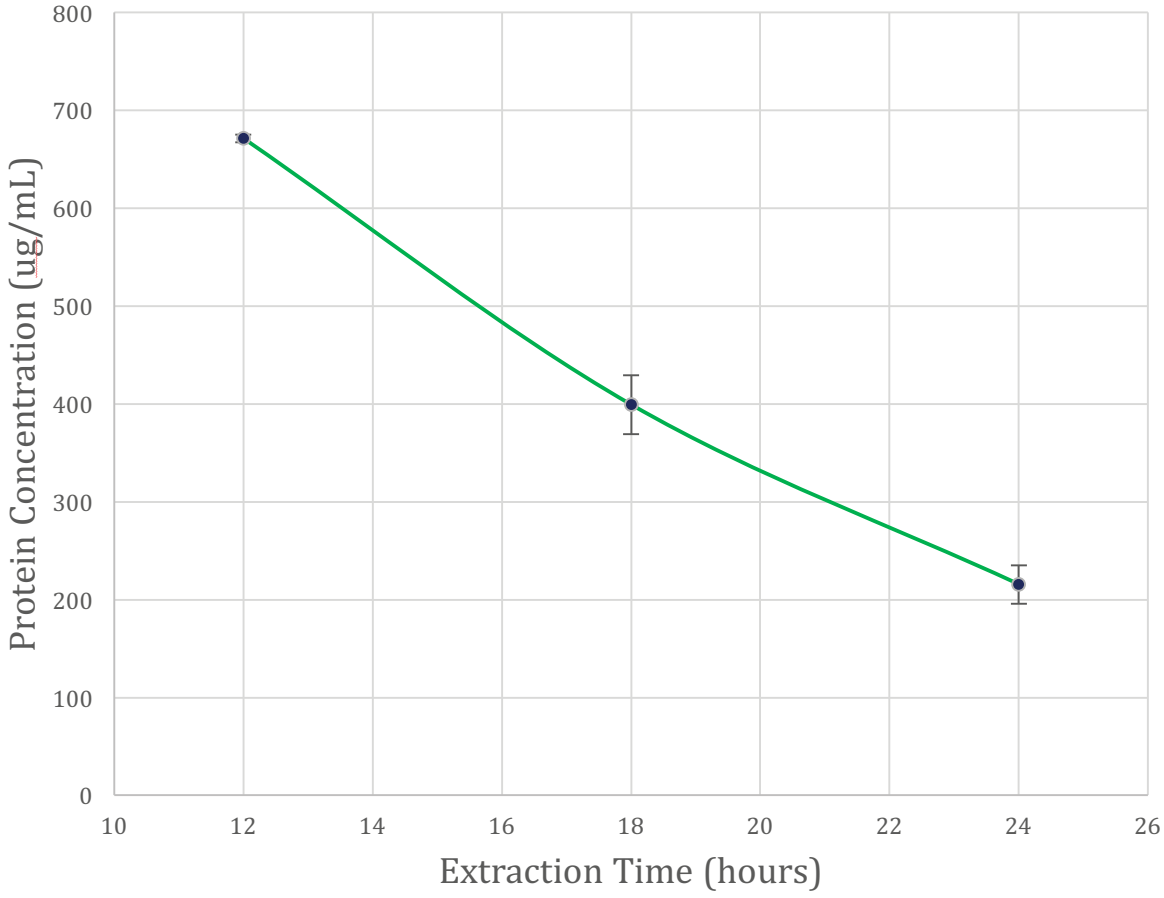

*Figure 4-8. Plot of protein concentration (µg/mL) vs. extraction duration (℃) *Reaction Conditions: 12-hour extraction using 25 U of pepsin/g of squid.*

The BCA analysis indicated that as exposure to heat increased, the protein concentration also decreased. Figure 4-8 shows that the highest protein concentration was obtained at an extraction duration of 12 hours with a value of 671.5µg/mL. As the extraction duration increased to 18 and 24 hours, the protein concentration dropped to 399.3µg/mL and then finally a concentration of 214.6µg/mL respectively. The decrease in protein concentration can be attributed to the fact that the prolonged exposure to heat resulted in higher quantities of protein unfolding resulting in an overall loss of gelatin.

However, the visual observations give rise to speculation that there is little to no gelatin extracted at both 18 and 24 hours and therefore, mostly salts were present in the samples. In order to confirm this as well to see what proteins were present, the molecular weight distribution was looked at by carrying out an SDS electrophoresis.

4.1.3.3 SDS Bands - Molecular Weight Distribution

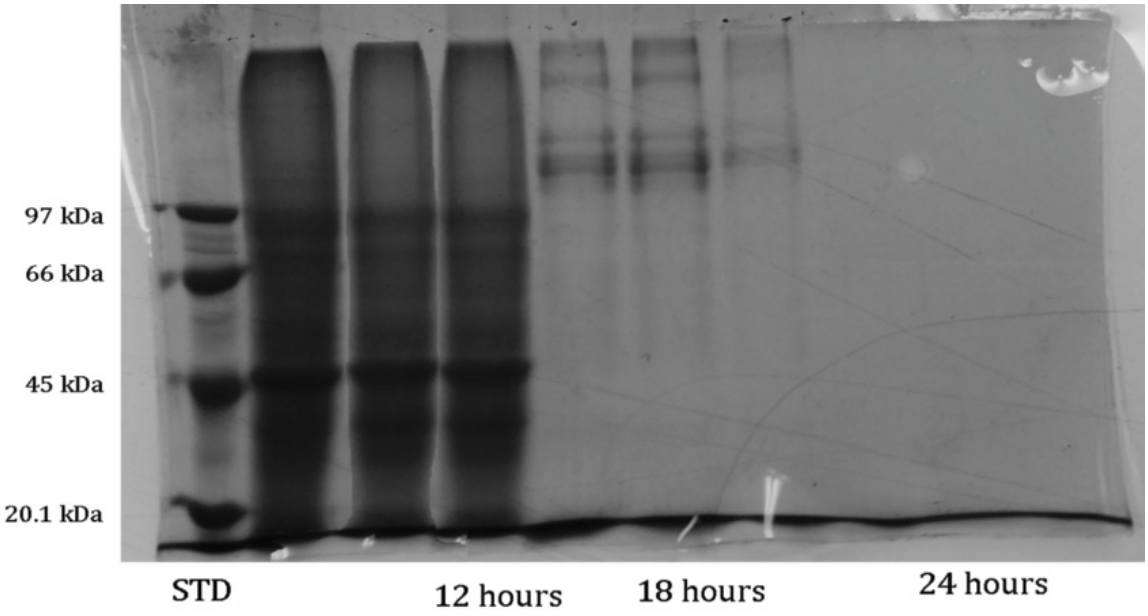

Figure 4-9. Electrophoretic analysis (SDS-PAGE) of gelatin preparations (triplicates of each sample with varying extraction durations were used) in the presence of 2-β Mercaptoethanol.

Figure 4-9 demonstrates that with the highest extraction duration of 24 hours, there were no protein bands found confirming that the sample did not contain gelatin and was mostly found to be freeze dried salts. At a temperature of 18 hours, there is a very faint protein band of ß-galactosidase seen at 116kDa. It was observed that this is the only sample with the highest molecular weight protein band extracted compared to all of the samples. However, since only one band was extracted it is most likely that this sample will not be efficient in forming films.

When looking at the sample extracted under a duration of 12 hours, there are more prominent protein bands present. The gelatin sample contains the proteins Phosphorylase B and Ovalbumin at 97kDa and 45kDa. There is also faint presence of the protein Serum Albumin at 66kDa. Aside from the gelatin sample extracted at 18 hours, for the most part, lower molecular weight bands were extracted. The SDS electrophoresis confirms that the gelatin sample extracted at a temperature of 12 hours is best suited for the development of edible films.

Overall, it was determined that under these conditions, prolonged heat extraction could result in the loss of gelatin, as at higher temperatures protein folding is favored. As Figure 4-8 depicts, the protein concentration decreased immensely as well as the gelatin yield. This could also be attributed towards the high presence of salts that were not efficiently washed off prior to the heat extraction. Despite the presence of the enzyme pepsin, it is noticed that low gelatin yields are obtained with the majority of the samples containing contaminants (mostly salts formed during neutralization). Overall, the extraction duration of 12 hours provided the best results between the different durations.

4.2 Determination of Optimal Conditions for Gelatin Extraction

By compiling the results of this study for objective 1, the best values for the different parameters being studied were determined. The gelatin samples obtained when studying the first parameter determined that the best pepsin concentration to use was 25U/g of dehydrated squid. For the second parameter, the gelatin samples determined that the best extraction temperature was 45℃. The gelatin samples obtained when studying the third parameter, determined that the best extraction duration was 12 hours. In comparison to the other gelatin samples obtained, the gelatin sample obtained under the optimal conditions expressed the highest gelatin yield, highest protein concentration and the most protein bands present. However, the gelatin obtained under these circumstances is still characterized as a low-quality gelatin. The sample shows no detection of higher molecular weight fragments of beta and alpha chains. It is clear that under these conditions gelatin is not efficiently extracted. From previous gelatin extractions, it is well accepted that more severe treatment conditions for gelatin extraction are detrimental to its physical properties. However, extreme heating conditions are commonly used to increase the yield in commercial

gelatins. Along with these extreme conditions, the incorporation of the enzyme pepsin has also been proven to increase the yield of gelatin extracted. However, the results of this study do not show any significant evidence of any increase in gelatin yield. According to other studies, this can possibly be because of the pepsin having a selective effect on the telopeptide region of collagen and only cleaving the alpha chains (Giménez et al., 2009). This would explain the extraction of lower molecular proteins. Another explanation for the lack of gelatin extracted could also be attributed towards the higher temperatures (higher than 45℃) at which the solubility of collagen was affected and hindered the conversion of collagen into gelatin. Similarly, the prolonged heat duration (higher than 12 hours) seemed to favor protein unfolding and resulted in the overall loss of protein during the extraction process.

All of the gelatin samples that were obtained for the different extraction conditions were than used to prepare 4% gelatin film forming solutions in order to test for film forming properties. The solutions were then cast into petri dishes and dried for 48 hours. After 48 hours, the solutions were observed to see which ones formed a film. Of the different samples, there was only one gelatin sample that formed a film; it was extracted under the optimal conditions of extraction duration of 12 hours, extraction temperature 45℃ and using a pepsin concentration of 25U/g. The film forming property of this sample confirmed that this sample was gelatin. All other samples were either not gelatin or contaminated with too much salt to form any films. For this reason, the gelatin extracted under the optimal conditions was then used for the completion of objective 2.

4.2.1 Hydroxyproline Content

Gelatin is a pure protein which contains 14% hydroxyproline, 16% proline and 26% glycine (A. J. Bailey & Light, 1989). Since the only other animal product containing hydroxyproline is elastic and at a lower concentration, hydroxyproline is used to determine the collagen or gelatin content (A. J. Bailey & Light, 1989). A unique feature of squid collagen is its high degree of cross-linking which is a result of its high amount of hydroxylysine and hydroxyproline content (Gómez-Guillén et al., 2002). Therefore, looking at the hydroxyproline concentration was critical. The stability of the triple-helical structure in re-natured gelatins is dependent on the imino acids hydroxyproline and proline that for the nucleation zones (Hill et al., 1998). However, hydroxyproline is believed to play a more singular role in the stabilization of the triple-stranded collagen helix (Burjanadze, 1979; Hill et al., 1998).

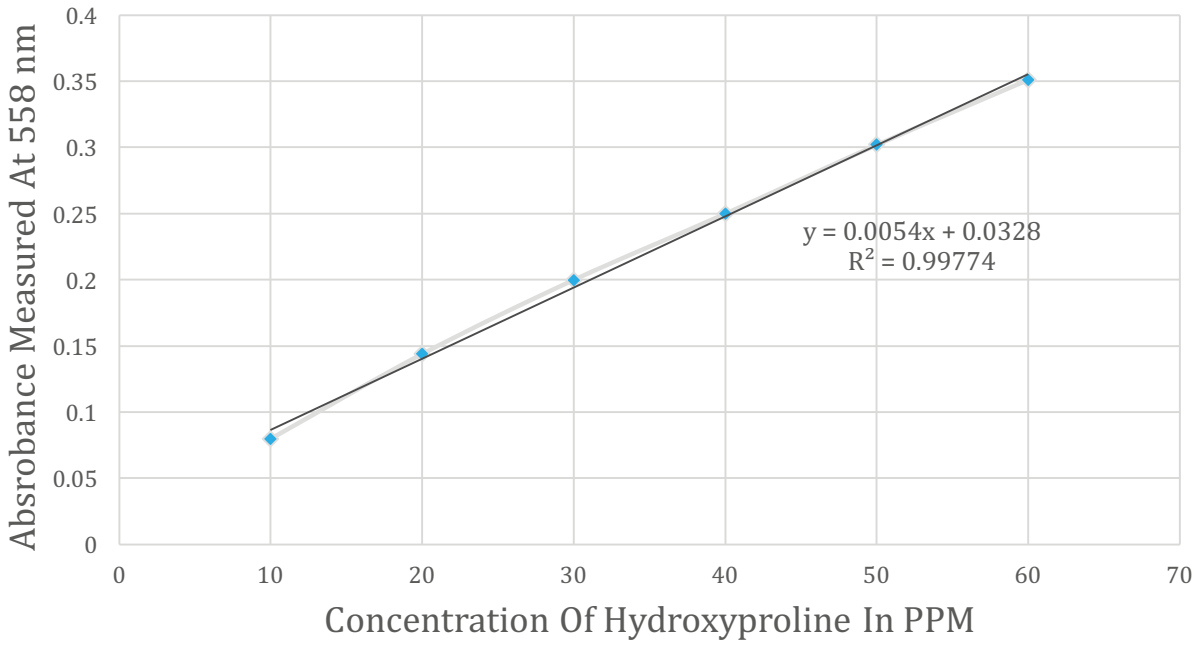

Figure 4-10. Standard curve prepared for the determination of hydroxyproline content.

Table 4-1. Results of average hydroxyproline content(%)in comparison to theoretical hydroxyproline content(Giménez et al., 2009).

Hydroxyproline Percentage of Extracted Gelatin (from *Illex argentinus)*	**Theoretical Hydroxyproline Percentage of Gelatin (*Dosidicus gigsas*)** (Giménez et al., 2009)
16.94% ± 0.1340	7.4%

The gelatin sample's hydroxyproline content was calculated to be 16.94%. This value is significantly higher than the value reported by Giminez et al. (2009) which is 7.4% hydroxyproline (Giménez et al., 2009). The high degree of hydroxyproline can account for the difficulty encountered during the extraction of gelatin. The lack of gelatin extracted could be due to the high stability of the triple-stranded collagen helix which was not efficiently cleaved by the enzyme pepsin. The functional properties of gelatin are greatly influenced by the amino acid composition and the molecular weight distribution (Gómez-Guillén et al., 2002; Kołodziejska et al., 2008; Muyonga et al., 2004a; Simon et al., 2003). The high hydroxyproline content could account for the low molecular weight distribution obtained which in return yielded low-quality gelatin.

4.3 Squid Gelatin-Based Films

For objective 2, edible films were formed from the gelatin sample extracted under the optimal conditions. There were two formulations used to form the films; 4% gelatin solutions and 8% gelatin solutions. It was observed that the gelatin samples dissolved readily in water before the solution was even heated. After the films were formed, they were characterized by testing first its mechanical properties, specifically the tensile strength and elongation break. The edible films' solubility and water uptake were also examined along with its opacity.

For the development of the films, the plasticizer glycerol was incorporated within the film forming solutions. Glycerol is a relatively small molecule that has hydrophilic characteristics (Gontard, Guilbert, & CUQ, 1993). This makes it easy for it to incorporate itself within protein chains by forming hydrogen bonds with amide groups and amino acid side chains of proteins (Gontard et al., 1993).

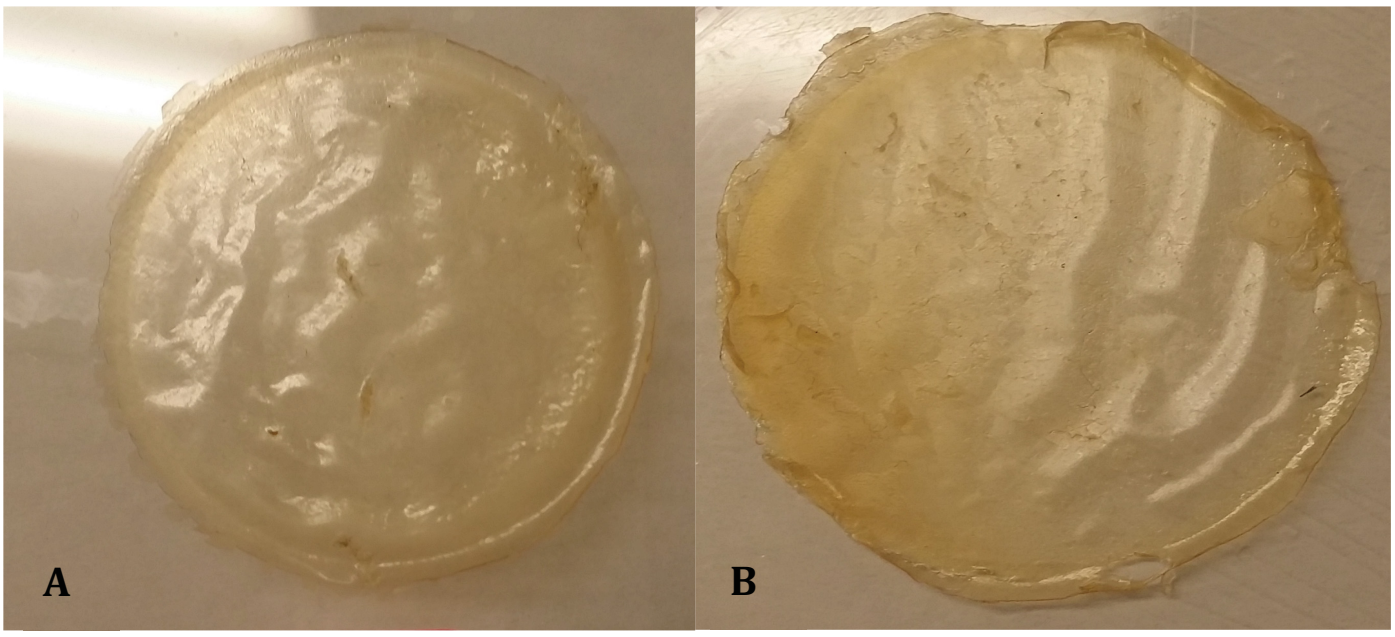

Figure 4-11. Image of dried and conditioned 8% gelatin film (A) and 4% gelatin film (B) respectfully.

4.3.1 Mechanical Properties

4.3.1.1 Tensile Strength

Table 4-2. Results for average tensile strength (N/mm^2) for 4% and 8% gelatin films.

Percent Gelatin Films	Tensile Strength (N/mm^2)
4%	0.229 ± 0.0037
8%	0.939 ± 0.0104

Tensile strength is defined as the maximum amount of tensile stress that a material can withstand before it breaks or becomes permanently deformed (Young & Budynas, 2002). This value specifies the point at which the material has transitioned from elastic to plastic deformation. The tensile strength of the 4% film and 8% film was calculated to be 0.229N/mm^2 ± 0.0037 and 0.939 N/mm^2 ± 0.0104 respectively. It is observed that as the gelatin concentration increases, so does the tensile strength. This is due to the higher percentage of protein present in the film which forms stronger films (Giménez et al., 2009).

In comparison to edible films formed from *Loligo formosana* (splendid squid), the tensile strength is significantly lower than their range between 12 and 33 MPa(N/mm^2) (Nagarajan, Benjakul, Prodpran, Songtipya, & Nuthong, 2013). The low tensile strength of the edible films prepared using *Illex argentinus* squid for our study are expected as there were no polymers of alpha chains that were extracted under the extraction conditions of this study. It is known that presence of the plasticizer glycerol makes films less resistant giving rise to its low tensile strength compared to other edible films formed from marine gelatin (Arnesen & Gildberg, 2007; Giménez et al., 2009; Gontard et al., 1993). With the incorporation of glycerol in the gelatin film network, there was a reduction in the direct interactions and the proximity between protein chains, resulting in lower tensile strength. It was reported by Lim, Mine & Tung (1999) that with increasing glycerol content, they also observed a decrease in tensile strength of gelatin films (Lim, Mine, & Tung, 1999).

4.3.1.2 Elongation

Table 4-3. Results for average elongation break (%) of 4% and 8% gelatin films.

Percent Gelatin Films	Elongation Break (%)
4%	27.2 ± 0.1979
8%	22.3 ± 0.0824

The elongation break is defined as the ratio between the changed length and the initial length after breakage of the material. Overall, it is the ability of a material to resist any changes in its shape before it breaks and deforms (Young & Budynas, 2002). The elongation break of the 4% and 8% gelatin films were relatively the same at 27.2% ± 0.1979 and 22.3% ± 0.0824 respectively. The slightly higher value observed in the 4% film can be attributed towards the addition of the plasticizer glycerol which can give rise to a higher breaking deformation.

Compared to the edible films formed by B. Giminez et al. (2009), who reported elongation breaks of 46.0 and 34.7%, the results of this study are lower (Giménez et al., 2009). When comparing the elongation break with the edible films formed from *Loligo formosana* (splendid squid), they had elongation breaks ranging from 5% to 21%. The values obtained in this study are closer to those of *Loligo formosana* squid than *Doscidicus gigas* squid. With the addition of the plasticizer glycerol, the elongation at break increases (Jongjareonrak, Benjakul, Visessanguan, Prodpran, & Tanaka, 2006). This is due to the glycerol reducing intermolecular interactions and improving the mobility of macromolecules which overall increases the elongation break (Gontard et al., 1993). Hence why higher elongation breaks were observed.

4.3.1.3 Film Thickness

Table 4-4. Results for Average Film Thickness and Average Film Weight.

Percent Gelatin Films	Average Film Thickness	Average Film Weight
4%	0.19 mm ± 0.0104	0.1356 g ± 0.0009
8%	0.30 mm ± 0.0062	0.4107 g ± 0.0002

The film thickness was measured for the two different formulations. As expected, the 8% gelatin film was thicker than the 4% gelatin film. Higher percentage of protein forms stronger and thicker films. It was noted that the more adhesive films were less sticky and easier to peel off.

4.3.2 Water Solubility

Table 4-5. Results for Film Solubility

Percent Gelatin Films	Initial Weight of the Film	Final Weight of the undissolved desiccated film residue	Solubility (%)
4%	0.0337g ± 0.00022	0 g	100%
8%	0.1026g ± 0.00005	0 g	100%

The gelatin films solubility in water was calculated to be 100% for both the 4% and 8% gelatin films. During the procedure, before the drying of the filtered paper, there appeared to be no residues that were collected during the filtration process. This was confirmed after the filter paper was dried and there was no residue present. In comparison to the study carried out by B. Giminez et al. (2009), the solubility of the edible films from our study is relatively similar to theirs which was calculated to be over 90% (Giménez et al., 2009). The water solubility of films can possibly

be related to the hydroxylysine content that is responsible for producing a high degree of cross-linking through covalent bonds that are insoluble (Giménez et al., 2009). Mammalian gelatin films contain high hydroxylysine residues which accounts for their low water solubility compared to fish gelatin. B. Giminez reported that both their gelatin preparations contained high amounts of hydroxylysine residues, however, there was still a high water solubility obtained. They concluded that since there was a lack of alpha chain polymers the amino acids were not covalently bonded (Giménez et al., 2009). Although the hydroxylysine content was not determined for this study, this could be a possible explanation. There is a lack of alpha chains in the gelatin extracted due to the pepsin enzyme hydrolyzing the collagenous material by breaking down the covalent links in the telopeptide regions. The telopeptide region is where the hydroxylysine residues establish their covalent bonds (Giménez et al., 2009).

4.3.3 Water Uptake

The water uptake was also tested by immersing pieces of the 4% and 8% edible films in water. After 5 minutes it was noted that both films had broken down and could not be removed after the time frame. The films make gel-like solutions in water at room temperature. There was an attempt to remove the fragments of the film from within the water, but the film came out in water blobs and had resembled the film forming solution it was before drying. This was to be expected as the films water solubility was 100%.

4.3.4 Opacity of Film - Effectiveness of UV and VIS light Blockage

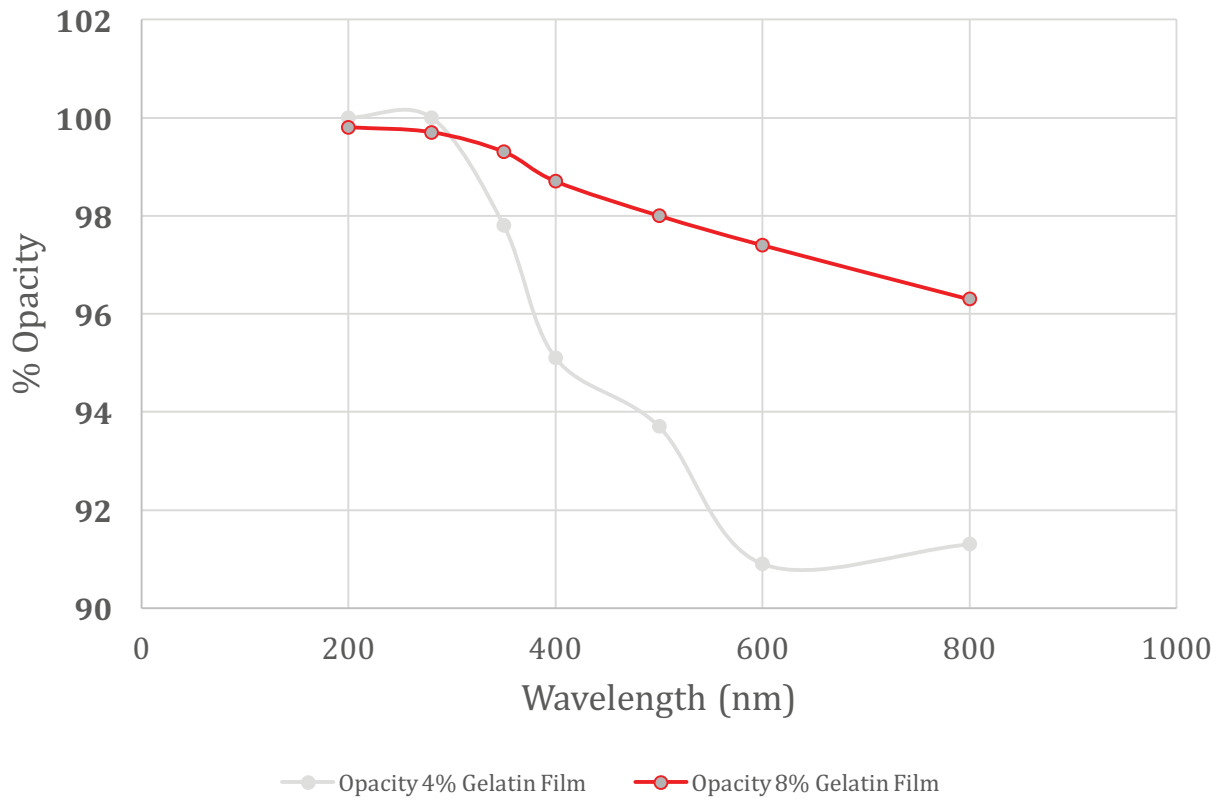

Figure 4-12. Graphical representation of opacity of 4% and 8% gelatin films.

The opacity of the films was measured at different UV and visible wavelengths in order to see if it would be possible for them to be effective in blocking lipid oxidation. As represented in the graph in Figure 4-12, both the 4% and 8% films effectively blocked at least 90% of the light. It is noted that in the visible spectrum, light is more effectively blocked at almost 100%. Within the UV range, it is observed that the opacity declines however it still remains relatively high (above 90%). Despite the fact that visually, the squid-skin gelatin films seemed to be relatively transparent, their opacity was quiet high. Compared to edible films made from *Dosidicus gigas* squid gelatin, the results are comparable as they also reported high opacity values (Giménez et al., 2009). They suggested that the high opacity can be attributed towards the presence of low molecular weight components that formed denser protein networks (Giménez et al., 2009). The high opacity, suggests the potential preventive effect of gelatin films on the retardation of lipid

oxidation that can be induced by UV light (Giménez et al., 2009) . In general, the amino acids tyrosine and phenylalanine are known to be more sensitive towards light and are capable of absorbing light at wavelengths below 300nm hence suggesting that the content of aromatic amino acids within the protein might play a major role in determining the UV barrier properties of edible films (Arnesen & Gildberg, 2007; Giménez et al., 2009; Li, Liu, Gao, & Chen, 2004).

CONCLUSION

For the production of edible and biodegradable gelatin films, most of the research and the food industry deals with utilizing mammalian extracted gelatin. However, many studies are being carried out in order to increase the application of fish gelatin based films. Fish gelatin has been observed to express good film forming properties, good transparency, near colorless and water solubility (Gómez-Guillén et al. 2009).

However, in the industry, a trend is being set in order to focus on the formulation of gelatin films with higher water resistance. This is due to the highly hygroscopic nature of gelatin that causes the swelling and dissolving when in contact with foods that have high moisture contents (Gómez-Guillén et al. 2009). Due to this main drawback, it is being suggested that fish gelatin based biofilms be an alternative for extending the shelf life of fresh meat, fish patties or post-harvest avocados (Gómez-Guillén et al. 2009). It was determined that from the results of this study, there is a potential for squid gelatin as edible films in the food industry. From the data presented in the results and discussion sections, the following conclusions were made.

With the use of an enzyme-aided process, it was possible to extract gelatin from shortfin squid (*Illex argentinus*) using an enzyme concentration of 15U/g, extraction temperature of 45°C and an extraction duration of 12 hours. These conditions were determined to be the optimal ones under which extraction took place. The gelatin that was extracted was heavily contaminated with salts that were formed during neutralization and were not washed off efficiently before extraction. For this reason, there was high presence of salt in the freeze-dried samples. The gelatin that was obtained was characterized as low quality due to their being a lack of high molecular weight fragments of beta and alpha chains. Lower molecular weight fragments were more efficiently extracted under these conditions. However, the gelatin sample was determined to have a high hydroxyproline content. Overall, the conditions of extraction that were used in this study need to be optimized in order to see higher yields obtained as well as high-quality gelatin that will be capable of forming films that have better properties.

Despite the low molecular weight gelatin extracted, it was observed that gelatin from shortfin squid (*Illex argentinus)* was still capable of forming edible films with adequate barrier properties. Although the films were 100% soluble in water, they were effective in blocking out both UV and VIS light. This suggests that these films could have a potential application as coatings on fresh produce. For example, their high water solubility will make it very easy for the films to be washed off of apples before being used. However, further tests need to be carried out on the microbial aspects of the films and its interactions within and on top of other food systems.

It is important for there to be further studies carried out to produce a biodegradable film from gelatin that is extracted from fish species. The key is carrying out a successful extraction of gelatin under the correct conditions, which will result in the highest yield of high-quality gelatin. It will also be important to determine which additives need to be added in order to have the desired barrier properties for a film that will produce active biomaterial packaging of marine origin. Furthermore, it is important to develop these films with high barrier properties in order to ensure the successful implementation of fish gelatin films in the food industry.

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