

AN ABSTRACT OF THE DISSERTATION OF

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Title: Immunological and Physiochemical Characterization of Fish Myosins

Abstract approved:

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Increased sales of surimi seafood, with the majority as crabstick in the United States indicates that surimi based products are becoming more popular. With growing popularity, there is increased competition for market share. Under these circumstances, some companies may be willing to sacrifice product quality in order to facilitate manufacture and reduce price as a means of gaining market share. During the last 10 years, poor quality imports, which exceeded nearly 2 million pounds a year, have negatively impacted the domestic market. The most important factor in surimi production is the textural properties imparted by the fish protein. Consequently, crabstick quality is directly related to the amount of fish protein from raw surimi that is used. A high quality crabstick product typically contains 40% or higher fish

protein. The protein content and quality of raw surimi are varied by fish species and surimi grade. As a way to increase profits, lower quality products can be sold while claiming they are made primarily of high quality fish protein surimi.

Indirect enzyme-linked immunosorbent assays (ELISA) technique was used to identify and quantify the use of dried egg white (DEW) and whey protein concentrate (WPC) in crabsticks. The use of SDS-PAGE for the quantification of protein additives has had limited success due to the high shear and high temperature processes of surimi crabstick. Monoclonal (anti-heat denatured ovalbumin) and polyclonal (anti- β -lactoglobulin) antibodies were used. Antibodies showed no significant cross-reactivity with non-target crabstick proteins. An optimized extraction solution of 10% SDS and 2.5% β ME yielded high extractability with improved consistency. Quantification of DEW and WPC was achieved using the optimized extraction solution and indirect ELISA. Estimated DEW values were within 7% of actual values, WPC samples were within 17%. Inter-assay coefficients of variance for DEW ranged from 0.9% to 3.1% and those of the WPC were 1.0% to 8.0%.

A competitive enzyme-linked immunosorbent assay (ELISA) was developed for quantification of Alaska pollock (AP) surimi in crabsticks. Identification of fish species is complicated by processing, cooking, and additional ingredients. ELISA is a powerful tool for identification and quantification of fish species. Polyclonal antibodies were raised in rabbits against a 15-amino-acid peptide (Ala-Pro-Lys-Lys-Asp-Val-Lys-Ala-Pro- Ala-Ala-Ala-Ala-Lys-Lys) determined from the myosin light chain 1 (MLC 1) of AP. Immunoblotting showed the anti-pep-AP antibody had no

significant cross-reactivity with protein additives. However, cross-reactivity of the MLC 1 from Pacific whiting, and threadfin bream surimi was observed. MLC 1 was purified from AP surimi and used as the coating protein in the competitive ELISA. MLC 1 was serially diluted and had a R^2 of 0.9845 following a logarithmic curve. All estimations of AP surimi were within 9% of the actual value. Inter-assay coefficients of variance ranged from 4.2 to 4.9%.

Antibodies were produced by injection of a synthesized 15-amino acid peptide or by whole myosin light chain 1 isolated from Alaska pollock (AP). A direct sandwich ELISA was tested using extracts prepared from AP, Pacific whiting (PW), true cod (TC), tilapia (T), and catfish (C). Fish extracts were studied using SDS-PAGE and Western blotting. A standard curve was created for each fish and used to estimate 3 different verification samples. All estimations were within 10, 37.5, 30, 43, and 34% of the actual value for AP, PW, TC, T, and C, respectively. When one or more fish species was mixed together with AP the estimation of the Alaska pollock content became much less accurate. This study confirms a direct sandwich ELISA accurately detects the quantity of AP. Testing found the sandwich ELISA developed exhibited cross-reactivity with other protein sources such as beef, chicken, pork, shrimp, and clam.

Purified Chinook salmon myosin was studied using SDS-PAGE and densitometric analysis to determine its purity, which was 94%. Myosin subjected to linear heating began to form aggregates at > 24 °C as measured by turbidity at 320 nm. Conformational changes, as measured by surface hydrophobicity (S_o), began at

18.5 °C and continued to increase up to 75 °C after which it decreased slightly. Total sulfhydryl content (TSH) showed similar trends from 18.5 to 50 °C after which point the TSH began to drop. Surface reactive sulfhydryl groups (SRSH) gradually increased as the temperature increased from 18.5 to 50 °C and then followed a similar trend as the TSH decreased from 55 to 80 °C. Differential scanning calorimetry showed four peaks, three endothermic (27.9, 36.0, 45.5 °C) and one exothermic (49.0 °C). Dynamic rheological measurements provided information concerning the gelation point of salmon myosin which was 31.1 °C as samples were heated at 2 °C/min.

Purified tilapia myosin was digested with α -chymotrypsin and purified to obtain heavy meromyosin (HMM) and light meromyosin (LMM). Tilapia myosin, HMM, and LMM were studied using SDS-PAGE. Myosin, HMM, and LMM were linearly heated from 10 to 90 °C and showed protein denaturation/aggregation during heating as measured by turbidity at 320 nm. Conformational changes as measured by surface hydrophobicity (S_o) showed a marked increase for myosin and HMM between 30 and 40 °C and reached a stable plateau at 70 °C. LMM, in an extremely small magnitude, also showed a continuous increase to 70 °C. Total sulfhydryl content (TSH) showed that the -SH residue content of HMM was nearly double that of LMM. Surface reactive sulfhydryl groups (SRSH) for myosin and HMM were relatively unchanged from 10 to 30 °C but increased significantly from 30 to 50 °C. SRSH content of LMM was lower than that of the TSH content of LMM but both showed a slightly decreasing trend as the sample was heated. Differential scanning calorimetry

showed 3 (17.5, 41.9, and 49.9 °C), 2 (43.0 and 67.1 °C), and 3 (40.4, 51.7, and 69.0 °C) major peaks for myosin, HMM, and LMM, respectively. Dynamic rheological measurements demonstrated crossover points, which are generally recognized as gelation point, 40.3 °C for myosin and 27.0 °C for HMM.

Obtaining antibodies that would bind the target protein in the highly processed crabstick proved to be a key for the success for assay development. The anti-pep-AP antibody proved to be very accurate in the qualification and quantification of Alaska pollock surimi used in crabsticks. Using the appropriate ELISA format, competitive vs. indirect, proved to be pivotal in obtaining accuracy and repeatability of the assay. Using a direct sandwich ELISA for the qualification and quantification Alaska raw fish fillets provided good estimation of Alaska pollock used in the verification sample. However, the Alaska pollock MLC 1 antibodies exhibited high levels of cross reactivity with other fish species.

It has been shown that salmon myosin can be affected by heating. As myosin was heated in solution myosin aggregates began to form as evidenced by the increased turbidity. Myosin denaturation and gel formation was also supported by the thermal transition points determined from differential scanning calorimetry and dynamic rheology. The exposure of buried hydrophobic and sulfhydryl groups of myosin was increased as the myosin was linearly heated. The study of tilapia myosin, HMM, and LMM carried out from chymotryptic digestion of myosin allowed for important insights into the thermo stability and gelation properties of tilapia.

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Immunological and Physiochemical Characterization of Fish Myosins

by

Zachary H. Reed

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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.

Zachary H. Reed, Author

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1 INTRODUCTION

Understanding immunological and rheological characteristics of fish myosins would be vital for the science of surimi and surimi seafood. This thesis consists of ten chapters including literature reviews and six research papers covering a wide scope of fish myosin for their immunological and rheological properties.

The most reliable determination for fish identification is made when the fish is still in its whole state, retaining all of its morphological characteristics. As the fish is processed by methods, such as heading, gutting, skinning, deboning, filleting, and mincing, these identifying characteristics are diminished or lost completely. Due to the difficulty in identifying fish species some unscrupulous commercial fish suppliers have been known to substitute lower priced fish species for those of higher value (Civera, 2003).

When attempting to identify fish species there are a number of challenges that must be overcome. Some methods developed have only been able to identify the fish species in its raw form (Mackie, 1996). While other methods have been developed for use on whole raw, cooked or marinated products (McNulty & Klesius, 2005). Other methods, such as DNA analysis, are capable of identifying fish species in its raw or cooked form, but can be costly to analyze and require highly skilled technicians to perform the analysis. DNA analysis is extremely accurate, however it does not provide a manner in which to quantify the species content used in the product.

The inherent difficulties in fish species product identification are due to a variety of factors. First, fish are extremely abundant in fresh and salt water all over the globe making the total number of species to identify extremely large. In 2003 Nature reported that 300 scientists from 53 countries have to date identified 20,000 different species of fish in the world's seas and oceans (Mason, 2003). In addition to the large number of fish species found in the world's oceans, the world's fresh bodies of water hold between 9,000 and 25,000 different fish species (Cosgrove & Rijsberman, 2000). The overall diversity of the world's fishes complicate the identification of each species, even more so when the fish is an important food source that is known throughout the world by different common names. Second, not all fish species are used for human consumption. Through processing the morphological characteristics of the fish are often removed thereby increasing the difficulty of species identification. Fish that have been processed into fillets or other products such as breaded fish sticks and fillets, have lost their morphological identity and therefore must rely on other methods for species identification.

Some people have intentionally substituted one species of fish for another of greater value (Martinez & James, 2005). In an attempt to encourage correct labeling of fish and seafood, the United States Food and Drug Administration (USFDA) Center for Food Safety and Applied Nutrition (CFSAN) has compiled a Seafood List that is available online that allows for the correct labeling of imported and domestically available seafood (<http://vm.cfsan.fda.gov/~frf/seaintro.html>). When food adulteration has taken place, it is a form of fraud committed against the consumer. Table 1

outlines some examples of the economic impacts that the substitution of fish can have on the consumer when it comes to their seafood purchasing power. Not only is the fraud perpetrated on the average customer in the market but has been found in restaurant entrees as well. While investigating the substitution of red snapper and swordfish in major U.S. cities, Hsieh (1998) found that 6 out 13 entrees represented as red snapper were indeed not red snapper. Of the 21 samples of swordfish 2 were found to be misrepresented as swordfish. Whether the restaurants were also victims of fraud or whether they themselves are the fraudulent party is not indicated.

Actual Seafood	Substituted Seafood	Price gain/lb
Red Snapper	Rockfish	\$3.06
Mahi Mahi	Yellowtail	\$0.87
Swordfish	Mako Shark	\$1.58
Cod (Atlantic)	Alaska Pollock	\$1.37
Halibut (Pacific)	Sea Bass	\$0.49
Dover Sole	Arrowtooth Flounder	\$0.30
Red Drum (Red Fish)	Black Drum	\$0.73
Yellow Perch (Great Lakes)	White Perch	\$1.55
Chum Salmon	Pink Salmon	\$0.24
Scallops	Skate Wings	\$6.74
Walleye	Alaskan Pollock	\$1.80

Table 1 – Examples of commonly substituted seafood in the USA (USFDA <http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/RegulatoryFishEncyclopediaRFE/ucm071528.htm>) Values represent the potential economic price gain per pound made by selling the lower priced substitute seafood as the higher priced item. Prices are given as the average ex-vessel price for 2008 of the species listed. Prices were obtained from the National Marine Fisheries Service, Fisheries Statistics Division, Silver Spring, MD.

In addition to the economic impact that fish substitution has on seafood consumers, fish substitution can also affect the particular fish species being substituted. In the United States the fish species *Lutjanus cumpechanus* is the only

species allowed to be sold under the name of red snapper (FDA, 2009b).

Unfortunately, red snapper has been overfished in the Gulf of Mexico and the South Atlantic (NOAA, 2009). Overfishing of red snapper has caused a high number of substitutions of other fish for red snapper (Hsieh, Chen & Nur, 1997; Hsieh, 1998; Huang, Marshall, Kao, Otwell & Wei, 1995; Renshaw, Saillant, Bradfield & Gold, 2006; Zhang, Huang, Cai & Huang, 2006), thus showing the importance of having a method that is capable of rapidly identifying the red snapper.

Fraud perpetrated against the consumer through the adulteration of food products may be objectionable for health and/or religious reasons as well (Jacquet & Pauly, 2008). A variety of testing methods are available for the detection of food contamination with known food allergens, most of which are directed towards proteins or glycoproteins. Techniques such as immunochemical detection and amplification of DNA allow for the detection and subsequent labeling of the offending allergens. The consumption of products containing, undeclared constituents may cause serious health risks and problems such as an allergy in sensitized individuals (Mackie, 1996). Fish is one of the most common causes of IgE-mediated food hypersensitivity. Extensive studies were done in the early and mid 1970s to try and determine the major cause of food hypersensitivity to fish. Elsayed and others (1971; 1983; 1975) determined that parvalbumin was the culprit for the majority of food hypersensitivity to fish. Even a minute intake of the fish allergen is sufficient to cause a severe reaction in those with hypersensitivity. Due to the potential danger for those with fish allergies many studies have been performed with the intent of identifying fish allergens and the detection of

those fish allergens in food (Chen, Hefle, Taylor, Swoboda & Goodman, 2006; Esteve-Romero, Yman, Bossi & Righetti, 1996; Fæste & Plassen, 2008; Gajewski & Hsieh, 2009; Kawase, Ushio, Ohshima, Yamanaka & Fukuda, 2001; Ma et al., 2008; Poulsen, Hansen, Nergaard, Vestergaard, Skov & Bindslev-Jensen, 2001; Sun, Lin, Deng, Liang & Gao, 2009; Yoshida, Ichimura & Shiomi, 2008). Along with fish, shellfish are also common cause of allergies than can be of concern for those with shellfish hypersensitivity (Fuller, Goodwin & Morris, 2006; Lu, Ohshima, Ushio, Hamada & Shiomi, 2007).

Myofibrillar proteins represent between 70 to 79% of total proteins in fish with myosin comprising 55 to 60% of the total myofibrillar proteins (Lanier, Carvajal & Yongsawatdigul, 2005). Myosin is the main protein responsible for the gelation properties of fish, with the gelation properties of the myosin being species specific (Chan, Gill & Paulson, 1993; Park, Yongsawatdigul, Choi & Park, 2008; Yongsawatdigul & Park, 1999). Myosin is a large asymmetric molecule that has a long α -helical coiled-coil tail and two globular heads with an approximate weight of 500 kDa (Hodge & Cope, 2000). The basic body plan of myosin consist of an N-terminal head or motor domain, a light chain-binding neck domain, and a class conserved, C-terminal tail domain and have been categorized into over twenty different classes (Mooseker & Foth, 2008).

Temperature plays an important role in the gelation properties of fish myosin (Visessanguan, Ogawa, Nakai & An, 2000). Heat induced gelation of myosin undergoes two steps, denaturation and aggregation (Stone & Stanley, 1992). The

process of denaturation involves the conformational changes of myosin, which can expose functional groups, such as hydrogen bonds and hydrophobic groups. During aggregation the denatured myosins align themselves to form a three-dimensional gel matrix, which provides the elastic nature of the heated myosin gels. The aggregation properties of myosin can be further studied by digesting the myosin with chymotrypsin, which will yield heavy and light meromyosin (HMM, LMM) (Ogawa, Miyagi, Tamiya & Tsuchiya, 1999a; Szent-Görgyi, 1953; Togashi, Kakinuma, Nayaka, Ooi & Watabe, 2002; Weeds & Pope, 1977). By studying the aggregation properties of the HMM and LMM we can determine which portion of the myosin molecule plays which role in the gelation process.

In this dissertation research, various scopes of fish myosin were thoroughly examined, namely immunological and biochemical. It was not possible to obtain fresh (< 4 h post mortem) Alaska pollock, also the physiochemical aspects of Alaska pollock has been extensively studied due to its important use in the production of surimi. The dissertation outline is as follows: 1) to create an assay capable of quantifying the amount of egg white and whey protein used in crabsticks; 2) develop a competitive ELISA to quantify the amount of Alaska pollock surimi used in crabstick; 3) investigate the use of a direct sandwich ELISA for the detection of raw Alaska pollock fillets; 4) to characterize the rheological and biochemical properties of Chinook salmon myosin as affected by heating; 5) to study the thermal aggregation of tilapia myosin, HMM, and LMM of tilapia.

2 LITERATURE REVIEW

2.1 Biochemical and immunochemical assays for species identification using myosin

2.1.1 Electrophoresis

2.1.1.1 SDS-PAGE

Electrophoretic techniques have proven to be useful in identifying food protein components. Researchers have had success in identifying a variety of fish species using SDS-PAGE and isoelectric focusing (IEF). (Chen & Hwang, 2002b; Etienne et al., 2001; Etienne et al., 2000; Hsieh et al., 1997; Piñeiro et al., 1999). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been used extensively for protein visualization and identification. In 1970, *Nature* published an article by Ulrich K. Laemmli titled “*Cleavage of structural proteins during assembly of the head of bacteriophage-T4*” (Laemmli, 1970) which described a discontinuous buffer system for the separation of proteins based solely on their molecular weight. This paper has become *Nature’s* most cited paper to date with over 192,022 citations and counting and was the second most cited paper between 1981 and 1995 (Garfield, 1998). SDS-PAGE works on the basic principle that as proteins are heated in the presence of SDS, an anionic detergent, they are denatured and evenly coated with SDS, which imparts a uniform negative charge to the protein that overwhelms the intrinsic protein charge (Nelson & Cox, 2000b). The denaturing effects of SDS remove all secondary, tertiary, and quaternary protein structures and the addition of β -

mercaptoethanol disrupts any disulfide bonds. When the denatured protein is loaded on the polyacrylamide gel and an electrical current is passed through the gel, the proteins begin to migrate away from the negatively charged cathode and towards the positively charged anode. The discontinuous SDS-PAGE gel is made up of two distinct polyacrylamide gel sections. The first section the protein enters is known as the stacking gel. The purpose of the stacking gel is to concentrate, or “stack” the proteins in a tight group so that all of the proteins enter the resolving gel at the same time. This is accomplished by the use of a Tris-HCl Glycine buffer with a pH of 6.8 and a lower concentration of acrylamide, which is less impeding on the movement of the proteins. Glycine is a zwitterion, a molecule that carries both a negative and a positive charge, and at pH 6.8 the neutral form is favored. As the electrical field is applied, the negatively charged chloride ions have a higher electrophoretic mobility than the neutral glycine ions and the mobility of the proteins is between that of the chloride and glycine. As the electrophoresis of the sample continues, the proteins are trapped in a very tight band between the chloride ions and the glycine ions, which allow for the proteins to be stacked and arrive at the resolving gel at the same time. As the protein sample enters the resolving, gel the pH is changed from 6.8 to 8.8, at which point the glycine ions are then changed predominantly to the negatively charged glycinate form. The glycinate mobility is increased and they pass the proteins in the gel and they migrate through PAGE gel, which acts as a molecular sieve. The electrophoretic mobility of the protein will increase as well, however the resolving gel has smaller pore sizes than that of the stacking gel so the movement of the proteins is

decreased. The separation at this point is due to the size of the protein as smaller proteins may move faster through the gel than larger ones, allowing for a separation based solely on molecular weight. Due to the nature of SDS-PAGE, it allows for consistent protein patterns to be separated, which can then be used for comparison of standard proteins and the molecular size of the protein can be estimated accurately (Weber, Pringle & Osborn, 1972). The visualization of the separated protein bands is typically done by staining and then de-staining the gels using a dye such as Coomassie brilliant blue.

SDS-PAGE has been used for fish species identification as well as endeavoring to quantify the amount of protein found in other food products made from fish. Reed and Park (2008) have used SDS-PAGE along with densitometry to quantify the amount of Alaska pollock surimi used in crabstick manufacture. However one major obstacle that has proven difficult to overcome when using SDS-PAGE as a means for identification is the overlapping of protein bands when migrating through the SDS gel matrix. Reed and Park found that when attempting to identify and quantify the amount of dried egg white (DEW) used in crabstick manufacture the issue was complicated due to the similar molecular weights of ovalbumin from dried egg white and actin from the Alaska pollock surimi. Ovalbumin is approximately 54% (Stevens, 1991) of the total protein found in DEW and has a molecular weight of 45 kDa, while actin comprises 15 to 30% (Lanier et al., 2005) of the myofibrillar protein and has a molecular weight of 43 kDa. This large quantity of proteins of similar molecular weight can cause the bands to overlap and not be resolved. Due to the limitations of

SDS-PAGE for qualification and quantification in complex food mixtures, other methods have also been explored out for protein identification.

2.1.1.2 Isoelectric focusing

Amino acids are the building blocks for proteins and each amino acid has a characteristic isoelectric point (pI). The isoelectric point is the pH at which the amino acid has an overall net charge of zero. The pI of amino acids can range from 2.77 for aspartic acid to 10.76 for arginine (Nelson & Cox, 2000a). When amino acids are linked together through peptide bonds they form proteins. The amino acid sequence imparts an overall characteristic pI for that individual protein, that is to say each protein will have a characteristic pH at which its overall net charge is zero. Isoelectric focusing (IEF) is an electrophoretic technique that takes advantage of the characteristic pI of individual proteins. When using IEF, a pH gradient is created between the anode and the cathode by allowing a mixture of low molecular weight organic acids and bases, known as ampholytes, to distribute themselves in an electric field that is generated across the gel (Gaál, Medgyesi & Vereczkey, 1980). The protein sample is then added to the gel and the electrical field is then reapplied and the proteins move along the pH gradient until they reach their pI at which point they have no net charge and they cease to move.

The Regulatory Fish Encyclopedia (FDA, 2009a) contains standardized isoelectric focus plates for 94 different commercially important fish species for North America. In 1990, the Association of Official Analytical Chemists adopted IEF as the official method for raw fish species identification (AOAC, 1990). Hsieh et al. (1997;

1998) demonstrated the ability of species identification using IEF for the commercially important fish species of red snapper (*Lutjanus cumpechanus*). Using isoelectric focusing Hsieh, Woodward & Blanco and (1995) tested 121 retail market snapper fillets for compliance with labeling regulations. Samples were tested against 12 authentic snapper species. They found that of the 81 samples labeled as red snapper only 24 (30%) were confirmed through IEF to be true red snapper. This meant that 57 (70%) of the fillet samples labeled as red snapper were actually other fish species. It was also reported that the majority of the fish species substituted as red snapper were actually scarlet snapper (*Lutjanus sanguineus*). This type of fish substitution can have a severe economical impact on the consumer. Species substitution has also been detected using IEF with a variety of other fish species such as European seabass (*Dicentrarchus labrax*), spotted seabass (*Dicentrarchus punctatus*), common pandora (*Pagellus erythrinus*) (Colombo, Colombo, Biondi, Malandra & Renon, 2000), European perch (*Perca fluviatilis*), Nile perch (*Lates niloticus*), European pikeperch (*Stizostedion lucioperca*), and sunshine bass (*Morone chrysops x saxatilis*) (Berrini, Tepedino, Borromeo & Secchi, 2006).

2.1.2 Chromatographic detection

In recent years chromatographic methods, such as gas chromatography coupled with mass spectroscopy, have been used for authentication of meat products (Sivadier, Engel, Bouvier & Ratel, 2008). Chromatographic methods, including gas chromatography (GC), thin-layer chromatography (TLC), high-performance liquid

chromatography (HPLC), are commonly used in food analysis. GC has been used to detect volatile compounds in fish sauce (Sanceda, 1983) as well as being used to predict sensory qualities of cold smoked salmon (Jónsdóttir, Ólafsdóttir, Chanie & Haugen, 2008). Morrison and others (2007) have used capillary GC and the isotopic composition of fish oil to distinguish between farmed and wild Gilthead Sea Bream. However, one of the drawbacks and difficulties associated with chromatographic methods is the extensive extraction process that often requires the use of hazardous chemicals. When using GC, a significant drawback is that most the molecules of interest must be volatile in order to be detected and the majority of molecules are not volatile or thermolabile and therefore cannot be analyzed by GC. In order to overcome some of the limitations of using a combination of gas chromatography and mass spectroscopy (MS) Tsutsumi et al. (2008) used ELISA to detect the concentrations of dioxins in retail fish. They found that the ELISA performed as well as the GC/MS analysis and was a more convenient method of detection.

2.1.3 DNA based identification

DNA testing methods have been used extensively in commercial fish and seafood species (Rasmussen & Morrissey, 2008). The use of DNA methods allows for a wide range of fish and fish products to be identified. Using restriction fragment length polymorphism (RFLP) Chakraborty and others (2007) were able to differentiate between 2 species of hairtail fillets (*Trichiurus japonicus* and *Trichiurus sp.*) sold at Japanese markets under the same name of Tachiuo. Along with identification of fish

species (Bossier, 1999; Espiñeira, Santaclara, Vieites & González-Lavín, 2008; Horstkotte & Rehbein, 2003), DNA methods have also been used for identification in various products such as caviar (Ludwig, Debus & Jenneckens, 2002; Rehbein, Molkentin, Schubring, Lieckfeldt & Ludwig, 2008), smoked fish products (Carrera et al., 2000; Rehbein, 2005; Smith, McVeagh & Steinke, 2008), bivalve species (Espiñeira, González-Lavín, Vieites & Santaclara, 2009), sterile fish mixtures (Asensio, González, Rodríguez, Hernández, Garcíá & Martín, 2004), and surimi based products (Pepe, Trotta, DiMarco, Anastasio, Bautista & Cortesi, 2007a).

DNA has certainly proven itself in the realm of species identification but it falls short when the need arises to quantify the amount of the identified species. Lockley and Bardsley (2000) have developed a DNA method by which they can discriminate between tuna (*Thunnus thynnus*) and bonito (*Sarda sarda*) but would require an additional steps if quantification of the fish was desired.

2.1.4 Immunochemical analysis

Even though DNA methods have been used for species identification, it lacks the ability to quantify the amount of protein present. Immunochemical methods, such as enzyme-linked immunosorbent assays (ELISA), are often much easier to perform and with optimization of the assay parameters, they allow for the detection of the species of interest. ELISA can also be set up in such a way as to quantify the amount of the protein of interest. In addition to species identification ELISA has proven to be extremely useful in the detection of many of the common allergens found in fish and

seafood such as the β' -component, which is a fragment of vitellogenin in salmonid roe (Shimizu, Watanabe, Saeki, Hara, Nakamura & Kishimura, 2009), tropomyosin from crustaceans (Fuller et al., 2006) and parvalbumin from fish (Chen et al., 2006; Fæste et al., 2008; Gajewski et al., 2009; Kawase et al., 2001; Ma et al., 2008; Yoshida et al., 2008).

2.1.4.1 Antibody production

The production of antibodies is a key component to successfully producing an immunological assay. Antibodies are derived from animals and are either polyclonal or monoclonal. Figure 2.1 shows the basic outline of polyclonal and monoclonal antibody production. Polyclonal antibodies are produced in live animals immunized with a specific antigen that is of interest. Polyclonal antibodies are antibodies that are derived from different B cell lines. They are a mixture of immunoglobulin molecules secreted against a specific antigen, each recognizing a different epitope (Sharon, 1998). Of the two types of antibodies produced, polyclonal antibodies are typically cheaper and easier to produce. Polyclonal antibodies for identification of fish and fish products, such as smoked salmon, trout, and bream (Carrera, Martin, Garcia, Gonzalez, Sanz & Hernandez, 1996) have been used extensively. They have also been used for the identification of grouper, wreck fish, and Nile perch (Asensio et al., 2003b). One group has used polyclonal antibodies in a sandwich ELISA format for the detection and quantification of tropomyosin from crustaceans in food (Werner, Egaas & Fæste, 2007). Lopata and others (2005) produced polyclonal antibodies for

the detection of aerosolized fish antigens in a fish processing factory, which made it possible to investigate exposure-disease response relationships among workers. The production of polyclonal antibodies has become very common and there are a number of companies that can custom produce polyclonal antibodies. However there are some disadvantages to the use of polyclonal antibodies. Due to the nature of polyclonal antibodies, multiple B cell lines recognize multiple epitopes of the antigen, the resulting cross reactivity can have advantages as well as disadvantages. Even though the likelihood of cross-reactivity with non target proteins is increased, there are also multiple antibodies that recognize multiple epitopes of the same antigen, which may allow for more sensitivity and be less affected by subtle changes of the antigen. Polyclonal antibodies may also require extensive purification steps to eliminate or reduce cross-reactivity. Even with extensive purification the polyclonal antibody may still exhibit high levels of cross reactivity.

Asensio and others (2003b) produced three polyclonal antibodies against the soluble protein fractions of three fish species, namely grouper, wreck fish, and Nile perch. In order to limit cross reactivity found in each polyclonal antibody, they blocked the cross-reacting antibodies by incubating each antibody with an aliquot of the soluble protein extract from the other two species. For example, the anti-grouper soluble protein antibody was blocked with 0.5 mg of each of the wreck fish and Nile perch soluble protein extract per milliliter of antiserum. This effectively blocked the cross-reactivity found in each of the polyclonal antibodies allowing for the definitive identification of each fish species when tested using an indirect ELISA assay.

However, it is important to note that in the study only three fish species were tested and therefore the assay can only be applied to these three fish under the prescribed testing conditions. Extensive testing for cross-reactivity would need to be done in order for the assay to be effective for a wide range of fish species.

Monoclonal antibodies are produced by a single B-lymphocyte that may produce two types of immunoglobulins (IgG and IgM) with IgG as the desired antibody class for immunoassays (Sharon, 1998). This is accomplished by a procedure developed by Kohler and Milstein (1980) whereby isolated B-lymphocytes (spleen cells) are fused with cancerous myelomas, which allows for continued growth forever in culture. Some of the advantages of monoclonal antibodies are that they can be very specific for a particular domain found on the antigen. The immortalized cell lines can be frozen for long term storage at -196°C and growth can be continued as needed making monoclonal antibodies a good choice for a commercial kit that will require a steady supply of consistent antibody reagents.

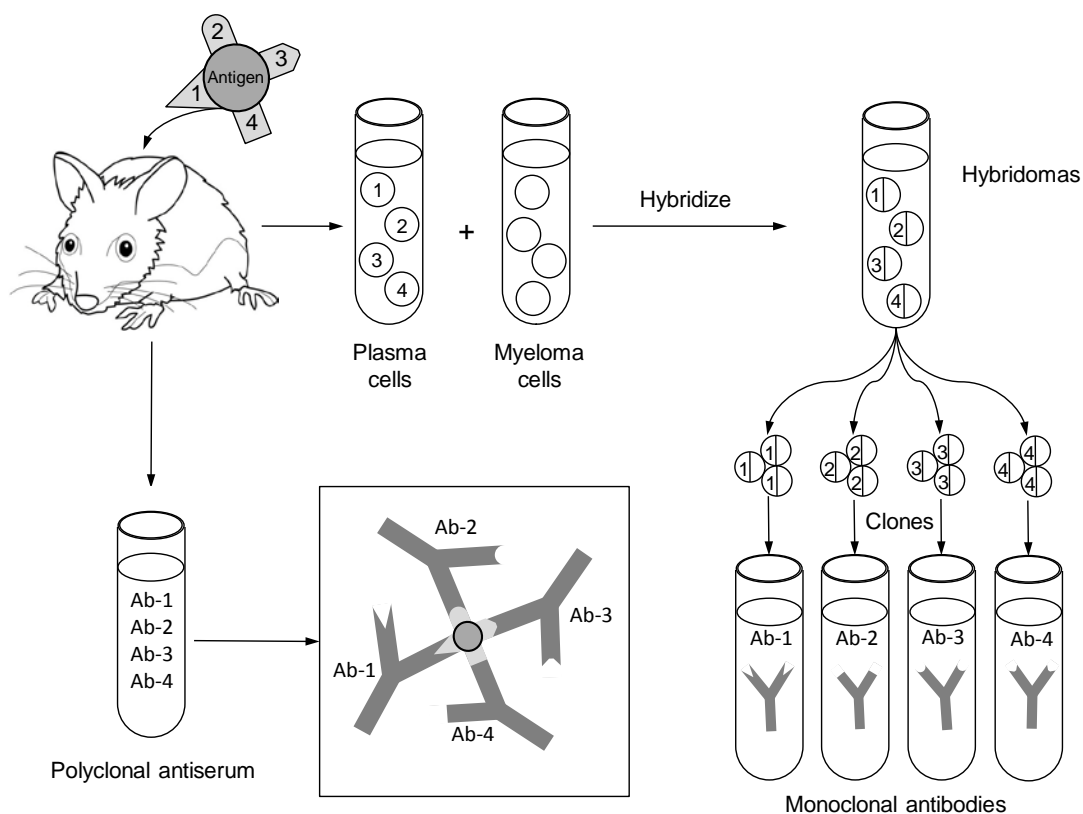


Figure 2.1 – General steps for polyclonal and monoclonal antibody production and selection (adapted from Kindt 2007)

2.1.4.2 Assay Development

When compared to many of the methods discussed so far, immunological assays have many advantages that others do not. Figure 2.2 outlines many of the advantages of ELISA. Some of these advantages are reduced assay time, which can be as little as minutes to several hours. It is easy to perform and does not require a highly skilled technician. Results may be quantifiable under controlled conditions as well as qualitative. ELISA takes advantage of the interaction and specificity that a given antibody has for an antigen.

ELISA

(Enzyme Linked Immunosorbent Assay)



Figure 2.2 – Advantages of using ELISA for protein identification

Figure 2.3 shows the three main types of ELISA schemes commonly used in food authentication namely direct and indirect antigen-coated ELISA, and a direct sandwich ELISA. Direct ELISA is often considered the simplest form of ELISA. The antigen is properly diluted and in a suitable buffer and passively adsorbed onto the surface of the well. Excess antigen is washed away and any remaining binding sites are blocked by

the addition of a blocking buffer. Blocking is followed by another washing step and then the enzyme labeled primary antibody is added to the plate. Immunological binding takes place between the enzyme-labeled antibody and the specific antigen. Excess labeled antibody is washed away and a suitable enzyme substrate/chromagen is added and color is developed. Color development is stopped by the alteration of the pH or by adding an inhibitory agent. The plate is then placed in a plate spectrophotometer and the absorbance is read. (Kindt, Goldsby & Osborne, 2007).

The indirect ELISA uses two antibodies; the primary antibody is specific for the antigen of interest known as the detection antibody and the secondary antibody is coupled to an enzyme that is made specifically to recognize the detection antibody. Often times the secondary enzyme labeled antibody is made to specifically recognize the IgG chain of the primary antibody. In the indirect ELISA the sample containing antigen is typically coated to the bottom of a well in a microtiter plate as in the direct ELISA method. The detection antibody is then used to detect the antigen of interest that is coated onto the plate, followed by the enzyme-labeled antibody that binds to the detection antibody. It is important to note that there are a large number of commercially available enzyme-labeled antibodies that are made that can be used to bind to the detection antibody. Once the excess enzyme-labeled antibody has been washed from the plate a suitable enzyme substrate is added that will give a chromogenic response. This response can then be recorded and if used in conjunction with a standard curve, the antigen may be quantified (Kindt et al., 2007).

In the sandwich ELISA, the antibody rather than the antigen is immobilized on a microtiter well. After the antibody is immobilized the antigen is added and allowed to react after which any unbound antigen is washed away. A second antibody, known as the detection antibody, is then added and allowed to react with the captured antigen. In the sandwich ELISA, the detection antibody can be labeled directly with an enzyme, making a direct sandwich assay, or a third antibody can be used which is bound to an enzyme and is specific for the detection antibody making an indirect sandwich ELISA. Regardless of which antibody is labeled with the enzyme, the enzyme substrate again is added and the color developed is measured (Kindt et al., 2007).

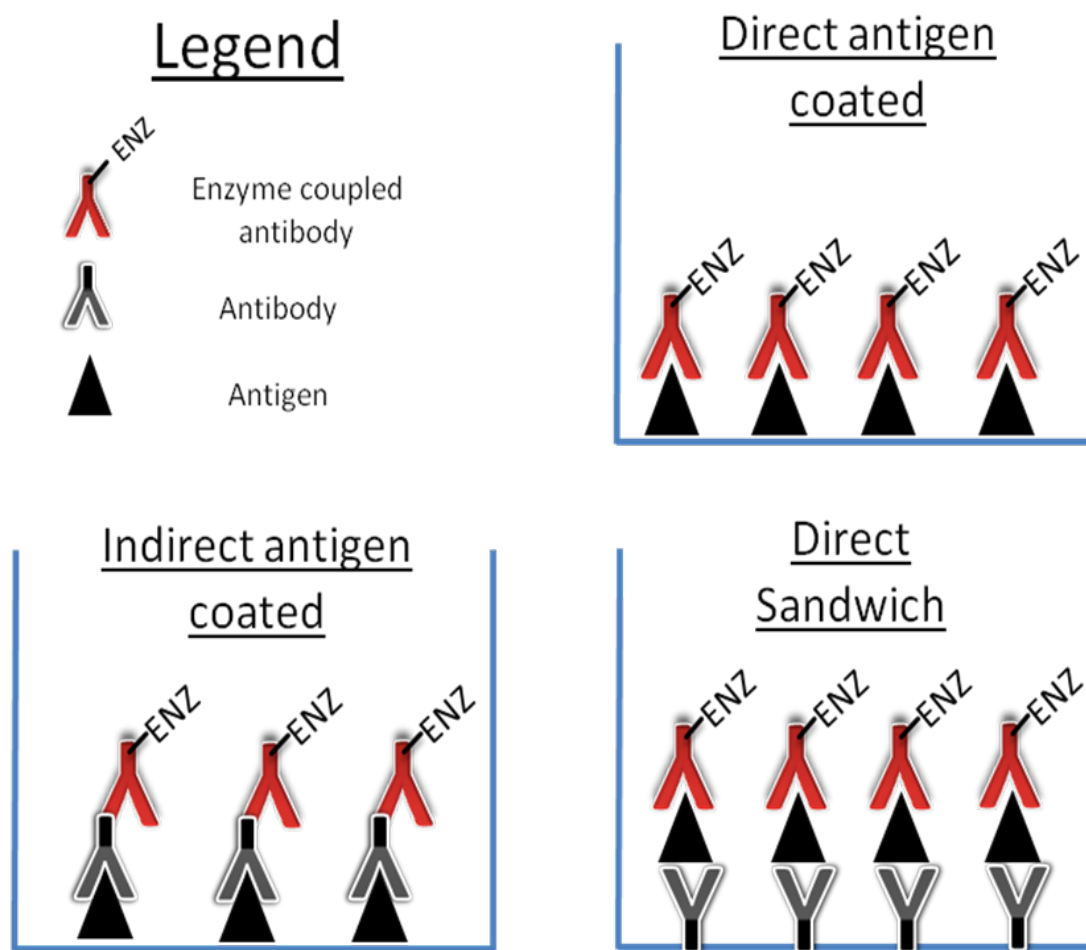


Figure 2.3 – Three commonly used ELISA schemes

2.1.4.3 ELISA used in food products

ELISA techniques have not been extensively used for fish species identification, this is due to the extensive cross reactivity found in fish. For some species such as sole, European plaice, flounder, Greenland halibut (Cespedes, 1999), grouper, wreck fish, Nile perch (Asensio et al., 2003a; Asensio et al., 2003b) red snapper (Huang et al., 1995) basa, tra (Hsieh, Chen & Gajewski, 2009), and catfish

(*Ictalurus sp*) (McNulty et al., 2005), ELISA assays have been developed. However the ELISA is only successful when used under the specific conditions outlined by each protocol. Many of these assays were developed in order to protect a specific fish species from unauthorized substitution. In the case of basa and tra Hsieh et al. (2009) developed a monoclonal antibody-based sandwich ELISA for reliable identification of imported *Pangasius* catfish. The assay developed was capable of a definitive “yes” or “no” when concerning the identification of the *Pangasius* catfish, raw or cooked. This assay can now be used to protect the American market of catfish from the *Ictaluridae* family of catfish. McNulty et al. (2005) developed an assay that was specific for *Ictalurus* sp. fillets. The monoclonal antibody was able to differentiate raw and cooked *Ictalurus* sp fillets from the proteins of the other fish tested. By using the assay developed by Hsieh et al. along with the assay developed by McNulty et al. absolute positive identification of American catfish being sold could be achieved.

Along with species identification of raw and cooked fish species ELISA has also been used for identification and quantification of highly processed fish and seafood products such as fushi (dried fish stick) (Ochiai & Watabe, 2003), mackerel dark muscle content in kamaboko (Ochiai, Ochiai, Hashimoto & Watabe, 2001), and a variety of clams (grooved carpet shell, yellow carpet shell, pullet carpet shell) (Fernandez et al., 2002). Reed and Park (2010b) were able to produce an indirect ELISA assay that was capable of quantifying the amount of dried egg white and/or whey protein added to crabstick made from Alaska pollock surimi. Verrez-Bagnis and Esriche-Roberto (1993) found that arginine kinase, which is present in most

invertebrates but absent in most vertebrates, could be used to produce a polyclonal antibody for detection of crab meat in surimi seafood. Using a direct ELISA their assay was capable of detecting 10-25 g of crab snow-crab flesh per kilogram of surimi-based product.

Carrera et al. (1996) devised an indirect ELISA assay for the identification of other highly prized fish products such as cold smoked salmon, trout, and bream. Using an indirect ELISA with polyclonal antibodies that were produced against the muscle soluble proteins from the 3 types of fish they were correctly able to identify all three species. However, it is important to note that when the antibodies were tested in an indirect ELISA and significant cross reactivity was exhibited by all three anti-fish serum samples. In order to overcome this obstacle and make the antibodies species specific, the authors used heterologous lyophilized fish extracts to block cross reactions. In this process known as blocking, the antibodies were rendered species specific and could be used for the identification of each individual species. In a later development, Carrera et al. (1997) produced an immunostick colorimetric ELISA assay for the detection of the same smoked salmon, trout, and bream. The advantage of the immunostick production for the detection of smoked fish is the ease of performing the assay, with no need for expensive plate readers, the development of a blue color was a clear indicator of the presence of the fish species being tested.

2.1.5 Summary

The illegal practice of mislabeling aquatic products not only represents a major fraud perpetrated on the consumer, it also can result in deleterious effects on the seafood industry. Methods for identification of fish species vary widely in accuracy, accessibility, and robustness. Enzyme linked immunosorbent assays (ELISA) provide a variety of formats for development such as direct, indirect, and sandwich ELISAs. The production of polyclonal and monoclonal antibodies allows for ELISAs to perform in a variety of species identification methods. These methods can be, and often are, adapted for use on either raw or cooked products. ELISAs have the capability of providing very sensitive and repeatable results from even complex food matrices such as surimi seafood. However, many of the antibodies produced have significant cross-reactivities with other fish species, which often times requires intensive purification processes to remove the cross-reactivity. One challenge that is still yet to be fully overcome is the production of a kit that can be used solely for the identification of any fish species when added to a mixed food item as either a contaminant or as an adulteration. Such contaminations and adulterations can cause severe allergic reactions in individuals that are hypersensitive to fish protein. For labeling and safety purposes, a standard is needed for the qualification and quantification of fish proteins found in food. Future trends will continue to lead development in the direction of species specific production of ELISA kits as well as kits for determining the presence of fish.

2.2 Rheological and biochemical properties of fish myosin

2.2.1 Myosin

Myosin is a large asymmetric molecule that has a long α -helical coiled-coil tail and two globular heads with an approximate weight of 500 kDa (Hodge et al., 2000). The basic body plan of myosin consists of an N-terminal head or motor domain, a light chain-binding neck domain, and a class conserved, C-terminal tail domain and has been categorized into over twenty different classes (Mooseker et al., 2008). The head or motor domain has a core sequence that is highly conserved in all myosin classes, and it contains the ATPase active site (Holmes, 2008). The neck region, also known as the lever arm, consists of a long α -helix of variable length and a tail region that is extremely variable in sequence, length, domain composition, and organization. The molecular weight of myosin heavy chain when dissociated in strong denaturing solutions is approximately 220 kDa (Lanier et al., 2005).

Myosin is the major muscle protein that is found in fish and comprises approximately 55-60% of the myofibrillar proteins (Lanier et al., 2005). Skeletal myosin can be broken up into six polypeptide chains, two heavy chains and four light chains. Myosin light chains typically range from 17 to 25 kDa. These amino acid chains are non-covalently attached to the myosin head (Lanier et al., 2005). Myosin can also be broken into fragments by proteolysis (Szent-Görgyi, 1953). When myosin is exposed to the proteolytic enzymes, trypsin or α -chymotrypsin, fragmentation occurs in the middle of the tail yielding heavy meromyosin (HMM, molecular weight about 350 kDa) and light meromyosin (LMM, molecular weight about 150 kDa).

Kasserra and Laidler (1969) performed studies on the mechanistic action of trypsin and α -chymotrypsin and found that the mechanism is nearly identical. In addition to the mechanism of trypsin and α -chymotrypsin digestion being similar, Margossian and Lowey (1982) showed that using trypsin or α -chymotrypsin produced similar proteolysis fragments. The HMM contains the head group and a short tail and can be further fragmented by the use of papain into subfragment 1 (S1, molecular weight 110 kDa) and subfragment 2 (S2, molecular weight 240 kDa).

There are two types of myosin light chains, the regulatory light chains, which are known for their role in phosphorylation and dephosphorylation (Sobieszek, 1988). The regulatory light chains can be selectively dissociated from myosin by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), thus they are often referred to as the DTNB light chains (Wagner, 1982). The role of phosphorylation and dephosphorylation corresponds to the contraction and relaxation of smooth muscle. The essential light chains are often referred to as alkali light chains because they are dissociated from myosin under alkaline conditions (Weeds et al., 1977). Each myosin head unit contains one DTNB subunit and one alkali unit. This means that there are four myosin light chains per myosin molecule. However, it is interesting to note that when whole myosin is analyzed using SDS-PAGE techniques, there are typically only three bands found for the myosin light chains. This occurs because each head unit has one light chain 2 (LC 2), the DTNB light chain, and then one essential light chain, which can be either light chain 1 or light chain 3 (Bechtel, 1986). Due to the repetition of the LC2

in both head units, the SDS-PAGE protein pattern shows only three myosin light chains.

2.2.2 Heavy meromyosin and light meromyosin

As previously mentioned, the myosin heavy chain can be enzymatically cleaved into HMM and LMM (Lowey & Cohen, 1962). It has been disputed as to which portion of the myosin molecule relates to which role in the thermal aggregation of myosin. Gill and Conway (1989) studied the chymotryptic cleavage of thermally aggregated cod myosin and came to the conclusion that the tail portion (LMM) of the myosin was involved in thermal aggregation rather than the head region (HMM). Sano, Noguchi, Matsumoto, and Tsuchiya (1990) examined HMM and LMM and found evidence that the initial stages of gel formation was indeed due to the gelation of LMM between 30 and 44 °C. When studying the thermal aggregation of HMM and LMM from cod and herring, Chan et al. (1993) found that the initial aggregates formed by an interaction of the HMM followed by compound aggregates being formed through the interaction of the LMM. They also found that the function of the HMM S-1 fragment in gelation was still unclear, but that it was only involved in temperatures above 55 °C.

2.2.3 Biochemical properties of fish myosin

The functional properties of myofibrillar proteins are extremely important in many muscle food products with myosin playing a significant role in the gel forming

ability (Macfarlane, Schmidt & Turner, 1977; Toshiyuki, Yoshio & Tsutomu, 1961). Purified myosin is solubilized well in salt water, but will form aggregates when heated and if the myosin concentration is high enough it can lead to gel formation (Chan et al., 1993; Fukushima, Satoh, Nakaya, Ishizaki & Watabe, 2003; Gill, Chan, Phonchareon & Paulson, 1992; Lefevre, Gill, Thompson & Fauconneau, 2007; Lin & Park, 1998; Yongsawatdigul et al., 1999). However purified fish myosin is very unstable and particularly sensitive to denaturation by heating or freezing. The stability of fish myosin has also been found to vary between fish species with some evidence of fish species habitat playing a role (Davies, Bardsley, Ledward & Poulter, 1988; Ogawa, Ehara, Tamiya & Tsuchiya, 1993).

Heat is one way in which proteins are denatured. A variety of chemical testing can be performed to understand the gelation process of myosin. The denaturation of proteins by heating can be monitored and studied by monitoring the degree of surface hydrophobicity of the protein. By measuring the surface hydrophobicity of fish myosin as affected by heat treatment, protein denaturation can be determined (Lin et al., 1998; Thawornchinsombut & Park, 2004; Visessanguan et al., 2000).

Hydrophobic bonding is thought to play a significant role in gel formation and typically increases the gel strength as it is heated with a maximum increase in strength up to approximately 60 °C (Gilleland, Lanier & Hamann, 1997; Lanier et al., 2005).

The three-dimensional structure of native myosin has a hydrophobic core, which allows hydrophobic amino acids, such as valine, leucine, isoleucine, methionine, phenylalanine, tryptophan and cysteine, to exclude water and to strengthen their

interaction (Betts & Russell, 2003). However, as the protein begins to denature the three-dimensional structure is disrupted and opens up where the hydrophobic portions of the protein are no longer protected from interaction with water and other substances (Benjakul, Visessanguan, Thongkaew & Tanaka, 2003). Therefore, studying the hydrophobicity of fish myosin can provide critical insight into the denaturation process. (Chan et al., 1993; Lin et al., 1998; Thawornchinsombut et al., 2004; Visessanguan et al., 2000; Wicker & Knopp, 1988; Yongsawatdigul & Park, 2003). Heat is one way in which proteins are denatured. The denaturation of proteins by heating can be monitored and studied by monitoring the degree of surface hydrophobicity of the protein.

Measuring the amount of reactive sulfhydryl (SH) groups can also serve to help understand the denaturation of myosin (Gilleland et al., 1997). When studying the amount of reactive sulfhydryl content there are two ways to approach, one measures the surface reactive sulfhydryl groups (SR-SH) and the other measures the total reactive sulfhydryl groups (TSH). It is useful to test for the SR-SH groups using a non-denaturing buffer to measure the amount of SH groups found on the surface of the native myosin. The SR-SH content of the protein can be compared to the TSH groups when the sample is tested using both a denaturing and non-denaturing buffer (Hoffmann & van Mil, 1997; Hsu, Hwang, Yu & Jao, 2007). Many of the SH groups are buried inside of the native myosin protein due to the protein conformation, and cannot react when tested for the SR-SH content. When 8 M urea is used, the myosin is completely denatured, thereby exposing the buried SH groups and allowing for their

quantification (Riddles, Blakeley & Zerner, 1979). When the myosin is heated it will begin to denature and the exposed sulfhydryl groups will begin to spontaneously form disulfide linkages as the temperature approaches 50 °C and above.

When looking at the physiochemical properties of fish myosin it can be useful to measure the turbidity of a protein solution to understand the denaturation and aggregation of the protein. Turbidity is a measure of the cloudiness or haziness created by the aggregation of suspended particles in a solution. As the sample is heated, the proteins begin to denature and with prolonged heating, they can begin to form larger myosin aggregates (Chan et al., 1993) thereby increasing the optical density (OD) at 320 nm. This change in OD is due to the aggregation of the suspended particles of myosin, which according to Yongsawatdigul et al. (1999) can be species specific. The OD of myosin measured in a linear heating pattern can help to provide information about the denaturation and aggregation characteristics of fish myosin.

2.2.4 Non-fracture rheology

The science of rheology revolves around the study of the deformation and flow of matter (Rao, 1999a). Rheology has applications in a wide variety of areas such as engineering, geophysics, physiology, pharmaceuticals, and foods. The study of rheology has become very important to measure the quality of food and the process fundamentals of food manufacturing particularly to measure the deformation behavior

of foods. Texture determination can be classified into fracture and non-fracture rheology.

2.2.4.1 Principles

There are three categories of rheology used to explain the behavior of matter: viscosity, elasticity, and viscoelasticity. The law of conservation of energy states that energy can neither be created nor destroyed. When measuring a viscous material the work energy that is applied to the material is dissipated into the sample as heat. In a purely elastic material all energy is stored in the material as elastic potential energy. When a material exhibits properties of both viscous and elastic behaviors it is said to be viscoelastic.

When a solid or semi-solid food exhibits viscoelastic properties a dynamic rheometer can be used to study the properties of the food under a variety of conditions. Dynamic rheology is also known as small amplitude oscillatory shear (SAOS) and can be used to determine viscoelastic properties of foods (Rao, 1999b). When testing a food using dynamic rheology, the food sample is subjected to a small sinusoidally oscillating strain or deformation. When a sample is tested using a rheometer a force is applied to the sample and as the plate or cone, depending on the geometry, is oscillated, this force or energy is transferred to the sample. Depending on the nature of the sample, the energy will either be transferred to and stored as elastic potential energy, or it will be dissipated as heat to the sample. As can be seen in Figure 2.4 when the sample is purely elastic the strain given to the sample and the stress of the

sample will be “in phase”. If, however the sample is a purely a liquid, the stress will be 90° “out of phase” with the strain applied to the sample. If the sample is of viscoelastic nature, as in the case of purified myosin, the strain will lag less than 90° “out of phase” with the stress.

When discussing the viscoelastic nature of food samples three main concepts and measurements are used. First, is G' , which is known as the storage modulus. The storage modulus G' is a measure of the magnitude of energy that is stored or recovered in the material per cycle of deformation. The loss modulus G'' is a measure of the energy lost as heat through the viscous component per cycle of deformation. The phase angle is given when the G' and G'' are taken together and they can be expressed as the $\tan-\delta$ (G''/G'). This phase angle can be used to help determine the nature of the sample. For a perfectly elastic solid, all the energy is stored, that is G'' is zero and the stress and strain will be in phase. When the phase angle is 0° it is considered a perfectly elastic solid. However, for a liquid with no elastic properties, all of the energy is dissipated as heat that is G' is zero and the stress and the strain will be out of phase by 90° . When studying viscoelastic foods the phase angle will be somewhere between 0° and 90° . The gelation point of a protein can be determined when $\log G' = \log G''$. This is when the protein changes states from a viscoelastic liquid to a solid state.

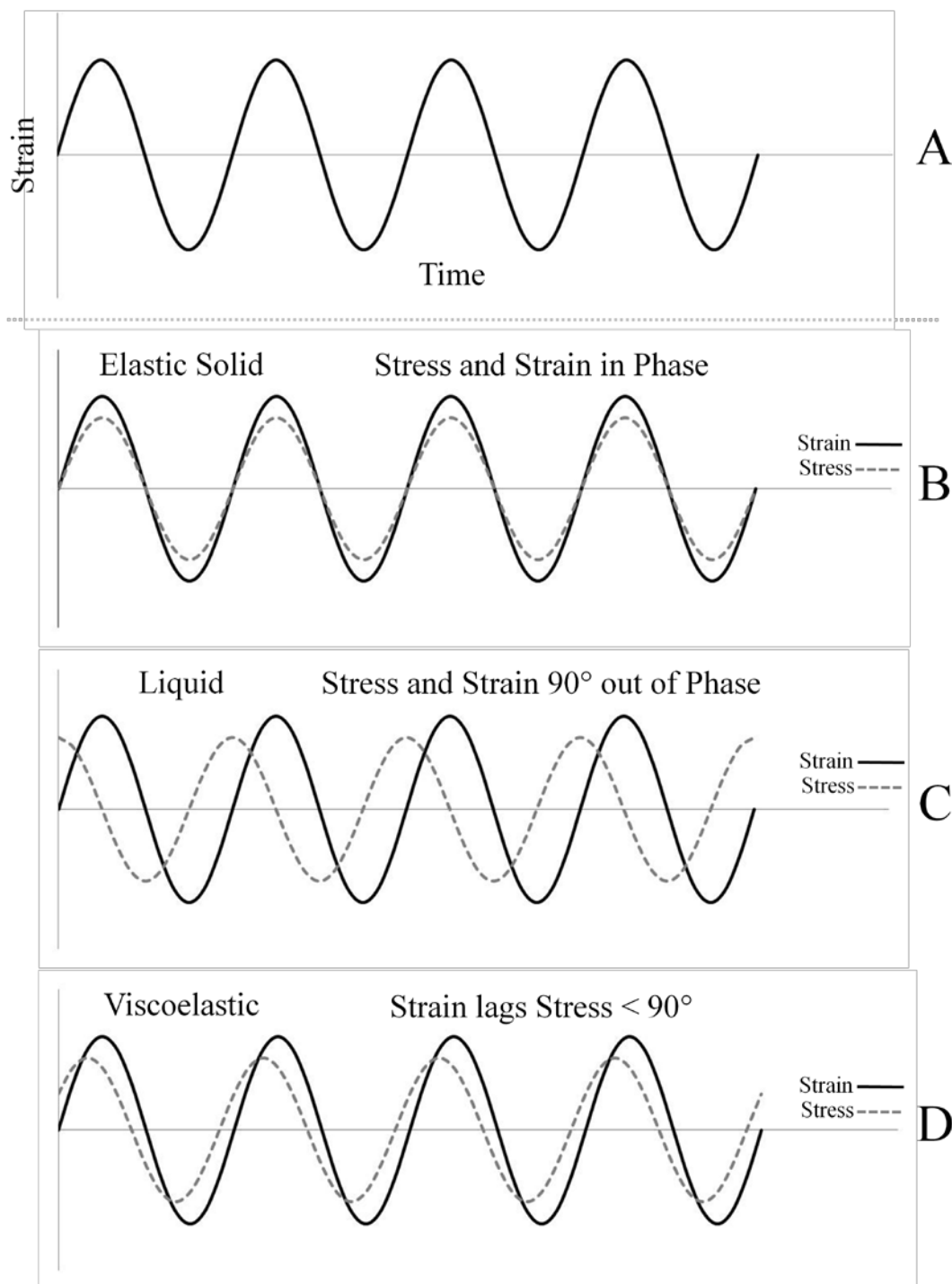


Figure 2.4 - Stress versus strain response of a Newtonian liquid (A) and a perfectly elastic solid (B), perfectly liquid sample (C), and a viscoelastic sample (D).

2.2.4.2 Application of dynamic rheology on fish proteins

Dynamic rheology is a powerful tool that can be used for the characterization of fish proteins, such as purified fish myosin. Many fish such as Alaska pollock, Pacific whiting, Southern blue whiting, Northern blue whiting, threadfin bream, lizardfish, and croaker are used to produce surimi (Guenneugues & Morrissey, 2005). In order to better understand the role that myosin plays in the gelation of surimi, many commonly utilized species have been studied using dynamic rheology. Fukushima et al. (2003) studied the rheological changes and differences for Alaska pollock, white croaker, and rabbit myosin. In their studies, they found that the thermal denaturation of myosin was species specific and yielded information on the stabilities of each species myosin. They also reported that the difference in the temperature at which the myosin denatured and formed a gel was an indication of their overall gel forming capability.

Not only can dynamic rheology be useful when studying different species, it can also provide valuable information about myosin that is obtained from different muscle portions in the same fish. Lefevre et al. (2007) studied the aggregation properties of salmon myofibrils and myosin purified from white and red muscle. When they compared myofibrils and myosin from white and red muscle, they found that the rheological profile for the red muscle proteins correlated to a shift to in denaturation and aggregation temperatures. The gel rigidity for both the red and white muscle myosin gels was similar, however the denaturation shown through dynamic rheology showed distinct differences in the proteins from the two muscle types.

2.2.5 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a method for physiochemical thermal analysis and can be used to determine the energy changes in substance under thermal treatments. The principle of the method is based on measuring temperature and spontaneous or compensating heat fluxes (Bershtein & Egorov, 1994). The main application of DSC is in studying the phase transitions, such as melting, glass transitions, or exothermic decompositions. These transitions involve energy changes that can be detected by DSC with great sensitivity.

2.2.5.1 Principles

The driving force behind the principle of DSC is the ability to measure extremely small changes in heat capacity as a function of electrical fluxes. DSC uses two vessels to measure the heat flow, one containing a reference material and the other a sample material. The sample and reference are heated linearly and the heat flow is measured. Conventional DSC systems use a system where the sample (typically 10-30 μg) and reference material are placed into a pan and heated. The heat that is added to each pan is measured. The difference in electrical fluxes is measured and collected during heating. One of the drawbacks of conventional DSC is that it does not efficiently measure all energy exchanges between the sample vessel and the calorimetric unit.

This is overcome in micro differential scanning calorimetry (MDSC) by the use of a Tian-Calvet type transducer. In the MDSC system the sample (typically 400-

500 mg) and reference vessels are enveloped by thermopiles. A thermopile is an electronic device that measures thermal energy and converts it to electrical energy. The thermopile is made up of a number of thermocouples, which are arranged around the cylinders that contain the sample and reference. These thermocouples convert the thermal energy to an electrical energy that can be measured (Figure 2.5). The use of a thermopile system allows for much more sensitive measurement of thermal energy from the sample and reference vessels.

As the sample and reference vessels are heated the sample can go through endothermic changes, such as melting or in the case of proteins, denaturation. Endothermic processes are such that they require heat from the furnace in order to maintain the sample vessel at the same temperature as the reference vessel. Therefore, more heat must be supplied to the sample vessel, which causes an electrical flux that can be recorded. As mentioned before, the thermopile will measure the temperature supplying a voltage, which is proportional to the temperature difference. DSC can also be used to measure exothermic phenomena such as gel formation.

In the case of exothermic events, the sample will require less heat from the DSC machine in order to maintain the same temperature as the reference material. The formation of a protein gel network, like that of purified myosin, is an exothermic process. As the denatured myosin protein begins to form chemical bonds, heat is generated. The heat generated in the sample lowers the amount of heat required from the furnace to maintain the sample and reference vessels at the same temperature. As the need for thermal energy to be transferred to the sample from the furnace is

lessened, due to the internal heat production, the signal is converted by the thermopile proportionally.

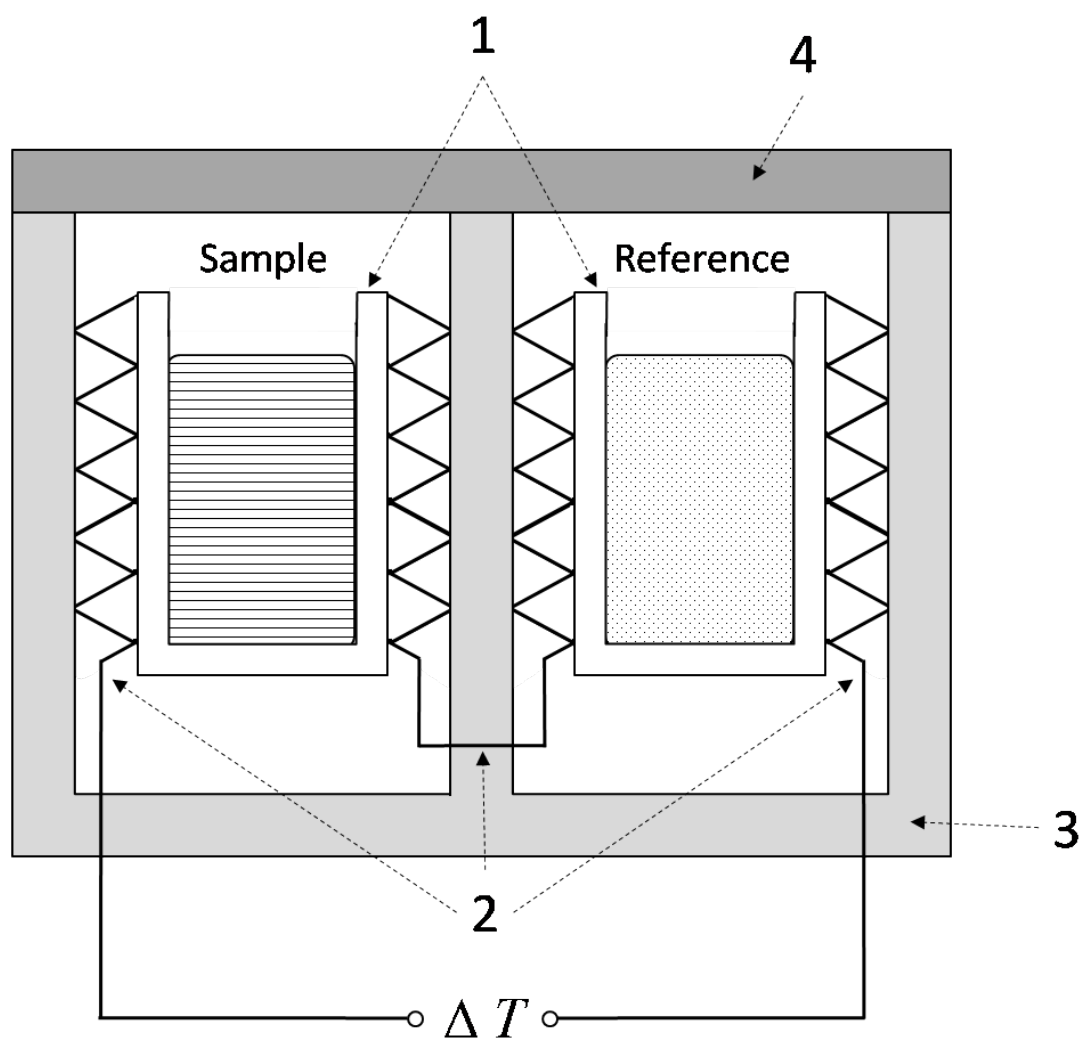


Figure 2.5 – Calvet type DSC transducer. 1 – sample and reference cylinders, 2 – thermocouples together comprising a thermopile, 3 – furnace (controlled by a programmable temperature controller), 4 – DSC transducer lid, ΔT is the measured difference between the sample and reference vessel (typically reported as millivolts).

2.2.5.2 Application of DSC methods on fish proteins

The information and data that can be obtained from DSC is of great importance in many areas of research, such as thermal behavior of proteins, and more specifically in the thermal behavior of muscle proteins. Myofibrillar proteins are the most important fish proteins in the gelation of surimi and surimi seafood. Of the myofibrillar proteins, myosin is known to be of key importance for the gelling properties of surimi (Carvajal, Lanier & Macdonald, 2005). Many key surimi fish species have been studied using DSC, such as Alaska pollock (Fukushima et al., 2003; Park, 1994; Wang & Kolbe, 1991), walleye pollack (Togashi et al., 2002), Pacific whiting (Thawornchinsombut & Park, 2007), and threadfin bream (Yongsawatdigul et al., 2003).

Beas, Wagner, Crupkin, and Anon (1990) studied the maximum temperatures of denaturation enthalpies of hake (*Merluccius hubbsi*) using DSC. They found that whole muscle free from connective tissue showed two distinct transitions. When they studied muscle that had the sarcoplasmic proteins removed, the enthalpy of the second transition was diminished. Yongsawatdigul et al. (2003) studied the effect of thermal denaturation and aggregation of threadfin bream actomyosin. They found that threadfin bream actomyosin exhibited three major transitions at 38.4, 51.0, and 80.7 °C with onset temperatures of 36.5, 47.0, and 76.2 °C, respectively. Enthalpies of denaturation of each major transition were 0.152, 0.169, and 0.251 J/g respectively. The protein gelation properties of surimi make it useful for surimi seafood, however the effects of freezing and storage can have a significant impact on the gelation

properties of surimi fish proteins. Moosavi-Nasab, Alli, Ismail, and Ngadi (2005) studied the effect of freezing methods and storage on the gelation properties of actomyosin from Alaska pollock surimi and fish mince. They found a shift in the DSC endothermic peak of actin for both fish mince and surimi to a lower temperature. This provides an indication that the proteins were destabilized through the freezing and frozen storage process.

Thermal stability of fish myosin was studied and compared to that of rabbit myosin (Ogawa et al., 1993) using DSC methods. The researchers found the structure of the fish myosin was much more unstable than rabbit myosin. By further studying fish myosin, they were able to determine that instability of the fish myosin was due to the myosin rod moiety.

Other forces can also cause denaturation of fish proteins besides heating and freezing. Ko, Hwang, Jao, and Hsu (2004) performed a study on tilapia myosin fragments and how they were affected by high hydrostatic pressure. After pressure treatment they used DSC methods and found that the native enthalpies for the S-1 and rod fragments were 0.18 and 0.09 J/g. As the pressure treatment increased to 200 MPa, only 6% of the enthalpy for the S-1 fragment remained and was completely removed by pressure treatments in excess of 250 MPa, indicating gel formation under high pressure. They also noted that there was no change in enthalpy values for the rod fragment due to pressure treatment.

Park, Yongsawatdigul, Choi, and Park (2008) found that the endothermic peaks of purified sardine myosin could be affected by pH and ionic strength. Testing

myosin at pH 2, 7, and 10 they found that at pH 2 the thermogram was not typical and that the myosin had been denatured. However at pH 7 and 10, sardine myosin exhibited the typical 3 endothermic peaks of fish myosin. Thawornchinsombut and Park (2004) studied the effects of pH and ionic strength on the number of endothermic peaks of acid and alkali treated Pacific whiting fish protein isolates. In their study they found that the control sample, fish protein isolate (FPI) only with 25 mM NaCl added, demonstrated five endothermic peaks. However, all pH-shift with ionic strength controlled treatments exhibited only three endothermic peaks. They also found that the actin peak completely disappeared. It appears that pH and ionic strength play an important role in the irreversible protein denaturation of some of the endothermic peaks.

Yongsawatdigul and Park (2004) found that acid-induced and alkali-induced denaturation of rockfish muscle occurred as confirmed by DSC thermograms. They found endothermic transition peaks in fish muscle and washed fish mince. However, they found that there was no net enthalpy that appeared in the acid-induced and alkali-induced samples. As the denaturation of proteins is an endothermic process and the aggregation of proteins is an exothermic process, it was shown in their report that the energy required for thermal denaturation of acid-induced and alkali-induced treatments was minimal because FPI was chemically denatured before thermal treatment.

As mentioned previously myosin can be digested which will produce a number of different fragments that can be tested using DSC. In a study performed by Nakaya,

Kakinuma, Watabe, and Ooi (1997) the differences in thermal unfolding of light meromyosin (LMM) from carp acclimatized to 10, 20, and 30 °C were studied. They found through the use of DSC that carp acclimatized to 30 °C showed the most stable acclimation of carp LMM. The peaks for 30 °C were 39.2 and 47.3 °C while those of the 10 °C carp were 32.5 and 39.5 °C, which was significantly lower than those of the 30 °C fish.

The results that can be obtained by DSC are extremely useful in identifying key components to protein denaturation. Kakinuma et al. (1998) investigated the thermal unfolding of three acclimation temperature-associated isoforms of carp LMM expressed by recombinant DNAs. In the study they used DSC techniques to study recombinant forms of LMM from 10 and 30 °C acclimatized carp. They found by studying the thermograms that the 10 and 30 °C endothermic peaks were at different temperatures. They also found that the thermodynamic properties of the chimerical LMMs isoforms are mainly determined by the C-terminal half of the LMM molecule. Liu, Foegeding, Wang, Smith, and Davidian (1996) showed thermal transition points at 35.3, 48.1, 49.9, and 67.0 °C for chicken breast myosin, however, none of the transitions were exothermic. When studying rabbit myosin head (subfragment 1) in 50 mM TRIS buffer (pH 8.0), 0.6 M KCl, along with heavy meromyosin in 0.1 M KCl, Shriver and Kamath (1990) found exothermic peaks at 48 and 65 °C.

CHAPTER 3

ESTIMATING THE QUANTITY OF EGG WHITE AND WHEY PROTEIN
CONCENTRATE IN PREPARED CRABSTICK USING ELISA

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3.1 ABSTRACT

Indirect enzyme-linked immunosorbent assays (ELISA) technique was used to identify and quantify the use of dried egg white (DEW) and whey protein concentrate (WPC) in crabsticks. The use of SDS-PAGE for the quantification of protein additives has had limited success due to the high shear and high temperature processes of surimi crabstick. Monoclonal (anti-heat denatured ovalbumin) and polyclonal (anti- β -lactoglobulin) antibodies were used. Antibodies showed no significant cross-reactivity with non-target crabstick proteins. An optimized extraction solution of 10% SDS and 2.5% β ME yielded high extractability with improved consistency. Quantification of DEW and WPC was achieved using the optimized extraction solution and indirect ELISA. Estimated DEW values were within 7% of actual values, WPC samples were within 17%. Inter-assay coefficients of variance for DEW ranged from 0.9% to 3.1% and those of the WPC were 1.0% to 8.0%.

3.2 INTRODUCTION

The crabstick industry does not have a standard of identity established for protein additives such as dried egg white (DEW) and whey protein concentrate (WPC). A lack of standard of identities can lead to increased use and/or abuse of protein additives in the crabstick formulation, which decreases surimi quantity and correlates to lower quality product if the usage is not properly optimized. Surimi refers to refined fish myofibrillar proteins used as an intermediate product in many seafood flavored products. Surimi is produced through heading, gutting, mincing, washing, dewatering and concentrating the fish myofibrillar proteins (Lee, 1984; Park & Lin, 2005a). The concentrated myofibrillar protein is mixed with cryoprotectants such as sugar, sorbitol, and polyphosphates that serve to stabilize fish myofibrillar protein during frozen storage (Park & Lanier, 1987; Park, Lanier & Green, 1988). Surimi is comminuted with other ingredients such as starch, DEW, WPC, and other flavors in the production of crab-flavored seafood (crabsticks). DEW has been used extensively as a functional food ingredient because of its gelling and foaming properties (Mine, 1995). Beta-lactoglobulin (β -LG) is largely responsible for the physicochemical properties as well as the functional behavior of food products that contain whey protein (Foegeding, Davis, Doucet & McGuffey, 2002). Burgarella, Lanier, Hamann and Wu (1985) showed that DEW and WPC can be used in low concentrations to provide an additive effect of increased gel strength and deformability.

One method used to quantify protein additives in crabstick is SDS-PAGE coupled with gel densitometry (Reed et al., 2008). We found that SDS-PAGE was useful for detection of DEW but was not appropriate for the quantification of DEW. Enzyme-linked immunosorbent assays (ELISA) take advantage of the interaction between proteins and antibodies and can be used to qualify and quantify protein additives in food. Commercial ELISA kits are available for the detection of egg, milk, wheat, buckwheat, and peanut proteins. It is mandatory for these five allergens to be labeled in Japan (Matsuda et al., 2006) and in most countries including United States. Due to the severity of some food allergies the use of ELISA has become widespread for detection of allergenic proteins (Faeste, Egaas, Lindvik & Lovberg, 2007; Fuller et al., 2006). ELISA has also been shown to be effective in quantifying egg white in processed pork products (Leduc, Demeulemester, Guizard, Le Guern, Polack & Peltre, 1999). Dupont et al. (2006) have developed an ELISA for bovine lactoferrin to determine lactoferrin concentrations in milk, whey, and cheese. Ovalbumin (OA) is the most abundant of egg white proteins, comprising 54% of the total proteins (Stevens, 1991). Of the whey proteins in bovine milk, β -lactoglobulin (β -LG) is the most abundant (Farrell et al., 2004).

Extraction of protein from the cooked gel sample is one of the most critical steps in obtaining a clean and reproducible ELISA assay. Commonly, Tris-buffered saline (TBS) and phosphate buffered saline (PBS) have been used to extract protein from the sample matrix. Asensio et al. (2003b) used 0.85% saline solution to extract proteins from grouper, wreck fish and Nile perch fillets. Hefle, Jeanniton and Taylor

(2001) used PBS to extract proteins from pasta for the detection of egg residues in food, while Holden, Fæste and Egaas (2005) used 0.1 M Tris, 0.5 M glycine at pH 8.7 for the extraction of lupine from several baked goods. Preliminary studies revealed that, when crabstick samples were extracted with PBS alone either overnight at room temperature or at 90 °C for an hour, results were not reproducible. Due to the strong gel matrix of high protein content, different extraction methods were investigated.

Watanabe et al. (2005) investigated the use of an extraction solution containing sodium dodecyl sulfate (SDS) and β -mercaptoethanol (β ME) for the detection of raw and processed egg. In their study they found the total protein extracted using TBS could be increased 10 to 100 fold with the addition of 1% SDS and 7% β ME to the TBS extraction solution. Ochiai and Watabe (2003) found that using an extraction solution of urea, SDS, and β ME, allowed for species identification of processed fish products.

The overall goal of this study was to develop an indirect ELISA assay for the qualification and quantification of dried egg white and whey protein concentrate when used as an additive to a cooked and pasteurized crabstick product. In addition, we attempted to develop an optimized extraction method for fish protein gels with strong texture.

3.3 MATERIALS AND METHODS

Food products and chemicals

Alaska pollock (*Theragra chalcogramma*) surimi (FA grade, 10 kg blocks), were obtained from Western Alaska Fisheries (Seattle, Wash., U.S.A.) and stored frozen at -18 °C. The 10 kg blocks were cut into approximately twenty 1 kg blocks, individually vacuum packed, and stored at -18 °C until used for surimi crabstick paste preparation. Ingredients used for surimi crabstick paste preparation were NaCl (Morton Iodized Salt, Morton International, Inc., Chicago, Ill, U.S.A.), corn starch (Corn Products International, Westchester, Ill, U.S.A.), wheat starch (Midsol 50, MGP Ingredients, Inc., Atchison, Kans., U.S.A.), modified waxy maize starch (Polartex 06727, Cargill, Inc., Cedar Rapids, Iowa, U.S.A.), sugar (used to represent all flavor components) (Pure Cane Sugar, C&H Sugar Company, Inc., Crockett, Calif., U.S.A.), and culinary tap water. The protein additives were dried egg white (DEW) (K-200, Henningsen Foods, Omaha, Nebr., U.S.A.) and whey protein concentrate (WPC 8600, Hilmar Ingredients, Hilmar, Calif., U.S.A.). All other chemicals were reagent grade.

Antibodies

Affinity purified rabbit polyclonal antibodies produced against bovine β -lactoglobulin (pAb anti- β -LG) and goat anti-mouse IgG coupled with horse radish peroxidase (HRP-anti-mIgG) were obtained from Bethyl Laboratories (Montgomery, Tex., U.S.A.). Mouse monoclonal antibodies against heat denatured hen ovalbumin (Ab anti-H-OA) were purchased from Abcam Inc. (Cambridge, Mass., U.S.A.). Goat anti-rabbit IgG coupled with HRP (HRP-anti-rIgG) was purchased from Sigma-Aldrich, Inc. (St. Louis, Mo., U.S.A.). All antibodies were delivered at a

concentration of 1 mg/mL. Antibodies were tested against a serial dilution of purified ovalbumin (OA) or β -lactoglobulin (β -LG) which produced a linear response. The antibodies were also tested against crabsticks. When tested against crabsticks, antibodies showed no reactivity with proteins other than the target protein.

Sample preparation

Basic surimi paste batches

Crabsticks were made with base ingredients including: surimi (40%), water (45%), salt (2%), starches (8%), and sugar (5%). Three formulations for crabstick were made containing base ingredients and protein additives such as DEW only, WPC only, and 50/50 mixture of DEW/WPC. For each of the 3 formulations a total of 9 batches per formulation were made. Six standard batches containing various protein additives were made (0.00, 0.25, 0.50, 1.00, 1.50, and 2.00% protein additives). Three additional verification batches were also made (0.375, 0.750, and 1.750% protein additives). For batches with protein additives, starch was replaced in a 1:1 ratio (w/w) by protein additive resulting in an equal moisture concentration of approximately 75%. Final batch sizes were approximately 1300 g. Frozen surimi was allowed to thaw at room temperature (≈ 23 °C) for approximately 1 h and then cut into small pieces. All chopping was performed using a Stephan vertical vacuum cutter (model UM 5 universal, Stephan Machinery Co., Columbus, Ohio, U.S.A.). Surimi pieces were added to the chopping bowl and chopped at 1,800 rpm for 1 min. Salt (26 g) was then added to the chopped surimi and chopping continued at 1,800 rpm for 1 min. Following the integration of the salt, 65 g of sugar was added in addition to starch

(50:40:10 corn:wheat:modified waxy maize), protein additive when required, and water followed by chopping at 1,800 rpm for 1 min. A vacuum of 40 – 60 kPa was applied to the surimi paste and chopping continued for 3 min at 3,000 rpm. Surimi paste was then placed in a plastic bag and refrigerated until samples were cooked.

Approximately 20-25 g of labeled surimi paste was placed into a sheet mold (stainless steel 25 cm x 7.5 cm x 1.4 mm) formed on top of a piece of aluminum foil, which had been sprayed with no-stick cooking spray (Pam® Original, ConAgra Foods, Inc., Omaha, Nebr., U.S.A.). Excess raw surimi paste was vacuum packed and frozen at -80 °C. Food grade plastic film was laid over the top of the paste and a stainless steel tube roller (11.5 cm x 4 cm dia.) was used to evenly spread out the paste to form a crabstick sheet. After the film was removed the aluminum foil containing the crabstick sheet was cooked in a steam bath (93 °C) for 90 sec. After initial cooking, crabstick sheets were cut in half, wrapped in plastic film and then placed back in the 93 °C steam bath for 30 min to simulate commercial pasteurization. Pasteurized samples were submerged in ice water to cool for 15 min. If samples were not to be used within 48 h they were frozen at -80 °C until needed.

For the verification process, 3 batches per formulation were made (0.375, 0.75, and 1.75% protein additive) and the protein concentrations were estimated using the linear equation produced from the 5 standard batches (0.25, 0.50, 1.00, 1.50, and 2.00% protein additives). Using Microsoft Excel software the standard curves were obtained by plotting the absorbance at 450 nm vs. the protein additive concentrations of 0.25, 0.50, 1.00, 1.50, and 2.00%. The standard curve was fitted to a linear

equation ($y = mx + b$), where y = absorbance at 450 nm, m = slope of the line, x = protein additive %, and b = intercept of the line. Calculation of the verification samples were given by the following equation: $x = (y - b)/m$.

Optimized sample extraction

During our preliminary experiments, we realized the optimization of the extraction solution was extremely important to obtain consistent data. In a preliminary optimization step 25 extraction solutions were tested. Extraction solutions contained various levels of SDS (2.5, 5, and 7.5% w/v), Tween 20 (0.05, 0.075, and 0.1% v/v) and β ME (5, 7.5, and 10% v/v). All extraction solutions were incubated for 1, 2, or 3 h at 90 °C (results not shown). A final optimized extraction protocol was achieved using 10% SDS w/v and 2.5% β ME v/v. Tween 20 was determined to have no significant effect on the extraction of the protein from the crabstick samples. This may be due to the much more substantial amount of SDS used as both are detergents. Samples used for the standard curve produced a linear response which then allowed for the estimation of the DEW and WPC content of the verification batches.

Approximately 2 g of sample was accurately weighed into a 50 mL centrifuge tube and 20 mL of solution containing 10% SDS and 2.5% β ME was added. Samples were homogenized for 1 min at speed 4 (Powergen 700, Fisher Scientific, Pittsburgh, Pa., U.S.A.) followed by extraction for 1 h at 90 °C. Extracted samples were centrifuged at 7,796 x g (Sorvall RC-5B, DuPont Co., Newton, Conn., U.S.A.) for 20 min. The supernatant was filtered (70 mm dia # 541, Whatman International Ltd,

Maidstone, U.K.) into a 25 mL volumetric flask and brought to volume using additional 10% SDS and 2.5% β ME solution.

Indirect ELISA

Ovalbumin (OA) from chicken egg white, grade VI (Sigma-Aldrich, Inc., St. Louis, Mo., U.S.A.) and β -Lactoglobulin (β -LG) from bovine milk (Sigma-Aldrich, Inc., St. Louis, Mo., U.S.A.) were serially diluted using PBS, containing 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer (Omnipur 10x PBS Concentrate, EMD Chemicals Inc., Gibbstown, N.J., U.S.A.) and assayed in both the OA and β -LG ELISA checking for linearity and cross-reactivity. OA and β -LG were solubilized in 5% SDS (w/v) and heated at 90 °C for 1 h. Total protein content was determined using the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951). Samples were serially diluted with PBS to 25 ng/mL and tested in triplicate. Indirect ELISA results were given as the average of the 3 serial dilutions.

Crabstick samples were serially diluted in PBS to a final dilution of 1:10,000. Polystyrene 96 well microtiter plates (Nalge Nunc International, Rochester, N.Y., U.S.A.) were coated with 100 μ L of serially diluted samples (6 wells/sample) and were incubated overnight at 4 °C. The plates were washed 3 times with PBS containing 0.5% (v/v) Tween 20 (PBS-T) and blocked with 100 μ L of Superblock® blocking buffer in PBS with 0.05% Tween 20 (Thermo Scientific, Rockford, Ill, U.S.A.) at 37 °C for 30 min. After the plates were washed 3 times with PBS-T, 100 μ L of diluted (1:5000, 1 mg/mL) Ab anti-H-OA or pAb anti- β -LG antibodies were

added to the wells and incubated at 37 °C for 1 h. After 3 PBS-T wash cycles, 100 µL of diluted (1:5000, 1 mg/mL) HRP-anti-mIgG when detecting Ab anti-H-OA and HRP-anti-rIgG when detecting pAb anti-β-LG were added to each well and incubated at 37 °C for 30 min. Plates were washed 3 times using PBS-T after which 100 µL of 3,3',5,5'-tetramethylbenzidine, 1-Step Ultra TMB ELISA (Thermo Scientific, Rockford, Ill, U.S.A.) was added and allowed to develop for 8 or 12 min at room temperature, for the DEW and WPC samples respectively. Color development was stopped by the addition of 100 µL of 2 N sulfuric acid per well. The plate was read at 450 nm using a Biolog Microlog (Biolog, Hayward, Calif., U.S.A.)

Immunoblotting

SDS-PAGE was performed as outlined by Laemmli (1970) using a 12.5% (%T) separating gel and a 4% (%T) stacking gel. The crabstick samples used for SDS-PAGE and immunoblotting contained both DEW and WPC. A 0.5 mL aliquot of extracted sample, that was used for the indirect ELISA, was mixed with 0.5 mL of Laemmli sample buffer (0.6 mL 1 M Tris-HCl (pH 6.8), 5.0 mL glycerol, 2.0 mL 10% (w/v) SDS, 0.5 mL βME, 1.0 mL 1% (w/v) bromophenol blue, 0.9 mL H₂O). Sample and buffer were vortexed and then heated for 3 min at 90 °C to disrupt any disulfide bonds. Samples were separated using a Mini-Protean III Cell (Bio-Rad, Hercules, Calif., U.S.A.) according to the operating manual.

After electrophoresis the gels were removed and equilibrated in Towbin solution (25 mM Tris, 192 mM glycine, 20% methanol (v/v) at pH 8.3) for 20 min and

transferred to a nitrocellulose membrane (Bio-Rad, Hercules, Calif., U.S.A.) using a Mini Trans-Blot[®] Electrophoretic Transfer Cell (Bio-Rad, Hercules, Calif., U.S.A.) using a slight modification of an earlier published method (Towbin, Staehelin & Gordon, 1979). The membrane was blocked using Superblock[®] blocking buffer in PBS with 0.05% Tween 20 (Thermo Scientific, Rockford, Ill, U.S.A.) for 30 min at 25 °C. The blocked membrane was washed 3 times for 5 min using PBS-T. The membrane was incubated with either Ab anti-H-OA or pAb anti- β -LG (1:15,000) for 60 min at 25 °C after which the membrane was washed 3 times. The membrane was incubated with HRP-anti-mIgG when detecting Ab anti-H-OA and was incubated with HRP-anti-rIgG when detecting pAb anti- β -LG antibodies (1:15,000) at 25 °C for 60 min followed by 3 additional wash cycles. Bands were visualized using 1-Step[™] TMB-Blotting (Thermo Scientific, Rockford, Ill, U.S.A.). Development was stopped by the addition of dd water. Molecular weight was determined by comparison to the Kaleidoscope[™] protein standard (Bio-Rad, Hercules, Calif., U.S.A.) also run in each SDS-PAGE.

Statistical methods

For determination of the inter-assay precision, the mean coefficients of variation (CV) were based on 3 extractions performed on separate days. Each day an extraction was performed and the extract was assayed in triplicate on the same day. All calculations were done using Microsoft Excel (Microsoft Corporation, Redmond, Wash., U.S.A.). Each assay contained 6 replicates of each sample. The lower limit of

detection (LLD) for the indirect assay was given as 3 times the standard deviation plus the background of blank wells. The lower limit of quantification (LLQ) was taken to be the lowest point (0.25% protein additive) of the standard curve.

3.4 RESULTS AND DISCUSSION

Optimized sample extraction

Extracting the proteins with 10% SDS and 2.5% β ME for 1 h at 90 °C was found to be the optimum condition yielding reproducible and reliable data for the ELISA assay. Figure 3.1 shows the increased accuracy and decreased variation obtained when the optimized extraction solution was used. The calculated protein was determined using the standard curve created from the standard crabstick batches. In Figure 3.1A, it is shown that the calculated % protein for DEW (calculated protein divided by protein added) ranges from 91.5% to 120.1% for the pre-optimized extraction solution (1% SDS (w/v) and 5% β ME (w/v)). It is our opinion that the addition of SDS and β ME was successful in disrupting the bonds formed during gelation. The gel matrix formed during the cooking of the crabsticks is believed to have caused steric hindrance during adsorption onto the ELISA plate. Due to the steric hindrance, some of the antibody epitopes were not available for interaction with the antibody. For the optimized extraction solution the range narrows to 100.0% to 101.7%. The standard deviation for the pre-optimized solution ranged from 4.6% to 24.0% and that of the optimized solution was 4.0% to 6.3%. The WPC extraction optimization showed a similar trend. The pre-optimized calculated % protein ranged

from 85.9% to 134.4% with a standard deviation range of 5.7% to 40.0%. The calculated % protein for WPC using the optimized extraction solution ranged from 95.6% to 116.7% with a standard deviation ranging from 1.7% to 11.4%.

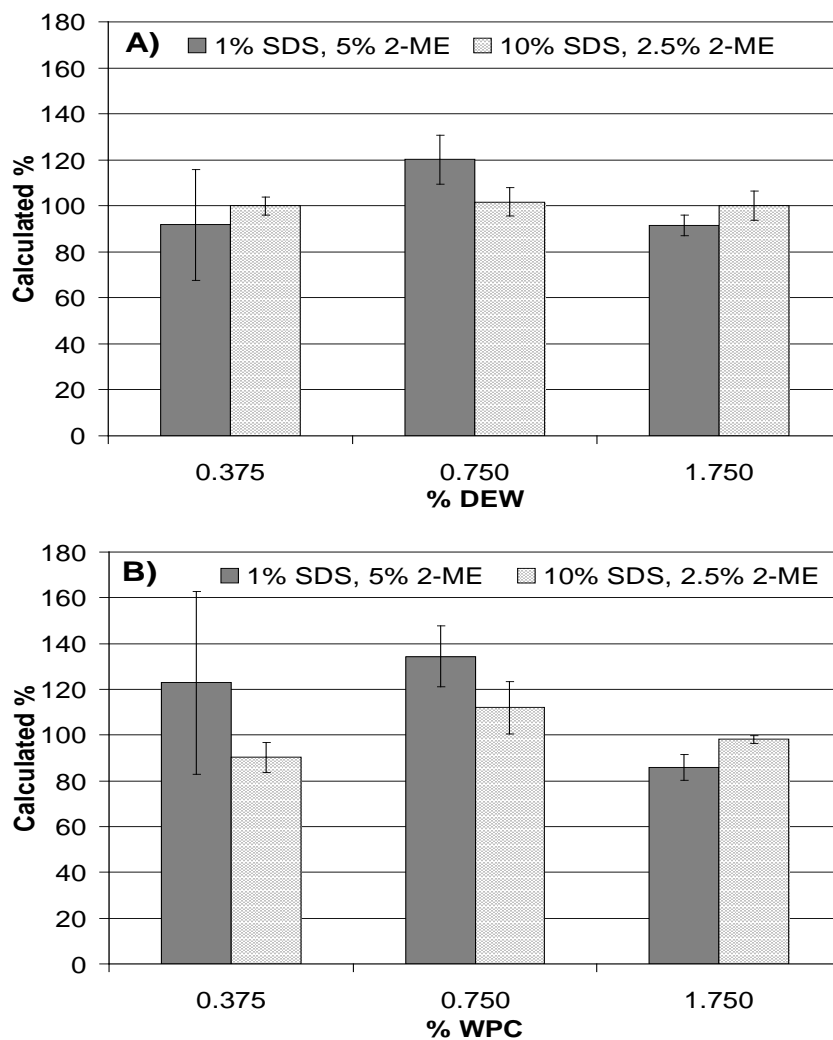


Figure 3.1 – Protein extraction using pre-optimized solution (1% SDS and 5% β ME) and optimized solution (10% SDS and 2.5% β ME) (A) Crabstick containing DEW, (B) crabstick containing WPC. The calculated protein was determined using the standard curve created from the standard crabstick batches.

Reactivity testing and immunoblotting

Purified OA and β -LG proteins were examined for reactivity and cross-reactivity with the Ab anti-H-OA and pAb anti- β -LG. Figure 3.2A, there is a linear response for OA passively absorbed to the microtiter plate and incubated with Ab anti-H-OA, while virtually no binding was observed with β -LG when incubated with the same Ab anti-H-OA. In Figure 3.2B, a linear response was observed for β -LG when incubated with pAb anti- β -LG. Again virtually no response was made for the OA incubated with pAb anti- β -LG. Immunoblotting of crabstick samples for DEW detection using Ab anti-H-OA showed similar patterns to those produced from purified ovalbumin and DEW only samples (Figure 3.3A). Figure 3.3B shows similar results for samples tested for the presence of β -LG using pAb anti- β -LG.

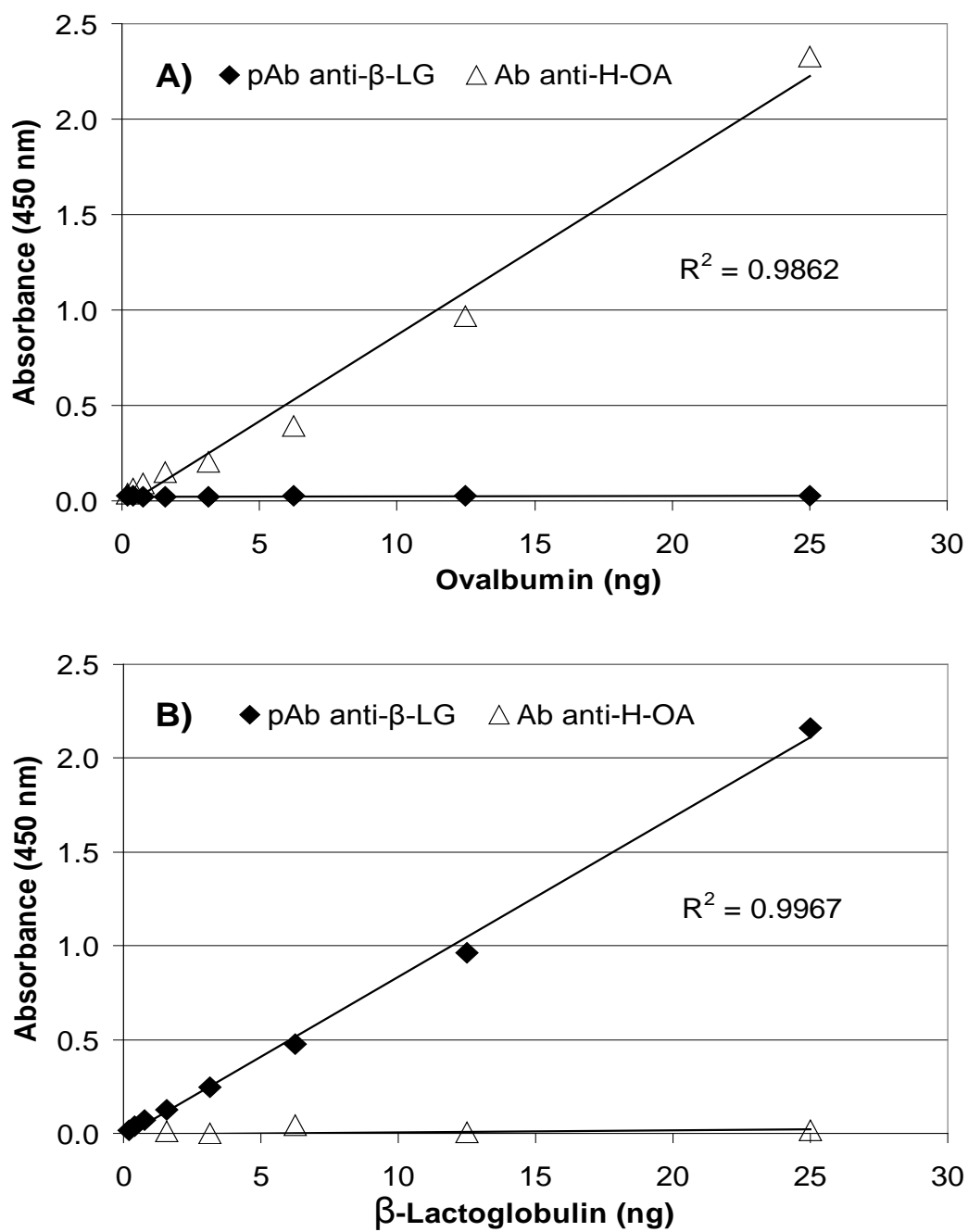


Figure 3.2 – Linear response of Mab H-OA (A) and Pab β -LG (B) tested against ovalbumin and β -lactoglobulin.

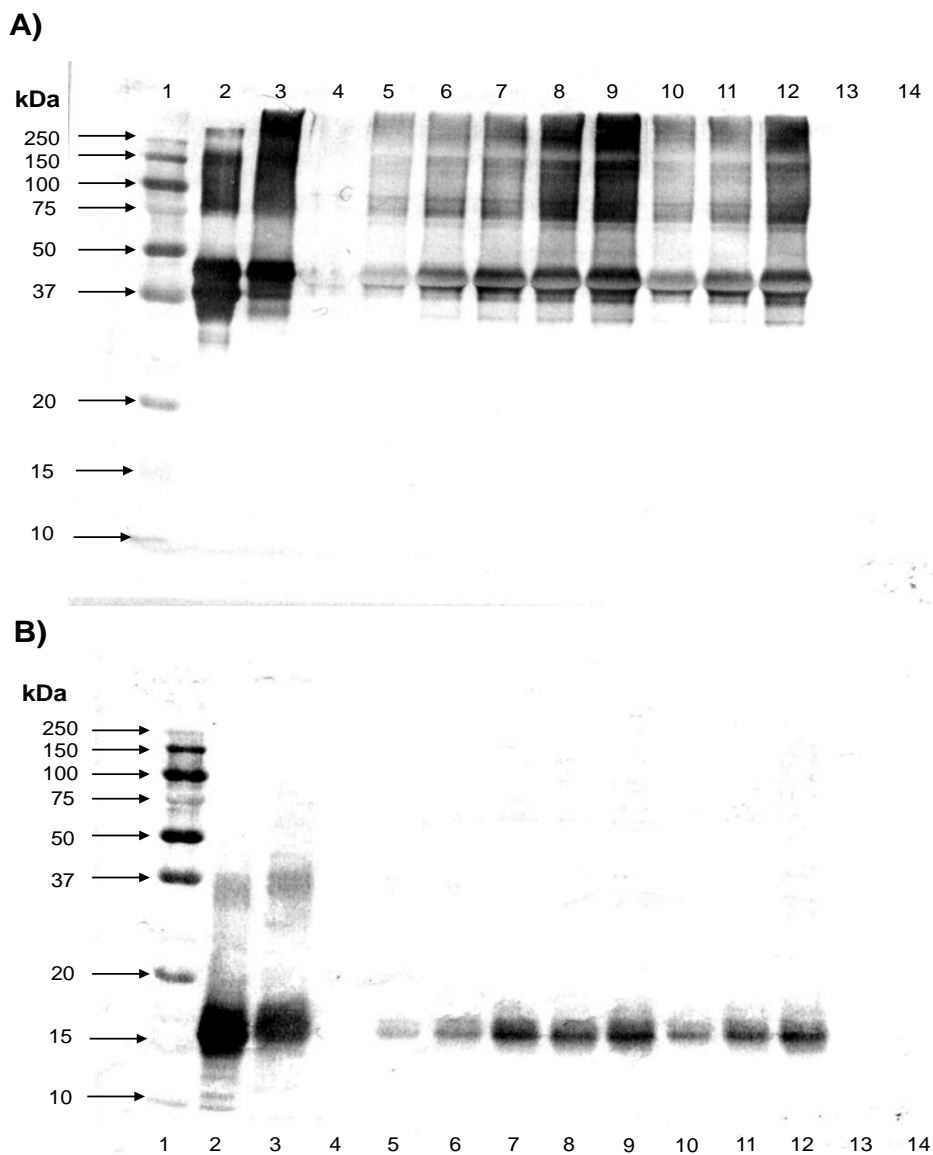


Figure 3.3 – Immunoblotting of DEW/WPC mixture tested using Mab H-OA (A) and Pab β -LG (B)

- (A) Lane (L) L1 Kaleidoscope marker, L2 Ovalbumin, L3 DEW, lanes 4 – 12 crabstick batches (% DEW/WPC) L4 0%, L5 0.25%, L6 0.5%, L7 1.0%, L8 1.5%, L9 2.0%, L10 0.375%, L11 0.75%, L12 1.75%, L13 β -LG, L14 WPC
- (B) Lane (L) L1 Kaleidoscope marker, L2 β -LG, L3 WPC, lanes 4 – 12 crabstick batches (% DEW/WPC) L4 0%, L5 0.25%, L6 0.5%, L7 1.0%, L8 1.5%, L9 2.0%, L10 0.375%, L11 0.75%, L12 1.75%, L13 Ovalbumin, L14 DEW.

Lower limit of detection vs. lower limit of quantification (LLD vs. LLQ)

Blank wells (36 total) were tested using Ab anti-H-OA with an average absorbance of 0.044 ± 0.006 and a LLD of 0.062 absorbance. The LLQ for the ELISA assay was calculated as the lowest point on the standard curve using 0.25% protein additives (16.5 and 12.4 ng/mL for DEW and WPC, respectively). The average absorbance value for the 0.25% DEW sample was 0.361 ± 0.034 . The control sample (0% DEW) had an average absorbance of 0.101 ± 0.021 which was 3.6 times lower than that of the 0.25% DEW sample. The absorbance of the control sample was higher than the LLD but still below the LLQ. The average absorbance for the blanks were calculated using the standard curve the resulting values were negative showing that the blank values lie outside of the linear range of the standard curve. A similar trend was found using the blank values and standard curve for the WPC. The blank wells for WPC had an average absorbance of 0.057 ± 0.007 and a LLD of 0.078. The absorbance of the LLQ for WPC was 0.476 ± 0.037 . The control sample (0% WPC) had an average absorbance of 0.085 ± 0.008 . Again the control sample was slightly higher than the LLD but still much lower than the LLQ. The range for the standard curve was chosen so as to cover the most commonly added amounts of protein additive.

Indirect ELISA of DEW and WPC in crabstick

Using the most abundant proteins found in the respective protein additives helped in creating an accurate assay. Finding antibodies that will bind well with the

heat denatured form of OA and β -LG was a key for the success of the assay development. An indirect ELISA was used to qualify and quantify the use of DEW and WPC in prepared crabstick. Due to the highly processed nature of the crabstick, the proteins found in the sample have been completely denatured. Chemical (addition of salt) and physical (chopping and cooking) processing made it even more difficult to find the antibodies that could detect the denatured protein. The Ab anti-H-OA was produced to bind strongly with OA in denatured and modified forms such as heat-denatured and reduced. The antibody used to detect β -LG was created using native β -LG. Table 3.1 shows the results of the verification batches for DEW, WPC, and their mixture (DEW/WPC). The calculated % protein was determined by dividing the determined protein % from the standard curve by the actual protein % added. Samples containing DEW had a calculated % protein that ranged from 98.8% to 106.6% and a standard deviation range of 2.5% to 6.3%. All calculated % protein values for the samples that contained DEW were within 7% of the actual added protein value. The WPC had a calculated % protein that ranged from 90.3% to 116.7% and a standard deviation range of 1.3% to 11.4%. All calculated % protein values for WPC samples were within 17%.

Table 3.1 – Protein additive estimation of verification batches using indirect ELISA

	Added protein (%)	Determined protein (%)	Sample recovery	
			% ^a	CV(%) ^a
DEW	0.375	0.375	100.0	3.1
	0.750	0.763	101.7	2.9
	1.750	1.749	100.2	1.0
WPC	0.375	0.339	90.3	7.6
	0.750	0.840	112.0	5.3
	1.750	1.717	98.1	1.0
DEW / WPC ^b (mixture)	0.375	0.371	98.8	2.8
	0.750	0.799	106.6	0.9
	1.750	1.755	100.3	1.8
DEW / WPC ^c (mixture)	0.375	0.383	102.2	8.0
	0.750	0.875	116.7	3.4
	1.750	1.674	95.6	4.0

^a Sample Recovery (%) and Coefficient of Variance (CV) as determined from 3 extractions done on 3 separate days in triplicate plates

^b Samples were tested with Mab H-OA detection antibodies

^c Samples were tested with Pab β -LG detection antibodies

To understand the precision of the assay, the CV were analyzed and reported in Table 3.2. Overall assay CV for DEW samples ranged from 0.9% to 3.1% while those for the WPC samples ranged from 1.0% to 8.0%. The difference in CV range for the two assays may be due, in part, to the nature of the antibodies used. The Ab anti-H-OA was developed against heat denatured OA. However the pAb anti- β -LG was created using the native form of β -LG. The difference in the CV may be because of

increased avidity of the Ab anti-H-OA with denaturation. An antibody produced using heat denatured β -LG may possibly lead to an antibody with a stronger avidity for heat denatured β -LG.

Table 3.2 – Inter-assay coefficient of variance^a (%) by indirect ELISA.

	Added protein (%)	Inter-assay (% CV)
DEW	0.375	3.1
	0.750	2.9
	1.750	1.0
WPC	0.375	7.6
	0.750	5.3
	1.750	1.0
DEW / WPC ^b (mixture)	0.375	2.8
	0.750	0.9
	1.750	1.8
DEW / WPC ^c (mixture)	0.375	8.0
	0.750	3.4
	1.750	4.0

^a Inter-assay CVs are given as the mean of 3 extractions done on 3 separate days. Each extract from each extraction was assayed in triplicate on the same day

^b Samples were tested with Mab H-OA detection antibodies

^c Samples were tested with Pab β -LG detection antibodies

Breton, Phan Thanh and Paraf (1988) found that affinity purified antibodies raised against native ovalbumin had higher avidity against heat-denatured ovalbumin than against the native ovalbumin molecule. Rumbo, Chirido, Fossati and Anon (1996) found that avidity peaked and then began to decrease as heating time increased. This trend held true for samples tested between 70 °C and 100 °C. They attributed the decrease in avidity to an increase in OA unfolding. Luis, Perez, Sanchez, Lavilla and Calvo (2007) showed that the use of native β -LG as an antigen, still allowed for the detection of highly denatured β -LG in food products including meat, bakery products, sauces, and snacks. Chicón, Belloque, Alonso and López-Fandiño (2008) found that high pressure treatment of β -LG at 200 and 400 MPa actually increased the binding of β -LG-specific rabbit IgG antibodies when tested in an indirect ELISA format. According to Svenning, Brynhildsvold, Molland, Langsrud and Vegarud (2000), β -LG antibody antigenicity was reduced due to the WPC processing. However, they stated that the reduction may be due to lower protein solubility. For WPC that was heated without any hydrolysis they showed 61% protein solubility and a 500-fold decrease in antigenicity. With pre-heated and hydrolyzed WPC, they showed 100% protein solubility and a 10-fold decrease in antigenicity. Their study pointed out that β -LG antibody antigenicity was affected not only by processing conditions, but also by protein solubility.

3.5 CONCLUSIONS

Obtaining antibodies that would bind the target protein in the highly processed crabstick proved to be a key for the success of the assay development. Ab anti-H-OA and pAb anti- β -LG were shown to be highly specific for the target protein and showed no cross-reactivity with non-target proteins found in the prepared crabstick. An optimized solution for extraction was made with 10% SDS and 2.5% β ME. This optimized extraction solution demonstrated high reproducibility for the prepared crabsticks. An indirect ELISA assay was tested and proved as an effective method for the qualification and quantification DEW and WPC in a cooked and pasteurized crabstick. All 3 levels of DEW protein additive were estimated to less than 7% of the actual value and those of the WPC were estimated to less than 17%. Accuracy of the WPC assay may be improved by producing an antibody from the heat denatured form of β -LG.

CHAPTER 4

QUANTIFICATION OF ALASKA POLLOCK SURIMI IN PREPARED
CRABSTICK BY COMPETITIVE ELISA USING MYOSIN LIGHT CHAIN 1
SPECIFIC PEPTIDE

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4.1 ABSTRACT

A competitive enzyme-linked immunosorbent assay (ELISA) was developed for quantification of Alaska pollock (AP) surimi in crabsticks. Identification of fish species is complicated by processing, cooking, and additional ingredients. ELISA is a powerful tool for identification and quantification of fish species. Polyclonal antibodies were raised in rabbits against a 15-amino-acid peptide (Ala-Pro-Lys-Lys-Asp-Val-Lys-Ala-Pro- Ala-Ala-Ala-Ala-Lys-Lys) determined from the myosin light chain 1 (MLC 1) of AP. Immunoblotting showed the anti-pep-AP antibody had no significant cross-reactivity with protein additives. However, cross-reactivity of the MLC 1 from Pacific whiting, and threadfin bream surimi was observed. MLC 1 was purified from AP surimi and used as the coating protein in the competitive ELISA. MLC 1 was serially diluted and had a R^2 of 0.9845 following a logarithmic curve. All estimations of AP surimi were within 9% of the actual value. Inter-assay coefficients of variance ranged from 4.2 to 4.9%.

4.2 INTRODUCTION

Surimi is stabilized myofibrillar proteins concentrated through heading, gutting, mincing, washing, and dewatering (Lee, 1984; Park & Lin, 2005b). The concentrated myofibrillar protein is then mixed with cryoprotectants, such as sugar, sorbitol, and polyphosphates, which serve to stabilize the surimi during frozen storage (Park et al., 1987; Park et al., 1988). Alaska pollock is abundant in the United States and is considered to be a premium fish for surimi. With the growing popularity of crabsticks in the United States, there is increased competition for market share. Under these circumstances, some companies may be willing to sacrifice product quality in order to facilitate manufacture and reduce price as a means of gaining market share. During the last 10 years, poor quality imports, which exceeded nearly 20 million pounds a year, have negatively impacted the domestic market (Park, 2005).

Crabsticks made with Alaska pollock support an extremely important commercial fishery in the United States. It is one of the world's largest fisheries and during 2006 made up 71.4% of the average ground fish catch off the coast of Alaska (NOAA, 2006). As of February 14th 2005, the Alaska pollock fishery was certified by the Marine Stewardship Council (2005). By having a well managed fishery, the Alaska pollock fishery has aided in sustained growth for the use of Alaska pollock. However there is some abuse of Alaska pollock, which comes mainly from the substitution of other cheaper and often lower quality fish that masquerade as Alaska pollock (Jacquet et al., 2008). Most substitution of pollock comes from the surimi industry where Alaska pollock is considered the premium species for surimi

production (Guenneugues et al., 2005). Consequently, crabstick quality is directly related to the amount of fish protein from surimi that is used. A high quality crabstick product typically contains surimi (mid-high grade) at 40% or higher. The protein content and quality of surimi vary with species and grades.

Due to the highly processed nature of surimi it is impossible to identify the species used for its production by the morphological characteristics of the fish. Surimi is mixed with other ingredients such as starch, egg white, whey protein concentrates, and other flavors for the production of crabsticks, which further complicates the identification of fish species used in its production. In order to maintain quality and comply with government labeling standards it is imperative that fish species and protein additives in crabsticks be identified and quantified. Pepe and others (2007a) studied 19 different cooked surimi based products using polymerase chain reaction and direct sequence analysis of the cytochrome b gene. They found that of the 19 products labeled as Alaska pollock (*Theragra chalcogramma*) 84.2% were shown to be prepared with species other than the one declared.

DNA identification of fish species is typically considered to be the most accurate, however it does not allow for the quantification of the fish protein used. Reed and Park (2008) recently used SDS-PAGE for the quantification of Alaska pollock surimi used in crabstick production. They found that it was possible to estimate the amount of surimi used but the issue was much more difficult when dried egg white (DEW) was added. The overlapping bands of ovalbumin from DEW and

actin from surimi made estimation of the surimi content difficult and accurate quantification of DEW exceedingly difficult.

Enzyme linked immunosorbent assay (ELISA) utilizes the interaction between antigens and antibodies and can be used to identify fish species (Asensio et al., 2003a; Cespedes, 1999). ELISA assays are extremely sensitive, with detection limits down to the nanogram level. Huang and others (1995) were able to identify red snapper (*Lutianus cumpechanus*) using monoclonal antibodies. Asensio and others (2003b) were able to unequivocally identify grouper (*Epinephelus guaza*), wreck fish (*Polyprion americanus*), and Nile perch (*Lates niloticus*) fillets using an indirect ELISA using polyclonal antibodies that were raised against species specific proteins. ELISA has also been used for quantification of a variety of food products including fish and fish products (Medina, 1988; Ochiai et al., 2001; Panheleux, Nys, Williams, Gautron, Boldicke & Hincke, 2000; Vidal, Gautron & Nys, 2005; Werner et al., 2007).

The overall goal of this study was to develop an antibody against a 15- amino-acid sequence of the myosin light chain 1 of Alaska pollock for the qualification and quantification of the Alaska pollock surimi used in the production of crabstick using a competitive ELISA. This would greatly benefit US crabstick manufacturers by protecting them against low quality imports. In addition, US fisheries would be able to maintain the proper use and identification of Alaska pollock.

4.3 MATERIALS AND METHODS

Food products and chemicals

Alaska pollock (*Theragra chalcogramma*) surimi (FA grade) and Pacific whiting (*Merluccius productus*) surimi (FA grade), were obtained from Trident Seafoods Corporation (Seattle, Wash., U.S.A.). threadfin bream (*Nempiterus sp.*) surimi (A grade) was obtained from Andaman Surimi Industries (Bangkok, Thailand). All surimi was stored frozen at -18 °C. The 10 kg blocks were cut into approximately twenty 1000 g blocks, individually vacuum packed, and stored at -18 °C until used in surimi crabstick paste preparation. Ingredients used for surimi crabstick paste preparation were NaCl (Morton Iodized Salt, Morton International, Inc., Chicago, Ill, U.S.A.), corn starch (Corn Products International, Westchester, Ill, U.S.A.), potato starch (Penplus 300, Penford Food Ingredients Co., Centennial, Colo., U.S.A.), sugar (used to represent all flavor components) (Pure Cane Sugar, C&H Sugar Company, Inc., Crockett, Calif., U.S.A.), culinary tap water, dried egg white (DEW) (K-200, Henningsen Foods, Omaha, Nebr., U.S.A.), whey protein concentrate (WPC 8600, Hilmar Ingredients, Hilmar, Calif., U.S.A.), and soy protein isolate (500E, Solae, St. Louis, Mo., U.S.A.). All other chemicals were reagent grade from VWR International (West Chester, Pa., U.S.A.)

Immunization protocol

Antibodies were produced by Antagene, Inc. (Sunnyvale, Calif., U.S.A.). Two New Zealand white rabbits were immunized with keyhole limpet haemocyanin (KLH) conjugated peptide (Ala-Pro-Lys-Lys-Asp-Val-Lys-Ala-Pro- Ala-Ala-Ala-Ala-Lys-Lys) in Complete Freund's Adjuvant (CFA) with all subsequent injections using

Incomplete Freund's Adjuvant (IFA). Data of total DNA nucleotide sequence has been also registered to the DDBJ/EMBL/GenBank DNA databases with accession number of AB042054. The immunogen was diluted to 1 mL with sterile saline and combined with 1 mL of the appropriate adjuvant. The antigen and adjuvant were then mixed to form a stable emulsion that was injected subcutaneously and provided enhanced immune response from the sustained presence of the immunogen. Day 1: 1st immunization with antigen in CFA. Day 20: 2nd immunization with antigen in IFA. Day 40: 3rd immunization with antigen in IFA. Day 60: 4th immunization with antigen in IFA. Day 70: final total bleed 50 mL from each rabbit. Blood was collected from the central ear artery with a 19 gauge needle and allowed to clot and retract at 37°C overnight. The clotted blood was then refrigerated for 24 h before the serum was decanted and clarified by centrifugation at 2,500 rpm for 20 min.

Antibodies

One hundred mL of rabbit antisera, from each rabbit, were delivered from Antagene, Inc. and affinity purified using a Nab Protein A/G Spin Kit (Pierce, Rockford, Ill, U.S.A.) according to the manufacturers' specification. Protein content of affinity purified antibodies rabbit anti-peptide from Alaska pollock (anti-pep-AP) was determined using the Lowry total protein determination method (Lowry et al., 1951) and then adjusted to a final concentration of 1 mg/mL using dd H₂O and placed in 100 µL aliquots and stored frozen at -80 °C until needed. Goat anti-rabbit IgG

coupled with HRP (HRP-anti-rIgG) was purchased from Sigma-Aldrich, Inc. (St. Louis, Mo., U.S.A.). All antibodies were stored frozen at a concentration of 1 mg/mL.

Crabstick sample preparation

Surimi crabstick paste batches, approximately 1,000 g, were made using 8 different surimi contents (25, 27.5, 30, 32.5, 35, 40, 42.5, and 45%) and 8 different starch contents (10, 9.5, 9, 8.5, 8, 7, 6.5, and 6%). The moisture content of each batch was adjusted to $\approx 75\%$ using ice water. Frozen surimi was allowed to thaw at room temperature for approximately 1 h ($\approx 23\text{ }^{\circ}\text{C}$) and then cut into small pieces. All initial surimi paste chopping was performed using a Stephan vertical vacuum cutter (model UM 5 universal, Stephan Machinery Co., Columbus, Ohio, U.S.A.). The surimi pieces were added to the chopping bowl and then chopped on low speed for 1 min. Twenty g of salt were then added to the chopped surimi and chopping continued on low speed for 1 additional min. Then 50 g of sugar, used to simulate all flavor ingredients, 10 g of DEW and 10 g of WPC, was added in addition to starch and water. For the 25, 27.5, 30, 32.5, 35, 40, 42.5, and 45% batches, 100, 95, 90, 85, 80, 70, 65, and 60 g of starch (50:50 mixture of corn and potato) along with 560, 540, 520, 500, 480, 440, 420, and 400 g of ice water, respectively, were added and then chopped on low for 1 min. A vacuum of 40 – 60 kPa was applied to the surimi paste and chopping continued for 3 min on high speed. Surimi paste was then placed in a plastic bag that was submerged in ice water slurry until ready to cook. Approximately 20-25 g of labeled surimi paste was placed into a sheet mold (stainless steel 25 cm x 7.5 cm x 1.4

mm) that was formed on top of a piece of aluminum foil, which had been sprayed with Pam® no-stick cooking spray (ConAgra Foods, Inc., Omaha, Nebr., U.S.A.). Excess raw surimi paste was vacuum packed and frozen at -80 °C. Food grade plastic film was then laid over the top of the paste and a stainless steel tube roller (11.5 cm x 4 cm dia.) was used to evenly spread out the paste to form a crabstick sheet. After the film was removed the aluminum foil containing the crabstick sheet was cooked on a rack in a steam bath (93 °C) for 3 min. After initial cooking, crabstick sheets were cut in half, wrapped in plastic film, placed inside a labeled Whirl Pak bag, and then returned to the 93 °C steam bath for 30 min to simulate commercial pasteurization. Pasteurized samples were submerged in ice water to cool for 15 min. If refrigerated samples were not to be used within 48 h, they were frozen at -80 °C until needed.

Protein extraction

All samples were extracted using the same extraction solution of 5% sodium dodecyl sulfate (w/v) (SDS) and 0.5% β -mercaptoethanol (v/v) (β ME) according to Reed and Park (2010b). For all samples (surimi, protein additives, and crabstick), 20 mL of extraction solution was added to the weighed sample and then homogenized for 1 min at speed 4 (Powergen 700, Fisher Scientific, Pittsburgh, Pa., U.S.A.) followed by incubation for 1 h at 90 °C. Incubated samples were then centrifuged for 20 min at 7,796 x g (Sorvall RC-5B, DuPont Co., Newton, Conn., U.S.A.). For surimi standards, (Alaska pollock, Pacific whiting, threadfin bream) approximately 3 g of surimi was used. For protein additives (DEW, WPC and SPI), 0.5806, 0.6181, and

0.6686 g were used respectively. Protein content of surimi and protein additive standards was adjusted to 3.75 mg protein/mL in preparation for subsequent separation by SDS-PAGE and transfer to nitrocellulose for immunoblotting. For the crabstick samples, approximately 5 g of each crabstick sample was used and extracted in the same manner as previously described; extracted solution was used for SDS-PAGE, immunoblotting, and competitive ELISA. After centrifugation, the supernatant of the crabstick sample was then poured into a 25 mL volumetric flask and brought to volume using additional extraction solution.

Purification of MLC 1 from Alaska pollock surimi

SDS-PAGE was performed as outlined by Laemmli (1970) using a 12.5% (stock solution of 29.2% acrylamide and 0.8% bis-acrylamide) separating gel and a 4% stacking gel (140 x 120 x 1.5 mm, w x h x thickness) using a Fisher Biotech Protein Electrophoresis System (FB-VE16-1, Fisher Scientific, Pittsburgh, Pa., U.S.A.). 800 μ L of 3.75 mg/mL Alaska pollock sample was mixed with 200 μ L of Laemmli sample buffer (0.6 mL 1 M tris-HCl (pH 6.8), 5.0 mL glycerol, 2.0 mL 10% (w/v) SDS, 0.5 mL β -mercaptoethanol, 1.0 mL 1% (w/v) bromophenol blue, 0.9 mL dd H₂O). Sample and buffer were vortexed for 5 s and then heated for 3 min at 90 °C in order for the β -mercaptoethanol in the sample buffer to disrupt any disulfide bonds. A 15 well comb was used and 30 μ L of AP sample was added wells 2 – 14, with 10 μ L of Kaleidoscope™ protein standard (Bio-Rad, Hercules, Calif., U.S.A.) in wells 1 and 15. Gels were electrophoresed until the dye front reached the bottom of the gel.

Four gels with the identical setup were run at one time using two electrophoresis machines. Gels were then removed from the plates and the gel portion containing the MLC 1 was excised. This portion was determined by a previously stained gel and we found that MLC 1 was the sole protein in the molecular weight range of 20 to 25 kDa. The Kaleidoscope™ standard uses pre-stained proteins and contains two markers at 20 and 25 kDa allowing for the precise and repeatable excision of the MLC1 from Alaska pollock surimi. The MLC 1 was then eluted out of the gel slices using a Bio-Rad Mini Gel Whole Eluter (Bio-Rad, Hercules, Calif., U.S.A.). The eluent was collected and then concentrated using Vivaspin 20 (Sartorius Stedim Biotech, Goettingen, Germany) protein concentration tube with a molecular weight cutoff at 10 kDa. Protein concentration was determined according to Lowry et al. (1951). Concentrated MLC1 in 25 µL aliquots was frozen at -80 °C.

Immunoblotting

The crabstick samples used for SDS-PAGE and immunoblotting contained both DEW and WPC. A 0.8 mL aliquot of extracted crabstick sample was mixed with 0.2 mL of Laemmli sample buffer. Sample and buffer were vortexed for 5 s and then heated for 3 min at 90 °C to disrupt any disulfide bonds. Samples were separated using a Mini-Protean III Cell (Bio-Rad, Hercules, Calif., U.S.A.) according to the operating manual. For the crabstick samples 10 µL of sample was loaded in each well. For the surimi and protein additive standards 7 µL was added to each well. On both SDS gels one lane was loaded with 15 µL of purified MLC 1. After

electrophoresis the gels were removed and equilibrated in Towbin solution (25 mM Tris, 192 mM glycine, 20% methanol (v/v) at pH 8.3) for 20 min and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, Calif., U.S.A.) using a Mini Trans-Blot[®] Electrophoretic Transfer Cell (Bio-Rad, Hercules, Calif., U.S.A.) using a slight modification of an earlier published method (Towbin et al., 1979). The membrane was blocked using Superblock[®] blocking buffer in PBS with 0.05% Tween 20 (Thermo Scientific, Rockford, Ill, U.S.A.) at 25 °C for 30 min. The blocked membrane was washed 3 times using PBS-T for 5 min. The membrane was incubated with either anti-Peptide Ab (1:500) at 25 °C for 60 min with shaking after which the membrane was washed 3 times. The membrane was incubated with HRP-anti-rIgG (1:5000) at 25 °C for 60 min with shaking followed by 3 additional wash cycles. Bands were visualized using 1-Step[™] TMB-Blotting (Thermo Scientific, Rockford, Ill, U.S.A.). Development was stopped by the addition of dd H₂O. Molecular weight was determined by comparison to the Kaleidoscope[™] protein standard also run in each SDS-PAGE.

Competitive ELISA

Purified MLC 1 from Alaska pollock was diluted to a concentration of 0.5 µg/mL and 100 µL was added to each well. Six wells per crabstick sample were used for the ELISA. The plate was coated with MLC 1 at 4 °C overnight and then washed 3 times using a PBS wash solution containing 0.25% (v/v) Tween 20 (PBS-T). The wells were then blocked with 100 µL of Superblock[®] blocking buffer in PBS with

0.05% Tween 20 (Thermo Scientific, Rockford, Ill, U.S.A.) at 37 °C for 30 min or at 4 °C overnight. Plates were then washed 3 times using PBS-T. Extracted crabstick was serially diluted to 1:1000 and 50 µL was added to each well. Then 50 µL of 1:250 diluted anti-pep-AP antibodies were added to each well containing sample and incubated at 37 °C for 1 h. This gave a final dilution of 1:2,000 for the crabstick sample and 1:500 for the anti-pep-AP antibody. After incubation the plate was washed 3 times using PBS-T. One hundred microliters of 1:5000 HRP-anti-rIgG were added to each well and incubated at 37°C for 30 min followed by 3 wash cycles. Following the wash, 100 µL of 1-step Ultra TMB ELISA (Thermo Scientific, Rockford, Ill, U.S.A.) were added and color was allowed to develop at room temperature for 18 min. Color development was stopped by the addition of 100 µL of 2 N sulfuric acid per well. The plate was then read at 450 nm using a Bio-tek EL312 plate reader (Bio-Tek Instruments, Inc., Winooski, Verm, U.S.A.)

Statistical methods

For determination of the inter-assay precision, the mean coefficients of variation (CV) were based on 3 extractions performed on 3 different days. Each day an extraction was performed and the extract was assayed on 3 separate plates on the same day. All calculations were done using Microsoft Excel (Microsoft Corporation, Redmond, Wash., U.S.A.). Each assay contained 6 replicates of each sample. The lower limit of detection (LLD) for the indirect assay was given as 3 times the standard

deviation plus the background of blank wells. The lower limit of quantification (LLQ) was taken to be the lowest point (25% surimi) of the standard curve.

4.4 RESULTS AND DISCUSSION

Protein extraction

In order to create a successful immunoassay the proteins must first be available for interaction with the detection antibody. Using a competitive ELISA, purified MLC 1 was coated on the wells and could thereby easily interact with the detection antibody. However in order for the antibody to detect the MLC 1 found in the crabstick, the protein first needed to be extracted into solution. Due to the nature of the bond types formed in the cooking process of crabstick, most importantly hydrophobic and disulfide bonds (Lanier et al., 2005), the use of a surfactant and reducing agent was necessary. Watanabe and others (2005) have shown that using a surfactant along with a reducing agent extracted more proteins. In our current study the use of 5% SDS (w/v) and 0.5% β ME (v/v) along with homogenization and extraction at 90 °C for 1 h we were able to obtain repeatable and accurate results.

Reactivity testing and immunoblotting

The anti-pep-AP was used to test for cross reactivity with other protein sources commonly used in crabstick manufacture as shown in Figure 4.1. The immunoblot showed that the anti-pep-AP antibody had no cross reactivity with DEW, WPC, or SPI. However, it revealed some cross reactivity with the MLC 1 of Pacific whiting

and that of threadfin bream. This cross reactivity could be due in part to a conserved protein motif specific to the myosin light chain 1 of fish. Asensio et al. (2003b) were able to overcome cross reactivity by pre-incubating the species specific anti-sera with fish extracts of the other fish in the study. This pre-incubation allowed the antibodies to be species specific. However, pre-incubation with a wide variety of fish species to remove cross reactivity would be very costly and time consuming. Antibody cross reactivity in fish species is a common problem. The use of a monoclonal antibody can help to limit cross reactivity but it is difficult to remove all cross reactivity. McNulty and Klesius (2005) found that using a specific monoclonal antibody they were able to differentiate several fish species but the mAb was cross reactive with many fish of the *Ictalurus* species. Even though the anti-pep-AP antibody shows some cross reactivity with Pacific whiting and threadfin bream it is suggested that it may be advantageous for use in the incorporation into a sandwich ELISA. Future production of an antibody against the entire MLC 1, specifically from Alaska pollock and Pacific whiting surimi, could provide antibodies that do not cross react with MLC 1 from different species. If this is the case a sandwich ELISA could be created that could use the anti-pep-AP antibody as a capture antibody for the MLC 1 of Alaska pollock and Pacific whiting while the second antibody would be species specific (Hsieh, 2009). This would allow for a single crabstick sample containing Alaska pollock and Pacific whiting to both be quantified.

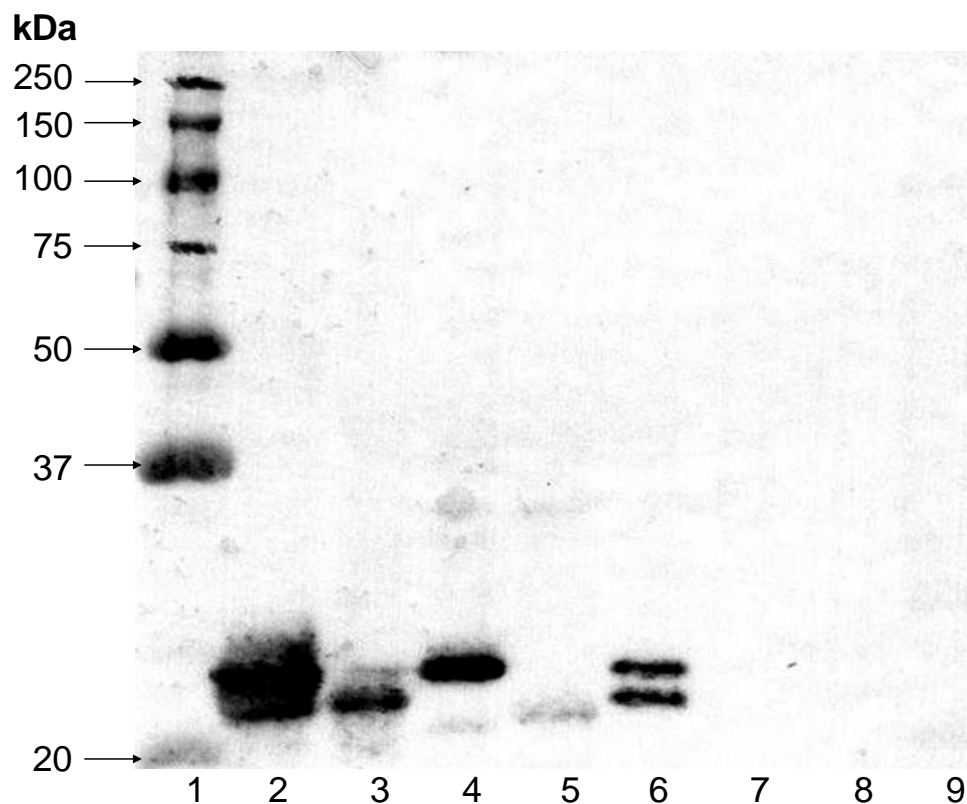


Figure 4.1 – Immunoblotting of common crabstick protein sources. Lane (L): (1) Kaleidoscope marker, (2) AP MLC 1, (3) Alaska pollock surimi, (4) Pacific whiting surimi, (5) threadfin bream surimi, (6) crabstick 50% Alaska pollock surimi, 50% Pacific whiting surimi, (7) DEW, (8) WPC, and (9) SPI.

Figure 4.2 shows the immunoblot of the crabstick batches compared to that of purified MLC 1 from Alaska pollock. The samples were loaded on an equal volume basis allowing for the visualization of the increasing intensity of the MLC 1 band in the crabstick samples. The crabsticks contained DEW and WPC at a final concentration of 1% each and again the immunoblot shows that there was no cross reactivity with the either of the protein additives.

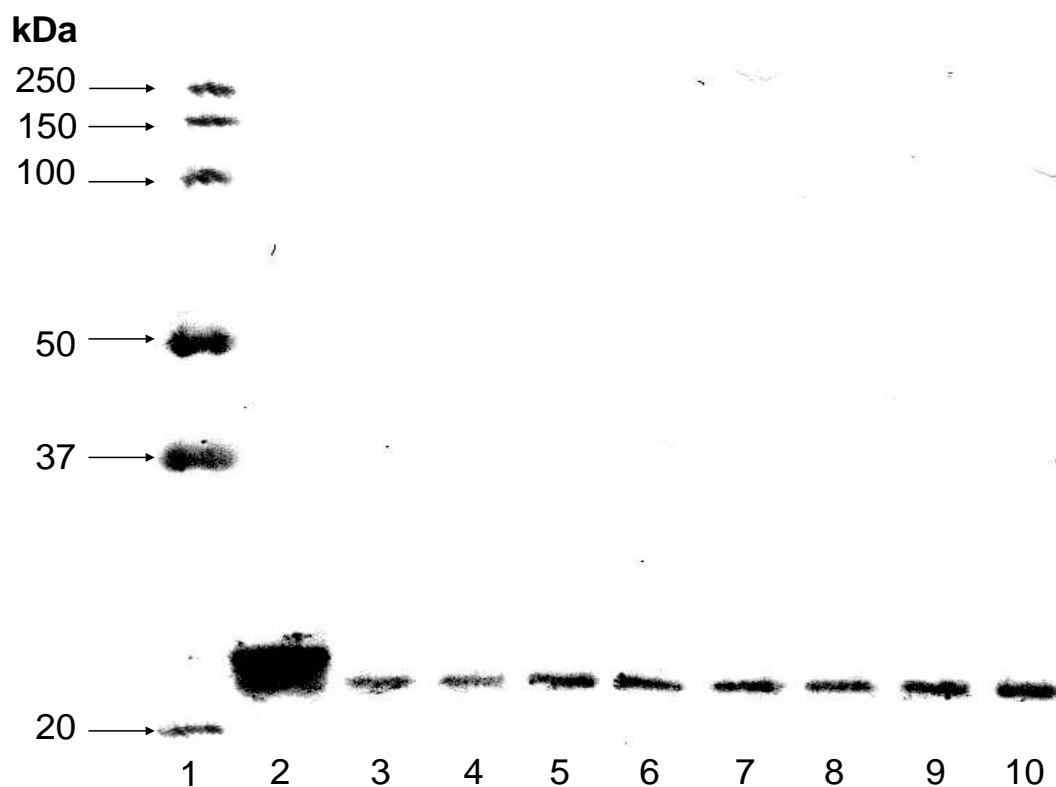


Figure 4.2 – Immunoblotting crabstick batches. Lane (L): (1) Kaleidoscope marker, (2) AP MLC 1, lanes 3-10 crabstick batches (% Alaska pollock surimi), (3) 25%, (4) 27.5%, (5) 30%, (6) 32.5%, (8) 40%, (9) 42.5%, and (10) 45%.

Lower limit of detection vs. lower limit of quantification (LLD vs. LLQ)

Blank wells (36 total) from 3 separate plates were tested using the anti-pep-AP antibody with an average absorbance of 0.063 ± 0.012 and a LLD of 0.0983. The LLQ for the competitive ELISA assay was taken to be the absorbance given by the crabstick batch containing 45% Alaska pollock surimi. This was chosen because the optical response in the competitive ELISA is inversely proportional to the antigen concentration. Therefore, the crabstick with the highest surimi content would give the

lowest absorbance reading. The average absorbance value for the 45% surimi crabstick was 0.659 ± 0.133 . This absorbance is 6.7 times higher than the LLD, indicating that even with 45% Alaska pollock surimi in the crabstick batch the absorbance readings would not be affected by any non-specific binding of the anti-pep-AP antibody.

Competitive ELISA

To begin the testing of the competitive ELISA assay purified MLC 1 from Alaska pollock was serially diluted to test the range of accuracy. Figure 4.3 shows how well the purified MLC 1 works in the competitive ELISA. Another key component to the success of the competitive ELISA is the ability of the anti-pep-AP antibody to recognize the denatured form of the MLC 1. Breton, Phan Trinh and Paraf (1988) found that heat denatured hen ovalbumin was more readily recognized by the with their anti-ovalbumin antibody due to the exposure of additional antigenic sites. Rumbo, Chirido, Fossati, and Anon(1996) found that the degree of heat denaturation can significantly affect the detection and quantification of hen ovalbumin. The MLC 1 was extracted under denaturing conditions (SDS, β ME) and was also separated out using denaturing SDS-PAGE methods.

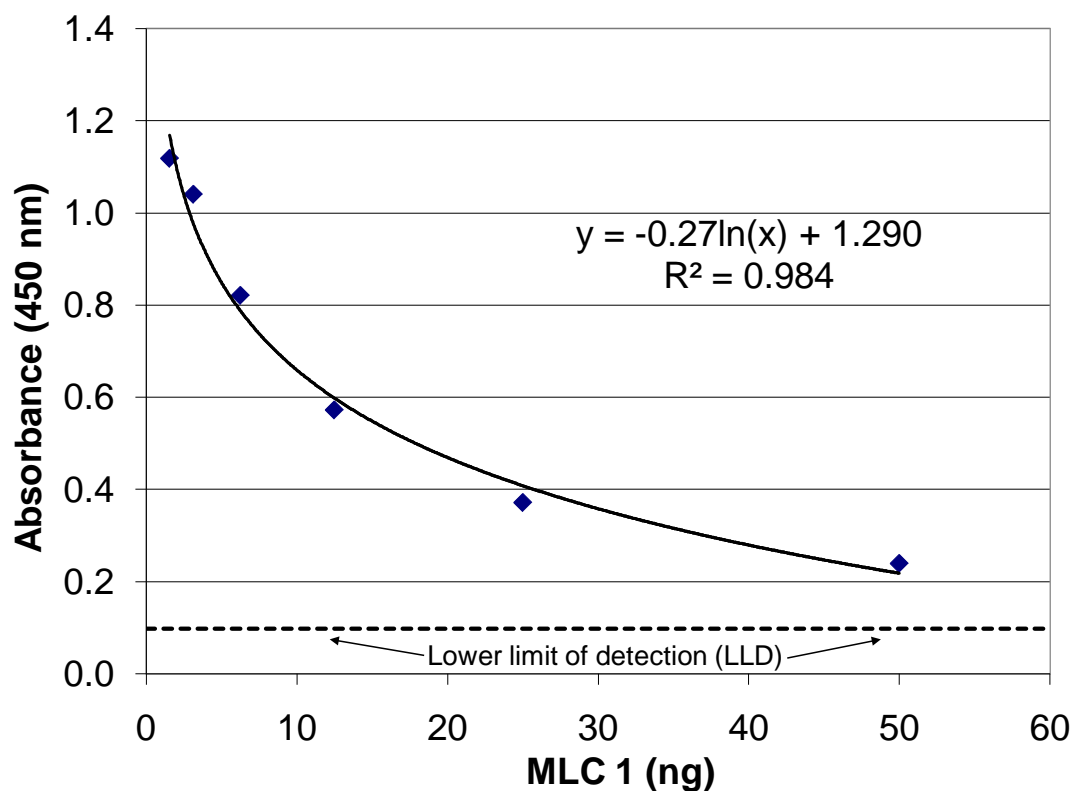


Figure 4.3 – Response of the competitive ELISA using purified MLC 1 from Alaska pollock in a serial dilution.

Our initial success using the purified MLC 1 was encouraging for our further studies using the prepared crabsticks. In order to have a relevant assay we chose to use a range of surimi content from 25% to 45% which allowed for the coverage of most common commercial crabsticks. Using the appropriate ELISA assay format was critical in creating a successful ELISA assay. Initial studies using the anti-pep-AP antibody were conducted using an indirect ELISA in which the crabstick extracts were used to coat the microtiter plate. Initial experiments were not accurate and repeatable

for quantifying the amount of MLC 1 from crabstick. This may have been due in part to the relatively low amount of MLC 1 compared to other proteins found in the crabstick. According to Reed and Park (2008), the MLC 1 of Alaska pollock makes up approximately 1% of the total protein found in surimi. When two separate antibodies are available that recognize the same target antigen a sandwich ELISA can be produced. Seiki et al. (2007) used a sandwich ELISA to determine the presence of crustacean proteins in processed food. However, their results were widely variable with a recovery range of 85 to 141% and intra- and inter-assay coefficients of variation that were 2.8 and 4.8% respectively.

Due to the lack of accuracy and repeatability of the indirect ELISA the competitive ELISA format was investigated. By purifying the MLC 1 from Alaska pollock surimi and using it as the coating protein, we were successful in designing an assay that could detect and quantify the MLC 1 from cooked and processed crabsticks. Table 4.1 shows the results of crabstick verification batches, containing 27.5, 32.5 and 42.5% of Alaska pollock surimi.

Table 4.1 – Alaska pollock surimi content estimated from verification batches using competitive ELISA

Added surimi (%)	Determined surimi (%)	Sample recovery (%) ^a
27.5	27.7	100.7
32.5	33.6	103.4
42.5	40.4	95.0

^a Sample recovery (%) and Coefficient of Variance (CV) as determined from 3 extractions done on 3 separate days in triplicate plates

The calculated % surimi was determined by dividing the determined surimi % from the standard curve by the actual surimi % added. For verification batches, the 27.5% surimi samples had a calculated % surimi content that ranged from 99.4 to 101.9% and a standard deviation of 1.2 to 1.7%. All calculated % surimi values for the 27.5% verification batch were within 2% of the actual added % surimi content value. The 32.5% samples had calculated % surimi content values that ranged from 101.0 to 104.8% and a standard deviation of 0.4 to 1.9%. All calculated % surimi values for the 32.5% verification batch were within 5% of the actual surimi content added. The 42.5% samples had calculated % surimi content values that ranged from 91.1 to 98.1% and a standard deviation of 0.7 to 1.9%. All calculated % surimi values for the 42.5% verification batch were within 9% of the actual added % surimi content

value. The overall precision of the assay was assessed by the analysis of the coefficient of variance. The CV was analyzed and reported in Table 4.2. The overall CV for all three crabstick verification batches ranged from 4.2 to 4.9%.

Table 4.2 – Inter-assay coefficient of variance^a (%) of surimi content estimated using competitive ELISA

Added surimi (%)	Inter-assay (% CV)
27.5	4.9
32.5	4.2
42.5	4.6

^a Inter-assay CVs are given as the mean of 3 extractions done on 3 separate days. Each extract from each extraction was assayed in triplicate on the same day.

4.5 CONCLUSIONS

The anti-pep-AP antibody proved to be very accurate in the qualification and quantification of Alaska pollock surimi used in crabsticks. Using the appropriate ELISA format, competitive vs. indirect, proved to be pivotal in obtaining accuracy and repeatability of the assay. The anti-pep-AP antibody worked very well for the

detection of MLC 1 from Alaska pollock surimi. However cross-reactivity was noted in the immunoblot with the MLC 1 of Pacific whiting and threadfin bream. This cross-reactivity could prove to be beneficial for future studies with possibilities of the production of a sandwich ELISA that could use one capture antibody for more than one species of fish. A competitive ELISA assay was tested and proven as an effective method for the qualification and quantification of Alaska pollock surimi used in cooked and pasteurized crabsticks. All 3 levels of Alaska pollock surimi used in the verification batches were estimated to less than 9% of the actual value of surimi content.

CHAPTER 5

5 IMMUNOLOGICAL INVESTIGATION OF RAW ALASKA POLLOCK
FILLETS FOR THE DETECTION OF MYOSIN LIGHT CHAIN 1 USING A
DIRECT SANDWICH ELISA

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5.1 ABSTRACT

Antibodies were produced by injection of a synthesized 15-amino acid peptide or by whole myosin light chain 1 isolated from Alaska pollock (AP). A direct sandwich ELISA was tested using extracts prepared from AP, Pacific whiting (PW), true cod (TC), tilapia (T), and catfish (C). Fish extracts were studied using SDS-PAGE and Western blotting. A standard curve was created for each fish and used to estimate 3 different verification samples. All estimations were within 10, 37.5, 30, 43, and 34% of the actual value for AP, PW, TC, T, and C, respectively. When one or more fish species was mixed together with AP the estimation of the Alaska pollock content became much less accurate. This study confirms a direct sandwich ELISA accurately detects the quantity of AP. Testing found the sandwich ELISA developed exhibited cross-reactivity with other protein sources such as beef, chicken, pork, shrimp, and clam.

5.2 INTRODUCTION

The ability to properly identify different species of fish has always been an important factor in the sale of seafood with proper identification becoming more and more difficult as the fish is processed. The substitution of one highly valued fish species for another has been documented and shown to have a significant impact on the consumer's buying power. It has been shown that substitution of fish can have an economic impact. In a study conducted by the FDA they found that gains in fraudulent substitutions of fish could translate to an economic gain of 24 cents a pound for pink salmon substituted as chum salmon and up to \$6.74 per pound of skate wings substituted for scallops (FDA, 2009c).

While investigating the substitution of red snapper and swordfish in major U.S. cities, Hsieh (1998) found that 6 out of 13 entrees represented as red snapper were indeed not red snapper. Of the 21 samples of swordfish 2 were found to be misrepresented as swordfish. Whether the restaurants were also victims of fraud or whether they themselves are the fraudulent party is not indicated. In another study conducted by Asensio and Samaniego (2009) the substitution of grouper and wreck fish was conducted in 24 school and university lunch rooms and 20 restaurants. The results of the testing concluded that 29 of the 44 meals that were represented as either grouper or wreck fish were indeed not.

In order to protect the consumer from the fraudulent substitution of one fish species for another it is important to find a method that can be used for fish species identification. A number of methods have been studied in an attempt to provide a

convenient method for fish species identification. Isoelectric focusing (IEF) is an electrophoretic technique that takes advantage of the characteristic pI of individual proteins. When using IEF a pH gradient is created between the anode and the cathode by allowing a mixture of low molecular weight organic acids and bases, known as ampholytes, to distribute themselves in an electric field that is generated across the gel (Gaál et al., 1980). The Regulatory Fish Encyclopedia (FDA, 2009a) contains standardized isoelectric focus plates for 94 different commercially important fish species for North America. In 1990 the Association of Official Analytical Chemists adopted IEF as the official method for raw fish species identification (AOAC, 1990). Hsieh et al. (1997; 1998) demonstrated the ability for species identification using IEF for the commercially important fish species of red snapper (*Lutjanus cumpechanus*). Using isoelectric focusing Hsieh et al. (1995) tested 121 retail market snapper fillets and were tested for compliance with labeling regulations. Samples were tested against 12 authentic snapper species. They found that of the 81 samples labeled as red snapper only 24 (30%) were confirmed through IEF to be true red snapper. This meant that 57 (70%) of the fillet samples labeled as red snapper were actually other fish species.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has also been used to test for fish species identification. Reed et al. (2008) used SDS-PAGE to separate the proteins isolated from crabsticks made using Alaska pollock and Pacific whiting surimi. In their study they were able to identify the samples that contained either one species or the other or a mixture of the two fish species.

However, due to the overlapping molecular weight of ovalbumin, which is a major protein found in egg white, and actin the efforts to quantify all protein sources in the crabstick were slightly hampered. In a collaborative study performed in Europe Piñeiro et al. (1999) worked on a method to produce a standard of reference for a number of fish and shellfish species. One of the breakthroughs that were used for the production of the standard of reference was to use SDS in the extraction solution which allowed for a more complete extraction of proteins. However even with a more completely extracted protein a major shortfall in using SDS-PAGE for identification is the vast number of fish species that are commonly used as a food source. The large number of fish species will inevitably lead to SDS-PAGE protein patterns that have overlapping bands which will not allow for absolute determination of fish species.

Due to the limitations and pitfalls that are included in using isoelectric focusing and SDS-PAGE electrophoretic techniques, other methods for fish species identification have been pursued. One major avenue of investigation is the use of ELISA techniques that use species specific antibodies for not only the identification but also the quantification of fish protein used in a particular product (Reed et al., 2010b).

Enzyme linked immunosorbent assay (ELISA) utilizes the interaction between antigens and antibodies and can be used to identify fish species (Asensio et al., 2003a; Cespedes, 1999). ELISA assays are extremely sensitive, with detection limits down to the nanogram level. Huang and others (1995) were able to identify red snapper (*Lutianus cumpechanus*) using monoclonal antibodies. Asensio and others (2003b)

were able to unequivocally identify grouper (*Epinephelus guaza*), wreck fish (*Polyprion americanus*), and Nile perch (*Lates niloticus*) fillets using an indirect ELISA using polyclonal antibodies that were raised against species specific proteins. ELISA has also been used for quantification of a variety of food products including fish and fish products (Medina, 1988; Ochiai et al., 2001; Panheleux et al., 2000; Vidal et al., 2005; Werner et al., 2007).

The overall goal of this study was to develop a direct sandwich ELISA for the detection of myosin light chain 1 from Alaska pollock. The detection of the myosin light chain 1 from Alaska pollock would allow for the qualification and quantification of the Alaska pollock fillets when tested against other fish species. This would greatly benefit US Alaska pollock fisheries by protecting them against species substitutions. In addition, US fisheries would be able to maintain the proper use and identification of Alaska pollock.

5.3 MATERIALS AND METHODS

Food products and chemicals

Alaska pollock (*Theragra chalcogramma*) whole fish was donated and received frozen (UniSea Inc., Dutch Harbor, Alaska, U.S.A.). Fresh Pacific whiting (*Merluccius productus*) was donated by Da Yang Seafoods (Astoria, Ore., U.S.A.). Fresh Pacific cod (*Gadus macrocephalus*) along with frozen fillets of tilapia (*spp*) and catfish (*Ictalurus punctatus*) were donated by Pacific Seafood Group (Warrenton, Ore, U.S.A.). Beef, chicken, pork, shrimp, and steamer clams were purchased at a local

supermarket. All samples were filleted, portioned, vacuum packed, and stored frozen at -80 °C until used. All other chemicals were reagent grade.

Purification of MLC 1 from Alaska pollock surimi

SDS-PAGE was performed as outlined by Laemmli (1970) using a 12.5% (stock solution of 29.2% acrylamide and 0.8% bis-acrylamide) separating gel and a 4% stacking gel (140 x 120 x 1.5 mm, w x h x thickness) using a Fisher Biotech Protein Electrophoresis System (FB-VE16-1, Fisher Scientific, Pittsburgh, Pa., U.S.A.). Alaska pollock sample (800 μ L of 3.75 mg/mL) was mixed with 200 μ L of Laemmli sample buffer (0.6 mL 1 M tris-HCl (pH 6.8), 5.0 mL glycerol, 2.0 mL 10% (w/v) SDS, 0.5 mL β -mercaptoethanol, 1.0 mL 1% (w/v) bromophenol blue, 0.9 mL dd H₂O). Sample and buffer were vortexed for 5 sec and then heated at 90 °C for 3 min in order for the β -mercaptoethanol in the sample buffer to disrupt any disulfide bonds. A 15 well comb was used and 30 μ L of Alaska pollock (AP) sample was added wells 2 – 14, with 10 μ L of Kaleidoscope™ protein standard (Bio-Rad, Hercules, Calif., U.S.A.) in wells 1 and 15. Gels were electrophoresed until the dye front reached the bottom of the gel. Four gels with the identical setup were run at the same time using two FB-VE16-1 electrophoresis units. Gels were then removed from the plates and the gel portion containing the MLC 1 was excised. This portion was determined by a previously stained gel and we found that MLC 1 was the sole protein in the molecular weight range of 20 to 25 kDa. The Kaleidoscope™ standard uses pre-stained proteins and contains two markers at 20 and 25 kDa allowing for the

precise and repeatable excision of the MLC1 from Alaska pollock surimi. The excised gel portions were placed in a 50 mL falcon tube along with 30 mL of phosphate buffered saline (PBS) and shipped to Antagene, Inc. (Sunnyvale, Calif., U.S.A.).

Immunization protocol

Two sets of polyclonal antibodies (Pab) were produced by Antagene, Inc. (Sunnyvale, Calif., U.S.A.). The first Pab (T382) was done using a 15 amino acid portion of the published DNA nucleotide sequence of the myosin light chain 1 (MLC1) of Alaska pollock (Ala-Pro-Lys-Lys-Asp-Val-Lys-Ala-Pro- Ala-Ala-Ala-Ala-Lys-Lys). Data of total DNA nucleotide sequence is registered to the DDBJ/EMBL/GenBank DNA databases with accession number of AB042054. The second Pab (T435) was produced using the entire MLC1 from Alaska pollock that was purified by separation using SDS-PAGE, as previously outlined. Two New Zealand white rabbits were immunized with keyhole limpet haemocyanin (KLH) conjugated peptide or purified MLC1 in Complete Freund's Adjuvant (CFA) with all subsequent injections using Incomplete Freund's Adjuvant (IFA). The immunogen was diluted to 1 mL with sterile saline and combined with 1 mL of the appropriate adjuvant. The antigen and adjuvant were then mixed to form a stable emulsion that was injected subcutaneously and provided enhanced immune response from the sustained presence of the immunogen. Day 1: 1st immunization with antigen in CFA. Day 20: 2nd immunization with antigen in IFA. Day 40: 3rd immunization with antigen in IFA. Day 60: 4th immunization with antigen in IFA. Day 70: final total bleed 50 mL from

each rabbit. Blood was collected from the central ear artery with a 19 gauge needle and allowed to clot and retract at 37°C overnight. The clotted blood was then refrigerated for 24 h before the serum was decanted and clarified by centrifugation at 2,500 rpm for 20 min.

Antibodies

One hundred mL of rabbit antisera, from each rabbit, were delivered from Antagene, Inc. and affinity purified using a Pierce[®] Chromatography Cartridge Melon[™] Gel Kit (Pierce, Rockford, Ill, U.S.A.) according to the manufacturers' specification. Protein content of affinity purified T382 and T435 antibodies were determined using the Lowry total protein determination method (Lowry et al., 1951) and then adjusted to a final concentration of 1 mg/mL using dd H₂O and placed in 100 µL aliquots and stored frozen at -80 °C until needed. All antibodies were stored frozen at a concentration of 1 mg/mL. Both Pabs were produced in rabbit so the use of a commercially produced anti-rabbit IgG antibody coupled to horse radish peroxidase (HRP) was not possible. A commercially available kit (EZ-Link[®] Plus Activated Peroxidase, Pierce, Rockford, Ill, U.S.A.) was used to couple HRP to the T435 (HRP-T435) antibody according to manufacturer's instructions. The un-reacted HRP was removed using a Vivaspin 20 (Sartorius Stedim Biotech, Goettingen, Germany) protein concentration tube with a molecular weight cutoff at 100 kDa. The purified HRP-T435 was adjusted to a final protein concentration of \approx 1 mg/mL and stored in 10 µL aliquots at -80 °C until used.

Sample preparation and protein extraction

Standard curves were prepared for Alaska pollock (AP), Pacific whiting (PW), true cod (TC), catfish (C), and tilapia (T). The standard was created by weighing out five samples of 1, 2, 3, 4, and 5 grams for each individual fish species. The samples were mixed with 20 mL of 5% sodium dodecyl sulfate (SDS) (w/v) and 0.5% β -mercaptoethanol (β ME) (v/v) and homogenized (Powergen 700, Fisher Scientific, Pittsburgh, Pa., U.S.A.) followed by incubation at 90 °C for 1 h. Incubated samples were then centrifuged at 17,000 x g (Sorvall RC-5B, DuPont Co., Newton, Conn., U.S.A.) for 20 min. The supernatant was then poured into a 25 mL volumetric flask and brought to volume using additional SDS- β ME solution. Three additional samples weighing 2.5, 3.5, and 4.5 g were used for quantification using the standard curve and were prepared in the same manner. Cross reactivity testing was performed using beef, chicken, pork, shrimp, and clams. Five gram samples were taken from these sources and homogenized and extracted in the same manner. For mixed species verification testing the Alaska pollock standard curve was used but verification samples were prepared by weighing out samples and extracting them in the same manner as outlined before as outlined in table below.

Table 5.1 – Experimental outline for mixed species

	#1	#2	#3	#4	#5	#6
Alaska pollock	0.0	1.5	2.0	0.0	1.5	2.0
Tilapia	3.0	1.5	1.0	1.5	0.8	0.5
Catfish	-	-	-	1.5	0.8	0.5

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as outlined by Laemmli (1970). Eight hundred μL of 1.25 mg/mL concentration of extracted sample were mixed with 200 μL of Laemmli sample buffer (0.6 mL 1 M tris-HCl (pH 6.8), 5.0 mL glycerol, 2.0 mL 10% (w/v) SDS, 0.5 mL β -mercaptoethanol, 1.0 mL 1% (w/v) bromophenol blue, 0.9 mL dd H₂O). Sample and buffer were vortexed and then heated at 90 °C for 3 min in order for the β -mercaptoethanol in the sample buffer to disrupt any disulfide bonds. Samples were electrophoresed using a Mini-Protean III Cell (Bio-Rad, Hercules, Calif., U.S.A.).

Immunoblotting

SDS-PAGE was performed as previously described. After electrophoresis the gels were removed and equilibrated in Towbin solution (25 mM Tris, 192 mM glycine, 20% methanol (v/v) at pH 8.3) for 20 min and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, Calif., U.S.A.) using a Mini Trans-Blot[®] Electrophoretic Transfer Cell (Bio-Rad, Hercules, Calif., U.S.A.) using a slight modification of an earlier published method (Towbin et al., 1979). The membrane

was blocked using Superblock[®] blocking buffer in PBS with 0.05% Tween 20 (Thermo Scientific, Rockford, Ill, U.S.A.) at 25 °C for 30 min. The blocked membrane was washed 3 times for 5 min using PBS-T. The membrane was incubated with either T382 or T435 (1:60) at 25 °C for 60 min after which the membrane was washed 3 times. The membrane was incubated with HRP-anti-rabbit-IgG (1:15,000) at 25 °C for 60 min followed by 3 additional wash cycles. Bands were visualized using 1-Step[™] TMB-Blotting (Thermo Scientific, Rockford, Ill, U.S.A.). Development was stopped by the addition of dd water. Molecular weight was determined by comparison to the Kaleidoscope[™] protein standard (Bio-Rad, Hercules, Calif., U.S.A.) also run in each SDS-PAGE.

Direct sandwich ELISA

A direct sandwich ELISA was created and used for all samples. Polystyrene 96 well microtiter plates (Nalge Nunc International, Rochester, N.Y., U.S.A.) were coated with 100 μ L of 25 μ g/mL T382 diluted using PBS, containing 137mM NaCl, 2.7mM KCl, 10mM phosphate buffer (Omnipur 10x PBS Concentrate, EMD Chemicals Inc., Gibbstown, N.J., U.S.A.) and were incubated overnight at 4 °C. The plates were washed 3 times with PBS containing 0.5% (v/v) Tween 20 (PBS-T), each washing step was performed the same. After washing the plates were blocked with 100 μ L of Superblock[®] blocking buffer in PBS with 0.05% Tween 20 (Thermo Scientific, Rockford, Ill, U.S.A.) at 37 °C for 30 min. The plates were washed 3 times and then 100 μ L of 100x serially diluted sample was added. Six wells per sample

were used. For each extraction three plates were used with the same setup allowing for statistical comparison. The plates containing the sample were incubated at 37 °C for 1 h. Following incubation the plates were washed and 100 µL of 1:1000 dilution of HRP-T435 antibody was added and incubated at 37 °C for 30 min. The plates were then washed a final time and 100 µL of 3,3',5,5'-tetramethylbenzidine, 1-Step Ultra TMB ELISA (Thermo Scientific, Rockford, Ill, U.S.A.) was added and allowed to develop at room temperature for 15 min. Color development was stopped by the addition of 100 µL of 2 N sulfuric acid per well. The plate was then read at 450 nm using a Bio-Tek EL312 plate reader (Bio-Tek Instruments, Inc., Winooski, Verm, U.S.A.).

Statistical methods

For determination of the inter-assay precision, the mean coefficients of variation (CV) were obtained based on the extractions performed and completed on three separate plates. All calculations were done using Microsoft Excel (Microsoft Corporation, Redmond, Wash., U.S.A.). Each assay contained 6 replicates of each sample. The lower limit of detection (LLD) for the direct sandwich ELISA assay was given as 3 times the standard deviation plus the background of blank wells.

5.4 RESULTS AND DISCUSSION

Direct sandwich ELISA

In order to test the direct sandwich ELISA (ds-ELISA) five fish species were used to identify the viability and accuracy of the assay with each fish. Figure 5.1 shows the results from the testing of Alaska pollock where a standard curve was created with a R^2 value of 0.991. The assay accurately calculated the value for the 2.5, 3.5, and 4.5 g verification samples at 2.6 ± 0.19 , 3.8 ± 0.10 , and 4.5 ± 0.03 g, respectively. These results showed that the ds-ELISA worked well for Alaska pollock and gave reliable results with values all within 10% of the actual value of Alaska pollock. The assay also shows that the protein extraction method consistently extracts the maximum amount of protein, providing repeatability of the assay.

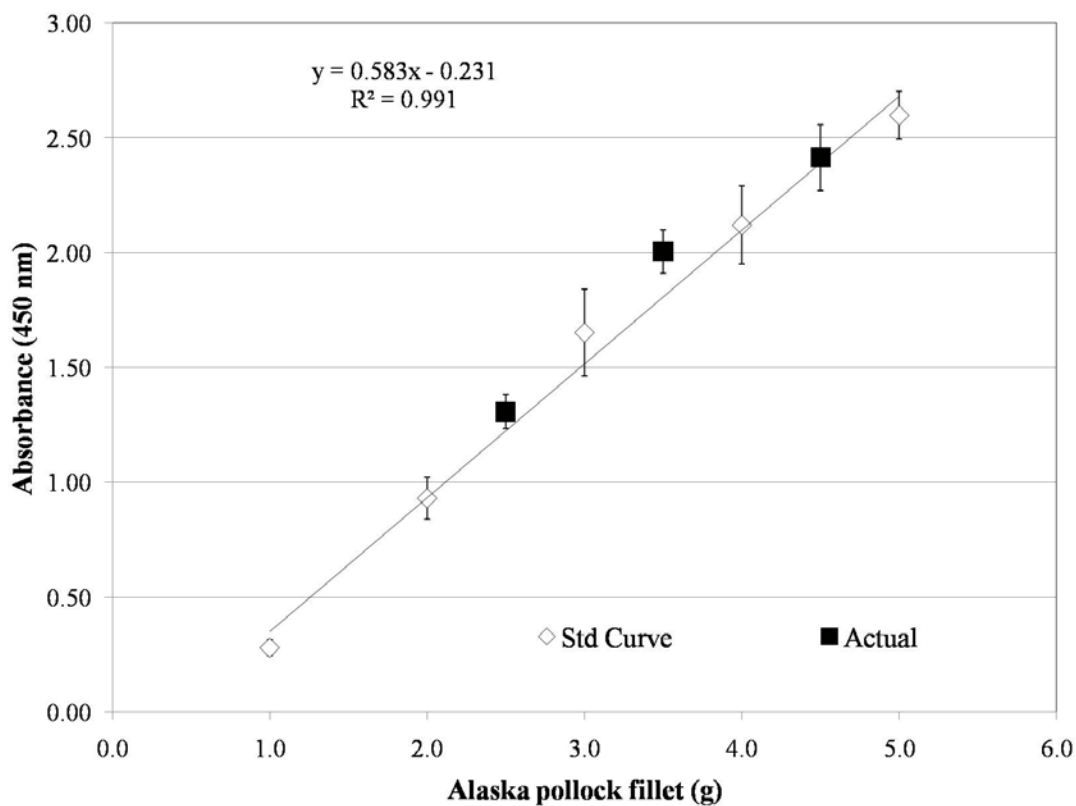


Figure 5.1 – Standard curve of Alaska pollock using ds-ELISA.

Further investigation to the usefulness of the ds-ELISA was performed using Pacific whiting (PW), true cod (TC), tilapia (T), and catfish (C). When these four species were tested for possible use it was shown that the different species did not perform in the same manner as the Alaska pollock. Figure 5.2 shows the results of the standard curve as tested on the four fish species.

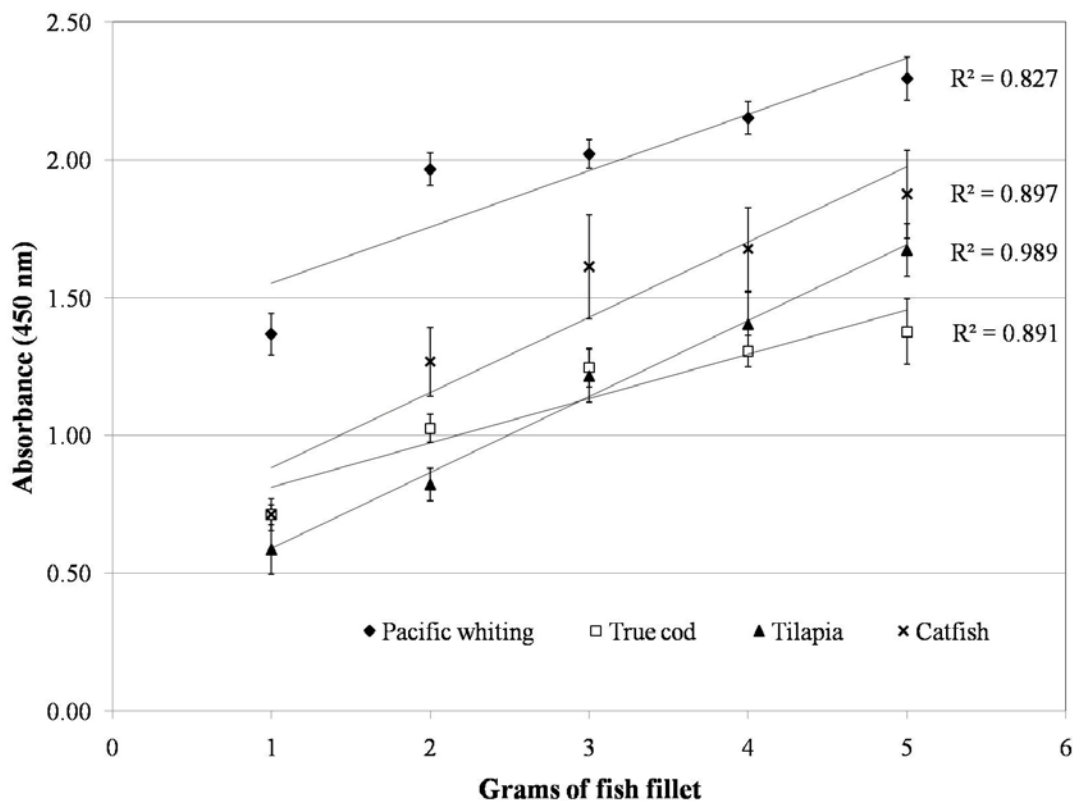


Figure 5.2– Standard curve for Pacific whiting, true cod, tilapia, and catfish using the ds-ELISA

The standard curves created for PW, TC, T, and C were found to have R^2 values of 0.827, 0.891, 0.989, and 0.897, respectively. However the estimated values for the verification samples covered a wide range and were over estimated for all samples and ranged from 92.4 to 142.5% of the actual value. The lack in accuracy for the other fish species may be due in part to the nature of the antibodies. The antibodies for the assay were created specifically for Alaska pollock and it stands to reason that they would recognize the Alaska pollock more readily. However it appears that there are some amino acid sequences in all of the fish species that allow for antibody recognition. The wide range of variability in the assay for fish species,

other than Alaska pollock, indicates that the affinity of the antibody for other proteins may not be as high for that of Alaska pollock. Figure 5.3 shows the results of the estimated values of the verification batches for AP, PW, TC, T, and C. Using Alaska pollock as the gold standard for assay performance, it can clearly be seen there is a wide variation in the estimated values of verification batches as well as the standard deviations of the samples.

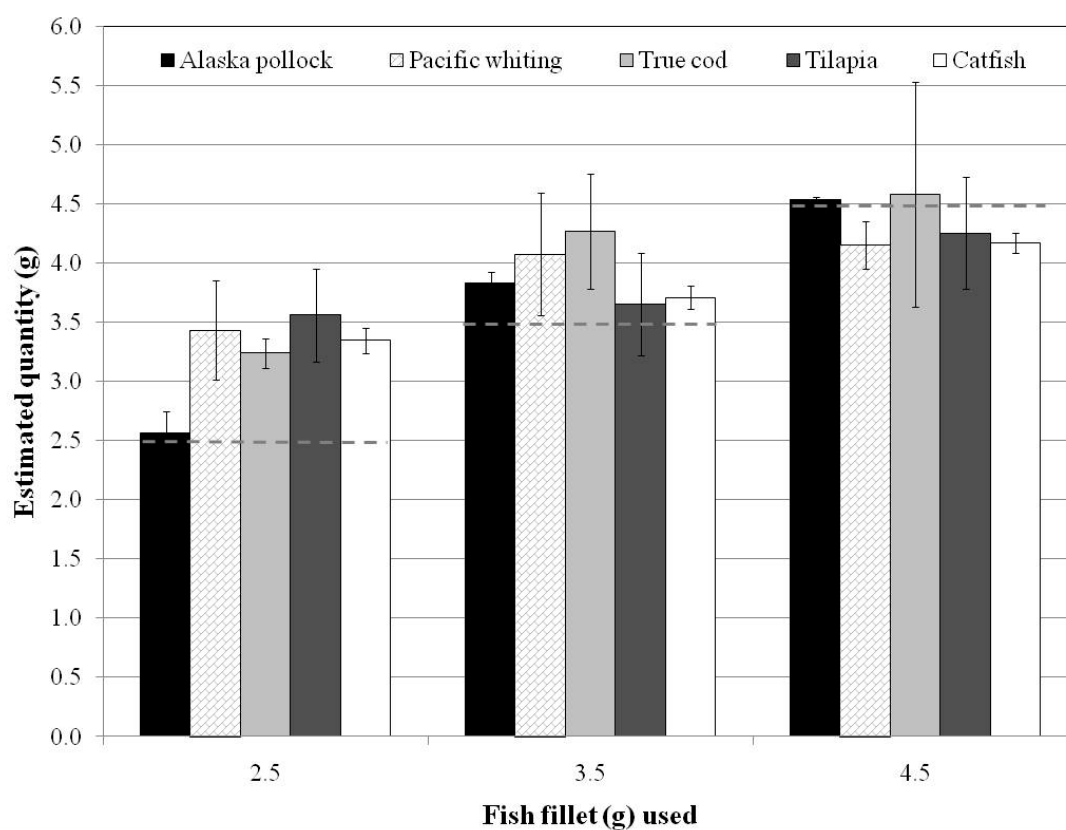


Figure 5.3 – Results of verification samples using corresponding individual standard curves. (Dashed line represents the actual grams of fish used in extraction and tested.)

In order to further understand why the ds-ELISA was detecting other fish species when the antibody was raised against the MLC 1 from Alaska pollock, the fish

samples were investigated using SDS-PAGE and immunoblotting. Figure 5.4 shows the results of the SDS-PAGE and immunoblotting experiments, and it is clearly seen that the Alaska pollock MLC 1 antibody is cross-reacting with a wide variety of proteins in all fish species tested including the MLC 1 of Alaska pollock

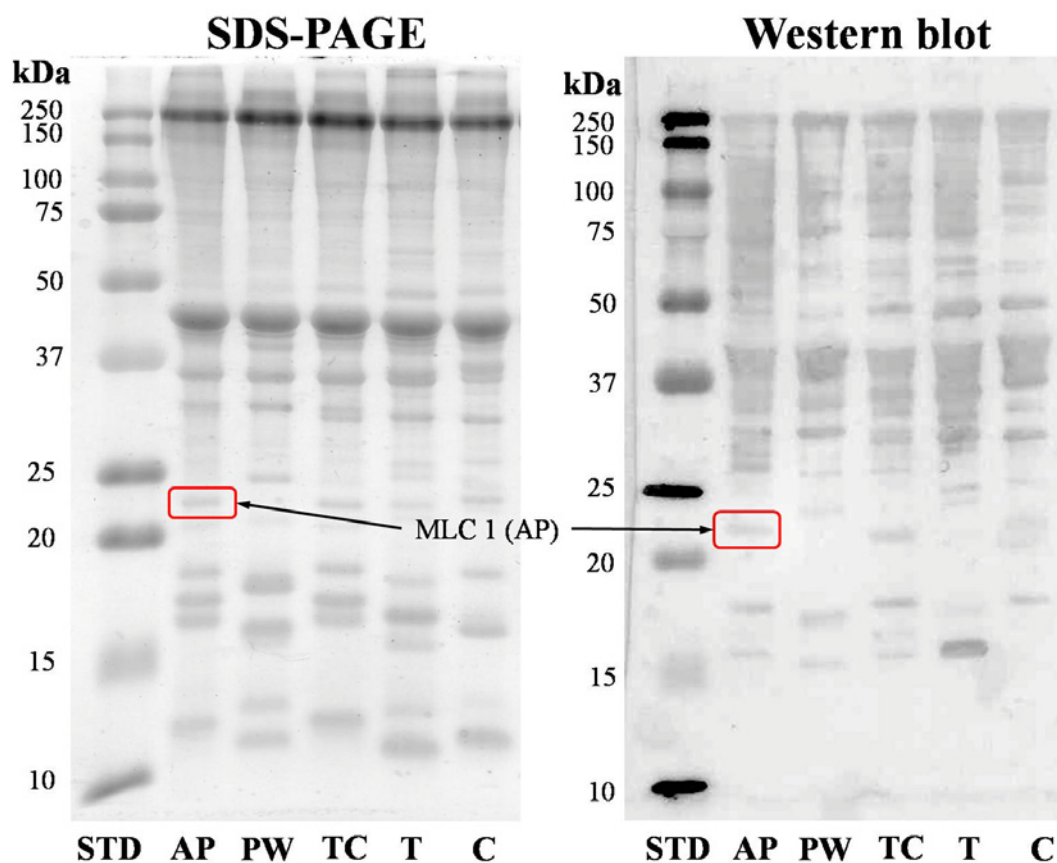
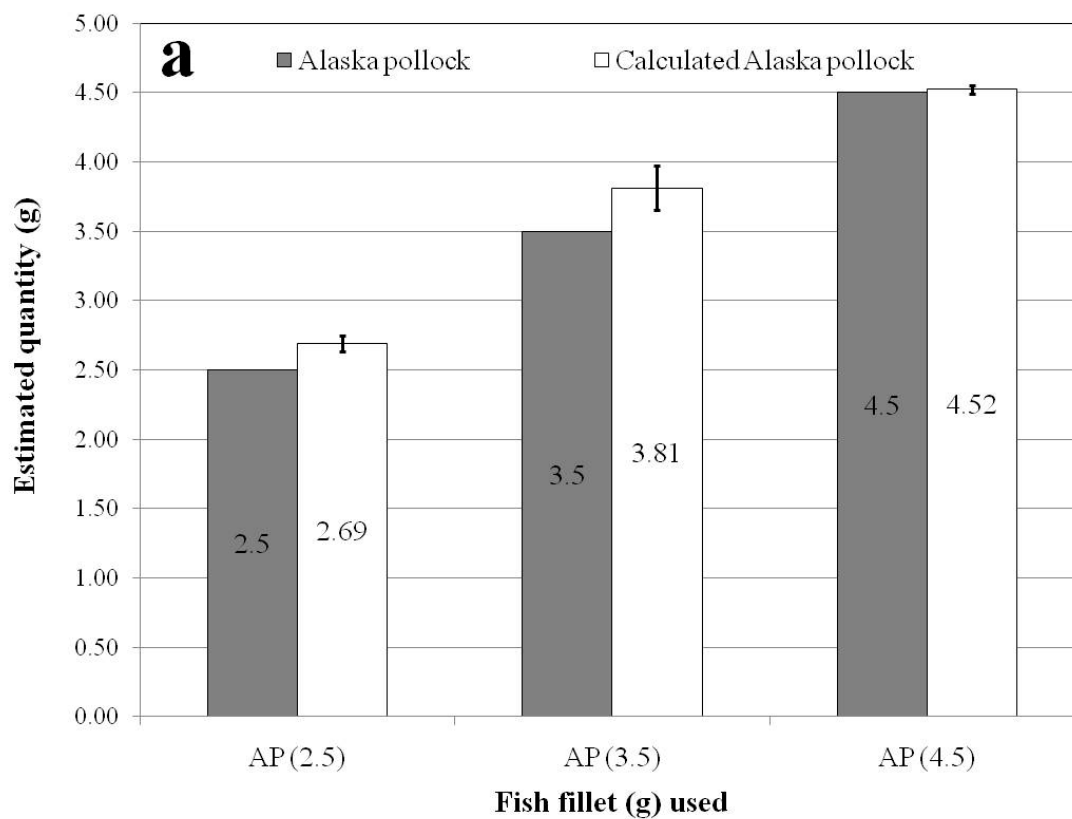
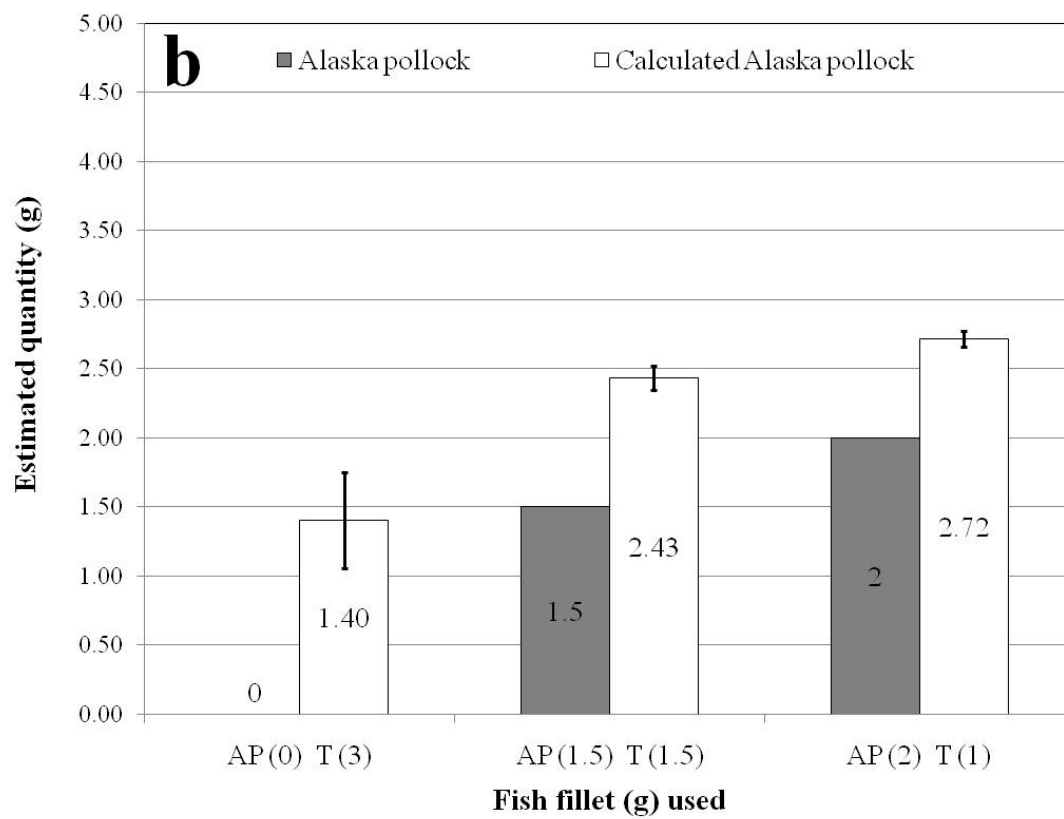


Figure 5.4 – Comparison of SDS-PAGE and western blot stained protein patterns. Kaleidoscope standard (STD), Alaska pollock (AP), Pacific whiting (PW), true cod (TC), and catfish (C)

Due to the cross-reactivity of the T382 and HRP-T435 antibodies used in ds-ELISA with all five fish species, a different approach was taken which used Alaska pollock mixed with other species in an attempt to quantify AP only. When Alaska

pollock fish was used alone and estimated using a standard curve of Alaska pollock fish the estimations were all within 10% of the actual value. When the assay was performed using Alaska pollock for the production of the standard curve, the sample that contained only tilapia showed an estimated 1.4 g of Alaska pollock. Similar trends were found for other samples containing Alaska pollock with tilapia (1.5:1.5, and 2.0:1.0; AP:T) and gave estimated values of 2.43 and 2.72 g, respectively. For the samples that contained Alaska pollock, tilapia, and catfish (AP:T:C) a similar trend of over estimation was followed. Figure 5.5 a-c show the results of the sample estimation of mixed samples.





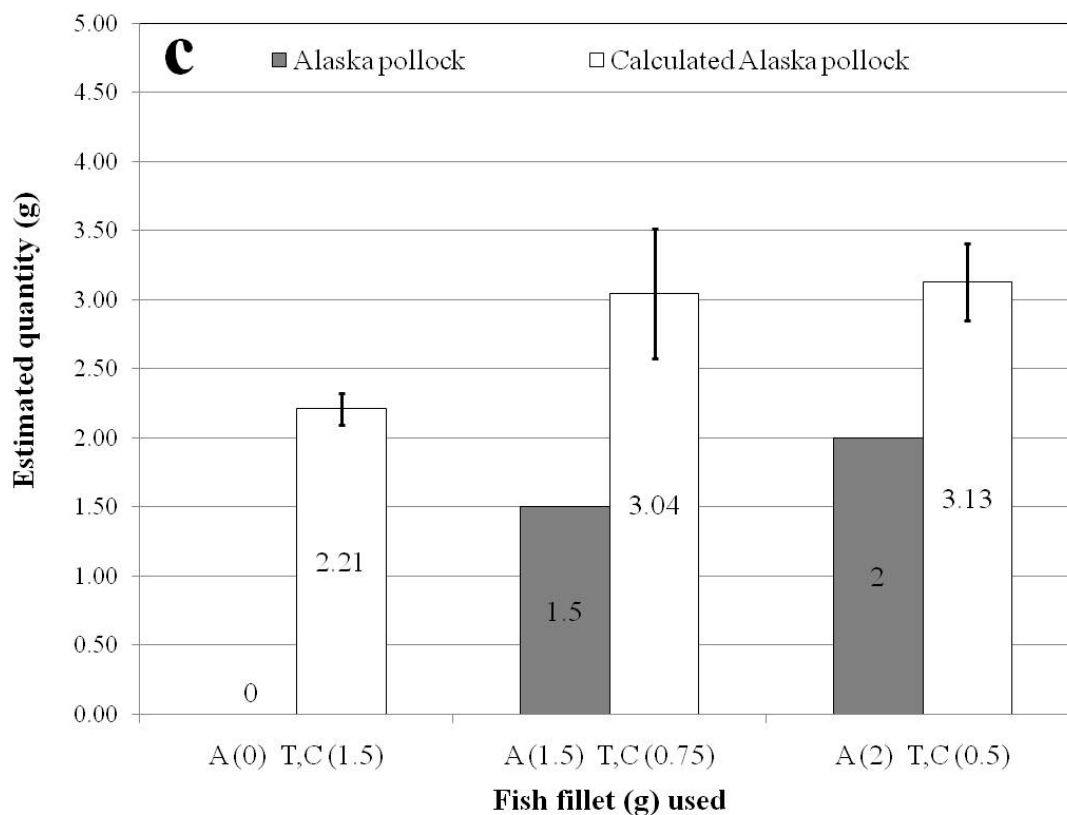


Figure 5.5 – Estimated amount of Alaska pollock fish (g) using an Alaska pollock standard curve and ds-ELISA. (a) Alaska pollock only, (b) Alaska pollock and tilapia, (c) Alaska pollock, tilapia, and catfish.

Our objective was to develop a ds-ELISA that could be used to identify and quantify Alaska pollock in its raw, cooked, and or processed forms to help protect the unlawful substitution of Alaska pollock with other lower cost fish species. However, as our study progressed we found that the Alaska pollock MLC 1 antibodies, T382 and HRP-T435, in the ds-ELISA format were prone to extremely high degrees of cross reactivity with other fish species. In 2003 Nature reported that 300 scientists from 53 countries have to date identified 20,000 different species of fish in the world's seas

and oceans (Mason, 2003). In addition to the large number of fish species found in the world's oceans, the world's fresh bodies of water hold between 9,000 and 25,000 different fish species (Cosgrove et al., 2000). The overall diversity of the world's fishes complicates the identification of each species, even more so when the fish is an important food source that is known throughout the world by different common names.

Due to the high cross reactivity of the Alaska pollock MLC 1 antibodies, we began to think that the antibodies and the ds-ELISA test could be used to differentiate fish protein from other species of animal proteins, such as beef, chicken, pork, shrimp, clams, and crab. ELISA kits that can be used for qualitative tests for the detection of beef, pork, poultry, and sheep in foods/feeds are available commercially from companies such as Neogen (Neogen Corporation, Lexington, Ky., U.S.A.). Extensive research has also been done for the identification of protein sources in food such as pork (Chen & Hsieh, 2000; Liu, Chen, Dorsey & Hsieh, 2006), raw and heat treated meats (Chen, Hsieh & Bridgman, 2002a; Hsieh, Sheu & Bridgman, 1998), chicken (Martin, Wardale, Jones, Hernandez & Patterson, 1989; Sheu & Hsieh, 1998), and beef (Hsieh, Zhang, Chen & Sheu, 2002).

To test for possible cross reaction with other animal protein sources (Hsieh, 2009) we performed the ds-ELISA using beef, chicken, pork, shrimp, crab, and clams along with six fish species, namely Alaska pollock, Pacific whiting, true cod, tilapia, catfish, yellowfin. In our test we used the average plus three times the standard deviation value of 12 blank wells as the cutoff for positive cross reactivity. Figure 5.6

shows the results of the ds-ELISA. All of the animal protein samples tested positive, thus eliminating the possibility that the Alaska pollock MLC 1 antibodies and ds-ELISA could be used to differentiate between animal protein sources.

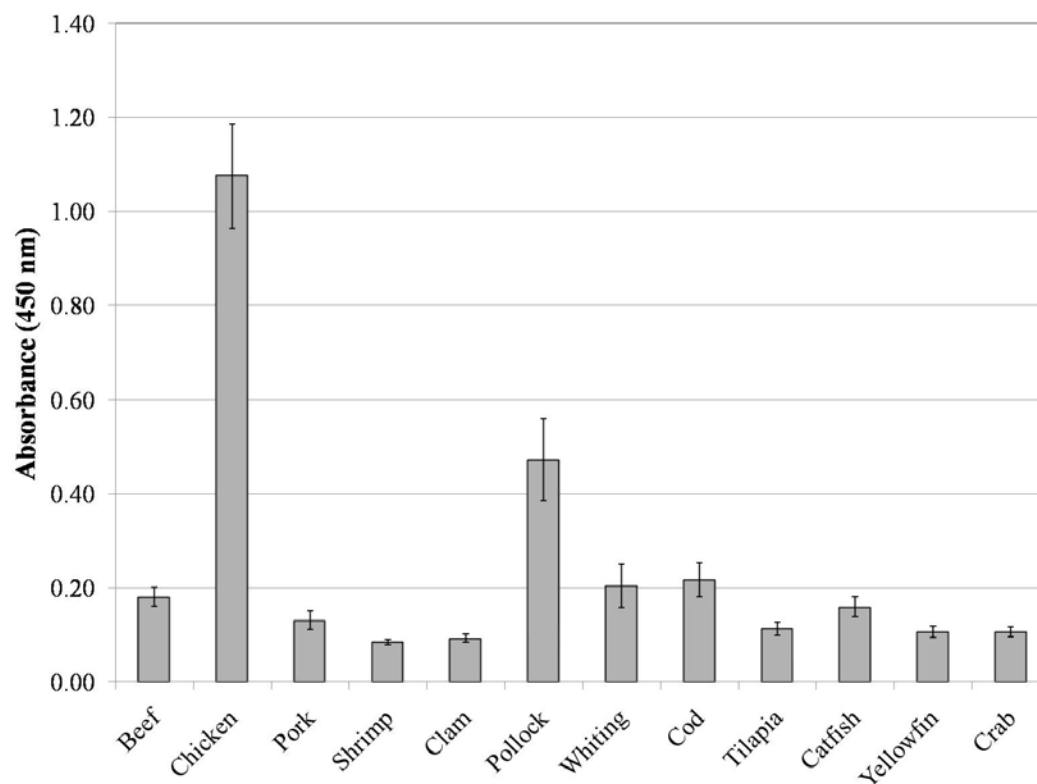


Figure 5.6 – ds-ELISA results for protein source cross reactivity.

5.5 CONCLUSIONS

Using a direct sandwich ELISA for the qualification and quantification Alaska pollock raw fish fillets provided good estimation of Alaska pollock used in the verification sample. However, the Alaska pollock MLC 1 antibodies exhibited high levels of cross reactivity with other fish species. When individual species were tested

for the possibility of using each species as its own standard curve, each of the other fish species exhibited a high degree of variability and a low degree of accuracy. Using the Alaska pollock standard curve to quantify the amount of Alaska pollock in a mixture of Alaska pollock, tilapia and catfish also had extremes in variability and accuracy proving unsuitable for use as a method to determine the amount of Alaska pollock in a mixture of fish. Expanding the scope of the study to test for the possibility of the MLC 1 antibodies being specific for fish only and not other animal species testing was done to check for cross-reactivity with a variety of animal species. Testing determined that the antibodies had sufficient cross-reactivity to rule out the possibility of using the MLC 1 ds-ELISA for differentiation between animal protein sources.

Acknowledgement

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CHAPTER 6

RHEOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF SALMON
MYOSIN AS AFFECTED BY LINEAR HEATING

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6.1 ABSTRACT

Purified Chinook salmon myosin was studied using SDS-PAGE and densitometric analysis to determine its purity (approximately 94%). Myosin subjected to linear heating began to form aggregates at > 24 °C as measured by turbidity at 320 nm. Conformational changes, as measured by surface hydrophobicity (S_o), began at 18.5 °C and continued to increase up to 75 °C after which it decreased slightly. Total sulfhydryl content (TSH) remained steady from 18.5 to 50 °C after which point the TSH began to drop. Surface reactive sulfhydryl groups (SRSH) gradually increased as the temperature increased from 18.5 to 55 °C and then followed a similar trend as TSH decreased. Presumably disulfide bond started to be formed at around 50-55°C. Differential scanning calorimetry showed four peaks, three endothermic (27.9, 36.0, 45.5 °C) and one exothermic (49.0 °C). Dynamic rheological measurements provided information concerning the gelation point of salmon myosin which was 31.1 °C as samples were heated at a rate of 2 °C/min.

6.2 INTRODUCTION

Myosin is a large asymmetric molecule that has a long α -helical coiled-coil tail and two globular heads with an approximate weight of 500 kDa (Hodge et al., 2000). The basic body plan of myosin consists of an N-terminal head or motor domain, a light chain-binding neck domain, and a class conserved, C-terminal tail domain. In addition, myosin has been categorized into over twenty different classes (Mooseker et al., 2008). The head or motor domain has a core sequence that is highly conserved in the myosin classes, and this region of myosin contains the ATPase active site (Holmes, 2008). The neck region, also known as the lever arm, consists of a long α -helix of variable length and a tail region that is extremely variable in sequence, length, domain composition, and organization.

Myosin is the major muscle protein that is found in fish and comprises approximately 55-60% of the myofibrillar proteins (Lanier et al., 2005). Skeletal myosin can be broken up into six polypeptide chains, two heavy chains and four light chains. Myosin light chains typically range from 17 to 25 kDa. There are two types of myosin light chains, the regulatory light chains, which are known for their role in phosphorylation and dephosphorylation (Sobieszek, 1988). The regulatory light chains can be selectively dissociated from myosin by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) thus they are often referred to as the DTNB light chains (Wagner, 1982). The role of this phosphorylation and dephosphorylation corresponds to the contraction and relaxation of smooth muscle. The essential light chains are often referred to as alkali light chains because they are dissociated from myosin under alkaline conditions

(Weeds et al., 1977). Each myosin head unit contains one DTNB subunit and one alkali unit. This means that there are four myosin light chains per myosin molecule. However, it is interesting to note that when whole myosin is analyzed using SDS-PAGE techniques, there are typically only three bands found for the myosin light chains. This occurs because each head unit has one light chain 2 (LC 2), the DTNB light chain, and then one essential light chain, which can be either the light chain 1 or the light chain 3 (Bechtel, 1986). Due to the repetition of the LC2 in both head units the SDS-PAGE protein pattern shows only three myosin light chains. These amino acid chains are non-covalently attached to the myosin head (Lanier et al., 2005).

Myofibrillar proteins play a major role in the gelling properties of muscle foods (Nakayama & Sato, 1971a; Nakayama & Sato, 1971b) and myosin is the most abundant protein found in myofibrillar proteins (Lanier et al., 2005). Myofibrillar proteins are the major component of surimi. Surimi is produced through heading, gutting, mincing, washing, dewatering and concentrating the fish myofibrillar proteins (Lee, 1984; Park et al., 2005a). The concentrated myofibrillar protein is mixed with cryoprotectants, such as sugar, sorbitol, and polyphosphates which serve to stabilize the fish myofibrillar protein during frozen storage (Park et al., 1987; Park et al., 1988). This concentrated and stabilized myofibrillar protein is used for surimi seafood products because of its excellent gelling ability.

In order to better understand the important role that myosin plays in the gelation of myofibrillar proteins, extensive studies have been performed on purified myosin from a variety of fish species. Myosin from many fish species used in the production

of surimi has been characterized such as Alaska pollock (Fukushima et al., 2003; Fukushima, Satoh, Yoon, Togashi, Nakaya & Watabe, 2005), Pacific whiting (Yongsawatdigul et al., 1999), cod (Brenner, Johannsson & Nicolai, 2009; Kristinsson & Hultin, 2003), threadfin bream (Hemung, Yongsawatdigul & Li-Chan, 2008; Toyohara & Shimizu, 1988; Yongsawatdigul et al., 2003). Due to the importance of myosin it has also been studied in many other fish that are not used specifically for surimi, such as chum salmon, herring, carp, Japanese stingfish, Pacific sardine, and catfish (Bouraoui, Fichtali, Pinder, Nakai & Bowen, 1997; Chan et al., 1993; Iwami, Ojima, Inoue & Nishita, 2002; Kakinuma et al., 1998; Nagai et al., 1999; Park et al., 2008; Raghavan & Kristinsson, 2008).

By studying physical and biochemical components of myosin, a better understanding of the gelling capability of myosin from a specific species is possible. Takahashi, Yamamoto, Kato, and Konno (2005) studied myosin from carp, rainbow trout, tilapia, yellowtail, pink salmon, brown sole, witch flounder, atka mackerel, and walleye pollock. They found in their study that the characteristics of myosin were species-specific and highly dependent upon the habitat of the fish.

In this study, we endeavored to elucidate the key characteristics of Chinook salmon myosin as affected by linear heating. Purified myosin was linearly heated in order to understand the gelling ability and determine the gelation point. Along with the gelling ability, some of the characteristics of myosin, such as surface hydrophobicity, surface reactive sulfhydryl content, and total sulfhydryl content, were

also investigated to determine the role that heat treatment plays on the thermal denaturation of salmon myosin.

6.3 MATERIALS AND METHODS

Fish

Chinook salmon (*Oncorhynchus tshawytscha*; body length: 16.9 ± 3.2 cm; weight: 39.9 ± 3.2 g) were obtained from Clatsop County Fisheries (Astoria, Ore., U.S.A.). Salmon were delivered live from the fisheries holding pens to the Oregon State University Seafood Research and Education Center (Astoria, Ore, U.S.A.) where they were placed in a 100 gallon tank. Fish were stored live until used, at which point the fish were sacrificed and the fish flesh was used immediately for myosin preparation. Pre-rigor muscle is known to be more effective for purification of myosin (Connell, 1962; Park, 1988; Trucco, Lupin, Giannini, Crupkin, Boeri & Barassi, 1982)

Myosin preparation

Myosin was purified according to the method of Martone, Busconi, Folco, Trucco, and Sanchez (1986) with slight modifications. All steps in the preparation of myosin were carried out in a 4 °C cold room and samples were stored in an ice-bath during all stirring and incubation steps. Freshly minced salmon was mixed with 10 volumes (1:10) of chilled solution A (0.1 M KCl, 1 mM phenylmethylsulfonyl fluoride, 0.02 % NaN₃, and 20 mM Tris-HCl, pH 7.5) before being homogenized at speed 1 (Powergen 700, Fisher Scientific, Pittsburgh, Pa., U.S.A.) for 1 min and

stirred in an ice bath for 15 min. The homogenate was centrifuged at 1,000 x g for 10 min. All centrifuge steps were performed using a Sorvall Ultra 80 centrifuge (DuPont Co., Newton, Conn., U.S.A.) with a Sorvall A625 rotor at 1°C. The pellet was recovered and homogenized in 5 volumes of solution B (0.45 M KCl, 5 mM β -mercaptoethanol, 0.2 M $\text{Mg}(\text{CH}_3\text{COO})_2$, 1 mM ethylene glycol-bis N, N, N', N'-tetraacetic acid (EGTA), 20 mM Tris-Maleate, pH 6.8). Adenosine 5'-triphosphate (ATP) was added to a final concentration of 10 mM. The homogenate was then stirred in an ice bath for 60 min followed by centrifugation at 10,000 x g for 15 min. The supernatant was then slowly filtered through 3 layers of cheese cloth and added to 25 volumes of pre-chilled (4 °C) 1 mM NaHCO_3 and stirred in an ice bath for 15 min followed by centrifugation at 12,000 x g for 15 min. The pellet was then re-suspended in 2.5 volumes of solution C (0.5 M KCl, 5 mM β -mercaptoethanol, 20 mM Tris-HCL, pH 7.5) and homogenized using a 55 mL tissue grinder (Potter-Elvehjem, Wheaton Instruments, N.J., U.S.A.) for 10 min before the addition of 2.5 volumes of 1 mM NaHCO_3 and MgCl_2 to a final concentration of 10 mM. The suspension was then placed in an ice bath and stirred overnight at 60 rpm. After stirring overnight, the suspension was centrifuged at 22,000 x g for 30 min. The myosin pellet was then kept on ice until used. The myosin pellet was re-suspended in 0.6 M NaCl and 20 mM phosphate buffer (pH 7.0) to a final concentration of 17 mg/mL and used for differential scanning calorimetry (DSC) and dynamic rheology. Before use in biochemical testing, myosin was re-suspended to a final concentration of 2 mg/mL, using 0.6 M NaCl and 20 mM phosphate buffer (pH 7.0). Suspended myosin sample

was used for measuring turbidity, total sulfhydryl content (T-SH), surface reactive sulfhydryl content (SR-SH), and surface hydrophobicity (S_o).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Myosin purity was determined using SDS-PAGE (Laemmli, 1970) with a 4% acrylamide stacking gel and 12.5% acrylamide separating gel. Protein concentration was determined using the Lowry method (Lowry et al., 1951). For SDS-PAGE, protein concentration of all samples was adjusted to 1.25 mg/mL with dd H₂O and then mixed with 5x sample buffer (0.6 mL 1 M tris-HCl (pH 6.8), 5.0 mL glycerol, 2.0 mL 10% (w/v) SDS, 0.5 mL β -mercaptoethanol, 1.0 mL 1% (w/v) bromophenol blue, 0.9 mL dd H₂O) at a ratio of 1:4, buffer:sample (v/v). Sample and buffer were vortexed and then heated at 90 °C for 3 min in order for the β -mercaptoethanol in the sample buffer to disrupt any disulfide bonds, after which samples were centrifuged for 2 min to remove any insoluble material. Samples were electrophoresed using a Mini-Protean III Cell (Bio-Rad, Hercules, Calif., U.S.A.). Electrophoresis gels were stained using Coomassie blue R-250 and de-stained in a 3 step process using an orbital shaker (50 °C). Step one used 50% methanol (v/v) and 10% acetic acid (v/v), followed by step 2, which used 10% methanol (v/v) and 10% acetic acid (v/v). The final step was to allow the gel to soak in dd water. The electrophoresis gels were scanned using a Gel Doc XR scanner (Bio-Rad, Hercules, Calif., U.S.A.) and were analyzed using Quantity One® (Bio-Rad Laboratories, Hercules, Calif., U.S.A.). The purity of myosin was determined using density readings from myosin heavy chain

(MHC) and myosin light chains divided by the total measured density of all protein bands in the lane.

Turbidity measurements

Myosin turbidity was tested using myosin suspension at a concentration of 0.5 mg/mL in 0.6 M NaCl and 20 mM phosphate buffer pH 7. Myosin suspension was added to a quartz cuvette that was capped and placed in a UV-2401PC spectrophotometer (Shimadzu Corp., Kyoto, Japan) equipped with a water-jacketed constant-temperature cell holder that was connected to a refrigerating/heating circulating bath (model 1167P, Polyscience, Niles, Ill., U.S.A.). The sample was heated from 10 °C to 80 °C at 1 °C/min and the absorbance at 320 nm was recorded every 30 sec during the experiment. Results were given from samples tested in triplicate. Turbidity experiments were done in duplicate from two separate myosin extractions.

Linear heating

The effects of samples that were linearly heated at 1 °C/min were studied. Myosin suspension was diluted to a concentration of 2 mg/mL using 0.6 M NaCl and 20 mM phosphate buffer pH 7. Approximately 500 mL of 2 mg/mL sample was placed in a 500 mL volumetric flask. A stir bar was placed in the flask and the flask was placed inside of a small cooler that was placed on top of a magnetic stirring plate. A thermocouple was placed inside the volumetric flask to monitor sample

temperature. The cooler was then filled with water and the water was circulated through a refrigerating/heating circulating bath (model 1167P, Polyscience, Niles, Ill., U.S.A.). The water was heated from 10 °C to 80 °C at 1 °C/min. As the sample was heated, 10 mL aliquots were removed from the volumetric flask and mixed immediately with 10 mL of 0.6 M NaCl that was pre-chilled to 1 °C in an ice bath to stop any further denaturation of the myosin from that aliquot. Samples were taken at 18.5, 22.5, 25.0, 27.5, 30.0, 32.5, 35.0, 40.0, 45.0, 50.0, 55.0, 60.0, 62.5, 65.0, 67.5, 70.0, 75.0, and 80.0 °C. Samples were then returned to the ice bath and stored in the cold room until used for testing. For each temperature tested, two aliquots were taken to perform the chemical tests in duplicate. All chemical tests (TSH, SRS_H, S_o) were completed within 48 h of myosin extraction and heating.

Surface hydrophobicity

The surface hydrophobicity (S_o) of the linearly heated myosin was determined using an ANS (1-anilinonaphthalene-8-sulfonate) probe according to the method of Alizadeh-Pasdar and Li-Chan (2000). Linearly heated myosin samples were diluted to various concentrations from 0.01 to 0.05 mg/mL in 0.6 M NaCl and 20 mM phosphate buffer (pH 7). After protein samples were diluted, 20 μ L of ANS probe (8 mM ANS in 0.1 M phosphate buffer pH 7.4) was added, samples were then vortexed and allowed to sit at room temperature for 10 min before being read on a luminescence spectrophotometer (Perkin Elmer LS-50B, Norwalk, Conn., U.S.A.). The samples were measured at wavelengths of 390 nm and 470 nm ($\lambda_{\text{excitation}}$, $\lambda_{\text{emission}}$),

respectively. The myosin S_0 was calculated from the initial slope of the net relative fluorescence intensity versus the protein concentration.

Surface reactive and total sulfhydryl determination

Surface reactive sulfhydryl content (SR-SH) was determined by the method established by Ellman (1959) using 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) with slight modifications. A volume of 0.5 mL of linearly heated myosin sample was added to 2 mL of 0.6 M NaCl and 20 mM phosphate buffer pH 7 along with 50 μ L of 10 mM DTNB in 0.1 M sodium phosphate buffer, pH 7.2 containing 0.2 mM EDTA. The samples were vortexed and incubated at 4 °C for 15 min after which they were read at 412 nm on a UV-2401PC spectrophotometer. The total sulfhydryl content (TSH) was determined in a similar manner as the SR-SH content with slight modifications. A volume of 0.5 mL of linearly heated myosin sample was added to 2 mL of 8 M Urea in 0.2 M Tris-HCl buffer pH 7 along with 50 μ L of 10 mM DTNB in 0.1 M sodium phosphate buffer, pH 7.2 containing 0.2 mM EDTA. The samples were vortexed and incubated at 40 °C for 15 min and read as described above. The TSH and SR-SH groups were determined using a molar extinction coefficient of 13,600 M⁻¹cm⁻¹ (Riddles et al., 1979).

Differential scanning calorimetry (DSC)

DSC studies of Chinook salmon myosin were performed using a Setaram micro differential scanning calorimeter (Setaram Co., Lyon, France). Temperature

calibrations were performed using naphthalene. Freshly prepared myosin was suspended in 0.6 M NaCl and 20 mM phosphate buffer pH 7 and was used for DSC studies. Approximately 500 mg of myosin suspension (17 mg/mL) was accurately weighed in a stainless steel vessel. De-ionized water was used in the reference sample vessel. The myosin suspension was scanned from 10 °C to 80 °C at 1 °C/min. Triplicate samples with reproducible thermograms were analyzed.

Dynamic rheological measurement

Non-destructive gel formation of myosin was measured as a function of temperature using a CS-50 dynamic rheometer (Bohlin Instruments, Inc., Cranbury, N.J., U.S.A.). Freshly prepared myosin was suspended in 0.6 M NaCl and 20 mM phosphate buffer pH 7 and was used for dynamic rheological studies. Myosin suspension (17 mg/mL) was tested using a 2 cm plate and plate geometry with a gap of 1 mm. To avoid sample drying during thermal testing, a moisture trap with a moistened sponge was used to cover the sample. Samples were tested using a heating rate of 2 °C/min and were heated from 10 °C to 80 °C. Unlike other testing (1 °C/min linear heating), temperature sweep using dynamic rheology was conducted at 2 °C/min because gel formed at 1 °C/min was too elastic causing slippage between the plate and plate. The oscillatory mode was applied with a fixed frequency of 0.1 Hz and a shear stress of 2.5 Pa, which was within the linear viscoelastic range of the myosin sample. Samples were tested in triplicate.

6.4 RESULTS AND DISCUSSION

SDS-PAGE

The purity of myosin was determined by SDS-PAGE (Figure 1) and the densitometric data showed that the purity of salmon myosin was approximately 94%. Figure 6.1 also shows that there was no significant change in myosin heavy chain (MHC) of the samples that were linearly heated. However, the measured density of MHC from the myosin standard that was not heated was significantly higher (10,123 pixel density (PD)) than those of the linearly heated samples (6972 – 7719 PD). The total amount of protein loaded in each well was the same. When analyzing the heated myosin sample, there was no significant difference noted in the pattern of myosin protein. However, there was a distinctive difference in cross-linked proteins above MHC where native myosin showed a light band and all other heat treated myosin samples showed a more intense band. This indicated the formation of large cross-linked protein when myosin was heated slowly (1 °C/min) (Figure 6.1).

There are four main types of linkages that are developed in the formation of myosin gel: ionic linkages, hydrophobic interactions, and covalent bonds (Lanier et al., 2005). When SDS sample buffer is added to the protein sample, the buffer will negate most of these bonds by the function of the SDS detergent and the reducing agent β -mercaptoethanol. However, there is a non-disulfide covalent bond initiated by endogenous transglutaminases and it is not disrupted by SDS sample buffer. This is how non-disulfide covalent bonds form larger protein aggregates during heating (more effectively when heated slowly). It is of interest to note that although the protein

pattern between samples does not vary significantly, there is a significant increase in the cross-linked protein band above the MHC band (Esturk, Park & Thawornchinsombut, 2004). This increased intensity of the protein band above MHC is likely due to aggregated proteins that preferably formed covalent bonds during slow heating and thus, increased their overall molecular weight. These aggregated proteins are not prevalent in the native non-set or non-heat treated myosin sample. It was interesting to note that the intensity of this high molecular weight cross-linked protein remained constant once it was formed at 18.5 °C.

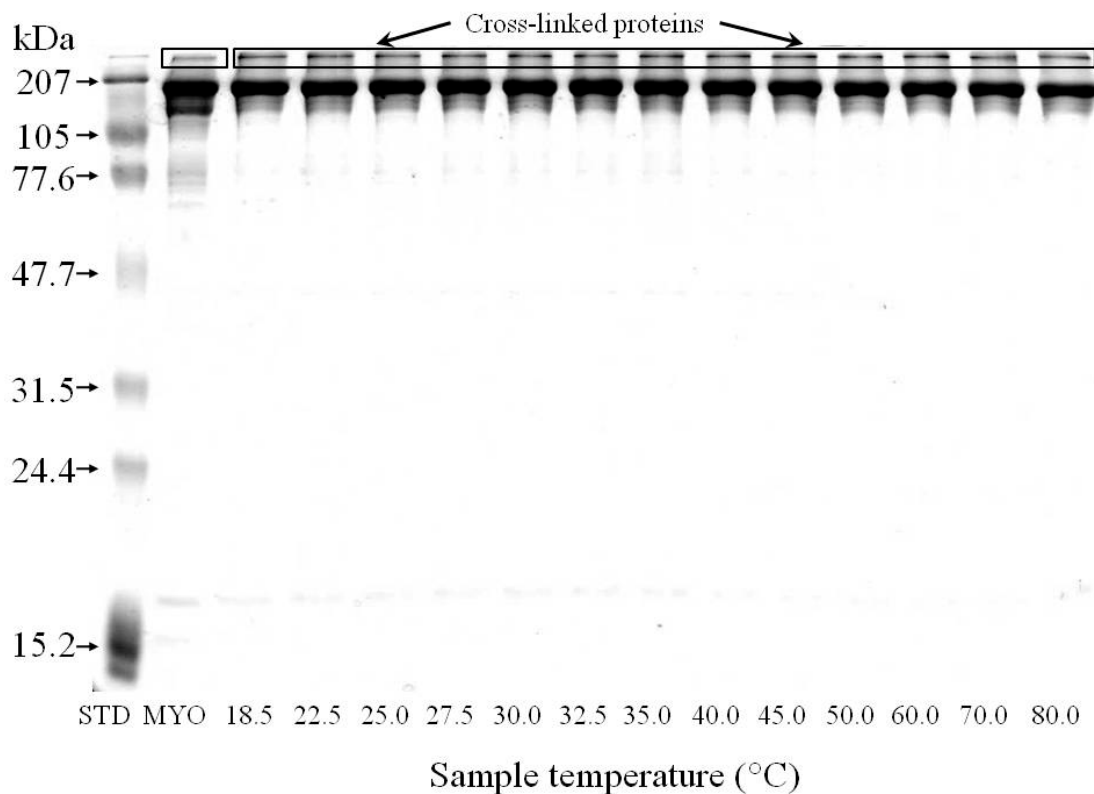


Figure 6.1 – SDS-PAGE pattern of purified salmon myosin (MYO) unheated, and samples linearly heated (1 °C/min) from 18.5 to 80 °C. STD: myosin (207), phosphorylase B (105), bovine serum albumin (77.6), ovalbumin (47.7), carbonic anhydrase (31.5), trypsin inhibitor (24.4), and lysozyme (15.2).

Turbidity measurements

As myosin was heated at 1 °C/min, it showed the process of myosin protein aggregation as measured by the increase in absorbance at 320 nm. Gill et al. (1992) demonstrated that increasing size of myosin aggregates in solution of heated fish

myosin increased absorbance (turbidity) of the solution. Figure 6.2 shows the increase in turbidity as a function of temperature increase. As the myosin sample was heated, the turbidity remained relatively stable and flat from 15 °C up through approximately 24 °C. At approximately 24 °C, the turbidity of the myosin sample began to increase, indicating the initiation of aggregation by unfolded proteins. Turbidity increased linearly from 24 °C to 52 °C at a rate of 0.0117 absorbance units per °C (AU/°C) with a R^2 value of 0.999. The turbidity of the heated myosin solution leveled off beginning at 53 °C and remained stable from 53 °C through 80 °C. The increase of turbidity at 24 °C was generally in agreement with the thermal transition point evidenced by differential scanning calorimetry (24.5 °C onset temperature) and dynamic rheology (25 °C transition point). Results are in agreement with those of Brenner et al. (2009) where turbidity increased as a result of myosin aggregation. However, in our literature search for myosin turbidity experiments we have not found any reports of a linear increase in myosin turbidity during heating.

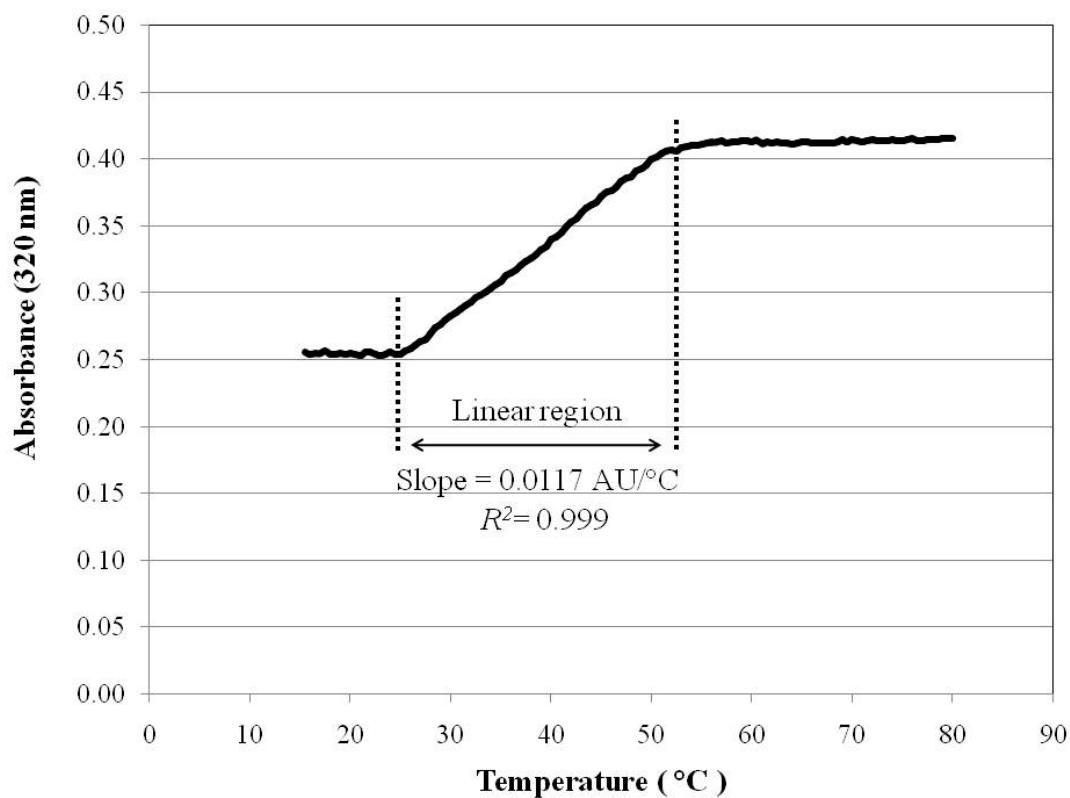


Figure 6.2 – Effect of temperature on turbidity of salmon myosin solution.

Differential scanning calorimetry

Chinook salmon myosin exhibited four major transition peaks at 27.9, 36.0, 45.5, and 49.0 °C with onset temperatures of 24.5, 32.6, 42.6, and 47.6 °C, respectively. As shown in the thermogram of salmon myosin (Figure 6.3) heated from 10 °C to 80 °C the first three peaks were endothermic in nature and the fourth was an exothermic peak. This may indicate myosin subunits started to unfold as low as 24.5 °C and completed aggregation (gelation) at 49 °C. The enthalpies of denaturation of

each major transition were 0.0285, 0.0167, 0.0197, and -0.0669 J/g, respectively. Liu et al. (Liu et al., 1996) showed thermal transition points at 35.3, 48.1, 49.9, and 67.0 °C for chicken breast myosin (0.6 M NaCl, 50 mM phosphate buffer pH 6.0), however, none of the transitions were exothermic. When studying rabbit myosin head (subfragment 1) in 50 mM TRIS buffer (pH 8.0), 0.6 M KCl, along with heavy meromyosin in 0.1 M KCl, Shriver and Kamath (1990) found exothermic peaks at 48 and 65 °C. They confirmed that the exothermic peak was attributed to aggregation and precipitation of the unfolded protein. Park and Lanier (1988) found that the exothermic peak near 50 °C was a likely indicator of fish myosin in the pre-rigor state. They explained that it was likely due to a rapid rise in ATP hydrolysis induced by rising temperatures. Like their tilapia study, we also used myosin prepared immediately after sacrificing live salmon and obtained an exothermic peak near 50 °C.

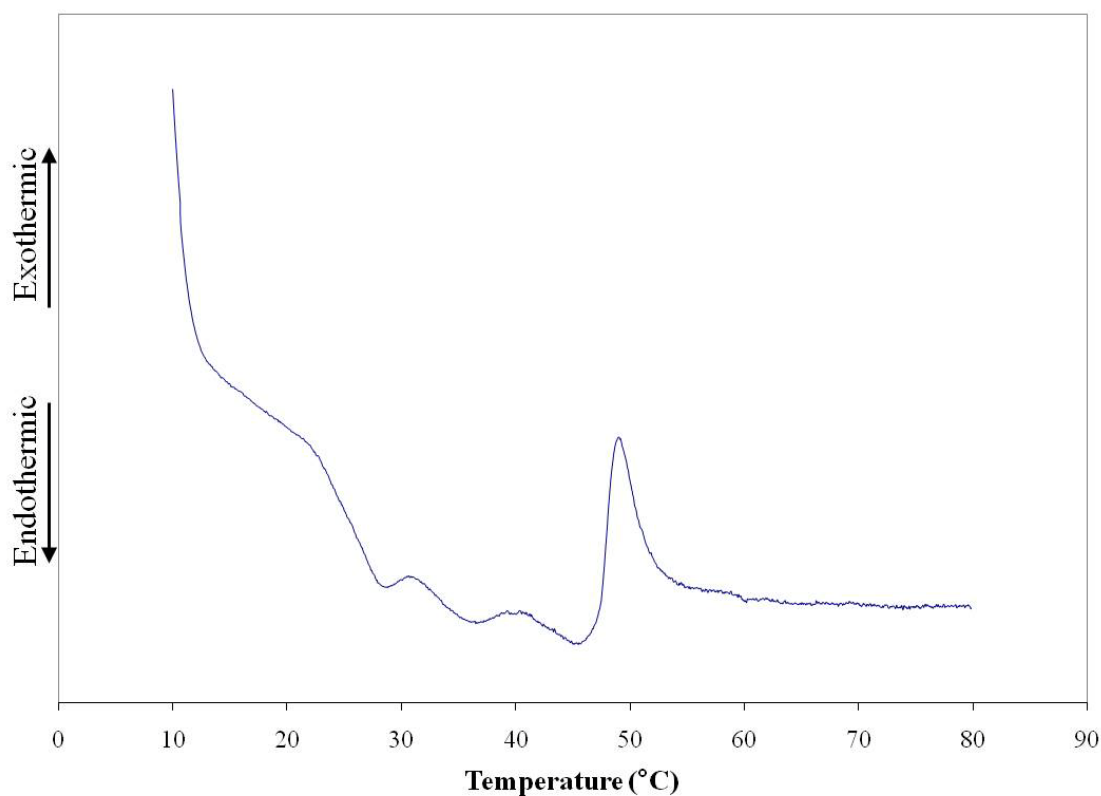


Figure 6.3 – DSC thermogram of salmon myosin heated from 10 to 80 °C at a heating rate of 1 °C/min.

In our study the DSC thermal transition peaks (27.9, 36.0, 45.5 °C) corresponded approximately to three of the transition points (25.0, 35.6, 44.7 °C) studied in dynamic rheological testing of salmon myosin. The thermal transition points could be specific to the different portions of the salmon myosin. Wright and Wilding (1984) indicated that the multiple transition peaks of the myosin molecule, namely the hinge, head, and rod regions are responsible for the multiple discrete transitions. When studying the S-1 fragment of black marlin (Lo, Iso, Taguchi,

Tanaka, Mochizuki & Nagashima, 1991) it was shown that there was one transition at 41 °C, but the rod subfragment gave two peaks at 41 and 62 °C, respectively.

We attempted to isolate heavy meromyosin (HMM) and light meromyosin (LMM) using chymotryptic digestion. But extensive damage was found to the HMM portion of the myosin (results not shown). This damage did not allow us to characterize the HMM and LMM from Chinook salmon. This was probably due to the species specific nature of salmon that often shows endogenous proteolytic degradation while farming, harvesting, processing, or transporting. However, in a subsequent study using tilapia, we successfully isolated HMM and LMM (Reed & Park, 2010a).

Dynamic rheological measurement

Salmon myosin was subjected to dynamic rheological measurements in order to better understand the gelling capability of purified myosin. G' is known as the storage modulus and is used to describe the elastic component of myosin gel, which is the energy that is applied to the sample and stored as elastic energy. G'' is the loss modulus, which describes the amount of energy dissipated to the gel as heat and is used to describe the viscous component of myosin gel. When the salmon myosin is heated from 10 to 80 °C it goes from a more viscous material to a more elastic material. This property of viscoelasticity is described in the form of the phase angle. The phase angle is given when G' and G'' are examined together and expressed as the $\tan-\delta$ (G''/G') (Rao, 1999b). This phase angle can be used to help determine the

nature of the sample, that is to say, where the sample may be in terms of its viscoelastic properties at a given temperature.

Figure 6.4 shows the rheogram for salmon myosin heated at 2 °C/min along with the thermal transition points that were of interest for further analysis using chemical testing methods. The rheogram of salmon myosin shows five distinctive transition points. Table 6.1 shows the values of the thermal transition points for G' the values were the average of three replicates. Transition point five shows that G' decreased as the temperature increased from 36.8 °C to 46.9 °C, after which G' increased as temperature increased to a final temperature of 80 °C.

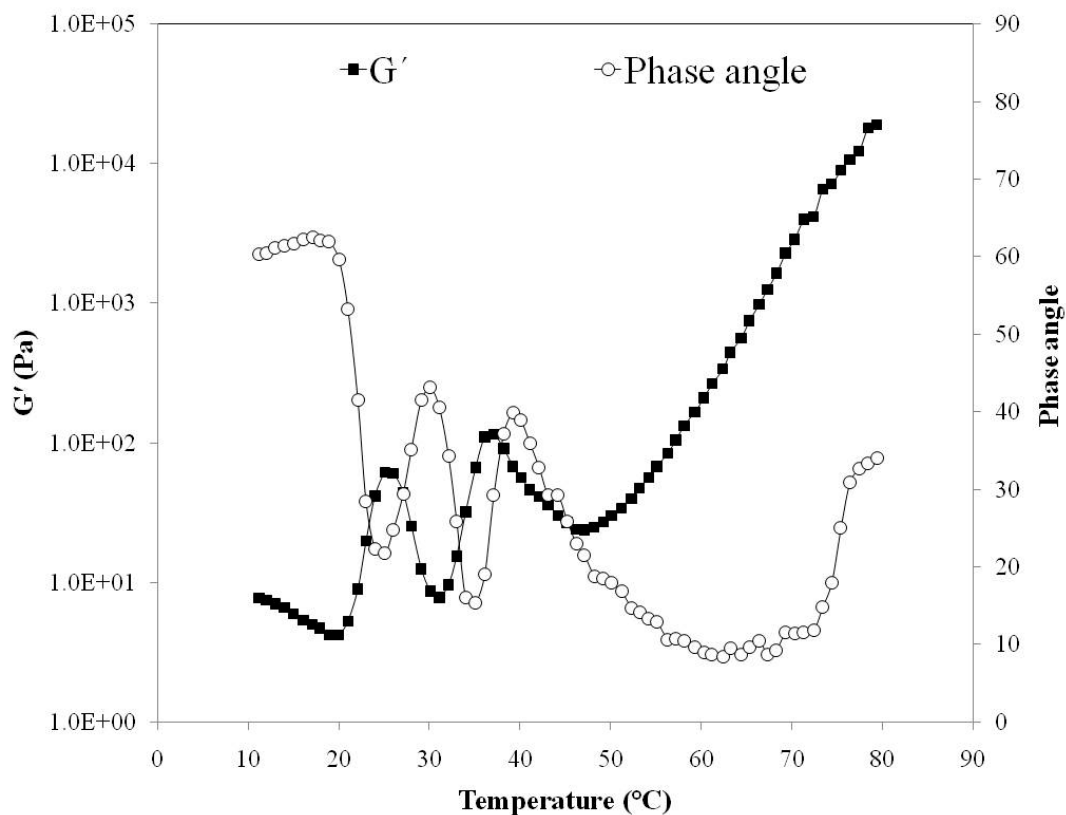


Figure 6.4 – Changes in storage modulus and phase angle of salmon myosin during temperature sweep (2 °C/min).

Transition points	
2 °C/min	
1	19.1
2	25.1
3	30.4
4	36.8
5	46.9

Table 6.1 – Thermal transition points of interest as determined from G' of salmon myosin heated at 2 °C/min.

The gelation point is often defined as the crossover of the storage (or elastic) modulus G' and the loss (or viscous) modulus G'' , which is the point at which $\log G' = \log G''$ (Friedrich & Heymann, 1988; Winter, 1987; Winter & Chambon, 1986). The gelation point for the 2 °C/min sample was approximately 31.1 °C. As shown in Figure 6.5, the first crossover at 21.7 °C was likely due to transformation of myosin subunits into a semi gel (gel-like), where the balance became more elastic mode than viscous mode. The second crossover was probably due to disruption of the semi gel, where the balance was more viscous mode than elastic mode. The third crossover was probably due to re-orientation of unfolded proteins to form a stronger gel.

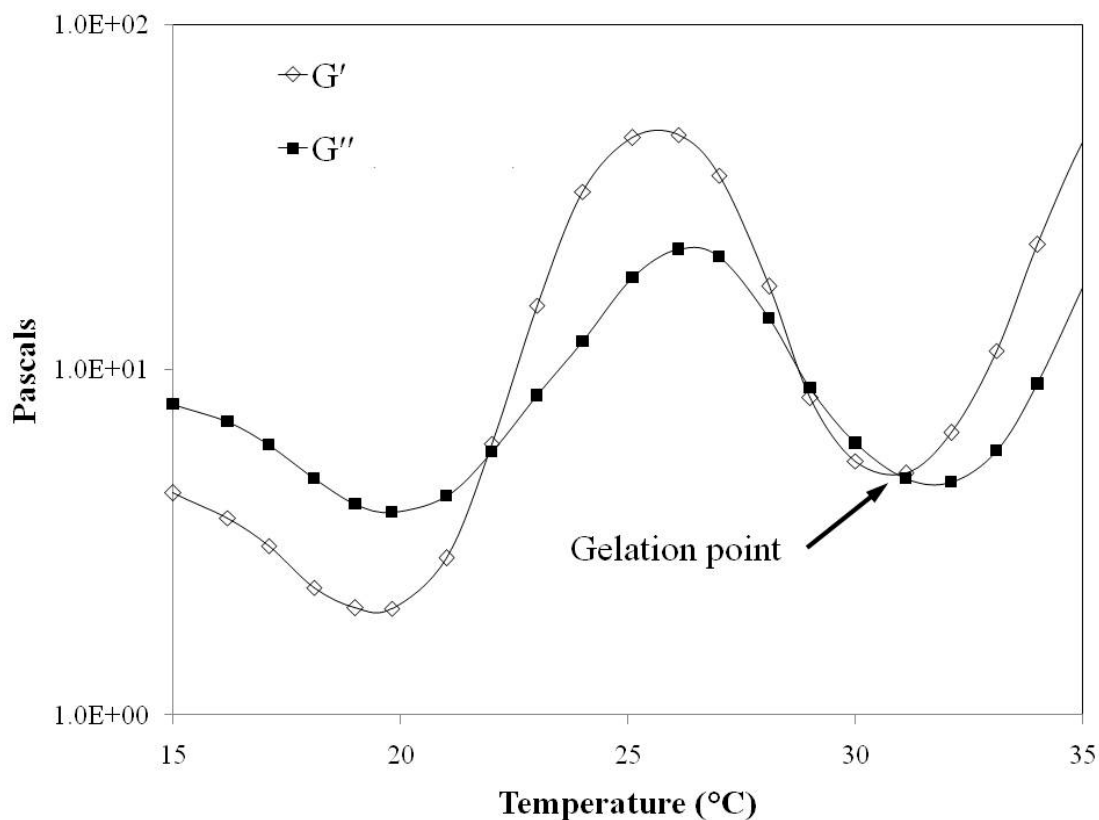


Figure 6.5 – Relationship between G' and G'' of salmon myosin during heating at 2 °C/min. The gelation point is defined as the point at which $\log G' = \log G''$.

Figure 6.6 shows the phase angle of the salmon myosin heated at 2 °C/min. These results are in agreement with studies done using Pacific whiting and cod myosin (Yongsawatdigul et al., 1999) vicilin and ovalbumin (Arntfield & Murray, 1992) and whey protein (Li, Ould Eleya & Gunasekaran, 2006). The phase angle presented a pattern that corresponds inversely to the changes in G' . The phase angle results show five transitions that occurred at similar temperatures as those determined from G' . We certainly noted an increase in phase angle after 70 °C. Once gel formation is completed, there is no reason for the transformation of the elastic and viscous phase.

This increase was thought to be due to slippage of the myosin gel positioned between the plate and plate geometry. Table 6.2 lists the corresponding temperatures for the phase angle transition points.

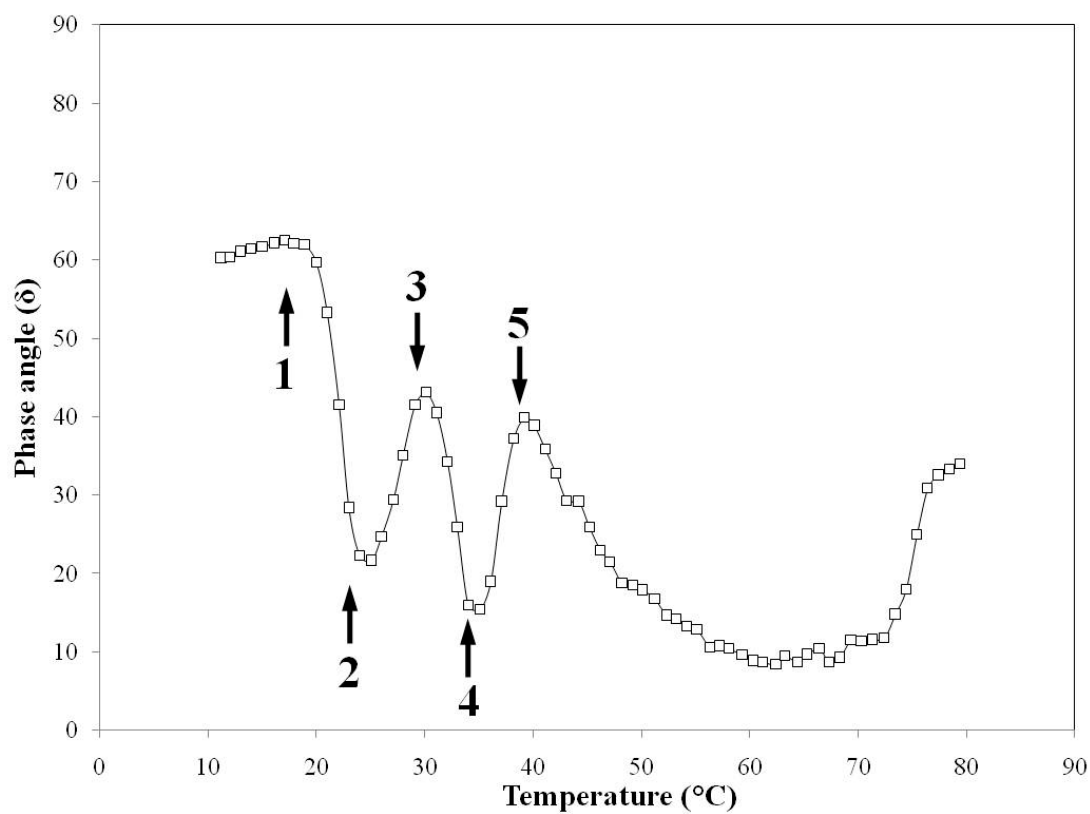


Figure 6.6 – Changes in phase angle (δ) during temperature sweep of salmon myosin as affected by heating rate ($2^{\circ}\text{C}/\text{min}$).

Transition points		
2 °C/min		
	Temperature (°C)	Phase angle (δ)
1	17.1	62.5
2	25.1	21.7
3	30.1	43.1
4	35.1	15.4
5	39.2	39.9

Table 6.2 – Phase angle transition points of salmon myosin during temperature sweep of dynamic rheology testing at 2 °C/min.

Surface hydrophobicity

In order to understand how salmon myosin interacts during heating, specifically at the thermal transition points as determined by dynamic rheology, the surface hydrophobicity (S_o) was measured during linear heating. Figure 6.7 shows how S_o of salmon myosin was affected by linear heating at 1°C/min. The S_o of salmon myosin continually increased from 18.5 to 75 °C and then remained unchanged.

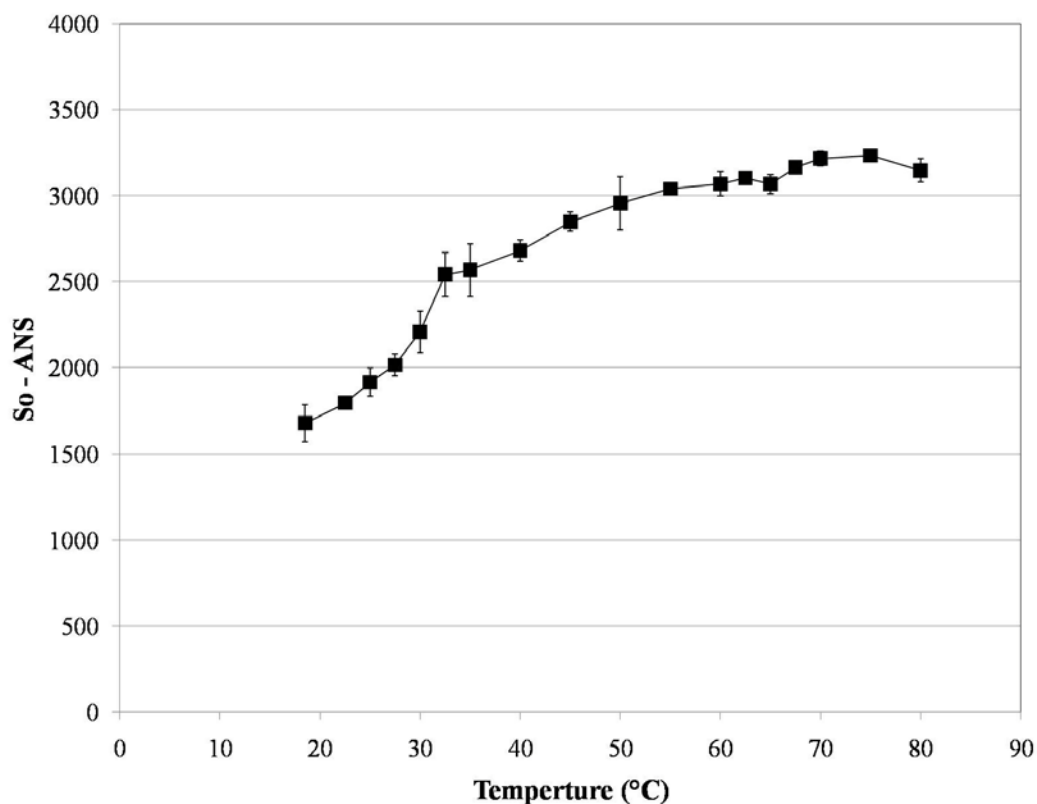


Figure 6.7 – Changes in surface hydrophobicity (S_0) of salmon myosin heated at 1 °C/min.

Hydrophobic interactions play an important role in gel formation (Lanier et al., 2005). Salmon myosin proteins can be denatured by heating. As proteins are denatured they begin to open up and expose their hydrophobic core. Consequently, they begin to interact with other hydrophobic regions on the same protein (intra) or with other denatured proteins (inter). The water hating (hydrophobic) regions of the proteins are excluded from water, which leads to a stronger interaction between them. It has been suggested that the exposure of hydrophobic domains is a prerequisite for the formation of large myosin aggregates (Egelanddal, Martinsen & Autio, 1995).

Lin et al. (1998) showed that the S_0 of salmon myosin increased as KCl content increased, which supports findings that hydrophobicity increases as proteins are denatured and allows for increased inter and intra protein hydrophobic bonding.

The rod (tail) portion of the myosin molecule consists of a long α -helical rod-shaped region (Lowey, Slayter, Weeds & Baker, 1969). Studies have shown that the α -helical structure consisting of a coiled-coil uses hydrophobic interactions as the dominant form for stability (Hoppe & Waterston, 1996). Due to denaturation of protein during heating, the hydrophobic core of myosin is increasingly exposed and therefore the surface hydrophobicity of myosin increased as temperature increased.

Surface reactive and total sulfhydryl content

The total sulfhydryl (TSH) and surface reactive sulfhydryl (SRSH) content was measured to study the effect of heating on the structure of salmon myosin (Figure 8). The TSH content of salmon myosin ranged from 5.5 to 6.2 mole/ 10^5 g protein, which was comparable to that of previous studies done using salmon myosin (6.5 mole/ 10^5 g protein) (Lin et al., 1998) and carp myosin (6.1 mole/ 10^5 g protein) (Tsuchiya & Matsumoto, 1975). The TSH content of myosin remained steady at around 6.0 mole/ 10^5 g protein from 18.5 to 50°C. From 50 - 55 °C the TSH values (6.2) began to drop gradually and reached 5.5 mole/ 10^5 g protein at 80°C. The gradual drop in TSH and SR-SH groups beginning at 50-55 °C is most likely due to the formation of disulfide linkages. Myosin can form these disulfide linkages between SH groups that are exposed upon heating (Lanier et al., 2005).

As the myosin is heated it begins to unfold and expose more of the SH bonds that are buried in the native state. Lin et al. (1998) studied the effect of KCl concentration on the SRSH of salmon myosin and found that as salt concentration increased and began to disrupt the ionic bonds in myosin, the myosin began to denature and expose previously buried SH groups. Yongsawatdigul et al. (2003) showed that the SRSH groups of threadfin bream actomyosin increased as the sample was tested at increasing temperatures. Taking the results of Lin and Yongsawatdigul together it is easily seen that many of the SH groups of proteins are buried and are exposed by denaturation with either chemical or physical denaturing processes. For salmon myosin heated linearly, the SRSH groups increased as temperature increased from 18.5 to 55 °C with an increase in SRSH groups of 4.2 to 6.1 mole/10⁵g protein. After 55 °C the SRSH groups began to decrease, as a result of the formation of disulfide bonds, to a final value of 5.7 mole/10⁵g protein at 80 °C (Figure 8).

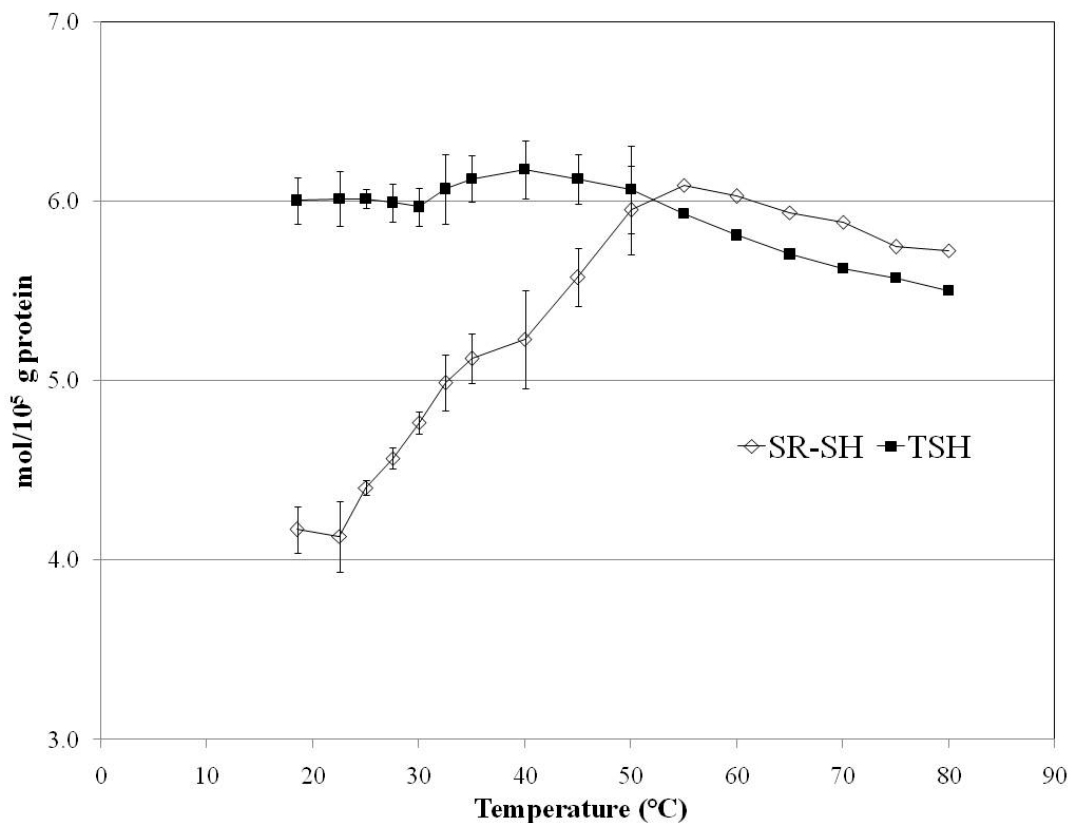


Figure 6.8 – Changes in total sulfhydryl content (TSH) and surface reactive sulfhydryl content (SRSH) of salmon myosin heated at 1 °C/min.

Similar to TSH content, SRSH content reached a maximum and then began to decline as the exposed sulfhydryl groups formed disulfide bonds. Chan, Gill, and Paulson (1992) found that some regions of the myosin molecule are less thermo stable than others and have a tendency to denature before the whole myosin molecule is completely denatured. The trend that is seen in the TSH and SRSH groups of declining sulfhydryl content can be explained by the formation of disulfide bonds as the protein is denatured. The decline in free sulfhydryl groups of the TSH samples begins at a lower temperature than that of the SRSH samples. The free sulfhydryl

group content begins to decline at a lower temperature because of the denaturing buffer that is used for the TSH testing. The 8 M urea and 40 °C temperature facilitates the denaturing and opening up of the protein thereby exposing the previously buried sulfhydryl groups. Once the sulfhydryl groups are exposed they are available to be measured or to react with other free sulfhydryl groups. This explains why the TSH content began to decline at a lower overall temperature than that of the SRSH.

6.5 CONCLUSIONS

As purified Chinook salmon myosin was heated in solution, myosin aggregates began to form at 24 °C as evidenced by increased turbidity. Myosin denaturation and gel formation was also supported by the thermal transition points determined from differential scanning calorimetry and dynamic rheology, respectively. The gelation point (31.1 °C) for salmon myosin was determined using the crossover point of $\log G'$ and $\log G''$. Changes in phase angle during the heating process exhibited similar trends as observed by the relationship between G' and G'' . An overall decrease in phase angle was observed from 10 °C to 70 °C, indicating an increase in overall elasticity. The exposure of buried hydrophobic and surface reactive sulfhydryl groups of myosin increased and maximized approximately at 75 °C and 55 °C as myosin was linearly heated, respectively. Subsequently hydrophobic interaction and disulfide bond played their major role in myosin gel formation.

CHAPTER 7

EFFECTS OF LINEARLY HEATED MYOSIN, HEAVY MEROMYOSIN,
AND LIGHT MEROMYOSIN FROM TILAPIA

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7.1 ABSTRACT

Purified tilapia myosin was digested with α -chymotrypsin and purified to obtain heavy meromyosin (HMM) and light meromyosin (LMM). Tilapia myosin, HMM, and LMM were studied using SDS-PAGE. Myosin, HMM, and LMM were linearly heated from 10 to 90 °C and showed protein denaturation/aggregation during heating as measured by turbidity at 320 nm. Conformational changes, as measured by surface hydrophobicity (S_o) showed a marked increase for myosin and HMM between 30 and 40 °C and reached a stable plateau at 70 °C. LMM, in an extremely small magnitude, also showed a continuous increase to 70 °C. Total sulfhydryl content (TSH) showed that the –SH residue content of HMM was nearly double that of LMM. Surface reactive sulfhydryl groups (SRSH) for myosin and HMM were relatively unchanged from 10 to 30 °C but increased significantly from 30 to 50 °C. SRSH content of LMM was lower than that of the TSH content of LMM but both showed a slightly decreasing trend as the sample was heated. Differential scanning calorimetry showed 3 (17.5, 41.9, and 49.9 °C), 2 (43.0 and 67.1 °C), and 3 (40.4, 51.7, and 69.0 °C) major peaks for myosin, HMM, and LMM, respectively. Dynamic rheology measurements demonstrated crossover points, which are generally recognized as gelation point, 40.3 °C for myosin and 27.0 °C for HMM.

7.2 INTRODUCTION

Myofibrillar proteins from fish have been studied extensively due to their ability to form an elastic gel network when heated. Myosin is the major muscle protein that is found in fish and comprises approximately 55-60% of the myofibrillar proteins found in fish (Lanier et al., 2005). Skeletal myosin can be broken up into six polypeptide chains, two heavy chains and four light chains. Myosin light chains typically range from 17 to 25 kDa. There are two types of myosin light chains, the regulatory light chains, which are known for their role in phosphorylation and dephosphorylation (Sobieszek, 1988). The regulatory light chains can be selectively dissociated from myosin by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) thus they are often referred to as the DTNB light chains (Wagner, 1982). The role of this phosphorylation and dephosphorylation corresponds to the contraction and relaxation of smooth muscle. The essential light chains are often referred to as alkali light chains because they are dissociated from myosin under alkaline conditions (Weeds et al., 1977). Each myosin head unit contains one DTNB subunit and one alkali unit. This means that there are four myosin light chains per myosin molecule. However, it is interesting to note that when whole myosin is analyzed using SDS-PAGE techniques, there are typically only three bands found for the myosin light chains. This occurs because each head unit has one light chain 2 (LC 2), the DTNB light chain, and then one essential light chain, which can be either the light chain 1 or the light chain 3 (Bechtel, 1986). Due to the repetition of the LC2 in both head units the SDS-PAGE

protein pattern shows only three myosin light chains. These amino acid chains are non-covalently attached to the myosin head (Lanier et al., 2005).

Myosin is a large asymmetric molecule that has a long α -helical coiled-coil tail and two globular heads with an approximate weight of 500 kDa (Hodge et al., 2000). The basic body plan of myosin consist of an N-terminal head or motor domain, a light chain-binding neck domain, and a class conserved, C-terminal tail domain and has been categorized into over twenty different classes (Mooseker et al., 2008). The head or motor domain has a core sequence that is highly conserved in all of the myosin classes, and it contains the ATPase active site (Holmes, 2008). The neck region, also known as the lever arm consists of a long α -helix of variable length and a tail region which is extremely variable in sequence, length, domain composition, and organization. The molecular weight of myosin heavy chain when dissociated in strong denaturing solutions is approximately 220 kDa. When subjected to digestion using chymotrypsin, myosin can be fragmented into two main portions, namely heavy meromyosin (HMM) and light meromyosin (LMM) (Lowey et al., 1962; Lowey et al., 1969; Szent-Görgyi, 1953). HMM contains the globular head unit along with a short tail portion and the LMM is made up of the α -helical coiled-coil tail with molecular weights of 350 and 125 kDa, respectively, for the dimers (Margossian et al., 1982).

In order to better understand the important roles that myosin, HMM, and LMM play in the gelation of myofibrillar proteins, extensive studies have been performed on purified myosin and its fragments from a variety of fish species. Fish such as Alaska pollock (Togashi et al., 2002), sardine (Ogawa et al., 1999a), Japanese stingfish

(Nagai et al., 1999), carp (Tsuchiya et al., 1975) cod, and herring (Chan et al., 1993). By studying myosin and its subfragments it is possible to elucidate the role that each fragment plays in myosin aggregation during heating. Chan et al. (1993) studied the aggregation of HMM and LMM subfragments from cod and herring, and found that 30 – 40 °C an initial aggregate formed by the interaction of the HMM portion of myosin. This was followed by compound aggregates being formed at 40 – 55 °C by the interaction of LMM.

In this study we endeavored to elucidate the key characteristics of tilapia myosin, heavy meromyosin, and light meromyosin as affected by linear heating. Purified myosin, HMM, and LMM were linearly heated in order to understand the gelling ability and determine the gelation point. Samples were also tested using differential scanning calorimetry to study the endothermic and exothermic patterns of each myosin subfragment. Some of the other key characteristics of myosin, HMM, and LMM, such as surface hydrophobicity, surface reactive sulfhydryl and total sulfhydryl content were also investigated to determine the role that heat treatment plays on the thermal denaturation/aggregation of tilapia myosin and its subfragments.

7.3 MATERIALS AND METHODS

Fish

Live fish was sacrificed and gutted at a supermarket in Portland, Oregon and was transported on ice to the Oregon State University Seafood Research and Education Center (Astoria, Ore, U.S.A.). The tilapia was filleted, skinned removed, and cut into strips. A Kitchenaid stand mixer (St. Joseph, MI, USA) fitted with a food

grinder and small grind plate (pre-chilled to 4 °C) was used to grind the tilapia strips. Estimated postmortem time before extraction was 4 h. It is generally known that pre-rigor muscle is more effective for obtaining purified muscle myosin (Connell, 1962; Park, 1988; Trucco et al., 1982)

Myosin preparation

Myosin was purified according to the method of Martone, Busconi, Folco, Trucco, and Sanchez (1986) with slight modifications. All steps in the preparation of myosin were carried out in a 4 °C cold room and samples were stored in an ice-bath during all stirring steps. Freshly minced tilapia was mixed with 10 volumes (1:10) of chilled solution A (0.1 M KCl, 1 mM phenylmethylsulfonyl fluoride, 0.02 % NaN₃, and 20 mM Tris-HCl, pH 7.5) and homogenized at speed 1 (Powergen 700, Fisher Scientific, Pittsburgh, Pa., U.S.A.) for 1 min and then stirred for 15 min. The homogenate was centrifuged at 1,000 x g at 4 °C for 10 min. All centrifuge steps were performed using a Sorvall Ultra 80 centrifuge (DuPont Co., Newton, Conn., U.S.A.) with a Sorvall A625 rotor. The pellet was recovered and homogenized in 5 volumes of solution B (0.45 M KCl, 5 mM β-mercaptoethanol, 0.2 M Mg(CH₃COO)₂, 1 mM ethylene glycol-bis N, N, N', N'-tetraacetic acid (EGTA), 20 mM Tris-Maleate, pH 6.8). Adenosine 5'-triphosphate (ATP) was added to a final concentration of 10 mM. The homogenate was then stirred in an ice bath for 60 min followed by centrifugation at 10,000 x g for 15 min. The supernatant was then slowly added to 25 volumes of pre-chilled 1 mM NaHCO₃ and stirred in an ice bath for 15 min followed by

centrifugation at 12,000 x g for 15 min. The pellet was then re-suspended in 2.5 volumes of solution C (0.5 M KCl, 5 mM β -mercaptoethanol, 20 mM Tris-HCL, pH 7.5) and homogenized using a 55 mL tissue grinder (Potter-Elvehjem, Wheaton Instruments, N.J., U.S.A.) for 10 min. before the addition of 2.5 volumes of 1 mM NaHCO₃ and MgCl₂ to a final concentration of 10 mM. The suspension was then placed in an ice bath and stirred overnight a 60 rpm. After the overnight stirring the suspension was centrifuged at 22,000 x g for 30 min. The myosin pellet was then kept on ice until resuspended immediately before performing the experiments. Before use, myosin was re-suspended using high salt buffer (0.6 M KCl and 50 mM phosphate buffer pH 7.0) and then used for all experiments and HMM and LMM preparation.

HMM and LMM preparation

Heavy meromyosin and light meromyosin was prepared essentially by the method outlined Margossian and Lowey (1982) with modifications outlined by Guo & Guilford (2004). Purified tilapia myosin was diluted to \approx 10 mg/ml using high salt buffer (0.6 M KCl and 50 mM phosphate buffer pH 7.0). Myosin digestion was performed by the adding of α -chymotrypsin (10 mg/mL in 0.001 N HCl, Sigma, St. Louis, MO, USA) to myosin (\approx 10 mg/mL) at a ratio of 1:130 [α -chymotrypsin (mg) : myosin (mg)] and incubating at 4 °C for 45 min with stirring. The digestion was stopped by the addition of 100 mM PMSF in 100% ethanol to a final concentration of 0.5 mM and allowed to stir for 10 min before dialysis. The digested sample was then dialyzed against low salt buffer (25 mM KCl, 25 mM Imidazole, 1 mM MgCl₂, pH

7.4) overnight at 4 °C with one changed of buffer followed by centrifugation at 180,000 x g for 1 h at 4 °C. The supernatant containing purified HMM was taken and stored on ice until used for further experiments. The pellet, which contained undigested myosin and LMM was then resuspended in 2 volumes of high salt buffer (described above) followed by the addition of 3 volumes of 95% ethanol (4 °C) and stirred on ice for 2-3 h to irreversibly denature the undigested myosin. The precipitate was collected by centrifugation at 20,000 x g for 30 min, and then resuspended in 5 volumes of high salt buffer overnight at 4 °C with at least 2 changes of buffer during dialysis. The solution was then centrifuged at 180,000 x g for 1 h at 4 °C where the supernatant (LMM) was collected and dialyzed overnight against frequent changes of low salt buffer in order to precipitate LMM. The precipitated LMM was collected by centrifugation at 20,000 x g for 45 min and resuspended in a small volume of high salt buffer. The LMM and HMM were used for subsequent experiments.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Myosin, HMM, and LMM purity was determined using SDS-PAGE (Laemmli, 1970) with a 4% acrylamide stacking gel and 12.5% acrylamide separating gel. The protein concentration was determined using the Bio-rad protein assay (Bio-Rad, Hercules, Calif., U.S.A.). Laemmli sample buffer (0.6 mL 1 M tris-HCl (pH 6.8), 5.0 mL glycerol, 2.0 mL 10% (w/v) SDS, 0.5 mL β -mercaptoethanol, 1.0 mL 1% (w/v) bromophenol blue, 0.9 mL dd H₂O) was mixed with protein samples. Sample and buffer were vortexed and then heated for 3 min at 90 °C. In order to study the effect

of disulfide bonds on the linearly heated samples (1°C/min) two SDS-PAGE gels were run for each set of samples (myosin, HMM, LMM). One set used Laemmli sample buffer as described above (with β ME), while the other sample was run with Laemmli sample buffer that had the β -mercaptoethanol replaced with water (without β ME: w/o β ME). The w/o β ME sample was non-reducing so that any disulfide bonds that were formed were maintained during electrophoresis. Samples were electrophoresed using a Mini-Protean III Cell (Bio-Rad, Hercules, Calif., U.S.A.). Electrophoresis gels were stained using Coomassie blue R-250 and de-stained in a 3 step process using an orbital shaker (50 °C). Step one used 50% methanol (v/v) and 10% acetic acid (v/v), followed by step 2 which used 10% methanol (v/v) and 10% acetic acid (v/v). The final step was to allow the gel to soak in dd water. The electrophoresis gels were scanned using a Gel Doc XR scanner (Bio-Rad, Hercules, Calif., U.S.A.) and were analyzed using Quantity One® (Bio-Rad Laboratories, Hercules, Calif., U.S.A.). The purity of myosin was determined using the density readings from the myosin heavy chain (MHC) and the myosin light chains divided by the total measured density of all protein bands in the lane.

Turbidity measurements

Turbidity was tested using samples (myosin, HMM, or LMM) at a concentration of 0.25 mg/mL in 0.6 M KCl and 50 mM phosphate buffer pH 7. A cuvette containing buffer only was used as a blank for the turbidity measurements. The sample was added to a quartz cuvette that was capped and placed in a UV-2401PC

spectrophotometer (Shimadzu Corp., Kyoto, Japan) equipped with a water-jacketed constant-temperature cell holder that was connected to a refrigerating/heating circulating bath (model 1167P, Polyscience, Niles, Ill., U.S.A.). The sample was heated from 10 °C to 90 °C at 1 °C/min and the absorbance at 320 nm was recorded every 30 sec during the experiment. Results were given from samples tested in triplicate. Turbidity experiments were done in duplicate.

Linear heating

The effects of samples that were linearly heated at 1 °C/min were studied. Myosin, HMM, and LMM samples were diluted to a concentration of 0.5 mg/mL using high salt buffer (0.6 M KCl and 50 mM phosphate buffer pH 7). Approximately 7 mL of adjusted sample was placed in a 15 mL conical tube and capped. There were 9 tubes in total used, one for each of the temperatures tested (10, 20, 30, 40, 50, 60, 70, 80, 90 °C). The tubes were then placed inside of refrigerating/heating circulating water bath (model 1167P, Polyscience, Niles, Ill., U.S.A.). The water was heated from 10 °C to 90 °C at 1 °C/min. As the samples were heated, a tube was taken from the water bath at each of the 9 temperatures and an equal volume of pre-chilled (4 °C) high salt buffer was added to stop heating. The samples were then placed in an ice bath until used for further experiments. All chemical tests (TSH, SRSH, S_o) were completed within 48 h of heating.

Surface hydrophobicity

The surface hydrophobicity (S_o) of the linearly heated samples (myosin, HMM, and LMM) was determined using an ANS (1-anilinonaphthalene-8-sulfonate) probe according to the method of Alizadeh-Pasdar and Li-Chan (2000). Linearly heated myosin samples were diluted to a final volume of 4 mL with various concentrations from 0.01 to 0.04 mg/mL in 0.6 M KCl and 50 mM phosphate buffer (pH 7). After protein samples were diluted, 20 μ L of ANS probe (8 mM ANS in 0.1 M phosphate buffer pH 7.4) was added and the samples were vortexed and allowed to sit at room temperature for 10 min before being read on a luminescence spectrophotometer (Perkin Elmer LS-50B, Norwalk, Conn., U.S.A.). Samples were measured at wavelengths of 390 nm and 470 nm ($\lambda_{excitation}$, $\lambda_{emission}$), respectively. The S_o was calculated from the initial slope of net relative fluorescence intensity versus protein concentration.

Surface reactive and total sulfhydryl determination

Surface reactive sulfhydryl content (SR-SH) was determined by the method established by Ellman (1959) using 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) with slight modifications. A volume of 0.5 mL of linearly heated sample (myosin, HMM, LMM) was added to 2 mL of 0.6 M KCl and 50 mM phosphate buffer pH 7 along with 50 μ L of 10 mM DTNB in 0.1 M sodium phosphate buffer, pH 7.2 containing 0.2 mM EDTA. The samples were vortexed and incubated for 15 min at room temperature after which they were read at 412 nm on a UV-2401PC spectrophotometer. Total sulfhydryl content (TSH) was determined in a similar

manner as the SR-SH content with slight modifications. A volume of 0.5 mL of linearly heated myosin sample was added to 2 mL of 8 M Urea in 0.2 M Tris-HCl buffer pH 7 along with 50 μ L of 10 mM DTNB in 0.1 M sodium phosphate buffer, pH 7.2 containing 0.2 mM EDTA. The samples were vortexed and incubated for 15 min at 40 °C and read as described above. The TSH and SR-SH groups were determined using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ (Riddles et al., 1979).

Differential scanning calorimetry (DSC)

DSC studies of whole muscle tilapia along with purified tilapia myosin, HMM, and LMM were performed using a Setaram micro differential scanning calorimeter (Setaram Co., Lyon, France). Temperature calibrations were performed using naphthalene. Whole muscle tilapia (\approx 500 mg) was excised from freshly sacrificed fish (within 2 h) and was used directly without further processing for DSC studies. Freshly prepared myosin was suspended in 0.6 M KCl and 20 mM phosphate buffer pH 7 and was used for DSC studies. Approximately 500 mg of sample was accurately weighed in a stainless steel vessel. The protein concentrations used for DSC studies were 21.6, 5.1, and 0.5 mg/mL for myosin, HMM, and LMM, respectively. De-ionized water was used in the reference sample vessel. The myosin suspension was scanned from 10 °C to 90 °C at 1 °C/min. Triplicate samples with reproducible thermograms were analyzed.

Dynamic rheological measurement

Non-destructive gel properties of myosin, HMM, and LMM was measured as a function of temperature using a Bohlin CVO dynamic rheometer (Malvern Instruments Ltd, Worcestershire, UK). Freshly prepared myosin or HMM, suspended in 0.6 M KCl and 50 mM phosphate buffer pH 7, was used for dynamic rheology studies. Myosin (21.6 mg/mL) and HMM (5.1 mg/mL) were tested using a cone and plate geometry (4° and 4 cm diameter) with a gap of 150 μm . To avoid sample drying during thermal testing, a moisture trap with a moistened sponge was used to cover the sample. Samples were tested using a heating rate of 1 $^{\circ}\text{C}/\text{min}$ and were heated from 10 $^{\circ}\text{C}$ to 90 $^{\circ}\text{C}$. The oscillatory mode was applied with a fixed frequency of 0.1 Hz and a shear stress of 0.1 Pa, which was within the linear viscoelastic range of the myosin and HMM sample. Samples were tested in triplicate.

7.4 RESULTS AND DISCUSSION

Turbidity measurements

As the tilapia myosin, HMM, and LMM were heated at 1 $^{\circ}\text{C}/\text{min}$, it showed the process of protein denaturation followed by aggregation as measured by the increased absorbance at 320 nm. Gill et al. (1992) demonstrated the increased absorbance (turbidity) as the size of myosin aggregates was enlarged upon heating. Figure 7.1 shows how the turbidity increases as a function of temperature. For all samples, (myosin, HMM, and LMM), the turbidity was relatively stable from 10 $^{\circ}\text{C}$ up to around 39.0, 37.7, and 37.3 $^{\circ}\text{C}$ for the myosin, HMM, and LMM, respectively. At

approximately 39.0 °C the turbidity of myosin began to increase indicating the initiation of protein unfolding and subsequent aggregation. This initial point when the myosin begins to denature is in agreement with results shown from DSC and dynamic rheological studies we performed on myosin. As the temperature approached 70 °C the turbidity of myosin began to level off and remained relatively unchanged from 70 to 90 °C.

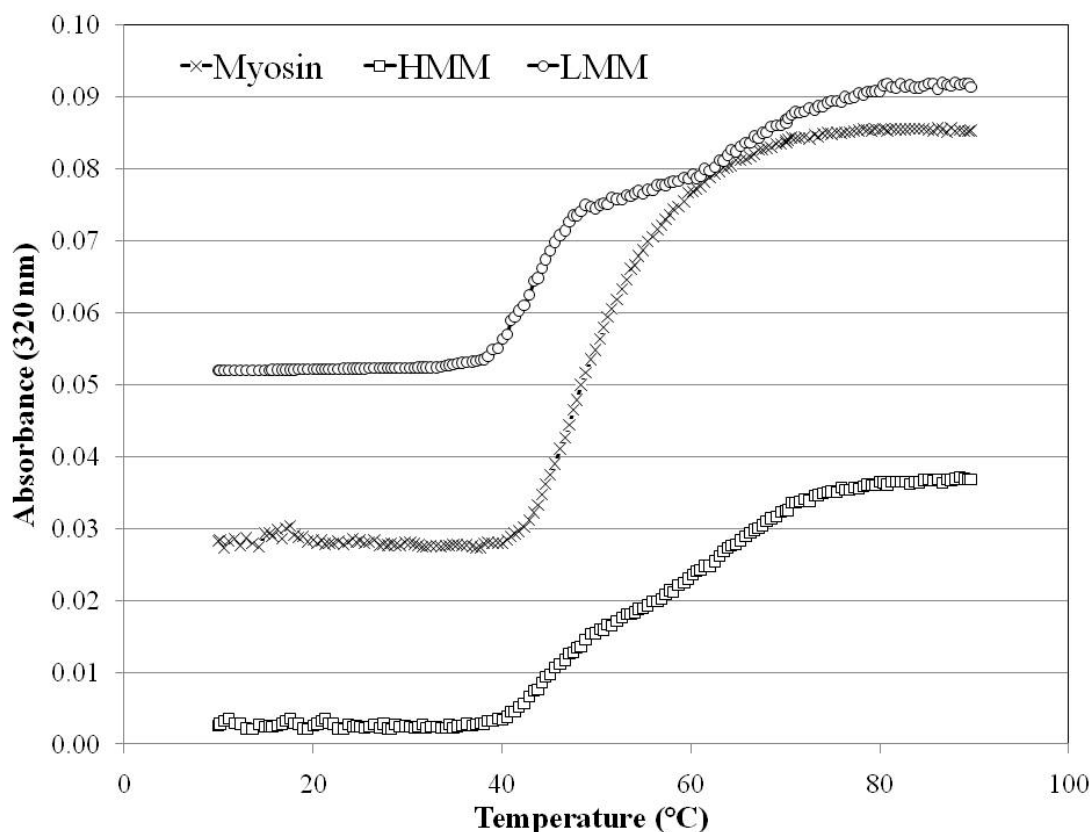


Figure 7.1 – Effect of linear heating (1 °C/min) on the turbidity of myosin, heavy meromyosin (HMM), and light meromyosin (LMM). All samples were tested at 0.25 mg/mL concentrations.

HMM showed an increase of turbidity beginning at around 37.7 °C and continued to increase until approximately 80 °C, at which point the turbidity leveled

off and remained relatively unchanged up to 90 °C. The delay in reaching the leveling off point of for HMM as compared to myosin may be partly explained by the continued aggregation of HMM at higher temperatures. This aggregation is in agreement with DSC data that showed an exothermic peak for HMM at approximately 67.1 °C, which indicates more protein aggregation than protein unfolding. Sano et al. (1990) also showed that the thermal aggregation of HMM corresponded to that of myosin alone in temperature ranges of 50 °C and above.

Figure 7.1 showed that the initial absorbance of the LMM at the same concentration of myosin and HMM was nearly double that of the myosin and almost five times higher than the initial absorbance of the HMM. Turbidity from the LMM began to increase at 37.3 °C and increased sharply from 37.3 to 47.5 °C at which point the turbidity increase continued but at a lower rate. However the rate began to increase again at 60.9 °C and continued to increase until it began to level off at 80 °C. Sasaki, Yuan, and Konno (2006) showed that increase in turbidity of LMM from carp was due largely in part to the unfolding of the α -helix structure, and that the complete unfolding of the structure was needed for protein aggregation.

All three samples showed that at temperatures below ≈ 37 °C, myosin, HMM, and LMM were extremely thermally stable. At these lower temperatures very little change in turbidity was seen. This thermal stability is most likely species specific to tilapia and due to its warm water habitat.

SDS-PAGE

The purity of myosin, HMM, and LMM was determined by SDS-PAGE (Figure 7.2) and the densitometric data showed that the purity of tilapia myosin, HMM, and LMM were ≈ 91.2 , 98.4 , and 93.2% pure, respectively.

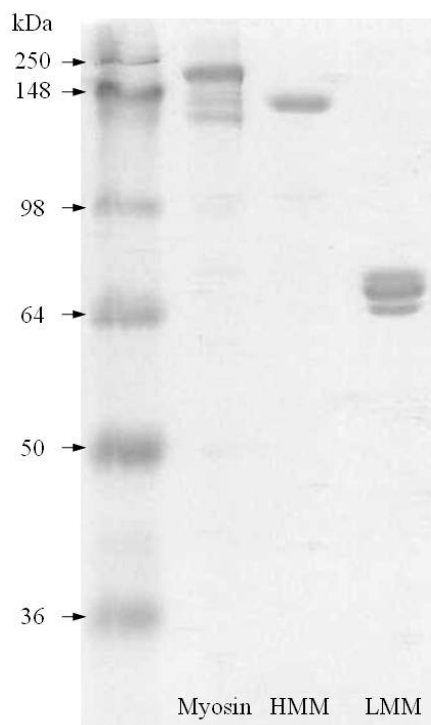


Figure 7.2 – SDS-PAGE of purified myosin, heavy meromyosin (HMM), and light meromyosin (LMM).

The optimal time for digestion of tilapia myosin was determined with the objective being to obtain HMM and LMM. Figure 7.3 shows the progression of myosin digestion at 4°C using α -chymotrypsin. As can be seen by Figure 7.3, even after 90 min of digestion not all of the myosin was digested. However, at 90 min two bands begin to form in the area of the LMM indicating extended damage is being done to the LMM causing unnecessary fragmentation of the LMM. Therefore purified myosin was digested for a total of 45 min in order to digest myosin and produce HMM

and LMM. Togashi et al. (2002) showed that the complete digestion of Alaska pollock myosin at 10 °C took two hours, which is in line with our digestion times showing that extensive digestion time is needed to completely digest fish myosin. However, they also showed that with the increased digestion time, more fragmentation of lower molecular weight protein was observed.

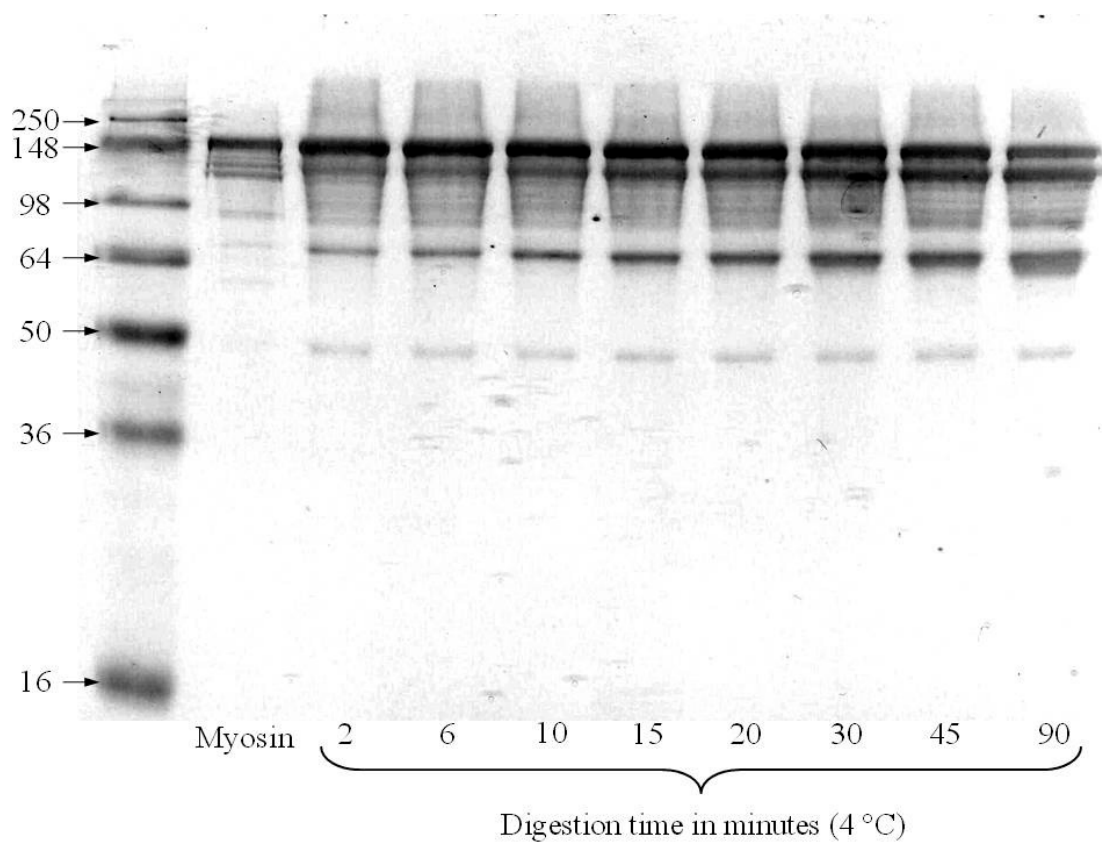


Figure 7.3 – SDS-PAGE of purified myosin digestion using α -chymotrypsin.

Appropriate denaturation is an essential step in heat-induced gelation and texture formation. As tilapia myosin, HMM, and LMM were heated, the proteins are denatured and exposed previously buried sulfhydryl groups are exposed to participate

in disulfide bonding and to aid in gel matrix formation (Tironi, Tomas & Anon, 2002; Yongsawatdigul et al., 2003). When linearly heated (1 °C/min) myosin samples were subjected to reducing (+ β ME) and non-reducing (- β ME) SDS-PAGE we found a difference in the protein patterns produced (Figure 7.4). When myosin was heated and subjected to reducing SDS-PAGE it was found that for samples heated up to 60 °C showed one major protein band that corresponded to the myosin heavy chain (MHC) of tilapia. Beginning at around 70 °C, a protein band in excess of 250 kDa began to appear indicating the formation of non-disulfide covalent bonding, while the MHC remained relatively unchanged. When linearly heated myosin was subjected to non-reducing SDS-PAGE (β ME) a completely different protein pattern was shown (Figure 7.4). The non-reducing SDS-PAGE showed that the myosin had formed very high molecular weight (>250 kDa) aggregates. These aggregates continued to increase in intensity as the treatment temperature increased with a corresponding decrease in MHC to 70 °C. As the myosin reached 80 and 90 °C, it appears that the protein aggregates were too large to enter into the SDS-PAGE gel. Myosin heated to 90 °C and subjected to non-reducing SDS-PAGE showed an almost complete disappearance of MHC and a marked decrease in overall protein content. This is most likely due the formation of very large protein aggregates during the heating of myosin that involve large numbers of disulfide bonds. For myosin the increase in disulfide bond formation as shown through non-reducing SDS-PAGE is in agreement with the results of our surface reactive sulfhydryl content results. We found that as the sample was linearly

heated that the number of surface reactive sulfhydryl groups increased, as a result of myosin denaturation.

Linearly heated HMM showed one major protein band at 109 kDa (Skaara & Regenstein, 1990) when subjected to reducing SDS-PAGE (+ β ME). As the treatment temperature increased the intensity of the HMM band had a very slight decrease indicating the possible formation of higher molecular weight HMM aggregates. However, no higher molecular weight protein bands appeared on the HMM SDS-PAGE gel. Linearly heated HMM subjected to non-reducing SDS-PAGE, like that of myosin, had a protein band pattern completely different than that of the reducing SDS-PAGE of HMM. Non-reducing SDS-PAGE of HMM showed that from 10 to 40 °C there was no clear HMM band indicated. However, at 50 °C a very high molecular weight (> 250 kDa) protein band began to appear and increased in intensity for the rest of the linearly heated samples. This indicates that the HMM was forming large aggregates that were composed mainly of disulfide bonds created during the heating and denaturation of the HMM molecules. SDS-PAGE pattern of non-reducing linearly heated HMM showed a correlation of high molecular weight protein band formation to the increase of surface reactive sulfhydryl groups in HMM.

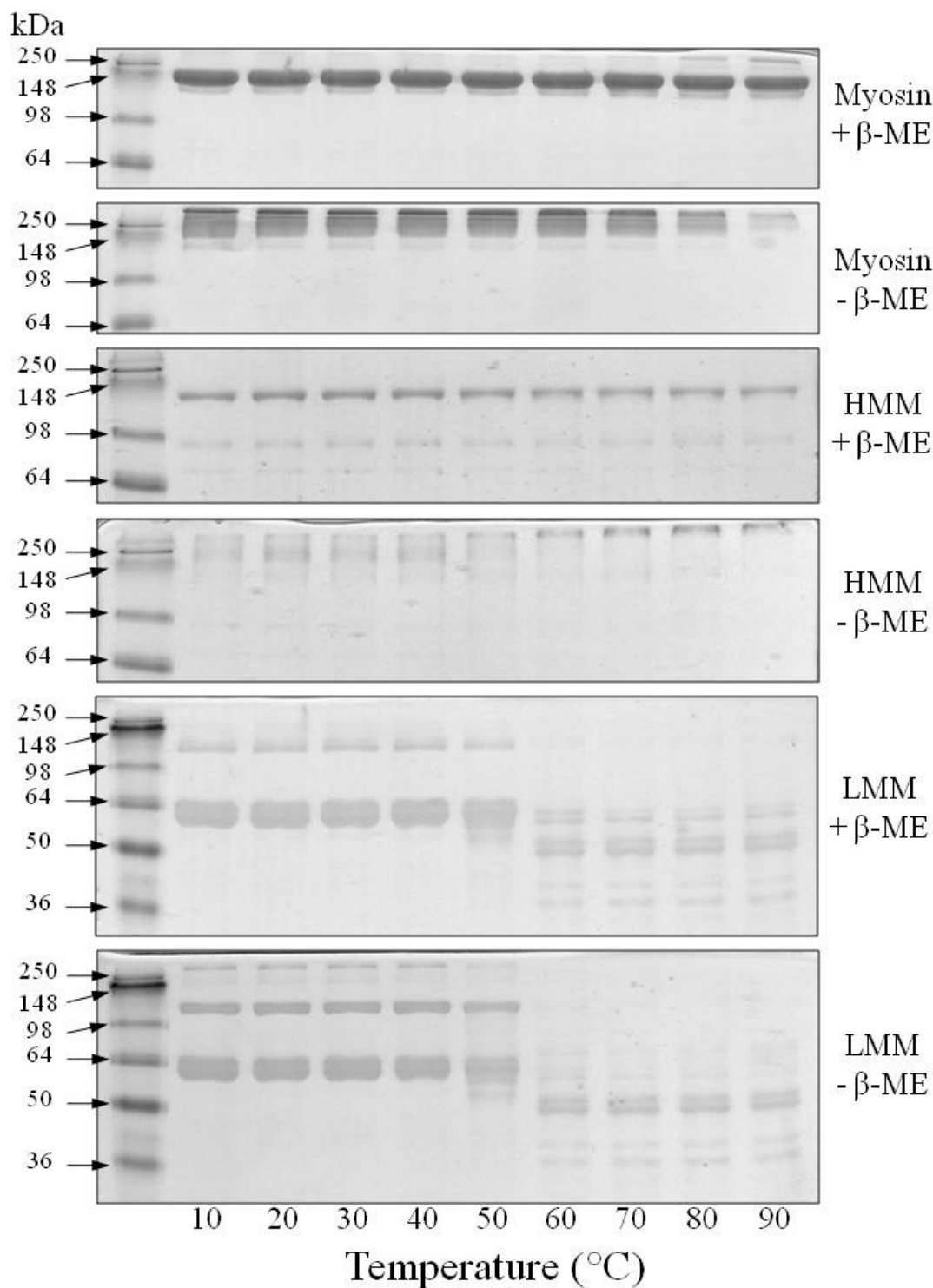


Figure 7.4 – Comparison of SDS-PAGE patterns for reducing (+βME) and non-reducing (-βME) linearly heated (1 °C/min) samples of myosin, HMM, and LMM

SDS-PAGE patterns of linearly heated LMM showed protein patterns that were very similar for reducing and non-reducing SDS-PAGE with the notable exception of a very high molecular weight (>250 kDa) protein band being seen on the non-reducing SDS-PAGE for samples heated from 10 to 40 °C. It is interesting to note the major change in protein band patterns of LMM under both reducing and non-reducing SDS-PAGE began at 50 °C. A significant reduction of the LMM band occurs at 60 °C with the appearance of a number of lower molecular weight protein bands (<64 kDa). As previously mentioned, the high α -helical content of LMM makes the –SH residues less available for interaction and aggregate formation while still in its native state. However, as can be seen by the rapid disappearance of the LMM at 60 °C what –SH residues are available appear to form very large (>250 kDa) aggregates. The unavailability of –SH residues in LMM would account for the lack of difference in protein band patterns for reducing and non-reducing SDS-PAGE.

Surface reactive and total sulfhydryl content

The total sulfhydryl (TSH) and surface reactive sulfhydryl (SRSH) content was measured to study the effect of heating on the structure of tilapia myosin, HMM, and LMM (Figure 7.5). The TSH content ranged from 6.3 to 8.2, 6.1 to 7.8, and 3.4 to 4.2 mole/10⁵g protein for tilapia myosin, HMM, and LMM, respectively while that of the SRSH ranged from 1.2 to 5.8, 4.7 to 6.6, and 2.6 to 3.3 mole/10⁵g protein for tilapia myosin, HMM, and LMM, respectively. These results are in agreement with previous studies done with tilapia (Ramirez, Martin-Polo & Bandman, 2000). The

gradual drop in TSH groups beginning at 30-40 °C is most likely due to the formation of disulfide linkages. Myosin, HMM, and LMM can form these disulfide linkages between SH groups that are exposed upon heating (Lanier et al., 2005). Figure 7.5 also shows the SRSH groups as affected by heating. The SRSH for myosin and HMM were relatively unchanged from 10 to 30 °C but increased significantly from 30 to 50 °C. The SRSH content of LMM was lower than that of the TSH content of LMM but both showed a slightly decreasing trend as the sample was heated.

As the proteins are heated they begin to unfold and expose more of the SH bonds that are buried in the native state. Lin et al. (1998) studied the effect of KCl concentration on the SRSH of salmon myosin and found that as salt concentration increased and began to disrupt the ionic bonds in myosin the myosin began to denature and expose previously buried SH groups. Yongsawatdigul et al. (2003) showed that the SRSH groups of threadfin bream actomyosin increased as the sample was tested at increasing temperatures. Taking the results of Lin et al. (1998) and Yongsawatdigul et al. (2003) together it is easily seen that many of the SH groups of proteins are buried and are exposed by denaturation with either chemical or physical denaturing processes.

HMM with a molecular weight of approximately 350 kDa has approximately 25 –SH residues per molecule (Asghar, 1985) while that of LMM has approximately 4-5 –SH residues per molecule (Lowey et al., 1969). The TSH content of tilapia HMM is nearly double that of tilapia LMM. It has been established that the α -helix content of LMM can be as high as 95% (Barker & Dayhoff, 1976) and this high

content of α -helices may account for low –SH content of LMM. Ogawa, Tamiya, and Tsuchiya (1996) found that the α -helical content of LMM from fish is highly species specific. Ogawa, Tsuchiya, Nakai, An, Nakamura, and Horimoto (1999b) studied the α -helix content of actomyosin (AM) from tilapia, lemon sole, ling cod, and rock fish and they found that heating at 40 °C for 30 min caused denaturation. They found that for all species except tilapia the α -helix content decreased considerably with heat treatment, noting that the tilapia α -helix content only decreased by 14% indicating its thermo stability. These findings correlate well with our TSH studies of LMM indicating a low level of –SH residues.

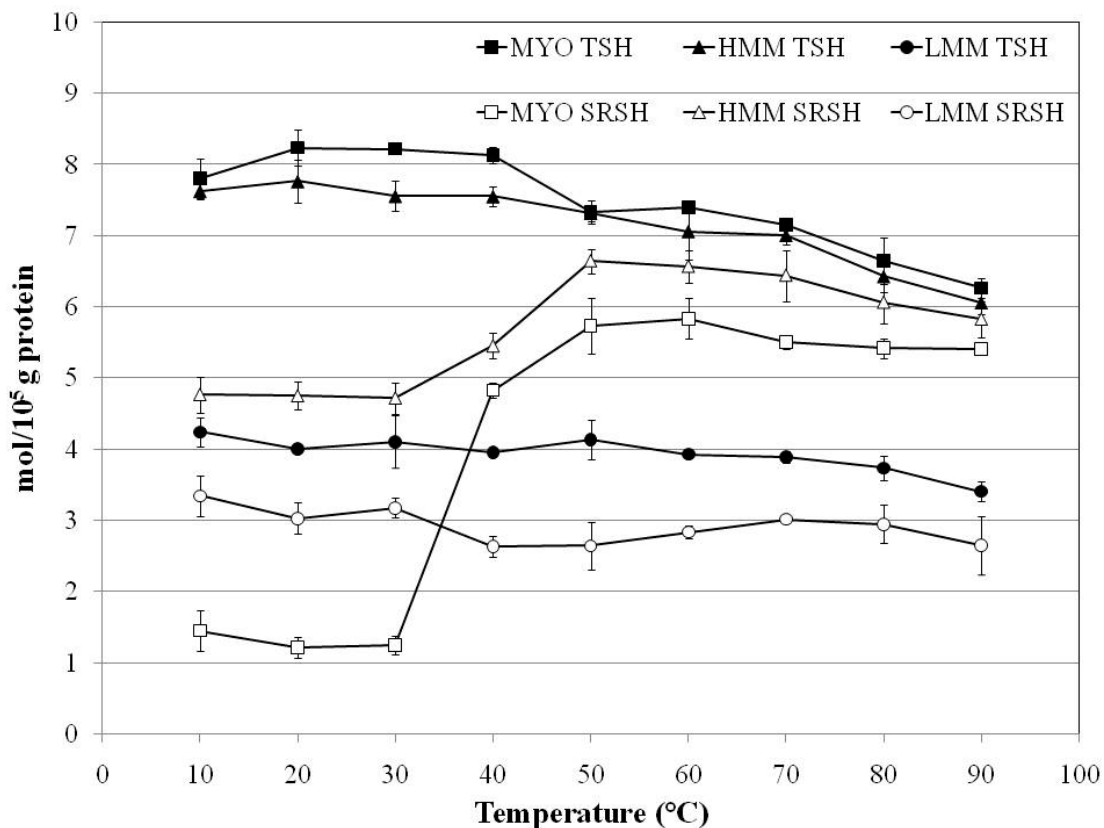


Figure 7.5 – Changes in total sulfhydryl content (TSH) and surface reactive sulfhydryl content (SRSH) of tilapia myosin, heavy meromyosin (HMM), and light meromyosin (LMM) heated at 1 °C/min.

Chan, Gill, and Paulson (1992) found that some regions of the myosin molecule are less thermo stable than others and have a tendency to denature before the whole myosin molecule is completely denatured. The trend that is seen in the TSH and SRSH groups of declining sulfhydryl content can be explained by the formation of disulfide bonds as the protein is denatured. The decline in free sulfhydryl groups of the TSH samples begins at a lower temperature than that of the SRSH samples. The free sulfhydryl group content begins to decline at a lower temperature because of the

denaturing buffer that is used for the TSH testing. The 8 M urea and 40 °C temperature facilitates the denaturing and opening up of the protein thereby exposing the previously buried sulfhydryl groups. Once the sulfhydryl groups are exposed they are available to be measured or to react with other free sulfhydryl groups.

Surface hydrophobicity

Hydrophobic interaction plays an important role in gel formation (Lanier et al., 2005). Tilapia myosin, HMM, and LMM proteins can be denatured by heating. As proteins are denatured they begin to open up and expose their hydrophobic core. Consequently, as the denatured proteins open they begin to interact with other hydrophobic regions on the same protein (intra) or with other denatured proteins (inter). The water hating (hydrophobic) regions of the proteins are excluded from water which leads to a stronger interaction between them. It has been suggested that the exposure of hydrophobic domains is a prerequisite for the formation of large myosin aggregates (Egelandsdal et al., 1995). Lin et al. (1998) showed that the S_0 of salmon myosin increased as KCl content increased, which supports findings that hydrophobicity increases as proteins are denatured and allows for increased inter and intra protein hydrophobic bonding.

In order to understand how tilapia myosin, HMM, and LMM interacts during heating the surface hydrophobicity (S_0) was measured using linearly heated samples. Figure 7.6 shows how S_0 of tilapia myosin, HMM, and LMM were affected by linear heating at 1°C/min. The S_0 of tilapia myosin and HMM showed a marked increase

between 30 and 50 °C and reached a stable plateau at 70 °C. LMM, in an extremely small magnitude, also showed a continued increase to 70 °C. All protein samples followed similar trends, however, the magnitude of increased S_0 of LMM was much lower than that of myosin and HMM.

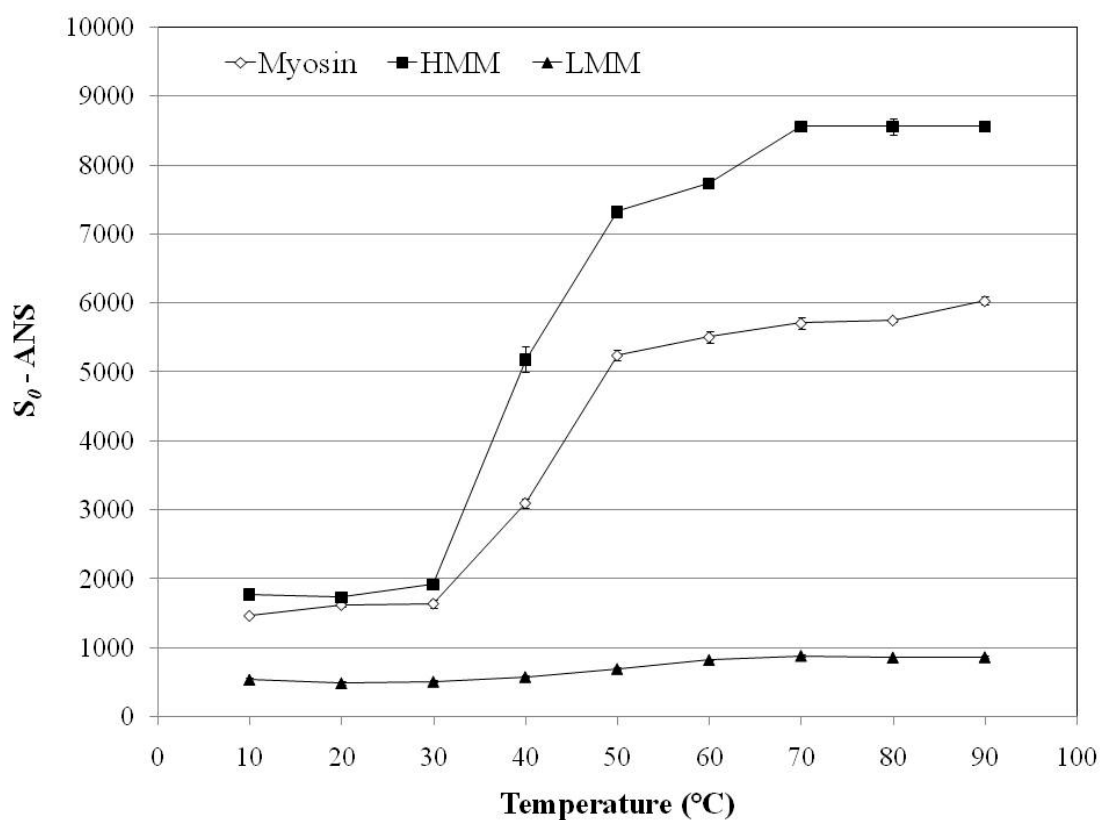


Figure 7.6 – Changes in surface hydrophobicity (S_0) of tilapia myosin, HMM, and LMM heated at 1 °C/min.

Differential scanning calorimetry

Whole muscle tilapia (Figure 7.7) exhibited five major peaks at 19.2, 52, 59.8, 71.4, and 81.9 °C. The first peak at 19.2 °C was exothermic with the other four peaks

being endothermic in nature. The enthalpies of denaturation of each major transition were -0.0269, 1.3205, 0.0368, 0.2467, 0.1707 J/g, respectively. Purified myosin showed three major peaks at 17.5, 41.9 and 49.9 °C, with enthalpies of denaturation - 0.0022, 0.0400, and 0.0146 J/g, respectively. HMM showed one endothermic peak at 43.0 °C and one major exothermic peak at 67.1 °C, with enthalpies of denaturation of 0.0143, -0.0517 J/g, respectively. LMM showed three endothermic peaks at 40.4, 51.7, and 69.0 °C, with enthalpies of denaturation of 0.0459, 0.0593, 0.0047 J/g, respectively (Figure 7.8).

Liu, Foegeding, Wang, Smith, and Davidian (1996) showed thermal transition points at 35.3, 48.1, 49.9, and 67.0 °C for chicken breast myosin (0.6 M NaCl, 50 mM phosphate buffer pH 6.0). When studying rabbit myosin head (subfragment 1) in 50 mM TRIS buffer (pH 8.0), 0.6 M KCl, along with heavy meromyosin in 0.1 M KCl, Shriver and Kamath (1990) found exothermic peaks at 48 and 65 °C. They also found that the exothermic peak was attributed to aggregation and precipitation of the unfolded protein. Park and Lanier (1988) found that this exothermic peak near 50 °C was a likely indicator of fish myosin in the pre-rigor state. They explained that it was likely due to a rapid rise in ATP hydrolysis induced by rising temperatures. Like their tilapia study, we also used myosin prepared from pre-rigor tilapia and obtained an exothermic peak near 67.1 °C in the HMM fraction.

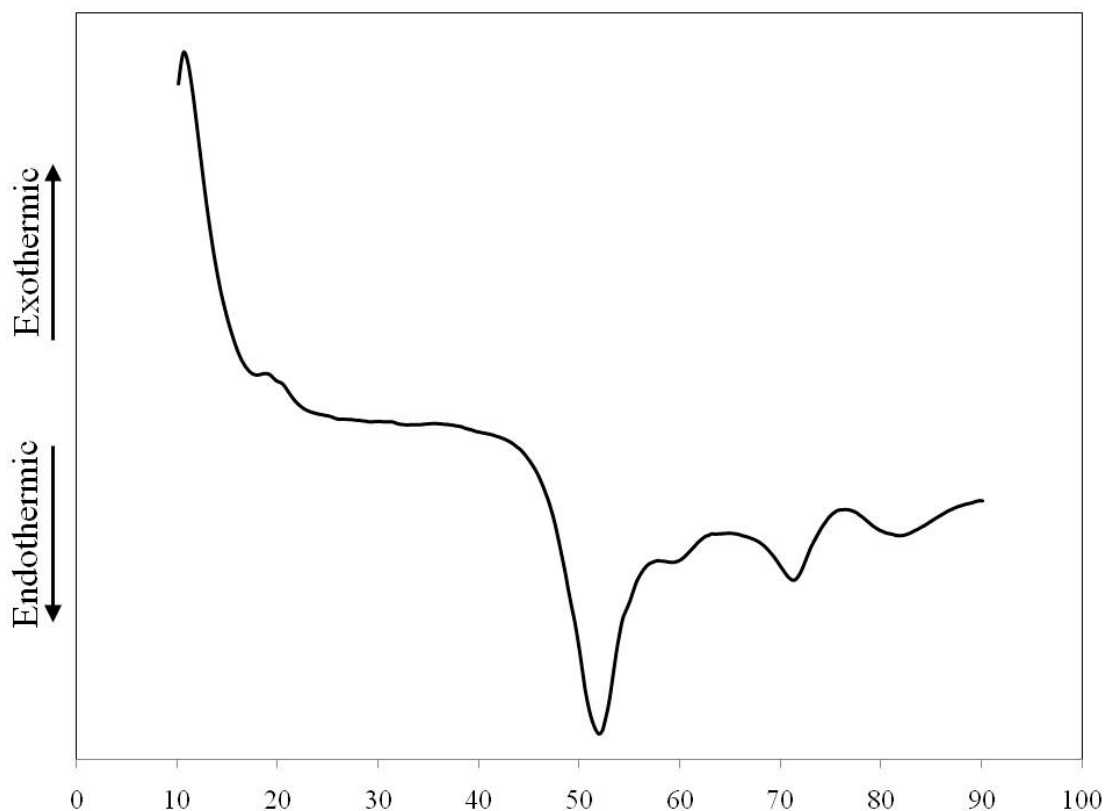


Figure 7.7 – DSC thermogram of whole muscle tilapia heated from 10 to 90 °C at 1 °C/min.

It has been shown that the thermal stability of fish myosin increases as the species adapts to increases in environmental temperatures (Davies et al., 1988). When carp was acclimatized to different holding temperatures (10, 20, 30 °C) DSC thermograms demonstrated a significantly increased thermal stability of myosin and LMM from carp stored at 30 °C (Nakaya et al. (1997; 2002). They showed thermal transition temperatures at 35.9, 39.7, and 49.1 °C for the carp acclimatized to 30 °C, whereas those for the 10 °C carp were lower at 32.8, 34.9, and 49.1 °C. Togashi et al. (2002) studied myosin and LMM from Alaska pollock and they found that for myosin there was two major endothermic peaks at 33.7 °C and 41.3 °C. The endothermic

peaks for our tilapia study were shown at higher temperature of 41.9 and 49.9 °C which may be explained by the habitat of the fish. Alaska pollock is cold water fish species while tilapia is a warm water species (Park, 2005). When studying the differences in gel forming ability of tilapia and Alaska pollock, Klesk, Yongsawatdigul, Park, Viratchakul, and Virulhakul (2000) found that there were significant differences in the optimal cooking temperatures for surimi produced from each fish. Due to its warm water habitat the tilapia showed increased shear stress and shear strain values when the surimi was set at 40 °C for 1 h before cooking at 90 °C for 15 min. These values were higher than those of Alaska pollock surimi gels. Our results coincide with a setting temperature of 40 °C, with endothermic peaks appearing at 41.9, 43.0, and 41.1 °C for myosin, HMM, and LMM respectively.

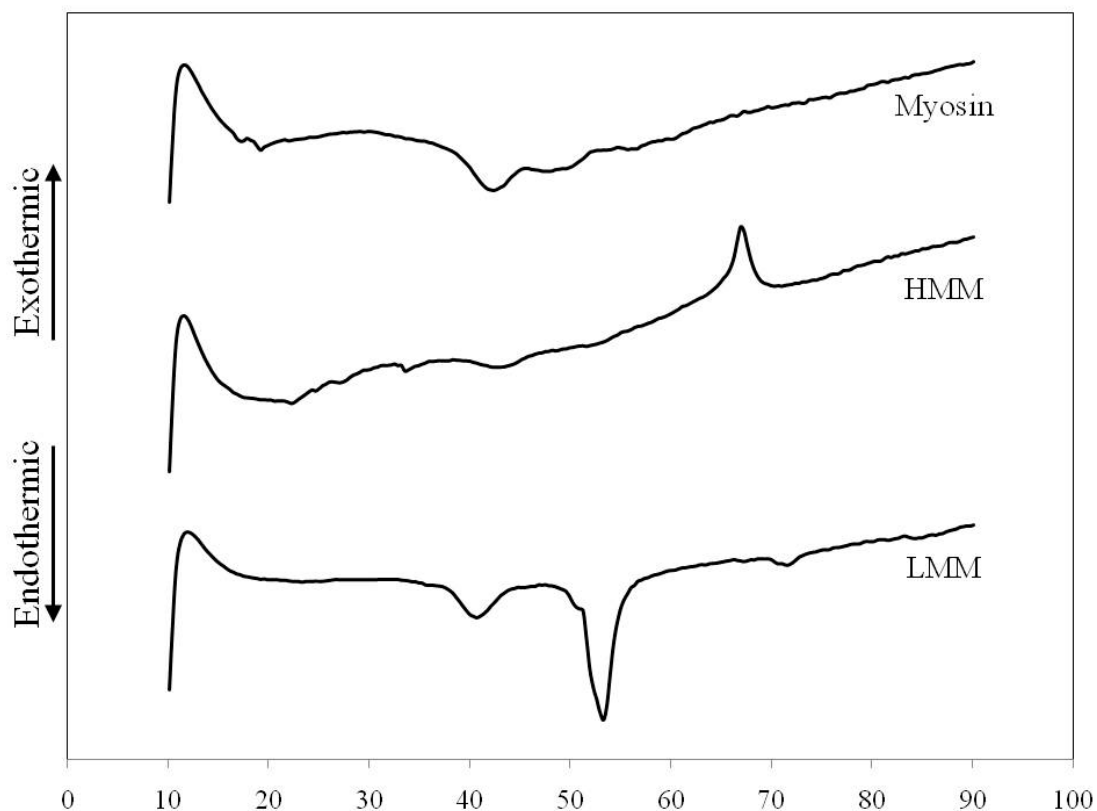


Figure 7.8 – DSC thermogram of tilapia myosin, HMM, and LMM heated from 10 to 90 °C at 1 °C/min.

The endothermic peaks noted at 42.4, 43.1, and 41.1 °C for myosin, HMM, and LMM corresponds well with the thermal gelation point (40.3 °C) of myosin as determined by dynamic rheology. As explained below, dynamic rheology revealed that the gelation point of HMM was 26.3 °C, however there was no corresponding endothermic peak produced from the DSC thermogram of HMM.

Dynamic rheological measurement

Tilapia myosin, HMM, LMM were subjected to dynamic rheology measurements in order to better understand the gelling capacity and the role that each

portion of myosin and its subfragments play. The storage modulus G' is a measure of the magnitude of energy that is stored or recovered in the material per cycle of deformation. The loss modulus G'' is a measure of the energy lost as heat through the viscous component per cycle of deformation. The phase angle is given when the G' and G'' are taken together and they can be expressed as the $\tan-\delta$ (G''/G'). This phase angle can be used to help determine the nature of the sample.

Myosin showed a gradual increase in G' as the sample was heated from 10 to 39 °C and at approximately 40 °C there was a dramatic increase for G' (Figure 7.9). The increase of myosin G' around 40 °C is in agreement with rheological studies performed by Ingadottir and Kristinsson (2010). G' for tilapia HMM showed a general increase from 10 to 42 °C and then became relatively flat from 42 to 90 °C.

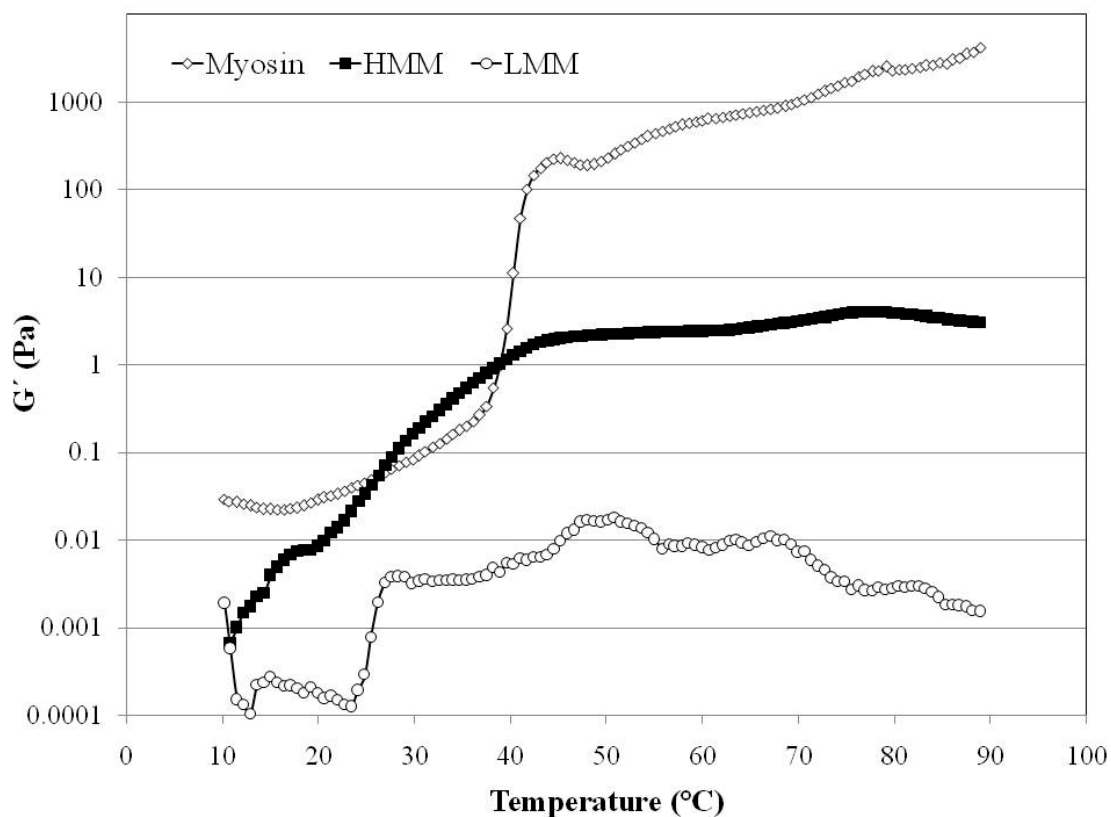


Figure 7.9 – Changes in storage modulus of myosin, HMM, and LMM during temperature sweep (1 °C/min)

The gelation point, which is often described as the crossover of G' and G'' ($\log G' = \log G''$) (Friedrich et al., 1988; Winter, 1987; Winter et al., 1986). The gelation points for myosin and HMM were 40.3 and 27 °C respectively (Figure 7.10 a-b).

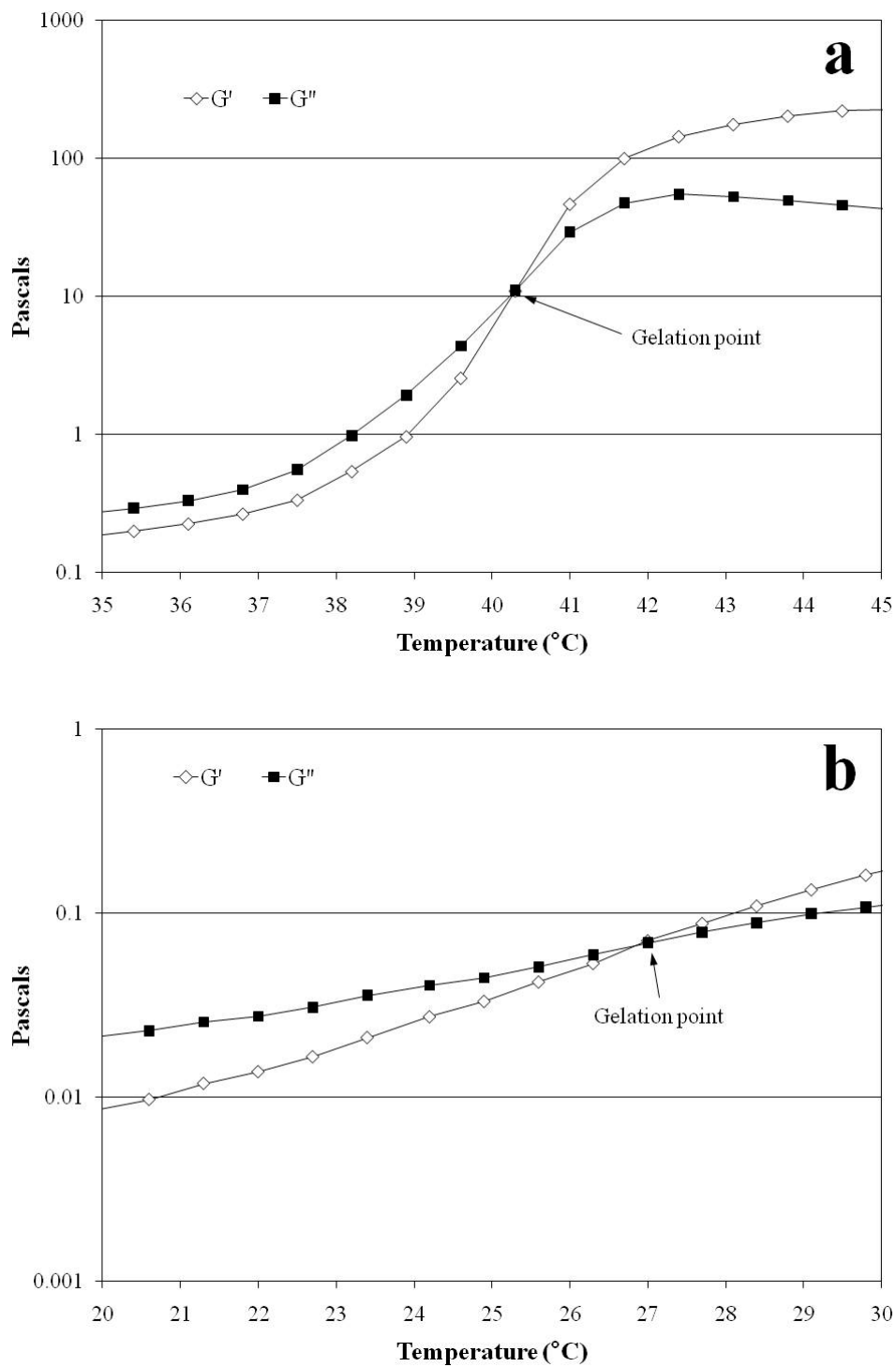


Figure 7.10 – Gelation points shown for tilapia myosin (a) and tilapia HMM (b) heated at 1 °C/min. The gelation point is defined as the point at which $\log G' = \log G''$.

LMM subjected to dynamic rheological testing never achieved a gelation point as defined by $\log G' = \log G''$. This may be due to the low concentration of LMM (0.7 mg/mL) that was obtained from the extraction and digestion process of tilapia myosin.

Figure 7.11 shows the phase angle of the tilapia myosin, HMM, and LMM heated at 1 °C/min. These results are in agreement with studies done using Pacific whiting and cod myosin (Yongsawatdigul et al., 1999). The phase angle presented a pattern that corresponds inversely to the changes in G' . The phase angle of LMM decreases significantly around 27 °C indicating that the LMM is involved in the gelation of myosin at low temperatures. When studying the myosin (Figure 7.11) it was shown that around 35 °C there may have been a partial rupture of the semi-gel being formed which allowed for the release of unbound water thereby increasing the phase angle. As heating continued the released water was restructured into a firmer gel texture as indicated by the continued decrease of the phase angle.

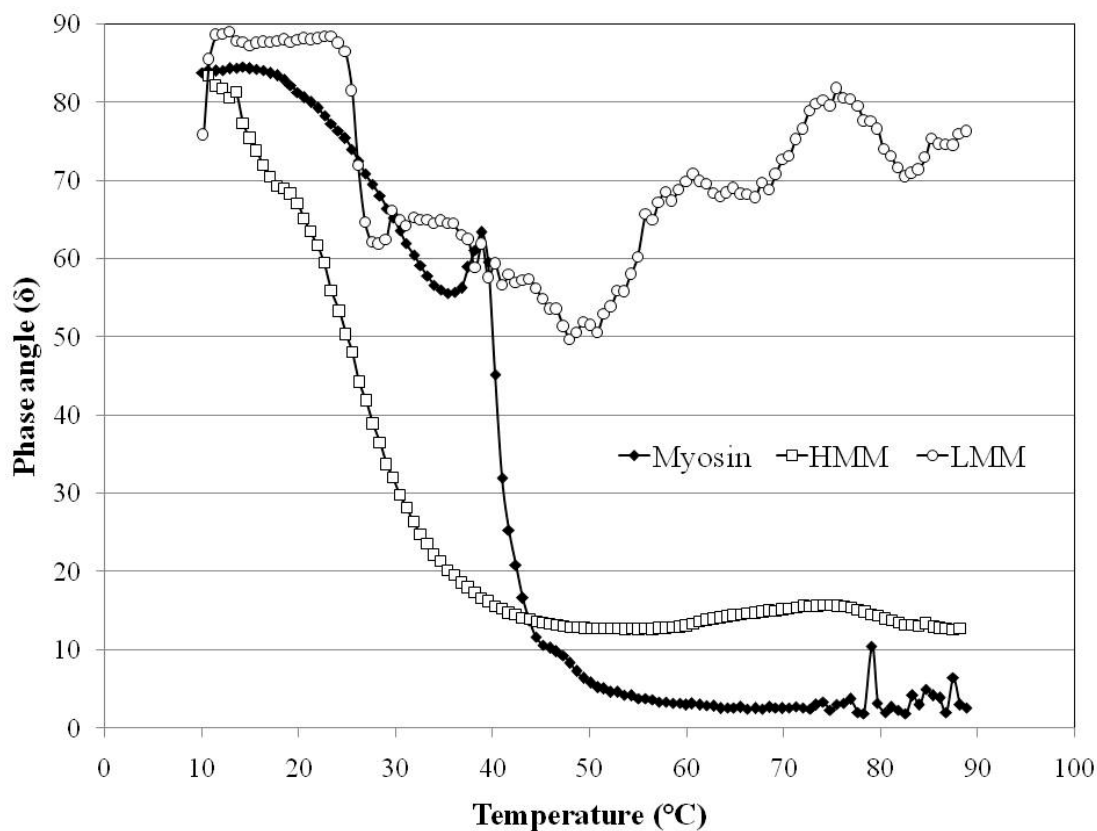


Figure 7.11 – Changes in phase angle (δ) during temperature sweep of tilapia myosin, HMM, and LMM as affected by heating rate (1 °C/min).

7.5 CONCLUSIONS

The study of tilapia myosin, HMM, and LMM carried out from chymotryptic digestion of myosin allowed for important insights into the thermo stability and gelation properties of tilapia. As myosin was heated in solution myosin aggregates began to form as evidenced by the increase of turbidity. Myosin denaturation and gel formation were also supported by the thermal transition points determined from differential scanning calorimetry and dynamic rheology. The exposure of buried hydrophobic and sulfhydryl groups of myosin and HMM increased as the myosin and

HMM were linearly heated. However the TSH and SRSH results indicated that the stability of LMM was likely due to its α -helix conformation. Studies using reducing and non-reducing SDS-PAGE helped to investigate the role that disulfide bonds play in the thermal aggregation of tilapia myosin, HMM, and LMM.

8 GENERAL CONCLUSIONS

Obtaining antibodies that would bind the target protein in the highly processed crabstick proved to be a key for the success of the assay development. Ab anti-H-OA and pAb anti- β -LG were shown to be highly specific for the target protein and showed no cross-reactivity with non-target proteins found in the prepared crabstick. An optimized solution for extraction was made with 10% SDS and 2.5% β ME. This optimized extraction solution demonstrated high reproducibility for the prepared crabsticks. An indirect ELISA assay was tested and proved as an effective method for the qualification and quantification DEW and WPC in a cooked and pasteurized crabstick. All 3 levels of DEW protein additive were estimated to less than 7% of the actual value and those of the WPC were estimated to less than 17%. Accuracy of the WPC assay may be improved by producing an antibody from the heat denatured form of β -LG.

The anti-pep-AP antibody proved to be very accurate in the qualification and quantification of Alaska pollock surimi used in crabsticks. Using the appropriate ELISA format, competitive vs. indirect, proved to be pivotal in obtaining accuracy and repeatability of the assay. The anti-pep-AP antibody worked very well for the detection of MLC 1 from Alaska pollock surimi. However cross-reactivity was noted in the immunoblot with the MLC 1 of Pacific whiting and threadfin bream. This cross-reactivity could prove to be beneficial for future studies with possibilities of the production of a sandwich ELISA that could use one capture antibody for more than one species of fish. A competitive ELISA assay was tested and proven as an effective

method for the qualification and quantification of Alaska pollock surimi used in cooked and pasteurized crabsticks. All 3 levels of Alaska pollock surimi used in the verification batches were estimated to less than 9% of the actual value of surimi content.

Using a direct sandwich ELISA for the qualification and quantification Alaska raw fish fillets provided good estimation of Alaska pollock used in the verification sample. However, the Alaska pollock MLC 1 antibodies exhibited high levels of cross reactivity with other fish species. When individual species were tested for the possibility of using each species as its own standard curve the other fish species exhibited a high degree of variability and a low degree of accuracy. Using the Alaska pollock standard curve to quantify the amount of Alaska pollock in a mixture of species also had extremes in variability and accuracy proving unsuitable for use as a method to determine the amount of Alaska pollock in a mixture of fish. Expanding the scope of the study to test for the possibility of the MLC 1 antibodies being specific for fish only and not other animal species testing was done to check for cross-reactivity with a variety of animal species. Testing determined that the antibodies had sufficient cross-reactivity to rule out the possibility of using the MLC 1 ds-ELISA for differentiation between animal species.

It has been shown that salmon myosin can be affected by heating. As myosin was heated in solution myosin aggregates began to form as evidenced by the increased turbidity. Myosin denaturation and gel formation was also supported by the thermal transition points determined from differential scanning calorimetry and dynamic

rheology. The exposure of buried hydrophobic and sulfhydryl groups of myosin was increased as the myosin was linearly heated.

The study of tilapia myosin, HMM, and LMM carried out from chymotryptic digestion of myosin allowed for important insights into the thermo stability and gelation properties of tilapia. As myosin was heated in solution myosin aggregates began to form as evidenced by the increase of turbidity. Myosin denaturation and gel formation were also supported by the thermal transition points determined from differential scanning calorimetry and dynamic rheology. The exposure of buried hydrophobic and sulfhydryl groups of myosin and HMM increased as the myosin and HMM were linearly heated. However the TSH and SRSH results indicated that the stability of LMM was likely due to its α -helix conformation. Studies using reducing and non-reducing SDS-PAGE helped to investigate the role that disulfide bonds play in the thermal aggregation of tilapia myosin, HMM, and LMM.

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APPENDIX

QUALIFICATION AND QUANTIFICATION OF FISH PROTEIN IN
PREPARED SURIMI CRABSTICK

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10.1 ABSTRACT

Species identification and protein quantification in surimi crabstick were achieved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). When Lowry and Kjeldahl protein determination methods were compared, the former showed more consistent results. Densitometric scanning of the gels was used for quantification of total fish protein as well as total egg white protein. The lower molecular weight proteins proved to be the most useful in fish species identification as well as egg white protein addition. Using a combination of the myosin heavy chain band and the species specific myosin light chain (Alaska pollock: 22.5 kDa; Pacific whiting: 24.4 kDa) proved the most accurate in calculating fish protein content of the crabstick sample, while those samples that contained egg white, quantification was accomplished from the densitometric analysis of the overlapping bands of actin (45 kDa) from fish and ovalbumin from egg white. Lysozyme (14.3 kDa) proved to be a unique protein band in determining the presence of egg white when the content of dried egg white was equal to or exceeded 0.5%.

10.2 INTRODUCTION

Surimi is the intermediate product produced through heading, gutting, mincing, washing, dewatering, and concentrating the myofibrillar proteins from fish (Lee, 1984). Surimi is primarily used as the base ingredient for crab-flavored seafood (crabstick). Crabstick quality is directly related to the quantity and quality of surimi present in the final product. Alaska pollock (AP) (*Theragra chalcogramma*) is considered by most manufacturers as the premium fish species for surimi production. Global surimi production in recent years has been close to 600,000 metric tons a year, according to Guenneugues and Morrissey (2005). For the last 25 years, consumption of U.S. surimi seafood has increased to 200 million pounds. However, recently, the market has suffered a trend of slower growth, due to increasing raw material costs and poor product quality.

Pacific whiting (PW) (*Merluccius productus*) is also used in the U.S.A. for surimi production. However special attention needs to be used due to endogenous proteolytic enzymes found in the fish (Morrissey, Hartley & An, 1995). Increasingly over the past 5 years, U.S. surimi seafood manufacturers have faced a significant challenge from low quality imports. Many imports claim they contain high quality Alaska pollock surimi when in fact they contain a lower quality species, higher amount of starches and/or protein additives, such as egg white, which are added to improve product texture at reduced cost. Currently, there are no standards for testing commercial crabstick products for verification and quantification of the fish species. However, one group has taken whole fish morphologically as a means to species

identification (Pepe, Trotta, DiMarco, Anastasio, Bautista & Cortesi, 2007b). A direct sequence analysis of the cytochrome b gene was applied to *Theragra chalcogramma*, *Merluccius merluccius*, *Merluccius hubbsi*, and *Merluccius capensis* along with 19 surimi seafood products labeled as *T. chalcogramma*. A phylogenetic analysis of surimi products demonstrated that mislabeling is a large-scale phenomenon. About 84.2% of surimi-based seafood products sold as *T. chalcogramma* were prepared with species different from the one declared. Only three samples were found to belong to *T. chalcogramma*. This method did not quantify the content of fish proteins.

Lower priced fish species are often substituted for more commercially important fish species. This practice has been documented in restaurant fish entrees (Hsieh, 1998). The identification of fish species for surimi is difficult because all morphological characteristics have been removed during processing of the fish. It has been shown, though, that isoelectric focusing can be used for the separation and identification of sarcoplasmic proteins (Hsieh et al., 1997; Mackie, 1996). Unfortunately, during surimi processing, the sarcoplasmic proteins are removed by repeated washings. Other methods such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) can be employed as a tool for the identification of fish species based on electrophoretic patterns of the respective fish proteins.

Species identification, primarily using sarcoplasmic proteins as the target proteins has been well documented in SDS-PAGE studies (An, Wei, Zhao, Marshall & Lee, 1989; Etienne et al., 2001; Piñeiro et al., 1999). However a method for the quantification of fish myofibrillar protein (surimi) using SDS-PAGE has not been

studied as intensely as species identification. Claeys and others (1995) have shown a linear relationship between densitometric readings and protein content of myofibrillar proteins in beef. In addition, Jin and Park (1996) have shown that densitometric readings can be used for quantification in goat milk cheese and the electrophoretic pattern can be used to differentiate it from cheese made of cow milk. Kolster and others (1992) have also shown that the high molecular weight wheat proteins of glutenin subunits are quantified using densitometry and Coomassie brilliant blue staining. Furthermore, Chen and Hwang (2002b) have shown that using the low molecular weight region (≤ 30.0 kDa) of myofibrillar extracts of 7 puffer fish species it was possible to identify the species.

The overall goal of this study was to develop standards for the qualification and quantification of not only the fish species, primarily using myofibrillar proteins, but to include other protein sources that are also used as additives in the manufacture of crabsticks. The potential outcome of this project could have a profound effect on the crabstick market here in the U.S. by allowing for the careful control of the quality of crabstick produced and imported.

10.3 MATERIALS AND METHODS

Alaska pollock (*Theragra chalcogramma*) surimi (FA grade, 10 kg block), was obtained from Western Alaska Fisheries (Seattle, Wash., U.S.A.) and stored frozen at -18 °C. Fresh Pacific whiting (*Merluccius productus*) surimi was obtained from Pacific Seafood Group (Warrenton, Ore., U.S.A.). Fresh surimi was put directly into

totes from the processing line and transported to the OSU Seafood Laboratory in a large cooler surrounded by ice. The fresh surimi was mixed with cryoprotectants (4% sugar, 5% sorbitol, 0.3% sodium polyphosphate) using a Hobart silent cutter (model VCM-40, Hobart Manufacturing Co., Troy, Ohio, U.S.A.) and formed into a 10 kg block, rapidly frozen to -20 °C using a plate freezer and stored frozen -18 °C. The 10 kg block was cut into approximately 1000 g blocks, individually vacuum packed, and stored at -18 °C until used in surimi crabstick paste preparation. Ingredients used for surimi crabstick paste preparation were NaCl (Morton Iodized Salt, Morton International, Inc., Chicago, IL, U.S.A.), corn starch (Corn Products International, Westchester, Ill, U.S.A.), wheat starch (Midsol 50, MGP Ingredients, Inc., Atchison, Kans., U.S.A.), Modified waxy maize starch (Polartex 06727, Cargill, Inc., Cedar Rapids, Iowa, U.S.A.), dried egg white (DEW) (K-200, Henningsen Foods, Omaha, Nebr., U.S.A.), sugar (used to represent all flavor components) (Pure Cane Sugar, C&H Sugar Company, Inc., Crockett, Calif., U.S.A.), and culinary tap water. Soy protein concentrate was provided by Solae (500E, Solae, St. Louis, Mo., U.S.A.). Whey protein concentrate was obtained from Hilmar Ingredients (WPC 8600, Hilmar Ingredients, Hilmar, Calif., U.S.A.)

Sample preparation

Basic surimi paste batches

Basic surimi crabstick paste batches, approximately 1100 g, were made using 4 different surimi contents (25, 30, 35, and 40%) and 4 different starch contents (12, 11,

10, and 9%). The moisture content of each batch was adjusted to $\approx 75\%$ using ice water. Frozen surimi was allowed to thaw at room temperature for approximately 1 h ($\approx 23\text{ }^{\circ}\text{C}$) and then cut into small pieces. All initial surimi paste chopping was performed using a Stephan vertical vacuum cutter (model UM 5 universal, Stephan Machinery Co., Columbus, Ohio, U.S.A.). The surimi pieces were added to the chopping bowl and then chopped on low speed for 1 min. Twenty-two g of salt were then added to the chopped surimi and chopping continued on low speed for 1 additional min. Then 55 g of sugar, used to simulate all flavor ingredients was added in addition to starch and water. For the 25, 30, 35, and 40% batches, 110, 100, 90, and 80 g of starch (50:40:10 corn:wheat:modified waxy maize) along with 616, 572, 528, and 484 g of ice water, respectively, were added and then chopped on low for 1 min. A vacuum of 40 – 60 kPa was applied to the surimi paste and chopping continued for 3 min on high speed. Surimi paste was then placed in a plastic bag which was submerged in a ice water slurry until portions were weighed for specific crabstick batches.

Specific surimi paste batches

Specific surimi paste batches were made to contain 0, 0.5, 1, and 2% DEW with a 1:1 direct replacement of starch with DEW. Approximately 196 g of surimi paste were weighed into the chopping bowl of a Kitchenaid Chef's Chopper (KFC3100WH, Kitchenaid, St. Joseph, Mich., U.S.A.). The weighed portion was brought to a final weight of approximately 200 g using 4:0, 3:1, 2:2, or 0:4 g ratios of

starch:DEW. Surimi paste was then chopped using 1 s pulses for 30 s after which the bowl was scraped with a rubber spatula. This procedure was repeated three times. The final surimi paste was then put into a labeled Whirl Pak bag (Fisher Scientific, Pittsburgh, Pa., U.S.A.) and the Whirl Pak bag was placed in ice water until formed into a sheet for cooking. Approximately 20-25 g of labeled surimi paste was placed into a sheet mold (stainless steel 25 cm x 7.5 cm x 1.4 mm) that was formed on top of a piece of aluminum foil, which had been sprayed with Pam® no-stick cooking spray (Pam® Original, ConAgra Foods, Inc., Omaha, Nebr., U.S.A.). Excess raw surimi paste was vacuum packed and frozen at -80 °C. Food grade plastic film was then laid over the top of the paste and a stainless steel tube roller (11.5 cm x 4 cm dia.) was used to evenly spread out the paste to form a crabstick sheet. After the film was removed the aluminum foil containing the crabstick sheet was cooked on a rack in a steam bath (93 °C) for 90 s. After initial cooking, crabstick sheets were cut in half, wrapped in plastic film, placed inside a labeled Whirl Pak bag, and then placed back in the 93 °C steam bath for 30 min to simulate a commercial pasteurization. Then samples were submerged in ice water to cool for 15 min. If samples were not to be used within 48 h they were frozen at -80 °C until needed.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as outlined by Laemmli (1970). Approximately 5.0 g of cooked and pasteurized crabstick sample were weighed into a 50 mL centrifuge tube and 20 mL of 5% (w/v) sodium dodecyl sulfate, which was heated to

90 °C, was added. Samples were homogenized for 1 min at speed 4 (Powergen 700, Fisher Scientific, Pittsburgh, Pa., U.S.A.) followed by incubation for 1 h at 90 °C. Incubated samples were then centrifuged for 20 min at 17,000 x g (Sorvall RC-5B, DuPont Co., Newton, Conn., U.S.A.). The supernatant was then poured into a 25 mL volumetric flask and brought to volume using additional 5% SDS solution. One mL of sample was mixed with 0.5 mL of Laemmli sample buffer (0.6 mL 1 M tris-HCl (pH 6.8), 5.0 mL glycerol, 2.0 mL 10% (w/v) SDS, 0.5 mL β -mercaptoethanol, 1.0 mL 1% (w/v) bromophenol blue, 0.9 mL dd H₂O). Sample and buffer were vortexed and then heated for 3 min at 90 °C in order for the β -mercaptoethanol in the sample buffer to disrupt any disulfide bonds. Samples were electrophoresed using a Mini-Protean III Cell (Bio-Rad, Hercules, Calif., U.S.A.).

Preliminary studies were conducted using 0.75 mm thick slab gels for protein separation but high quantities of myosin and actin in the crabstick caused these bands to spread and smear resulting in lower protein resolution. In order to overcome the smearing of the protein bands slab gels of 1 mm thickness were used and the desired resolution was achieved. Slab gels, 1 mm thick, consisted of a separating gel (12.5% T), which was polymerized for 45 min to 1 h, and a stacking gel (4% T), which was poured 15 min before sample application. A 15 well comb was used to ensure sufficient lanes would be available for sample loading. All samples were loaded with a total volume of 2 μ L using a 10 μ L Hamilton syringe (Hamilton Company, Reno, Nev., U.S.A.). The nature of the SDS samples, namely high concentrations of detergent, appeared to cause capillary like uptake of excess sample when being loaded

using the plastic tips along with the micropipette. The Hamilton syringe was used because it gave consistent results in loading the gels. Electrophoresis conditions were identically maintained for all gels, constant voltage of 200 V with an initial current of 30 mA was applied for the first 15 min and then current increased to 60 mA for 50 min. Completed electrophoresis gels were placed in 50 mL of 0.125% (w/v) Coomassie dye (Brilliant Blue Coomassie R-250, Bio-Rad, Hercules, Calif., U.S.A.) and gently shaken on an orbital shaker heated to 52 °C for exactly 30 min, The gels were then removed from the stain and de-stained using 100 mL of 50% (v/v) methanol and 10% (v/v) glacial acetic acid for 1 h while being heated to 52 °C on an orbital shaker. After 1 h, gels were placed into 100 mL of 10% methanol (v/v) and 10% (v/v) glacial acetic acid for 30 min, after which the gels were placed in 100 mL of dd H₂O for 30 min. Molecular weights were determined by comparing the relative band mobility of a molecular standard as outlined by Weber and others (1972). The protein standard used was the Kaleidoscope precision plus obtained from Bio-Rad (Bio-Rad, Hercules, Calif., U.S.A.). All samples were loaded in triplicate and the average trace quantity for each band was used for protein quantification.

Gel densitometry

The electrophoresis gels were scanned using a Gel Doc XR scanner (Bio-Rad, Hercules, Calif., U.S.A.) and were analyzed using Quantity One® (Bio-Rad Laboratories, Hercules, Calif., U.S.A.). The scanned images were stored and molecular weight was determined by comparing the relative front (Rf) values on the

gel with those of the protein standard. In order to obtain higher accuracy 2 lanes per gel were dedicated to the Kaleidoscope standard. For each sample, the total band area of three proteins, myosin heavy chain (MHC), actin (A), actin/ovalbumin (A/O), and myosin light chain (MLC) the samples were calculated as a percentage of the sum of major band areas. Lysozyme represents only 3.4% of total proteins found in egg white (Stevens, 1991) therefore lysozyme was used for qualification only due to the low levels and variability of the band intensity.

Kjeldahl protein assay

Total nitrogen content was calculated using the Kjeldahl method as outlined in AOAC (1995). Approximately 1.3 g of cooked and pasteurized crabstick sample was accurately measured. Samples were then digested in triplicate using a heating block (model BD 20, Fisher Scientific, Pittsburgh, Pa., U.S.A.) and distilled using a distillation unit (model 100, Fisher Scientific, Pittsburgh, Pa., U.S.A.). Samples were then titrated using 0.1 N HCl until a sample remained a light pink hue. Final protein concentration was calculated using a factor of 6.25 multiplied by total nitrogen. All samples were done in triplicate and the average reported.

Lowry protein assay

Supernatant from the SDS-prepared crabstick sample was diluted with dd H₂O at a ratio of 0.25:4.75 mL and then tested for protein content (Lowry et al., 1951). The supernatant was diluted for Lowry testing using dd H₂O due to the high concentration

of SDS interfering with protein determination (Peterson, 1983). Protein determination was also performed on the individual protein sources namely AP and PW surimi and DEW. All samples were conducted in triplicate. All Lowry experiments were performed using a UV-2401PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and data was obtained and analyzed using a UV Probe v1.11 (Shimadzu Corporation, Kyoto, Japan).

Calculation of protein content

Protein calculations were made by first measuring the density of each band followed by comparing it with the total protein applied to each well. Based on our preliminary experiments, we found the combination of using the band of interest area (BIA) compared to the major bands area (MBA, 13 bands total) gave consistent results.

BIA = Band of interest area

MBA = Major bands area

Protein = Concentration of protein as determined by Lowry (mg/mL)

$$\left(\frac{\text{BIA}}{\text{MBA}} \right) = \% \text{ BIA}$$

$$(\% \text{ BIA}) \times (\text{Protein}) = \text{Protein}\% \text{ BIA}$$

The protein calculated as Protein% BIA was plotted on the X-axis and the theoretical surimi content of crabstick was plotted on the Y-axis. In order to create an equation that could be used for calculating the percent surimi used in an unknown sample, the protein % BIA for each level of surimi content, 25, 30, 35, and 40% was plotted for the AP, PW, and AP/PW (50/50) surimi batches. The resulting lines for AP, PW, and AP/PW (50/50), all with 0% DEW, ($y = 3.44x + 1.91$, $y = 5.60x + 16.46$, $y = 5.13x + 16.82$, respectively) are given in Fig. 1. Similar lines and equations were created for all levels of DEW additive (graphs not shown). For the prepared crabstick samples that contained no DEW the myosin heavy chain, actin, and species specific myosin light chain band areas were used for the data in Figure 10.1. For samples that contained DEW only, the myosin heavy chain and species specific myosin light chain were used for creating the graphs and subsequent equations due to the overlap of ovalbumin and actin.

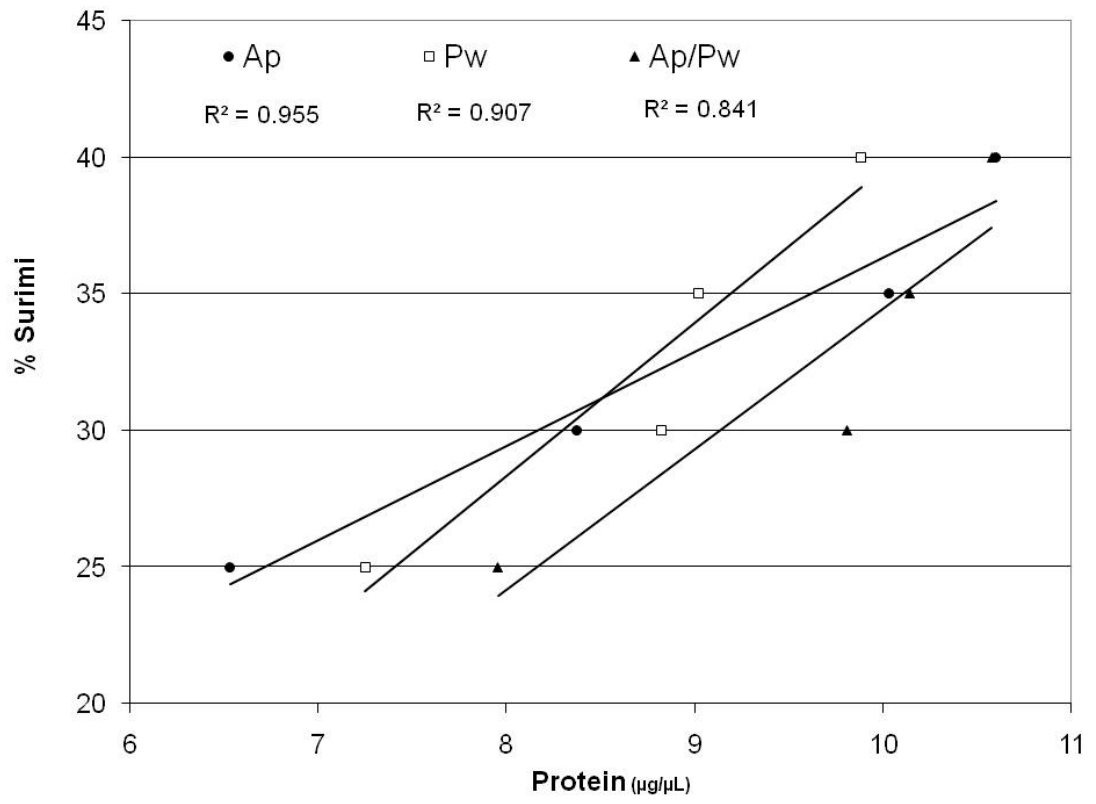


Figure 10.1 – Graph of Lowry determined protein concentration compared with theoretical percent surimi protein.

Statistical analysis

Triplicate data for protein content, determined using the Kjeldahl and Lowry methods, were averaged and reported as a mean and standard deviation. Triplicate trials, done on each SDS-PAGE run, for each crabstick sample were averaged and reported as the mean and standard deviation, which were then used for protein quantification. Surimi content for samples that contained DEW additive, and egg white content alone were calculated with the mean and standard deviation being

reported. All mean and standard deviations were calculated using Microsoft Excel (Microsoft Corporation, Redmond, Wash., U.S.A.).

10.4 RESULTS AND DISCUSSION

Protein determination

Total protein was determined using two common methods, Kjeldahl total nitrogen determination and Lowry total protein determination. The values used for the determination of linearity were given by calculating the total percentage of protein in each species specific crabstick sample with 3 levels of surimi content and 3 levels of DEW content. For example, the R^2 value for the Alaska pollock 30% surimi batch was given by plotting the percent protein content (Kjeldahl: 5.1, 5.5, 5.8, 6.1; Lowry: 4.6, 5.4, 5.7, 6.4) for the crabsticks containing 0.0, 0.5, 1.0, and 2.0% DEW, respectively. The R^2 values of both methods used for the crabstick samples for linearity were compared (Table 10.1). It was determined that the Lowry method proved to be more accurate and therefore only the Lowry method was used for protein determination of the verification batches. The protein content for AP and PW surimi was measured using the Lowry method and determined to be $14.5 \pm 0.02\%$ and $13.5 \pm 0.19\%$ respectively. The protein content of DEW used was calculated as $82.4 \pm 3.2\%$.

%	Alaska pollock		Pacific whiting		Alaska pollock and Pacific whiting	
	Lowry	Kjeldahl	Lowry	Kjeldahl	Lowry	Kjeldahl
25	0.9171	0.9947	0.9857	0.9554	0.9993	0.9209
30	0.9943	0.9607	0.9300	0.9218	0.9523	0.9639
35	0.9928	0.9637	0.9998	0.9607	0.9568	0.9158
40	0.9799	0.9660	0.9553	0.9277	0.9792	0.9724

Table 10.1 – Comparison of R^2 values for Lowry and Kjeldahl protein determination methods for standard prepared crabstick samples.

SDS-PAGE

The electrophoretic patterns of AP and PW surimi, along with common protein additives (DEW, WPC, and SPI) were compared (Figure 10.2). In the surimi industry, we found almost all of commercial surimi seafood contains egg white, while WPC and SPI are used on a limited scale. Therefore, WPC and SPI were not included in our introductory study, but were used for electrophoretic pattern comparison only.

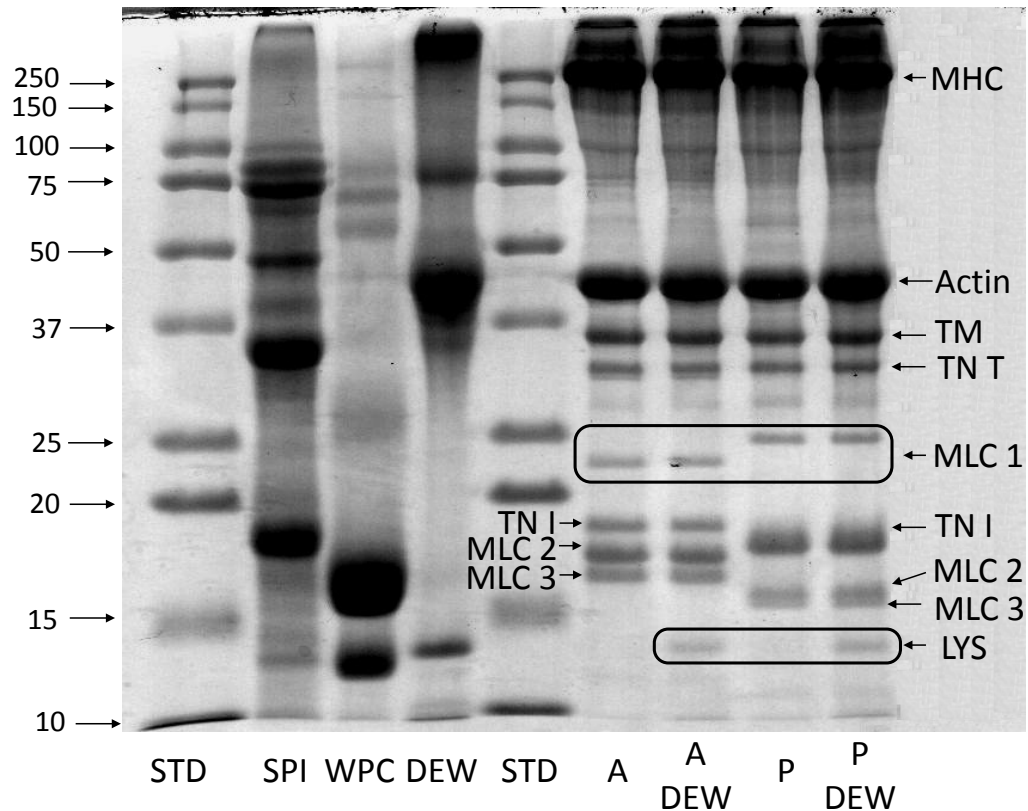


Figure 10.2 – SDS-PAGE pattern of protein sources. STD = kaleidoscope protein standard (kDa); SPI = soy protein isolate; WPC = whey protein concentrate; DEW = dried egg white; AP = Alaska pollock; A DEW = Alaska pollock crabstick with DEW; PW = Pacific whiting; P DEW = Pacific whiting crabstick with dried egg white. MHC = myosin heavy chain, AC = actin, TM = tropomyosin, TN T = troponin T, MLC (1, 2, and 3) = myosin light chain, TN I = troponin I, LYS = Lysozyme.

Two primary WPC bands were observed at 18.1 and 14.7 kDa for β -lactoglobulin and α -lactalbumin, respectively (Farrell et al., 2004). SPI showed two distinctively thick bands at 33.2 and 18 kDa for β -conglycinin and glycinin, respectively (Brooks & Morr, 1985; Samoto et al., 2007). There were 6-8 thinner bands as well. DEW, which was used as the primary target protein additive in our study, showed three distinctive bands at 76.6, 45, and 14.3 kDa indicating conalbumin,

ovalbumin, and lysozyme respectively (Stadelman & Cotterill, 1977). When surimi samples were compared to the DEW the primary band of DEW, ovalbumin overlapped the actin band from the fish, presenting a difficulty in quantifying and qualifying egg white content. However DEW contains a unique band around 14.3 kDa that is not seen in the pure surimi samples (Fig. 2) by comparing the 2 lanes for AP (AP and A DEW) and the 2 lanes for PW (PW and P DEW). This band can be used as a target protein in determining the presence of egg white.

Figure 10.3, representing 1 of 12 SDS-PAGE runs (Alaska pollock: 25, 30, 35, and 40%; Pacific whiting: 25, 30, 35, and 40%; 50/50 Alaska pollock/Pacific whiting: 25, 30, 35, and 40%), demonstrates the SDS-PAGE patterns of 30% Alaska pollock surimi gels containing 0-2% DEW. It clearly showed that the density of the lysozyme band increased as the content of DEW increased from 0 to 2% with a detection threshold of 0.5% DEW. We also noted the gradual increase of the density of the 45 kDa band, which we believe is the result of the combined proteins of actin and ovalbumin. Similar results were observed for all 12 SDS-PAGE runs.

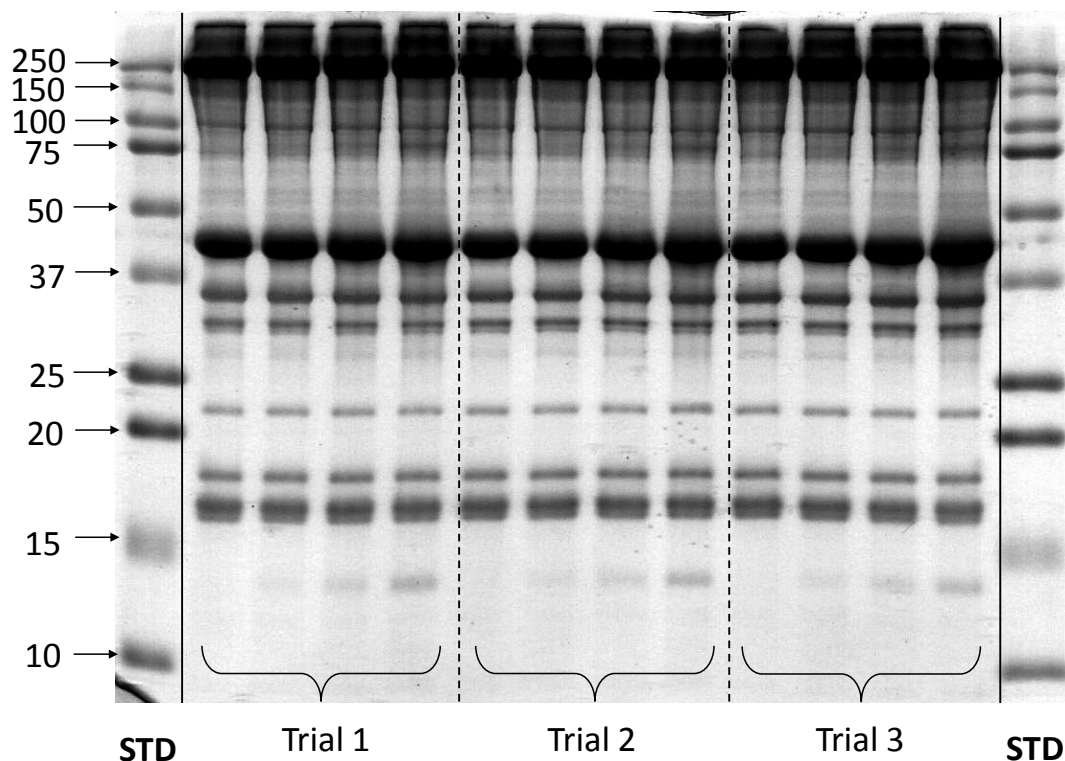


Figure 10.3 – SDS-PAGE pattern of prepared crabstick with 30% Alaska pollock and DEW. STD = kaleidoscope standard (kDa); Samples were loaded in triplicate trials, the value for the sample was given as the average of the 3 trials for each sample, and each trial contained the following percentages of DEW: 0%, 0.5%, 1%, and 2%, respectively.

Verification

Surimi content

In order to verify our qualification and quantification of proteins, two unknown samples, A and B, containing, surimi percentages at 27.5% and 32.5%, respectively, were randomly created. From each of these formulations, two batches were made, one with 0% DEW addition and the other with 1% DEW addition. Results for all surimi content estimations are shown in Table 10.2. For the unknown batches A and B that

were shown to contain no DEW the protein, by visual inspection of the SDS-PAGE run, protein % BIA was calculated for the myosin heavy chain, actin, and myosin light chain as in the prepared crabstick samples batches. The value of the protein % BIA was then substituted into the batch specific equation and the resulting y value was the percent surimi used in the unknown batch. Each species batch, AP, PW, and AP/PW (50/50) created one equation from the 0% DEW additive; therefore no statistical analysis was done on these results. The surimi estimation for AP, PW, and AP/PW (50/50) unknowns for the A verification batches (27.5% surimi) containing no DEW were 30.5%, 24.9%, and 27.6%, respectively, while those for the B surimi verification batches (32.75% surimi) were 31.6%, 32.5%, and 31.5%, respectively. Our verification confirmed that the quantity of surimi for samples containing no egg white can be estimated with an accuracy of 90-100%.

Table 10.2 – Estimated surimi protein content of verification batches.

	Verification batches (surimi)			
	A		B	
	(27.5%) ^a	(27.5%) ^{a, c}	(32.5%) ^a	(32.5%) ^{a, c}
Alaska pollock	30.5 ^b	30.7 ± 0.83 ^{b, c, d}	31.6 ^b	33.2 ± 1.33 ^{b, d}
Pacific whiting	24.9 ^b	31.1 ± 3.31 ^{b, c, d}	32.5 ^b	31.7 ± 1.99 ^{b, d}
Alaska pollock & Pacific whiting (50/50)	27.6 ^b	30.2 ± 2.39 ^{b, c, d}	31.5 ^b	33.8 ± 1.72 ^{b, d}

^aTheoretical percent surimi.

^bCalculated surimi percent.

^cSamples containing DEW.

^dStandards were calculated using 0.5%, 1%, and 2% DEW. With 3 levels of DEW, 3 lines were created each with their respective slopes and intercepts. To calculate the surimi protein content, each of the 3 equations was used and the resulting values were averaged and a standard deviation reported.

The calculation for unknown samples that contained DEW was slightly more challenging to calculate. For unknown samples that contained DEW only the myosin heavy chain and myosin light chain band areas were used for calculating the protein % BIA. For each species batch there were 3 levels of DEW used 0.5, 1.0, and 2.0%. Three equations were then determined from the graph of these crabstick samples. Not knowing the level of DEW in the unknown batches the surimi content was calculated using each of the 3 equations given from the 3 levels of DEW. The surimi content was reported as the mean with a standard deviation for the unknown samples containing DEW (Table 2). The surimi estimation for AP, PW, and AP/PW (50/50) unknowns for the A verification batches (27.5% surimi) containing DEW was 30.7 ± 0.8%, 31.1 ± 3.3%, and 30.2 ± 2.4% respectively, while those for the B verification

batches (32.5% surimi) were $33.2 \pm 1.3\%$, $31.7 \pm 1.9\%$, and $33.8 \pm 1.7\%$, respectively. Our verification confirmed that the quantity of surimi can be estimated with an accuracy of 87-98%.

DEW content

Calculation of the DEW content was performed using the same equations as shown above. However for the DEW calculation only the combined actin/ovalbumin band was used. Because each level of surimi content, 25, 30, 35, and 40% contained a sample with 0.5, 1, and 2% by weight of DEW, three different standard curves were created for each of the DEW level contents, respectively. For the final calculation of DEW, the DEW content was calculated using each equation and then taking the average of the three results along with the standard deviation as the final calculated DEW content. The verification batches contained 0.82% by weight DEW protein. The DEW estimation for AP, PW, and AP/PW (50/50) unknowns for the A verification batches were $1.05 \pm 0.92\%$, $0.41 \pm 0.49\%$, and $1.02 \pm 0.62\%$ respectively, while those for the B verification batches were $0.97 \pm 0.91\%$, $0.89 \pm 0.40\%$, and $1.17 \pm 0.59\%$, respectively. Results show that the band intensity did vary as the DEW content increased but the combined band intensity given by the overlap of actin/ovalbumin did not yield linear results. As seen in Fig. 3 the lysozyme band (14.3 kDa) intensity increases as the DEW content increases. However, it could not be used for quantification due variations in band intensities of small quantities. This study

confirmed that the use of SDS-PAGE for qualification of egg white was appropriate, but not appropriate for the quantification of egg white.

10.5 CONCLUSIONS

The development of standards for the qualification and quantification of fish protein as a result of surimi content was achieved. Myosin light chains were key elements in determining species identification. Estimation of surimi protein content was attained using SDS-PAGE methods that had previously not been applied to surimi and provided an overall accuracy within 87-98%. Protein identifications for additives were visibly determined and DEW content qualification was successful however the DEW protein estimation was not as accurate as that of the surimi protein content. This may be due to the difficulties in detecting low levels of DEW added. Further investigation is needed to obtain a more reliable method for quantification of DEW. A study utilizing enzyme-linked immunosorbent assays, which uses antibodies raised against specific proteins, with quantification by spectrophotometry would therefore be needed.