Characterization of Myosin, Myoglobin, and Phospholipids Isolated from Pacific Sardine (*Sadinops sagax*)

Joo Dong Park

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Jae W. Park

Myoglobin (Mb) was extracted from Pacific sardine and added to Pacific whiting surimi to measure its effects on protein gelation. The purity of Mb extract was determined by SDS-PAGE. Mb extracted using ethanol showed higher purity than Mb extract using ammonium sulfate. The addition of 0.2% Mb significantly reduced breaking force of Pacific whiting surimi gel. However, a synergistic effect of 1.0% Mb was observed with 1.0% beef plasma protein (BPP) to increase surimi gel strength. The highest storage modulus of gels was observed at 1.0% Mb addition, which corresponded with the non-fracture gel and also supported a synergistic interaction

between 1.0% Mb and 1.0% BPP. Differential scanning calorimetry showed Mb addition might have affected myosin denaturation and aggregation.

Biochemical and conformational changes of purified sardine myosin were investigated at various pHs. The greatest myosin protein solubility was observed at pH 7 and remained constant up to pH 11. Three endothermic peaks were obtained for samples prepared at pH 7 and 10, while no peaks were shown for pH 2 samples, indicating chemical denaturation of myosin occurred before thermal treatment. The greatest Ca^{2+}-ATPase activity was observed at pH 7, while no activity was observed between pH 2-5 and pH 11. Total sulfhydryl content showed low activity at pH 2.5-4 while the greatest measure was obtained for samples at pH 5.5. Surface hydrophobicity was not detected from pH 2.5 to 5.0 because of low protein solubility, thereafter, the content remained consistent through pH 11.

Phospholipids (PL) were extracted from Pacific sardine and added to Alaska pollock surimi to measure its effects on protein gelation. Sep-pak silica column was used with hexane and methanol for PL extraction. The purity of PL extract was determined by thin-layer chromatography and results demonstrated high purity of obtained PL extract. The PL fraction contained higher levels of unsaturated fatty acids than neutral lipids (NL) and total lipids (TL) fractions, respectively. Freeze-thaw process was conducted to rapidly induce changes to see the effects of phospholipids during a long period of storage. Storage modulus was affected by 1% PL addition compared to control during freeze-thaw study. However, PL did not affect to color of Alaska pollock surimi gel.

Characterization of Myosin, Myoglobin, and Phospholipids Isolated from Pacific Sardine (*Sadinops sagax*)

by

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APPROVED:

Major Professor, representing Food Science and Technology

Head of the Department of Food Science and Technology

Dean of the Graduate School

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

Joo Dong Park, Author

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CONTRIBUTION OF AUTHORS

Dr. Jae W. Park was involved in the design, experiment, and writing of each chapter. Dr. Jin S. Kim was involved in the design and analysis of chapter 3. Dr. Yeung J. Choi and Dr. Jirawat Yongsawatdigul was involved in the analysis and writing of chapter 4. Dr. Tomoko Okada was involved in the design and analysis of chapter 5.

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CHAPTER 1

INTRODUCTION

The landings of pacific sardines (*Sardinops sagax*) have increased recently in the Oregon-Washington coastal area. A new Pacific sardine fishery began for the Oregon-Washington coastal area in 1999 and the catch volume increased from 776 metric tons (M/T) in 1999 to over 45,000 M/T in 2005. However, most of the landings in the Astoria area are processed as a bait for the long-line fishery and exported to Asian countries at low prices (McCrae and Smith 2006). Sardines are known to be a good source of protein having a range of 15-20% depending on catch location and species (Nunes and others 1992; Castrillon and others 1997; Marti de Castro and others 1997; Gokodlu and others 1998; Caponio and others 2004). Sardine also contains high levels (range 38% to 42%) of n-3 polyunsaturated fatty acid (PUFA) such as eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3) (Okada and Morrissey 2007).

The pH-shift method has been demonstrated as a practical process for protein isolation from a variety of fish species. The pH-shift method is a novel process in which proteins are isolated from animal muscle using acid or alkaline solubilization of the proteins (Hultin and Kelleher 1999; Choi and Park 2002; Perez-Mateos and others 2004; Yongsawatdigul and Park 2004; Batista and others 2007). With this method, fish muscle protein can first be solubilized at extreme pH values (either pH 2 or 10) and then precipitated at the isoelectric point (pI) of the muscle protein before

removing free water by centrifugation. This approach has several advantages over the conventional surimi processing method. These advantages include higher yield, higher protein quality, and efficient removal of insoluble impurities.

Sardines contain a higher amount of sarcoplasmic proteins compared to demersal, bottom dwelling fish (Haard and others 1994). Sarcoplasmic proteins comprise about 18-25% of total fish muscle proteins (Suzuki 1981; Lanier and others 2005). Among the sarcoplasmic proteins, myoglobin (Mb) is presumably the most important protein because it is primarily responsible for meat color, which is related to product quality (Kijowski 2001).

The effect of Mb on the gel forming ability of myofibrillar proteins (surimi) is debatable. Some researchers have indicated that sarcoplasmic proteins, including Mb, have an adverse effect on the formation of heat-induced gels by interfering with myosin cross-linking during gel formation (Chaijan and others 2006). It has been suggested that these proteins may interfere with myosin cross-linking during gel matrix formation because they do not form gels and have poor water-holding capacity (Smith 1991). On the other hand, other researchers believe that sarcoplasmic protein fractions from fish do not interfere with heat-induced gelation of myofibrillar proteins (Morioka and others 1992; Ko and Hwang 1995; Karthikeyan and others 2004; Kim and others 2005) and suggest that sarcoplasmic proteins may actually enhance gelation (Hultin and others 2005).

Myosin is the major protein in fish muscle, comprising approximately 55-60% of the myofibrillar protein and is the component responsible for the contractile as well as gelling properties of muscle (Lanier and others 2005). Myosin is composed of two

220 kDa heavy amino acid chains and 4 light chain subunits, ranging from 17 to 22 kDa. These amino acid chains are non-covalently attached to the myosin head (Lanier and others 2005).

Since solubility of myosin is an important factor for protein gelation, it is necessary to understand how pH affects the conformation of myosin in relation to its solubility. Protein solubility has been shown to be strongly affected by pH (Yang and Froning 1990; Turgeon and others 1992; Stefansson and Hultin 1994; Monahan and others 1995; Choi and Park 2002; Yongsawatdigul and Park 2004). Many studies have been performed on the biochemistry of myosin, most of which examined rabbit myosin at extreme pHs (Cross and others 1984; Ozog and Bechet 1995; Chang and others 2001). Also, there are many studies on fish myosin at extreme pHs (Watabe and Hashimoto 1980; Choi and Pyeun 1987; Lin and Park 1998).

Phospholipids (PL) are a major cause of quality deterioration on lipid oxidation (Pazos and others 2005). It is known that PL have a critical role in the oxidation process since PL have high PUFA concentration. However, Jeong and others (1995) reported that PL extracted from squid viscera has an antioxidant effect on fish oil. King (1992) also reported the antioxidant properties of PL in salmon oil. Similarly, Chen and Nawar (1991) reported that PL have an antioxidative effect on milk fat.

Therefore, experiments were conducted based on these following objectives: 1) to determine an effective means of Mb extraction and to understand the role of Mb in the gelation mechanism of myofibrillar proteins; 2) to measure conformational changes of Pacific sardine myosin at various pHs; 3) to study the characteristic of PL

from Pacific sardine and their effects on Alaska pollock surimi by repeated freeze-thaw.

CHAPTER 2

LITERATURE REVIEW

2.1 PACIFIC SARDINE

Pacific sardine (*Sardinops sagax*) is a pelagic fish harvested from southeastern Alaska to the southern tip of Baja California (Hammann and others 1988; Hammann 1998; Schweigert 2002). Pacific sardines were first landed commercially in Oregon during 1935–36, and the majority of catches were landed in Astoria and Coos Bay, Oregon for oil and fish meal production (Emmett and others 2005). However, sardine fisheries declined in the early 1940s and they were rarely observed north of the California-Oregon border over the next 50 years (Wing and others 2000). It appears that this occurrence was associated with warm ocean conditions as well as anomalous northward movement of southern species related to El Niños (Pearcy and Schoener 1987). In the early 1980s, fishermen began to take sardine incidentally with Pacific mackerel and jack mackerel in the southern California mackerel fishery. The sardines by-catch was primarily canned for pet food, while small quantities were canned for human consumption as well (Hill and others 2007).

Since 1992, Pacific sardines have again been observed off southern and central British Columbia and a directed purse-seine fishery has been reestablished as sardine continue to increase in abundance. Pacific sardines have also made a strong biological comeback in the past decades in the Oregon and Washington coastal area, with an increase in catch volume from 776 metric tons (mt) in 1999 to 42,052.3 mt in 2007

(Wiedoff and Smith 2007). However, most of the landings are quickly frozen and sold overseas, especially to Asian countries, such as Korea and Japan, at low prices as bait and aquaculture feed. Limited quantities of the sardine harvest are canned or sold fresh for human consumption and animal food (McCrae and Smith 2006). This evidence of increased sardine supply prompted us to investigate utilization of Pacific sardines for maximizing its use for human consumption. Research in this area has been in progress in our laboratory since 2003.

Several studies have shown the importance of the inclusion of sardines in the human diet (Andrade and others 1995; Bandarra and others 1997; Tarley and others 2004). A study of Sanchez-Muniz and others (2003) demonstrated that serum cholesterol level of rats fed olive oil-fried sardines diet greatly decreased compared to the control group. Also, liver fat and total, free and etherified cholesterol levels were lower in the sardine fed group in comparison with the control group. This study also demonstrated that intake of whole sardines fried in olive oil normalizes cholesterol metabolism in hypercholesterolemic rats more quickly than consumption of only the extracted fat from sardines (Sanchez-Muniz and others 2003).

Sardines contains fairly high levels of protein ranging 15-20% of whole fish depending on catch location and species (Nunes and others 1992; Castrillon and others 1997; Marti de Castro and others 1997; Gokodlu and others 1998; Caponio and others 2004). An animal study using rats demonstrated that sardine protein possesses beneficial effects on blood coagulation and/or fibrinolysis. The activated partial thromboplastin time, which is an assay for blood coagulation time in the intrinsic blood coagulation pathway was reported to be prolonged in rats fed the 20% sardine

protein diet compared to that of the control group (Murata and others 2004). Sardines are considered to represent a fatty fish, although the lipid content varies between 0.5 and over 20% depending on the season, stage of sexual maturity, and body size. Sardine is also a good source of n-3 polyunsaturated fatty acids (n-3 PUFAs) especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which have been known to posses numerous health benefits (Nettleton 1995; Nettleton and Katz 2005). Pacific sardines contain relatively high levels of n-3 PUFAs which is approximately 40% of total fatty acids (Okada and Morrissey 2007).

2.1.1 Myoglobin

Myoglobin (Mb) contributes to the redness of muscle. Mb is a complex molecule, which includes a protein and a non-peptide portion. Heme, the non-peptide portion of Mb molecules, is composed of two parts, an iron atom and porphyrin. Porphyrin is made up of a tetrapyrrole, linked together by a methyne bridge. The color change of Mb is related to the complex status of heme, globin and ligands that surround iron. It has been shown that apomyoglobin in solution with pH close to neutral has a compact and unique spatial structure with an extended hydrophobic core. This structure is extremely stable at approximately 30 °C. The stability of apomyoglobin varies in fish species due to different amino acid sequence and secondary structure of globin.

Sarcoplasmic proteins comprise about 18-25% of the total muscle proteins (Suzuki 1981; Lanier and others 2005). Among sarcoplasmic proteins, Mb is presumably the most important protein because it is primarily responsible for meat

color, which is related to product quality (Kijowski 2001). Muscle and meat color are primarily determined by the concentration and chemical state of the Mb pigments (Han and others 1994). Sardines contain particularly high amounts of Mb due to the abundance of dark muscle (Chaijan and others 2004). Some researchers have indicated that sarcoplasmic proteins, including Mb, have an adverse effect on the formation of heat-induced gel by interfering with myosin cross-linking during gel formation (Chaijan and others 2006). It has been suggested that these proteins may interfere with myosin cross-linking during gel matrix formation because they do not form gels and have poor water-holding capacity (Smith 1991). On the other hand, other researchers believe that sarcoplasmic protein fractions from fish do not interfere with heat-induced gelation of myofibrillar proteins (Morioka and others 1992; Ko and Hwang 1995; Karthikeyan and others 2004; Kim and others 2005) and suggest that sarcoplasmic proteins may actually enhance gelation (Hultin and others 2005). Therefore, if the role of Mb in the protein gelation is found, it will be very useful for utilization of Pacific sardines which contain predominantly high amounts of Mb.

2.1.2. Myosin

Myosin is the major protein in fish muscle, comprising approximately 55-60% of the myofibrillar protein and is the component responsible for the contractile as well as gelling properties of muscle (Lanier and others 2005). Fish myosins from ordinary and dark skeletal muscle are similar in structure to mammalian and avian myosin. Myosin is composed of two 220 kDa heavy chains and 4 light chain subunits, ranging

from 17 to 22 kDa. These amino acid chains are non-covalently attached to the myosin head (Lanier and others 2005).

There are two types of myosin light chain-the nonessential or 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) light chains, which dissociate from the myosin molecule after treatment with DTNB, and the essential or alkali light chains (A1 and A2), which dissociate at pH 11. There are 4 different light chains, existing as three types of different light chains. Therefore, only three light chains are shown in the sodium dodecylsulfate-polyacrylamide gel electrophoresis test. Mathew and Prakash (2006) reported that the molecular weights of the A1, A2, and DTNB chains of sardine myosin were 31, 23, and 22 kDa, respectively. Watabe and Hashimoto (1980) also reported that myosin from white mackerel muscle had three light chain subunits with molecular weights of 26.5, 20, and 17.5 kDa.

Since solubility of myosin is an important factor for protein gelation, it is necessary to understand how pH affects the conformation of myosin in relation to its solubility. Protein solubility has been shown to be strongly affected by pH (Yang and Froning 1990; Turgeon and others 1992; Monahan and others 1995). However, limited studies have been performed on the biochemistry of myosin, most of which examined rabbit myosin at extreme pHs (Dreizen and others 1967; Gershman and Stracher 1969; Cross and others 1984; Ozog and Bechet 1995). They reported that the secondary structure of rabbit myosin was not significantly affected by pH and the tertiary structure of rabbit myosin was slightly changed. Lin and Park (1998) reported that myosin solubility increased as KCl concentration increased until 0.5M KCl. Most recently, Kristinsson (2002) measured conformational changes of cod myosin rods

over an extreme range of pH. After the treatment at extreme pH, the myosin rod was unfolded. It was refolded to its native conformation through its secondary structure, when returned to neutral pH. However, the structure of the myosin head did not reassemble its native conformation. Therefore, to utilize sardine protein using the pH-shift method, the conformational changes of sardine myosin were studied.

2.1.3. Phospholipids

Although sardine is considered to be a fatty fish, its lipid content is known to be widely varied depending on environmental and physiological differences, including diet availability, temperature, and the sexual maturity of sardines (Caponio and others 2004).

It has been suggested that fatty and lean sardines have different lipid composition where it is affected by total lipid content. Phospholipids (PL) can be considered structural lipids whereas neutral lipids are principally reserve lipids related to changes in dietary and physiological state (Bandarra and others 1997; Leonardis and Macciola 2004). Content of PL in sardine oil is reported to be approximately 3.65 % and 11.25% in lean and fatty sardines, respectively (Bandarra and others 1997). This represent that the relative percentage of PL decreased in the fatty season and increased in the lean season. Among PL, phosphatidylcholine (PC) seems to be the predominant fraction, and it has been reported that PC has an important role in fish lipid degradation.

PL, in general, are highly enriched with EPA and DHA (20 to 50% of each fatty acid). In fish, lipid class composition, including PL, is known to vary

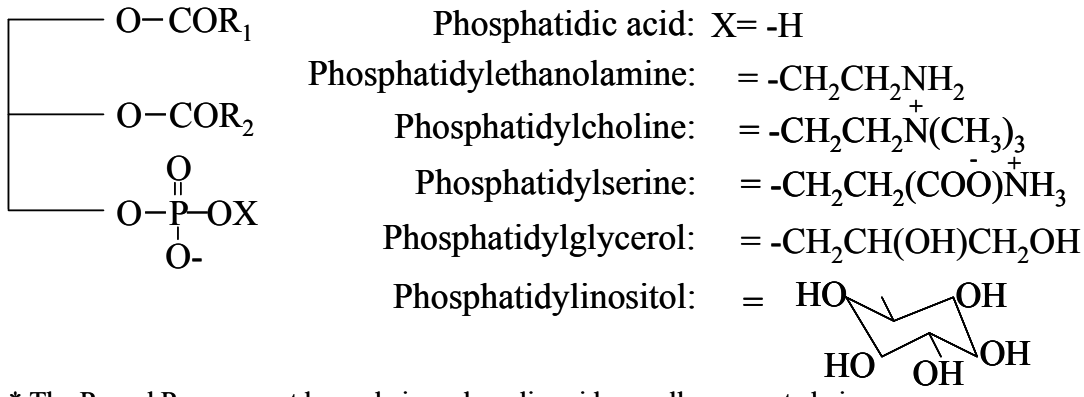
considerably depending on the fat content of the fish. The total PL content of fish usually remains between 1 and 1.5% as based on total fish wet weight, which is considerably lower than triglyceride (TG) (Haraldsson and Thorarensen 1999). A study by Bandarra and others (1997) demonstrated that PL content of lean sardine lipids was significantly higher than that of fatty sardine, which is rational as most of adipose fat is consisted of TG while PL is a the main component of cell membranes. Among PL, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant fractions in fish flesh and PC commonly comprised approximately 50-60% of the total PL (Haraldsson and Thorarensen 1999).

PL are ubiquitous in nature and are key components of the lipid bilayer of cells (Fahy and others 2005). PL molecules consist of a glycerol backbone having a polar phosphate-head group attached to *sn*-3 position, and acyl, alkyl, or alkenyl moieties attached at the *sn*-1 and *sn*-2 positions. They are categorized as distinct classes according to the nature of the polar head group along with incorporated fatty acids (R_1 and R_2). Basic structures of phospholipids classes are shown in Figure 2.1 (adapted and modified from Forrester and others (2004)).

More than 400 PL species from biological extracts have been directly identified due to recent improvements in electrospray ionization mass spectrometry (Ivanova and others 2004). The measure classes of phospholipids found in the membrane of mammalian cells include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PE), phosphatidylinositol (PL) and phosphatidylglycerol (PG). Of these, PC is the most ubiquitous and widely used

in food, pharmaceutical, and cosmetic products as a highly proficient emulsifiers (Haraldsson and Thorarensen 1999).

It is widely recognized that phospholipids play multiple roles in cell processes. Their primary function is to define the permeability barrier of cells and organelles by forming a phospholipids bilayer (Dowhan 1997). In addition to their physiological roles in cells, phospholipids have also been reported to exhibit antibacterial, antiviral and antitumoral activities (Dumay and others 2006).



* The R_1 and R_2 represent long-chain carboxylic acids usually connected via an ester bond to the primary and secondary alcohol residues of glycerol

Figure 2.1 Structures of glycerophospholipid classes.

PL are a major cause of quality deterioration from lipid oxidation (Pazos and others 2005). There are many studies regarding how lipid oxidation affects surimi gelation (Shimizu and others 1992; Kelleher and others 1994; Murakawa and others 2003; Eymard and others 2005). Lipid oxidation products can interact with proteins resulting in quality deteriorations, such as reduced rheological properties of surimi products.

Lipid oxidation in fish is one of the most important factors for quality loss during frozen storage. Lipid oxidation can affect the appearance, flavor, texture, and nutritional value of fish, which results in quality deterioration. Lipid oxidation varies where rate and degree of lipid degradation in fish depends on fish species and muscle type. Fish lipids have a high degree of unsaturation in the form of multiple double bonds in the fatty acids and are highly susceptibility to attack by molecular oxygen (Flick and others 1992). The oxidation reaction contains the following three steps: initiation, propagation, and termination.

A free radical is generated at the initial stage of lipid oxidation, and carbon and oxygen-centered (ROOH) molecultes are formed followed by hydroperoxides formation. These radicals undergo carbon-carbon cleavage to form breakdown products, such as aldehydes, ketons, alcohols, hydrocarbons, esters, furans and lactones (Kanner and Rosenthal 1992). Oxidation of fatty acids linked to the *sn*-1 and *sn*-2 positions of glycerophospholipids lead to many different reaction products depending on chain length and degree of unsaturation. In general, the *sn*-2 position of diacylglycerophopholipids is frequently linked to PUFAs that are prone to oxidation (Fruhwirth and others 2007). When the *sn*-2 fatty acids of PL are oxidized by radicals, several types of oxidative products are formed. These include PL containing fatty acid oxidation products, lysophospholipids, and fragmentation products of fatty acid oxidation. Some of these products, lysophosphatidic acid or lysophospholipids, can be formed both enzymatically and nonenzymatically (Subbanagounder and others 2000).

2.2 FISH PROTEIN ISOLATE PRODUCTION USING pH-SHIFT

Recently many studies were conducted to produce fish protein isolate (FPI) using a variety of species, including red muscle fish. Complete utilization of all fish parts is the ultimate goal of industry. The surimi industry has changed dramatically over the past decade with reduced landings of several white muscle species beginning in 2000. As a result surimi prices have continuously increased prompting some researchers to explore the utilization of new fish species for surimi production (Chen and others 1996; Park and others 2003). Expanding the range of species to include red muscle fish, however, has presented some obstacles due to proteolytic enzymes and color pigments (Park and Lin 2005).

The pH-shift method is a novel process in which proteins are isolated from animal muscle using acid or alkaline solubilization followed by isoelectric isolation to improve gelation properties and yield (Hultin and Kelleher 1999; Yongsawatdigul and Park 2004). This method has several advantages, such as higher yield, higher protein quality, and efficient removal of insoluble impurities (lipids, membranes, skin, and bones) (Park and Lin 2005). Almost all myofibrillar proteins and sarcoplasmic proteins are recovered when the pH is adjusted to the isoelectric point after homogenization and solubilization at extreme pHs. Yields have been reported as high as 40% for some species leading to less protein loss in waste water thereby decreasing disposal costs (Kim and others 2003). With this method, fish muscle protein can first be solubilized at extreme pH values (either pH 2 or 10) and then precipitated at the isoelectric point (pI) of the muscle protein before removing free water by centrifugation.

Choi and Park (2002) reported that acidic or alkaline solubilization processes with Pacific whiting yielded 20% more proteins than the conventional 3-washing process. Alkali treated protein isolates have also shown better gel quality than those prepared from acid solubilization or conventional surimi processing (Yongsawatdigul and Park 2004). In addition, the use of high speed centrifugation removes neutral and membrane lipids, which result in significantly reduced oxidation and fishy odor. Lipid oxidation can affect the appearance, flavor, texture, and nutritional value of surimi, which ultimately leads to quality deterioration. The pH-shift method can also reduce lipid-soluble toxins such as polychlorinated biphenyls and polyaromatic hydrocarbons, in the final product.

The process by which fish protein isolate (FPI) are produced is distinctively different from conventional surimi production. FPI is normally produced by solubilization of fish mince at pH 2 or 11 using HCl or NaOH, respectively, and subsequent precipitation at pH 5.5. Due to the nature of the process, FPI undergoes some conformational changes (unfolding and aggregation) during pH treatments. Fish protein isolate is currently ready to be used commercially as a functional ingredient, but has to be utilized differently from conventional surimi.

2.3 METHODOLOGY TO DETERMINE PROTEIN FUNCTIONALITY

2.3.1 Fracture analysis by penetration

The most important property of surimi gel as a surimi seafood product is texture. The texture of muscle foods may be very complex due to the presence of

fibers of varying sizes and properties. To measure the rheological properties of surimi gel, there are two main categories; fracture and non-fracture gel analysis.

The penetration test is a most popular fracture gel measurement technique used in the surimi industry and academic area. This empirical test was initially developed by Matsumoto and Arai (1952) and later modified by Okada and Yamazaki (1958). In the penetration test, force (g) and depth of penetration (cm) at the gel break point are used to describe the gel properties (Hamann and MacDonald 1992). Force indicates the strength of gel measured as force values at rupture of the sample during probe penetration. Deformation indicates cohesiveness or elasticity denoting displacement from the point when the probe first touches the sample to the point where the probe penetrates the sample by rupturing surface. Often these two values are multiplied together to give the jelly strength, which is the value used in Japanese grading standards. However, it is important to know that multiplying force by deformation is rheologically wrong. The same values of force ultiplied by deformation can denote totally different gel properties if they are not used independently. The independent use of these two values is therefore highly recommended (Kim and others 2005).

2.3.2 Non-fracture rheological analysis

Rheology is the study of material deformation and flow of matter (Rao 1999). The science of rheology began about 70 years ago. It was founded by two scientists meeting in the late 1920's having a shared dilemma which was a need for describing fluid flow properties. The scientists were Professor Marcus Reiner and Professor Eugene Bingham. All materials, from gases to solids, can be divided into the

following three categories of rheological behavior: first, viscous materials; in a purely viscous material all energy added is dissipated into heat. Second, elastic materials; in a purely elastic material all energy added is stored in the material. Third, viscoelastic materials; a viscoelastic material exhibits viscous as well as elastic behavior.

A dynamic rheometer applied to solid or semi-solid foods generally applies small strains in sinusoidal oscillation. There are three main rheometer concepts. Measurements are obtained based on three main rheometer concepts: storage modulus (G'), the amount of energy stored in the system as mechanical energy after a deforming force is applied; loss modulus (G"), the amount of energy not recoverable after a deforming force is applied; and phase angle (δ), often expressed as tan-δ (G"/G'). From this information, the gel point (at log G'= log G") can be determined, where the transition from a viscoelastic liquid to a solid state occur (Figure 2.2).

A rheometric measurement normally consists of a strain (deformation) or a stress analysis at a constant frequency (normally 1 Hz). It is often combined with a frequency analysis, e.g. between 0.1 and 100 Hz. For such a product like surimi, the phase angle is also small, e.g. 20 ° (a phase angle of 0 ° means a perfectly elastic material and a phase angle of 90 ° means a perfectly viscous material). Tan-δ values <1 (G"<G') indicate more solid like behavior and values >1 (G">G') indicate more liquid-like behavior. Therefore, dynamic tests can be used to explain the gelation phenomena and to predict the fracture gel properties.

In surimi and surimi seafood products, fish myofibrillar proteins are first extracted by comminuting with salt and then form a three-dimensional structure called a gel upon thermal processing. A gel is an intermediate structure between a solid and

a liquid, exhibiting both viscous and elastic properties (Hamann and MacDonald 1992). When fish myosin solution was subjected to a dynamic test, the tan-δ was initially about 70 ° and then dropped significantly to 10 °. As a viscous sample is transformed into a gel matrix with heating, myosin sample even with low protein concentration showed a cross over point at log G'= log G", indicating the gel point is around 29 °C (Kim and others 2005) (Figure 2.2).

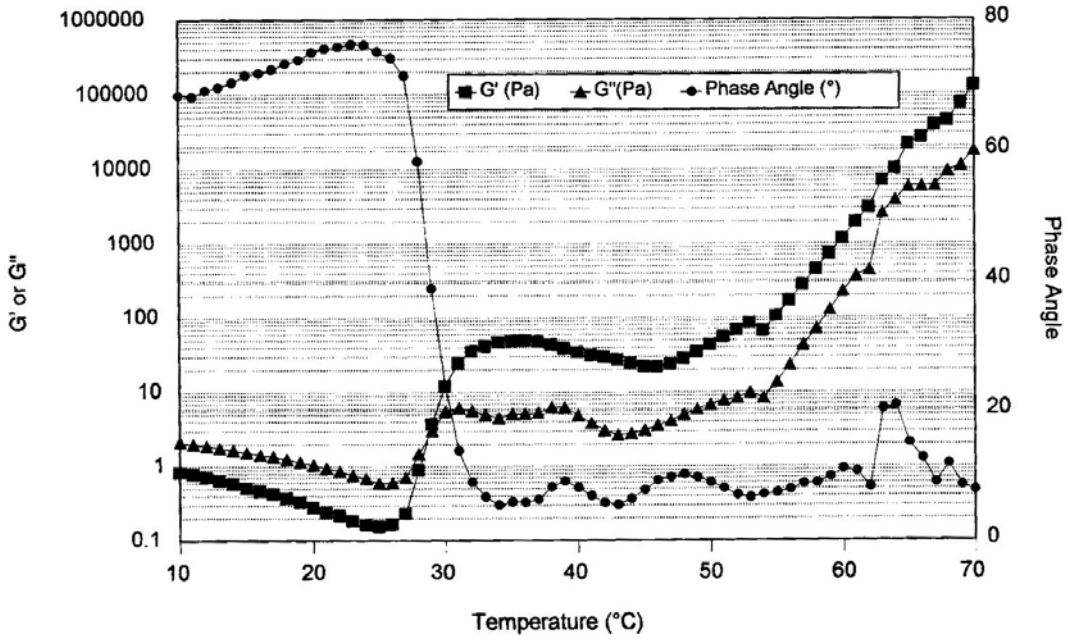


Figure 2.2 Dynamic test curves of Pacific whiting myosin (Kim and others 2005).

2.3.3 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a thermoanalytical technique in which the difference of energy inputs into a substance and a reference material is measured as a function of temperature while the substance and reference material are

subjected to a controlled temperature program. When thermal transition occurs in the sample, DSC provides a direct calorimetric measurement of the transition energy at the temperature of the transition (Wright 1984).

DSC is often used to characterize the thermal transitions in polymers such as the glass transition temperature and melting point. Organic liquids or solids, and inorganic materials can also be analyzed. Advantages include rapid analysis time and small sample size, typically 1-500 mg.

There are two types of DSC, conventional DSC and micro DSC. Micro DSC is highly sensitive and can accommodate larger sample sizes (20 mg vs. 500 mg). The micro DSC can also detect very weak heat generation in a temperature range from room temperature to 120 °C. The main difference between conventional DSC and micro DSC is that the micro DSC has a Tian-Calvet type fluxmetric probe that envelopes the sample and is capable of measuring almost all the energy exchanges between sample vessel and the calorimetric unit (MicroDSC IIIa, Setaram Inc., Lyon, France).

2.3.3.1 Applications

A wide variety of applications are applicable for polymer, organic, and inorganic analysis to measure 1) glass transition temperature, 2) heats of crystallization and fusion, 3) degree of cure of thermosets, 4) heats of curing reactions, 5) oxidative stability, 6) heats of decomposition (dehydration), and 7) heats of vaporization and solution.

2.3.3.2 Parameters of DSC

Enthalpy is a key thermodynamic parameter. Enthalpy is a thermodynamic quantity equal to the internal energy of a system plus the product of its volume and pressure. DSC is used to characterize phase transitions with respect to enthalpy of transition (Koyama and others 1999).

Heat capacity is specific heat that is not often measured or studied in food research or on food components. Since heat capacity forms the basis of enthalpic measurements, it is therefore appropriate to consider the theoretical basis of specific heat determination by DSC. Thermograms depict differential heat flow versus temperature. Most substances undergo a change in heat capacity with temperature reflecting a change in structure and therefore a change in their capacity to absorb energy. Privalov and Khechinashvili (1974) observed that prior to the denaturation transition, there was a steady increase in the heat capacity of a protein. This pre-denaturation change was ascribed to a gradual loosening of the protein structure resulting in a change in the accessibility of side-chain groups to water molecules.

Temperature and heat of transition are two parameters probably represent the most important information yielded by DSC analyses. In the area of food research, the observed DSC transitions mainly relate to processes such as protein denaturation, starch gelatinization and fat crystal melting. The reason why these processes are discerned by DSC as a transition or peak is because of their highly co-operative nature, i.e. they all involve near simultaneous rupture of inter- and intra-molecular bonding (Wright 1984).

2.3.3.3 Principle of DSC

The substance and an inert reference are heated at a programmed rate, and any thermally induced changes occurring in the sample are then recorded as a differential heat flow, which is normally displayed as a peak on a thermogram. Figure 2.3 is schematic of a DSC IIIa. The sample and reference are both maintained at a predetermined temperature by the program during a thermal event in the sample.

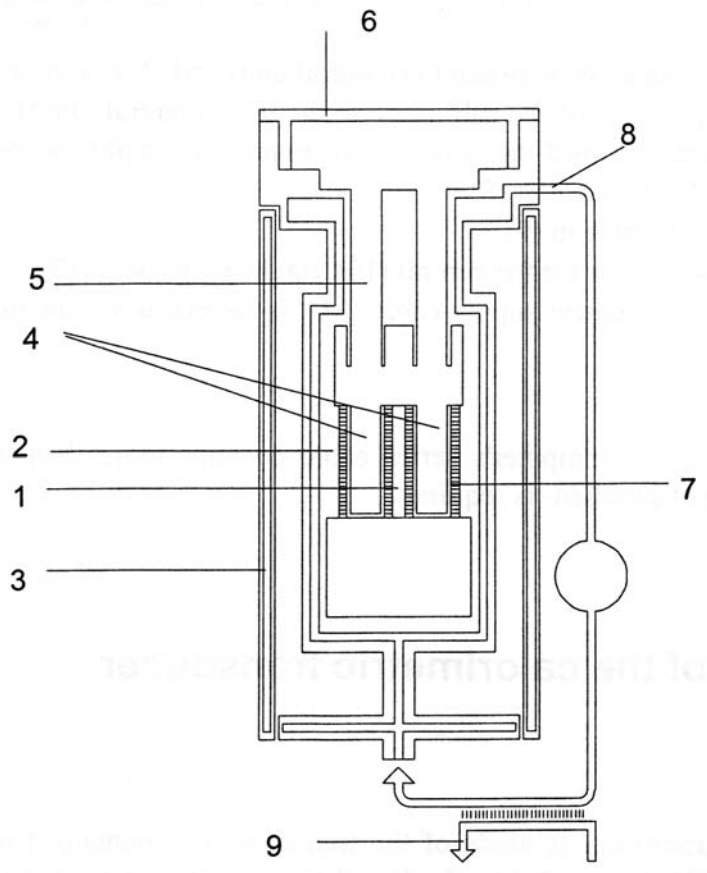


Figure 2.3 Micro DSC IIIa Calorimetric transducer. (User manual, Setaram Co., France)

1, metallic cylinder; 2, first metallic chamber; 3, second metallic chamber; 4, measurement and reference vessel; 5, thermal buffer for external temperature; 6, gilt cover; 7, plane fluxmeter; 8, a loop of liquid circulating; 9, water circulating.

The amount of energy that must be supplied to or withdrawn from the sample to maintain zero temperature differential between the sample and the reference is the experimental parameter displayed as the ordinate of the thermal analysis curve. The sample and reference are placed in identical environments, metal pans on individual bases each of which contain a platinum resistance thermometer and a heater.

Any transition accompanied by a change in specific heat produces a discontinuity in the power signal, and exothermic or endothermic enthalpy changes give peaks with areas proportional to the total enthalpy change. The measuring principle is to compare the rate of heat flow to the sample and to an inert material, which are heated or cooled at the same rate.

Changes in the sample are associated with absorption or evolution of heat cause a change in the differential heat flow, which is then recorded as a peak. The area under the peak is directly proportional to the enthalpic change and its direction indicates whether the thermal event is endothermic or exothermic. For proteins, the thermally induced process detectable by DSC is the structural melting or unfolding of the molecule. During a thermal event in the fish protein, denaturation (endothermic) and aggregation (exothermic) of fish protein occur. As the DSC records the differential heat flow values, the heat flow data only shows the net values, which are the positive values after calculating the endothermic and exothermic values.

Therefore, in case of fish protein, normally endothermic peaks are shown in the results. Because the denaturation happens during the heating process endothermic peaks are a greater than exothermic peaks. The transition of protein from native to denatured conformation is accompanied by the rupture of inter- and intra-molecular bonds, and the process must occur in a cooperative manner to be discerned by DSC (Ma and Harwalkar 1991).

2.3.3.4 Evaluate DSC data on fish protein polymers

The study of the thermal behavior of myofibrillar proteins is of technological importance to determine and predict the final quality of fish products because functional and textural characteristics of fish protein mainly depend on their myofibrillar proteins. DSC offers a direct method to study the thermal transition of muscle proteins *in situ.* The use of thermal analysis and in particular of DSC, to monitor conformational changes is relatively new in terms of food proteins (Kijowski and Mast 1988).

DSC has been intensively used in fish protein research primarily to focus on actin and myosin. DSC analysis was used to determine protein characteristic (actin and myosin) characteristics of flesh fishes, including black marline (*Makaira mazara*) (Lo and others 1991) and carp (Nakaya and Watabe 1995). DSC can also be a useful tool to compare any protein characteristics between different species as shown in a comparison study of bigeye tuna myoglobin and myosin of other scombridae fish, such as mackerel (Ueki and Ochiai 2004). Determination of protein denaturation of frozen fish has been also studied for various species, including hake fillet (Careche

and others 2002), mackerel (Howell and Saeed 2004), cod fillets (Schubring 2004), and eel (Huang and Ochiai 2005). These studies focused on changes of fish quality after long storage. DSC is used to determine protein denaturation of the samples with long storage time such as 6–12 months. Fish fillet quality/freshness can be evaluated by determining whether actin and myosin are denaturated along with other quality parameters such as color, texture and water retention ability. In addition, DSC can be used to determine protein stability of fish fillet during high pressure treatment (100-200 MPa) as shown in study using turbot (Chevalier and Le Bail 2001).

Among fish protein research areas, surimi or fish protein isolate is the most intensively studied. DSC is used to monitor protein denaturation in fish protein gel. Species in these studies include tilapia (Barreto and others 2000), blue whiting (Herrera and others 2001), silver hake and mackerel (Belibagli 2003), and Pacific whiting (Mossavi-Nasab and others 2005), walleye pollack (Togashi 2002), and Alaska pollock (Wang 1991; Park 1994).

Belibagli (2003) studied determination of thermal diffusivity values obtained using a transient method both from heating and cooling information and apparent specific heat of surimi made from silver hake and mackerel as a function of temperature (60–110 °C) and added potato starch content (3% and 7%, w/w). The study demonstrated that DSC thermograms of silver hake and mackerel surimi showed three endothermic peaks, and an endothermic peak having a large area was observed, which overlapped the third highest temperature peak of surimi samples, when starch was added to surimi samples. No significant shifts in the endothermic peaks of myofibrillar proteins were detected with increasing starch content. Transition

temperatures for starch–surimi systems were higher than those for starch–water systems. There were deviations in the apparent heat capacity function calculated from DSC measurements in surimi samples containing starch. These are attributed to gelatinization of starch and modification of water structure.

CHAPTER 3

EXTRACTION OF SARDINE MYOGLOBIN AND ITS EFFECT ON GELATION PROPERTIES OF PACIFIC WHITING SURIMI

J.D. Park and J.W. Park

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3.1 ABSTRACT

Myoglobin (Mb) was extracted from Pacific sardine and added to Pacific whiting surimi to measure its effects on protein gelation. The purity of Mb extract was determined by SDS-PAGE. Mb extracted using ethanol showed higher purity than Mb extract using ammonium sulfate. The addition of 0.2% Mb significantly reduced breaking force of Pacific whiting surimi gel. However, a synergistic effect of 1.0% Mb was observed with 1.0% beef plasma protein (BPP) to increase surimi gel strength. The highest storage modulus of gels was observed at 1.0% Mb addition, which corresponded with the fracture gel analysis and 1.0% Mb also showed a possible synergistic interaction with 1.0% BPP for improving gel force and deformation values. Differential scanning calorimetry showed Mb addition possibly affected myosin denaturation and aggregation as demonstrated by increased enthalpy values (ΔH).

Key words: ethanol extraction, gelation, myoglobin, Pacific sardine, Pacific whiting

3.2 INTRODUCTION

Pacific sardines (*Sardinops sagax*) have made a strong biological comeback in the Oregon-Washington coastal area. A new Pacific sardine fishery began for the Oregon-Washington coastal area in 1999 and the catch volume increased from 776 metric tons (M/T) in 1999 to over 45,000 M/T in 2005. However, most of the landings in the Astoria area are processed as bait for the long-line fishery and exported to Asian countries with low prices (McCrae and Smith 2006). Sardines are known to be a good source of protein having a range of 15-20% depending on location and species (Nunes and others 1992; Castrillon and others 1997; Marti de Castro and others 1997; Gokodlu and others 1998; Caponio and others 2004).

The pH-shift method has been demonstrated as a practical process for protein isolation from a variety of fish species. The pH-shift method is a novel process in which proteins are isolated from animal muscle using acid or alkaline solubilization of the proteins (Hultin and Kelleher 1999). With this method, fish muscle protein can be first solubilized at extreme pH values (either pH 2 or 10) and then precipitated at the isoelectric point (pI) of the muscle protein before removing free water by centrifugation. This approach has several advantages over the conventional surimi process method. These include higher yield, higher protein quality, and efficient removal of insoluble impurities.

Various research efforts were made to characterize the biochemical and functional properties of fish proteins isolated by the pH-shift method. However, only limited species, such as Pacific whiting (Choi and Park 2002; Thawornchinsombut and

Park 2005), Alaska pollock (Kim and others 2005) , rockfish (Yongsawatdigul and Park 2004), Atlantic cod (Kristinsson and Hultin 2003a; Liang and Hultin 2005), and herring (Undeland and others 2002), have been well documented. Sardines contain a higher amount of sarcoplasmic proteins compared to demersal, bottom dwelling, fish (Haard and others 1994). Sarcoplasmic proteins are proteins that are located inside the sarcolemma and are soluble in water or low molarity saline (< 50 mM) solutions. Sarcoplasmic proteins comprise about 30-35% of the total muscle proteins (Xiong 1997). Among sarcoplasmic proteins, Mb is presumably the most important protein because it is primarily responsible for meat color, which is related to product quality (Kijowski 2001). Muscle and meat color are primarily determined by the concentration and chemical state of the Mb pigments (Han and others 1994). Sardines contain particularly high amounts of Mb due to the abundance of dark muscle (Chaijan and others 2004).

The effect of Mb on the gel forming ability of myofibrillar proteins (surimi) is debatable. Some researchers have indicated that sarcoplasmic proteins, including Mb, have an adverse effect on the formation of heat-induced gel by interfering with myosin cross-linking during gel formation (Chaijan and others 2006). It has been suggested that these proteins may interfere with myosin cross-linking during gel matrix formation because they do not form gels and have poor water-holding capacity (Smith 1991). On the other hand, other researchers believe that sarcoplasmic protein fractions from fish do not interfere with heat-induced gelation of myofibrillar proteins (Morioka and others 1992; Ko and Hwang 1995; Karthikeyan and others 2004) and suggest that sarcoplasmic proteins may actually enhance gelation (Hultin and others 2005). In

conventional surimi processing, because of its water soluble characteristics, sarcoplasmic proteins, including Mb, are removed during the washing step. However, the pH-shift method retains sarcoplasmic proteins, including Mb, in the isolated protein. Therefore, determining the role of Mb on the gelation properties of surimi is greatly needed. Our objectives were to determine effective means of Mb extraction and to understand the role of Mb in the gelation mechanism of myofibrillar proteins.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Pacific sardines (*Sardinops sagax*; body length: 23.5 ± 0.3 cm; weight: 119.9 ± 7.8 g) were obtained from Pacific Seafood Co. (Warrenton, Oreg., U.S.A.) July through September 2005. Sardines were randomly chosen, packed in ice, and delivered to the laboratory on the day of catch. After removing head and viscera, each fish was minced using a vertical-cutter/mixer (Stephan Machinery Corp., Columbus, Ohio, U.S.A.) for 2 min and used immediately for Mb extraction. Commercial Pacific whiting (*Merluccius productus*) surimi, without enzyme inhibitors, was obtained from American Seafood Co. (Seattle, Wash., U.S.A.). Beef plasma protein (BPP) was obtained from Proliant Inc. (Ames, Iowa, U.S.A.). A commercially available horse Mb was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

3.3.2 Sample preparation

3.3.2.1 Mb extraction using ammonium sulfate

The Mb extraction method using ammonium sulfate was adapted from Ponce-Alquicira and Taylor (2000). Sardine mince was mixed (1:3, w/v) with 0.01 N sodium phosphate buffer (pH 6.3) and homogenized. The homogenate was centrifuged at 10,000 x g for 20 min and the supernatant was collected. Ammonium sulfate was added in small increments until its concentration reached 20% before centrifuging at 5,000 x g for 15 min. Once the supernatant was obtained, ammonium sulfate was continuously added to reach 80% concentration in the solution. After centrifugation, the precipitate was mixed with deionized water and subjected to dialysis overnight. Recovered Mb was freeze-dried using freeze dryer (model Freezone 12L; Labconco Corp., Kansas City, Mo., U.S.A.) and stored at -70 °C until tested. One kg of sardine mince yielded 0.5-1.0g of freeze-dried Mb.

3.3.2.2 Mb extraction using ethanol

To sardine mince, 0.01 N sodium phosphate buffer (pH 6.3) was added (1:2, w/v) and then the mixture was centrifuged at 10,000 x g for 20 min. Ethanol (100%) was added in small increments until the mixture was comprised 80% of ethanol. The solution was then centrifuged at 5,000 x g for 15 min and the supernatant was obtained. Ethanol was continuously added to the supernatant until reaching 90% in solution. The precipitate was mixed (1:100, w/v) with deionized water. Recovered Mb was freeze-dried using freeze dryer (model Freezone 12L; Labconco Corp., Kansas city, Mo., U.S.A.) and stored at -70 °C until tested.

3.3.3.Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Extracted Mb was subjected to SDS-PAGE using 5% stacking gel and 15% separating gel applied with 20 μg of protein to verify the purity of Mb. The protein concentration was determined using Lowry method (Lowry and others 1951). Gel was stained in 0.125% Coomassie blue R-250 and destained in 40% methanol and 10% acetic acid (Laemmli 1970). A low-range molecular weight standard mixture (Sigma Chemical Co., St. Louis, Mo., U.S.A.) included bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), glyceraldehydes-3-phosphate dehydrogenase of rabbit muscle (36 kDa), carbonic anhydrase of bovine erythocyte (29 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), bovine α-lactalbumin (14.2 kDa), and aprotinin of bovine lung (6.5 kDa). As evidenced by SDS-PAGE, Mb extracted with ethanol was used, due to its purity, for characterization of biochemical and incorporated into surimi for measuring functional properties.

3.3.4 Buffering capacity of Mb extracted using ethanol

Buffering capacity was determined according to the modified method of Jyothirmayi and others (2006). Two sets of Mb (1 g) extracted with ethanol were solublized in deionized water (30 mL). An aliquot (0.1 mL) of 0.5 M HCl was added continuously to one set and 0.1 mL of 0.5 M NaOH was added continuously to the other set. The pH was measured using a pH meter (model AR15; Fisher Scientific, Pittsburgh, Pa., U.S.A) while being stirred by magnetic stir bar. The changes of pH, while HCl and NaOH were added, were precisely monitored. The amount of added

hydrochloric acid and sodium hydroxide solution was plotted against pH and the buffering capacity was expressed as the mean value of mmol of hydrochloric acid and sodium hydroxide per g of Mb required to change one unit of pH.

3.3.5 Gel preparation and evaluation

Various levels (0.2, 0.4, 0.6 and 1.0%) of freeze dried Mb were added to Pacific whiting surimi to determine its effect on surimi gels. Gels of the Pacific whiting surimi were prepared containing 2% NaCl, with or without 1% BPP, and adjusted to 78% moisture using ice/water as follows; Pacific whiting surimi (partially thawed) was cut into small cubes (3cm) and placed in a mixer (model SC400; Black & Decker Corp., Towson, Md., U.S.A.). Surimi was chopped at low speed for 1 min, then salt was added, and the mixture was chopped at high speed for 1 min. The mixture was moved to a mortar before adding ice/water and BPP, and ground using a pestle for 3 min. The paste was packed into a polyethylene bag and the air pockets from the surimi paste were removed using a vacuum machine (model Reiser VM-4142; Roescher Werke GMBH, Osnabrueck, Germany). The paste was extruded, using a sausage stuffer (model 14208, The Sausage Maker, Buffalo, N.Y., U.S.A.), into stainless steel cooking tubes (inner diameter=1.9 cm, length=17.5 cm). The interior wall of the tubes was coated with a film of pan coat spray (Western Family Foods, Inc., Portland, Oreg., U.S.A.). The paste was cooked at 90 °C for 15 min in a circulating water bath and then chilled in ice/water for an additional 15 min. The chilled gels were stored overnight in a refrigerator (5 °C) before gel analysis. Gels were equilibrated to room temperature before testing for an hour. Gels were cut into 30 mm

long and then subjected to the punch test using a Texture Analyzer (TA-XT plus, Texture Technologies Corp., N.Y., U.S.A.). A spherical probe (5 mm diameter) was used with a crosshead speed set at 1 mm/sec. Breaking force (g) and deformation (mm) were recorded to determine the strength and cohesiveness of the gel, respectively.

3.3.6 Color

A Minolta Chroma Meter CR-300 (Minolta Camera Co., Osaka, Japan) was used to measure the color of the surimi gels. Results were reported in CIE L* and b* values, where L* represents lightness and b* blue/yellow. The whiteness was then calculated using the equation L* - 3b* (Park 1994).

3.3.7 Oscillatory dynamic measurement

Development of a gel network was measured as a function of temperature using a CS-50 rheometer (Bohlin Instruments, Inc., Cranbury, N.J., U.S.A.). The raw paste (2% NaCl, 78% moisture) was placed between a cone and plate (4 °) with a gap of 1 mm. To avoid sample drying during heating, a plastic cover (trapper) with moistened sponge was used. The sample was heated from 15 to 85 °C at a heating rate of 1 °C/min. The oscillatory mode was used with a fixed frequency of 0.1 Hz and shear stress of 160 Pa, which was in the linear viscoelastic range.

3.3.8 Differential scanning calorimetry (DSC)

DSC studies were performed using a Setaram micro differential scanning calorimeter (Setaram Co., Lyon, France). The temperature calibrations were performed using napthalen. Samples containing 2% NaCl and 78% moisture content were accurately weighed to approximately 500 mg in a stainless vessel. In the reference vessel deionized water was added. Samples were scanned at 1 °C/min over the range of 15-85 °C. Triplicate samples with reproducible thermograms were analyzed.

3.3.9 Statistical Analysis

The results were presented as the average and standard deviation of each experiment conducted in triplicate. Statistical comparisons were made between treatments by ANOVA and Tukey's test using SPSS (version 13.0) software package (SPSS Inc., Chicago, Ill., U.S.A.). A statistical significance was reported at $P < 0.05$.

3.4 RESULTS AND DISCUSSION

3.4.1 Mb extraction and SDS-PAGE

To determine the purity of extracted Mb, samples were subjected to SDS-PAGE analysis (Figure 3.1). From the left, low molecular weight standard, commercially available horse heart Mb, sardine Mb extracted using ammonium sulfate, and sardine Mb extracted using ethanol are shown. It has been reported that Mb from mackerel and two varieties of sardines (*Sadinops melanosticta* and *Sardinella gibbosa*) also had a molecular weight of 14.9, 15.3, and 15.6 kDa, respectively (Yamaguchi and others 1979; Chaijan and others 2007). There was a distinctive difference of the Mb

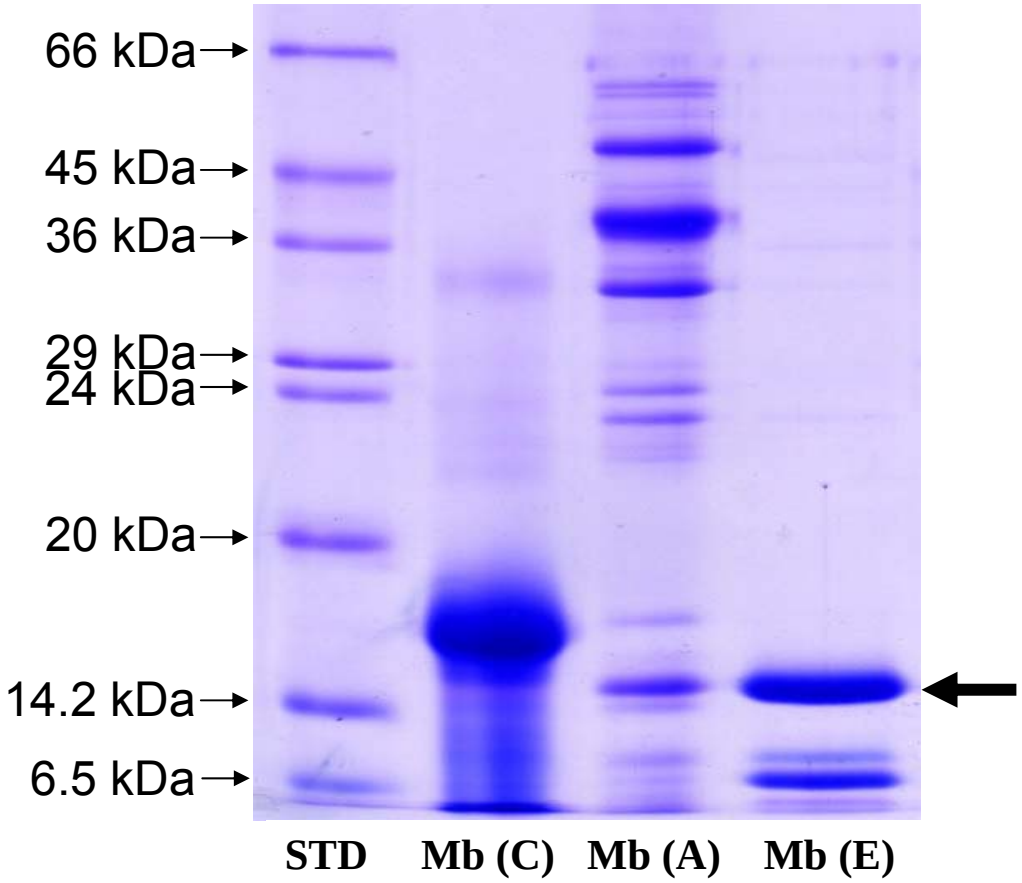

Figure 3.1 SDS-PAGE pattern of Mb extracted from Pacific sardine using ammonium sulfate and ethanol. STD, low standard molecular weight; Mb (C), commercial horse heart Mb; Mb (A), Mb extracts using ammonium sulfate; Mb (E), Mb extracts using ethanol.

purity between the two extraction methods using ammonium sulfate and ethanol. Recovered Mb by the ammonium sulfate method contained numerous protein bands at 47, 39, 33, and 15 kDa. Sarcoplasmic proteins in fish muscle consist of a large number of soluble proteins, primarily enzymes, heme proteins, and other albumins (Haard and others 1994). Kim and others (2005) reported that the major sarcoplasmic bands were 43, 40, 17, 11, and 8 kDa in rockfish (*Sebastes flavidus*). It can be

assumed that numerous protein bands in samples extracted by the ammonium sulfate method observed in this study might have been other sarcoplasmic components. On the other hand, the Mb fraction obtained with the ethanol method showed a higher concentration of the Mb band and also bands at 9, 7, and 5 kDa. This suggests that the purity of Mb extracted with the ethanol method was much higher compared to the ammonium sulfate method. Even though smaller bands below the Mb band have yet to be characterized, the ethanol method certainly showed effectiveness in isolating and recovering Mb. Sardine Mb isolated with ethanol was more pure than Mb isolated by ammonium sulfate precipitation, and thus was used for further studies.

3.4.2 Buffering capacity

Buffering capacity of Mb showed that an average of 0.48 mmol HCl or 1.32 mmol NaOH per g of Mb was required to change the pH by one unit (Figure 3.2). The highest buffering capacity of Mb was measured at pH between 6 and 7. It has been reported that the most effective buffers are those with pKs close to that of the physiological pH, around pH 6-8. Mb is a histidine-rich protein where histidine contains an imidazole group having its pK at 6.5 (Wittnich and others 2006). This could be the reason the extracted Mb showed a high buffering capacity in this study. Even though the highest buffering capacity of sardine Mb was shown at pH 6-7, certainly other amino acids than histidine might have contributed to buffer capacity of Mb over the entire range shown.

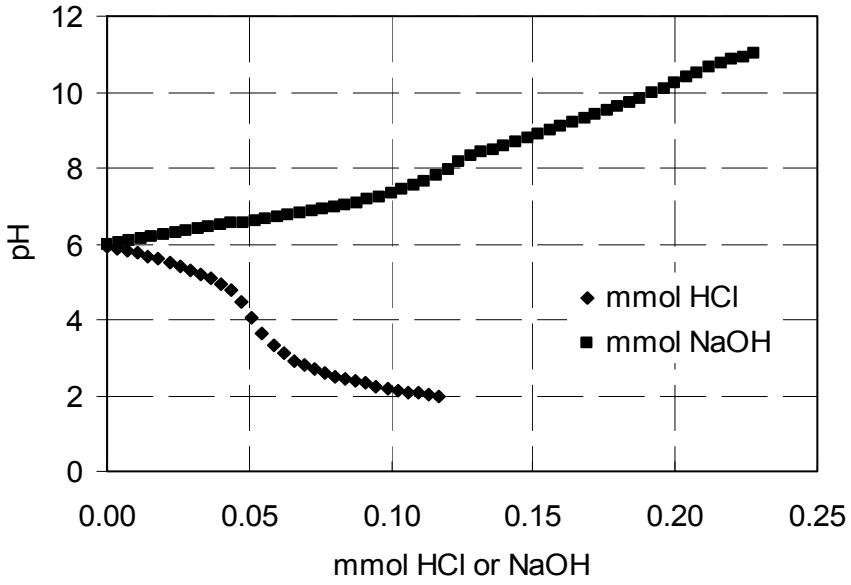

Figure 3.2 Buffering capacity of Mb extracted from Pacific sardines

3.4.3 Fracture gel analysis

It is uncertain whether sarcoplasmic proteins, including Mb, affect surimi gelation positively or negatively. Several studies have shown that the sarcoplasmic fraction interferes with the gelling ability of surimi when sarcoplasmic protein was not removed (Okada 1964; Shimizu and Nishioka 1974; Smith 1991; Chaijan and others 2006) while other studies have demonstrated that sarcoplasmic proteins might instead contribute to gel formation of myofibrillar protein when isolated sarcoplasmic protein was added (Morioka and others 1992; Ko and Hwang 1995; Karthikeyan and others 2004; Kim and others 2005). High breaking force and deformation value are desirable for good surimi gel. Changes in breaking force values (g) and deformation values (mm) of surimi gel prepared with different concentrations of sardine Mb addition are shown in Figure 3.3.

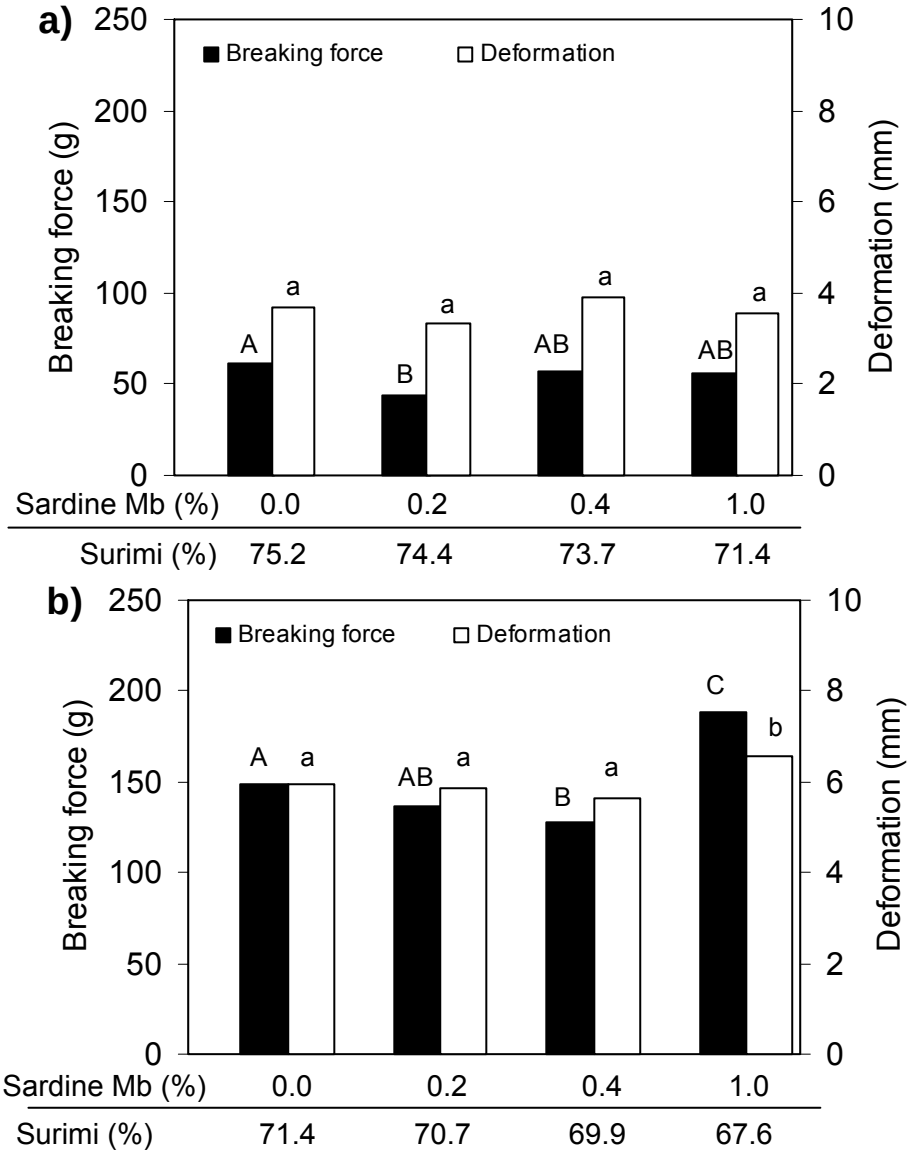

Figure 3.3 Textural properties of Pacific whiting gels prepared at various levels of sardine Mb contents in the absence of BPP (a) and in the presence of BPP (b). Different letters indicate a significant difference ($P < 0.05$).

There was no significant difference ($P > 0.05$) found on the strength (force) between samples except at 0.2% Mb addition where breaking force values were significantly reduced ($P < 0.05$). On the other hand, gel deformability was not

affected by Mb addition. When BPP was included, the effect of Mb on Pacific whiting surimi gels was the opposite compared to gels prepared without BPP (Figure 3.4). Addition of Mb at 0.4% concentration significantly reduced ($P < 0.05$) the force value although it did not affect gel deformation.

Kim and others (2005) reported that adding sarcoplasmic protein from rockfish (*Sebastes flavidus*) to Alaska pollock surimi did not interfere with the gelation of myofibrillar proteins, and instead positively contributed to gelation at 2.0% concentration of sarcoplasmic protein. However, it was also confirmed that the gel forming ability of sarcoplasmic proteins was much weaker than myofibrillar proteins.

In our study, 1.5% surimi or 3.8% surimi can be successfully replaced by 0.4% or 1.0% Mb (Figure 3.3a). In the presence of BPP, 1.0% Mb successfully replaced 3.8% surimi in a large magnitude (Figure 3.3b). No significant effectiveness of BPP was found until Mb concentration reached 1.0%, indicating a synergistic interaction between 1.0% Mb and 1.0% BPP in terms of gel forming ability of Pacific whiting surimi.

3.4.4 Color

Color, particularly whiteness is an important factor to determine surimi quality. Whiteness was calculated using the L*-3b* equation to differentiate effects of protein additives on visual colors of surimi gels (Park 1994). L* indicates lightness to darkness and b* denotes yellowness to blueness. As shown in Figure 3.4, Mb from sardine showed a negative effect on the whiteness of Pacific whiting surimi regardless of BPP inclusion. There was an inverse correlation found between Mb content and

whiteness. Equations showing the relationship between Mb concentration (X) and whiteness (Y) of Pacific whiting surimi were obtained as:

$$Y_1 = -5.7321X_1 + 74.072 \ (R^2 = 0.9934),$$

$$Y_2 = -3.3964X_2 + 69.057 \ (R^2 = 0.9656)$$

where Y_1 was calculated in the absence of BPP and Y_2 in the presence of BPP.

Muscle and meat color are primarily determined by the concentration and chemical state of the Mb pigments (Han and others 1994). Mb is known to contribute to the redness of muscle (Chen and others 2004). The lower whiteness in Pacific whiting surimi gels made with Mb observed in this study suggests that Mb negatively contributes to the color of surimi gels. This tendency was in agreement with the study of Chaijan and others (2006), which reported that removal of Mb with water improved whiteness of surimi from sardine (*Sardinella gibbosa*).

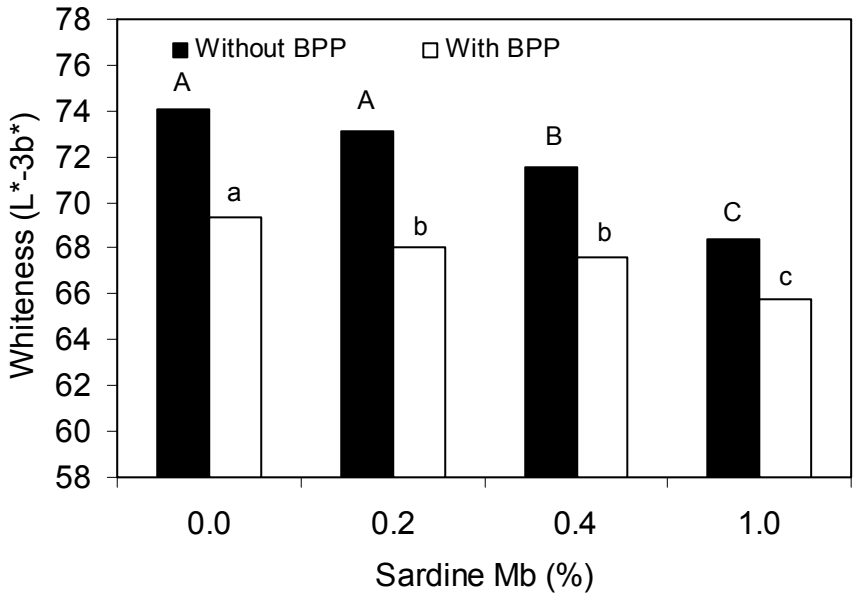

Figure 3.4 Whiteness of Pacific whiting gels prepared at various Mb contents with or without BPP.

3.4.5 Oscillatory dynamic measurement

Non-fracture gel analysis was conducted to measure the effect of Mb addition during the heat gelation of Pacific whiting surimi. Storage modulus, G', indicates an elastic element of surimi gel. High elasticity is desired for good quality surimi. G' values during liner heating rate, regardless of BPP, showed a similar trend, but the effect of Mb concentration was significantly affected by the presence of BPP (Figure 3.5, 3.6). As shown in Figure 3.5, without BPP, G' values decreased as Mb concentration increased. However, in Figure 3.6, with BPP, G' values increased as Mb concentration increased, thereby showing a synergistic effect of Mb with BPP.

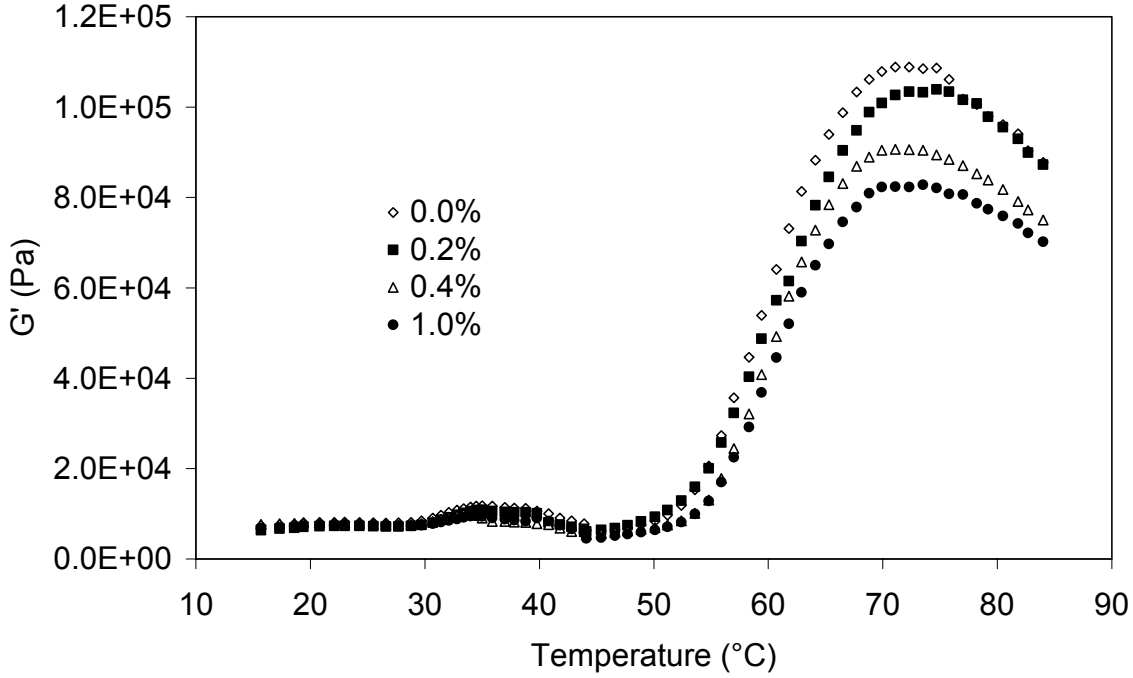

Figure 3.5 Changes in storage modulus during temperature sweep during heating of Pacific whiting surimi with various Mb contents.

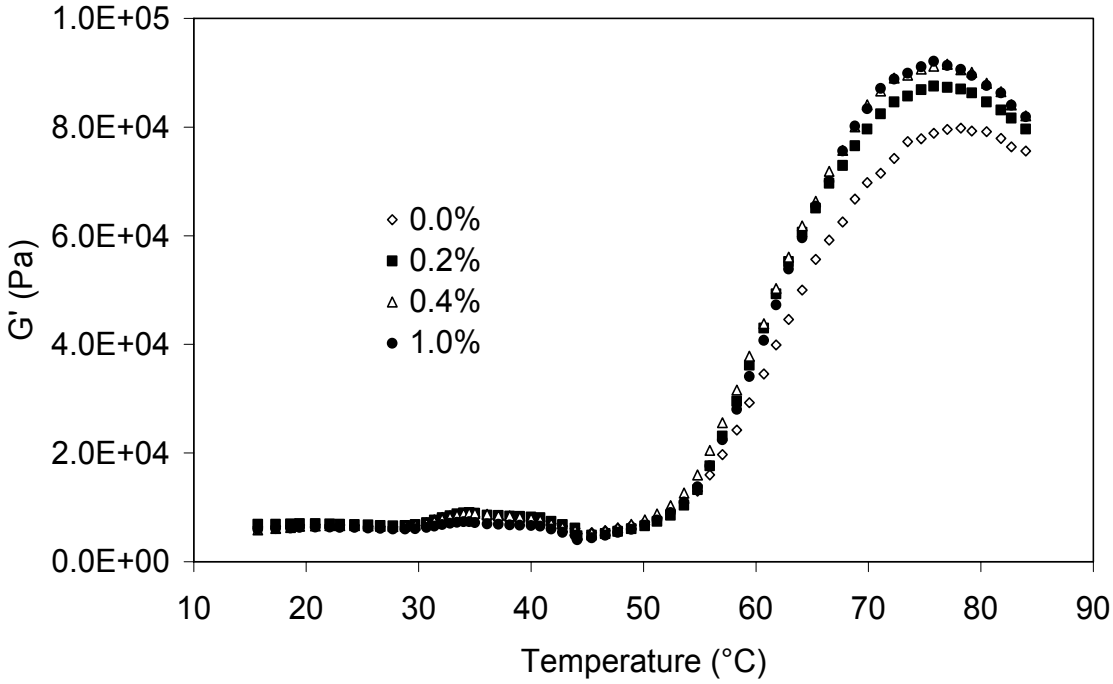

Figure 3.6 Changes in storage modulus during temperature sweep during heating of Pacific whiting surimi with various Mb contents in the presence of BPP.

This was in agreement with Kim and others (2005) using sarcoplasmic protein from rockfish (*Sebastes flavidus*) and Alaska pollock surimi, which demonstrated that the higher the sarcoplasmic protein content, the deeper the valley at around 45 °C. They suggested the trend could be correlated with the lower myofibrillar protein concentration. Starting at 45 °C, gelation was completed at 75 °C. G' values were significantly reduced around 70 °C as Mb concentration increased (Figure 3.5). Without BPP, the highest G' value was shown at 0% Mb addition in Pacific whiting surimi, indicating higher viscoelasticity. With BPP, on the other hand, the highest G' value was observed at 1.0% Mb addition (Figure 3.6) while the other tendency (with gelation starting at 45 °C and completing at 70 °C) was similar to the sample prepared

without BPP. This result indicated a synergistic interaction of 1.0% Mb and 1.0% BPP.

3.4.6 DSC

DSC analysis has been used to determine thermal behavior of muscle proteins in terms of protein folding and stability in biochemical systems. Changes in protein structure during DSC analysis are referred to as "transition". A lower transition temperature indicates that the protein is more susceptible to thermal denaturation (Jittinandana and others 2003).

Mb added to Pacific whiting surimi, regardless of BPP inclusion, exhibited four major transition temperatures at around 30, 37, 45, and 60 °C, respectively, depending on Mb concentration (0.0, 0.2, 0.4, 1.0%). Angsupanich and others (1999) reported cod myofibrillar proteins exhibited four endothermic transitions at 34.2, 43.8, 50.6, and 64.9 °C, respectively and concluded that the first three peaks were attributed to the myosin subunits while the last peak was attributed to the actin transition. There was no significant difference ($P > 0.05$) found with regards to peak 1, 2, and 4. However, as shown in peak 3, there was a trend where enthalpy values increased as Mb content increased (Table 3.1). This result implies that Mb addition increased the delta H value of Peak 3 of surimi gels during heating, suggesting that sardine Mb addition may affect surimi myosin denaturation and subsequent myosin cross-linking properties.

Table 3.1 - Thermal properties of sardine Mb measured using micro DSC

		Peak 1		Peak 2		Peak 3		Peak 4	
Mb%	BPP%	Peak Temp (°C)	ΔH J/g	Peak Temp (°C)	ΔH J/g	Peak Temp (°C)	ΔH J/g	Peak Temp (°C)	ΔH J/g
0.0	0	30.44^{b}	0.0355^{a}	37.35^{a}	0.0823^{abc}	45.07^{a}	0.0187^{a}	60.01^{abc}	0.1644^{bcd}
0.2	0	30.15^{a}	0.0395^{a}	37.16^{a}	0.0871^{bc}	45.11^{a}	0.0209^{a}	61.14^{abc}	0.1172^{ab}
0.4	0	30.87^{cd}	0.0324^{a}	36.59^{a}	0.0839^{abc}	44.86^{ab}	0.0320^{b}	61.27^{bc}	0.1527^{bc}
1.0	0	30.86^{cd}	0.0315^{a}	36.59^{a}	0.0805^{abc}	44.43^{c}	0.0545^{c}	61.79^{cd}	0.1727^{bcd}
0.0	1	30.58^{b}	0.0268^{a}	36.57^{a}	0.0914^{bc}	44.63^{ab}	0.0167^{a}	59.23^{a}	0.2395^{d}
0.2	1	30.52^{b}	0.0339^{a}	36.67^{a}	0.0996^{c}	44.78^{abc}	0.0157^{a}	59.82^{ab}	0.2202^{cd}
0.4	1	30.71^{bc}	0.0326^{a}	36.95^{a}	0.0852^{ab}	44.66^{cd}	0.0351^{b}	60.46^{ab}	0.1919^{cd}
1.0	1	31.08^{d}	0.0306^{a}	37.43^{a}	0.0548^{a}	44.74^{abc}	0.0416^{b}	63.43^{d}	0.0821^{a}

45

3.5 CONCLUSION

Ethanol extraction was more effective than ammonium sulfate extraction in the isolation of Mb. Mb obtained from Pacific sardine fillet alone did not have a positive effect on Pacific whiting surimi gelation. However, 1.0% Mb addition showed a synergistic interaction with BPP as shown by the highest force and deformation values of the prepared Pacific whiting gels. For color analysis, addition of Mb obtained from Pacific sardine negatively affected the whiteness of Pacific whiting surimi gel. The results from DSC analysis showed that Mb addition resulted in myosin denaturation and aggregation. Based on the results, it would not be necessary to remove Mb when preparing surimi from Pacific sardines.

CHAPTER 4

BIOCHEMICAL AND CONFORMATIONAL CHANGES OF MYOSIN PURIFIED FROM PACIFIC SARDINE AT VARIOUS pHS

Joo D. Park, Jirawat Yongsawatdigul, Yeung J. Choi, and Jae W. Park

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4.1 ABSTRACT

Biochemical and conformational changes of purified sardine myosin were investigated at various pHs. The purity of myosin, as determined by SDS-PAGE, was approximately 94.6%. One major band at 205 kDa, corresponding to myosin heavy chain and three light chains at 31, 24, and 23 kDa were observed on the SDS-PAGE gel. The greatest myosin protein solubility was observed at pH 7 and remained constant up to pH 11. Sardine myosin showed no solubility at pH 2.5-5.0. Three endothermic peaks were obtained for samples prepared at pH 7 and 10, while no peaks were shown for pH 2 samples, indicating chemical denaturation of myosin occurred before thermal treatment. The greatest Ca^{2+}-ATPase activity was observed at pH 7, while no activity was observed between pH 2-5 and pH 11. Total sulfhydryl content was not measured at pH 2.5-4 while the greatest measure was obtained for samples at pH 5.5. Surface hydrophobicity was not detected from pH 2.5 to 5.0, thereafter, the content remained consistent through pH 11. Storage modulus, indicating the elastic element of myosin gels, was minimally affected at pH 2, indicating myosin was chemically denatured before the temperature sweep treatment. However, at pH 10, the thermal exposure of myosin, as evidenced by dynamic thermograms with deeper valleys at 40-60 °C, was noted indicating myosin was not damaged by adjustment to pH 10 and therefore was still able to undergo thermal gelation.

Key words: Pacific sardine, myosin, pH, solubility

4.2 INTRODUCTION

Pacific sardines (*Sardinops sagax*) are a good source of protein, ranging from 15-20% protein, depending on fishing ground, species, and fishing season (Nunes and others 1992; Castrillon and others 1997; Marti de Castro and others 1997; Gokodlu and others 1998; Caponio and others 2004). Recently, sardines have returned to the Pacific Northwest, with wild animal landings exceeding over 40,000 metric tones in 2005 (McCrae and Smith 2006). However, most of this harvest is processed as bait for the long-line tuna fishery and is therefore underutilized as a fish protein source for human consumption.

Producing surimi, which is stabilized fish protein, would be one option for utilizing Pacific sardines for human consumption. Park and Park (2007) previously studied the effect of sardine myoglobin on the gel forming ability of myofibrillar protein and determined the pH-shift method is a viable way to obtain sardine protein for surimi production. Unlike conventional surimi processing, however, where myofibrillar proteins are refined by removing sarcoplasmic proteins, a novel pH-shift method, which employs the isolation of both fish myofibrillar and sarcoplasmic proteins using acid or alkaline solubilization and subsequent precipitation could be used to utilize a greater portion of sardine proteins (Hultin and Kelleher 1999; Kim and others 2003; Yongsawatdigul and Park 2004; Thawornchinsombut and Park 2006).

With this method for fish protein isolates, sardine muscle proteins would first be solubilized at extreme pH values (either pH 2 or 10) and then precipitated at the isoelectric point (pI) of sardine muscle protein before dewatering by centrifugation. This method has several advantages over the conventional surimi processing method.

These include greater yield, greater protein quality, and efficient removal of insoluble impurities (Park and Lin 2005).

Since the pH-shift method has recently received attention from both industry and academia, a study on the biochemical behavior of sardine myosin at a wide range of pH would enhance the understanding of myosin gelation. However, only a few studies have been conducted regarding the effect of various pHs on myosin (Kaminer and Bell 1966; Kristinsson 2002).

Myosin is the major protein in fish muscle, comprising approximately 55-60% of the myofibrillar protein and is the component responsible for the contractile as well as gelling properties of muscle (Lanier and others 2005). Myosin is composed of two 220 kDa heavy amino acid chains and 4 light chain subunits, ranging from 17 to 22 kDa. These amino acid chains are non-covalently attached to the myosin head (Lanier and others 2005).

Since solubility of myosin is an important factor for protein gelation, it is necessary to understand how pH affects the conformation of myosin in relation to its solubility. Protein solubility has been shown to be strongly affected by pH (Yang and Froning 1990; Turgeon and others 1992; Monahan and others 1995). However, limited studies have been performed on the biochemistry of myosin, most of which examined rabbit myosin at extreme pHs (Dreizen and others 1967; Gershman and Stracher 1969; Cross and others 1984; Ozog and Bechet 1995).

The structural stability and flexibility, however, of myosin rod and its regions have been studied (Harrington and Rodgers 1984). Most recently, Kristinsson (2002) measured conformational changes of cod myosin rod over an extreme range of pH. It

was suggested that after treatment at extreme pH, the myosin rod refolded to its native conformation through its secondary structure, when returned to neutral pH. However, the structure of myosin head did not reassemble to its native conformation. Thawornchinsombat (2004) also reported fish protein refolding does not give full recovery of its conformation after acid and alkaline pH treatments. With our long term interest in upgrading Pacific sardine to human food, a better understanding of myosin chemistry subjected to a wide range of pH is greatly needed.

The objective of the present study was to measure conformational changes of Pacific sardine myosin at various pHs. The biochemical properties of myosin at various pH values were also compared to clarify gelation characteristics of myosin from sardine muscle.

4.3 MATERIALS AND METHODS

4.3.1 Fish

Pacific sardines (*Sardinops sagax*; body length: 23.5 ± 0.3 cm; weight: 119.9 ± 7.8 g) were obtained from Astoria Holding Co. (Astoria, OR, U.S.A.). Sardines were caught off the OR-WA coast by commercial purse seiners and samples were collected 3-6 hrs post harvest July through October 2006. Sardines were randomly collected, packed in ice, and delivered to the laboratory on the day of catch. Sardine myosin was purified immediately after delivered.

4.3.2 Myosin preparation

Myosin was purified according to the method of Martone and others (1986) with slight modifications. All steps in the preparation of myosin were carried out at 4 °C to minimize myosin denaturation. Pacific sardine mince was mixed with 10 volumes (1:10) of solution A (0.1 M KCl, 1 mM phenylmethylsulfonyl fluoride, 0.02% NaN_3, and 20 mM Tris-HCl buffer, pH 7.5) before homogenizing for 1 min (model GLH-115, PG 700, Fisher Scientific, Pittsburgh, Pa., U.S.A.). The homogenate was stirred for 15 min and centrifuged at 1,000 x g for 10 min. The pellet was collected and suspended in 5 volumes of solution B (0.45 M KCl, 5 mM β-mercaptoethanol (β-ME), 0.2 M $Mg(CH_3COO)_2$, 1 mM ethylene glycol bis N, N, N',N'-tetraacetic acid (EGTA), 20 mM Tris-HCl buffer, pH 7.5). Adenosin 5'-triphosphate (ATP) was added to a final concentration of 10 mM.

The mixture was then kept for 60 min with stirring and centrifuged at 10,000 x g for 15 min. Supernatant was obtained and 25 volumes of 1 mM $NaHCO_3$ was added to supernatant and kept for 30 min. The pellet was obtained by centrifugation at 12,000 x g for 15 min. The pellet was then resuspended in 2.5 volumes of solution C (0.5 M KCl, 5 mM β-ME, 20 mM Tris-HCl buffer, pH 7.0 before homogenizing with a tissue grinder (55 mL, Potter-Elvehjem, Wheaton Instruments, N.J., U.S.A.). The homogenate was kept for 10 min before mixing with 2.5 volumes of 1 mM $NaHCO_3$. Both $MgCl_2$ and sodium pyrophosphate were added to a final concentration of 10 mM, respectively.

The solution was then kept overnight and centrifuged at 22,000 x g for 15 min. The myosin pellet was kept on ice until used. Before use, myosin was re-suspended in 2 volumes of solution C. Myosin sample was used for measuring solubility, Ca-

ATPase activity, total sulfhydryl content, and surface hydrophobicity. For the oscillatory dynamic measurement and micro differential scanning calorimetry test, myosin pellet was mixed with 50% glycerol and kept frozen at -70 °C until used.

4.3.3 Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

To determine the purity of extracted myosin, prepared myosin extract was subjected to SDS-PAGE (Laemmli 1970) using 5% stacking acrylamide gel and 12% separating acrylamide gel. Protein concentrations were determined by the method of Lowry and others (1951). SDS-PAGE gel was stained in 0.125% Coomassie blue R-250 and destained in 40% methanol and 10% acetic acid. A wide-range molecular weight standard mixture (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was used, which included myosin from rabbit muscle (205 kDa), β-galactosidase from *E. coli* (116 kDa), phosphorylase b from rabbit muscle (97 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase from bovine liver (55 kDa), chicken egg ovalbumin (45 kDa), glyceraldehydes-3-phosphate dehydrogenase of rabbit muscle (36 kDa), carbonic anhydrase of bovine erythocyte (29 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), bovine α-lactalbumin (14.2 kDa), and aprotinin of bovine lung (6.5 kDa).

The 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.).

4.3.4 Solubility

To evaluate the effect of pH on myosin solubility, 0.5 mL of myosin solution (4 mg/mL) was added to a buffer solution [pH 2, 2.5 (glycine, IS = 0.05), 3, 4, 5, 5.5, 6 (potassium hydrogen phthalate, IS = 0.15), 7 (sodium phosphate, IS = 0.05), 8 (sodium bicarbonate, IS = 0.05), 9 (boric acid and potassium chloride, IS = 0.05), 10 (sodium carbonate, IS = 0.05) and 11 (sodium hydrogen phosphate, IS = 0.1)]. Buffer solutions were added to myosin solution at a 1:1 ratio. All buffers, except pH 5.5, were purchased from VWR International Inc. (West Chester, Pa., U.S.A.) Buffer pH 5.5 was made by adjusting pH 5 buffer solution to pH 5.5 using 1N NaOH. The mixtures were continuously stirred at 4 °C for 3 h.

The homogenates were then centrifuged at 18,000 x g for 15 min. Protein remaining in the supernatant after centrifugation was measured by the method of Lowry and others (1951) using bovine serum albumin (BSA) as a standard. The resulting measurement yielded the protein concentration of the soluble fraction of myosin in the supernatant, which indicated myosin solubility.

4.3.5 Ca-ATPase activity

A volume (0.125 mL) of 0.5M Tris-maleate buffer (pH 7.0), 0.125 mL of 0.1 M $CaCl_2$, and 1.875 mL of deionized water were added to 0.25 mL of myosin solution (4 mg/mL) at various pHs (2, 2.5, 3, 4, 5, 5.5, 6, 7, 8, 9, 10 and 11). The mixture was incubated at 25 ^{o}C for 5 min before adding 0.125 mL of 20 mM ATP solution. The myosin solution was incubated at 25 ^{o}C for 8 min before stopping the reaction by adding 1.25 mL of chilled 15% trichloroacetic acid. The mixture was then centrifuged

at 3,000 x g at 4 ^{o}C for 5 min and the supernatant was analyzed for inorganic phosphate released according to the method of MacDonald and Lanier (1994).

4.3.6 Total sulfhydryl content

Total sulfhydryl group was determined by the method established by Ellman (1959) using 5-5'-dithiobis-(2-nitrobenzoic acid) (DNTB). A volume of 9 mL of 0.2 M Tris-HCl (pH 7.0) containing 8 M urea, 2% SDS, and 10 mM ethylene diamine tetraacetic acid (EDTA) was added to 1 mL of myosin solution (4 mg/mL). The resulting myosin mixture (4 mL aliquot) was mixed with 0.1% DNTB (0.4 mL). The reaction mixture was then incubated at 40 ºC for 25 min before measuring absorbance at 412 nm. Total sulfhydryl groups were determined using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$.

4.3.7 Surface hydrophobicity

Surface hydrophobicity (S_o) of myosin at various pHs was determined by the method of Kato and Nakai (1980). Myosin solution at each pH condition (2-11) was diluted to various concentrations from 0.1 to 1 mg/mL in 0.6 M KCl. After stabilizing at 25 ºC, 10 µL of 1-anilinonaphthalene-8-suflonic acid (ANS) (8 mM in 0.1 M phosphate buffer, pH 7.0) was added to 2 mL of the diluted protein solutions, respectively. The relative fluorescent intensity of ANS-protein conjugates was measured using a luminescence spectrophotometer (Perkin Elmer LS-50B, Norwalk, CT, U.S.A.) at wavelengths (λ_{ex}, λ_{em}) of 374 nm and 485 nm, respectively. Protein hydrophobicity was calculated from the initial slope resulting from the plots between

relative fluorescence intensity and protein concentration (w/v) using linear regression analysis for each dilution set, respectively.

4.3.8 Micro differential scanning calorimetry (micro DSC)

DSC studies were performed using a SETARAM micro differential scanning calorimeter (SETARAM Co., Lyon, France). Temperature calibrations were performed using naphthalene. Myosin samples kept with 50% glycerol were dialyzed (MW 12,000-14,000) overnight against 0.5 M KCl, 5 mM β-ME, 20 mM Tris-HCl buffer, pH 7.0 at 4 °C and then centrifuged. Myosin (5 mg/mL), approximately 500 mg, was accurately measured in a stainless steel vessel. Deionized water was then added to the reference vessel. The myosin solutions at pH 2, 7, and 10 were scanned at 1 °C/min over a range of 10-80 °C. Since myosin is a salt soluble protein, 0.6 M KCl was added to the pH 7 sample to observe its thermal gelation. Triplicate samples with reproducible thermograms were analyzed.

4.3.9 Oscillatory dynamic measurement

Myosin solutions at various pHs (2, 7, and 10) were measured as a function of temperature using a CS-50 dynamic rheometer (Bohlin Instruments, Inc., Cranbury, N.J., U.S.A.). Myosin samples kept with 50% glycerol were dialyzed (MW 12,000-14,000) overnight using 0.5 M KCl, 5 mM β-ME, 20 mM Tris-HCl buffer, pH 7.0 at 4 °C and then centrifuged. Myosin pellet was obtained and the pH was properly adjusted to 2, 7, and 10. The myosin solution (5 mg/mL) was then placed between a

cone and plate (4 °) with a gap of 1 mm. To avoid sample drying during heating, a plastic cover (trapper) with moistened sponge was used. The samples were heated from 10 to 80 °C at a heating rate of 1 °C/min. The oscillatory mode was applied with a fixed frequency at 0.1 Hz and shear stress at 1 Pa, within the linear viscoelastic range demonstrated by the samples. Samples were tested in duplicate.

4.3.10 Statistical analysis

The results were presented as the average and standard deviation of solubility, Ca^{2+} ATPase activity, total sulfhydryl content, and surface hydrophobicity measurement conducted in triplicate. Statistical comparisons were made between treatments by ANOVA and Tukey's test using SPSS (version 13) software package (SPSS Inc., Chicago, Ill., U.S.A.). A statistical significance was reported at $P < 0.05$.

4.4 RESULTS AND DISCUSSION

4.4.1 SDS-PAGE pattern of sardine myosin

To determine the purity of extracted myosin, the sample was subjected to SDS-PAGE analysis. The electrophoretic patterns of the wide molecular weight standard and sardine myosin are shown in Figure 4.1. The results revealed the presence of one major band with a molecular weight of 205 kDa, which was determined as myosin heavy chain. Minor bands, with molecular weights of 31 kDa followed by 24 and 23 kDa were also observed and determined to be myosin light chains.

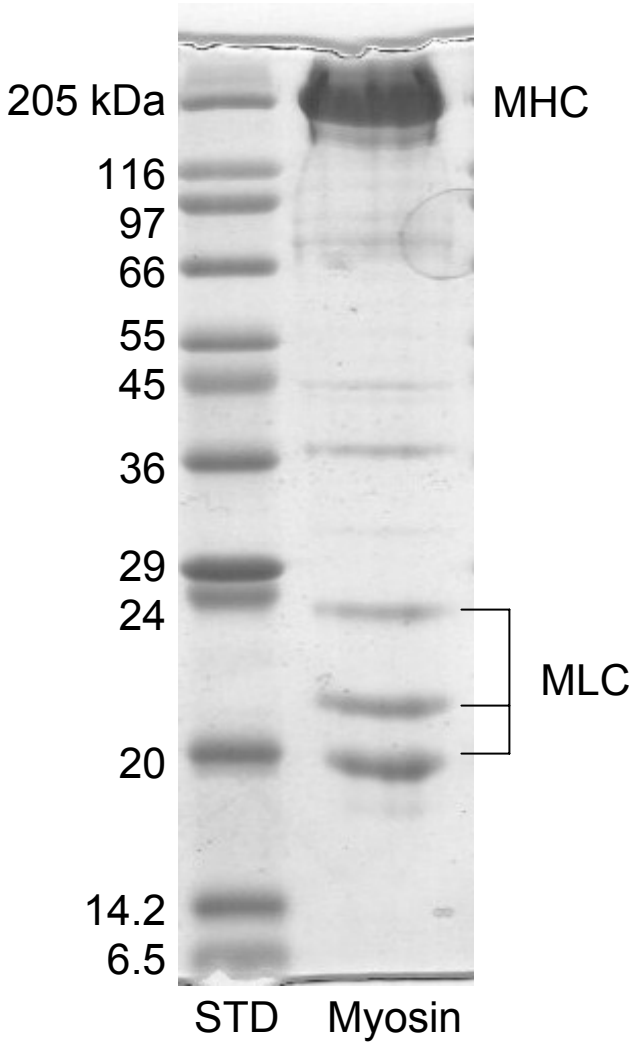

Figure 4.1 SDS-PAGE pattern of purified sardine myosin. Purified myosin was applied to the 12% acrylamide separating gel. STD: low standard molecular weight, MHC: myosin heavy chain, MLC: myosin light chains

Okagaki and others (2005) reported that molecular weight of light chains of carp ranged between 16 and 26 kDa. Based on the accumulated density of all bands measured using a gel scanner, the purity of myosin was found to be approximately 94.6%.

These results were generally in agreement with previously reported studies. Mathew and Parkash (2006) reported that myosin heavy chain and light chains from sardines (*Sardinella longiceps*) showed molecular weights of 205, 31, 23, and 22 kDa, respectively. Watabe and Hashimoto (1980) also reported that myosin from white mackerel muscle had three light chain subunits with molecular weights of 26.5, 20, and 17.5 kDa. For our myosin purification process, in addition to the presence of trace amounts of actin [(45 kDa), (Figure 4.1)], one additional minor band at 37 kDa was also observed. This minor band, according to the study on African catfish (*Heterobranchus longifilis*) by Huriaux and others (1999), is likely from tropomyosin.

4.4.2 Effect of pH on solubility of sardine myosin

The solubility of Pacific sardine myosin was determined at various pHs (Figure 4.2). The results showed that myosin was sparingly soluble at low pH values ranging between pH 2.5 to 5.0. A sharp increase in solubility occurred at pH 5.5 and continued through pH 11.0. The greatest solubility was observed at pH 7.0. Good solubility was also measured at pH 2.0, but a significant drop was made at pH 2.5. Insolubility between pH 2.5 to 5.0 was probably due to the pI of myosin, which is known to be pH 4.8 to 6.2 (Kinsella 1984) depending on the salt concentration used (Lin and Park 1998).

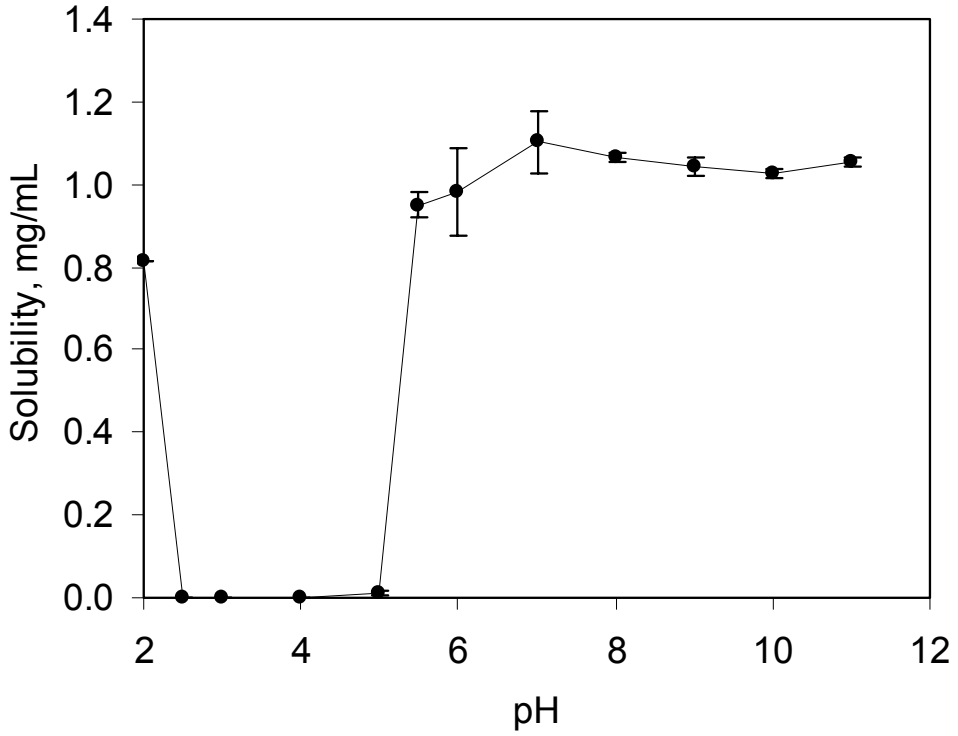

Figure 4.2 Solubility of sardine myosin as affected by pH. Buffer solution [pH 2, 2.5 (glycine, IS = 0.05), pH 3, 4, 5, 5.5, 6 (potassium hydrogen phthalate, IS = 0.15), 7 (sodium phosphate, IS = 0.1), pH 8 (sodium bicarbonate, IS = 0.05), pH 9 (boric acid and potassium chloride, IS = 0.05), pH 10 (sodium carbonate, IS = 0.05) and pH 11 (sodium hydrogen phosphate, IS = 0.1)]. Buffer solutions were added to myosin solution at a 1:1 ratio.

4.4.3 Effect of pH on Ca^{2+}-ATPase of sardine myosin

Ca^{2+}-ATPase activity is known as an important index for evaluating the quality of protein and is positively related to gelation properties (Katoh and others 1979), which are affected by conformational changes of myosin. To better understand the conformational changes of sardine myosin, Ca^{2+}-ATPase activity was evaluated (Figure 4.3). Ca^{2+}-ATPase activity showed the smallest activity at pH <5 and > pH 10. The greatest Ca^{2+}-ATPase activity was observed at pH 7 ($P < 0.05$).

According to other studies, these results were typical because Ca^{2+}-ATPase activity is highly pH dependent (Katoh and others 1977). Watabe and Hashimoto (1980) observed that Ca^{2+}-ATPase of mackerel myosin had high activity at pH 6.5 and 9.5. Kameyama and others (1985) also reported that Ca^{2+}-ATPase of rabbit myosin had high activity at pH 6 and 9. The loss of ATPase activity does not necessarily imply the unfolding of myosin head. It merely indicates that the myosin head has undergone conformational changes (Chan and others 1995).

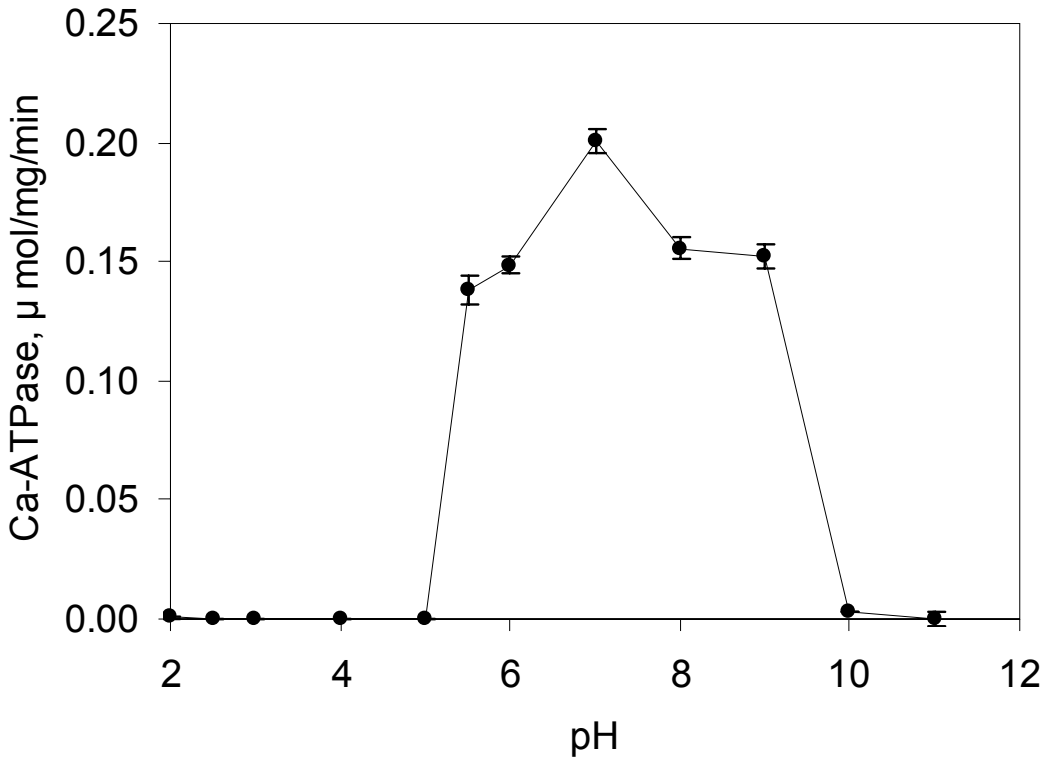

Figure 4.3 Ca^{2+}-ATPase activity of sardine myosin as affected by pH. Buffer solution [pH 2, 2.5 (glycine, IS = 0.05), pH 3, 4, 5, 5.5, 6 (potassium hydrogen phthalate, IS = 0.15), pH 7 (sodium phosphate, IS = 0.1), pH 8 (sodium bicarbonate, IS = 0.05), pH 9 (boric acid and potassium chloride, IS = 0.05), pH 10 (sodium carbonate, IS = 0.05) and pH 11 (sodium hydrogen phosphate, IS = 0.1)]. Buffer solutions were added to myosin solution at a 1:1 ratio.

4.4.4 Effect of pH on total sulfhydryl content of sardine myosin

The pH effect on the total sulfhydryl (SH) content of sardine myosin was determined (Figure 4.4). At pH 3 and 4, there was no measurable SH. This was due to aggregation of sardine myosin at pH 3 and 4 as well as a reduced effectiveness of Ellman's reagent, which is highly pH dependent (Hofmann and Hamm 1978). The reactive SH groups in the native state of myosin are located on the protein surface while some are buried within the protein structure. A decrease in SH content is considered to be due to the formation of disulfide bonds through oxidation of SH groups or disulfide intrachanges, which stabilize the protein structure (Hayakawa and Nakai 1985).

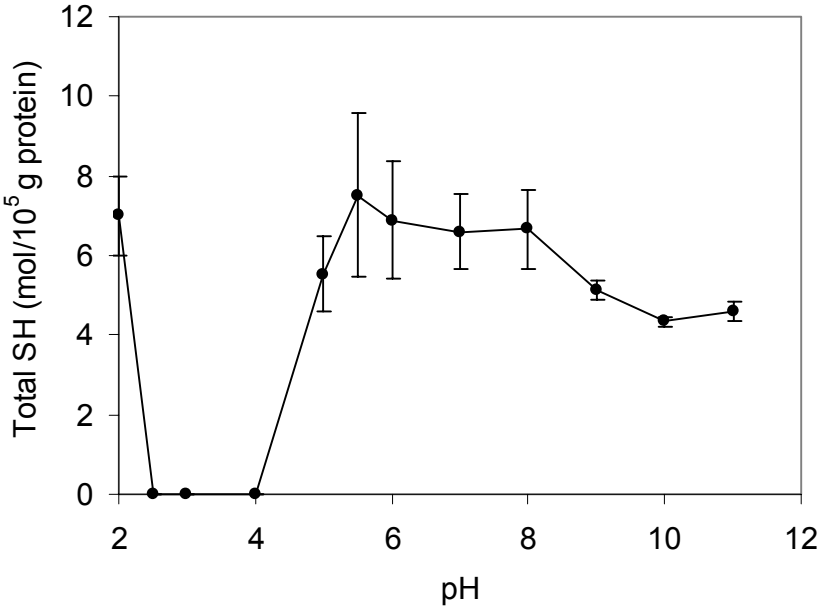

Figure 4.4 Total SH content of sardine myosin as affected by pH. Buffer solution [pH 2, 2.5 (glycine, IS = 0.05), pH 3, 4, 5, 5.5, 6 (potassium hydrogen phthalate, IS = 0.15), pH 7 (sodium phosphate, IS = 0.1), pH 8 (sodium bicarbonate, IS = 0.05), pH 9 (boric acid and potassium chloride, IS = 0.05), pH 10 (sodium carbonate, IS = 0.05) and pH 11 (sodium hydrogen phosphate, IS = 0.1)]. Buffer solutions were added to myosin solution at a 1:1 ratio.

The results revealed a trend similar to that observed for sardine myosin solubility (Figure 4.2). This trend is also in agreement with a previous study using salmon myosin (Lin and Park 1998). A greater SH content was observed at pH above 5.5. When pH was greater than 5.5, an increased SH content was observed. Thawornchinsombut and Park (2004) also reported that under alkaline condition, regardless of ionic strength (10 and 600 mM NaCl), the SH content significantly decreased as pH increased.

4.4.5 Effect of pH on surface hydrophobicity of sardine myosin

Surface hydrophobicity was determined to monitor the conformational changes of sardine myosin at various pH values (Figure 4.5). The greatest hydrophobicity value appeared at pH 6.0. Similar values of hydrophobicity were observed at pH 2.0, 5.5 and from 7.0 to 11.0. This implies that alkaline pHs might have minimal effect on the globular structure of the myosin. Kristinsson and Hultin (2003a) reported that myosin partially unfolded in a different manner when subjected to acid and alkali conditions, respectively. In addition, under both acid and alkali pH-shift treatments the conformation of the myosin head did not revert to its native state upon refolding. In the current study, surface hydrophobicity results indicate a smaller change in the conformation of alkali treated myosin than that of acid treated myosin. We speculated that alkali treated myosin did not undergo significant conformational changes. Surface hydrophobicity was not detectable at pH 2.5 to 5.0. This was likely due to the insolubility of myosin at these pH levels (Figure 4.2).

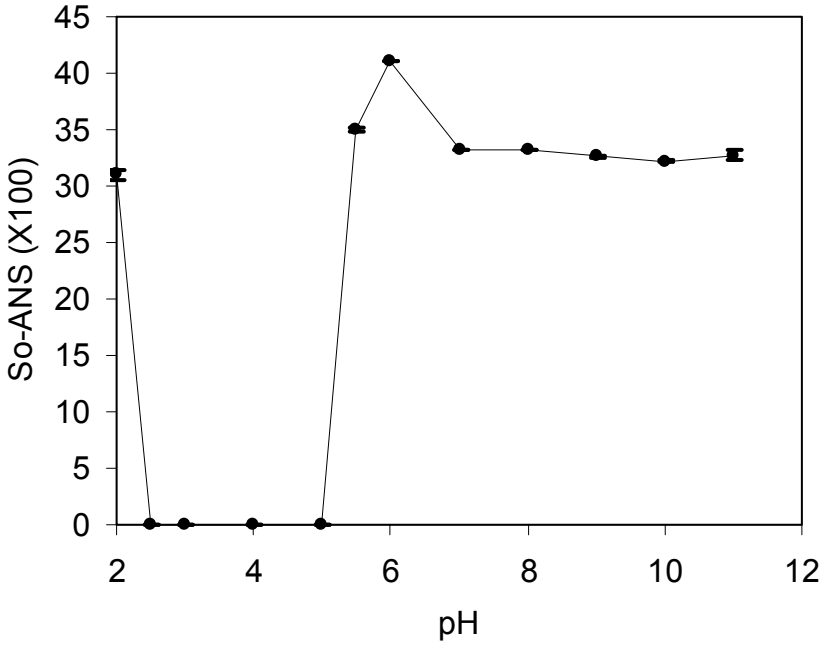

Figure 4.5 Surface hydrophobicity of sardine myosin as affected by pH. Buffer solution [pH 2, 2.5 (glycine, IS = 0.05), pH 3, 4, 5, 5.5, 6 (potassium hydrogen phthalate, IS = 0.15), pH 7 (sodium phosphate, IS = 0.1), pH 8 (sodium bicarbonate, IS = 0.05), pH 9 (boric acid and potassium chloride, IS = 0.05), pH 10 (sodium carbonate, IS = 0.05) and pH 11 (sodium hydrogen phosphate, IS = 0.1)]. Buffer solutions were added to myosin solution at a 1:1 ratio.

4.4.6 Effect of pH on micro DSC of sardine myosin

Micro DSC thermograms obtained in this study are shown in Figure 4.6. The greatest enthalpy value of denaturation for myosin was observed at 0.6M KCl and pH 7. Myosin is a salt soluble protein, therefore, in order to be compared at pH 2 and 10, myosin at pH 7 needed to be solubilized using 0.6M KCl. The DSC can be used to monitor the unfolding process of the protein as it undergoes temperature-induced transitions, with lower transition temperatures indicating that the protein is more susceptible to thermal denaturation (Shriver and Kamath 1990; Jittinandana and others

2003). For the pH 7 sample in 0.6 M KCl, three typical endothermic transition peaks were observed at 31, 51, and 65 °C. This was in agreement with Sano and others (1988) who reported that carp myosin showed multiple transitions at pH 6.8. Togashi and others (2002) also reported that walleye pollock myosin had three endothermic transitions at 28, 34, and 41 °C, respectively. In addition, Lo and others (1991) reported myosin from black marlin muscle had three endothermic transitions at 40, 43, and 50 °C, respectively, at pH 6.8. With the addition of 0.6 M KCl, the onset temperatures were shifted slightly to lower temperatures, indicating the destabilization of myosin conformation by 0.6 M KCl.

In the current study, pH 10 samples, at lesser ionic strength (IS value=0.05) revealed three endothermic peaks, which is similar to the findings of Sano and others (1988), who found three endothermic transitions at 31, 41, and 61 °C at pH 7 and low ionic strength (IS value=0.05). This implies that myosin was not completely unfolded at pH 10 (Figure 4.6), which is also supported by data shown in surface hydrophobicity (Figure 4.5). However, at pH 2, typical thermograms were not observed. It can therefore be assumed that myosin was already unfolded chemically at pH 2 treatment. Total enthalpy value of each treatment showed 0.035 (pH 2), 0.215 (pH 7), 0.609 (pH 7, 0.6 M KCl), and 0.229 (pH 10) indicating that myosin at pH 10 is less unfolded than pH 2.

4.4.7 Effect of pH on rheological properties of sardine myosin

The effect of pH on the storage modulus (G') of sardine myosin is shown in Figure 4.7. G' value indicated the viscoelastic behavior of sardine myosin at various

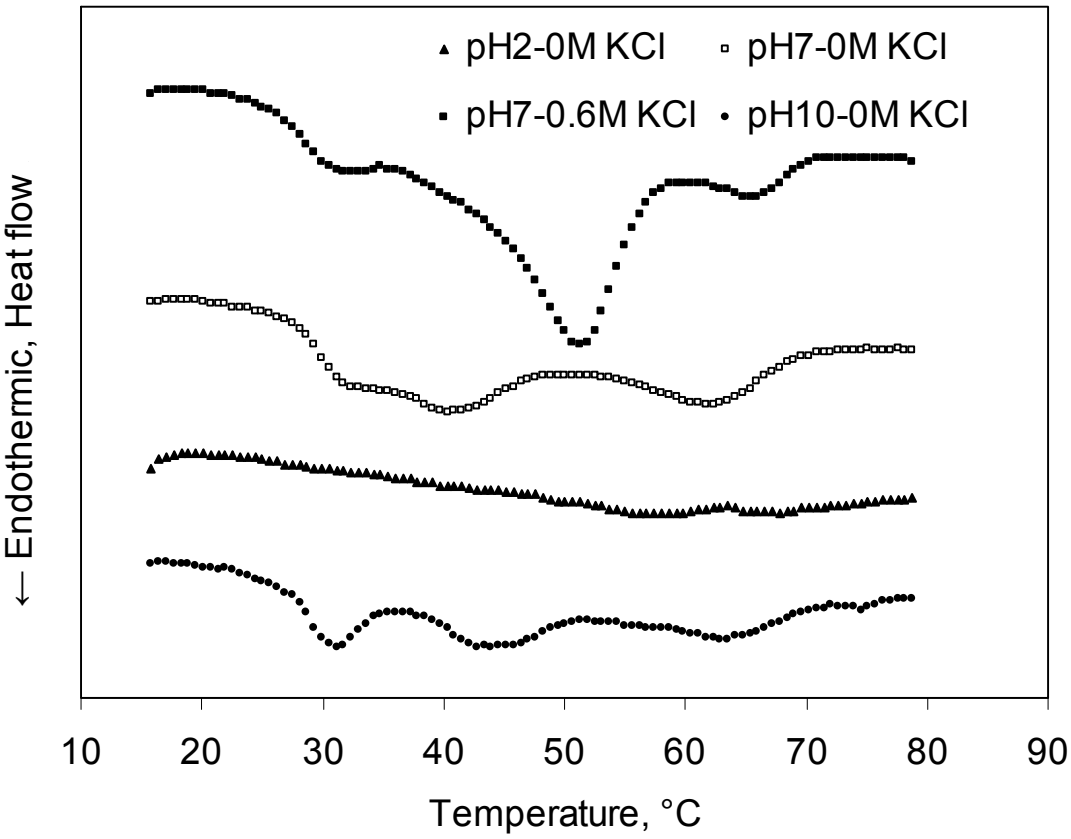

Figure 4.6 DSC thermogram of sardine myosin as affected by pH. Myosin at various pHs were heated from 10 °C to 80 °C at 1 °C/min.

pH values. Since myosin is a salt soluble protein, 0.6M KCl was applied to the pH 7 sample to measure the effects of low and high ionic strength on the viscoelastic properties. G' values were significantly affected by changing pH from 2 to 10. According to Lou and others (2000), a typical temperature sweep pattern demonstrates an initial increase in G' followed by a decrease in G' due to the denaturation of light meromyosin followed by the increase in the fluidity of myofibrillar filaments. However, myosin at pH 2 exhibited significantly different viscoelasticity compared to native myosin at pH 7. G' of myosin at pH 2 gradually increased without large transthermal peaks during heating from 10 °C to 80 °C, indicating that myosin at pH 2

was chemically unfolded before subjecting to the temperature sweep of the dynamic rheology test. Therefore, the rheogram (pH 2) did not show the typical pattern of myosin [(pH 7, 0.6M KCl), (Figure 4.7)]. This result supports the micro DSC test results, which also indicated myosin at pH 2 was conformationally damaged and not thermally reactivated. At pH 2, therefore, myosin may have already been unfolded and formed a random coil structure. Kristinsson and Hultin (2003b) also reported that acid-treated myosin (pH 2.5 → 7.5) did not show a peak in the storage modulus during the heating process.

At pH 10, the initial G' value of myosin showed the greatest value. G' decreased gradually until around 60 °C, indicating the thermal unfolding of myosin, and then increased significantly, indicating most of the myosin molecules were not damaged at pH 10. At pH 10, myosin molecule possesses negative charges. When it was heated, entanglement would be limited due to electrostatic repulsion. This could be a reason why it decreased as heated starting at 35°C. For pH 7 myosin sample treated with 0.6 M KCl, the pattern of the G' value was similar to that of the myosin suspension (0.6 M NaCl, 20 mM sodium phosphate, pH 7.0) from Pacific whiting (Yongsawatdigul and Park 1999). Myosin at pH 7 without salt showed comparatively low G' values up to 30 °C. After the rapid increase of G' between 30 and 40 °C, storage modulus did not change.

4.5 CONCLUSION

It is well accepted that the pH-shift method will be useful for the industrial production of high quality fish protein isolates in the near future. This method

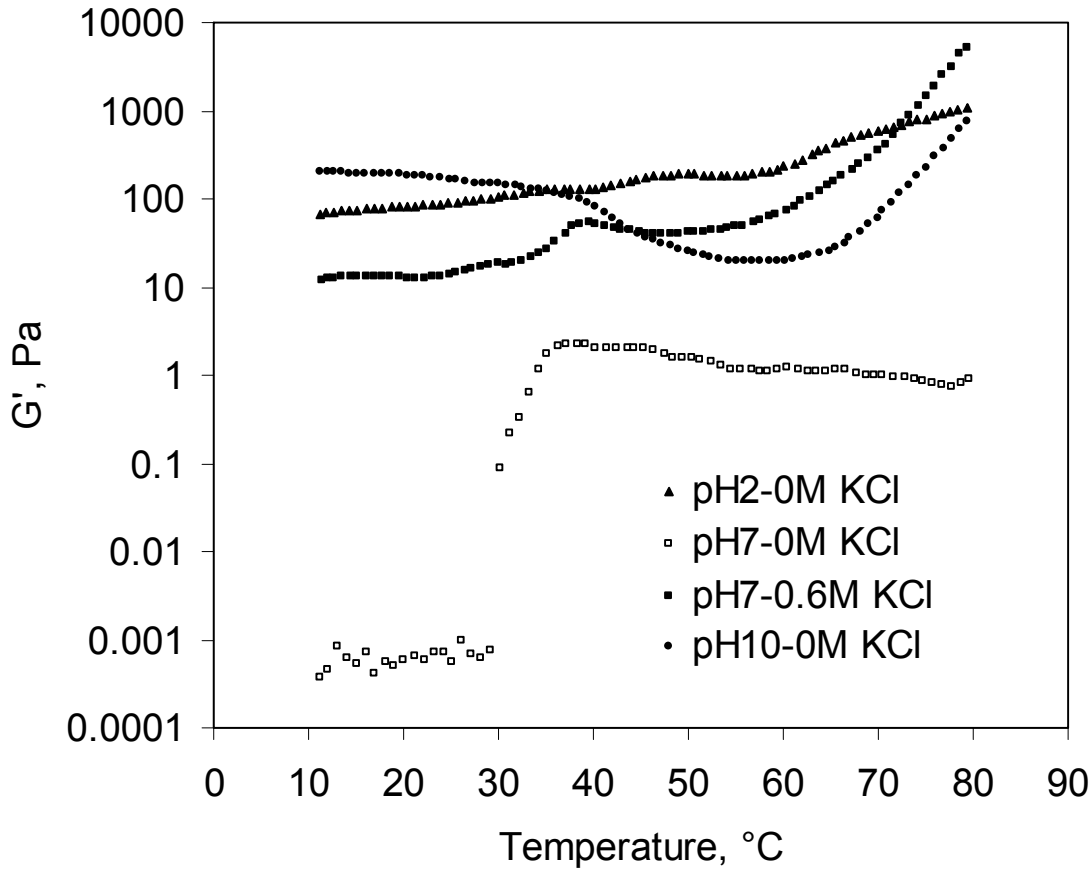

Figure 4.7 Storage moduli (log G') of sardine myosin as affected by pH. Myosin at various pHs were heated from 10 °C to 80 °C at 1 °C/min.

possesses several advantages over the conventional surimi processing method, providing greater yield and greater protein quality. The current study revealed that the alkaline process resulted in myosin gel with similar elastic values when prepared at neutral pH and better than myosin gel prepared using the acidic process. Therefore, the alkaline pH-shift method appeared to be effective in isolating high quality functional fish proteins from Pacific sardines.

CHAPTER 5

EXTRACTION OF PHOPHOLIPIDS FROM PACIFIC SARDINE AND THEIR EFFECT ON GELATION PROPERTIES OF ALASKA POLLOCK SURIMI

Joo D. Park and Jae W. Park

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5.1 ABSTRACT

Phospholipids (PL) were extracted from Pacific sardine and added to Alaska pollock surimi to measure their effects on protein gelation. Sep-pak silica column was used with hexane and methanol for PL extraction. The purity of PL extract was determined by thin-layer chromatography and results demonstrated high purity of obtained PL extract. Fatty acid composition of three different lipid fractions, total lipids (TL), neutral lipids (NL), and PL, was determined by gas chromatography. The PL fraction contained higher levels of unsaturated fatty acids than NL and TL fractions. The predominant fatty acid in TL and NL fractions was 16:0 (palmitic acid) while 22:6n-3 (docosahexaenoic acid, DHA) was the major fatty acid in PL fraction. Freeze-thaw cycles were applied to rapidly induce the biochemical changes of phospholipids and mimic long term frozen storage. Lipid oxidation measured by TBARS increased with the addition of 1% PL and was further enhanced when freeze-thaw cycles extended to 6. Storage modulus (G') of Alaska pollock surimi gel was negatively affected by 1% PL addition particularly after 2 or 4 freeze-thaw cycles. However, G' values of sample with 1% PL increased at 6 and 8 cycles, indicating possible gelation enhancement through protein oxidation. PL at 1% level did not affect lightness of Alaska pollock surimi gel during extended freeze-thaw cycles, but increased yellowness slightly.

Key words: phospholipids, Pacific sardine, Alaska pollock surimi

5.2 INTRODUCTION

Surimi is traditionally manufactured from lean fish like Alaska pollock, Pacific whiting, or threadfin bream. The surimi industry, however, has changed dramatically over the past decade. Most recently, the harvest of several white muscle fish has decreased. As a result surimi prices have continuously increased. In response to the price increase, some researchers have explored the utilization of new fish species for surimi production (Chen and others 1996; Alvarez and others 1999; Park and others 2003), expanding the range of species to include dark muscle fish. In addition, the overexploitation of white muscle fish species has led manufacturers to consider fatty fish species, like sardine and mackerel, as possible resources for surimi production.

However, dark muscle fish are difficult to process because they exhibit great seasonal variability, particularly in lipid contents. Moreover, due to their high content of dark muscle and fat, these fish are sensitive processing materials. They easily lose freshness and functional properties of their proteins (Shimizu and others 1992). In addition, there also exist pro-oxidants, such as myoglobin and oxidizing enzymes, in most commercially available fatty fish, which contribute to lipid oxidation.

Lipid oxidation in fish is one of the most important factors for quality loss during frozen storage. In fish, lipids are mainly in the unsaturated form, exhibiting multiple double bonds in the fatty acids, which are readily susceptibility to attack by molecular oxygen (Flick and others 1992). Lipid oxidation can affect the appearance, flavor, texture, and nutritional value of fish, which results in quality deterioration. The extent of lipid oxidation varies with season and species.

The oxidation reaction contains three steps: initiation, propagation, and termination. Peroxides (primary products) are formed in the propagation step of the reaction. After peroxides are formed, the non-conjugated double bonds, present in natural unsaturated lipids, are converted to conjugated double bonds. The primary products are then broken down into secondary products, such as malonaldehydes. Therefore, lipid oxidation can be determined by measuring the primary or secondary products in the oil or fat (DeMan 1999).

The total content of lipids varies by fish species of fish. Lean fish such as Alaska pollock and cod, generally contains < 2% lipids, whereas in fatty species, such as sardine and mackerel, values raging from 4 up to 26% have been reported (Ackman 1994; Caponio and others 2004; Okada and Morrissey 2007). In addition, lipids of fatty fish are rich in long-chain polyunsaturated fatty acids (PUFA), which possess excellent nutritional value (Hultin and others 2005).

Fish lipids can be roughly divided into two main groups; neutral lipids (NL) and polar lipids. The former consist of triacylglycerols (TG), which occur as large droplets within the adipose tissue or as smaller droplets within the muscle cells or intercellular spaces. Polar lipids consist mainly of phospholipids (PL), which are important constituents of membranes. PL are reported to be more susceptible to oxidation than TG due to high PUFA concentration. In addition, the total amount of PL is relatively independent of environmental factors although it has been reported that intrinsic factors such as total lipid (TL) level affects the amount of PL (Bandarra and others 1997). Similarly, the fatty acid composition of sardine phospholipids is minimally effected by season (Shirai and others 2002).

PL are a major cause of quality deterioration in lipid oxidation (Pazos and others 2005). It is known that PL play a critical role in the oxidation process since PL have a high PUFA concentration. However, Jeong and others (1995) reported that PL extracted from squid viscera had an antioxidant effect on fish oil. King (1992) also reported antioxidant properties of PL in salmon oil. Similarly, Chen and Nawar (1991) reported that PL have an antioxidative effect on milk fat.

Several studies were conducted regarding how lipid oxidation affects surimi gelation (Shimizu and others 1992; Kelleher and others 1994; Murakawa and others 2003; Eymard and others 2005). However, there is no research available in terms of how much PL affect the stability of fish proteins during frozen storage and gelation during the heating process. The objectives of this study were to extract PL from Pacific sardine, to characterize PL, and to determine their effects on Alaska pollock surimi during frozen storage. For the frozen storage experiment, freeze-thaw cycles were used in order to mimic longterm frozen storage and to measure quality changes of surimi as affected by PL.

5.3 MATERIALS AND METHODS

5.3.1 Materials

Pacific sardines (*Sardinops sagax*; body length: 26.91 ± 1.6 cm; weight: 190.34 ± 17.34 g) were obtained from West Bay Co. (Astoria, Oreg., U.S.A.) from July through September 2006. Fresh sardines were randomly chosen, packed in ice, and delivered to the laboratory on the day of catch. Commercial FA grade Alaska

pollock (*Theragra chalcogramma*) surimi, which was 7 mo old, was obtained from Trident Seafood Co. (Seattle, Wash., U.S.A.).

5.3.2 Methods

5.3.2.1 Isolation of PL from Pacific sardine

Whole fish was minced using a vertical-cutter/mixer (Stephan Machinery Corp., Columbus, Ohio, U.S.A.) for 2 min at high speed. Mince was mixed with hexane:methanol (2:1) to obtaine crude oil. NL and PL were isolated from sardine mince according to the method of Christie (1992) with a slight modification. The isolation of NL and PL was done using a solid phase extraction (SPE) column (Sep-Pak Vac 35cc, C18; Waters, Milford, Mass. U.S.A.). Crude oil with hexane (1:2) was subjected to SPE column and then two volumes of hexane were used to elute the NL. Finally methanol was used to isolate PL. Isolated lipids were subjected to thin layer chromatography (TLC) for verification as described below. Fatty acid profiles in isolated lipids were determined by gas chromatography (GC).

5.3.2.2 Determination of lipid composition by thin-layer chromatography separation

NL and PL extracted from sardine were subjected to thin layer chromatography (TLC) to verify the purity of NL and PL, respectively. TLC was determined according to the method of Okada and Morrissey (2007). Samples were dissolved in hexane (10% solution, v/v), and an aliquot of 5 µl sample mixture was loaded onto the silica plate (K6 silica gel 60, 200 x 200 x 0.25 mm, Whatman Inc., Brentford, UK).

The developing solvent was hexane:diethylether:acetic acid (60:40:2), v/v). Spots were visualized by spraying 5% sulfuric acid solution followed by drying at 100 °C on the hot plate for 30 min. TLC-reference standard containing cholesteryl oleate (CO), triolein (TO), oleic acid (OL), and lecithin (PL) (Cat. No. 18-5, Nu Check Prep, Inc., Elysian, Minn., U.S.A.), was used for qualification. Lecithin, consisting of three types of PL; phosphatidylcholine (PC), phosphatidylethanolamin (PE), and phosphatidylinositol (PI), was used to verify the presence of PL.

5.3.2.3 Gas chromatography (GC)

Fatty acids in NL and PL were converted into fatty acid methyl esters (FAME) according to AOAC method 991.29 (1998), and their composition was determined by GC. A Shimadzu GC-2010 (Shimadzu Corp., Kyoto, Japan), equipped with a flame-ionization detector and capillary column (Omegawax^{TM} 250 capillary column, 30 m × 0.25 × 0.25 μm film thickness, Supelco, Bellefonte, Pa., U.S.A.) was used for analyzing FAME. GC system parameters were set as follows: injector and detector temperatures of 250°C and 270°C, respectively; column temperature of 170°C with 8 min hold time; gradual heating to 245°C at a rate of 1°C/ min with 2 min hold time; and helium as a carrier gas. The fatty acid concentrations were calculated by comparison of their retention time with that of the reference standards (Supelco, Bellefonte, Pa., U.S.A.).

5.3.2.4 Freeze-thaw cycle

PL, membrane lipids, are not removed through conventional surimi processing and are often known to give fishy odor and enhance the denaturation of fish proteins during frozen storage. PL (0 and 1%) were added to Alaska pollock surimi to determine their effect on surimi gels during freeze-thaw cycles. A small portion (~300 g) of Alaska pollock surimi (partially thawed) was cut into small pieces and placed in a mixer (model SC400; Black & Decker Corp., Towson, Md., U.S.A.). Surimi was first chopped at low speed for 1 min. After adding 1% of PL or water (0% PL) the mixture was chopped at high speed for 1 min. The mixture was then moved to a mortar and ground using a pestle for 3 min. Chopping and grinding were conducted at 4 °C. The paste was packed into a polyethylene bag and air pockets from the surimi paste were removed using a vacuum machine (model Reiser VM-4142; Roescher Werke GMBH, Osnabrueck, Germany). A set of experiments was conducted before freezing (0 ft cycle). The remaining packages were stored at -18 °C. Each remaining portion (~30g) was placed in a plastic bag, vacuum sealed, and subjected to the following freeze-thaw schedule: the portions were placed in a -18 °C freezer overnight (16 hr) and then in a 4 °C cooler for 3 hr to thaw. They were again placed in a -18 °C freezer overnight to refreeze and repeated for 2, 4, 6, and 8 freeze-thaw cycles.

5.3.2.5 Oscillatory dynamic measurement

Development of a gel network was measured as a function of temperature using a CS-50 rheometer (Bohlin Instruments, Inc., Cranbury, N.J., U.S.A.). Paste containing two levels of PL [(1% PL and 1% water (0% PL)]was mixed with 2% NaCl and placed between a cone and plate (4 °) with a gap of 150 μm. To avoid sample

drying during heating, a plastic cover (trapper) with moistened sponge was used. The sample was heated from 20 to 80 °C at a heating rate of 1 °C/min. The oscillatory mode was used with a fixed frequency of 0.1 Hz and shear stress of 50 Pa, which was in the linear viscoelastic range.

5.3.2.6 Color

Due to the limited amount of PL, gels prepared after measuring oscillatory dynamic properties were used for color measurement. A Minolta Chroma Meter CR-300 (Minolta Camera Co., Osaka, Japan) was used to obtain at least 5 color measurements of surimi gel samples. Results were reported in CIE L* and b* values, where L* represents lightness and b* yellow/blue.

Measurement of thiobarbituric acid-reactive substances

Thiobarbituric acid-reactive substances (TBARS) measurement is the most widely used test for measuring the extent of lipid oxidation. The advantage of this method is that the lipids do not have to be extracted from the tissue. TBARS assay was performed as described by Buege and Aust (1978) with slight modification. Thiobarbituric acid reacts with two molecules of malonaldehyde and generates a stable product with strong adsorption at 535 nm (Vaya and Aviram 2001). Surimi sample (3g) with/without 1% PL addition, respectively, was homogenized with 15 mL of a solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl, using a homogenizer (model GLH-115, PG 700, Fisher Scientific, Pittsburgh, Pa., U.S.A.) for 1 min at speed level 3. The mixture was heated in a boiling water bath

(100 °C) for 10 min to develop a pink color, cooled with running tap water and centrifuged (Sorvall RC-5B, Newtown, Conn., U.S.A.) at 3,600 x g at 25 °C for 20 min. The absorbance of the supernatant was measured at 532 nm. TBARS was calculated and expressed as mg malonaldehyde/kg (mg MA/kg) sample.

5.3.2.7 Statistical analysis

The results were presented as the average and standard deviation of each experiment conducted at least in triplicate. Statistical comparisons were made between treatments by ANOVA (Tukey's test) and one sample T-test using SPSS (version 13.0) software package (SPSS Inc., Chicago, Ill., U.S.A.). A statistical significance was reported at $P < 0.05$.

5.4 RESULTS AND DISCUSSION

5.4.1 Thin layer chromatography (TLC)

Figure 5.1 showed the quantitative analysis of lipid composition of total lipids (TL), neutral lipids (NL) and phospholipids (PL) fractions, respectively. The predominant fraction of TL and NL was triglyceride (TG). PL fraction, obtained using SPE column with attached aminopropyl residue, showed significantly higher levels of phospholipids. Solid-phase extraction chromatography is known to separate all of the major classes of lipids found in a biological system (Subbanagounder and others 2000). Aminopropyl SPEC columns with sequential solvent elution carried out in this study appeared to be an efficient technique for lipid class separation of Pacific sardine lipids based on the high purity of PL fraction (Figure 5.1).

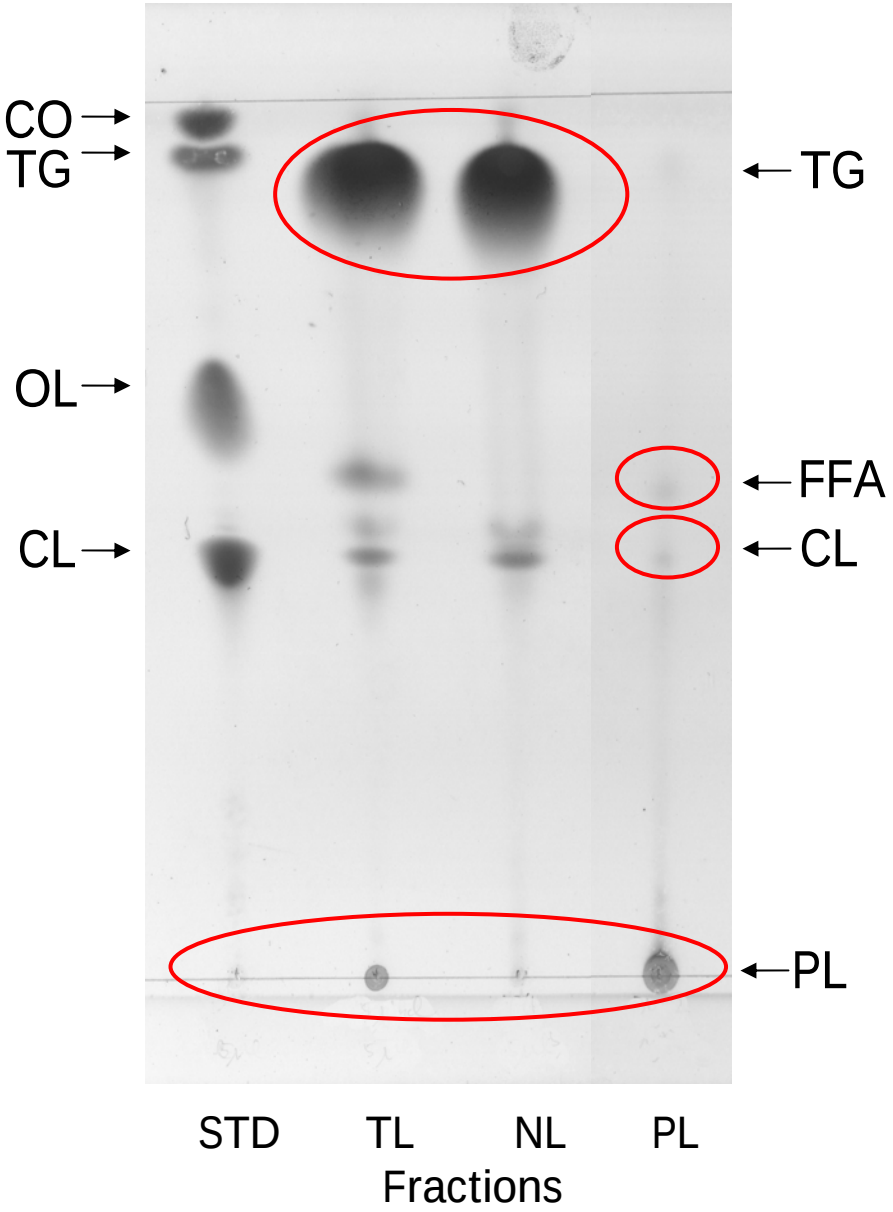

Figure 5.1 Separation of lipid fractions of Pacific sardine by thin layer chromatograpy. STD, TLC reference standard [cholesteryl oleate (CO), triolein (TG), oleic acid (OL), cholesterol (CL), and lecithin (PL)]; TL, total lipids; NL, neutral lipids; PL, phospholipids; TG, triacylglycerols; FFA, free fatty acid; MG, monoacylglycerols.

The lipid class composition varies greatly with fat content of the fish. A study by Bandarra and others (1997) demonstrated that PL content of lean sardine with 1.20% of total lipids was significantly higher (11.25% PL) than that of fatty sardine

with 18.4% of total lipids (3.65% PL). Pacific sardines used in the present study contained 17.6% of total lipid, which were considered as fatty sardines. As expected, the PL recovery from total lipid of whole sardines was very low (0.4% ± 0.1%).

5.4.2 Fatty acid profile

The fatty acid composition of Pacific sardine oil (Table 5.1) showed a larger proportion of n-3 PUFA in the PL fraction (52.72%) as compared with the TL (40.14%) and NL (40.09%) fractions, respectively. The high level of n-3 PUFAs in PL was derived from significantly high concentration of DHA (35.60%), which accounts for approximately 67.5% of total n-3 PUFAs. Bandarra and others (1997) showed similar tendency that the fatty acid distribution of the polar fraction was more unsaturated than the neutral fraction. A study by Passi and others (2002) also demonstrated a similar trend in fatty acid profiles of the TG and PL fraction from sardine oil.

The fatty acid profile of sardine is influenced by season, with the level of PUFAs being negatively correlated to the fat content, whereas a reverse correlation is observed for saturated and monounsaturated fatty acids (Bandarra and others 1997; Mendes and others 2000). There is an inverse relationship between EPA and DHA, with DHA being more abundant during the lean season. When the lipid content is low, it becomes an important component of membrane structural lipids. On the other hand, EPA is more abundant when fish fat content is high because of its greater involvement in the composition of reserve lipids (Mendes and others 2000). In the present study, it was clearly noted that DHA was more abundant in the membrane lipid. For the TL

and NL fractions, EPA was more abundant (20.72% and 21.15%, respectively) compared to DHA (14.01% and 13.51% respectively).

Table 5.1. Fatty acid profiles (wt/wt%) of total lipid, neutral lipid, and phospholipids from sardine

Fatty acids	TL	NL	PL
C14:0	7.75 ± 0.04	7.98 ± 0.31	1.35 ± 0.37
C15:0	0.56 ±0.00	0.57 ± 0.01	0.37 ± 0.00
C16:0	22.68 ± 0.19	23.19 ± 0.22	28.17 ± 0.07
C17:0	1.30 ± 0.23	0.81 ± 0.40	0.51 ± 0.17
C18:0	6.16 ± 0.15	6.62 ± 0.51	2.21 ± 1.84
SFA	**38.46 ± 0.15**	**39.17 ±0.27**	**32.62 ± 2.71**
C14:1n5	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.00
C16:1n7	8.15 ± 0.02	8.02 ± 0.31	2.11 ± 0.05
C17:1n9	1.81 ± 0.42	1.58 ± 0.15	0.24 ± 0.25
C18:1n9	6.43 ± 0.02	6.29 ± 0.14	3.92 ± 1.66
C20:1n9	0.16 ± 0.01	0.10 ± 0.08	0.14 ± 0.24
C22:1n9	0.03 ± 0.02	0.01 ± 0.00	0.02 ± 0.00
C24:1n9	0.67 ± 0.00	0.67 ± 0.02	2.10 ± 0.08
MUFA	**16.60 ± 0.40**	**16.02 ± 0.51**	**8.58 ± 1.29**
C18:2n6	2.00 ± 0.01	2.01 ± 0.02	4.44 ± 6.34
C18:3n6	0.18 ± 0.00	0.17 ± 0.17	0.20 ± 0.26
C18:3n3	0.99 ± 0.01	1.03 ± 0.04	0.30 ± 0.22
C20:2n6	0.47 ± 0.00	0.40 ± 0.10	0.06 ± 0.02
C20:3n6	0.16 ± 0.00	0.09 ± 0.01	0.06 ± 1.07
C20:3n3	0.71 ± 0.01	0.68 ± 0.00	1.07 ± 0.88
C20:5n3	20.72 ± 0.13	21.15 ± 0.33	14.44 ± 0.38
C22:2n6	0.77 ± 0.02	0.79 ± 0.02	0.31 ± 0.01
C22:5n3	4.25 ± 0.03	4.31 ± 0.11	2.32 ± 0.07
C22:6n3	14.01 ± 0.11	13.51 ± 0.32	35.60 ± 1.02
PUFA	**44.74 ± 0.30**	**44.55 ± 0.71**	**58.87 ± 3.97**
n-3 PUFA	**40.14 ± 0.27**	**40.09 ± 0.78**	**52.72 ± 1.66**

TL: Total lipid; NL: Neutral lipid; PL: Phospholipids, SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid; n-3 PUFA: n-3 polyunsaturated fatty acid.

These results, therefore, seem reasonable as the Pacific sardines used in this study were caught from July through September 2006, when the lipid content of sardines ranges from medium to high.

Among saturated fatty acids (SFA), the predominant fatty acid was 16:0 in all fractions: TL (22.68%), NL (23.19%) and PL (28.17%). The constantly high values of 16:0 found in this study is in agreement with other studies (Bandarra and others 2001; Passi and others 2002). It has been suggested that this fatty acid is a key metabolite that is not affected by feeding behavior.

Passi and others (2002) demonstrated a significant reduction of 14:0 in PL (1.8%) compared to the TG fraction (6.5%). Similar to their study, our study showed a lower level of 14:0 in PL (1.35%) than in TL (7.75%) and in NL (7.98%), respectively. The low level of 14:0 in the PL fraction, which consists of structural lipids, suggests its exogenous origin from dietary phytoplankton as supported by its high percentage in the TL fraction (Bandarra and others 1997).

5.4.3 Thiobarbituric acid-reactive substances (TBARS)

Figure 5.2 shows TBARS of both surimi with and without 1% PL addition, respectively, as affected by freeze-thaw cycles. Initially, TBARS of surimi without PL addition was 0.05 mg malonaldehyde/kg while the level of TBARS of surimi with 1% PL addition was 0.15 mg malonaldehyde/kg. Overall, surimi with 1% PL showed relatively higher levels of TBARS except after 8 freeze-thaw cycles. TBARS continued to increase as freeze-thaw was repeated by 6 cycles regardless of PL addition.

Pacheco-Aguilar and others (2000) reported that TBARS of the same species of sardine (*Sardinops sagax*) muscle increased from an initial value of 4.3 mg malonaldehyde/kg at 2 days of frozen storage to a maximum of 37.2 at 11 days of storage. They reported that no objectionable odor was detected at these concentrations. Having 0.521 mg malonaldehyde/kg at the highest value, TBARS detected in our samples were relatively low level (the initial stages of reaction). As shown in the fatty acid profile (Table 5.1), PL fraction of sardine oil contained significantly high levels of n-3 PUFA that are susceptible to oxidation.

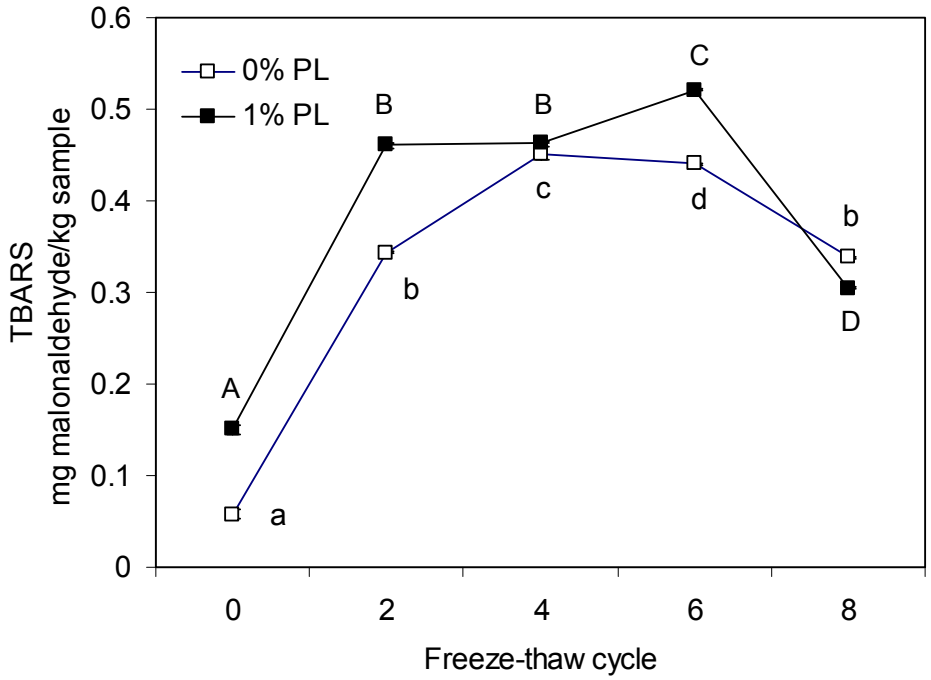

Figure 5.2 Changes in TBARS values of Alaska pollock surimi as affected by freeze-thaw cycle with/without phospholipids (1%). A~D and a~d: values with different alphabets are significantly different at $P < 0.05$, respectively.

Changes, such as lipolysis, lipid oxidation, as well as interactions with nonlipid components like proteins, in fish lipids are directly and indirectly responsible for the quality deterioration of fish muscle (Pacheco-Aguilar and others 2000). During chilled storage of fatty fish species including sardines, a significant ($P < 0.05$) effect of lipid deterioration has been detected, which can cause a loss of fish quality (Aubourg and others 1997).

Malonaldehyde is an important secondary product of auto-oxidation and is typically used as an indicator of lipid peroxidation. However, this secondary products (malonaldehyde) can be decomposed to, monohydroperoxides and form volatile breakdown products. Lipid hydroperoxides can also be condensed into dimers and polymers that can further break down and produce volatile materials (Frankel 1984). Cornforth and West (2002) reported that TBARS values of cooked ground pork had decreased after 10 days of storage at 2 °C. Therefore, the reduction of the TBARS values observed at 8 freeze-thaw cycles was thought due to the decomposition of the malonaldehyde into volatile compounds regardless of PL addition.

5.4.4 Oscillatory dynamic measurement

Non-fracture gel analysis was conducted to measure the effect of PL addition during heat gelation of Alaska pollock surimi. Storage modulus, G', indicates an elastic element of surimi gel. High elasticity is desired for good quality surimi. As shown in Figure 5.3, G' increased slightly from around 30 to 36 °C and then decreased from 44-46 °C. This is a typical G' pattern of Alaska pollock surimi (Yoon and others 2004). According to Lou and others (2000), the initial increase is due to denaturation

of light meromyosin. The decrease is due to the fluidity release fluidity from myofibrillar filaments. Then G' gradually increased until becoming steady around 70 °C.

G' values around 70 °C for samples without PL were higher than their counterparts containing PL. This implies that PL negatively affects Alaska pollock surimi during frozen storage and gelation. The difference in final G' values was extended as freeze-thaw cycles increased.

The primary or secondary products of PL oxidation are likely to affect protein gelation during freeze-thaw and/or the linear heating process. When Alaska pollock surimi with 1% PL was examined as affected by freeze-thaw cycles (Figure 5.4), the final G' values decreased significantly by repeating freeze-thaw up to 4 cycles. However, the G' values then increased when freeze-thaw cycles further extended to 6 and 8. This is probably due to gel enhancement through protein oxidation. This was in agreement with the results of Srinivasan and Xiong (1996) who reported that G' values of beef heart surimi at around 70 °C increased after 7 days storage at 0 °C. When samples were assayed for carbonyl content, they found a relationship between G' and protein oxidation during storage. Park (2005) reported that formation of S-S bonds through the oxidation of sulfhydryl (-SH) groups using oxidizing agents, such as L-ascorbic acid, sodium ascorbic acid, and erythrobic acid, can enhance gel strength. However, Saeed and Howell (2004) reported that Atlantic mackerel protein gelation showed a negative relationship with lipid oxidation. Saeed and Howell (2002) also reported that oxidized lipids interact with proteins inducing reduction of textural properties.

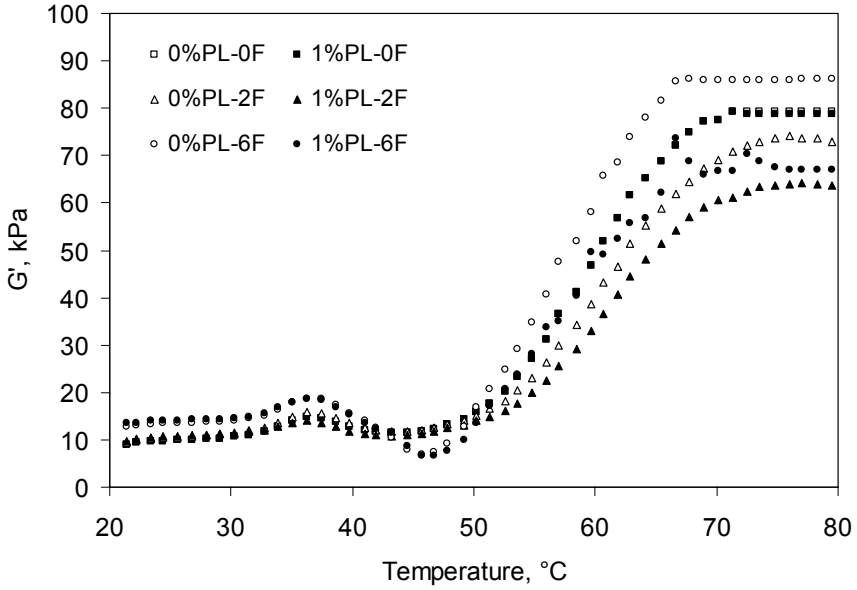

Figure 5.3 Storage moduli (log G') of Alaska pollock surimi as affected by freeze-thaw with/without phospholipids. Surimi paste was heated from 20 to 80 °C at 1 °C/min.

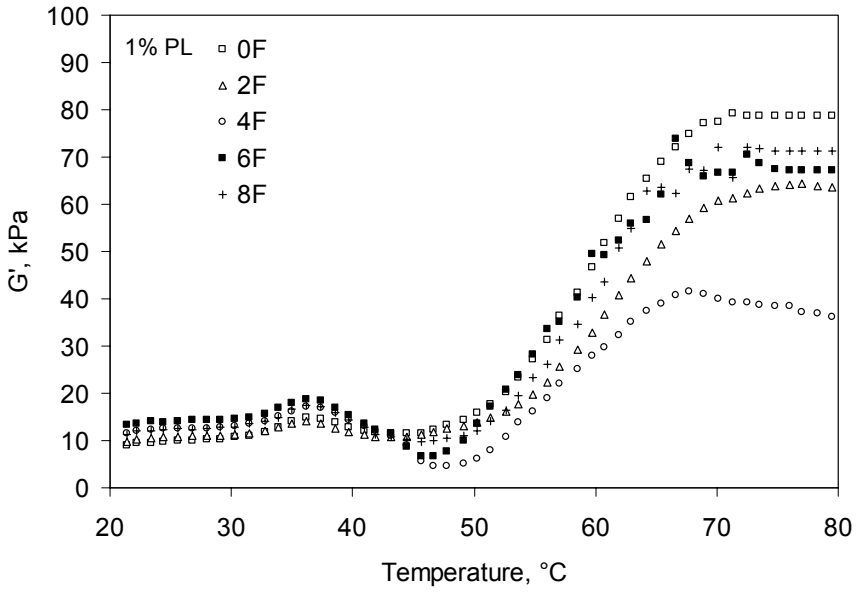

Figure 5.4 Storage moduli (log G') of Alaska pollock surimi as affected by freeze-thaw cycle in the presence of phospholipids (1%). Surimi paste was heated from 20 to 80 °C at 1 °C/min.

5.4.5 Color

Like gel texture, color, particularly whiteness, is an important factor to determine surimi quality. L* indicates lightness (100) to darkness (0) and b* denotes yellowness (+50) to blueness (-50). The color of PL was colorless after extracting from TL because all pigment stayed with NL during the separation process using a sep-pak column. As shown in Table 5.2, PL from sardine showed no significant effect ($P > 0.05$) on the L* and b* values of Alaska pollock surimi during freeze-thaw treatment.

Table 5.2. L* and b* values of Alaska pollock surimi gels affected by freeze-thaw cycles with/without phospholipids (0 and 1%)

	PL (%)	Freeze-thaw cycle				
		0	2	4	6	8
L*	0	88.51±0.80[a]	88.98±0.52[a]	89.15±0.11[a]	89.22±0.64[a]	88.92±0.44[a]
	1	89.98±0.36[b]	89.16±0.65[a]	89.68±0.42[a]	89.48±0.34[a]	89.92±0.39[b]
b*	0	4.49±0.12[a]	4.79±0.32[a]	4.88±0.11[a]	4.92±0.58[a]	4.79±0.46[a]
	1	5.58±0.32[b]	5.70±0.33[b]	6.12±0.56[b]	5.87±0.35[b]	6.06±0.25[b]

L* or b* values in the same column at each freeze-thaw cycle followed by different letters are significantly different ($P < 0.05$, one sample T-test). There was no significant difference ($P > 0.05$, ANOVA) during the freeze-thaw process, as represented in each row, therefore significant difference was not noted.

Our results indicated that PL at 1% addition level did not significantly ($P > 0.05$) affect the color of surimi during storage. However, surimi gels with 1% PL showed a greater L* and b* values compare to gels without PL ($P < 0.05$). Park

(2005) showed a similar trend. When vegetable oil was added to surimi, L* and b* values of surimi gels increased.

5.5 Conclusion

Recovery yield of PL was 0.4% from total lipid of whole sardine. However, PL from Pacific sardines possessed a high level of PUFA indicating great potential to induce lipid oxidation. Therefore, although PL contained nutritionally valuable PUFA, the removal of PL would likely improve the storage stability and possibly improve the value of the sardine as a food source.

CHAPTER 6

GENERAL CONCLUSIONS

The role of myoglobin (Mb) in the gelation mechanism of myofibrillar proteins was determined in Study 1. Ethanol extraction was more effective than ammonium sulfate extraction for the isolation of Mb. Mb obtained from Pacific sardine fillet alone did not have a positive effect on Pacific whiting surimi gelation. However, 1.0% Mb addition showed a synergistic interaction with beef plasma protein (BPP), as shown by the highest breaking force and deformation values of the prepared Pacific whiting surimigels. For color analysis, addition of Mb obtained from Pacific sardine negatively affected the whiteness of Pacific whiting surimi gels. The results from DSC analysis showed that Mb addition resulted in myosin denaturation and aggregation.

The conformational changes of Pacific sardine myosin at various pHs were determined in Study 2. The results revealed that the alkaline process produced fish protein gels with even greater elasticity as compared to using acidic processes. Therefore, the alkaline pH-shift method appeared to be effective for isolating high-quality functional fish proteins from Pacific sardines.

Study 3 covers the extraction of phospholipids (PL) and their characterization. The effects of PL on Alaska pollock surimi during freeze-thaw cycles were determined. Recovery yield of PL was 0.4% from total lipid of whole sardine. However, PL from Pacific sardines possessed a high level of PUFA indicating a great

potential to induce lipid oxidation. Therefore, the removal of PL would improve the value of the sardine as a food source due to improved lightness of gel color and possibly by increased storage stability from removing these lipid oxidizing substrates.

In conclusion, the current study revealed that the alkaline process resulted in fish protein gel with even greater elasticity as compared to acidic processes. Also, Mb isolated from sardine did not have a positive effect on fish protein gelation. However, PL isolated from sardine had a negative effect on fish protein gelation and color (yellowness) of gel. Also, myosin chemically less changed at alkaline pH. Therefore, the alkaline pH-shift method appeared to be most effective for isolating high quality functional fish proteins from Pacific sardines.

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