There are presently two successful methods used to refine fish muscle proteins: surimi and fish protein isolate (FPI). Both surimi and FPI have the ability to form an elastic gel upon comminution and heating. However, their gelation behaviors are different as they are refined in a biochemically opposite way based on the nature of protein denaturation. The focus of this study was to compare surimi and FPI under various processing conditions, such as rigor mortis, frozen storage, comminution conditions, and blending effect, and how these conditions can affect their functional properties including gel texture.

The structural changes and rheological properties of tilapia protein prepared using FPI and surimi with pre- and post-rigor muscle were evaluated. No rigor effect was observed on the gel-forming ability of FPI, although higher storage modulus (G’) and better gel texture were obtained in surimi made from pre-rigor tilapia compared to surimi made from post-rigor tilapia. Results suggested pre-rigor processing may
improve gel-formation properties of surimi, but not as much for the gelation of FPI.

Storing fish in a freezer for extended periods of time can adversely affect the gel-forming ability of muscle proteins. The effect of frozen storage (0, 1, and 3 mo) on the biochemical and physical characterization of FPI and surimi made from tilapia was elucidated. The Ca\(^{2+}\)ATPase activity of tilapia fillet continuously reduced throughout the frozen storage; however, the decline trend of its activity was slower than cold or temperate water species. As reported by storage modulus (G’), storing whole fish frozen for 3 mo did not affect the gelling ability of FPI and surimi. The results from surface hydrophobicity, surface reactive sulphydryl (SRSF) content, and differential scanning calorimetry also corresponded to the results from storage modulus. Thus, frozen tilapia, if stored up to 3 mo, may be used like fresh fish in the processing of FPI and surimi and no negative effects on gel qualities. The uniqueness of tropical fish tilapia was thought due to its high thermal stability.

The quality of surimi gels was affected more so under various rigor stages and frozen storage compared to FPI gels. Conversely, the addition of salt into FPI induced a higher degree of unfolding protein structure prior to gelation compared to surimi. In addition, comminution conditions affected the quality of FPI gel more than that of surimi gel. A significant increase in puncture gel texture was observed when FPI and surimi were chopped at 25°C for 18 min compared to samples chopped at 5°C for 6 min. The comparable results were detected as measured by storage modulus. FPI chopped with 3% salt at 5°C for 6 min showed the lowest gel texture among all treatments, possibly because protein structure was not disintegrated appropriately and formed larger protein aggregates and coarser gels demonstrated by microscopic
analyses. Results suggested controlling chopping temperature and time, and the addition of salt, may be significant factors to enhance production of high quality gel in FPI and surimi.

Moreover, the effect of various comminution conditions on structural changes were investigated using Fourier transform infrared (FT-IR) and Raman spectroscopy. Both procedures exhibited increasing chopping temperature and time, adding salt, promoted a higher degree of unfolding protein structure in FPI and surimi paste made from tilapia, when they were chopped at 25°C for 18 min compared to samples chopped at 5°C for 6 min. Also, FPI and surimi gels prepared after chopping at 25°C for 18 min revealed higher β-sheet contents and more chemical bonds such as hydrophobic interactions and disulfide bonds than those at 5°C for 6 min. Controlling comminution conditions may be one of the important factors to produce high quality gels from FPI and surimi using tropical fish like tilapia. Additionally, FT-IR and Raman spectroscopy are useful complementary tools, allowing a better interpretation of the structural changes in FPI and surimi under various comminution conditions.

The gelation properties of blending two different fish proteins obtained from surimi and FPI at different ratios was evaluated. Effects of blending surimi and FPI on gel functionality (whiteness, hardness, and cohesiveness) demonstrated a linear pattern when the proportion of surimi is larger than or equal to that of FPI. Also, breaking force and penetration distance decreased significantly when the ratio of surimi to FPI decreased. Results indicated gels cooked in a water bath tended to exhibit a higher breaking force than gels cooked ohmically. On the other hand, a higher penetration distance was observed for gels cooked ohmically compared to gels
cooked in a water bath. Blending surimi and FPI did not affect the inter-molecular interactions of protein in a linear pattern, like mixing various grades of surimi, but this might be feasible only when the proportion of FPI does not exceed 50%.
Biochemical and Physical Characterization of Fish Protein Isolate and Surimi for their Compatibility

by

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Major Professor, representing Food Science and Technology

Head of the Department of Food Science and Technology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Yuka Kobayashi, Author
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Fish muscle proteins have unique functional and nutritional characteristics. Depending upon the type of fish, handling and processing conditions, their functional properties and qualities can be varied. Currently, the demand for fish muscle proteins is escalating faster than it can be fulfilled with traditional resources, which is putting greater pressure on fish stocks. Improved use of this natural resource is crucial, especially with low-value materials, which are presently not being maximally utilized for human food production (Park & Lanier, 2000; Kristinsson et al., 2014).

Currently, two effective methods of refining fish muscle proteins are being used: surimi and fish protein isolate (FPI). Surimi processing has over 900 years of history and is one of the most successful commercial procedures used to refine fish proteins. Surimi processing consists of various steps including heading, gutting, mincing, washing, dewatering, refining, and freezing with cryoprotectants. During the production of surimi, both chemical and physical denaturation of proteins must be prevented (Park et al., 2014a,b). Conversely, FPI has less than 2 decades of research and commercialization. FPI processing involves chemically inducing fish proteins to unfold and then partially refold. Chemical denaturation is induced to maximize soluble proteins (myofibrillar and sarcoplasmic proteins) using pH-shift and insoluble components (stroma proteins and neutral fat) are removed. Most of the soluble proteins are subsequently collected at the isoelectric point using centrifugation.
FPI can be effectively made from underutilized raw materials, such as pelagic species and by-products from fish processing, which are challenging to utilize as raw materials for surimi production.

Both refined proteins (surimi and FPI) have the ability to form an elastic gel upon heating. The gelation of surimi or FPI is physicochemically a complex process involving structural and functional changes of myofibrillar proteins (Lanier et al., 2014; Park, 2012). Previous studies demonstrated comparative studies of the gel-forming behavior of surimi and FPI and characterized the biochemical and physical properties of the two protein forms prepared from the same species. Protein gels prepared using FPI from several species have shown similar and/or better gelation properties compared to those produced from surimi (Hultin & Kelleher, 2000; Choi & Park, 2002; Undeland et al., 2002; Thawornchinsombut & Park, 2004, 2007; Ingadottir & Kristinsson, 2010; Azadian et al., 2012). Kristinsson and Hultin, (2003a, b) and Thawornchinsombut et al. (2006) reported alkali- or acid-treated proteins indicated substantial changes in the globular part of the myosin molecules, leading to less stability in FPI compared to surimi during heating and/or frozen storage.

In addition, the effect of salt on the gelation properties of FPI and surimi has been evaluated, but remains debatable. In surimi, salt helps enhance gelation; in FPI, the effect on gelation when adding salt varies. Perez-Mateo et al. (2004) showed FPI gels made from Atlantic croaker chopped with salt acquired higher gel texture than surimi with salt. Conversely, results obtained by Kim and Park (2008) suggested the negative role of salt on the gel-forming ability of FPI made from Alaska pollock surimi, and FPI chopped with salt indicated lower gel texture than FPI chopped with
salt. This may be because the addition of salt induces protein aggregation, which then contributed to low quality gel products similar to the salting-out effect when excess salt is added to denature protein. These varied results in FPI, with respect to salt addition, may be related to species and/or different methods used for gel preparation.

Early studies comparing gel qualities of FPI and surimi are valuable to learn the protein characteristics, functional properties, and stability of the products. The rheological and biochemical characteristics of the gel are affected by refining/processing methods, as well as by intrinsic (species, age, rigor mortis, and degree of stress before harvest) and extrinsic (protein concentration, pH, ionic strength, and temperature) factors (Park et al., 2014b). However, limited technical information is available for understanding the compatibility of these two proteins. In addition, a comparative study, regarding the above mentioned parameters, between FPI and surimi prepared using the same species has not previously been published. Understanding the factors affecting gelation properties is essential to improve the qualities of gelled protein products.

Freshness of fish is the most vital factor determining the quality and stability of fish protein gel. The functional properties of the muscle proteins can be affected by the biochemical changes that occur during rigor mortis (Park et al., 2014b). Park et al. (1990) revealed surimi prepared from pre-rigor tilapia improved gel-forming abilities and resulted in a stronger gel compared to surimi prepared from post-rigor tilapia. Frozen storage is another important factor to consider as fish products are one of the most highly traded food commodities. Early research reported storing fish in a freezer for an extended period of time can adversely affect the gel-forming ability of muscle
proteins (Okazaki, & Kimura, 2013; Benjakul, Visessanguan, Thongkaew, & Tanaka, 2005). Therefore, more research is needed to explore the ideal processing stage (rigor mortis and frozen storage) and further develop the relationship between FPI and surimi. This information may be useful to develop new technologies and utilize fishery resources more effectively.

Moreover, the gelation properties of the muscle proteins can be affected by comminution conditions. The comminution process is used to maximize protein extraction and to combine other ingredients homogeneously into the paste. Two of the most important variables affecting protein quality in gel preparation are temperature and time (Poowakanjana and Park, 2014; Park et al., 2014b). These factors can be controlled during comminution. Poowakanjana and Park (2014) demonstrated the gel-forming behavior of surimi is altered by chopping temperature, chopping duration, and salting time and protein functionality depends on the species. Given the crucial factors of chopping settings, a better perception of the functionality of protein in gelation between FPI and surimi should be explored. Additionally, in order to find the optimum use of fish proteins to enhance gel qualities and develop cost-effective production procedures, the gelation behavior of blends of surimi and FPI must be investigated. This approach can be used as a guide to develop a procedure to obtain the best quality products for each type of seafood.

In this dissertation research, various processing factors in refining fish proteins (FPI and surimi) were thoroughly studied and compared in a systematic manner to seek their compatibility. The dissertation is outlined: 1) to evaluate gelation properties of FPI and surimi prepared from tilapia at pre- and post-rigor and further assess how
the two protein refining methods contribute differently to the structure and texture of the gel; 2) to elucidate the relationship between fresh and frozen whole fish and their effect on the biochemical and rheological properties of gels prepared using FPI and surimi from tilapia; 3) to investigate the gelling ability of FPI and surimi made from tropical fish tilapia as affected by chopping temperature and time; 4) to study the effect of comminution conditions with regard to the structure of the fish protein paste and the corresponding gels prepared from FPI and surimi, respectively, using FT-IR and Raman spectroscopy; and 5) to examine the gelation behavior of blends of surimi and FPI at different ratios based on biochemical and rheological properties.
LITERATURE REVIEW

2.1 Surimi and fish protein isolate

Fish muscle proteins have unique functional and nutritional qualities. Their functional properties and qualities are greatly dependent upon the type of fish, handling, and processing conditions, since fish muscle proteins are sensitive to denaturation. Therefore, research to increase the use of fish muscle proteins as a food source and/or a food ingredient has been growing (Park & Lanier, 2000; Hultin, 2002). Presently, surimi and fish protein isolate (FPI) are two successful methods used to refine fish proteins (Figure 2.1).

![Diagram of surimi and fish protein isolate production]

**Figure 2.1** Scheme of process to produce surimi (a) and fish protein isolate using pH shifts (b) (Park, 2009).
Surimi is a Japanese word meaning washed fish mince that has over 900 years of history. This Japanese term has become a universal term for stabilized fish myofibrillar proteins. Surimi is an intermediate product prior to being processed into various secondary products such as kamaboko, chikuwa, hanpen, crab-flavored sticks, and so on. Surimi processing is one of the most effective commercial methods of refining fish proteins. The processing goal is to remove undesirable components (sarcoplasmic proteins, fat, connective tissues, skin, bone, etc.) and concentrate functional myofibrillar proteins. The manufacturing consists of various steps including: heading, gutting, mincing, washing, dewatering, refining, and freezing with cryoprotectants. Over the past 50 years, there were several key innovations to improve the utilization of surimi as a functional, profitable, and sustainable ingredient. Such innovations include the discovery of cryoprotectants, productivity enhancements of heading and gutting machines, and the discovery of decanter technology. Cryoprotectants helped prevent freeze denaturation of surimi and improve its quality, while the other two innovations increased production yield (Park, 2012; Park et al., 2014a,b).

Numerous species have been used in the production of surimi. For a species to be considered a viable surimi resource, it must be currently abundant, underutilized, and economically competitive. Hence, the surimi industry is constantly changing because of the fluctuation of fish stocks around the world. This variation can occur as a result of environmental conditions, overfishing, the global economic situation, and the supply of raw materials for surimi. In addition, new technologies have allowed new resources to be utilized as raw material for surimi. For instance, the application of
protease inhibitors made it feasible to utilize Pacific whiting for surimi production (Guenneugues & Ianelli, 2013; Park, 2012).

However, using undesirable raw materials to make surimi results in low recoveries and low product quality. This implies that using surimi technologies to process fish and creating value-added fish products could lead to limited utilization of the animal. At present, the demand for fish protein is expanding faster than can be satisfied with traditional resources, putting great pressure on fish stocks. Better use of these materials is crucial, particularly with low-value raw materials, which are currently not being maximally utilized for human food use. The examples of these materials are fatty, pelagic fish and deboned muscle tissue from fish processing. The use of these materials is difficult because functionality of the proteins can be easily lost during processing, the product is unstable due to lipid oxidation, and there are unappealing aspects such as dark colors, strong flavors, unsightly appearance, and poor texture. Protein functionalities that can affect product quality include solubility, water holding capacity, gelation, fat binding ability, foam stabilization, and emulsification properties (Hultin & Kelleher, 1999; Hultin et al., 2003; Kristinsson et al., 2014)

The FPI process, using acid- and alkali-aided solubilization/precipitation, was developed by Hultin and Kelleher (1999). Unlike surimi, which originated in Japan in 1115, FPI has less than 2 decades of research and commercialization. The first step of the extraction mechanism of the FPI process is to solubilize muscle proteins at low (pH 2 to 3.5) or high (pH 10.5 to 11) pH. Muscle tissues are disrupted by grinding and/or homogenizing with enough water to form particles, which are used to
solubilize a major proportion of the available proteins and reduce viscosity. This process allows easy separation of insoluble materials, such as bones, skin, connective tissue, cellular membranes, and neutral storage lipids, from solubilized components.

A significant decrease in solution viscosity is essential to separate insoluble compositions from soluble proteins by centrifugation. As viscosity of protein solutions generally increases exponentially with protein concentration, appropriate dilution of the proteins is necessary. The muscle proteins rapidly solubilize during this process, since the acid and alkaline conditions used in the process are far enough from the muscle protein’s isoelectric points (approximately pH 5 to 6) that protein side chains gain a net positive (at low pH) or negative (at high pH) charge. This subsequent high charge causes the proteins to repel each other and solubilize.

Then, the recovery of the essentially membrane-free and lipid-free soluble proteins is achieved by precipitating the proteins via adjusting its pH to the isoelectric point. Different raw materials may respond differently to the same pH conditions; thus, it is important to evaluate how the proteins in the raw material react to changes in pH for maximizing recovery of the proteins (Kristinsson & Demir, 2003; Kristinsson et al., 2014; Undeland et al., 2002; Park, 2009).

Refining fish proteins using FPI procedures offer several advantages, including higher yields, improved functional properties, removal of most lipids, reduction of pollutants, and efficient removal of insoluble impurities. The FPI process increases yield of proteins compared to the surimi process because FPI contains both myofibrillar proteins and sarcoplasmic proteins while surimi contains only myofibrillar proteins. The improved protein yield resulting from the FPI process
signifies there are fewer portions to remove in the waste water and by-product pollution is decreased. As mentioned in the previous paragraph, removal of substantially all of the lipids helps to stabilize the product against oxidation. Also, the pH shift process improves product safety, by removing lipid-soluble toxins and reducing cholesterol levels. Kristinsson and Demir (2003) reported FPI showed a significantly slower growth of aerobic bacteria during cold storage compared to surimi or the starting raw material. Moreover, all substances used in the process are generally recognized as safe (GRAS). The FPI process eliminates the need for a refiner machine since it does not depend on diffusion processes to extract the water-soluble materials from broken muscle. This allows the processing of whole fish rather than fillets, resulting in a faster process than standard surimi processing (Hultin & Kelleher, 1999; Kristinsson & Liang, 2006; Kristinsson et al., 2014; Chen & Jaczynski, 2007a, b).

The three-dimensional structure of a protein determines not only its size and shape, but also its function. FPI and surimi refining methods are chemically opposite with regards to protein denaturation. FPI is refined through chemical denaturation using pH-shifting while surimi protein is concentrated by avoiding protein denaturation. Hence, the structures of these refined proteins are different, which means their functionalities are different (Kristinsson et al., 2014).

Fish protein has the ability to form an elastic gel upon setting and/or heating, which helps produce the desired texture for many seafood products (Lanier et al., 2014). The gel-forming ability of surimi or FPI is a complex physicochemical process involving structural and functional changes of proteins. Various researches
investigated and compared gelling properties of surimi, alkali-, and acid-extracted FPI. Results demonstrated gel made from FPI exhibited equal or superior gelling ability compared to gel made from surimi (DeWitt et al., 2007; Ingadottir & Kristinsson, 2010; Kim et al., 2003; Kristinsson & Ingadottir, 2006; Kristinsson & Liang, 2006; Kristinsson et al., 2005; Perez-Mateos et al., 2004; Rawdkuen et al., 2009, Thawornchinsombut & Park, 2007). The superior gelling ability is likely because of a combined effect of better charge distribution through conformational changes, partial refolding at neutrality, homogeneous dispersion of myofibrilar proteins disintegrated through mechanical homogenization, and retained sarcoplasmic proteins (Park, 2009). Furthermore, alkaline extraction appears to yield better gel-forming ability than acid extraction (Kim et al., 2003; Yongsawatdigul & Park, 2004; Kristinsson & Liang, 2006; Chen & Jaczynski, 2007a; Thawornchinsombut & Park, 2007). According to Yongsawatdigul and Park (2004), oxidation of sulfhydryl groups could occur and form disulfide bonds during acid solubilization and possibly result in lower sulfhydryl content, lower protein extractability, and poorer gel quality.

2.2 Factors affecting gelation properties of FPI and surimi

The rheological and biochemical characteristics of the gel can be affected intrinsically (species, freshness, and degree of stress before harvest) and extrinsically (protein concentration, pH, ionic strength, and temperature) (Park et al., 2014b). This section will review aspects that can influence gel qualities made from FPI and surimi, such as rigor-mortis, frozen storage, and comminution conditions.
2.2.1 Rigor mortis

Rigor mortis is the phenomenon describing the irreversible interaction between actin and myosin, causing the muscle of an animal to become non-extensive and stiff shortly after death when adenosine triphosphate (ATP) is depleted (Foegeding et al., 1996). This section will review current knowledge of rigor mortis and the mechanisms involved in filleting and heat-induced gelation using pre-, in-, and post-rigor seafood processing. It is important to understand how these processing practices contribute to the structure and texture of fillets and gels.

2.2.1.1 Biochemical changes during rigor mortis

After an animal’s death, the cessation of oxygen supply begins and the production of ATP is restricted. The muscle can remain metabolically active for hours in the pre-rigor state. There are two methods of breaking down glucose: anaerobic and aerobic. Once the heart stops beating, the anaerobic method is the only way to produce energy. The anaerobic method can only produce 2 moles of ATP for each mole of glucose oxidized, compared to 36 moles of ATP during aerobic breakdown. Thus, the synthesis of ATP ceases when the creatine or arginine phosphates are depleted.

One of the consequences of post mortem glycolysis is the accumulation of lactic acid in the muscle, which results in the lowering of the pH (Foegeding et al., 1996). Pawar and Magar (1965) studied biochemical changes in catfish, tilapia, and mrigal during rigor mortis. The pH dropped from 6.7, 6.6, and 6.8 for freshly killed fish to 6.1, 5.8, and 6.0 for fish in full rigor for catfish, tilapia, and mrigal, respectively. This
decline of pH can affect the quality of the fish muscle tissues. The net surface charge on the muscle protein changes and this causes the original structure to partially denature and lose some of its water-holding capacity (Foegeding et al., 1996; Kristoffersen et al., 2006; Duran et al., 2008).

The amount of lactic acid formed depends on the glycogen reserves of the muscle prior to death. Some studies showed the levels of stored glycogen and the ultimate post mortem pH are related to the amount of stress and exercise faced before death, as well as the nutritional status of the fish. Generally, well-rested and well-fed fish contain more glycogen compared to stressed fish (Roth et al., 2006; Duran et al., 2008). Korhonen et al. (1990) demonstrated simple tests to follow rigor mortis development in fish such as the measurements of the relative ATP/inosine monophosphate (IMP) content ratio, muscle pH, and visual measurements of stiffness in whole fish. These tests demonstrated the effects of ante-mortem stress on the rate of onset of rigor mortis and related biochemical changes in the muscle.

2.2.1.2 Filleting fish in relation to rigor mortis

Many seafood products are filleted in order to satisfy consumer demands for more convenient and high-quality products. One of the strategies to produce high-quality and value-added products is to utilize pre-rigor processing. Many studies have shown advantages of pre-rigor processing in cold and/or temperate water species (Skjervold et al., 2001; Roth et al., 2002, 2006; Kristoffersen et al., 2006; Duran et al. 2008). The first advantage of pre-rigor fillets is placing the product on the market faster, thereby reducing the amount of degradation compared to post-rigor fillets.
Kristoffersen et al. (2006) and Skjervold et al. (2001) studied the effects of pre-rigor filleting on the quality of Atlantic cod and Atlantic salmon, respectively. Both studies showed pre-rigor filleting resulted in significantly less gaping compared to in-rigor or post-rigor filleting. This may be because pre-rigor processing leaves the fillets free to contract during rigor mortis, thus avoiding tension build-up that may occur in the myocommata. Moreover, filleting and skinning post-rigor may more easily result in gaping since the strength of the connective tissues is lower at reduced pH (Love et al., 1972; Foegeding et al., 1996). There was also a significant difference in visual color by filleting Atlantic salmon during pre-rigor processing. According to Skjervold et al. (2001), rigor influences the structure and smoothness of the fillet surface, which affects the reflection of light. Duran et al. (2008) found that pre-rigor rainbow trout and mirror carp fillets had significantly firmer texture compared to in-rigor and post-rigor fillets. This appeared to be a similar reason for reducing the incidence gaping, and it is likely to be associated with the ability of pre-rigor fillets to contract freely from the vertebrate.

Although there were positive effects on pre-rigor processing, these effects did not apply to every species. For instance, water binding capacity and fillet weight loss did not differ among pre-, in-, or post-rigor filleting for Atlantic salmon (Skjrtvold et al., 2001). Furthermore, there were some negative quality aspects to pre-rigor filleting. Kristoffersen et al. (2006) indicated a significantly higher liquid loss and lower water content in cod fillets produced from pre-rigor muscle. Loss of water is important because it is economically equivalent to the loss of meat and water soluble nutrients (Foegeding et al., 1996).
2.2.1.3 Gel-forming properties in fish muscle as influenced by rigor mortis

Fish muscle can form a sol (paste) and turn into a viscoelastic gel upon heating. The rheological and biochemical characteristics of the gel can be affected by species, freshness, and processing parameters, such as protein concentration, pH, ionic strength, and temperature. The gelling ability of myofibrillar protein is highly important to the production of surimi and/or surimi seafood, such as crabsticks and fish sausage (Kristinsson et al., 2014).

Early studies showed the functional properties of muscle proteins can be affected by different species and processing methods during rigor mortis. Saffle and Galbreath (1964) reported an increase in extractability of salt soluble protein (SSP) when using pre-rigor beef and pork compared to post-rigor beef and pork. However, extractability of SSP from chicken white tissue between pre- and post-rigor did not show significant differences (Sayre, 1968). Chicken myofibrils extracted from pre-rigor leg showed greater protein extractability and stronger gel than myofibrils extracted from post-rigor leg, while the reverse result was found for breast myofibrils (Xiong & Brekke, 1991). Improved gel formation of myofibrils from post-rigor chicken breast may be due to the slow denaturation of protein until proteolysis increases (Yongsawatdigul et al., 2014).

Park et al. (1990) found surimi produced from pre-rigor tilapia had significantly higher protein content and yield, reduced cooking loss, stronger gel, and improved gel-forming ability. Authors observed mince from pre-rigor muscle was easier to dewater compared to mince from in- or post-rigor muscle. The higher amount of
protein recovery was possibly detected due to the compact particles in pre-rigor muscle. This may have helped reduce the release of fine particles and swelling during the water leaching process. The thermal behavior of actomyosin during the transformation from the sol to the gel was examined by differential scanning calorimetry (DSC) (Park & Lanier 1989; Park et al., 1990). The results showed greater enthalpy of denaturation in surimi prepared from pre-rigor fillets, indicating protein from pre-rigor surimi was a more “native” protein, required higher energy to denature the protein structure, and formed a strong ordered gel (Park et al., 1990).

Considerations of rigor-mortis during processing may become a good processing technique to improve the quality of seafood products, such as fillets and gels. Physical conditions, including size, temperature, and the level of stress before death, can influence properties of the products. However, different species can respond differently to the same processing method. Thus, it is important to develop a procedure for each type of seafood in order to obtain the best quality products.

2.2.2 Freezing and frozen storage

The process of commercial freezing has been established since the early 1900’s. Freezing is an outstanding process for preserving the quality of seafood. Frozen storage has several valuable benefits; for example, fishing vessels that freeze fish at sea can travel farther from port and catch enough fish to make the trip profitable. Also, the increase in preservation and storage time allows processors and wholesalers to hold inventories longer. With the increased shelf-life, products can be distributed greater distances, potentially opening up new markets at an optimum price. Frozen
storage also enables year-round marketing of species that are harvested during a limited time period, resulting in a reduction of seasonal fluctuations and greater choice of products for consumers. When freezing is performed immediately after harvest, better quality products result, compared to fish kept in chilled storage until marketed. Additionally, freezing products is an advantage to processing in some cases, such as when shelling prawns. Frozen storage can also effectively inhibit microbial spoilage effectively (Brown, 1986; Kramer et al., 2012).

However, storing fish and/or seafoods in a freezer for extended periods of time can negatively affect the functionality of proteins, since freezing cannot terminate structural and physicochemical changes (Brown, 1986; Kramer et al., 2012). As reported by Benjakul and Visessanguan (2010), changes of fish muscle proteins that take place during frozen storage include partial dehydration of protein during freezing; an increase in inorganic salts in an unfrozen phase; interaction of lipids, free fatty acids, and/or products from lipid oxidation with proteins; and trimethylamine oxide demethylase (TMAOase) activity.

2.2.2.1 Protein denaturation and structural changes

The change in the state of water causes undesirable changes in the food system since the bulk volume of most foods is water. Denaturation of fish proteins during frozen storage causes a decrease in the amount of available liquid water, diminished molecular mobility, an increase in solute concentration, and physical impairment of protein structures arising from ice crystal formation. Lowering product temperature can lead to moisture migration as crystals in regions of low temperature grow faster
than those in regions of higher temperature. The three-dimensional protein structures are surrounded by a hydration sphere and the structure is markedly reliant on the network of hydrogen bonds. Consequently, any internal dehydration (ice crystal formation) or recrystallization will induce a disruption of the hydrogen bonding system, weaken intramolecular hydrophobic interactions, break up the water-mediated hydrophobic-hydrophilic interactions, and leave unprotected and vulnerable regions of the protein structures. This prompts intermolecular interactions that contribute to protein-protein interactions and eventually aggregation. Moreover, the concentration of solutes can accelerate protein denaturation and aggregation, leading to loss of functionality due to the destabilization of proteins. Muscle may then become tougher, drier, and lose its water-binding capability (Brown, 1986; MacDonald et al., 2014; Shenouda, 1980; Taborsky, 1979).

Various factors can affect deterioration of fish muscle and gel-forming ability during frozen storage such as fish species, freezing temperature and time, and enzymatic degradation (Ang & Hultin, 1989; Badii & Howell, 2001; Hsieh & Regenstein, 1989, Benjakul et al., 2003). Benjakul et al. (2005) showed that, under extended frozen storage, lizardfish was most susceptible to denaturation and loss in gelation compared to threadfin bream, bigeye snapper, and croaker. Results indicated the formaldehyde level in lizardfish muscle was the highest among these four tropical species, which contributed to increased protein denaturation.

Storing fish in a freezer for extended periods of time can negatively affect the gel-forming ability of muscle proteins (Okazaki, & Kimura, 2013; Benjakul et al., 2005). The quality of surimi gels made from frozen hoki, Alaska pollock, and
lizardfish determined by MacDonald, et al. (1992); Scott et al. (1988); and Kurokawa (1979), respectively, demonstrated a declining trend as frozen storage induced denaturation. Thawornchinsombut and Park (2006) investigated the effect of pH and cryoprotectants on the functional properties of alkali-extracted FPI. Results showed the factors responsible for decreased quality during frozen storage were freeze/thaw cycles and/or the absence of cryoprotectants. Consequently, authors suggested FPI needs cryoprotectants to prevent freeze-induced aggregation during frozen storage.

2.2.3 Comminution

It is important to understand the aspects that affect gel-forming properties in order to develop the qualities of gelled protein products. Muscle proteins must be initially liberated prior to gelation. Hence, comminution is a crucial step and there are two main functions that take place during the process. The purposes of comminution are to maximize protein extraction and to homogeneously combine other ingredients into the paste. Also, one of the steps to evaluate quality for both sellers and buyers of surimi or FPI is comminution. The majority of comminuted products made from muscle proteins are produced by two-step comminution including salting and then mixing. Numerous factors, including time, temperature, and other mechanical functions of the silent cutters, such as blade configuration, sharpness, and/or vacuum, can affect the maximum extraction of available proteins (Poowakanjana & Park, 2014). Understanding these parameters would provide vast knowledge on the effect of various comminution conditions on the properties of protein gels. This section will review several comminution factors affecting the quality of gelled products.
2.2.3.1 Thawing and tempering

In general, surimi and FPI are stored below -18°C; therefore, they need to be partially thawed or broken into smaller pieces prior to comminution. This step helps reduce the load on the equipment during the following fine comminution step. In order to produce and maintain the quality of thawed products, it is essential to understand the thawing and/or tempering process and ensure the system is effective. The difference between thawing and tempering is the complete product temperature after its process. Thawing is the reverse process of freezing; during thawing, the product temperature is raised above 0°C. Conversely, the completed product temperature remains below the freezing point during the tempering process. The thawing process is lengthy and takes longer than freezing because the surface of the food and thawing medium is likely to be smaller, and the thermal conductivity of the unfrozen food is lower than that of the frozen food, resulting in a slower rate of heat transfer. Therefore, processors must pay special attention to avoid over-thawing and sustain a consistent rise in temperature for all parts of the material. Otherwise, the thawing process may allow the multiplication of food poisoning microorganisms to recommence and/or induce protein denaturation causing low quality gels (Poowakanjana & Park, 2014).

2.2.3.2 Salting

The roles of salt are to facilitate extraction of myofibrillar proteins, control microorganism as preservation, and improve taste (Poowakanjana & Park, 2014). Sun
and Holley (2011) demonstrated the concentration of salt affects protein solubility and ultimately dictates the ability of protein gelation. Salt assists unfolding of myofibrillar protein, favoring more water molecules to come in contact, such as salting-in, and improves gelation. However, adding salt beyond an optimum concentration can adversely influence gel quality. Adding excess salt can, in fact, induce the shift of unfolded proteins to aggregates, since proteins fail to entrap water molecules under high ionic strength called salting-out (Poowakanjana & Park, 2014). Poowakanjana et al. (2015a) demonstrated the time of salt addition during chopping could contribute differently to the gelation properties of fish myofibrillar protein. Different pH conditions can also change protein solubility as a result of salt content (Thawornchinsombut & Park, 2005).

The effect of salt on gelation properties of FPI and surimi are different. In surimi, salt contributes to improved gelation, while for FPI the effect of salt addition on gelation changes, depending on the species. Kim and Park (2008) revealed the negative role of NaCl on the gel-forming ability of FPI prepared from Alaska pollock surimi. Their results indicated FPI obtained higher gel texture when NaCl was not added during chopping. In contrast, Perez-Mateos et al. (2004) demonstrated FPI gels made from Atlantic croaker chopped with NaCl attained higher gel texture than conventional surimi with NaCl.

As mentioned previously, protein solubility highly depends on different ions. Various research groups have studied the substitution of salt in surimi and FPI products. Potassium chloride with stabilizing agents has been primarily used as a salt substitute (Arfat & Benjakul, 2013; Poowakanjana & Park, 2014; Tahergorabi et al.,
Tahergrabi and Jaczynski (2012) reported there were no significant differences in shear stress and shear strain from Alaska pollock surimi gel when natural salt and sodium-free salt at similar levels of ionic strength were used. However, negative results of gel quality were observed in Alaska pollock surimi gel as the salt substitute content increased (Tahergrabi et al., 2012a). Tahergrabi et al. (2012b) used KOH for FPI preparation instead of NaOH. Results showed that FPI prepared using KOH had better texture compared to FPI prepared with NaOH.

2.2.3.3 Protein and moisture content

Moisture content of surimi or FPI during comminution is critical. However, moisture content of surimi or FPI differs depending on fish species, harvest season of the fish, and other processing conditions such as mincing, washing, dewatering, refining, and screw press (Poowakanjana & Park, 2014). Also, the density of protein molecules in a food system can be altered by adjusting moisture content. Higher protein content can lead to smaller space among protein molecules inside the matrix and more condensed protein structures. With different densities, the degree of protein unfolding can vary under similar comminution conditions. Proteins with lower density unfold to a greater extent compared to those with higher concentration because of more space and less internal friction (Chakravarty & Varadarajan, 2002; Robinson-Rechavi et al, 2006; Soranno et al., 2012).

Controlling surimi or FPI moisture concentration is necessary to determine gelation properties on a laboratory scale, since moisture content is a key function in gelation. Yoon et al. (1997) demonstrated effects of moisture content on gel
functionality and there was a linear or nonlinear pattern, depending on the type of surimi and a specific range of moisture content. Poowakanjana et al. (2015b) investigated rheological properties of Alaska pollock surimi as affected by heating rates and moisture content. Results suggested slow completion of gelation in surimi with high moisture content. Thus, protein and moisture content can impact the degree of unfolding during comminution.

2.2.3.4 Time and temperature control

Two of the most important variables determining protein quality in gel preparation are temperature and time control (Poowakanjana & Park, 2013a). Controlling chopping conditions also plays a key role in different types of comminuted products such as frankfurter, pork bologna sausage, beef sausage, and pork meat (Hensley & Hand, 1995; Colmenero et al., 1996, Boles et al., 1998; Tyszkiewicz et al., 1997). Understanding the thermal stability of fish proteins is a prerequisite to optimizing production techniques.

Previous publications reported chopping temperature and time, and salting time can impact the quality and stability of fish protein gels prepared from surimi. These studies revealed protein functionality depends on species, and that protein thermal stability is closely related to fish habitat temperature (Douglas-Schwarz & Lee, 1988; Hemung et al., 2008; Poowakanjana & Park, 2013, 2014). Esturk et al. (2004) and Poowakanjana et al. (2015a) investigated the effect of chopping conditions on gelation properties of surimi from various species, such as cold water species Alaska pollock, temperate water species Pacific whiting, and warm water species big eye.
snapper, lizardfish, and threadfin bream. Results indicated Alaska pollack was more susceptible to denaturation and aggregation of proteins, and led to lower gel qualities, during chopping at a higher temperature for longer duration than Pacific whiting, big eye snapper, lizardfish, or threadfin bream. The optimum chopping condition for big eye snapper and lizardfish was 20°C while the recommended chopping method for threadfin bream was to continuously chop surimi until the temperature reached near their habitat temperature between 25–30°C (Esturk et al., 2004; Poowakanjana et al., 2012, 2015a).

2.3 Structural biochemical and rheological analysis

2.3.1 Vibrational spectroscopy

Spectroscopy is the study of the interaction of electromagnetic radiation with matter. There are many different types of spectroscopy. The types vary in regard to the particles to be analyzed (atom or molecule), the nature of the radiation-matter interaction to be observed (absorption, emission, or diffraction), and the regions of the electromagnetic spectrum (wavelength and frequency) used in the analysis (Penner, 2010). Vibrational spectroscopy, the infrared region, measures the energy change involving transitions between the vibrational levels of molecules. It is commonly known as a combined term used to represent two analytical techniques such as infrared (IR) and Raman spectroscopy. Vibrational spectroscopy is a valuable instrument for the investigation of molecular structure, including proteins, carbohydrates, nucleic acids, cell membrane, and tissues. Both spectroscopies are non-destructive, non-invasive instruments that provide information about the
intramolecular forces acting between the atoms in a molecule, the intermolecular forces in condensed phase, and the nature of the chemical bonds. In addition, each vibrational spectrum is unique like a fingerprint, so these tools can be utilized for identification, structure interpretation, reaction monitoring, quality control, and quality assurance (Wehling, 2010; Reichenbacher & Popp, 2012; Damez & Clerjon, 2013).

IR and Raman spectroscopies have been extensively used to determine the structural change of proteins, since these techniques do not require chemical modification and the sample preparation is simple. Another positive aspect of these techniques is that there is no heat generation during the measurement (Surewicz et al., 1993; Herrero, 2008; Li-Chan, 1996).

There are other available techniques to elucidate protein structures, such as circular dichroism (CD), x-ray crystallography, and nuclear magnetic resonance (NMR). However, these techniques have weaknesses. Although CD is an effective method to study protein structures, particularly peptide backbone, the sample must be in a clear liquid form, which requires laborious sample preparation (Pelton & McLean, 2000). The x-ray diffraction method provides structural information of protein molecules, yet samples must be crystallized and molecules must be solitary. The structures of protein molecules are likely to change prior to testing; thus, it may not be a suitable method to detect the conformational change of proteins (Damez & Clerjon, 2013; Dogan et al., 2010; Surewicz et al., 1993). Likewise, NMR measurements must be performed with a solid probe, which involves difficult sample preparation skills. Moreover, the molecular weight of the sample has to be less than
30 kD; hence, this technique is not capable of determining myofibrillar protein with a myosin or actin subunit, which are 205 kD and 45 kD, respectively (Pelton & McLean, 2000; Reuhs & Simsek, 2010; Damez & Clerjon, 2013). Consequently, vibrational spectroscopy is an important technique because it has broad applications and the information contained in a spectrum is substantial.

2.3.1.1 Comparison between Fourier transform infrared and Raman spectroscopy

FT-IR and Raman spectroscopies are complementary techniques that allow differences among structural macromolecular changes to be detected (Flores-Morales et al., 2012; Sivam et al., 2013; Yuen et al., 2009). Both of these techniques assess vibration in different ways. FT-IR spectroscopy is based on the change in the electrical dipole moment. The sample is irradiated with polychromatic light of suitable wavelength and a photon of light is absorbed. This light can be identified when the frequency (energy) of the absorbed light equals the energy required for a specific bond to vibrate within the sample. In Raman spectroscopy, the vibration gives rise to irradiation of a sample with monochromatic light of a suitable wavelength and the photons are either inelastically or elastically scattered. A very small portion, one in every million photons, of the absorbed radiation energy is scattered with lower (stokes) or higher (anti-stokes) frequency than the incident light. These inelastically scattered lights are called Raman scattering (Figure 2.2). In contrast, the elastically scattered light, known as Rayleigh scattering, has the same absorbed radiation energy as the incident laser light. Current Raman instruments are designed to collect Raman scattering and filter out Rayleigh scattering. There is
another requirement in order for a vibration to be Raman active. There must be changes in polarizability of chemical bonds when the irradiated energy is transformed into molecular vibrations (Kong & Yu, 2007; Surewicz et al., 1993; Van de Voot, 1992; Li-Chan & Qin, 1998; Herrero, 2008; Li-Chan, 1996).

**Figure 2.2** Transition of energy for Rayleigh and Raman scattering, and IR absorption

Both FT-IR and Raman data are presented in a spectrum, but how the information is presented is different. The FT-IR spectrum represents the molecular transmission or absorption by the sample versus energy, which is signified by its wavenumbers (cm\(^{-1}\)), the reciprocal of wavelength (1/\(\lambda\)). Raman spectrum is obtained by plotting the intensity of the Raman scattered light versus the energy difference (Raman shift), which is also indicated as wavenumbers (Li-Chan, 1996; Sivam et al., 2013). It is essential to be familiar with how the intensity of the bands in the FT-IR and Raman spectra vary, considering both instruments are acquiring the vibrational frequencies of the functional groups within the sample. This is because the vibrational modes derive from different mechanisms (i.e., changes in dipole moment or
polarizability). Polar bonds such as C=O, N-H, and O-H show strong IR stretching vibrations, while nonpolar bonds such as C=C, C-C, and S-S reveal intense Raman bands (Reichenbacher & Popp, 2012; Li-Chan, 1996).

2.3.1.2 Application of FT-IR and Raman spectroscopy

Several publications applied both vibrational spectroscopies in food product analyses and results indicated a better interpretation of the spectra and an increase of the accuracy in the analyses (Flores-Morales et al., 2012; Fontecha et al., 1993; Pereira et al., 2009; Kizil et al., 2002; Holse et al., 2011). Sivam et al. (2013) demonstrated the complementarity of the two techniques for examining the secondary conformations and structure of gluten proteins and polysaccharides in finished bread systems. Pereira et al. (2009) reported the identification of the principal sea vegetable colloids in ground seaweed samples with a minimum of handling, and treatment was achievable by using a combination of two vibrational spectroscopic methods (FT-IR-ATF and FT-Raman). This denotes that the determined composition describes the native composition of the phycocolloids as precisely as possible. Yuen et al. (2009) conducted comparative studies of traditional wet chemistry procedures and FT-IR and Raman spectroscopic methods for the determination of the degree of substitution (DS) in carboxymethylated non-starch polysaccharides, including cellulose, guar gum, locust beam gum, and xanthan gum. Results suggested there is a great potential to replace the use of conventional wet chemistry methods with the application of both Raman and FT-IR spectroscopic methods for measuring DS. In addition, the
spectroscopic procedure established in this study was relatively simple, fast, did not use toxic chemicals, and required small amounts of sample.

FT-IR and Raman spectroscopy have been used to analyze the structural changes of solid-state FPI and surimi paste and gel under different storage, comminution, and cooking conditions (Bouraoui et al., 1997; Cando et al., 2016; Herranz et al., 2013; Herrero et al., 2004; Larrea-Wachtendorff et al., 2015; Liu et al., 2015; Moosavi-Nasab et al., 2005; Poowakanjana et al., 2012; Tadpitchayangkoon et al., 2010; Thawornchinsombut et al., 2006). Moosavi-Nasab et al. (2005) studied the structural changes during preparation and storage of surimi by FT-IR spectroscopy. FT-IR spectrum exhibited an increase in α-helix content with an increased number of washing cycles. Additionally, results suggested that fast freezing is more effective than slow freezing in maintaining the secondary structure of proteins during long-term frozen storage. Cando et al. (2016) demonstrated an increase in β-sheet components in surimi paste at the expense of α-helix structure as the NaCl content increased using FT-IR spectroscopy. Similarly, Raman spectroscopy showed salting resulted in increased β-sheet content of surimi paste (Bouraoui et al., 1997; Barrett et al., 1978).

Thawornchinsombut et al. (2006) investigated the stability of alkali-treated protein isolate at various frozen storage conditions using Raman spectroscopy. Results indicated that alkali-treated protein isolate was slightly less stable than conventional surimi under frozen storage conditions. FT-IR and Raman data could clarify the degree of protein unfolding through microenvironment of hydrophobic,
aliphatic, aromatic side chains, and disulfide formation (Li-Chan, 1996; Herrero et al., 2004; Poowakanjana et al., 2012; Kong & Yu, 2007; Surewicz et al., 1993). Therefore, application of FT-IR and/or Raman spectroscopies for elucidation of protein structures in FPI and surimi is beneficial.

2.3.2 Surface hydrophobicity

Hydrophobic interactions are one of the main bonding mechanisms for protein folding among nonpolar groups. When two separated nonpolar groups are present in an aqueous environment, which is thermodynamically unfavorable, nonpolar groups tend to aggregate to minimize the area of direct contact with water/polar groups (Lanier et al., 2014; Damodaran, 2007). Nakai (2000) illustrated two definitions of protein hydrophobicity, such as average hydrophobicity and surface hydrophobicity. According to Bigelow (1967), the average hydrophobicity was defined as the total hydrophobicity of all amino acid residues consisting of a protein divided by the amount of amino acid in the protein. Although there is no approved definition for surface (effective) hydrophobicity, the concept applies to hydrophobic regions on the molecular surface that play a key role in protein function, including binding with small ligands, protein-lipid interactions, and interactions with other biological macromolecules (Nakai & Li-Chan, 1988). Therefore, assessment of the hydrophobicity is crucial to understand protein structure and functionality.

Research groups have proposed several methods for measuring surface hydrophobicity, such as partition in aqueous two-phase systems, high performance liquid chromatography (HPLC), binding methods, contact angle measurement,
intrinsic fluorescence, derivative spectrophotometry, and probe spectrofluorometry (Nakai et al., 1996; Nakai 2000; Kirk et al., 1996). However, no standard procedure has been established. The most popular techniques for examining the surface hydrophobicity of proteins is using fluorescent probes due to their simplicity, rapidity, ability to predict functionality, and use of small quantities of purified protein for analysis (Nakai, 2000).

In probe spectrofluorometry, samples with fluorescent probes are analyzed and fluorescence increases under hydrophobic environments. Fluorescent spectra and quantum yield are generally correlated to the environment, particularly solvent polarity. 1-anilinonaphthalene-8-sulfonic acid (ANS) has been the most popular fluorescent probe to determine the surface hydrophobicity of proteins (Figure 2.3). The ANS is an anionic probe of the aromatic sulfonic acid class, and this probe is widely used for protein membranes (Nakai et al., 1996). The binding site appears between sulfonate groups of ANS anion and cationic charge of the polar amino acids (Matulis & Loverien, 1998). ANS anions become fluorescent when they are not surrounded by hydrophilic molecules. The degree of fluorescence increases proportionally to the number of ANS anions that are surrounded by nonpolar groups. Therefore, the higher quantum yields of fluorescence represent more hydrophobic groups exposed on the surface of proteins (Nakai, 2000; Wicker & Knopp, 1988).
Cis-parinaric acid (CPA) is another group of anionic fluorescent probe. This probe is important in protein-lipid interactions in food systems, since the probe is non-aromatic, similar to native fatty acids, and unable to fluoresce in water (Nakai, 2000). Kato and Nakai (1980) observed a correlation between the relative hydrophobicity values of proteins determined by CPA fluorescence and properties related to protein-lipid interactions, which were interfacial tension and emulsifying activities.

A disadvantage of using these anionic probes is that electrostatic and hydrophobic interactions may influence the interaction between the protein and the probe (Nakai, 2000; Alizadeh-Pasdar & Li-Chan, 2000). Hence, the application of these anionic probes may result in an overestimation of the hydrophobicity value. Charged but neutral or uncharged probes may be used in order to overcome the charged effects observed in anionic probes. 1,6-diphenyl-1,3,5-hexatriene (DPH) is a neutral lipophilic fluorescent probe that is a popular hydrophobic probe for membrane proteins with phospholipids. However, the application as a fluorescent probe for
proteins is restricted because the nonpolar nature of the probe limits its solubility in aqueous circumstances. 6-propionyl-2-(dimethylamino)-naphthalene (PRODAN) contains both electron-donor and electron-acceptor subunits within the molecule. Thus, it will exclude potential inclusion of electrostatic interaction and it is much easier to dissolve in more polar solvents compared to DPH (Nakai, 2000; Nakai et al., 1996). In addition, Alizadeh-Pasdar & Li-Chan (2000) reported PRODAN may be a better choice over ANS or CPA to determine the surface hydrophobicity of proteins over a wider range of pH values.

2.3.3 Sulfhydryl content

Disulfide bonds (S-S) and sulfhydryl (SH) groups play important roles in the heat-induced gelation of proteins. The SH groups are known to be the most reactive functional groups in proteins. Disulfide bonds are covalent bonds thought to attribute to gel formation of protein, and they can take place both intramolecularly and intermolecularly. An intramolecular disulfide bond is formed when two cysteine residues are brought into proximity with proper orientation. These disulfide bonds support the stabilization of the folded structure of proteins. An intermolecular disulfide bond can occur by oxidation of two cysteine molecules on neighboring protein chains, which contain reactive (free) SH groups. Additionally, intramolecular disulfide bonds can be altered to intermolecular disulfide bonds. For instance, protein mixtures comprising cystine and cysteine residues can promote sulfhydryl-disulfide interchange reactions. The development of gel networks is a process involving unfolding of proteins followed by orientation of unfolded molecules during
aggregation. Therefore, monitoring the degree of protein unfolding/aggregation upon heating may help understand the gelling quality of proteins. One of the parameters to determine protein configuration and interaction is measuring the amount of reactive SH groups (Kim et al., 2003; Nurkhoeriyati et al., 2001; Lanier et al., 2014; Damodaran, 2007).

5,5\textsuperscript{'}-dithio-bis-(2-nitrobenzoic acid) (DTNB), known as Ellman’s reagent, as a versatile water-soluble compound has been widely used to quantify the free SH groups in the solution developed by Ellman (1959). DTNB reacts with conjugate base of free SH groups of cysteine to yield a colored product (yellow), such as 2-nitro-5-thiobenzonate anion (TNB\textsuperscript{2\textsuperscript{-}}) (Figure 2.4). This reaction is quick and stoichiometric; thereby, the amount of TNB\textsuperscript{2\textsuperscript{-}} is estimated by absorption spectroscopy at 412 nm. SH groups can be assayed using a molar extinction coefficient of TNB rather than using a standard curve. However, the rate of the reaction of DTNB with free SH groups can vary depending on several factors, including the solvent, the reaction pH, the pKa’ of the SH, and steric and electrostatic effects. Hence, it is essential to consider which molar extinction coefficient to use prior to testing in order to properly calculate the amount of SH groups (Ellman, 1959; Riddles et al., 1979, 1983).
There are two ways to determine the amount of reactive SH content, such as surface reactive sulfhydryl group (SRSH) and total reactive sulfhydryl groups (TSH). In SRSH content assay, no denaturing agent is added in a buffer solution, so the amount of SH groups found on the surface of proteins are measured. On the other hand, a denaturing agent, such as urea solution, is added in a buffer in order to quantify TSH content. Urea helps Ellman’s reagent to access all of the SH groups that were buried inside the protein structure. Therefore, comparing SRSH and TSH contents is a useful test to observe unfolding/aggregation (refolding) and gelling behavior of fish proteins (Reddles et al., 1979; Reed & Park, 2011a,b; Hsu et al., 2007). Yongswatdiful and Park (2003) and Sano et al. (1994) demonstrated that the SRSH groups of threadfin bream and carp actomyosin, respectively, increased as sample temperature increased. Results suggested aggregation, corresponding to

**Figure 2.4** The reaction of Ellman’s reagent
disulfide bond formation, began to appear after 60°C, resulting in a decrease of SRSH content. Similarly, Reed and Park (2011b) observed the exposure of buried SRSH groups of salmon myosin increased and was maximized at 55°C as myosin was continuously heated.

2.3.4 Rheological analysis

Rheology is a science that measures flow ability and deformation of materials in liquid, semi-solid, or solid conditions when force is applied. Because the perceived texture of food products extends beyond rheological properties, these properties should be viewed as a subset of the textural properties of foods. There are three classes of rheological tests using instruments, including fundamental, empirical, and imitative tests. Fundamental tests measure true physical properties that are familiar to engineers. Results are comparable, since the same results are obtained within the experimental error regardless of the methods used. However, fundamental testing may not give simple answers due to laborious and time-consuming processes. Empirical tests are typically descriptive in nature and correlate with a property of interest. Also, these methods are quicker and simpler compared to fundamental methods. The drawbacks of these methods are that results are specific to a particular instrument. Lastly, imitative methods use instruments to imitate the conditions for sensory evaluation of texture (Kim et al., 2000; Ross, 2006; Foegeding, 2007; Tabilo-Munizaga & Barbosa-Cánovas, 2005).

Efforts to measure food texture by fundamental methods have introduced two types of rheological tests using small or large strains. Small strain rheological
measurements provide strains less than required for structural breakdown (non-fracture test), while large strain rheological testing use forces required to bite through the structure (fracture test) (Kim et al., 2000). This section will focus on reviewing the oscillatory dynamic test which is a non-fracture test.

2.3.4.1 Oscillatory dynamic test

All materials can be categorized by three rheological behaviors: viscosity, elasticity, and viscoelasticity. The law of conservation of energy is used to illustrate the behavior of matter. With viscous materials, all energy added is dissipated into heat. When measuring elastic materials, all energy added is stored in the material. Viscoelastic materials exhibit both properties of viscous and elastic behaviors. Dynamic rheology, also known as small amplitude oscillatory shear (SAOS), allows viscoelastic properties of foods to be understood and provides insights about chemical composition and physical structure. Dynamic rheometers apply small strains in sinusoidal oscillation when testing solid or semi-solid foods. In addition, dynamic rheometers can classify the elastic and viscous responses of a material during measurement of forces by applying deformation. These force values are then mathematically transformed to define fundamental rheological properties, such as storage modulus, loss modulus, and phase angle (Kim et al., 2000; Ross, 2006).

The storage modulus (G’), indicating the elastic component, is a measurement of the amount of energy that is stored or recovered in the material per cycle of deformation. The loss modulus (G’’), indicating the viscous component, is a measurement of the amount of energy lost as heat in the material per cycle of
deformation. The phase angle \( \frac{G''}{G'} \) can be expressed as \( \tan \delta \) and varies between 0 and 90°. This measurement can be used as an indicator of the nature of the sample. For instance, the phase angle decreases as the elastic component becomes stronger, whereas the phase angle increases as the material becomes viscous (Kim et al., 2000; Ross, 2006). Prior to testing a sample, it is necessary to determine the linear viscoelastic region (LVR). A deforming force is continuously applied to the sample, but this force never exceeds a strain large enough to destroy the structure. If the structure of the sample is destroyed, the elastic structure will also be destroyed. Another measuring system to be considered is the frequencies applied when examining the dynamic rheology of samples. Low frequencies should be applied in order to give sufficient time for long range motion of chains to occur (Poowakanjana et al., 2015b).

Three methods of measuring systems exist in the oscillatory dynamic test. These methods are cone and plate, parallel plates, and cup and bob (Figure 2.5). Both cone and plate and parallel plate configurations require relatively small sample volumes and are easy to clean. The cone and plate configuration permits the shear stress and shear rate to remain constant at any location of the sample, unlike parallel plates. The cone angle setting should be small, since a large cone angle can cause the shear rate across the gap to vary. The advantage of using the cup and bob measuring system is to be able to work with low viscosity materials and mobile suspensions. However, this configuration requires large sample volumes, and can also produce issues when performing high frequency measurements (CVO User Manual, 1999).
The oscillatory dynamic test is a valuable test to investigate composition/structure/function relationships in fish proteins. Many publications have utilized dynamic rheology to understand the role that fish proteins play during gelation. Fukushima et al. (2003) found that the thermal denaturation and stability of Alaska pollock, white croaker, and rabbit myosin were different. The results suggested gel forming ability was species specific, and closely associated with habitat environments. Lefevre et al. (2007) demonstrated denaturation and aggregation temperatures of white and red muscle in salmon were distinctively different. In addition, dynamic rheology is a useful tool to observe how different factors can affect gel forming capabilities including refining methods of proteins (surimi and FPI), salt concentration, moisture content, heating rate, and so on (Ingadottir & Kristinsson, 2010; Poowakanjana et al., 2015b).
2.3.5 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a thermal technique to study how material heat capacity is changed by temperature. Changes in the heat flow are used to track the changes in its heat capacity. The principle aspect of a DSC system contains two cells (a reference cell and a sample cell). The instrument is designed to maintain the two cells at the sample temperature as they are heated linearly, and the heat added to each pan is monitored.

DSC allows a wide variety of applications to measure the physical and chemical properties and structure of most ingredients applied in the food products, including glass transition temperature of amorphous structure; melting temperature of crystalline structure; crystallization of amorphous material; denaturation of proteins; gelatinization of starch; analysis of frozen solutions used for freeze drying; and oxidative stability of fats and oils. By understanding these properties and structures, desired formulations and processing methods can be developed (Thomas & Schmidt, 2010).

The two types of available DSC devices are conventional DSC and micro DSC (MDSC). One of the disadvantages of using a conventional DSC is that it does not effectively detect all energy exchanges between the sample vessel and the calorimetric unit. In order to improve the sensitivity, MDSC uses a Tian-Calvet type transducer. Sample and reference vessels are fully surrounded by a thermopile that measures thermal energy and converts it to electrical energy (Figure 2.6). The thermopile comprises from a number of thermocouples. The sensitivity of measuring thermal energy from the sample and reference vessels becomes much better by the
use of a thermopile system. In addition, MDSC can accommodate a larger sample size (400-500 mg) compared to conventional DSC (10-30 μg) (Hu et al., 2009).

![Calvet type DSC](image)

**Figure 2.6** Calvet type DSC. 1 – sample and reference cylinders, 2 – thermopile, ΔT represents the measured difference between the sample and reference vessel, 3 – furnace, 4 – DSC transducer lid

DSC can be used to measure changes in endothermic and exothermic phenomena, signifying protein denaturation and aggregation, respectively. Changes in the sample are related to absorption or discharge of heat resulting in a change in the variance heat flow, which is then recorded as a peak. The area under the peak is directly proportional to the enthalpic change, and the direction of the peak represents either endothermic or exothermic behavior. Endothermic processes need heat from the
furnace to preserve the sample vessel at the same temperature as the reference vessel. In contrast, heat is generated as the denatured protein begins to form chemical bonds. Thus, the sample will require less heat (exothermic) from the DSC device to maintain the sample and reference vessels at the sample temperature (Biliaderis, 1983; Arntfield & Murray, 1981; Johnson, 2013).

The accurate and high quality data obtained from DSC provides vital information on thermal denaturation and thermodynamics involved with proteins. Examples of ways to measure muscle proteins include monitoring conformational changes and thermal behaviors; determining characteristics of actin and myosin; designing processing equipment; estimating heating and cooling rate; and analyzing the energy requirement of the process (Biliaderis, 1983). Ueki and Ochiai (2004) compared protein characteristics of different species, such as bigeye tuna and mackerel myoglobin and myosin using DSC. Ogawa et al. (1993) used DSC methods to study thermal stability of fish myosin and rabbit myosin. Results indicated the structure of the fish myosin was much more unstable than rabbit myosin.

Numerous studies have shown negative effects of frozen storage on fish qualities by DSC (Saeed & Howerll, 2004; Schubring, 2004; Thawornchnsombut & Park, 2006). Park et al. (1990) demonstrated higher enthalpies of denaturation in surimi prepared from pre-rigor fish compared to surimi prepared from post-rigor fish. The results characterize destabilization of protein structures and demonstrated the lower the energy required to denature the proteins. Park and Lanier (1989) investigated the thermal behavior of tilapia muscle proteins at various stages in the processing of surimi and purification of myosin and actin by DSC. Authors observed a shift to
lower transition temperature with further processing, indicating denaturation of proteins.

Other factors can lead to denaturation of fish proteins, which can be detected by DSC. Kim et al. (1986) reported the reduction of endothermic peaks from four to three from Alaska pollock surimi after the addition of 3% sodium chloride, which indicates protein denaturation. Park et al. (2008) reported the endothermic peaks of purified sardine myosin could be influenced by pH and ionic strength. Similar results using Pacific whiting fish protein isolate were observed by Thawornchinsombut and Park (2004). DSC can be used to determine protein stability of fish fillets during high pressure treatment (Chevalier & Le Bail, 2001). Moreover, DSC can help understand the effect of ingredients, such as starch, on thermal properties of surimi (Belibagli et al., 2003).
CHAPTER 3

GELATION PROPERTIES OF TILAPIA FISH PROTEIN ISOLATE AND SURIMI PRE- AND POST-RIGOR

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3.1 Abstract

The structural changes and rheological properties of tilapia proteins prepared using two refining methods (pH shift processing and surimi processing, respectively) with pre- and post-rigor muscle were investigated. Higher storage modulus ($G'$) and better gel texture were observed in surimi produced from pre-rigor fish than surimi made from post-rigor fish, but no rigor effect was noted on the gel-forming ability of fish protein isolate (FPI). The effect of salt on the gelation of tilapia muscle with different rigor states or refining method was also determined. The addition of salt appeared to readily unfold protein structures in FPI as measured by surface hydrophobicity, surface reactive sulfhydryl content, dynamic rheology, and Raman spectroscopy. However, the effect of salt on the degree of unfolding in surimi was not as sensitive as in FPI.
3.2 Introduction

Two successful methods are currently being used to refine fish muscle proteins: surimi and fish protein isolate (FPI). During the production of surimi, both chemical and physical denaturation of proteins must be avoided to obtain good quality surimi (Park et al., 2014b). On the other hand, FPI is produced through chemical-induced denaturation by pH shift. The pH shift procedure first maximizes the solubility of fish muscles and recovers most of soluble proteins subsequently acquired at the isoelectric point by centrifugation. FPI is different compared to surimi, since sarcoplasmic proteins remain along with the myofibrillar proteins (Hultin & Kelleher, 2000).

The gel-forming ability of FPI or surimi is a complex physicochemical process involving structural and functional changes of myofibrillar proteins. The rheological and biochemical characteristics of the gel can be affected intrinsically (species, freshness, and degree of stress before harvest) and extrinsically (protein concentration, pH, ionic strength, and temperature). In addition, the functional properties of the muscle proteins can be affected by the biochemical changes that occur during rigor mortis (Park et al., 2014b).

Early research has reported that the functional properties of muscle proteins can be affected by different species and processing methods during rigor mortis. Extractability of salt soluble protein (SSP) in pre-rigor beef and pork was higher compared to SSP in post-rigor beef and pork (Saffle & Galbreath, 1964). However, extractability of SSP from chicken white tissue between pre- and post-rigor did not show significant differences (Sayre, 1968).
Xiong and Brekke (1991) found that chicken myofibrils extracted from post-rigor breast showed greater protein extractability and stronger gel than myofibrils extracted from pre-rigor breast, however the reverse was found for leg myofibrils. One possible reason for improved gel formation of myofibrils from post-rigor chicken breast was the slow denaturation of the protein until proteolysis was increased (Yongsawatdigul et al., 2014).

Park et al. (1990) reported surimi produced from pre-rigor tilapia had significantly higher protein content and yield, reduced cook loss, stronger gel, and improved gel-forming ability. The higher amount of protein recovery possibly occurred due to the compact particles in pre-rigor muscle. This may have helped reduce the release of fine particles and swelling during the water leaching process. Moreover, the results of differential scanning calorimetry showed greater enthalpy of denaturation in surimi prepared from pre-rigor fillets, indicating the protein from pre-rigor surimi was more “native” protein and needed higher energy to denature the protein structure (Park et al., 1989). Xiong and Brekke (1991) and Park et al. (1990) obtained different results for the gel-forming ability by rigor using chicken and tilapia muscle, respectively. Different species may have different rates and ways of protein denaturation during rigor mortis. The sample preparations to refine functional proteins using washing and pH shift are different, which likely affect the results. Thus, it is necessary to find the optimum processing stage (pre- and post-rigor) and further develop their relationship with FPI and surimi. No information regarding the biochemical and gelling properties of FPI prepared using pre- and post-rigor fish has been reported. The objectives of this study were to investigate the gelation behavior
of FPI and surimi made from tilapia processed during pre- and post-rigor stages and further to understand how the two protein refining methods contribute differently to the formation of the fish protein gel.

3.3 Materials and methods

Materials

Live tilapia (*Oreochromis niloticus*), 130 fish with a size of 0.9-1.4 kg, were obtained from a fish farm (Green Hill Gardens Inc., Eugene, OR, USA), and kept in aerated holding tanks (24-26°C) for 36 hr. The fish were removed from the tank and batch-weighed on an electronic scale to determine total weight, and then chill-killed using an ice-water bath. The fish were packed in ice and filleted at different times: immediately after harvest or 120 hr after harvest for the pre- and post-rigor stage, respectively. The fillets were ground using a meat grinder (Electric 2.6 HP 2000 Watt Industrial Meat Grinder, City of Industry, CA, USA) with 4.5 mm perforations.

Ingredients used for gel preparation of surimi or FPI were NaCl (Morton Iodized Salt, Morton International, Inc., Chicago, IL, USA), sugar (Pure Cane Sugar, C&H Sugar Company, Inc., Crockett, CA, USA), and oil (PAM canola oil, ConAgra Food Inc., Omaha, NE, USA). Bradford reagent for protein analysis was purchased from Bio-Rad Laboratory (Hercules, CA, USA). All other chemicals were reagent grade (GR).

Preparation of fish protein isolates

Tilapia mince was homogenized (model GLH-115, PG700, Fisher Scientific, Pittsburgh, PA, USA) with chilled tap water (1:9 ratio) for 1 min at speed level 3. The
homogenates were adjusted to pH 11.0 ± 0.01 using 2N HCl and/or 2N NaOH. Samples were then centrifuged at 8,000 x g for 20 min at 4°C (Sorvall RC-5B, Newtown, CT, USA and Beckman Coulter, Fullerton, CA, USA). The supernatant, separated from neutral lipids, skin, bone, and collagen, was filtered using two layers of cheesecloth. The pH of the filtered supernatant was adjusted to the nominal isoelectric point (pH 5.5) (Ingadottir & Kristinsson, 2010). Aggregated precipitates in four layers of cheesecloth and one layer of screen mesh (2 mm) were dewatered using a washing machine (Kenmore 20022, Kenmore, Chicago, IL, USA) at 700 rpm for 12 min. The pH of the protein isolates was adjusted to approximately 7.0 using 2N NaOH. The protein isolates were mixed with cryoprotectants (5% sorbitol, 4% sugar, and 0.3% sodium tripolyphosphate) using a mixer (VCM 40, Hobart Corp, Troy, OH, USA) for 2 min, placed in a plate freezer overnight (-25°C), vacuum-packed, and stored at -18°C until tested within 2 month. Temperature was maintained below 5°C during processing. Two batches of FPI were made using pre-rigor fish, and two additional batches were made using post-rigor fish.

Preparation of surimi

Ground meat was washed at a 1:2 ratio (mince:cold water) with manual stirring for 10 min. Washed mince was filtered using the screen mesh (2 mm) and dewatering was done manually by repeated pendulum swings. These washing-dewatering steps were repeated once more. The washed mince was then wrapped in two layers of cheesecloth and one layer of screen mesh before dewatering in the washing machine
as described above for preparation of FPI. Lastly, the dewatered meat was mixed with cryoprotectants using the mixer as explained above for preparation of FPI before placing in a plate freezer overnight. Samples were vacuum-packed and stored at -18°C until tested within 2 months. All steps in the preparation of surimi were done in a 4°C cold room or on ice to maintain the temperature below 5°C. Two batches of surimi were made using pre-rigor fish, and two additional batches were made using post-rigor fish.

Paste and gel preparation

Approximately 40 g of frozen surimi or FPI were partially thawed at room temperature for 10 min and cut into small pieces. The sample was chopped at speed level 5 for 1 min with a blender (Osterizer 4172, Sunbeam-Oster Co., Inc., Fort Lauderdale, FL, USA). Chopping continued for 1 min after the addition of 0, 2, and 3% salt, respectively. Before continuing to chop for another 1 min, moisture content was adjusted to 79% using ice based on the moisture content of original FPI and surimi. The sample was then chopped for an additional 2 min for a total chopping time of 5 min. The final temperature of the surimi or FPI paste was less than 10°C. Paste (28.0 g) was molded into a thin sheet using a homemade stainless sheet molding frame (7.5 x, 25.5 x 0.1 cm thick) positioned on aluminum foil sprayed lightly with a lecithin anti-stick coating. The sheet was cooked on a wire rack under steam at 90°C for 20 min. The sheet of gel was held at a room temperature (approximately 20°C) for 5 min
and stored overnight at 4°C. Gel preparation was done twice for each treatment at 3 different salt levels.

Surface hydrophobicity

Using the procedure described by Alizadeh-Pasdar and Li-Chan (2000), protein surface hydrophobicity (S₀) of the supernatant from the paste was determined. The supernatant was collected by homogenizing 3 g of paste and 27 ml of 0.6M KCl in 20 mM Tris-HCl buffer pH 7.0 for 1 min and centrifuging the mixture at 20,000 x g for 30 min at 4°C (Beckman Coulter). An ANS (1-anilinonaphthalene-8-sulfonate) probe was used with the ANS stock solution containing 8 mM ANS in 0.1 M phosphate buffer pH 7.4. Protein concentration was determined by the Bradford dye-binding method with bovine serum albumin (BSA) as purchased. The protein concentration of the supernatant was adjusted to 0.05, 0.1, 0.2, and 0.4 mg/ml using 0.6M KCl in 20 mM Tris-HCl buffer pH 7.0. Then, 20 μl of ANS stock solution was added into 4 ml of samples and held at room temperature for 10 min. The samples were measured at a wavelength of 390 nm and 470 nm (λ_{excitation}, λ_{emission}), respectively, using a luminescence spectrophotometer (PerkinElmer LS-50B, Norwalk, CT, USA). The initial slope (S₀) of the net relative fluorescence intensity versus the protein concentration was used as the protein surface hydrophobicity. The greater slope indicates more unfolded protein structures.

Surface reactive sulphydryl content
Surface reactive sulfhydryl (SRSH) content was determined using Ellman’s reagent (5, 5'-dithiobis (2-nitrobenzoic acid); DTNB) according to the method of Hamada et al. (1994). Salt soluble protein was extracted by homogenizing 3 g of paste with 27 ml of 0.6M KCl in 20 mM Tris-HCl buffer pH 7.0 for 1 min and centrifuging them at 20,000 x g at 4°C for 30 min (Beckman Coulter). The protein concentration of salt soluble protein from the paste was diluted to 1 mg/ml protein with 0.6M KCl in 20 mM Tris-HCl buffer pH 7.0. Diluted sample (0.5 ml) was mixed with 2 ml of 0.6M KCl in 20 mM Tris-HCl buffer pH 7.0, and 50 μl of 0.1M sodium phosphate buffer pH 7.2 containing 10 mM DTNB and 0.2 mM EDTA. The sample was vortexed (Vortex Genie 2, Scientific Industries, INC., Bohemia, NY, USA) and incubated at room temperature for 15 min before reading the absorbance at 412 nm (UV-VIS Spectrophotometer, UV 2401PC, Shimadzu Co., Kyoto, Japan). The SRSH content was calculated based on absorbance using a molar extinction coefficient of 13,600 M⁻¹cm⁻¹ (Hamada et al., 1994).

Oscillatory dynamic measurement

Storage modulus (G’) was measured as a function of temperature using a CVO rheometer (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The paste sample was applied between a cone (4° and 40 mm diameter) and plate with a gap of 150 μm. A trapper with moistened sponge covered the sample during heating to avoid the sample drying out. Temperature sweeps were done using a heating rate of 2°C/min at a fixed frequency of 0.1 Hz. The sample was heated from 20 to 90°C and
the oscillatory mode was applied at a shear stress of 50 Pa. This was within a linear viscoelastic region determined from a stress sweep. Duplicate samples per treatment were run.

Fracture gel evaluation

Cold gels (4°C) wrapped in aluminum foil were kept at room temperature for 2 hr prior to gel testing to allow them to reach at room temperature. The gel sheet was placed in an extensibility fixture with 10 mm (diameter) openings (TA-108s5). Puncture force (g) and puncture distance (mm) were measured using a texture analyzer (TA-XT plus, Texture Technologies Corp, Hamilton, MA, USA) using a spherical probe (5 mm diameter) moving at 1 mm/sec speed. Eight measurements were collected for each treatment.

Raman Spectroscopy

The structural changes of surimi and FPI paste were analyzed following the method of Poowakanjana et al. (2012) using a FT-Raman spectrometer (NXR FT-Raman module, Thermo Scientific Inc., Waltham, MA, USA) at room temperature with the use of the dynamically aligned Vecta-plus interferometer installed in the FT-IR bench (Nicolet 6700, Thermo Scientific Inc.). A glass microscope slide was used to place approximately 0.4 g of sample, and the sample was then placed into the instrument.

Raman spectral data were collected using 1064 nm incident light from the fundamental of a Nd:YLF laser with an InGaAs detector. The setting used was a
spectral resolution of 4.0 cm\(^{-1}\) from 499 to 4002 cm\(^{-1}\), and the final spectra were an average of 64 scans. OMNIC Professional software version 8.3 was used to smooth and analyze the recorded spectra. Each individual spectrum was normalized using the peak near 2933 cm\(^{-1}\) as an internal standard and this peak is attributed to the C-H stretching vibration. Assignments of peaks in the spectra to specific vibrational modes of proteins were made as stated in the published literature as summarized by Ogawa et al. (1999). Four measurements from duplicate samples were collected to obtain the spectra average of spectral data.

Statistical analysis

Response data was subjected to a mixed model in a standard split-split-plot design (PROC MIXED) with contrasts using Tukey’s studentized range (honestly significant difference) test. All statistical analyses were done using the SAS program (SAS\(^{\circledR}\) University Edition, SAS Institute Inc., Cary, NC, USA). Significant level \(\alpha\) was set at 0.05.

3.4 Results and discussion

Surface hydrophobicity

Hydrophobic interactions play a critical role in stabilizing the structure of native proteins. Measurement of protein surface hydrophobicity is valuable, since it can observe any protein structural alternation during different processing methods using different raw materials (Lanier et al., 2014). Surface hydrophobicity was not significantly different between pre- and post-rigor treatments for both FPI and surimi
(Fig. 1). The results of surface hydrophobicity in FPI were in agreement with Thawornchinsombut and Park (2007) and Raghavan and Kristinsson (2008) who demonstrated that FPI made from Pacific whiting and catfish had increased surface hydrophobicity when ionic strength increased. Moreover, surface hydrophobicity of FPI was higher than surimi after the addition of salt. Surface hydrophobicity of FPI was significantly affected by salt addition. This may be attributed to the chemical process in FPI preparation that enhanced the degree of protein unfolding using pH shift.

However, surface hydrophobicity in surimi decreased as salt content increased to 3%. This result was in contrast to other reports in which the addition of salt helped unfold protein structures resulting in an increase of surface hydrophobicity (Lanier et al., 2014; Lin & Park, 1998, Park & Lanier, 1989; Sun & Holley, 2011). This disagreement was possibly because the chopping time (5 min) in the paste preparation was short and/or the final chopping temperature (5-10°C) was probably too low for more thermally stable tropical fish like tilapia. Egelandsdal et al. (1995) indicated an increase in exposed hydrophobic amino acid and sulfhydryl groups may enhance the aggregation of protein network and improve gel qualities during gelation. Thus, it is important to disintegrate protein structure prior to gelation.

Poowakanjana and Park (2013) suggested optimum chopping for threadfin bream, which is most common tropical fish for surimi, could be achieved by continuously chopping surimi until the temperature reached between 25 and 30°C. They also illustrated the thermal sensitivity of protein is closely related to fish habitat temperature. Runglerdkriangkrai et al. (1999), Sano et al. (1994), and Benjakul et al.
(2001) reported that surface hydrophobicity of carp, flying fish, and bigeye snapper started to increase at temperatures above 30°C. Tilapia could possibly behave similar to other tropical species. The subsequent study will soon clarify the effect of chopping temperature.

Figure 3.1 – Surface hydrophobicity ($S_0$) of fish protein isolate (FPI) and surimi paste prepared from pre- and post-rigor tilapia at various salt concentrations. Error bars represent the standard deviation of 2 determinations. Different letters (A–E) on the bars indicate a significant difference at $P < 0.05$. Values not sharing a letter are significantly different. Fpre: FPI from pre-rigor fish. Fpost: FPI from post-rigor fish. Spre: Surimi from pre-rigor fish. Spost: Surimi from post-rigor fish.

Surface reactive sulphydryl content

The denaturation of proteins can be studied by measuring the concentration of SRSH, and SRSH groups are considered to be the most reactive functional group in proteins (Runglerdkriangkrai et al., 1999). No significant effect of rigor was found
between pre- and post-rigor treatments for both FPI and surimi (Figure 3.2). The SRSH content in FPI was significantly higher (P < 0.05) than in surimi for each respective salt concentration. Yongsawatdigul and Park (2004) and Nurkhoeriyati et al. (2011) suggested that SH-groups become more reactive on FPI made from alkali treatment compared to FPI prepared from acid treatment. Buried sulfhydryl groups were exposed more as the chemical process in FPI preparation enhanced the degree of unfolding. Therefore, alkaline solubilization was used since oxidation of sulfhydryl groups could occur and form disulfide bonds during acid solubilization (Yongsawatdigul & Park, 2004).

The SRSH content in FPI at 3% salt was significantly higher (P < 0.05) compared to FPI at 0 and 2% salt. On the other hand, no significant effect of salt addition was noted for surimi. Lin and Park (1998) studied the effect of KCl concentration on the SRSH of salmon myosin and suggested that the myosin began to denature when the ionic linkages of myosin were disrupted by salt. Considering the results of Poowakanjana et al. (2015a) and Sano et al. (1994), the chopping time and temperature for surimi in the current study may be a reason for the similar SRSH content at different salt concentrations.
Figure 3.2 – Surface reactive sulphydryl (SRSH) content of fish protein isolate (FPI) and surimi paste prepared from pre- and post-rigor tilapia at various salt concentrations. Error bars represent the standard deviation of 2 determinations. Values not sharing a same letter are significantly different (P < 0.05). Abbreviations are same as Figure 3.1.

Oscillatory dynamic measurement

The change of protein conformation and the nature of thermal denaturation and subsequent association can be monitored by testing the viscoelastic properties. The storage modulus (G’) can be referred to the elastic property and a higher G’ describes the nature of gel-like (elastic) samples. The rheograms (Figure 3.3A-C) showed surimi prepared from post-rigor fish showed lower G’ compared to surimi made from pre-rigor fish throughout the gelation process. These results were consistent with Park et al. (1990), who studied the effect of the state of rigor on the gel forming ability using tilapia muscle. However, G’ in FPI during gelation were similar between pre- and post-rigor treatments when 2 or 3% salt was incorporated. When no salt was
added, G’ of pre-rigor FPI was significantly higher than post-rigor FPI up to approximately 70°C.

Kristinsson and Hultin (2003a) found that major changes occurred with myosin during FPI processing, such as denaturation, dissociation, and aggregation of the muscle proteins. This could lead to the similar protein structures in FPI regardless of rigor stage. There was a distinctive difference in G’ during heating between FPI and surimi because chemical treatment in FPI induced unfolding and refolding. Similar curves were observed by Kristinsson and Liang (2006) and Ingadottir and Kristinsson (2010) using Atlantic croaker and tilapia muscles, respectively.

The paste transformation from sol to gel can occur when the temperature begins to rise, causing the onset of G’. This point is helpful to understand the stability of proteins, and the higher onset of G’ represents the higher stability of protein structure (Egelandsdal et al., 1986). The increase of onset temperature by the addition of salt was observed in surimi. The first increase of G’ around 42 and 43°C until peaking around 46 and 48°C with 2 and 3% salt in surimi, respectively indicating the formation of gel-like structure which is attributed to cross-linking of myosin (Ingadottir & Kristinsson, 2010).

The delayed onset by salt addition, even though clearly demonstrated in this study, is not in agreement with previous reports (Lanier et al., 2014; Poowakanjana et al., 2015a; Sun & Holley, 2011). Authors again speculate the difference is likely due to incomplete chopping of tropical surimi. Conversely, the decrease of onset temperature was observed in FPI as salt increased. It is important to speculate that extending chopping time and increasing chopping temperature can decrease the onset
temperature of G’ (Poowakanjana et al., 2012). This result correlated with the results from surface hydrophobicity and the SRSH content: thus, suggesting a more unfolded structure of protein in FPI requires a lower temperature and a lower salt content to further unfold and form an ordered gel.
Figure 3.3 – Storage modulus (G’) of fish protein isolate (FPI) and surimi paste prepared from pre- and post-rigor tilapia as affected by temperature sweep at 0, 2, and 3% salt (A, B, and C, respectively). PreF: FPI from pre-rigor fish. PostF: FPI from post-rigor fish. PreS: surimi from pre-rigor fish. PostS: surimi from post-rigor fish.

Fracture gel evaluation
There was no significant difference in puncture force and puncture distance between pre- and post-rigor FPI for each respective salt content (Figure 3.4A & 3.4B). However, puncture force in surimi prepared from pre-rigor fish at 0% salt was significantly higher (P < 0.05) compared to surimi processed from post-rigor fish (Figure 3.4A). Likewise, puncture distance in surimi made from pre-rigor fish at 0 and 2% salt were significantly higher (P < 0.05) than surimi using post-rigor treatment (Figure 3.4B).

In the present study, puncture force and puncture distance decreased as salt content increased in surimi while force and distance increased by the addition of salt in FPI. These data differed from Kim and Park (2008) and Perz–Mateos et al. (2004) who demonstrated the negative effects of salt on gelation of alkali-treated FPI. As the authors used frozen surimi and frozen stabilized mince as the raw material to make FPI, fresh fish used for the preparation of FPI in this study might have been a contributing factor to the different results.

Both puncture force and puncture distance in FPI were higher at 2% salt compared to 3% salt in the present study. An increase in exposed hydrophobic amino acids exposed and sulfhydryl groups buried is believed to enhance the aggregation of the protein network and improve gel qualities (Egelandsdal et al. 1995).
Figure 3.4 – Puncture force (A) and puncture distance (B) of fish protein isolate (FPI) and surimi gel prepared from pre- and post-rigor tilapia at various salt concentrations. Error bars represent the standard deviation of 8 determinations. Values not sharing a same letter are significantly different (P < 0.05). Abbreviations are same as Figure 3.1.

Raman spectroscopy
The higher intensity of the peak at $I_{1448}$ suggested an increase in hydrophobic interaction of aliphatic residues. (Herrero, 2008). Although there was no significant difference in hydrophobic interaction of aliphatic residues between pre- and post-rigor treatments in both FPI and surimi paste, surimi prepared from pre-rigor fish showed higher intensity of $I_{1448}$ than surimi prepared from post-rigor fish (Figure 3.5). The Raman band at 1448 cm$^{-1}$ in surimi at 0% salt was significantly lower (P < 0.05) compared to surimi at 2 and 3% salt, respectively. This may be due to the effect of relatively short chopping time (5 min) and relatively low final chopping temperature (5-10°C) in the present study.

Poowakanjana et al. (2012) demonstrated a significant decrease in the intensity of $I_{1450}$ when chopping time of threadfin bream was extended from 6 to 21 min. Furthermore, extending chopping time of Alaska pollock and Pacific whiting at cold temperature did not influence the hydrophobic interaction of the protein. Ogawa et al. (1999) found structural changes in actomyosins (AMs) isolated from tilapia were less sensitive to heating at 40°C than lemon sole, ling cod, and rockfish AMs. Therefore, it is likely that higher final temperature and ionic strength and also longer chopping time are necessary for decreasing hydrophobic interaction in tilapia proteins. However, a slight decrease at $I_{1448}$ was observed in FPI as salt concentration increased. It was postulated that this was because protein structures in FPI were different from surimi, and hydrophobic interaction of aliphatic residues may easily be separated by the higher ionic strength.

Secondary structural changes were estimated by examining the amide I region (1645-1685 cm$^{-1}$) and the amide III region (1220-1350 cm$^{-1}$) shown in Table 3.1. The
amide I mode corresponds to amide carbonyl C=O stretching, with partial involvement of C-N stretching and N-H bending. The amide III mode mainly assigns to C-N stretching, N-H bending, and C-C stretching. Amide I band near 1650-1660 cm\(^{-1}\) and amide III band near 1260-1350 cm\(^{-1}\) correlate with \(\alpha\)-helix content, and Amide I band near 1662-1668 cm\(^{-1}\) and amide III band near 1230-1240 cm\(^{-1}\) contribute to \(\beta\)-sheet structure (Tadpitchayangkoon et al., 2010b). The values are slightly low at 3% salt, since random coil may start to form by adding more salt (Herrero, 2008).

Secondary structure fractions estimated from amide bands in FPI paste were similar to previously reported values (Thawornchinsombut et al., 2006). In general, \(\alpha\)-helical structures were more intense than \(\beta\)-sheet structures in surimi paste. The results in surimi were in agreement with Poowakanjana et al. (2012) who stated chopping time and temperature did not affect the majority of secondary protein structure.

Both FPI and surimi showed a slight increase in the amount of \(\beta\)-sheet structure as the salt content increased. The alternation of \(\alpha\)-helix to \(\beta\)-sheet and/or random structure may occur during setting or the gelation process (Barrett et al., 1978; Bouraui et al., 1997; Poowakanjana et al., 2012). However, an increase in the content of \(\beta\)-sheet and random coil structures may also form from some unknown structures. Moreover, the results in surimi at different concentrations of salt differ from the results of Barrett et al. (1978) and Bouraoui et al. (1997) who found a slight decline in
α-helical content after the addition of salt. This may be due to tilapia proteins having different thermal stability and intermolecular interactions involving ionic strength.

Figure 3.5 – The Raman band at 1448 cm$^{-1}$ of fish protein isolate (FPI) and surimi paste prepared from pre- and post-rigor tilapia at various salt concentrations. Error bars represent the standard deviation of 4 determinations. Values not sharing a same letter are significantly different ($P < 0.05$). Abbreviations are same as Figure 3.1.
Table 3.1 – Normalized intensity at selected regions of Raman spectra of fish protein isolate (FPI) and surimi paste prepared from pre- and post-rigor tilapia at various salt concentrations.

<table>
<thead>
<tr>
<th>Peak assignment</th>
<th>Wavenumber (cm⁻¹)</th>
<th>Pre-rigor FPI</th>
<th>Post-rigor FPI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Salt 0%</td>
<td>Salt 2%</td>
</tr>
<tr>
<td>CH2 bending</td>
<td>1448</td>
<td>1.09 ± 0.02 a</td>
<td>1.03 ± 0.08a</td>
</tr>
<tr>
<td>Amide I (α-helix)</td>
<td>1648</td>
<td>0.65 ± 0.07</td>
<td>0.75 ± 0.05</td>
</tr>
<tr>
<td>Amide I (β-sheet)</td>
<td>1668</td>
<td>0.63 ± 0.08</td>
<td>0.70 ± 0.08</td>
</tr>
<tr>
<td>Amide III (α-helix)</td>
<td>1336</td>
<td>0.67 ± 0.04</td>
<td>0.83 ± 0.01</td>
</tr>
<tr>
<td>Amide III (β-sheet)</td>
<td>1236</td>
<td>0.64 ± 0.03</td>
<td>0.78 ± 0.01</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Peak assignment</th>
<th>Wavenumber (cm⁻¹)</th>
<th>Pre-rigor surimi</th>
<th>Post-rigor surimi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Salt 0%</td>
<td>Salt 2%</td>
</tr>
<tr>
<td>CH2 bending</td>
<td>1448</td>
<td>0.96 ± 0.04 a</td>
<td>1.24 ± 0.03 b</td>
</tr>
<tr>
<td>Amide I (α-helix)</td>
<td>1648</td>
<td>0.70 ± 0.02</td>
<td>0.83 ± 0.07</td>
</tr>
<tr>
<td>Amide I (β-sheet)</td>
<td>1668</td>
<td>0.51 ± 0.04</td>
<td>0.74 ± 0.05</td>
</tr>
<tr>
<td>Amide III (α-helix)</td>
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<td>0.75 ± 0.02</td>
<td>0.90 ± 0.11</td>
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<tr>
<td>Amide III (β-sheet)</td>
<td>1236</td>
<td>0.66 ± 0.06</td>
<td>0.87 ± 0.17</td>
</tr>
</tbody>
</table>

Mean from 4 determinations. Values within a row having different superscripts are significantly different (P < 0.05).
3.5 Conclusion

The results suggested pre-rigor processing may improve gel-formation properties of surimi, but had little effect on the gelation of FPI. The inclusion of 2% salt into FPI improved gel strength, although the addition of 3% salt into FPI decreased G’ as well as gel strength. However, the addition of salt did not enhance gelation properties in tilapia surimi, possibly due to the chopping conditions in the present study. Therefore, the degree of protein unfolding prior to gelation is an important factor to improve gel qualities. Additional study is in progress to verify the effect of chopping time and temperature for tilapia FPI and surimi.
CHAPTER 4

BIOCHEMICAL AND PHYSICAL CHARACTERIZATIONS OF FISH PROTEIN ISOLATE AND SURIMI PREPARED FROM FRESH AND FROZEN WHOLE FISH

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4.1 Abstract

The effect of frozen storage on the biochemical and physical characterization of fish protein isolate (FPI) and surimi made from tilapia was elucidated: tilapia frozen for 3 mo can be used like fresh fish in the processing of FPI and surimi. The Ca\textsuperscript{2+}ATPase activity continuously reduced throughout the frozen storage up to 3 mo; however, the diminishing trend of Ca\textsuperscript{2+}ATPase activity was slow. According to storage modulus (G’), storing whole fish frozen for 3 mo did not affect the gelling ability of FPI and surimi with 0% salt. The comparable results were observed by surface hydrophobicity, surface reactive sulfhydryl content, and differential scanning calorimetry. The addition of salt into FPI induced higher degrees of denaturation before gelation compared to surimi. Our results suggested that frozen tilapia, if stored up to 3 mo, can be used to make good quality FPI and surimi.
4.2 Introduction

Two procedures have been successfully utilized to refine fish proteins: fish protein isolate (FPI) and surimi. However, the processes of each are quite different. The FPI method induces chemical unfolding of fish proteins to solubilize both myofibrillar and sarcoplasmic proteins, recovers these soluble proteins at the isoelectric point by centrifugation, and then the unfolded proteins are refolded by neutralization (Hultin & Kelleher, 2000). The conventional surimi process, however, avoids or minimizes chemical and physical denaturation to obtain good surimi quality and primarily recovers myofibrillar proteins.

In general, fresh fish are used for FPI and surimi production. Freshness of fish is considered one of the decisive factors for the quality and stability of the fish protein gel. Storing fish in a freezer for extended periods of time can negatively affect the gel-forming ability of muscle proteins (Okazaki, & Kimura, 2013; Benjakul, Visessanguan, Thongkaew, & Tanaka, 2005). The quality of surimi gels made from frozen hoki, Alaska pollock, and lizardfish determined by MacDonald, Lelievre, and Wilson (1992); Scott, Porter, Kudo, Miller, and Koury (1988); and Kurokawa (1979), respectively, demonstrated a declining trend as frozen storage induced denaturation.

Factors affecting gel-forming ability during frozen storage are fish species, freezing methods, storage temperature, and enzymatic degradation (Ang & Hultin, 1989; Badii & Howell, 2001; Hsieh & Regenstein, 1989, Benjakul, Visessanguan, Thongkeaw, & Tanaka, 2003). Benjakul et al. (2005) found that, under extended frozen storage, lizardfish was most susceptible to denaturation and loss in gelation compared to threadfin bream, bigeye snapper, and croaker. Results indicated the
formaldehyde level in lizardfish muscle was the highest among these four tropical species, which contributed to increased protein denaturation.

Denaturation of fish proteins during frozen storage is affected by various factors: partial dehydration of protein during freezing; an increase in inorganic salts in the frozen phase; interaction of lipids, free fatty acids, and/or products from lipid oxidation with proteins; and trimethylamine oxide demethylase (TMAOase) activity (Benjakul & Visessanguan, 2010). These reactions lead to significant reduction in gel-forming ability. However, frozen seafood products are imperative since fish products are one of the most highly traded food commodities. If frozen fish could be used for FPI or surimi production, logistics for raw fish handling would be much easier. No information exists on fish protein gels made from FPI and surimi using fresh and frozen whole fish of the same species. The purpose of this study was to evaluate the relationship of the biochemical and physical properties of fish protein gels prepared using differently refined proteins (FPI and surimi) from fresh and frozen tilapia.

4.3 Materials and methods

Materials

Live tilapia (Oreochromis niloticus) with a size of 0.9–1.4 kg were obtained from Green Hill Gardens Inc. (Eugene, OR, USA). Whole fish were packed in ice for 120 h in a cold room (4 °C). A half was used as fresh fish and the other half was stored in a -18 °C freezer for 1 and 3 mo and used as frozen fish. To process frozen fish, the fish
were thawed in the cold room for 36 h, filleted, and ground using a meat grinder (Electric 2.6 HP 2000 Watt Industrial Meat Grinder) with 4.5 mm perforations before subjecting to two refining processes. Ingredients used for gel preparation of surimi or FPI were NaCl (Morton Iodized Salt, Morton International, Inc., Chicago, IL, USA), phosphate blend (SD BNI CO., LTD., Ansan-shi, Kyeonggi-do, Korea), sugar (Pure Cane Sugar, C&H Sugar Company, Inc., Crockett, CA, USA), and sorbitol (sorbitol NF/FCC, Roquette American Inc., Gurnee, IL, USA). Bradford reagent for protein analysis was purchased from Bio-Rad Laboratory (Hercules, CA, USA). All other chemicals were reagent grade.

Measurement of Ca-ATPase activity

Actomyosin (AM) was extracted according to the method of Benjakul et al. (2005) with slight modifications. A filleted sample (8 g) was mixed with 80 mL of 0.6 M KCl (4°C, pH 7.0) and homogenized at speed 5 for 4 min (Tissue Tearor Homogenizer, BioSpec Products Inc., Bartlesville, OK, USA). The homogenized solution was centrifuged at 5,000 x g at 0°C for 30 min. The supernatant was filtered through two layers of cheesecloth and three volumes of chilled deionized water were added. Then, AM was collected by centrifuging at 9,000 x g at 0°C for 20 min. The pellet was recovered and stirred in an equal volume of 1.2 M KCl (4°C, pH 7.0) for 20 min. After stirring, the suspension was centrifuged at 9,000 x g at 0°C for 20 min.

ATPase activity was measured using the AM solution at a concentration of 2.0 mg/mL in 0.6 M KCl (pH 7.0) (Benjakul, Seymour, Morrissey, & An, 1997). One quarter mL of 0.5 M Tris-maleate (pH 7.0), 0.25 mL of 0.1 M CaCl$_2$, and 3.75 mL of
deionized water were added to the 0.5 mL of AM solution. To this mixture, 0.25 mL of 20 mM ATP solution was added to start the reaction and then incubated for 8 min at 25°C. The reaction was stopped by the addition of 2.5 mL chilled 15% trichloroacetic acid. The concentration of inorganic phosphate released during incubation was measured using a UV – VIS spectrophotometer (UV – 2401PC spectrophotometer, Shimadzu Corp., Kyoto, Japan) described by Fiske and Subbarow (1925). The ATPase specific activity was presented as μ mole inorganic phosphate (Pi) released/mg protein/min. Both pH and ATPase activity were measured in triplicate from three different fish. All steps in the preparation of samples were performed in a 4°C cold room or by keeping samples in ice in order to maintain temperature below 5°C.

Preparation of fish protein isolates

Fish mince, prepared above (pH 6.9 at 0 mo, 6.8 at 1 mo and 6.7 at 3 mo frozen storage), was homogenized (model GLH-115, PG700, Fisher Scientific, Pittsburgh, PA, USA) with chilled tap water (1:9 ratio) for 1 min at speed level 3. The pH of the homogenates was adjusted to 11.0 ± 0.01 using 2N NaOH. Samples were then centrifuged at 8,000 x g at 4 °C for 20 min (Sorvall RC-5B, Newtown, CT, USA and/or Beckman Coulter, Fullerton, CA, USA). After centrifugation, two layers of cheesecloth were used to filter the supernatant from neutral lipids, skin, bone, and connective tissues. The pH of the filtered supernatant was adjusted to the isoelectric point (pH 5.5) using 2N HCl. Once the pH was adjusted, aggregated precipitates were wrapped in four layers of cheesecloth and one layer of screen mesh (2 mm) before
being dewatered by using a washing machine (Kenmore 20022, Kenmore, Chicago, IL, USA) at 700 rpm for 12 min. The pH of the protein isolates was adjusted to approximately 7.0 using 2N NaOH.

Preparation of surimi

Ground meat was washed by manually stirring at a 1:2 ratio (mince:cold water) for 10 min. Washed mince was filtered using a screen mesh and dewatering was manually performed by repeated pendulum swings. These washing-dewatering steps were repeated once. Then, two layers of cheesecloth and the above mentioned screen mesh was used to dewater the washed mince in the washing machine as described above for FPI.

Freezing FPI and surimi with cryoprotectants

Fish protein isolates and surimi, independently, were mixed with cryoprotectants (5 g/100g sorbitol, 4 g/100g sugar, and 0.3 g/100g of a mixture (50:50) of sodium tripolyphosphate and tetrasodium pyrophosphate), and placed in a plate freezer overnight (-25°C). Samples were vacuum-packed and stored at -18 °C until tested. All steps were conducted in a 4°C cold room or in ice in order to maintain a temperature below 5°C. Two batches of FPI and surimi were made using whole fish frozen for 0, 1, or 3 mo.

Paste preparation
A frozen FPI or surimi block (approximately 50 g) was partially thawed at room temperature for 10 min and cut into small pieces. The sample was chopped using a blender (Osterizer 4172, Sunbeam-Oster Co., Inc., Fort Lauderdale, FL, USA) at speed level 5 for 1 min. Once the sample was chopped for 1 min, 0, 2, and 3 g/100g salt for three treatments, respectively, were added before chopping for an additional 1 min. Before continuing to chop for another 1 min, moisture content was adjusted to 79% (g/100g) using ice/cold water. The sample was then chopped until the total chopping time reached 5 min. The final temperature of the FPI or surimi paste was less than 10°C. The paste was used for biochemical and physical analyses, such as surface hydrophobicity, surface reactive sulfhydryl content, oscillatory dynamic measurement, and differential scanning calorimetry.

Surface hydrophobicity

Protein surface hydrophobicity (S₀) of the supernatant from the paste was measured according to the method described by Alizadeh-Pasdar and Li-Chan (2000) with slight modification. An ANS (1-anilinonaphathalene-8-sulfonate) probe was used with the ANS stock solution containing 8 mM ANS in 0.1 M phosphate buffer pH 7.4. Protein concentration of the supernatant was adjusted to 0.05, 0.1, 0.2, and 0.4 mg/mL using 0.6M KCl in 20 mM Tris-HCl buffer pH 7.0. ANS stock solution (20 µL) was then added to four milliliters of diluted samples and incubated at room temperature for 10 min. A wavelength of 390 nm and 470 nm (λ_excitation, λ_emission), respectively, was used to measure the samples by a luminescence spectrophotometer.
(Perkin Elmer LS-50B, Norwalk, Conn., USA). The initial slope ($S_0$) of relative fluorescence intensity versus protein concentration was calculated by linear regression analysis and used as the protein surface hydrophobicity.

Surface reactive sulfhydryl content

The procedure of Hamada, Ishizaki and Nagai (1994) with slight modification was followed to determine surface reactive sulfhydryl (SRSH) content using Ellman’s reagent (5, 5’-dithiobis (2-nitrobenzoic acid); DNTB). Salt soluble protein (SSP) was measured from the paste, and the protein concentration of the SSP supernatant was diluted to 1 mg/mL protein with 0.6M KCl in 20 mM Tris-HCl buffer pH 7.0. Diluted sample (0.5 mL) was mixed with 2 mL of 0.6M KCl in 20 mM Tris-HCl buffer pH 7.0, and 50 µL of 0.1M sodium phosphate buffer (pH 7.2) containing 10 mM DTNB and 0.2 mM EDTA. The sample was vortexed and held at room temperature for 15 min before reading the absorbance at 412 nm (UV-VIS Spectrophotometer, UV 2401PC, Shimadzu Co., Kyoto, Japan). The SRSH content was calculated based on absorbance using a molar extinction coefficient of 13,600 M$^{-1}$cm$^{-1}$.

Oscillatory dynamic measurement

Heat-induced gelation of FPI and surimi was measured through temperature sweep using a Bohlin CVO-100 rheometer (Malvern Instruments Ltd., Worcestershire, UK). The paste sample was placed between a cone (4° and 40 mm diameter) and plate leaving a gap of 150 µm. Sample drying during heating was prevented by covering
the sample using a trapper with a moistened sponge. A stress sweep was conducted using a range of 1 to 1500 Pa at 20°C to determine a linear viscoelastic range and, based on the results; shear stress was set at 50 Pa during temperature sweep testing. The sample was heated from 20°C to 90°C at a heating rate of 2°C/min and a fixed frequency of 0.1 Hz.

**Differential scanning calorimetry**

FPI and surimi pastes obtained from various storage times were analyzed in duplicate by a micro differential scanning calorimeter (Micro DSC III, Setaram Inc., Lyon, France). Transition temperatures and enthalpy were observed. Naphthalene was used to calibrate the instrument for temperature accuracy. De-ionized water was used in a reference stainless steel sample vessel, and approximately 500 mg of sample paste was accurately weighed in a stainless steel vessel. The sample was scanned over a temperature range of 20-80°C at 1°C/min.

**Statistical analysis**

Response data was subjected to a mixed model in a standard split-split-plot design (PROC MIXED) with contrasts using Tukey’s studentized range (honestly significant difference) test. SAS program (SAS Institute Inc., Cray, NC, USA) was used to perform all statistical analyses. Significant level α was set at 0.05.

**4.4 Results and discussion**
Ca-ATPase activity

The Ca$^{2+}$ATPase activity continuously decreased from 43.3 (0 mo) to 38.6 (1 mo) and 34.8 μmol Pi/mg protein/min (3 mo) during extended frozen storage. Previous studies suggested the change in Ca$^{2+}$ATPase activity can be utilized as an indicator for the integrity of the myosin molecule (Dey & Dora, 2011; Benjakul et al. 2003, 2005). A decline in activity associated with the increase in frozen storage time represented the denaturation of myosin, specifically in the head region (Ochiai & Chow, 2000; Benjakul & Bauer, 2000). Thus, it is assumed that the rearrangement of protein through protein–protein interaction in myosin during frozen storage corresponded to the loss in Ca$^{2+}$ATPase activity.

In the present study, the degree of diminishing Ca$^{2+}$ATPase activity was slower than other findings in croaker (Dey & Dora, 2011), as well as in Japanese jack mackerel, chub mackerel, and silver jellyfish (Khan, Hossain, Hara, Osatomi, Ishihara, & Nozaki 2003). In addition, Benjakul et al. (2003) found Ca$^{2+}$ATPase activity of NAM in lizard fish was less stable compared to croaker, threadfin bream, and bigeye snapper during frozen storage. Hence, the time dependence of freeze-denaturation of fish myofibrillar protein varies among species. This is correlated with intrinsic factors, such as protein native structures and concentration of denaturation-inhibiting substances. Extrinsic features can also affect the Ca$^{2+}$ATPase activity, including temperature of frozen storage (Wang, Xiong, & Srinivasan, 1997), the addition of cryoprotectants (Khan et al., 2003; Li, Kong, Xia, Liu, & Li, 2013), and the method of packaging during extended frozen storage (Leelapongwattana, Benjakul, Visessanguan, & Howell, 2005). It is presumed that tilapia, with the procedure used
in our study, was able to maintain stable protein structures and did not drastically
denature the structures; consequently, only a slight decrease of Ca\(^{2+}\)ATPase activity
was detected during frozen storage.

Surface hydrophobicity

Surface hydrophobicity of both FPI and surimi paste was not significantly
different among the 3 different frozen storage times (Figure 4.1). Results were in
disagreement with other studies in which an increase of surface hydrophobicity due to
unfolding of protein structures was observed as frozen storage time increased
(Benjakul et al., 2003; Leelapongwattana, et al., 2005; Li et al., 2013). This may be
due to species specific and different sample preparation methods. However, similar
results to the present study were found when surimi was prepared from frozen beef
heart or frozen deboned duck meat (Wang et al., 1997; Parkington, Xiong, Blanchard,
& Xiong, 2000; Ramadhan, Huda, & Ahmad, 2012). Thus, our results suggested that
tilapia muscle proteins remained stable during frozen storage.

Another reason for the thermal stability of tilapia muscle during frozen storage
may be related to TMAOase activity. Careche and Li-Chan (1997) reported the
presence of formaldehyde (FA) during frozen storage of cod myosin led to more
extensive protein denaturation resulting in more interactions between hydrophobic
groups and the formation of covalent bonds. The content of TMAO and the activity of
TMAOase are likely to be low in tilapia (Sotelo & Rehbein, 2000); possibly
stabilizing tilapia during frozen storage in a similar way as mammal muscles.
Surface hydrophobicity in FPI at 3% salt was significantly higher than FPI at 0% and 2% salt, respectively (Figure 4.1). These results are consistent with previous studies (Thawornchinsombut & Park, 2007; Raghavan & Kristinsson, 2008) that evaluated the effect of ionic strength on surface hydrophobicity from FPI made from Pacific whiting and catfish, respectively. FPI may be more susceptible to chopping temperature and time compared to surimi, since FPI was refined through chemical unfolding and refolding of protein structures.

Results of the present study showed that surface hydrophobicity in surimi decreased as salt content increased. Previous publications stated the addition of salt during chopping aided unfolding of protein structures causing increased surface hydrophobicity (Lanier, Yongsawatdigul, & Carvajal-Rondanelli, 2014). Different outcomes in the present study may be due to chopping time and temperature. As mentioned in previous paragraphs, tilapia is probably more thermally stable similar to other tropical species. An increase in surface hydrophobicity could be obtained when chopping was done at high temperature for a longer period (Poowakanjana & Park, 2013).
Surface hydrophobicity ($S_0$) of fish protein isolate (FPI) and surimi paste prepared from fresh and frozen whole tilapia at various salt concentrations. Error bars represent the standard deviation of at least 2 determinations. Different letters (A–D) on the bars indicate a significant difference at $P < 0.05$. 0mF: FPI prepared from fresh tilapia. 1mF: FPI prepared from tilapia frozen for 1 mo. 3mF: FPI prepared from tilapia frozen for 3 mo. 0mS: Surimi prepared from fresh tilapia. 1mS: Surimi prepared from tilapia frozen for 1 mo. 3mS: Surimi prepared from tilapia frozen for 3 mo.

Surface reactive sulfhydryl content

The surface reactive sulfhydryl (SRSH) content in FPI was consistent among the 3 different frozen storage times and was significantly higher than surimi (Figure 4.2). This may result from the chemical process of pH shift used during FPI refining. Like the previous report (Youngsawatdigul & Park, 2004) that SH groups become more reactive when FPI was prepared by alkali treatment than acid treatment, a significantly higher SRSH in FPI was also observed.
SRSH content did not show a significant effect in surimi paste as frozen storage time of whole tilapia increased (Figure 4.2). However, early research conducted on ling cod (Sultanbawa & Li-Chan, 2001), Greenland halibut (Lim & Haard, 1984), common carp (Lim & Haard, 1984), and lizardfish (Leelapongwattana et al., 2005) reported frozen storage reduced SH groups. The decrease in SRSH content was considered to be due to the formation of disulfide bonds through the oxidation of SH groups or disulfide interchanges (Sultanbawa & Li-Chan, 2001). This disparity was likely due to the difference in species and sample preparation between the two studies.

Wang et al. (1997) reported the SRSH content of beef heart surimi was consistent after 12 weeks of frozen storage. Endoo and Yongsawatdigul (2014) showed an increase in SRSH content of surimi from silver carp, Nile tilapia, and rohu for up to 9 mo of frozen storage. Although an increase in SRSH content was observed, indicating unfolding of actomyosin, the formation of disulfide bonds induced by protein denaturation and aggregation probably did not occur while whole tilapia was stored in a freezer for 3 mo. Therefore, our results suggested tilapia muscle proteins was thermally stable during frozen storage.

SRSH content in FPI at 3% salt was significantly higher (P<0.05) than FPI at 0% or 2% salt, respectively. In contrast, the effect of salt addition in surimi was lower. Lanier et al. (2014) stated myosin began to denature and SRSH content of myosin increased when the ionic linkage of myosin was disrupted by salt. The chopping time and temperature for surimi in the current study may affect the similar SRSH content at different salt concentrations (Poowakanjana & Park, 2013).
Figure 4.2 – Surface reactive sulphydryl (SRSH) content of fish protein isolate (FPI) and surimi paste prepared from fresh and frozen whole tilapia at various salt concentrations. Error bars, different letters, and abbreviations on each bar indicate the same as those in Figure 4.1.

Oscillatory dynamic measurement

Fig. 4.3A-I showed the thermal gelation profiles of FPI and surimi gels in terms of the thermal behavior of the $G'$ storage modulus (Fig. 4.3A-C), $G''$ loss modulus (Fig. 4.3D-F), and phase angle (Fig. 4.3G-I). The rheograms of FPI with 0% salt were similar among three different frozen storage periods. However, $G'$ above 55°C of FPI with 3% salt was slightly lower as frozen storage increased, even though $G''$ of FPI samples showed a similar trend among three different frozen storage periods. This suggested that the addition of salt may negatively affect the gelation as frozen storage of FPI increased.

$G'$, $G''$, and phase angle in surimi with 0% salt was similar among the different frozen storage times. This observation was in contrast to Lin, Chen, and Chen (2005)
in which G’ of surimi made from horse mackerel decreased as frozen storage periods of whole fish increased. A possible explanation for no effect of surimi processing from fresh or frozen whole fish on G’ is that tilapia may be thermally stable during 3 mo of frozen storage and does not suffer major protein structure changes. Moreover, Parkington et al. (2000) found that the gelling capacity from beef heart surimi improved during 12 mo of frozen storage. Thus, producing surimi from 3 mo frozen-stored tilapia without adverse effects on gel qualities is possible. It was also noted surimi with 3% salt indicated higher G’ at 0 mo compared to 1 or 3 mo, although G” was similar among the different storage periods. This trend was an opposite of FPI and suggested that the addition of salt improved the gelation behavior as frozen storage of surimi increased.

There was a distinctive difference between FPI and surimi during heating. Hultin and Kelleher (2000) revealed major changes occurred with myosin during FPI processing, such as denaturation, dissociation, and aggregation of the muscle proteins. This pH-shifting treatment in FPI induced unfolding followed by refolding, which may be responsible for the resemblance of rheograms in FPI without salt and the difference of rheograms in surimi among different storage periods.

The onset of G’ is a point where the temperature begins to rise due to the transformation from paste to gel and the higher onset of G’ represents the higher stability of the protein structure (Ingadottir & Kristinsson, 2010). The onset of G’ in surimi with 0% salt was lower than FPI with 0% salt (Fig. 4.3A). Conversely, a steady increase of G’ in surimi with 2% and 3% salt was observed at higher temperatures (around 55 °C) compared to FPI with 2% and 3% salt (Fig. 4.3B&C).
The results of phase angle (Fig. 4.3G-I) demonstrated that surimi with 0% salt was the only sample that showed a phase angle lower than 20° at 20°C, indicating a solid-like characteristic. This indicated that surimi with 0% salt caused the higher onset of $G'$. Furthermore, the onset of gelation, where phase angle starts dropping, was similar between FPI and surimi samples with salt added (near 40°C) (Fig. 3H, I). Several studies have demonstrated a similar trend for FPI and surimi at different salt concentrations (Kristinsson, & Liang, 2006; Thawornchinsombut & Park, 2007). It is postulated that the effect of salt addition into FPI induced protein aggregation as if pH-induced unfolding and salt addition would make proteins behave like salting-out and form a weaker gel. Proteins in FPI were unfolded during pH-shift (even though they were partially refolded back as the pH neutralized) prior to gelation, and their gel formation started earlier than surimi (Poowakanjana & Park, 2014). Also, results from phase angles showed that all FPI samples had lower than 30° at 20°C, indicating a solid-like structure. However, the onset of gelation was lower in FPI compared to surimi, when salt was added. This implies that FPI structure was more unfolded compared surimi to prior to gelation. The changes in phase angle during heating were more notable in surimi compared to FPI (Fig. 3G-I). The smaller changes in phase angle in FPI were possibly because FPI was more unfolded compared to surimi prior to gelation and therefore, less protein structural changes during heating.
Figure 4.3 – Storage modulus (G’) (A–C), loss modulus (G”) (D–F), and phase angle (δ) (G–I) of fish protein isolate (FPI) and surimi paste prepared from fresh and frozen whole tilapia as affected by temperature sweep at 0%, 2%, and 3% salt (A–C, respectively). Abbreviations on each treatment indicate the same as those in Figure 4.1.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a useful tool to monitor protein denaturation and aggregation. A shift to lower transition temperature characterizes destabilization of protein structures, lowering the energy required to denature the proteins. Also, the disappearance of specific peaks denotes structural destabilization of protein constituents (Kim, Hamann, Lanier, & Wu, 1986). The changes in the thermal stability of FPI and surimi made from fresh and frozen whole fish are presented in Table 4.1.

FPI showed one major peak at all three salt concentrations. Transition point and peak temperature shifted to lower temperature as a result of salt addition. The
endothermic patterns of FPI for all storage treatments were similar, exhibiting no difference in transition points and peak temperature regardless of raw materials used: fresh or frozen fish. This result was consistent with the results from surface hydrophobicity, SRSH content, and oscillatory dynamic measurement.

Surimi exhibited three major transition peaks at 0% salt. When 2% and 3% salt were added to the surimi, only two endothermic peaks were observed. Similarly, Kim et al. (1986) reported the reduction of endothermic peaks from four to three from Alaska pollock after the addition of 3% sodium chloride. Moreover, the enthalpies of denaturation at 2% and 3% salt showed a slight decrease, which signified structural destabilization of proteins as the salt concentration increased. However, the transition point and peak temperature were not significantly different as the frozen storage of whole fish extended to 3 mo. These results showed that utilizing frozen whole tilapia to produce surimi did not seem to have a greater effect on the transition temperature and the enthalpies of denaturation, while salt addition had a larger impact.
Table 4.1 – Effect of salt (0-3%) on peak temperatures (T1–T3) and enthalpies (ΔH1–ΔH3) of fish protein isolate (FPI) and surimi paste prepared from fresh and frozen whole tilapia. Abbreviations indicate the same as those in Figure 4.1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Parameters</th>
<th>T1 (°C)</th>
<th>ΔH1 (J/g)</th>
<th>T2 (°C)</th>
<th>ΔH2 (J/g)</th>
<th>T3 (°C)</th>
<th>ΔH3 (J/g)</th>
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<tr>
<td>0mF 0% salt</td>
<td></td>
<td>52.2</td>
<td>0.52</td>
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<td></td>
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<tr>
<td>0mF 2% salt</td>
<td></td>
<td>45.2</td>
<td>0.15</td>
<td></td>
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<tr>
<td>0mF 3% salt</td>
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<td>45.2</td>
<td>0.14</td>
<td></td>
<td></td>
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<tr>
<td>1mF 0% salt</td>
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<tr>
<td>3mF 0% salt</td>
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<td>0.66</td>
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4.5 Conclusion

The gel quality of FPI and surimi made from fresh tilapia were not significantly better than that made from frozen fish stored for 3 mo. Our results suggested tilapia frozen for 3 mo can be successfully used as a raw material for both FPI and surimi production without sacrificing gelling quality. As gel-forming ability is dictated by the degree of protein denaturation, FPI was more sensitive to salt addition compared to surimi.
CHAPTER 5

PHYSICOCHEMICAL CHARACTERIZATIONS OF TILAPIA FISH PROTEIN ISOLATE UNDER TWO DISTINCTIVELY DIFFERENT COMMINUTITION CONDITIONS

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5.1 Abstract

The effect of two distinctively different comminution conditions on biochemical, rheological, and microscopic characterization of fish protein isolate (FPI) and surimi prepared from tilapia was investigated. A significant increase in puncture gel texture was observed when FPI and surimi were chopped at 25°C for 18 min compared to samples chopped at 5°C for 6 min. Comparable results were observed as measured by storage modulus (G’) in oscillatory dynamic rheology. FPI chopped with 3% salt at 5°C for 6 min exhibited the lowest gel texture among all treatments. This treatment was represented well by larger protein aggregates and coarser gels in microscopic analyses and correlated well with puncture test results. This is the first study to report the gelling property of FPI as affected by comminution conditions and suggests that controlling chopping temperature and time is important to produce high quality gels from FPI, like from surimi.
5.2 Introduction

Understanding the factors affecting gelation properties is necessary to improve the qualities of gelled protein products. Comminution is one of the critical steps to maximize protein extraction and to combine other ingredients homogenously into the paste (Jones and Mandigo 1982; Colmenero et al. 1996; Boles et al. 1998; Fukushima et al. 2007; Poowakanjana and Park 2014). Understanding the thermal stability of fish proteins is also a perquisite in order to optimize production techniques. Previous studies demonstrated the gel forming behavior of surimi is altered by chopping temperature, chopping duration, and salting time. These studies also showed protein functionality depends on species and thermal sensitivity of these proteins is closely related to fish habitat temperature (Douglas-Schwarz and Lee 1988; Fukushima et al. 2005; Hemung et al. 2008; Poowakanjana and Park 2013). Esturk et al. (2004) and Poowakanjana et al. (2012) investigated the effect of chopping conditions on gelation properties of surimi from various species, such as cold water species Alaska pollock (AP), temperate water species Pacific whiting (PW), and warm water species big eye snapper (BE), lizardfish (LZ), and threadfin bream (TB). Results indicated the highest gel strength of AP and PW were obtained at 0°C chopping for 15 min and at 15-20°C for 15 min, respectively. The optimum chopping temperature for BE and LZ was 20°C while the recommended chopping method for TB was to continuously chop surimi until the temperature reached between 25 and 30°C.

Surimi processing is one of the most effective commercial methods to refine fish proteins. Unlike surimi, which has over 900 years of history, originating from ancient
Japan, fish protein isolate (FPI), which is processed differently to refine fish proteins, has less than 2 decades of research and commercialization. FPI can also be made from underutilized raw materials, such as pelagic species and by-products from fish processing. Structural changes occurring during comminution and cooking of FPI and surimi are different because refining methods are chemically opposite with regards to protein denaturation. FPI is refined through chemical denaturation followed by refolding using pH-shifting and centrifugation while myofibrillar protein is concentrated as surimi by avoiding protein denaturation (Hultin and Kelleher 2000).

Protein gels prepared using FPI from several species have shown similar and/or better gelation properties compared to those produced from surimi (Hultin and Kelleher 2000; Choi and Park 2002; Undeland et al. 2002; Ingadottir and Kristinsson 2010;). However, the effect of salt on gelation properties of FPI and surimi are different. In surimi, salt contributes to improved gelation, while for FPI the effect of salt addition on gelation varies. Kim and Park (2008) demonstrated the negative role of NaCl on gel forming ability of FPI prepared from Alaska pollock surimi. Their results showed FPI obtained higher gel texture when NaCl was not added during chopping. The addition of NaCl may induce protein aggregation since FPI is already chemically unfolded during extraction. The addition of salt then contributes to low quality gel products similar to the salting-out effect when excess salt is added to denature protein. Conversely, Perez-Mateos et al. (2004) reported FPI gels made from Atlantic croaker chopped with NaCl obtained higher gel texture than conventional surimi with NaCl. Varied results, with respect to salt addition, may be correlated with species and/or different methods used for gel preparation.
As mentioned earlier, two of the most important variables affecting protein quality in gel preparation are temperature and time control. However, little information exists on chopping parameters for FPI, and there is no comparative study between FPI and surimi prepared using the same species. Comparing the functionality of protein in gelation between FPI and surimi prepared with optimum comminution procedures may enhance the effective utilization of fish proteins refined from the two different methods. Therefore, the objective of the present study was to elucidate the gelation properties of FPI and surimi made from tropical fish tilapia as affected by comminution temperature and time.

5.3 Materials and methods

Materials

Tilapia (*Oreochromis niloticus*), with a size of 1.0 – 1.5 kg, were obtained from a fish farm (Green Hill Gardens Inc., Eugene, OR, USA). Fresh fish were placed in an ice cooler before delivering to the Oregon State University Seafood Research and Education Center (Astoria, OR, USA) and stored in ice in a cold room (4°C). Ingredients used for gel preparation of FPI or surimi were NaCl (Morton Iodized Salt, Morton International, Inc., Chicago, IL, USA), phosphate blend Polymix GA (SD BNI CO., LTD., Hwaseong-si, Kyeonggi-do, Korea), sugar (Pure Cane Sugar, C&H Sugar Company, Inc., Crockett, CA, USA), and sorbitol (sorbitol NF/FCC, Roquette America Inc., Gurnee, IL, USA). Bradford reagent for protein analysis was purchased from Bio-Rad Laboratory (Hercules, CA, USA). All other chemicals were reagent grade.
Preparation of fish protein isolate

FPI was prepared according to Kristinsson et al. (2014) with a slight modification. Fresh tilapia stored in ice, 28-32 h postharvest, was filleted and ground using a meat grinder (Electric 2.6 HP 2000 Watt Industrial Meat Grinder, City of Industry, CA, USA) with 3.0 mm perforations. Mince was washed with chilled water (1:5 ratio) for 1 min by gentle stirring and storing for 2 min. Then, the floating fat was removed and washed mince was mixed for 5 min using a mixer (BIG STIX™ Immersion Blenders WSB50, Waring Commercial, Torrington, CT, USA) at speed level 7. The ground meat was filtered using two layers of screen mesh (2 mm in diameter) to eliminate connective tissues before the filtered ground meat was homogenized (model GLH-115, PG700, Fisher Scientific, Pittsburgh, PA, USA) for 2 min at speed level 3. The homogenates were adjusted to pH 11.0 ± 0.05 using 2M NaOH to solubilize myofibrillar and sarcoplasmic proteins. Then, the pH of the samples was adjusted to the isoelectric point (pH 5.5) using 2M HCl while gently stirring. Aggregated precipitates were drained using two layers of screen mesh (2 mm in diameter) and dewatered by repeated pendulum swings before being dewatered by a screw press dehydrator (model SD–8, Ikeuchi Tekkosho, Sano, Japan) at speed 5. Lastly, the pH of the protein isolates was adjusted to approximately 7.0 using 2N NaOH.

Preparation of surimi

Using fresh tilapia from the same batch used for FPI, tilapia mince was obtained by grinding fillets using a meat grinder with 4.5 mm perforations. The ground meat
was washed by gentle stirring with cold tap water (3-5°C) for 10 min at a mince/water ratio of 1:2. Washed mince was filtered and dewatered by repeated pendulum swings using a screen mesh (2 mm). The washing-dewatering process was repeated. The washed mince was subjected to a strainer (model S1, Ikeuchi Tekkosho, Sano, Japan) to refine myofibrillar proteins from connective tissues and pin bones. The washed mince was then placed in the screw press dehydrator, the same used for FPI processing, at speed 5 and dewatered.

Freezing fish protein isolate and surimi with cryoprotectants

Cryoprotectants (5% sorbitol, 4% sugar, and 0.3% of a mixture (50:50) of sodium tripolyphosphate and tetrasodium pyrophosphate) were mixed into FPI and surimi using a mixer (VCM 40, Hobart Corp, Troy, OH, USA) for 2 min, packed in a plastic container (approximately 500-600 g) before placing in a plate freezer (-25°C) overnight. Vacuum-packed samples were stored at -18°C until tested. During the preparation of FPI and surimi, the samples were kept in a 4°C cold room or on ice to ensure the temperature was maintained below 10°C. Two batches of FPI and surimi were produced, respectively.

Paste and gel preparation

Approximately 800 g of frozen FPI or surimi block was partially thawed at room temperature for 40 min, allowing the core temperature to reach about –5°C. Partially thawed blocks were cut into small cubes (3-5 cm). Two different chopping (5°C for 6
min and 25°C for 18 min) were conducted at room temperature using a chopping bowl equipped with circulating coolant set for −10°C and without circulating coolant, respectively. These two chopping conditions were chosen: first, low chopping temperature condition (5°C for 6 min) chosen as it is universally used for FPI or surimi.; second, the higher chopping temperature and longer chopping time (25°C for 18 min) was used as tilapia’s habitat temperature was about 25°C. Previous studies (Poowakanjana et al. 2013, 2014, 2015; Esturk et al., 2004) confirmed that protein functionality depends on species, and thermal sensitivity of these proteins is closely related to fish habitat temperature. In addition, Poowakanjana et al. (2014, 2015) demonstrated that chopping at longer time (18-21 min) can help unfold protein structures, especially for tropical species.

Cubes were chopped for 1 min using a silent cutter (UM 5 universal, Stephan Machinery Corp, Columbus, OH, USA) at 1,800 rpm. Two different salt concentrations (0% and 3%) were added before chopping for an additional 1 min. After chopping for 2 min total, moisture content was adjusted to 78% using ice water. Sugar was added to compensate for the salt content in order to maintain protein concentrations equally between 0 and 3% salt treatments. Poowakanjana and Park (2014) reported that sugar neither enhances nor diminish gelation as it was used as an inert ingredient. After chopping for another 1 min, the chopping bowl was then connected to a vacuum pump (40–60 kPa). The sample was chopped at 3,600 rpm until chopping time totaled 6 min for low chopping temperature and 18 min for high chopping temperature.
FPI and surimi paste were packed into a polyethylene bag and subjected to a vacuum machine (Reiser VM-4142; Roescher Werke, Osnabrueck, Germany) in order to eliminate air pockets introduced during packing. A sausage stuffer was then used to stuff the paste into metal tubes (3.0 cm I.D. and 25.3 cm length). The sample was cooked at 90°C for 30 min and submerged in ice water to cool for 15 min. Gels were refrigerated (4°C) overnight. Gel preparation was performed twice for each treatment at 2 different salt concentrations (0% and 3%).

Surface hydrophobicity

Protein surface hydrophobicity ($S_0$) of the supernatant from the paste was determined using an ANS (1-anilinonaphthalene-8-sulfonate) probe according to the procedure of Alizadeh-Pasdar and Li-Chan (2000) with slight modification. The stock solution of 8 mM ANS was prepared in 0.1 M phosphate buffer (pH 7.4). The protein concentration of supernatant was serially diluted with 0.6 M KCl in 20 mM Tris-HCl buffer (pH 7.0) from 0.01 to 0.2 mg/ml. Four ml of each diluted sample were mixed with 20 μl of ANS stock solution. After incubation at room temperature for 10 min, the relative fluorescence intensity (RFI) of each solution was measured using a wavelength of 390 nm and 470 nm ($\lambda_{\text{excitation}}, \lambda_{\text{emission}}$), respectively by a luminescence spectrophotometer (Perkin Elmer LS-50B, Norwalk, Conn., USA). The initial slope ($S_0$) of RFI versus protein concentration was calculated by linear regression analysis and used as an index of protein surface hydrophobicity.
Surface reactive sulfhydryl content

Surface reactive sulfhydryl (SRSH) content was measured using Ellman’s reagent (5, 5’-dithiobis (2-nitrobenzoic acid); DNTB) as described by Hamada et al. (1994) with slight modification. Paste was solubilized in a solution of 0.6 M KCl in 20 mM Tris-HCl buffer (pH 7.0), and the protein concentration of the supernatant was adjusted to 1 mg/ml using the same solution. Two ml of 0.6 M KCl in 20 mM Tris-HCl buffer (pH 7.0) and 50 μL of 0.1M sodium phosphate buffer (pH 7.2) containing 10 mM DTNB and 0.2 mM EDTA were added to 0.5 ml of the diluted sample. The sample was then vortexed and held at room temperature for 15 min before reading absorbance at 412 nm (UV-VIS Spectrophotometer, UV 2401PC, Shimadzu Co., Kyoto, Japan). The SRSH concentration was calculated based on absorbance using a molar extinction coefficient of 13,600 M⁻¹cm⁻¹.

Rheological measurements

The rheological properties of FPI and surimi pastes were monitored as a function of temperature using dynamic oscillation with a CVO rheometer (Malvern Instruments Ltd., Worcestershire, UK). A cone (4° and 40 mm diameter) and plate arrangement with a gap of 150 μm was used in this study. A trapper with a moistened sponge inside was applied to prevent moisture evaporation during measurements. A stress sweep was conducted to determine a linear viscoelastic region, and shear stress of 50 Pa was selected for temperature sweep testing. Storage modulus (G’) and phase
angle (δ) were measured from 20°C to 90°C at a heating rate of 2°C/min at a fixed frequency of 0.1 Hz.

Fracture gel evaluation

The day after cooking, gels were removed from refrigerated storage and set at room temperature for 2 h before fracture analysis. The gels (3 cm diameter) were cut into 25-mm long pieces and subjected to a penetration test using a texture analyzer (TA-XT plus, Texture Technologies Corp, Hamilton, MA, USA) with a spherical probe (5 mm diameter) at 1 mm/s. Ten measurements were collected for each treatment.

Color analysis

A CIE Lab color scale was used to determine L*, a*, and b* values of gels from 25 mm of samples using a Minolta colorimeter (CR-310; Minolta Camera Co. Ltd., Osaka, Japan). Using the procedure described by Kim and Park (2008), a Minolta calibration plate and a Hunter Lab standard hitching tile were used to standardize the instrument, and whiteness was calculated using the equation L* – 3b*.

Scanning electron microscopy

All gel samples were fixed for 24 h and rinsed in sodium cacodylate. Osmium tetroxide (2 g/100 ml) was then used to stain samples for 1 h. Samples were rinsed in water three times for 10 min each before being dehydrated in acetone with serial dilutions of 30, 50, 70, 90, and twice in 100% (v/v) followed by critical point drying.
Dried samples were sputter-coated with gold and palladium (60:40 ratio) and observed in a Quanta 600 FEG field emission scanning electron microscope (FEI Inc., Hillsboro, OR, USA). This microscopy work was conducted at the Oregon State University Electron Microscope Facility (Corvallis, OR, USA).

Transmission electron microscopy

Gels were fixed for 24 h and rinsed in a 0.1 mol/l sodium cacodylate buffer containing 2.5 g/100 ml glutaraldehyde and 1 g/100 ml paraformaldehyde. Samples were then stained in 2 g/100 ml osmium tetroxide for 1 h and rinsed three times in water for 10 min each. Samples were incubated with 0.2 g/100 ml tannic acid for 10 min, 1 g/100 ml osmium tetroxide for 1 h, 1 g/ml thiocarbohydrazide for 20 min, 1 g/100 ml osmium tetroxide for 60 min, 0.5 g/100 ml uranylacetate in 25 g/100 ml methanol for 2 h, and lead aspartate for 30 min. All incubation steps were performed at room temperature and samples were rinsed with water after each incubation step. Subsequently, samples were dehydrated in a graded series of acetone (10, 30, 50, 70, 90, 95, and twice in 100%). Samples were then infiltrated with Araldite resin (3:1, 1:1, 1:3, and twice in 100% resin), and polymerized overnight at 60 °C. Thin trapezoidal sections (200 μm W x 200 μm L by 50–90 nm thick) were prepared by a microtome. The specimens were viewed using a FEI Titan 80-200 TEM/STEM with ChemiSTEM Technology (FEI Inc., Hillsboro, OR, USA). This microscopy work was conducted at the Oregon State University Electron Microscope Facility (Corvallis, OR, USA).
Statistical analysis

Data were evaluated using a mixed model in a standard split-split-plot design (PROC MIXED) with contrasts using Tukey’s studentized range (honestly significant difference) test. Statistical analysis was performed using the SAS program (SAS Institute Inc., Cary, NC, USA). Significant level was set at 0.05, and two different batches were made for each treatment.

5.4 Results and discussion

Surface hydrophobicity

Protein surface hydrophobicity is a useful test to measure protein structural alterations during different processing methods, since hydrophobic interactions play a crucial role in stabilizing the structure of native proteins. Surface hydrophobicity in FPI was significantly higher (p < 0.05) than surimi among all treatments (Figure 5.1). In agreement with other studies (Kristinsson and Hultin 2003a; Chaijan et al. 2006; Thawornchinsombut and Park 2007), surface hydrophobicity increased as the chemical process of pH shift unfolded protein structures during FPI refining.

A significant increase (p < 0.05) in surface hydrophobicity was observed when FPI were chopped at 25°C for 18 min compared to chopping at 5°C for 6 min. Poowakanjana et al. (2015a) demonstrated the degree of protein unfolding in surimi was affected differently by chopping time and temperature, and salting time. Marcos et al. (2010) also showed denaturation of sarcoplasmic protein by increasing pressurization temperatures. Our results confirmed that chopping temperature and time could influence changes of protein conformation in FPI. Moreover, the present
study showed the addition of 3% salt in FPI increased surface hydrophobicity significantly \( (p < 0.05) \). Similar results were observed in FPI prepared from catfish (Raghavan and Kristinsson 2008) and Pacific whiting (Thawornchinsombut and Park 2007). Bombrum et al. (2014) evaluated protein-protein interactions in pork muscle by measuring the number of hydrophobic sites. Their results suggested that increased surface hydrophobicity was caused by salt and mechanical action, such as tumbling period.

One of the important aspects of protein network aggregation related to gel quality is the exposition of hydrophobic amino acids (Egelandsdal et al. 1995). The mechanism for modifying surface area could be attributed to changes of particle size. Smaller particle size can migrate more easily within the protein network and increase its reactivity (Orliac et al. 2002; Sun et al. 2011a; Sun et al. 2011b). This suggested that higher chopping temperature and longer chopping time, in addition to the homogenization used in FPI refining, may enhance protein unfolding in paste prior to gelation and increase protein inter-molecular interactions during gelation in FPI.
Figure 5.1 – Surface hydrophobicity ($S_0$) of fish protein isolate (FPI) and surimi paste prepared from tilapia at various salt concentrations (0% and 3%) and comminution conditions (5°C for 6 min and 25°C for 18 min). Error bars represent the standard deviation of at least 2 determinations. Different letters (A–G) on the bars indicate significant difference at $p < 0.05$. F5: FPI chopped at 5°C for 6 min. F25: FPI chopped at 25°C for 18 min. S5: Surimi chopped at 5°C for 6 min. S25: Surimi chopped at 25°C for 18 min.

Surface reactive sulfhydryl content

Figure 5.2 illustrates the surface reactive sulfhydryl (SRSH) contents of samples. Sulhydryl groups are considered to be the most reactive functional groups in proteins. FPI, particularly when refined through alkaline treatment, can promote oxidation of sulhydryl groups and result in the formation of disulfide groups (Kim et al. 2003; Nurkhoeriyati et al. 2011). In fact, SRSH contents in FPI paste was significantly higher ($p < 0.05$) than surimi paste for all treatments. This was probably because the chemical process in FPI preparation enhanced the degree of protein unfolding (Ingadottir and Kristinsson 2010) and increased surface hydrophobicity.
The addition of NaCl had a significant effect (p < 0.05) on SRSH contents in both FPI and surimi. An increase in SRSH content in surimi by adding salt and increasing chopping temperature was also found in other studies (Sano et al. 1994; Poowakanjana et al. 2015a). Lanier et al. (2014) and Lin and Park (1998) stated myosin began to denature and the SRSH content of myosin increased as the ionic linkage of myosin was disrupted by salt. It was interesting to note that the SRSH content in FPI without salt significantly increased (p < 0.05) when chopped at higher temperature for longer time. However, the opposite trend was seen in FPI with 3% salt and the decrease in SRSH content was observed when chopped at the higher temperature for longer time. These results could be attributed to the formation of new disulfide bonds because alkaline pH treatment and 3% salt probably induced unfolded proteins to aggregate, like the salting-out effect. Similar findings with pork muscle and fish water-soluble proteins were also reported (Iwata et al. 2000; Bombrun et al. 2014).

Shiku et al. (2005) studied biodegradable films prepared from myofibrillar proteins of blue marlin flesh, and they found that the important chemical bonds involved in protein-protein interactions were disulfide bonds and hydrophobic interactions in alkaline conditions. In addition, Omana et al. (2010b) reported a positive correlation between myofibrillar protein hydrophobicity and free sulphydryl content, in which protein unfolding exposed more SH groups at the protein surface. In the current study, the SRSH content decreased when chopped with 3% salt at 25°C for 18 min as salt and high temperature caused the acceleration of the protein
disruption and the subsequent formation of disulfide bonds (Sano et al. 1994; Iwata et al. 2000; Bombrun et al. 2014; Poowakanjana et al. 2015a).

Figure 5.2 – Surface reactive sulphydryl (SRSH) content of fish protein isolate (FPI) and surimi paste prepared from tilapia at various salt concentrations (0% and 3%) and comminution conditions (5°C for 6 min and 25°C for 18 min). Error bars, different letters, and abbreviations on each bar indicate the same as those in Figure 5.1.

Oscillatory dynamic rheology

Testing the viscoelastic properties can illustrate the change of protein conformation in thermal denaturation and association (Kim et al. 2003). Exploring oscillatory dynamic properties of fish proteins as affected by different refining methods and by various comminution conditions reveals the nature of thermal denaturation and subsequent association. The rheograms of FPI and surimi during
heating were quite different (Figure 5.3a and b). Major changes occurred with myosin during FPI processing: denatured, disassociated, and aggregated (Hultin and Kelleher 2000). The changes of storage modulus (G’) from FPI followed similar patterns as observed by Ingadottir and Kristinsson (2010). G’ patterns of surimi with 3% salt showed different patterns, particularly in denaturation of light meromyosin (around 30–40°C) compared to other species, such as Alaska pollock or Pacific whiting (Fukushima et al. 2005; Poowakanjana et al. 2012). There was a slight peak, indicating light meromyosin denatured around 30–40°C, but the peak was smoother than other species. This may be because tilapia proteins have high thermal stability and the degree of unfolding of light meromyosin may be slower or less compared to cold or temperate water species. In FPI treatments with 3% salt, there was no sign of light memromyosin due to chemical denaturation during the refining process.

The shape of the rheograms between FPI and surimi without salt around 50°C was different: FPI showed a V-shape and surimi showed a U-shape. FPI exhibited lower G’ than surimi around 50°C, indicating more viscous, and this may explain that protein particles in FPI could easily migrate, entangle each other, and form protein networks. Hence, this may lead to a faster change in G’ in FPI than surimi.

The effect of chopping conditions on G’ for FPI and surimi were also different after 55°C. FPI treatments clearly showed the effect of chopping temperature and time, but not in surimi. This was probably because the differences in protein structures prior to comminution. The protein structures of FPI were denatured and refolded during the refining process. On the other hand, the native stage was
maintained for the protein structures of surimi. This may have caused a difference in sensitivity to chopping conditions.

Figures 5.3c and 5.3d show the phase angle of FPI and surimi heated from 20 to 90°C. The phase angle can be expressed as tan-δ (G’’/G’) and is useful to determine the thermal transition of the sample from a sol-like (viscous) to a gel-like (elastic) state (Reed and Park 2011a). Distinctive differences of the phase angle were observed between FPI and surimi, and the changes in phase angle inversely corresponded to the changes in G’.

FPI paste prepared with 3% salt at 25°C for 18 min demonstrated lower G’ at 20°C than samples prepared at 5°C for 6 min, denoting the paste chopped at higher temperature was more viscous. Conversely, higher G’ was detected in FPI chopped at 25°C for 18 min when gelation was completed beyond 75°C compared to samples chopped at 5°C for 6 min. These observations may be related to higher thermal stability of tilapia. Comparable results were presented by Fukushima et al. (2007) and Poowakanjana et al. (2012, 2015a), which demonstrated chopping temperature and time affected denaturation of the myosin tail region.

The phase angles of surimi with 3% salt at 20°C were significantly higher than FPI with 3% salt for both comminution conditions, indicating proteins in surimi were more salt-sensitive and became more viscous (Figure 5.3c-d). However, significantly higher (p < 0.05) phase angle was observed at 20°C when FPI paste was chopped at 25°C for 18 min compared to FPI paste chopped at 5°C for 6min. Therefore, our results suggested that chopping FPI made from tilapia at a higher temperature for a
longer time may help unfold protein structures when salt was added than when salt was not added. The increase in surface hydrophobicity and SRSH contents correlates to increased protein unfolding as demonstrated by lower G’ and higher phase angle at 20°C.

The onset of G’ is the point where the temperature initiates an increase in G’ as thermally unfolded proteins start to aggregate. The lower the onset temperature for G’ implies the lower thermal stability of the protein. G’ onset can also be used to determine the extent of protein unfolding or aggregation (Ingadottir and Kristinsson 2010). In the present study, the onset of G’ from FPI with 3% salt was lower than FPI with 0% salt. This may indicate that chopping FPI with salt enhanced thermal unfolding and subsequent aggregation. A similar trend was also observed for surimi chopped with 3% salt.

The phase angle of FPI and surimi with 3% salt showed a slight increase around 40°C and 48°C, respectively, possibly indicating a partial rupture of the semi-gel being formed. These results were similar to other studies conducted using tilapia myosin (Reed and Park 2011a), salmon myosin (Reed and Park 2011b), and Pacific whiting myosin (Yongsawatdigul and Park 1999). Lower onset temperature for FPI as compared to surimi is likely due to chemical denaturation occurring earlier during FPI preparation.

It was noted that the continued decrease of the phase angle (forming firmer gel texture) of tilapia started at a higher temperature than Pacific whiting myosin due to the thermal stability of tilapia proteins. Different patterns of phase angle between FPI and surimi without salt addition were also observed. A transition of phase angle in
FPI without salt started as the temperature rose beyond 25°C, perhaps demonstrating the release of free water from FPI paste. Phase angle then remained constant until temperature reached around 39°C. Continued heat induced structure rupture, possibly releasing a large quantity of trapped water when phase angle was 33–35 degree. The transition of phase angle in FPI without salt was larger (around 13 to 35 degree phase angle) compared to surimi without salt (around 14 to 26 degree phase angle). This is probably because proteins in FPI were structurally changed during the pH shift.
Figure 5.3 – Storage modulus ($G'$) and phase angle ($\delta$) of fish protein isolate (FPI) (a, c) and surimi (b, d) paste prepared from tilapia as affected by temperature sweep at various salt concentrations (0% and 3%) and comminution conditions (5°C for 6 min and 25°C for 18 min). Abbreviations are same as those in Figure 5.1.

Fracture analysis
A significant increase ($p < 0.05$) in breaking force (Figure 5.4a) and penetration distance (Figure 5.4b) was observed when FPI and surimi gels were chopped at 25°C for 18 min compared to chopping at 5°C for 6 min. Results suggested the optimum chopping condition for FPI and surimi made from tropical fish tilapia may be obtained by chopping at a higher temperature for a longer period of time. These results were in agreement with surimi research done by Douglas-Schewarz and Lee (1988), Esturk et al. (2004), and Poowakanjana et al. (2012, 2015a). They demonstrated that protein thermostability of each species determined the optimum chopping condition. In other words, whether fish proteins are in the form of surimi or FPI, optimum final chopping temperature is recommended to be relatively close to the fish habitat temperature with chopping time long (15-20 min) for tropical species and short (5-10 min) for cold water species using 1500-3500 rpm. In addition, this may be correlated to results from surface hydrophobicity and SRSH content. An increase in exposed hydrophobic amino acids and sulfhydryl groups may enhance the aggregation of the protein network and improve gel qualities during gelation (Egelandsdal et al. 1995).

FPI chopped at 5°C for 6 min with 3% salt showed slightly lower breaking force than FPI chopped at 5°C for 6 min without salt, while no change for penetration distance was observed. Similarly, previous studies (Kim and Park 2008) showed the addition of salt in alkali-treated FPI from Alaska pollock adversely influenced gel qualities. However, Perez-Mateos et al. (2004) reported a significant increase of
breaking force when salt was added to FPI made from Atlantic croaker. Salt sensitivity in FPI appeared to be different based on the thermal stability of species. The present study indicated the inclusion of 3% salt in FPI from tropical fish tilapia significantly (p < 0.05) improved gel-forming ability when chopped at a higher temperature for longer time. Degree of fish protein unfolding during pH shifting likely varies by species as sensitivity against salt changes in ascending order of tropical fish tilapia, temperate water fish Atlantic croaker, and cold water fish Alaska pollock. Chaijan et al. (2006) demonstrated differences in protein integrity and bonding could affect gel-forming ability. Therefore, it is postulated that chopping FPI prepared from tropical fish tilapia with salt may need higher temperature and longer chopping time to finely denature protein structure for the formation of a more ordered gel network.
Figure 5.4 – Breaking force (a) and penetration distance (b) of fish protein isolate (FPI) and surimi gel prepared from tilapia at various salt concentrations (0% and 3%) and comminution conditions (5°C for 6 min and 25°C for 18 min). Error bars represent the standard deviation of at least 10 determinations. Different letters and abbreviations on each bar indicate the same as those in Figure 5.1.

Color measurement

Color is one of the important quality factors for consumer acceptance. Whiteness in FPI was significantly lower (p < 0.05) than surimi, although there were no significant differences (p < 0.05) among chopping procedures and salt content (Table 5.1). This was because FPI contained both sarcoplasmic proteins, such as heme proteins and enzymes, and myofibrillar proteins, whereas surimi only contained myofibrillar proteins. Fowler and Park (2015) found that whiteness of Pacific whiting surimi decreased as salmon plasma protein concentration increased. Several authors have also demonstrated the lightness (L*) of FPI, surimi, or meat decreased as the
amount of sarcoplasmic proteins increased (Jafarpour and Gorczyca 2009; Joo et al. 1999; Kim et al. 2005; Marcos et al. 2010). In the present study, L* was significantly lower (p < 0.05) in FPI after 3% salt was added (Table 5.1). This may be due to the oxidation of myoglobin retained in the samples, which possibly yielded brown metmyoglobin.

In FPI, higher chopping temperature and longer chopping time decreased a* (more negative values indicating more red hue) and b* (less yellow hue) (Table 5.1). It is presumed that samples chopped at a higher temperature for longer time may have resulted in smaller particle size and changed the surface area for interaction, specifically in the presence of sarcoplasmic proteins. In addition, Park (1995) studied surimi gel colors prepared from Alaska pollock and Pacific whiting as affected by physical conditions. The results showed that a* and b* of gels decreased as freeze-thaw cycle increased, signifying denaturation of proteins. However, a* and b* of surimi gels in the present study did not decrease by increasing chopping temperature and extending chopping time, except the surimi gel chopped with 3% salt at 25°C for 18 min. It is assumed that tilapia proteins are more thermostable than cold water species and therefore did not affect a* and b* to the same degree as in FPI.
Table 5.1 – Whiteness, L* (lightness), a* (redness), and b* (yellowness) of fish protein isolate (FPI) and surimi gel prepared from tilapia at various salt concentrations (0% and 3%) and comminution conditions (5°C for 6 min and 25°C for 18 min). The standard deviation was obtained from at least 5 determinations. Different letters (a–e) and abbreviations on each column indicate the same as those in Figure 5.1.

<table>
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<th></th>
<th>Whiteness</th>
<th>L*</th>
<th>a*</th>
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<tr>
<td>F0%5C</td>
<td>53.5 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.0 ± 0.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-1.49 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.11 ± 0.23&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>F0%25C</td>
<td>55.6 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.7 ± 0.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-1.66 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.69 ± 0.28&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>F3%5C</td>
<td>56.3 ± 1.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.5 ± 0.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.04 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.48 ± 0.65&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>F3%25C</td>
<td>60.2 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.9 ± 0.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.49 ± 0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.84 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>S0%5C</td>
<td>63.6 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.6 ± 0.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-3.38 ± 0.05&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.65 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>S0%25C</td>
<td>65.2 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>5.43 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>S3%5C</td>
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<td>82.4 ± 0.51&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
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<td>6.20 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
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Scanning electron microscopy

Scanning electron microscopy (SEM) images showed both FPI and surimi gels chopped at 25°C for 18 min (Figure 5.5b, d, f, and h) revealed fewer numbers of voids and more compact structure than gels chopped at 5°C for 6 min (Figure 5.5a, c, e, and g). These results were associated with the results from the puncture test. FPI gel with 3% salt chopped at 5°C for 6 min (Figure 5.5c) had the roughest structure among the samples tested as its fracture gel texture values were also lowest (Figure 5.4). Kim and Park (2008) found that the addition of salt negatively affected the gel properties of FPI from pollock where the paste was prepared at 5°C with 6 min chopping.

However, FPI gel with 3% salt chopped at 25 °C for 18 min (Figure 5.5d) showed a denser gel network comprised of aggregated proteins in the form of samll
globular beads compared to all other treatments. Yin et al. (2014) reported surimi gels with nano-scaled fish bone formed continuous networks, while gels with micro-scaled fish bone particles formed discontinuous porous networks with large pores. Hermansson (1979) demonstrated gel matrixes tend to be rough and coarse when aggregation occurred rapidly. Hence, results indicated chopping FPI at higher temperature for longer time may help reduce particle size of FPI, leading to stronger protein-protein interactions.
Figure 5.5 – Scanning electron microscope image of fish protein isolate (FPI) and surimi gels. a: FPI chopped with 0% salt at 5°C for 6 min (F-0-5-6). b: FPI chopped with 0% salt at 25°C for 18 min (F-0-25-18). c: FPI chopped with 3% salt at 5°C for 6 min (F-0-3-6). d: FPI chopped with 3% salt at 25°C for 18 min (F-3-25-18). e: surimi chopped with 0% salt at 5°C for 6 min (S-0-5-6). f: surimi chopped with 0% salt at 25°C for 18 min (S-0-25-18). g: surimi chopped with 3% salt at 5°C for 6 min (S-3-5-6). h: surimi chopped with 3% salt at 25°C for 18 min (S-3-25-16).
Transmission electron microscopy

In transmission electron microscopy (TEM) images, darker color represents protein-protein interactions, such as association, aggregation, or polymerization (Ryan et al. 2012). Network strands of FPI gels (Figure 5.6a–d) were larger than surimi gels (Figure 5.6e–h) for all treatments. The granular and coarse structure of FPI gels may be due to protein aggregation during FPI processing and also the presence of globular sarcoplasmic proteins. FPI gels chopped at 25°C for 18 min (Figure 5.6b and d) showed smaller protein aggregates and greater homogeneity of protein dispersion compared to FPI gels chopped at 5°C for 6 min (Figure 5.6a and c). The images are correlated well with puncture tests and SEM results.

FPI gels with 3% salt chopped at 5°C for 6 min (Figure 5.6c) exhibited the roughest and largest protein aggregates among all treatments. Comparable results were observed by Ryan et al. (2012), who suggested the association rate of protein aggregates was related to particle size, salt concentration, and protein concentration. Kim et al. (1987) studied surimi gel structures by TEM and the results showed that gel strength increased with increased regularity of the dispersed phase in the gel network. Hence, our results indicated the uniform alignment of disintegrated particles in the gel may correspond to gel strength. In addition, chopping FPI made from tilapia with salt at higher temperature for longer time may help enhance disintegration of proteins and decrease particle size, leading to stronger gel.
Figure 5.6 – Transmission electron microscope image of fish protein isolate (FPI) and surimi gels. Different letters indicate the same as those in Figure 5.5.
5.5 Conclusion

The gel texture of FPI and surimi prepared from tilapia significantly increased (p < 0.05) when chopped at 25°C for 18 min as opposed to chopping at 5°C for 6 min. Our results indicated chopping FPI and surimi from tilapia with salt enhanced gel texture as chopping temperature and time increased. However, a significant decrease of breaking force was observed when FPI was chopped at 5°C for 6 min because tilapia is tropical species. Properly controlled comminution may increase the disintegration of protein structure, decrease particle size, and enhance gelation. Under optimized chopping conditions, cooked gels from two different fish protein extraction methods (FPI and surimi made from tilapia) were comparable. Based on the current study, our future research will evaluate how two different fish proteins obtained from FPI and surimi, respectively, can be mixed with regards to gelation properties.
CHAPTER 6

FT-IR AND RAMAN SPECTROSCOPIES DETERMINE STRUCTURAL CHANGES OF TILAPIA FISH PROTEIN ISOLATE AND SURIMI UNDER DIFFERENT COMMUNUTION CONDITIONS

Yuka Kobayashi, Steven G Mayer, and Jae W. Park

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6.1 Abstract

Tilapia proteins refined by pH shift and conventional water washing were chopped under various comminution conditions and their structural changes were investigated using Fourier transform infrared (FT-IR) and Raman spectroscopies. Both techniques revealed the degree of unfolding in protein structure increased when fish protein isolate (FPI) and surimi were chopped at 25°C for 18 min compared to samples chopped at 5°C for 6 min. Results indicated both hydrophobic interactions and disulfide bonds were significantly enhanced during gelation. FPI and surimi gels prepared after chopping at 25°C for 18 min exhibited higher β-sheet contents and more chemical bonds such as hydrophobic interactions and disulfide bonds than those at 5°C for 6 min. Our results suggested that controlling comminution conditions may be one of the important aspects to improve gel qualities in FPI and surimi from tropical fish like tilapia. Moreover, FT-IR and Raman spectroscopies are useful complementary tools for elucidating the change in the structure of protein during comminution and gelation.
6.2 Introduction

Surimi and fish protein isolate (FPI) are two current refining methods for fish proteins. Surimi is stabilized myofibrillar proteins obtained from deboned fish mince that is washed with cold tap water and mixed with cryoprotectants. FPI is made through maximizing soluble proteins (myofibrillar and sarcoplasmic proteins) using pH-shift and removing insoluble components (stroma proteins and neutral fat) and all soluble proteins are subsequently obtained at the isoelectric point (Hultin & Kelleher, 2000). One of the various factors affecting gel-forming ability is comminution condition (Colmenero, Carrascosa, Barreto, Fernandez, & Carballo, 1996; Fukushima, Okazaki, Fukuda, & Watabe, 2007; Poowakanjana & Park, 2014).

Comminution maximizes protein extraction and homogeneously mixes other ingredients into the paste. Previous publications reported chopping temperature and time, and salting time can impact the quality and stability of the fish protein gels prepared from surimi. The functionality of proteins was closely associated with thermal stability of each species (Douglas-Schwarz & Lee, 1988; Hemung, Li-Chan, & Yongsawatdigul, 2008; Poowakanjana & Park, 2013, 2014). Cold-water species was more susceptible to heat-induced denaturation and aggregation of proteins. Chopping surimi from cold-water species at a relatively higher temperature can easily lead to lower gel texture compared to temperate- or warm-water species. The optimum chopping condition for surimi from warm-water species was to continuously chop surimi until the temperature reached near their habitat temperature between 25–30°C (Esturk, Park, & Thawornchinsombut, 2004; Poowakanjana, Mayer, & Park, 2012; Poowakanjana, Park, & Park, 2015).
Vibrational spectroscopy is useful to investigate the changes on chemical composition and molecular structure of proteins, carbohydrates, nucleic acids, cell membrane, and tissues. Fourier transform infrared absorption spectroscopy (FT-IR) and Raman spectroscopy are complementary techniques that reveal differences among structural macromolecular changes (Flores-Morales, Jiménez-Estrada, & Mora-Escobedo, 2012; Sivam, Sun-Waterhouse, Perera, & Waterhouse, 2013; Yuen, Choi, Phillips, and Ma, 2009). Molecular vibrations are infrared-active when the dipole moment of the molecule changes as the molecule vibrates; whereas, vibrations are Raman-active when the polarizability of the molecule changes as the molecule vibrates. Both techniques can be used to nondestructively analyze, with higher sensitivity, very small amounts of dry and wet samples (Li-Chan, 1996; Sivam et al., 2013).

Several studies used both vibrational spectroscopies in food products and results indicated a better interpretation of the spectra and an increase of the accuracy of the analysis (Flores-Morales et al., 2012; Fontecha, Bellanato, & Juarez, 1993; Pereira, Amado, Critchley, Van de Velde, & Ribeiro-Claro, 2009). Pereira et al. (2009) demonstrated it is possible to identify the principal colloids in ground sea veggie samples with a minimum of handling and treatment using a combination of two vibrational spectroscopic methods (FT-IR-ATR and FT-Raman). This signifies that the composition determined illustrates the native composition of the phycocolloids as precisely as possible. Sivam et al. (2013) showed the complementarity of the two techniques for examining the secondary conformations and structure of gluten proteins and polysaccharides in finished bread systems. Yuen et al. (2009) studied
carboxymethylated non-starch polysaccharides including cellulose, guar gum, locust beam gum, and xanthan gum and compared traditional wet chemistry methods to Raman and FT-IR spectroscopic methods to determine the degree of substitution (DS). Results suggested there is a great potential to replace the use of conventional wet chemistry methods with the application of both Raman and FT-IR spectroscopic methods for measuring DS. In addition, the spectroscopic procedure established in this study was relatively simple and fast, and did not use toxic chemicals, but required only small amounts of sample.

FT-IR and Raman spectroscopy have been used to analyze the structural changes of solid-state FPI and surimi paste and gel under different storage, comminution, and cooking conditions (Bouraoui, Nakai, & Li-Chan, 1997; Cando, Herranz, Borderías, & Moreno, 2016; Herranz, Tovar, Borderías, & Moreno, 2013; Herrero, Carmona, & Careche, 2004; Larrea-Wachtendorff, Tabilo-Munizaga, Moreno-Osorio, Villalobos-Carvajal, & Pérez-Won, 2015; Liu, Hu, Guo, Wang, Sun, & Xu, 2015; Moosavi-Nasab, Alli, Ismail, & Ngadi, 2005; Poowakanjana et al., 2012; Tadpitchayangkoon, Park, Mayer, & Yongsawatdigul, 2010; Thawornchinsombut, Park, Meng, & Li-Chan, 2008). However, a comparative study on the use of both FT-IR and Raman spectroscopy for analyzing structures of the refined fish protein from FPI and surimi has not yet been attempted. Also, there is no information on the structural changes of FPI by different comminution conditions. The combination of both spectroscopic techniques may be valuable to understand the structural changes of proteins during comminution. Hence, the objective of the present study was to investigate how comminution conditions affect the structure of the fish protein paste and their
corresponding gels prepared from FPI and surimi using FT-IR and Raman spectroscopy.

6.3 Materials and methods

Materials

Tilapia (*Oreochromis niloticus*), with a size of 1.0 – 1.5 kg, were obtained from a fish farm (Green Hill Gardens Inc., Eugene, OR, USA). The fish were packed in an ice cooler and transported to the Oregon State University Seafood Research and Education Center (Astoria, OR, USA) where they were stored in a cold room (4°C) and covered with ice until processed. NaCl (Morton Iodized Salt, Morton International, Chicago, IL, USA), phosphate blend Polymix GA (SD BNI CO, Hwaseong-si, Kyeonggi-do, Korea), sugar (Pure Cane Sugar, C&H Sugar Company, Crockett, CA, USA), and sorbitol (sorbitol NF/FCC, Roquette America, Gurnee, IL, USA) were used as ingredients for producing FPI or surimi and preparing their gels. All other chemicals were reagent grade.

Preparation of fish protein isolate

Tilapia fillets, 28-32 h postharvest, were ground with a meat grinder (Electric 2.6 HP 2000 Watt Industrial Meat Grinder, City of Industry, CA, USA) with 3.0 mm perforations. Using a 1:5 ratio (mince: chilled tap water), mince was stirred gently for 1 min and sat for 2 min. After the floating fat was discarded, washed mince was mixed for 5 min using a mixer (BIG STIX™ Immersion Blenders WSB50, Waring Commercial, Torrington, CT, USA) at speed level 7. Then, connective tissues were
removed by filtering through two layers of screen mesh (2 mm), and the filtered
mince was homogenized (model GLH-115, PG700, Fisher Scientific, Pittsburgh, PA,
USA) for 2 min at speed level 3. The pH of the filtered homogenates was adjusted to
11.0 ± 0.01 with 2N NaOH. Protein aggregates were collected by adjusting pH of the
samples to the isoelectric point (pH 5.5) using 2N HCl. Dewatering of the aggregated
precipitates was performed by draining in two layers of screen mesh with pendulum
swings and using a screw press dehydrator (model SD-8, Ikeuchi Tekkosho, Sano,
Japan) at speed 5. The pH of the protein isolates was adjusted to approximately 7.0
using 2N NaOH. Sorbitol (5%), sugar (4%), and a mixture (50:50) of sodium
tripolyphosphate and tetrasodium pyrophosphate (0.3%) as cryoprotectants were
added to the protein isolates and placed in a plate freezer overnight (-25°C). Vacuum-
packed samples were stored in a freezer (-18°C) until tested, within 3 mon. All steps
in the preparation of FPI were conducted on ice or in a 4°C cold room to keep the
temperature below 10°C. This procedure was repeated once more to produce another
batch of FPI.

Preparation of surimi

A meat grinder with 4.5 mm perforations was used to obtain the tilapia mince.
Washing was performed by stirring mince and water gently at a ratio of 1:2 for 10
min. The washed mince was filtered and dewatered by repeated pendulum swings
using a screen mesh described in FPI preparation. The process of washing-dewatering
was repeated once more. The washed mince was placed in a strainer (model S1,
Ikeuchi Tekkosho, Sano, Japan) to remove connective tissues and pin bones. Then, the washed mince was dewatered by the screw press dehydrator at speed 5, as explained in FPI preparation. The same proportions of cryoprotectants used in FPI preparation were mixed into the dewatered mince for 2 min. Finally, the samples were vacuum-packed and stored at -18°C until tested, within 3 mon. All steps in the preparation of surimi were completed on ice or in a 4°C cold room to keep the temperature below 5°C. This procedure was repeated once more to produce another batch of surimi.

Paste and gel preparation

Approximately 50 g of frozen FPI or surimi was partially thawed at room temperature for 10 min and cut into small pieces. The sample was ground for 1 min using a mortar and pestle. Salt (0% or 3%) was added before grinding for an additional 1 min. Once the sample was ground for 2 min total, sugar was added as an inert ingredient to balance the sugar-salt concentration for 0% salt treatment and to maintain protein concentrations equally. Moisture content was adjusted to 78% using cold water (3–4°C) and ground for another 1 min. The sample was then ground until the comminution time totaled 6 min or 20 min for a low (5°C) or high (20°C) comminution temperature, respectively. Paste preparation using the mortar and pestle was performed in an ice bath or at room temperature to control the final comminution temperature for 5°C and 20°C, respectively.
FPI or surimi paste (approximately 22 g) was applied on aluminum foil sprayed lightly with oil and a molding sheet frame (7.5 cm wide, 25.5 cm long and 0.1 cm thick) was used to shape the paste into a thin sheet. The sheet-shaped paste was cooked in steam at 90°C for 1 min inside a water bath. Immediately after cooking, a small piece of the sheet of gel was removed for measurement, and the rest of the sheet of gel was stored in a refrigerator overnight to be tested the next day. Gel preparation was conducted twice for each treatment at 2 different salt levels. Samples (paste and gel) prepared for Raman and FT-IR spectroscopy were from the same batch.

FT-IR spectroscopy

Protein’s secondary structures in FPI and surimi paste and their gels were studied using a FT–IR spectrometer (Nicolet 380, Thermo Scientific Inc., Waltham, MA, USA) equipped with an attenuated total reflectance (ATR) accessory. The samples were scanned 64 times from 500 to 4000 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\). The spectra were collected at room temperature using approximately 0.5 g of sample that was placed on the surface of the ATR crystal. Background interference was removed and the recorded spectra were analyzed using OMNIC Professional software version 8.3. Four measurements from duplicate samples were collected to obtain an average of spectral data.

Raman spectroscopy

Using the procedure described by Poowakanjana et al. (2012) with slight modification, the Raman spectra of FPI and surimi paste and gel were collected using
a FT-Raman spectrometer (NXR FT-Raman module, Thermo Scientific Inc., Waltham, MA, USA) at room temperature with the use of the dynamically aligned Vecta-plus interferometer installed in the FT-IR bench (Nicolet 6700, Thermo Scientific Inc., Waltham, MA, USA). Approximately 0.4 g of sample was applied on a glass microscope slide and the sample was placed into the instrument. Raman spectral data were recorded using 1064 nm incident light from the fundamental of a Nd:YLF laser with a thermoelectrically cooled InGaAs detector. A spectral resolution was set to 4.0 cm\(^{-1}\) from 499.41 to 4001.56 cm\(^{-1}\), and the final spectra were an average of 64 scans. The recorded spectra were analyzed using OMNIC Professional software version 8.3.

Bands near 1003 cm\(^{-1}\) or 1450 cm\(^{-1}\) are popular bands used as internal standards for the relative peak heights or areas rather than on absolute signals. These peaks are attributed to the phenylalanine band at 1003 cm\(^{-1}\) and the CH\(_2\) bending vibration mode at 1450 cm\(^{-1}\). However, these bands are sensitive to the protein composition and/or the environment of peptide backbone. This shows that the band is sensitive to the relative abundance of phenylalanine or CH\(_2\) bending units; hence, the relative height of these bands will change accordingly if the protein composition changes (Beattie et al., 2004). Thus, each individual spectrum was not normalized in the present study. In place of normalization, we collected spectral data four different times from duplicate samples for each treatment to ensure the results were consistent. Raman frequencies and approximate description of specific vibrational modes of proteins were chosen as stated in published literature (Ogawa, Nakamura, Horimoto, An, Tsuchiya, & Nakai, 1999; Herrero 2008).
Statistical analysis

A mixed model in a standard split-split-plot design (PROC MIXED) was used to evaluate response data. Mean separation was attained using Tukey’s studentized range (honestly significant difference) test. The SAS program (SAS Institute Inc., Cary, NC, USA) was used to carry out all statistical analysis with the significance level set at 0.05.

6.4 Results and discussion

Amide conformation regions

The FT-IR and Raman spectra of FPI and surimi paste, gel, and gel stored overnight in a refrigerator among different comminution conditions are presented in Table 6.1 and Table 6.2, respectively. Both methods provide information on molecular vibrations; therefore, specific vibrations can be found in similar frequency regions. Secondary structural changes in FT-IR spectra were estimated by examining the amide I region (1600–1700 cm\(^{-1}\)) and the amide II region (1500–1600 cm\(^{-1}\)). The amide I band was assigned to C=O stretching/hydrogen bonding coupled with COO, and the amide II band corresponds to the bending vibrations of N-H groups and stretching vibrations of C-N groups. The amide I band near 1639 cm\(^{-1}\) was assigned to \(\beta\)-sheet structure and the amide II band near 1550 cm\(^{-1}\) attributed to \(\alpha\)-helical structure (Böcker, Ofstad, Wu, Bertram, Sockalingum, Manfait, Egelandsdal, & Kohler, 2007; Tongnuanchan, Benjakul, Prodpran, & Songtipya, 2013; Hernández-

The pronounced differences in secondary structure were observed in paste treatments. FPI paste chopped with 0% salt did not show the band at 1550 cm\(^{-1}\), and this band disappeared after gelation for all treatments (Figure 6.1a and Table 6.1). The \(\alpha\)-helical band in FPI paste appeared after the addition of salt, possibly because the salt enhanced protein solubilization and the unfolding of protein structures that were aggregated during the FPI refining process (Cando et al., 2016). The disappearance of the \(\alpha\)-helix band after heating was also observed in other studies (Boye, Alli, Ismail, Gibbs, & Konishi, 1995; Murayama & Tomida, 2004; Saguer, Fort, Alvarez, Sedman, & Ismail, 2008), indicating the unfolding of protein structures during heating and the formation of \(\beta\)-sheets as an ordered network. The FT-IR band at 1550 cm\(^{-1}\) showed a slight decrease of absorbance when samples were chopped at 25\(^{\circ}\)C for 18 min compared to samples chopped at 5\(^{\circ}\)C for 6 min (Table 6.1). This suggested that chopping at a higher temperature for a longer time period may enhance the disintegration of protein structures before gelation since tilapia is a warm water fish species (Poowakanjana et al., 2012; Poowakanjana & Park, 2014).

At the 1639 cm\(^{-1}\) band, there were no significant differences between FPI and surimi at different comminution conditions in paste (Figure 6.1b). However, FPI gels showed significantly higher (p < 0.05) absorbance at 1639 cm\(^{-1}\) compared to surimi gels, and surimi gels with 0% salt exhibited significantly higher (p < 0.05) absorbance than surimi gels with 3% salt. After gels were stored overnight in the refrigerator, the
band at 1639 cm\(^{-1}\) in surimi gels increased, and there were no significant differences among all treatments for gels stored overnight. The increase of \(\beta\)-sheet band in surimi gels after storing overnight may suggest that surimi gels take longer to form a more ordered network with a higher density of cross-links than FPI gels. Moreover, surimi gels may be more involved with the formation of H-bonding networks than FPI gels (Cando et al., 2016; Saguer et al., 2008). The change of \(\beta\)-sheet band between FPI and surimi gels may be related to the change of storage modulus (\(G'\)) during heating from 20 to 90\(^\circ\)C as previously studied in the thermal gelation profile (Kobayashi & Park, unpublished). The shape of rheograms between FPI and surimi without salt around 50\(^\circ\)C were V-shaped and U-shaped, respectively. These rheograms show that protein particles in FPI may be easier to migrate, entangle with each other, and form protein networks faster than surimi, resulting a faster change in \(G'\) in FPI than surimi.

The amide I (1645-1685 cm\(^{-1}\)) and the amide III (1220-1350 cm\(^{-1}\)) regions were observed in the Raman spectra for assessing the secondary structure changes shown in Figure 6.2 and Table 6.2. The amide I mode was assigned to amide carbonyl C=O stretching, with partial involvement of C-N stretching and N-H bending. The amide III mode corresponds to C-N stretching, N-H bending, and C-C stretching. The \(\alpha\)-helical and \(\beta\)-sheet contents are illustrated by the amide I band near 1648 cm\(^{-1}\) and 1660 cm\(^{-1}\) and the amide III band near 1324 cm\(^{-1}\) and 1243 cm\(^{-1}\), respectively (Tiadipitchyangkoon et al., 2010; Poowakanjana et al., 2012).

Similar to FT-IR spectra, Raman bands at 1648 cm\(^{-1}\) and 1324 cm\(^{-1}\) indicating the \(\alpha\)-helical structure disappeared after heating (data not shown). These results were
consistent with other studies demonstrating the transition of \( \alpha \)-helical structure to \( \beta \)-sheet structure after gelation (Beattie, Bell, Farmer, Moss, & Patterson, 2004; Bouraoui et al., 1997; Ogawa et al., 1999; Sánchez-González, Carmona, Moreno, Borderías, Sanchez-Alonso, Rodríguez-Casado, & Careche, 2008). Figure 6.2 shows amide I and III Raman spectra of FPI and surimi paste under different chopping condition (5°C for 6 min and 25°C for 18 min) and salt content (0% and 3%). Once the chopping temperature and time increased, and with the addition of salt, the intensity of the 1648 cm\(^{-1}\) and 1324 cm\(^{-1}\) bands shifted to 1660 cm\(^{-1}\) and 1243 cm\(^{-1}\), respectively. This indicated that both comminution condition and salt content could promote protein structure in FPI paste to undergo the unfolding from \( \alpha \)-helical structure to random coil and refolding from random coil to \( \beta \)-sheet structure.

Likewise, surimi paste showed a decrease in intensity and a shift from the band near the \( \alpha \)-helical structure to bands near the \( \beta \)-sheet structure after increasing chopping temperature and time, as well as the addition of salt. Bouriaoui et al., 1997 and Li-Chan 1996 reported the addition of salt could induce a trend toward uncoiling of helices, and the increase in the amount of \( \beta \)-structure. In addition, Poowakanjana et al. (2012) showed the amount of disruption on secondary protein structure could be different by changing chopping temperature and time in surimi paste. Thus, the present study suggested that a higher chopping temperature, for a longer time period, in FPI and surimi prepared from warm-water species (e.g., tilapia) may improve protein unfolding and enhance protein-protein interactions during gelation.
The Raman bands at 1660 cm\(^{-1}\) and 1243 cm\(^{-1}\) in FPI gel and gel stored overnight in a refrigerator were more intense than surimi gel and gel stored overnight (Table 6.2). A similar trend was observed in FT-IR spectra; however, the increase of the \(\beta\)-sheet band in surimi from gels to gels stored overnight in a refrigerator was not detected in the Raman spectra. This may be associated with the fundamental difference between FT-IR and Raman spectroscopy.
Table 6.1 – Mean (±SD) absorbances at selected regions of FT-IR spectra of fish protein isolate and surimi prepared from tilapia as affected by various comminution conditions. F0-5: FPI chopped with 0% salt at 5°C for 6 min. F0-25: FPI chopped with 0% salt at 25°C for 18 min. F3-5: FPI chopped with 3% salt at 5°C for 6 min. F3-25: FPI chopped with 3% salt at 25°C for 18 min. S0-5: surimi chopped with 0% salt at 5°C for 6 min. S0-25: surimi chopped with 0% salt at 25°C for 18 min. S3-5: surimi chopped with 0% salt at 5°C for 6 min. S3-25: surimi chopped with 3% salt at 25°C for 18 min.

<table>
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<th>F3-25</th>
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<th>S0-25</th>
<th>S3-5</th>
<th>S3-25</th>
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<td>0.109 ± 0.002 a</td>
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<td>0.107 ± 0.006 a</td>
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<td>0.037 ± 0.007 b</td>
<td>0.032 ± 0.001 b</td>
<td>0.023 ± 0.001 c,y</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gel ON</td>
<td>0.043 ± 0.007 a</td>
<td>0.038 ± 0.005 a</td>
<td>0.040 ± 0.001 a,x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2927</td>
<td>Gel</td>
<td>0.078 ± 0.001 a,x</td>
<td>0.061 ± 0.001 b</td>
<td>0.052 ± 0.003 c,y</td>
<td>0.037 ± 0.001 d,y</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gel ON</td>
<td>0.087 ± 0.005 a,y</td>
<td>0.083 ± 0.001 a,x</td>
<td>0.083 ± 0.001 a,x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1739</td>
<td>Gel</td>
<td>0.059 ± 0.008 a</td>
<td>0.060 ± 0.009 a,x</td>
<td>0.036 ± 0.002 b,y</td>
<td>0.068 ± 0.002 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gel ON</td>
<td>0.061 ± 0.001 a</td>
<td>0.046 ± 0.001 b,y</td>
<td>0.062 ± 0.006 a,x</td>
<td>0.068 ± 0.001 a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means in a row with different superscript (a, b) are significantly different (p < 0.05) among treatments. Means between paste, gel, and gel stored overnight (Gel ON) in the same column (treatment) for each respective variable with different superscript (x, y, and z) are significantly different (p < 0.05).
Table 6.2 – Mean (±SD) intensities at selected regions of Raman spectra of fish protein isolate and surimi prepared from tilapia as affected by various comminution conditions. Abbreviations indicate the same as those in Table 1.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>F0-5</th>
<th>F0-25</th>
<th>F3-5</th>
<th>F3-25</th>
<th>S0-5</th>
<th>S0-25</th>
<th>S3-5</th>
<th>S3-25</th>
</tr>
</thead>
<tbody>
<tr>
<td>1660</td>
<td>Gel</td>
<td>0.294 ± 0.030 a</td>
<td>0.296 ± 0.030 a</td>
<td>0.289 ± 0.030 a</td>
<td>0.295 ± 0.010 a,y</td>
<td>0.241 ± 0.010 b</td>
<td>0.253 ± 0.030 ab</td>
<td>0.269 ± 0.010 ab</td>
</tr>
<tr>
<td></td>
<td>Gel ON</td>
<td>0.302 ± 0.020 a</td>
<td>0.325 ± 0.040 a</td>
<td>0.293 ± 0.010 a</td>
<td>0.337 ± 0.030 a,x</td>
<td>0.235 ± 0.010 b</td>
<td>0.254 ± 0.020 ab</td>
<td>0.259 ± 0.020 ab</td>
</tr>
<tr>
<td>1243</td>
<td>Gel</td>
<td>0.316 ± 0.010 a</td>
<td>0.327 ± 0.030 a,y</td>
<td>0.294 ± 0.020 a</td>
<td>0.308 ± 0.020 a</td>
<td>0.272 ± 0.020 a</td>
<td>0.272 ± 0.020 a</td>
<td>0.275 ± 0.030 a</td>
</tr>
<tr>
<td></td>
<td>Gel ON</td>
<td>0.333 ± 0.030 a</td>
<td>0.375 ± 0.010 a,x</td>
<td>0.291 ± 0.010 a</td>
<td>0.324 ± 0.020 a</td>
<td>0.247 ± 0.030 b</td>
<td>0.270 ± 0.020 b</td>
<td>0.281 ± 0.070 a</td>
</tr>
<tr>
<td>540</td>
<td>Gel</td>
<td>0.342 ± 0.010 a</td>
<td>0.365 ± 0.030 a</td>
<td>0.343 ± 0.001 a</td>
<td>0.376 ± 0.020 a</td>
<td>0.276 ± 0.020 b</td>
<td>0.306 ± 0.010 b</td>
<td>0.334 ± 0.030 a</td>
</tr>
<tr>
<td></td>
<td>Gel ON</td>
<td>0.348 ± 0.030 a</td>
<td>0.372 ± 0.020 a</td>
<td>0.347 ± 0.030 a</td>
<td>0.394 ± 0.020 a</td>
<td>0.318 ± 0.030 a</td>
<td>0.340 ± 0.030 a</td>
<td>0.353 ± 0.040 a</td>
</tr>
<tr>
<td>850</td>
<td>Paste</td>
<td>0.305 ± 0.030 a</td>
<td>0.283 ± 0.040 a</td>
<td>0.256 ± 0.010 b</td>
<td>0.235 ± 0.040 b,y</td>
<td>0.252 ± 0.010 b</td>
<td>0.233 ± 0.001 b</td>
<td>0.221 ± 0.010 b,y</td>
</tr>
<tr>
<td></td>
<td>Gel</td>
<td>0.308 ± 0.040 a</td>
<td>0.315 ± 0.040 a</td>
<td>0.263 ± 0.020 a</td>
<td>0.298 ± 0.030 a,y</td>
<td>0.235 ± 0.010 b</td>
<td>0.254 ± 0.010 b</td>
<td>0.260 ± 0.001 b</td>
</tr>
<tr>
<td></td>
<td>Gel ON</td>
<td>0.319 ± 0.030 a</td>
<td>0.324 ± 0.001 a</td>
<td>0.275 ± 0.040 ab</td>
<td>0.310 ± 0.020 ab,x</td>
<td>0.249 ± 0.030 b</td>
<td>0.259 ± 0.010 b</td>
<td>0.261 ± 0.040 b</td>
</tr>
<tr>
<td>1077</td>
<td>Paste</td>
<td>0.388 ± 0.060 ab,x</td>
<td>0.351 ± 0.070 ab</td>
<td>0.347 ± 0.020 ab</td>
<td>0.330 ± 0.030 ab</td>
<td>0.439 ± 0.050 a,x</td>
<td>0.362 ± 0.020 ab,x</td>
<td>0.330 ± 0.070 ab</td>
</tr>
<tr>
<td></td>
<td>Gel</td>
<td>0.336 ± 0.010 a,xy</td>
<td>0.350 ± 0.030 a</td>
<td>0.304 ± 0.001 ab</td>
<td>0.315 ± 0.010 ab</td>
<td>0.287 ± 0.020 b,y</td>
<td>0.293 ± 0.020 b,y</td>
<td>0.292 ± 0.010 b</td>
</tr>
<tr>
<td></td>
<td>Gel ON</td>
<td>0.329 ± 0.020 a,y</td>
<td>0.341 ± 0.030 a</td>
<td>0.287 ± 0.010 a</td>
<td>0.349 ± 0.010 a</td>
<td>0.290 ± 0.040 a,y</td>
<td>0.293 ± 0.020 a,y</td>
<td>0.286 ± 0.070 a</td>
</tr>
</tbody>
</table>

Means in a row with different superscript (a, b, and c) are significantly different (p < 0.05) among treatments. Means between paste, gel, and gel stored overnight (Gel ON) in the same column (treatment) for each respective variable with different superscript (x, y) are significantly different (p < 0.05).
Figure 6.1 – Various FT-IR spectra of tilapia fish proteins at various salt concentrations (0 and 3%) and comminution conditions (5°C for 6 min and 25°C for 18 min). a: Spectra (1470-1980 cm⁻¹) of fish protein isolate and surimi paste chopped with 0% salt at 25°C for 18 min. F0-25: FPI chopped with 0% salt at 25°C for 18 min. S0-25: Surimi chopped with 0% salt at 25°C for 18 min. b: Means absorbance of the FT-IR band at 1639 cm⁻¹. Error bars represent the standard deviation of 4 determinations. Different letters (a-c) on the bars indicate a significant difference at p < 0.05 among comminution treatments. Different letters (x-z) in the bars indicate a significant difference at p < 0.05 among paste, gel, and gel stored overnight (Gel ON).
for each respective chopping condition. No letters on or in the bars indicate no significant difference. Abbreviations indicate the same as those in Table 6.1.
Figure 6.2 – Various Raman spectra of tilapia fish proteins at various salt concentrations (0% and 3%) and comminution conditions (5°C for 6 min and 25°C for 18 min). a: Spectra (1550-1800 cm\(^{-1}\)) of fish protein isolate, b: Spectra (1550-
1800 cm$^{-1}$) of surimi, c: Spectra (1100-1400 cm$^{-1}$) of fish protein isolate, d: Spectra (1100-1400 cm$^{-1}$) of surimi. Abbreviations indicate the same as those in Table 6.1.

Aliphatic bending vibrations

The FT-IR band at 1450 cm$^{-1}$ refers to CH$_2$ or CH$_3$ bending vibrations arising from protein side chain and/or lipids (Böcker et al., 2007; Hernandez-Martinez et al., 2014; Long, Ji, Pan, Sun, Li, & Qin, 2015). Although this band was not found in FPI samples, the band was observed in surimi gel or surimi gel stored overnight in a refrigerator (Table 6.1), possibly because the lipid content in FPI was lower than surimi (Hultin & Kelleher, 2000). Another possible reason of no absorbance in FPI treatments was because FT-IR spectroscopy depends on the changes in the dipole moment representing less sensitive to homo-nuclear molecular bonds (Li-Chan, 1996; Sivam et al., 2013).

The higher absorbance at 1450 cm$^{-1}$ signifies an increase in hydrophobic interaction of protein side chain (Li-Chan, 1996). Surimi gel stored overnight in the refrigerator revealed significantly higher (p < 0.05) absorbance at 1450 cm$^{-1}$ than surimi gel. Among surimi gel treatments, surimi chopped at 5°C for 6 min with 0% salt exhibited significantly higher absorbance compared to the other surimi gel treatments (p < 0.05). This may be correlated with the degree of unfolding protein structure during chopping. Chopping surimi at a higher temperature for a longer time period with the addition of salt indicated a higher degree of protein unfolding compared to a lower chopping temperature without salt by amide band regions. In addition, Kirschner, Ofstad, Skarpeid, Høst, and Kohler (2004) and Long et al. (2015) demonstrated the increase of absorbance at 1450 cm$^{-1}$ after heat treatment in aged
beef loin and soy glycinin, respectively. Hence, chopping at a lower temperature without salt may have had a lesser degree of unfolding protein structure and more hydrophobic interactions as shown in Table 6.1.

In Raman spectra, aliphatic amino acid residues show CH bending modes near 1440-1465 cm\(^{-1}\), and this band is suggested to monitor hydrophobic interactions between aliphatic residues. The higher intensity of this band denotes an increase in hydrophobic interaction of aliphatic residues (Herrero, 2008; Li-Chan, 1996). Beattie et al. (2004) reported that Raman spectroscopy can be used to predict sensory-perceived beef quality, and hydrophobicity of the myofibrillar environment is one of the important aspects contributing to the shear force, tenderness, and texture of the beef.

There were no significant differences among paste treatments; however, a slight decrease in the intensity of the band at 1448 cm\(^{-1}\) was observed in FPI and surimi paste after increasing chopping temperature and time with the addition of salt (Figure 6.3). Poowakanjana et al. (2012) presented a comparable result that a significant decrease in the intensity of the band at 1450 cm\(^{-1}\) was obtained when chopping time of threadfin bream (warm water species) was extended from 6 to 21 min. Tadpitchayangkoon et al. (2010a) and Thawornchinsombut et al. (2006) also observed a decline of the intensity of this band upon the extreme pH extraction and freeze-thaw cycling of fish proteins, respectively. Therefore, it is presumed that the longer chopping time and the addition of salt could enhance the opening of the tertiary structure of FPI and surimi paste.
The intensity of the Raman band at 1448 cm\(^{-1}\) in FPI and surimi gel and gel stored overnight in the refrigerator was higher than FPI and surimi paste, indicating the increase of hydrophobic interactions after gelation. This suggested hydrophobic interaction plays one of the crucial roles during gelation (Poowakanjana et al., 2012; Poowakanjana & Park, 2013, 2014). With respect to the band intensity at 1448 cm\(^{-1}\), longer comminution time and higher final comminution temperature always resulted in higher intensity of gels made from FPI and surimi as well as those gels stored overnight. However, different direction was observed when comparing intensity of this particular band between FPI gel to FPI gel stored overnight and between surimi gel to surimi gel stored overnight. In FPI samples, gels stored overnight exhibited no significant change in intensity (\(p > 0.05\)) when compared with FPI gel which was similar to surimi samples with the addition of salt. In contrast, with the absence of salt, surimi gel stored overnight demonstrated lower intensity than that of the surimi gel. This may be because hydrophobic interactions in surimi gels were getting weak during refrigerated storage (Howe et al. 1994). The higher degree of protein unfolding (by the addition of salt) prior to gelation may help form stronger hydrophobic interactions. In addition, these results may be related to results from breaking force (Douglas-Schewarz & Lee, 1988; Esturk et al., 2004; Poowakanjana et al., 2012, 2015a), since hydrophobic interactions are one of the major chemical bonds for protein-protein interactions in gel-formation. Furthermore, FPI chopped at 5°C for 6 min with 3% salt showed lower intensity of the band at 1448 cm\(^{-1}\) for FPI gel and gel stored overnight. This result was in agreement with previous studies in breaking force. Kobayashi and Park (unpublished data) observed FPI chopped at 5°C for 6 min with 3%
salt showed slightly lower breaking force than that without salt. Kim and Park (2008) demonstrated the addition of salt in alkali-treated FPI prepared from Alaska pollock surimi negatively affected gel qualities. Thus, the intensity of the Raman band at 1448 cm\(^{-1}\) may be one of the key Raman bands to distinguish the quality of the gel. The results of aliphatic bending vibrations were similar between FT-IR and Raman spectra, but Raman spectroscopy may be more sensitive to this band, since the band was not identified in all FPI treatments and surimi paste treatments in FT-IR spectroscopy.

Figure 6.3 – Means intensity of the Raman band at 1448 cm\(^{-1}\). Error bars and different letters are sample as those in Figure 1b and abbreviations indicate the same as those in Table 6.1.

C-H stretching vibrations
The FT-IR bands at 2854 cm\(^{-1}\) and 2927 cm\(^{-1}\) were assigned to methylene symmetric and asymmetric stretching vibrations, respectively, appearing from protein side chain and/or lipids (Böcker et al., 2007; Muyonga, Cole, & Duodu, 2004; Hernandez-Martinez et al., 2014). These bands were seen in surimi gel and surimi gel stored overnight in the refrigerator (Table 6.1). There was no detection of these bands in surimi paste possibly because these bands are sensitive to denatured structures upon heating. Böcker et al. (2007) studied pork muscle tissue subjected to different processing factors, including aging, salting, and heat treatment, in order to induce the necessary degree of vibration of the spectra. They found both FT-IR and Raman spectra were mostly affected by heat treatment, followed by the different salt concentrations. However, these bands did not appear in FPI treatments. This was likely because FPI contained lower lipid content compared to surimi (Hultin & Kelleher, 2000). When Tongnuanchan et al. (2013) compared to film made from FPI and film prepared from unwashed mince, the FT-IR bands at 2854 cm\(^{-1}\) and 2924 cm\(^{-1}\) were found in film made from unwashed mince but not in film made from FPI. They assumed the disappearance of these bands may be due to the removal of lipid content during FPI processing.

The bands at 2854 cm\(^{-1}\) and 2927 cm\(^{-1}\) in surimi gel decreased significantly after increasing chopping temperature and time and salt content, though there were no significant differences among surimi gel stored overnight (Table 6.1). The decrease in absorbance of the bands at 2854 cm\(^{-1}\) and 2927 cm\(^{-1}\) may imply increasing the degree of conformational disorder and level of flexibility of protein subunits (Long et al.,
2015). This suggested comminution condition and salt content may influence the degree of protein unfolding in surimi gel.

The Raman band assigned to the C-H stretching mode is observed near 2929 cm\(^{-1}\) (Figure 6.4). It has been established that the exposure of aliphatic hydrophobic side chains of proteins would lead to an increase of the intensity of the band (Herrero, 2008; Li-Chan, 1996). FPI and surimi paste chopped at 25°C for 18 min presented higher intensity of the band at 2929 cm\(^{-1}\) compared to the paste chopped at 5°C for 6 min, signifying the exposure of hydrophobic residues of proteins by increasing chopping temperature and time. FPI paste chopped at 5°C for 6 min with 3% salt indicated the lowest intensity of this band, perhaps because chemical denaturation of FPI occurred prior to comminution and the addition of 3% salt may have induced the proteins to aggregates like salting-out, resulting less exposure of hydrophobic residues (Poowakanjana & Park, 2014).

Additionally, the intensity of the band at 2929 cm\(^{-1}\) decreased in a descending order of paste, gel, and gel stored overnight, except FPI chopped at 5°C for 6 min with 3% salt. Bouraoui et al. (1997), Ogawa et al. (1999), and Sanchez-Gonzalez et al. (2008) demonstrated similar trends; the decrease of C-H stretching vibrations from surimi paste to gel. FPI chopped at 5°C for 6 min with 3% salt did not show the same trend. The addition of salt in FPI may have produced protein aggregates like salting-out as mentioned previously. Then, these aggregates may have been loosened by heating during gelation, indicating a slight increase in the intensity of the band at 2929 cm\(^{-1}\). This may have occurred since FPI was chemically denatured and refolded.
prior to comminution. As FPI contained both sarcoplasmic and myofibrillar proteins and it was made from thermally stable tilapia, the effect of salt on the unfolding of proteins was probably not large at low temperature/short time chopping. Therefore, with the addition of salt, FPI made from tilapia may require chopping longer to reach higher temperature, so that hydrophobic residues are exposed more and form ordered gel networks. Similar to the results from aliphatic bending vibrations, C-H stretching vibrations are more sensitive in the Raman spectra than the FT-IR spectra, especially in FPI samples.

Figure 6.4 – Means intensity of the Raman band at 2929 cm\(^{-1}\). Error bars and different letters are same as those in Figure 1b and abbreviations indicate the same as those in Table 6.1.
Other FT-IR and Raman bands

The FT-IR band near 1740-1760 cm\(^{-1}\) attributed to stretching vibration of aldehyde or ester carbonyl groups, indicating lipid content (Hernandez-Martinez et al., 2014; Holes et al., 2011; Liu et al. 2015; Sivam et al. 2013). No band at 1739 cm\(^{-1}\) was found in FPI and paste treatments (Table 6.1). The results indicated that chemical denaturation occurred during the FPI refining process and more lipids were removed than the surimi refining process (Hultin & Kelleher, 2000). Tongnuanchan et al. (2013) revealed the washing process in red tilapia surimi could remove lipids to some extent and caused the disappearance of the band at 1746 cm\(^{-1}\). Liu et al. (2015) confirmed the correlation between the band at 1745 cm\(^{-1}\) and the lipid content determined by proximate analysis to differentiate three marine fish surimi. However, this FT-IR band was not observed in Raman spectra. Flores-Morales et al. (2012) demonstrated a similar result of structural changes obtained by FT-IR and Raman spectroscopy on retrograded starch of maize tortillas. They reported the appearance of the 1743 cm\(^{-1}\) band in the FT-IR spectra, but not in the Raman spectra.

A band at 540 cm\(^{-1}\) was present in the Raman spectra (Table 6.2), corresponding to the S-S stretching vibration of the disulfide bonds. The location of the S-S stretching band near 540 cm\(^{-1}\) assigned to be a trans-gauche-trans conformation. There was no appearance of the band near 540 cm\(^{-1}\) in FT-IR spectra, since the symmetric and/or non-polar vibration is IR-inactive (Herrero, 2008; Li-Chan, 1996). The band at 540 cm\(^{-1}\) was observed in neither FPI nor surimi paste. The heating resulted in an appearance of the band at 540 cm\(^{-1}\), indicating the formation of disulfide bonds during the gelation process. Similar results were obtained by Bouaoui
et al. (1997) and Ogawa et al. (1999) demonstrating the increase in the intensity of the band near 530 cm\(^{-1}\) when fish proteins were heated. A slight increase of the band at 540 cm\(^{-1}\) was seen as chopping temperature and time increased for both FPI and surimi treatments. These results were comparable with a fracture test (Douglas-Schewarz & Lee, 1988; Esturk et al., 2004; Poowakanjana et al., 2012); however, the present study did not show significant differences between treatments, excluding surimi gel after the addition of salt. Hence, FPI and surimi prepared from tilapia may achieve a better gelation process when chopped at a higher temperature for a longer time period.

In the Raman spectra, the ratio of doublet bands at 850 and 830 cm\(^{-1}\) are known to monitor the hydrogen bonding of the phenolic hydroxyl group and to determine the microenvironment around tyrosine residues, representing buried and exposed tyrosine groups (Herrero 2008; Li-Chan 1966). In the present study, the band near 830 cm\(^{-1}\) was not seen in the Raman spectra. This may be because the tyrosine doublet was generally observed as two weak bands, and the band at 850 cm\(^{-1}\) may overlap the 830 cm\(^{-1}\) band or the intensity of the band at 830 cm\(^{-1}\) could have been extremely low (Sanchez-Gonzalez et al., 2008). The intensity of the band at 850 cm\(^{-1}\) decreased as chopping temperature and time increased in FPI and surimi paste (Table 6.2). Also, the decreased intensity of the band at 850 cm\(^{-1}\) was found after the addition of salt in paste treatments. These results could indicate the exposure of the tyrosine residues due to the increase in chopping temperature and time, and the addition of salt. An increase in the intensity of the band at 850 cm\(^{-1}\) was observed from paste to gel; this
result may signify that the tyrosine residue is refolded and buried within the gel network (Li-Chan, 1996; Thawornchinsombut et al., 2006).

The Raman band near 1060-1100 cm\(^{-1}\) is attributed to C-N and C-C stretch modes. This band could be valuable as an indicator of conformational change that broadens and loses intensity upon denaturation (Herrero, 2008; Thawornchinsombut et al., 2006). The intensity of the band at 1077 cm\(^{-1}\) decreased when samples were chopped at 25°C for 18 min compared to samples chopped at 5°C for 6 min (Table 6.2). The intensity of this band also decreased as the salt content increased; suggesting a diminution in the intensity of this band is related to disintegration of protein structures consequent to comminution. Thawornchinsombut et al. (2006) found a decline of the band intensity at 1072 cm\(^{-1}\) after freeze-thaw was repeated for FPI and surimi, suggesting protein unfolding resulting from the storage condition. In the present study, the intensity of the band at 1077 cm\(^{-1}\) reduced from paste to gel samples, indicating conformational change due to thermal denaturation. Ogawa et al. (1999) reported that the Raman intensity in the region of 1050-1100 cm\(^{-1}\) decreased upon heating (setting at 40°C) of rockfish actomyosin.

6.5 Conclusion

FT-IR and Raman spectroscopies provided compatible information, allowing a better understanding of the structural change in FPI and surimi under various comminution conditions. However, the FT-IR spectra showed fewer bands in FPI samples compared to surimi samples. Results showed the degree of unfolding protein structure in FPI and surimi paste prepared from tilapia became higher by increasing
chopping temperature and time, or by adding salt. In addition, both FT-IR and Raman spectra indicated the increase of hydrophobic interactions, disulfide bonds, and $\beta$-sheet contents in FPI and surimi gels as chopping temperature and time increased from 5°C for 6 min to 25°C for 18 min. This spectroscopic information may be correlated with the gel texture of FPI and surimi. The present study suggested controlling comminution conditions is one of the important factors as protein structures are disintegrated in order to improve gel forming ability in FPI and surimi.
CHAPTER 7

OPTIMAL BLENDING OF TWO Refined FISH PROTEINS PREPARED USING DIFFERENT METHODS

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7.1 Abstract

Two different mixtures (Alaska pollock surimi with carp fish protein isolate (FPI) and carp surimi with carp FPI) were investigated for their compatibility and functionalities. As the proportion of FPI increased, it was observed surface hydrophobicity and surface reactive sulfhydryl (SRSH) content increased significantly, indicating the degree of fish protein unfolding prior to gelation was much higher than surimi alone. Comparable results were shown as measured by storage modulus (G’) in oscillatory dynamic rheology, demonstrating the gelling temperature was reduced when the proportion of FPI increased. Effects of mixing surimi and FPI on gel functionality (hardness, cohesiveness, and whiteness) exhibited a linear pattern when the proportion of surimi was larger than or equal to that of FPI. However, there were no linear relationships when the proportion of FPI exceeded that of surimi.
7.2 Introduction

The demand for fish protein is growing substantially throughout the world due to population growth, an increase in the relative preference for fish, the health advantages associated with consumption of fish, and an increase in expendable income (Elyasi et al., 2010; Kristinsson et al., 2014). Surimi and fish protein isolate (FPI) are two major methods for refining fish proteins, and their refining processes are opposite with respect to protein denaturation. Protein denaturation must be avoided during surimi processing, although FPI chemically induces denaturation by altering pH during the process. Also, the composition of proteins is different between surimi and FPI. Surimi contains only myofibrillar proteins, while FPI contains myofibrillar proteins as well as sarcoplasmic proteins (Hultin & Kelleher, 2000).

Both surimi and FPI have the ability to form an elastic gel upon heating, which makes them a key ingredient for surimi seafood. Gelling properties are significantly affected by biological factors (species, freshness, and degree of stress before harvest) and processing factors (protein concentration, pH, ionic strength, comminution conditions, and temperature) (Park et al., 2014). In addition, the gel-forming ability of muscle proteins can be affected by the addition of gel strength enhancers such as starch, egg white, whey protein, or soybean protein (Hsu & Chiang, 2001). Research in effective utilization of fish proteins is important for sustainable fisheries. Moreover, formulation optimization with various ingredients is critical in order to improve product quality and/or profitability (Park & Beliveau, 2014).

One way to maintain consistent functionality of proteins and minimize production costs is to blend various grades of fish proteins in an appropriate manner.
Yoon et al. (1997) showed linear relationships of shear stress, shear strain, and whiteness of surimi gels made with high and low grades of Alaska pollock and Pacific whiting. Hsu and Chiang (2010) determined low-grade hairtail surimi could be used to produce surimi seafood when blending with high-grade Alaska pollock and/or golden threadfin bream surimi. Several studies have focused on the effects of surimi or fish mince on the properties of meat and fish products (Cavenaghi-Altemio et al., 2013; Desmond & Kenny, 1998; Elyasi et al., 2010). For instance, Murphy et al. (2004) found the potential benefits of surimi as a meat filler or fat replacement in pork sausage. In addition, Amiza and Ng (2015) examined the possibility of reducing the cost of producing fish sausage by replacing surimi with fish mince. Furthermore, different protein sources, such as FPI made from silver carp and surimi made from Alaska pollock, demonstrated that different additives have different effects on gel texture based on the refining methods. The addition of sarcoplasmic proteins into surimi negatively affected the gel qualities; however, gels prepared from silver carp FPI did not show as many adverse effects as in surimi gels (Paker & Matak, 2015). Kristinsson et al. (2013) reported sarcoplasmic proteins did not interfere with gel formation of fish protein isolate produced from pH shift, and gels also showed greater stability to lipid oxidation.

Therefore, it would be extremely valuable if the optimum utilization of various fish proteins is determined. It would enhance gel qualities and develop cost-effective production methods. However, there is no information on how two different fish proteins (FPI and surimi) are blended and utilized in making surimi seafood. The objectives of this study were to investigate the gelation properties of a fish protein
blend (FPI and surimi) at different ratios and further to understand how two fish proteins prepared using two refining methods contribute to the structure and texture of the gel.

7.3 Materials and methods

Materials

Alaska pollock (Gadus chalcogrammus, formerly Theragra chalcogramma) (NOAA, 2013) surimi (AA grade) was obtained from Glacier Fish Company (Seattle, WA, USA) and stored frozen at -18°C. The 10 kg blocks were cut into smaller blocks (approximately 1,000 g), individually vacuum-packed, and stored at -18°C until used. Grass carp (Ctenopharyngodon idella) were donated by Riverine Fisheries International LLC. (Hickman, KY, USA). Fresh fish were packed in an ice cooler and delivered to the Oregon State University Seafood Research and Education Center (Astoria, OR, USA), where they were stored in a cold room (4°C) and covered with ice. Fish were processed into surimi and FPI at approximately 36 hours postharvest. Ingredients used for surimi and FPI making and gel preparation were sorbitol (sorbitol NF/FCC, Roquette America Inc., Gurnee, IL, USA), phosphate blend Polymix GA (SD BNI CO., Hwaseong-si, Kyeonggi-do, Korea), sugar (Pure Cane Sugar, C&H Sugar Company, Inc., Crockett, CA, USA), and NaCl (Morton Iodized Salt, Morton International, Inc., Chicago, IL, USA). Bradford reagent for protein analysis was purchased from Bio-Rad Laboratory (Hercules, CA, USA). All other chemicals were reagent grade.
Preparation of carp surimi

Fresh carp was filleted and ground using a meat grinder (Electric 2.6 HP 2000 Watt Industrial Meat Grinder, City of Industry, CA, USA) with 4.5 mm perforations. The ground meat was washed by gentle stirring with chilled water at a ratio of 1:2 for 10 min. The washed meat was dewatered by repeated pendulum swings using a screen mesh (2 mm). After connective tissues were removed using a strainer (model S1, Ikeuchi Tekkosho, Sano, Japan), the washed mince was dewatered with a screw press dehydrator (model SD-8, Ikeuchi Tekkosho, Sano, Japan) at speed level 5. Then, cryoprotectants (5% sorbitol, 4% sugar, and 0.3% of a mixture (50:50) of sodium tripolyphosphate and tetrasodium pyrophosphate) were added to surimi using a mixer (VCM 40, Hobart Corp, Troy, OH, USA) for 2 min. Finally, samples were packed in a plastic container (approximately 500-600 g) and placed in a plate freezer (-25°C) overnight. Vacuumed-packed samples were stored at -18°C until tested. All steps in the preparation of surimi were performed on ice or in a 4°C cold room.

Preparation of carp fish protein isolate

Similar to surimi preparation, ground carp meat was prepared with a meat grinder with 3.0 mm perforations. The ground meat was washed by gentle stirring with chilled water at a ratio of 1:5 for 1 min and sat for 2 min in order to remove the floating fat. A mixer (BIG STIX™ Immersion Blenders WSB50, Waring Commercial, Torrington, CT, USA) at speed level 7 was used to mix washed mince for 5 min, and connective tissues were discarded by filtering through two layers of screen mesh. The
filtered ground meat was homogenized (model GLH-115, PG700, Fisher Scientific, Pittsburgh, PA, USA) for 2 min at speed level 3. The solubilization of myofibrillar and sarcoplasmic proteins were performed by adjusting the pH of the homogenates to 11.0 ± 0.05 with 2 N NaOH. The pH of the samples was then adjusted to the isoelectric point (pH 5.5) using 2N HCl for collecting protein aggregates. Aggregated precipitates were dewatered using two layers of screen mesh with pendulum swings prior to being dewatered with a screw press dehydrator at speed level 5. The pH of the protein isolates was adjusted to approximately 7.0 using 2N NaOH, and the same proportions of cryoprotectants used in surimi preparation were mixed into FPI using a mixer (VCM 40) for 2 min. Samples were stored with the same method used in surimi preparation. All steps in the preparation of FPI were performed on ice or in a 4°C cold room.

Paste and gel preparation

Surimi and FPI samples were combined at different ratios (100:0, 75:25, 50:50, 25:75, and 0:100, surimi to FPI, respectively). Paste samples (approximately 850 g) were prepared at 78% moisture with 2% salt. Frozen surimi and/or FPI blocks were allowed to be partially thawed at room temperature for 40 min and its core temperature was approximately -5°C. Partially thawed blocks were cut into small cubes (2-4 cm), and paste chopping was performed using a silent cutter (UM 5 universal, Stephan Machinery Corp, Columbus, OH, USA) equipped with circulating chiller set at -15°C. Cubes were added to the chopping bowl and chopped on low
speed (at 1,800 rpm) for 1 min. Then, 2% salt was added and chopping continued on low speed for an additional 1 min. Then, the moisture content was adjusted to 78% with ice water. After chopping for another 1 min, the sample was chopped on high speed (at 3,600 rpm) while applying a vacuum (40-60 kPa) to remove air pockets formed during comminution. Chopping continued until the chopping time totaled 18 min. Paste temperature was kept below 8°C.

Surimi and/or FPI paste were packed into a polyethylene bag and subjected to a vacuum machine (Reiser VM-4142; Roescher Werke, Osnabrueck, Germany) to remove air pockets developed during packing. The paste was then stuffed into metal tubes (3.0 cm I.D. and 25.3 cm length) and nylon tubes (3.0 cm I.D. and 15 cm length) using a sausage stuffer for cooking in a water bath and an ohmic cooker, respectively. Sample pastes were then cooked either in a 90°C water bath for 30 min or ohmically at a voltage gradient of 12.62V/cm in which the core temperature of 90°C was obtained in 30 sec. Gels were chilled in ice water before storing in a refrigerator (4°C) overnight. Gel preparation was conducted twice for each treatment.

Surface hydrophobicity

Protein surface hydrophobicity (S₀) of the supernatant from the paste was measured using an ANS (1-anilinonaphthalene-8-sulfonate) probe containing 8 mM ANS in 0.1 M phosphate buffer (pH 7.4) (Alizadeh-Pasdar & Li-Chan, 2000). The supernatant of the paste was prepared by homogenizing 3 g of paste with 27 ml of 0.6 M KCl in 20 mM Tris-HCl buffer (pH 7.0) for 1 min and centrifuging at 20,000 x g at
4 °C for 30 min. After centrifugation, protein concentration of the supernatant was serially diluted with the same buffer from 0.01 to 0.2 mg/ml. Then, 20 µL of ANS stock solution was added into 4 ml of each diluted sample and held at room temperature for 10 min. A luminescence spectrophotometer (Perkin Elmer LS-50B, Norwalk, Conn., USA) was used to measure the relative fluorescence intensity (RFI) of each solution at a wavelength of 390 nm and 470 nm (\(\lambda_{\text{excitation}}, \lambda_{\text{emission}}\)), respectively. An index of the protein \(S_0\) was calculated from the initial slope of the net RFI versus the protein concentration.

**Surface reactive sulphydryl content**

Ellman’s reagent (5, 5’-dithiobis (2-nitrobenzoic acid); DNTB) was used to measure surface reactive sulphydryl (SRSH) content of paste samples according to the procedure of Hamada et al. (1994). Paste was homogenized in a solubilizing solution of 0.6 M KCl in 20 mM Tris-HCl buffer (pH 7.0), and the protein concentration of the supernatant was adjusted to 1 mg/ml with the same buffer. Diluted sample (0.5 ml) was mixed with 2 ml of 0.6M KCl in 20 mM Tris-HCl buffer (pH 7.0), and 50 µL of 0.1M sodium phosphate buffer (pH 7.2) containing 10 mM DTNB and 0.2 mM EDTA. The mixture was then vortexed and incubated at room temperature for 15 min. The absorbance at 412 nm (UV-VIS Spectrophotometer, UV 2401PC, Shimadzu Co., Kyoto, Japan) was measured and the SRSH content was calculated based on absorbance using a molar extinction coefficient of 13,600 M\(^{-1}\)cm\(^{-1}\).
Oscillatory dynamic rheology

Viscoelastic changes during gelation were observed through a temperature sweep using a CVO rheometer (Malvern Instruments Ltd., Worcestershire, UK). The paste sample was positioned between a cone (4° and 40 mm diameter) and plate with a gap of 150 μm. A moisture trap with a moistened sponge was used during heating in order to minimize drying of the sample. A stress sweep was performed prior to temperature sweep to determine a linear viscoelastic region, and shear stress of 30 Pa was chosen for the oscillatory mode. The samples were heated from 10 °C to 90 °C at a heating rate of 2 °C/min with a constant frequency set at 0.1 Hz.

Fracture analysis

After storing the gels at 4°C overnight, they were held at room temperature for 2 h allowing them to equilibrate to room temperature. The gels were then cut into 25-mm lengths and subjected to a fracture test using a Texture Analyzer (TA-XT plus, Texture Technologies Corp, Hamilton, MA, USA) with a spherical probe (5 mm diameter) at 1 mm/s. Breaking force (g) and penetration distance (mm) were recorded. Ten measurements were collected for each treatment.

Color measurement

$L^*$, $a^*$, and $b^*$ values of gels from 25 mm samples were measured using a Minolta colorimeter (CR-310; Minolta Camera Co. Ltd., Osaka, Japan). According to the procedure of Kim and Park (2008), the standardization of the instrument was
performed using a Minolta calibration plate and a Hunter Lab standard hitching file. Whiteness was calculated based on the equation $L^* - 3b^*$.

Statistical analysis

Response data was subjected to a mixed model analysis of variance (PROC MIXED) with contrasts using Tukey’s studentized range (honestly significant difference) test. Two different batches were produced for each treatment, and all statistical analyses were performed using the SAS program (SAS Institute Inc., Cary, NC, USA) with the significant level set at 0.05.

7.4 Results and discussion

Surface hydrophobicity

Hydrophobic interactions are one of the major factors promoting protein network aggregation related to gel quality, and they also play a crucial role in stabilizing the structure of native proteins (Egelandsdal et al., 1995; Kristinsson & Rasco, 2000; Lanier et al., 2014). Protein surface hydrophobicity was measured in order to investigate the degree of unfolding of protein structure under different ratios of surimi and FPI paste. Surface hydrophobicity in paste became significantly higher ($p < 0.05$) when the proportion of FPI was larger than or equal to that of surimi. Results indicated the protein structures were more unfolded when the ratio of FPI increased, possibly due to the effect of NaOH-induced pH shifting during FPI preparation. Kristinsson and Hultin (2003a,b) studied conformational and structural changes of cod myosin at pH 11 and after subsequent pH readjustment to pH 7.5. They found an
irreversible change in the globular head region caused the failure of light chains to reassemble onto it. In contrast, the conformation of the myosin rod reverted to its native state upon refolding. This suggested that the lack of structure recovery in the myosin head during FPI processing caused an increased exposure of hydrophobic clusters. Likewise, Yongsawatdigul and Park (2004) assumed that light meromyosin had undergone denaturation during alkali treatments as indicated by differential scanning calorimetry (DSC) results.

Other studies showed higher surface hydrophobicity in FPI paste compared to surimi paste because of the chemical process in FPI preparation (Chaijan et al., 2006; Thawornchinsombut & Park, 2007). Raghavan and Kristinsson (2008) reported alkali-treated myosin from catfish exhibited increased surface hydrophobicity compared to control samples. In addition, Tadpitchayangkoon et al. (2010a) observed gradual increases of surface hydrophobicity in soluble sarcoplasmic proteins isolated from striped catfish as pH increased from 6 to 12. Therefore, the present study suggested that blending a higher concentration of FPI compared to surimi can demonstrate increased surface hydrophobicity.
Surface reactive sulfhydryl contents

The surface reactive sulfhydryl (SRSH) content of samples were shown in Fig. 2. Regarding functional groups in proteins, sulfhydryl groups are considered to be the most reactive (Nurkhoeriyati et al., 2011). A significant increase (p < 0.05) in SRSH content was observed as FPI concentration increased. This may be because the chemical process in FPI preparation induced protein unfolding to a higher degree. Kristinsson and Hultin (2003b) demonstrated pH-shift processing in cod myosin led to significant conformational changes in the globular head fraction of the myosin...
heavy chains. They also noted a large part of the myosin light chains were lost during the pH-shift processing. These structural changes possibly caused more exposure of reactive thiol groups. Similar results were reported by Lowery (1965) using myosin prepared from rabbit muscle.

Moreover, the conformational change involving sulphydryl groups is more important in alkali-treated FPI than acid-treated FPI, since they can promote oxidation of sulphydryl groups resulting in the formation of disulfide groups. These groups can provide the stability of proteins and improve the textural properties of the recovered proteins upon gelation (Monahan et al., 1995; Omana et al., 2010a). Kristinsson and Hultin (2003b) observed an increase in reactivity of accessible thiol groups on myosin after pH treatment, and more for alkali-treated samples than the acid-treated ones. Yongsawatdigul and Park (2004) showed alkaline-treated muscle proteins from rockfish promoted the formation of disulfide linkages upon heating, providing gels with higher breaking force and deformation than acid-treated samples.

The proteins recovered with FPI processing revealed increased SRSH content compared to the native proteins (Gehring et al., 2011; Nolsoe & Undeland, 2009). Hrynets et al. (2011) and Omana et al. (2010a) found an increase in SRSH content for pH-treated samples compared to mechanically separated turkey meat and chicken dark meat, respectively. The study of edible films prepared from fish water-soluble proteins (FWSP) showed that disulfide linkages were an important chemical bonding involved in protein-protein interactions. Authors reported adjusting the pH of FWSP solution to pH 12 facilitated in increasing SRSH groups and forming films which were flexible. Furthermore, Monahan et al. (1995) showed thiol oxidation reactions
between exposed SH groups occurred under alkaline conditions. The environmental factors, such as pH, enhanced protein-protein interactions resulting in favorable gelation from whey protein isolate (Monahan et al., 1995). Protein structures in surimi are closer to native structures than samples in FPI; thus, increasing the ratio of FPI to surimi may promote increased SRSH content when mixing surimi and FPI at different concentrations.

SRSH content was significantly higher (p < 0.05) in blending Alaska pollock surimi with carp FPI (PS-CF) than blending carp surimi with carp FPI (CS-CF). Results indicated that PS-CF treatments had a higher rate of protein unfolding compared to CS-CF treatments under the same comminution conditions. This was possibly because PS is less thermostable than CS (Poowakanjana & Park, 2014). Additionally, Poowakanjana and Park (2013) demonstrated a longer chopping time alone could unfold the protein structures, indicating PS may be more sensitive to mechanical action compared to CS.
Figure 7.2 – Surface reactive sulphydryl (SRSH) content of paste prepared from blending surimi (S) and fish protein isolate (F) at different ratios: S100, S75/F25, S50/F50, S25/F75, and F100. Abbreviations, error bars, and different letters on and/or in each bar indicate the same as those in Figure 7.1.

Oscillatory dynamic rheology

Testing the viscoelastic properties of fish proteins as affected by blending different ratios of surimi and FPI helps to understand the change of protein conformation, the nature of thermal denaturation and subsequent association. The rheograms demonstrated the gelation processes were different as the concentration of surimi and FPI changed. The changes of storage modulus (G’) at surimi to FPI ratios of 100:0, 75:25, and 50:50 showed a small peak, indicating light meromyosin denatured around 37-47°C (Fig. 3A and 3B). However, there was no sign of light meromyosin in the other two treatments, likely because the chemical process of pH shift unfolded protein structures during FPI refining. Kristinsson and Hultin (2003a),
Thawornchinsombut and Park (2007), and Yongsawatdigul and Park (2004) showed similar G’ patterns of pH-untreated samples versus pH-treated samples, particularly in denaturation of light meromyosin in cod, Pacific whiting, and rockfish, respectively. Major conformational changes occurred with the myosin head region during FPI processing. The pH treatments may cause the loss of contact between the light chains and the globular head regions of the protein due to the result of electrostatic repulsion and distortion of the neck region (Kristinsson & Hultin, 2003a,b; Hultin & Keller, 2000). Gershman and Dreizen (1970) also documented alkali disassociation of light chains in rabbit myosin. Moreover, results indicated the peak, representing light meromyosin, became smaller as the ratio of surimi to FPI decreased. This suggested that the conformation of samples had been changed into a more unfolded state when the FPI to surimi ratio increased.

A point where the temperature begins to rise as thermally unfolded proteins start to aggregate is called the onset of G’. The lower the onset temperature for G’ denotes the lower thermal stability of the proteins (Ingadottir & Kristinsson, 2010). The onset of G’ decreased as FPI concentration increased. This was probably accompanied by a higher degree of protein unfolding in FPI compared to surimi and/or a loss of light meromyosin during FPI processing (Kristonsson & Hultin, 2003b; Undeland et al., 2002). Similar trends were observed in other studies using cod, Pacific whiting, rockfish, and tilapia, respectively (Kristinsson & Hultin, 2003a; Thawornchinsombut & Park, 2007; Yongsawatdigul & Park, 2004; Ingadottir & Kristinsson, 2010).

G’ was higher at 10°C and 90°C when the amount of surimi was higher than FPI. This may be because protein structures in FPI were more unfolded than surimi prior
to thermal treatment, corresponding to results from surface hydrophobicity and SRSH content, respectively. In addition, the shapes of the rheograms were different among treatments, and the transformations of paste to gel were smoother when the concentration of surimi was higher than FPI. Protein particles in FPI may easily move, entangle each other, and form protein network, leading to a faster change in $G'$. Omana et al. (2010b) reported denatured FPI became aggregates during the formation of a gel network, which caused an increase in protein-protein interactions. However, they observed that protein-water binding was reduced, leading to high breaking force with low deformation. This suggested that FPI samples may have had lower protein-water interactions compared to surimi samples. As a consequence, the alteration in $G'$ became sharper as the ratio of FPI to surimi increased.

In the present study, blending PS-CF appeared to have lower thermal stability than blending CS-CF. PS-CF samples showed small peaks, indicating light meromyosin was denatured, at lower temperature than CS-CF samples (Fig. 3A and 3B). The onset $G'$ in PS-CF was also lower than that of CS-CF, implying lower stability of the protein structures. Furthermore, the differences between $G'$ at 10°C and 90°C among treatments were larger in PS-CF compared to CS-CF. It may be hypothesized that blending PS-CF was more sensitive to heat than blending CS-CF, and led to a higher degree of unfolding and conformational changes. Comparable results were presented by Fukushima et al. (2007) and Tadpitchayangkoon et al. (2010b), which demonstrated the thermal stability of samples affected denaturation and aggregation temperatures.
The phase angle of blending surimi and FPI at different ratios heated from 10 to 90°C is presented in Fig. 3C and 3D. The phase angle is useful to determine the thermal transition of the sample from a sol-like (viscous) to a gel-like (elastic) state. The transition of the phase angle became larger as the ratio of surimi to FPI increased, possibly because proteins in FPI were structurally changed during pH-shifting. Yongsawatdiful and Park (2004) illustrated a decrease in phase angle, indicating the formation of an elastic material, started at a relatively lower temperature in FPI compared to washed mince. The lowest phase angle was obtained at a ratio of 25:75, surimi to FPI. This may be correlated to a lower breaking force at ratio of 25:75, surimi to FPI.
Figure 7.3 – Storage modulus ($G'$) (A, B) and phase angle ($\delta$) (C, D) of paste prepared from blending surimi (S) and fish protein isolate (F) at different ratios (S100, S75/F25, S50/F50, S25/F75, and F100) as affected by temperature sweep. Abbreviations are same as those in Figure 7.1.
Fracture analysis

A significant decrease (p < 0.05) in breaking force and penetration distance was observed when gels were prepared from blending surimi and FPI (Fig. 4A and 4B). Gels prepared from a blend of surimi and FPI showed linear responses in texture until the proportion of FPI exceeded 50%. Results indicated gels prepared from surimi or FPI alone showed significantly higher (p < 0.05) breaking force than gels prepared from blended samples. Cavenaghi-Altemio et al. (2013) reported breaking force is most likely due to the functional performance of the protein type rather than the protein content. Likewise, Paker and Matak (2015) showed different additives have different effects on gels based on the composition of the protein used. Chen (2000) and Park (1994) demonstrated the addition of wheat gluten or whey protein concentrate made surimi gels more brittle, while plasma protein concentrate and egg white seemed to enhance the binding functionality of surimi through protein-protein interactions. Park (1994) and Tadpitchayangkoon et al. (2010b) revealed some protein additives and sarcoplasmic proteins, respectively, appeared to delay denaturation (unfolding) of fish proteins measured by DSC. As it is observed in this study that blending FPI with surimi certainly increased more unfolded proteins, it is postulated that adding unfolded proteins in a large quantity may interfere with protein-protein interactions and cause adverse effects on the quality of gels prepared from surimi. Similar results were perceived by the addition of soy protein isolate (SPI) into carp surimi (Luo et al., 2008).
Another reason for a decrease in breaking force by blending surimi and FPI may be because the ratio of surimi and FPI was not optimum. Samejima et al. (1986) showed lower gel strength when adding actin to myosin, whereas Yasui et al. (1980) illustrated a positive effect of actin on gel formation with an optimal ratio of actin and myosin. This suggested that the ability of protein to form gels depends on a favorable balance of attractive and repulsive forces and different protein structures.

In the current study, gels with a higher amount of surimi indicated a higher penetration distance. Also, some gels prepared from the blends of surimi and FPI showed higher penetration distance than gels prepared from FPI alone. Omana et al. (2010b) described that higher breaking force with low deformation in FPI gels may be due to the reduction of protein-water binding during gelation. Perez-Mateos and Lanier (2007), Nurkhoeriyati et al. (2011), Luo et al. (2008), Thawornchinsombut and Park (2007), Chaijan et al. (2006) obtained similar results using Atlantic menhaden, spent duck meat, SPI, Pacific whiting, sardine and mackerel, respectively. Park (1994) stated that deformation, as an indicator of protein interactions, was strongly affected by protein functionality. Thus, in order to develop an optimization procedure for gel making with consistent quality, it is important to consider not only protein-protein interaction, but also protein-water binding.

The results indicated gels cooked in a water bath (slow heating) demonstrated higher breaking force than gels cooked ohmically (fast heating) possibly due to the role of endogenous transglutaminase. However, higher penetration distance was observed when gels were cooked ohmically compared to gels cooked in a water bath. When comparing PS-CF and CS-CF samples, the highest breaking force was found in
100% surimi gel among PS-CF samples, but it was found in 100% FPI gel among CS-CF samples. Results suggested both heating rate and species can affect gel qualities when surimi and FPI are mixed at different ratios.
Figure 7.4 – Breaking force (A) and penetration distance (B) of paste prepared from blending surimi (S) and fish protein isolate (F) at different ratios: S100, S75/F25, S50/F50, S25/F75, and F100. W-PS-CF: gels prepared from Alaska pollock surimi and carp fish protein isolate and cooked at 90°C for 30 min in a water bath. O-PS-CF: gels prepared from Alaska pollock surimi and carp fish protein isolate cooked ohmically. W-CS-CF: gels prepared from carp surimi and carp fish protein isolate and cooked at 90°C for 30 min in a water bath. O-CS-CF: gels prepared from carp surimi and carp fish protein isolate cooked ohmically. Error bars, and different letters on and/or in each bar indicate the same as those in Figure 7.1.

Color measurement

Color is one of the important quality factors for consumer acceptance. Whiteness of gel decreased significantly (p < 0.05) as the ratio of FPI increased (Fig. 5). This was because FPI treatments contained both sarcoplastic proteins (such as heme proteins and enzymes) and myofibrillar proteins, whereas surimi treatments only contained myofibrillar proteins. Comparable results were detected by Yongsawatdiful and Park (2004), Parker and Mata (2015), and Omana et al. (2010) indicating a decrease of whiteness due to the presence of sarcoplastic proteins. Park (1994) and Chen (2000) also found that protein additives affected the whiteness and the values varied among different additives. Yoon et al. (1997) studied the linearity of relationships between high and low grades of Alaska pollock and Pacific whiting when they were blended proportionally. Results indicated that blending different grades exhibited linearity of whiteness, possibly because lower grade surimi includes more impurities, such as heme proteins, enzymes, skin, and connective tissues.

Results indicated gels cooked ohmically demonstrated higher whiteness than gels cooked in a water bath. Ohmic cooking generates uniform heat and provides even temperature distribution, since both the liquid and solid phases are simultaneously
heated (Park & Beliveau, 2014). Dai et al. (2013) examined the effects of ohmic cooking on color attributes and sarcoplasmic changes of pork muscle. They found that degradation of the sarcoplasmic proteins was slower in ohmic cooking compared to water bath cooking, resulting in brighter color and higher water holding capacity.

Figure 7.5 – Whiteness of paste prepared from blending surimi (S) and fish protein isolate (F) at different ratios: S100, S75/F25, S50/F50, S25/F75, and F100. Abbreviations, error bars, and different letters on and/or in each bar indicate the same as those in Figure 7.4.

7.5 Conclusion

Results from surface hydrophobicity and SRS content indicated the degree of unfolding in fish proteins prior to gelation increased as the ratio of FPI to surimi increased. Effects of blending surimi and FPI on gel functionality (hardness, cohesiveness, and whiteness) showed a linear pattern when the proportion of surimi is
larger than or equal to FPI. However, when the proportion of FPI exceeds more than 50%, these relationships were no longer linear. It was noted that breaking force and penetration distance decreased significantly when the ratio of surimi to FPI decreased. Blending Alaska pollock surimi with carp FPI or carp surimi with carp FPI can be achievable through intermolecular interaction only when the proportion of FPI does not exceed 50%.
For the wholesome quality and stability of surimi seafood, understanding the functional properties of fish muscle protein and their gelation process are important. The gel formation of fish protein isolate (FPI) and surimi is a complex process comprising structural and functional changes of proteins. The degree of protein unfolding prior to gelation is a crucial factor to improve gel qualities. Surimi gels prepared from pre-rigor tilapia had higher rigidity values and lower degrees of denaturation compared to gels prepared from the post-rigor stage. However, the effect of rigor mortis on FPI gels was not as strikingly pronounced as on surimi. In addition, the effect of salt on the gelation of tilapia muscle with different rigor states or refining methods was determined. The addition of salt seemed to easily unfold protein structure in FPI as measured by surface hydrophobicity, surface reactive sulfhydryl (SRSH), storage modulus (G’), and Raman spectroscopy, whereas the effect of salt in surimi was not as sensitive as in FPI.

The relationship between fresh and frozen whole fish when refined differently as FPI or surimi, and their effect on the biochemical and rheological properties of gels from the same species, were investigated. The quality of FPI and surimi made from fresh tilapia were not significantly better than that made from frozen whole tilapia stored for 3 mo. Frozen storage did not affect the quality of FPI gel as much as surimi. The results suggested tilapia frozen for 3 mo can be successfully used as a raw material for both FPI and surimi production without losing gelling quality. The
addition of salt to FPI also induced a higher degree of denaturation prior to gelation than surimi.

The effect of rigor and frozen storage on the gel-forming ability was not noted in FPI as much as surimi. However, FPI was more sensitive than surimi to differences in chopping time and temperature. Results indicated both FPI and surimi prepared from tilapia chopped at 25°C for 18 min showed a higher breaking force and penetration distance compared to chopping at 5°C for 6 min. Likewise, higher storage modulus ($G'$) was obtained from FPI and surimi chopped at 25°C for 18 min than samples chopped at 5°C for 6 min. Although chopping FPI and surimi from tilapia with salt improved gel texture as chopping temperature and time increased, a significant decrease of breaking force was observed when FPI was chopped at 5°C for 6 min. This treatment demonstrated by larger protein aggregates and coarser structure in microscopic analyses. Properly controlled comminution may increase the disintegration of protein structure, decrease particle size, and enhance gelation. Under optimized chopping conditions, cooked gels from two fish proteins prepared by different refining methods (FPI and surimi made from tilapia) were comparable.

Furthermore, Fourier transform infrared (FT-IR) and Raman spectroscopy provided compatible information, allowing a better interpretation of the structural changes in FPI and surimi under various comminution conditions. Both techniques revealed the degree of unfolding in FPI and surimi paste prepared from tilapia became higher when chopping temperature and time increased, or when salt was added. Results showed that both hydrophobic interactions and disulfide bonds were
significantly increased during gelation. Also, both FT-IR and Raman spectra indicated the increase of hydrophobic interactions, disulfide bonds, and β-sheet contents in FPI and surimi gels as chopping temperature and time increased from 5°C for 6 min to 25°C for 18 min. This spectroscopic information may be correlated with the gel texture of FPI and surimi. The study suggested controlling comminution conditions is one of the important factors as protein structures are disintegrated in order to improve gel-forming ability in FPI and surimi. FT-IR and Raman spectroscopy are useful complementary tools for elucidating the change in the structure of protein during comminution and gelation. The FT-IR spectra showed fewer bands in FPI samples compared to surimi samples.

The dissertation research and previous research suggested that the sensitivity of myofibrillar proteins to salt depends on the species. For those species with high thermal stability like tilapia or Atlantic croaker, their myofibrillar proteins were not easily unfolded like cold water species, which are sensitive to temperature, during pH-induced chemical process.

Blending various grades of fish proteins is one way to manage consistent functionality of proteins and minimize production costs. The study of blending carp FPI and Alaska pollock surimi as well as carp FPI and carp surimi demonstrated the compatibility of fish proteins prepared using two distinctively different methods as a resource for fish protein gel products. The blending effects of FPI and surimi on gel functionality, including hardness, cohesiveness, and whiteness, exhibited a linear pattern when the proportion of surimi is larger than or equal to that of FPI. When the
ratio of FPI exceeds more than 50%, the relationships were no longer linear. Moreover, results from surface hydrophobicity and SRSH content indicated the degree of fish protein unfolding prior to gelation increased as the ratio of FPI to surimi increased. Results suggested blending FPI and surimi did not affect the inter-molecular interactions of protein in a linear pattern and might be attainable only when the proportion of FPI does not exceed 50%.
CHAPTER 9

BIBLIOGRAPHY


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