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Title: Physicochemical Properties of Alaska Pollock Surimi as Affected by Salinity and Freeze-Thaw Cycles

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The effects of the residual salt of surimi on biochemical and physical properties as affected by various freeze and thaw cycles were examined. Fresh Alaska pollock (*Theragra chalcogramma*) surimi was mixed with 4.0% sugar, and 5.0% sorbitol, along with eight combinations of salt (0.4, 0.6, 0.8, and 1.0 % NaCl) and sodium polyphosphate (0.25 and 0.5%). Surimi was then vacuum packed and stored at -18°C until used. Freeze-thaw (FT) cycles (0, 3, 6, and 9) were used to mimic long term frozen storage. At the time of gel preparation, each treatment was adjusted to maintain 2% salt and 78% moisture. The pH decreased as the residual salt increased during frozen storage. Salt extractable protein (SEP) and Ca^{2+}-ATPase activity decreased as FT cycles extended. Regardless of residual salt and phosphate during frozen storage, whiteness value (L*-3b*) decreased as FT cycles extended. Water

retention ability (WRA) and texture significantly decreased at higher salt content (0.8 and 1.0 %) after 9 FT cycles.

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Physicochemical Properties of Alaska Pollock Surimi as Affected by Salinity and Freeze-Thaw Cycles

by

Ey Jung Kang

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

Ey Jung Kang, Author

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PHYSICOCHEMICAL PROPERTIES OF ALASKA POLLOCK SURIMI AS AFFECTED BY SALINITY AND FREEZE-THAW CYCLES

CHAPTER 1

INTRODUCTION

Surimi, which is concentrated myofibrillar proteins, is a primary ingredient for surimi seafood such as kamaboko, crabstick, and fish ball. Crabstick was first introduced to the U.S. market during the late 1970s and has since become increasingly popular (Park and Lin 2005). According to Guenneugues and Morrissey (2005), global surimi production in recent years is close to 600,000 metric tones. Even though new fish species, such as Pacific whiting (*Merluccius productus*) and threadfin bream (*Nemipterus* spp.), are utilized for surimi production, Alaska pollock (*Theragra chalcogramma*) is still the most popular fish used. Alaska pollock provides the largest harvest of whitefish in the world with approximately 250,000 metric tons of surimi produced annually (Guenneugues and Morrissey 2005).

Surimi was first produced in Japan in the 12th century, but was not globalized until the discovery of cryoprotectants in 1960. This technique required the addition of low molecular weight carbohydrates, such as sucrose and sorbitol, in the dewatered myofibrillar proteins prior to freezing (Park and Lin 2005). Also, sodium phosphate

was mixed along with cryoprotectant to improve the pH of surimi as well as to chelate mineral compounds in surimi.

Traditionally, fresh surimi was produced on a daily basis depending on the availability of fresh fish. In the U.S., due to the geographical distance of Alaska pollock surimi plants to surimi users, the use of fresh surimi was almost impossible (Pipatsattayanuwong and others 1995). The discovery of cryoprotectant, however, overcame the limitations of the traditional surimi process (Lee 1984) by enabling the stabilization of frozen surimi and consequent globalization of frozen surimi (Matsumoto 1978). However, cryoprotectants, can not completely eliminate the denaturation of surimi during frozen storage. Even with cryoprotectants added, surimi can still undergo severe physicochemical changes during frozen storage depending on various factors, such as mixing procedures, freezing methods, and the ingredients used as cryoprotectants.

During the last 15 years, surimi production yield increased from 12-15% to 26-30% due to improved cutting methods of fish and implementation of meat recovery from the frames and wash water (Guenneugues and Morrissey 2005). The objective of surimi processing is to remove all unnecessary compounds such as fat, blood, pigments, odorous substances, and undesirable matters, including salt. When salt is not properly removed during washing, it can act as a denaturing agent against myofibrillar proteins. However, today’s production-driven process often employs sea water and/or adds salt to facilitate the water removal process at the expense of maintaining surimi quality. As a result, rapidly decreased surimi gel texture during

frozen storage has become a problem. Chung and others (1993) investigated the effect of pH and NaCl addition during gel preparation on the gel strength of Pacific whiting surimi. However, no research has been done to determine the effect of the salinity of surimi during frozen storage.

The objective of this study was to investigate the effects of various salt and phosphate concentrations on the physicochemical properties of surimi during 0, 3, 6, and 9 freeze and thaw (FT) cycles.

CHAPTER 2

LITERATURE REVIEW

E.J. Kang

What is surimi?

Surimi is concentrated myofibrillar proteins from fish muscle produced by continuous processing steps of heading, deboning, mincing, washing and dewatering, and then mixing with cryoprotectants, such as sugar, sorbitol, and phosphate to improve frozen shelf life (Park and Lin 2005). The washing and dewatering process refines fish myofibrillar proteins from the sarcoplasmic proteins and undesirable materials such as fat, blood, pigments, and odorous substances. Therefore, high quality surimi is naturally odorless and colorless (Pipatsattayanuwong and others 1995).

Surimi seafood, most popularly crabsticks, was first introduced to the U.S. in the late 1970s. The market rapidly increased in the 1980s and since has shown slow, but steady growth. In 2003, consumption was approximately 90,000 metric tons (Park 2005a) and in 2005 the surimi market in the U.S. was worth over $400-million (Voorhees 2007).

For a long period of time, Alaska pollock had been the main resource for surimi. However, the Alaska pollock harvest steadily decreased from approximately 6.5 million metric tons in the late 1980s to less than 3 million metric tons in 2000 primarily due to the collapse of Russian fisheries. This change inevitably initiated a global effect to use new species for surimi production. At the same time, the global surimi industry made an effort to recover more proteins from the surimi process. As a result, the surimi supply has been annually maintained at close to 600,000 metric tons

by including surimi produced from various resources from around the world (Guenneugues and Morrissey 2005).

Frozen storage of surimi

Fish muscle proteins tend to lose their gel-forming ability during frozen storage. High quality surimi can only be made from fish whose myofibrillar proteins have not been denatured (Matsumoto 1979; Suzuki 1981; Acton and others 1983; MacDonald and others 1990). A study was conducted by Pipatsattayanuwong and others (1995), in order to investigate gel functionality and shelf life of fresh Pacific whiting surimi verses frozen surimi. Fresh surimi was found to have higher gel strength than frozen surimi. Also, the gel-forming ability of surimi made from fresh fish in good condition does not change significantly for up to one year when held at a constant temperature below -20°C (Iwata and others 1971). However, when less-fresh fish was used, surimi quality deteriorated at a faster rate than when fresh fish was used. Therefore, it is important to prepare surimi from the freshest fish and store it below -20°C without significant temperature fluctuations (Lee 1984; Scott and others 1988).

Due to the seasonal availability of fish, surimi production is limited. Alaska pollock *(Theragra chalocogramma)* is the largest white fish biomass producing about 250,000 metric tons of surimi per year (Guenneugues and Morrissey 2005). The discovery of cryoprotectants in 1960 enabled surimi to be manufactured in larger

quantities and kept frozen for a longer period of storage with minimum change in quality. Before 1960, there was no method available to control freeze denaturation. Therefore, only fresh surimi was used in the Japanese kamaboko industry until the discovery of cryoprotectants (Lee 1984).

Commercially, surimi is formed in 10 kg blocks (56 x 310 x 590 mm) in plastic bags and frozen using a contact plate freezer with ammonia as the refrigerant until the core temperature reaches -25°C (~ 2.5 hr) (Park and Lin 2005). Although freezing surimi significantly decreases the undesirable changes, such as microbial spoilage and the rate of biochemical reactions in muscle, some deterioration of muscle protein functionality associated with frozen storage is inevitable (Powrie 1973; Matsumoto 1979, 1980; Park and others 1987; Reynolds and others 2002; Shenouda 1980). Since deterioration is known to be affected by several factors such as salt concentration, pH, ionic strength, surface tension, and the physical effects of ice and dehydration (Park 1994; Reynolds and others 2002), it is important to determine the optimum conditions that will minimize deterioration of surimi during frozen storage.

Denaturation during frozen storage

Extended frozen storage can produce profound effects on the structural and chemical properties of muscle proteins, which can, in turn, significantly influence the quality attributes of muscle food products (Park and Lanier 1989; Reynolds and others

2002). The addition of cryoprotectants is important to ensure maximum functionality of frozen surimi because freezing induces protein denaturation and aggregation. Cryoprotectants are uniformly incorporated into the dewatered meat using a kneader or silent cutter before formation of the surimi blocks. Sucrose (4%) and sorbitol (4-5%) serve as the primary cryoprotectants and sodium polyphosphate (0.2-0.3%) is also added as both a chelating agent by making metal ions in surimi inactive and as a pH enhancing agent. Combining theses ingredients together protects fish myofibrillar proteins and minimizes protein denaturation during long periods of frozen storage (Pipatsattayanuwong and others 1995).

The loss of protein functionality and, in particular, the gel-forming ability of frozen fish is due to freeze-denaturation and aggregation of the myofibrillar proteins (Grabowska and Sikorski 1976; Sikorski and others 1976; Matsumoto 1980; Suzuki 1981; Reynolds and others 2002). During frozen storage of fish muscle, denaturation, ice crystallization, and dehydration lead to conformational changes of myofibrillar proteins, causing the loss of functional properties and affecting the quality of surimi (Shenouda 1980). These properties, often expressed in terms of salt soluble protein and gel forming ability, are essential for developing the proper texture of surimi-based products (Sych and others 1990; Park 1994). Factors influencing protein denaturation during freezing and frozen storage include salt concentration, pH, ionic strength, surface tension, and the physical effects of ice and dehydration (Park 1994; Reynolds and others 2002).

The sharp decrease in gel forming ability, water holding capacity, and fat emulsifying capacity reflects the deterioration of proteins during frozen storage. It has been known that denaturation of actomyosin, the main constituent of the myofibrillar protein, occurs during frozen storage. Freezing and frozen storage denature actin and myosin, the component proteins of the actomyosin complex, as well as heavy meromyosin (HMM) and light meromyosin (LMM), the subunits of myosin. Denaturation of myosin and actomyosin has so far been ascribed to intermolecular aggregation, but some investigations have shown that intramolecular transconformation, the unfolding of the polypeptide chains, occurs in globular proteins and in subunits within the globular structures. The enzymes of the sarcoplasmic fluid also undergo denaturation during frozen storage (Matsumoto 1980).

Chemical properties

Salt (NaCl)

Texture is one of the most important factors used to determine the quality of surimi. Textural properties are controlled by the internal structure of the gel; composed mainly of myofibrillar proteins (Kubota and others 2006). After fish are subjected to 1-3 washing cycles, the ionic strength of the surimi decreases

dramatically (Lin and Park 1996). However, when the textural properties are measured, salt is necessary for extracting myofibrillar proteins that will then form a three-dimensional gel structure upon thermal processing. Without adding salt, surimi does not have gel-forming ability due to the insolubility of myofibrillar proteins at low ionic strength (Wu and Smith 1987; Stefansson and Hultin 1994).

It has been known that 1.7-3.5% NaCl (0.29-0.6 M) is required for surimi to form an adequate gel (Suziki 1981; Lee 1984, 1986; Shimizu 1985; Roussel and Cheftel 1990). In commercial practice, 1.2-2.5% NaCl (0.21-0.43 M) is commonly used to solubilize myofibrillar proteins, especially myosin, the major component responsible for surimi gelation (Samejima and others 1981; Yasui and others 1982; Wang and Smith 1995). At this salt concentration, myosin molecules are released from the thick filaments of the myofibrils and dispersed in the solution as monomers (Ishioroshi and others 1983). An ordered three dimensional gel network is then formed from solubilized myofibrillar proteins upon setting and/or heating (Niwa 1992; Lanier 1986).

Nakai and Li-Chan (1988) indicated that salt concentrations were important in determining the functional properties of proteins by changing the protein conformation through electrostatic and hydrophobic forces. Park and Lanier (1989) showed that addition of salt shifted the denaturation transitions to lower temperatures and decreased the enthalpies of heat denaturation. These results suggested that addition of salt caused a partial unfolding of proteins, increasing their sensitivity to heat denaturation. When the salt concentration was higher than 1 M, the gel forming ability

of myosin gradually decreased (Suziki 1981). When salt concentration increases, the salt ions attract some of the water molecules which decreases the number of water molecules available to interact with the charged part of the protein. As a result of the increased demand for solvent molecules, the protein-protein interactions are stronger than the solvent-solute interactions therefore the protein molecules coagulate by forming hydrophobic interactions with each other and decreases the solubility of myosin (Regenstein 1984; Stefansson and Hultin 1994).

During extended frozen storage, as ice crystals continue to grow, the relative concentration of salt increases leading to protein denaturation and subsequent loss of protein functionality as well as texture hardening (Shenouda 1980; Xiong 1997). Therefore, the effect of salt would be different depending on whether the surimi is fresh or frozen for a long period. Retained salt in frozen surimi is likely to act negatively on the functional properties of fish protein.

Phosphate and pH

Phosphates are used along with cryoprotective agents (sugar/sorbitol) and affect the pH of both water and meat. Due to the high buffering capacity of meat, the effect of phosphates on the pH of meat is somewhat less than that of water. Alkaline phosphates increase meat pH in the range of 0.1 to 0.6 units, depending on the phosphate (0.1-0.3%) used. The effects of phosphate to change the pH are in

descending order of: pyrophosphates, tripolyphosphates, and hexametaphosphates. Hexametaphosphates are more neutral and do not significantly affect the pH of meat. Acid pyrophosphates, in contrast, often decrease meat pH (Knipe 1992).

Phosphates are added to surimi as cryoprotectant at 0.25 to 0.3%, traditionally as a mixture (1:1) of sodium tripolyphosphate or tetrasodium pyrophosphate (Park and Lin 2005). Even though the mechanism of how phosphates work as a cryoprotectant is unclear, the most likely explanation of the function is its antioxidant properties on actomyosin as a metal chelator. Minced fish flesh goes through extensive washing and dewatering, nevertheless, there are still small quantities of metal ions remaining in the dewatered meat. If these metal ions are not completely removed or inactivated (chelated), they can accelerate the denaturation of fish myofibrillar proteins during freezing and frozen storage (Park 2005b).

In addition, due to the strength of the phosphates to raise pH, the water binding ability of the gel improves and more salt-soluble myofibrillar proteins are extracted. When fish undergo rigor mortis the pH decreases from neutral 7.0 to 6.5. However, the pH of washed fish meat can be maintained between 7.0 and 7.2 by adding 0.25 to 0.3% polyphosphate. This pH neutralization is a critical step for retaining the texture-forming ability of fish proteins (Park 2005b).

When it comes to surimi seafood, the use of phosphates reduces paste viscosity, allowing better machinability. Also, texture and water retention increase when phosphates are properly added. However, traditional phosphates have been known to have limited solubility in cold water, therefore, it is recommended that phosphates be

added as a pre-prepared solution. Hunt and others (2004) showed that the use of potassium phosphates, which are highly soluble, could enhance the shelf life of frozen surimi, as indicated by the gel forming ability of surimi after 9 FT cycles. Chemical blends (1:1) of tetrasodium pyrophosphate and sodium tripolyphosphate also proved to be better as a cryoprotectant than the conventional mechanical blend. Either addition of pre-prepared sodium phosphate solution or direct addition of potassium phosphate would guarantee the full function of phosphate in the comminuted meat system. However, there is no strict limit on the level of phosphate for use in seafood in the U.S., whereas meat and poultry products are allowed to contain a maximum residual concentration of 0.5% added phosphate (Park 2005b).

Salt extractable protein (SEP)

Protein denaturation is one of the primary factors that affect the gel forming ability of surimi. A truer indication of cryoprotection against protein denaturation is maintenance of the extractability of salt soluble proteins during frozen storage (Park and others 1988). Since myofibrillar proteins are generally classified as salt soluble proteins (Lanier and others 2005) the quality and quantity of salt soluble proteins are the factors that affect the elasticity of surimi-based gels (Kawashima and others 1973; Nishioka and others 1983). Therefore, solubility can be considered as the most influential property of a protein molecule (Nakai and others 1991).

During frozen storage fish muscle suffers deteriorative quality changes (Ohnishi and Rodger 1979). Rapid texture deterioration occurred during frozen storage and these changes are connected to protein changes, especially of the myofibrillar proteins, myosin and actin, which are responsible for the two-step process of gel formation. In this process, proteins are partially unfolded and become more reactive in the first step by addition of salt during chopping and subsequent heating. The main function of the salt is to solubilize the myofibrillar proteins. In the second step, the proteins aggregate in an ordered manner and form a continuous three-dimentional network or they aggregate randomly and form lumps (Madsen 1984). Actomyosin in a monomeric form is soluble, but the solubility is lost when a dimer or higher polymers are formed (Rodger and others 1979). A decrease in the amount of salt soluble actomyosin is generally regarded as a primary criterion of freeze denaturation and/or aggregation (Matsumoto 1980; Park and others 1988).

Ca^{2+}-ATPase activity

Actomyosin constituents are the main components contributing to gelation and textural properties of surimi (Lanier and others 2005). Ca^{2+}-ATPase activity can be used to evaluate the gel forming capacity of actomyosin as an indicator of the biochemical quality of muscle proteins (MacDonald and Lanier 1994; Carvajal and others 1999). A decrease of Ca^{2+}-ATPase activity, which measures the conformational

changes of myosin, relates to the oxidation of sulfhydryl groups at the active sites of myosin (Jiang and others 1988; Sompongse and others 1996). It also decreases with increased time of frozen storage, due to the loss of ATPase activity of the myosin component. The rate of ATPase reduction, however, is slower in actomyosin compared to free myosin. Connell (1959) and Kawashima and others (1973) have also detected some ATPase activity in insoluble aggregated actomyosin.

Inactivation of Ca^{2+}-ATPase is species-dependent (Hashimoto and others 1982). Myosin of warm-water fish exhibits higher stability than those of cold-water fish (Howell and others 1991). Sulfhydryl groups at active sites of myosin are responsible for the actomyosin Ca^{2+}-ATPase activity (Yamaguichi and Sekine 1966). Katoh and others (1979) used ATPase activity to evaluate the quality of frozen surimi from Alaska pollock. High Ca^{2+}-ATPase activity was noted for high quality surimi, corresponding well to gel strength. He also found that Mg^{2+}-ATPase activity was an excellent index for assessing quality of surimi as well as for estimating the freshness of raw material used for surimi preparation.

Therefore, Ca^{2+}-ATPase activity can be used to evaluate the gel forming capacity of actomyosin as an indicator of the biochemical quality of muscle proteins and monitor conformational changes of myosin (MacDonald and Lanier 1994; Carvajal and others 1999).

Surface hydrophobicity (S_o)

Hydrophobicity is the physical property of non polar solutes to repel from a mass of water and cluster together. Hydrophobic interactions in proteins have a major role in defining conformation and mediating protein-protein interactions. In addition, the number and the relative size of hydrophobic sites on the protein surface usually dictate its solubility and propensity to aggregate under physiological conditions of pH, temperature, and ionic strength (Cardamone and Puri 1992). In addition, conformation of protein can also be monitored through the changes of surface hydrophobocity. Many hydrophobic groups are exposed at the molecular surface as proteins unfold (Kato and Nakai 1980). Surface hydrophobicity was applied to follow the unfolding of various proteins, including milk, soy, and muscle proteins (Kato and Nakai 1980; Hayakawa and Nakai 1985; Wicker and others 1989; Yongsawatdigul and Park 1999). Changes of protein conformation and degree of unfolding have a significant effect on protein functionality. Surface hydrophobicity was reported to correlate well with interfacial properties of food proteins (Hayakawa and Nakai 1985). Roura and others (1992) also found that surface hydrophobicity of fish actomyosin increased as viscosity decreased.

Since the importance of hydrophobic interactions for the stability, conformation, and function of proteins is well recognized, hydrophobicity must be taken into consideration to explain the functional properties of food protein texture and form (Li-Chan 1991). Due to the macromolecular structure of proteins, surface

hydrophobicity is more influential for functionality than total hydrophobicity. Surface hydrophobicity influences intermolecular interactions, such as binding of small ligands or association with other macromolecules. Therefore information on surface hydrophobicity is essential to understand protein functionality (Haskard and Li-Chan 1998).

Interest in the hydrophobicity of proteins has long been focused on its contribution to the stabilization of molecular structure of native proteins and on the mechanisms of molecular folding. In a folded protein, many hydrophobic residues pack against each other in the core of the protein. To achieve the minimum free energy in folding of macromolecules, hydrophobic groups should be restricted to the interior of folded molecules, thus secluded from the solvent water molecules. In fact, a crystallographic study of the three-dimensional structures of proteins has revealed that many hydrophobic groups are at least partly exposed on the surface of the protein molecules and play a key role in intermolecular interactions (Nakai and others 1996).

Despite the important roles of surface hydrophobicity in the mechanisms of protein functionality, the quantification and measurement of surface hydrophobicity is still quite controversial and no consensus has been reached on a standard method (Nakai and others 1996). Several methods have been proposed for the quantitative estimation of protein hydrophobicity such as calculated values using hydrophobicty scales, partition methods, binding methods, contact angle measurement, and spectroscopic methods (Nakai and others 1991).

Methods using fluorescent probes have been most popular due to their simplicity, speed, ability to predict functionality, and use of small quantities of purified protein for analysis. Fluorescence probe methods (Kato and Nakai 1980; Hayakawa and Nakai 1985) measure hydrophobic groups on the protein surface that are able to bind the probe; the probe's quantum yield of fluorescence and wavelength of maximal emission depend on the polarity of its environment. Fluorescence probe methods are accepted as a means for assessing hydrophobic sites available on the surface of protein molecules, such as to reflect their overall three dimensional structure in solution. Another advantage of these methods is their applicability to complex systems composed of several interacting molecular species, providing the average surface hydrophobicity of a protein mixture (Haskard and Li-Chan 1998).

The most popular types of probes used for spectrofluorometric measurement include the anionic probes of the aromatic sulfonic acid class, such as 1-(anilino)-naphthalene-8-sulfonate (ANS^-), its dimeric form (bis-ANS^{2-}), and 6-(p-toluidinyl) naphthalene-2-sulfonate (TNS^-). These probes have been used extensively to quantify protein hydrophobicity, to monitor conformational changes in biological macromolecules, and to study protein binding sites. Limitations using such ionic probes to determine protein hydrophobicity include the possibility that electrostatic as well as hydrophobic interactions may contribute to the probe-protein interaction (Haskard and Li-Chan 1998).

Methods such as the fluorescence probe or the other various ligand binding techniques measure hydrophobic groups on the surface of the protein molecule that are

able to bind the probes or ligands, and thus are expected to yield parameters that correlate with the functionality of proteins. In terms of methodology simplicity, hydrophobic probe methods using ANS, cis-parinaric acid (CPA) and other fluorescence probes are probably the most popular for hydrophobicity determination (Nakai and others 1991).

Various hydrophobicity parameters have been used to develop equations explaining functionality. Although the fluorescence probe methods with ANS and CPA have proven most popular due to ease of measurement and ability to predict functionality, application of the hydrophobicity values obtained by these methods may be limited due to: 1) the presence of anionic groups on these probes that may interact with the protein molecules through charge effects, and 2) the low protein concentrations used for measurement of fluorescence in contrast to the much higher concentrations usually encountered in real food systems (Nakai and others 1991).

Formaldehyde (FA) content

Formaldehyde (FA) is usually known as a toxic and colorless gas with a pungent smell that readily solubilzes in water and forms formalin, which is used for preservation. However, a trace amount of FA is produced as a metabolic byproduct in most organisms, including fish. FA, which is an unstable and highly reactive compound has been reported in fresh gadoid fish (Amano and others 1963; Amano

and Yamada 1965; Yamagata and others 1995) such as hoki (MacDonald and others 1992). The level of FA is a useful index of frozen storage deterioration (MacDonald and others 1992; Benjakul and others 2005).

Trimethylamine oxide demethylase (TMAOase), an enzyme distributed in various organs (Benjakul and others 2003; Rehbein and Schreiber 1984; Gill and Paulson 1982; Yamagata 1995) and muscle (Kimura and others 2000; Phillippy and Hultin 1993), degrades trimethylamine oxide (TMAO) to dimethylamine (DMA) and FA during frozen storage of gadoid fish (Amano and Yamada 1965) as shown in the following reaction (Carvajal and others 2005):

$$(CH_3)_3NO \rightarrow (CH_3)_2NH + HCOH$$

With regard to fish species, differences in storage temperature, muscle integrity and reducing conditions (Parking and Hultin 1982), packaging and atmosphere (Lundstrom and others 1982), and TMAOase activity (Benjakul and others 2005), result in different reaction rates of FA formation. For red hake, packaging method and atmosphere were found to primarily affect FA formation (Lundstrom and others 1982).

FA accumulated during frozen storage may react with different functional groups of the myofibrillar protein side chains to form intra- and intermolecular methylene crosslinks, resulting in toughness of fish flesh (Regenstein and others 1982; Sikorski and Kolakowska 1990). The myofibrillar proteins of fish that react with FA

denature and aggregate during frozen storage (MacDonald and others 1992; Benjakul and others 2005; Parking and Hultin 1982), causing textural alterations and reducing protein extractability (Amano and Yamada 1965; Tokunaga 1964, 1965; Castell and others 1973, Sikorski and Kolakowska 1990). A decrease in extractable protein coupled with a proportional increase in DMA and FA have been observed by Tokunaga (1974). The FA formation was associated with the decrease in gel-forming ability of hoki during frozen storage at -29°C (MacDonald and others 1992; Benjakul and others 2005).

Protein denaturation by FA in frozen fish tissue is not a simple mechanism. FA may function by modifying surface groups of proteins making them more susceptible to denaturation, particularly when they are stressed by freezing or heating (Hultin 1992; Yamagata and Low 1995). Sikorski and Kolakowska (1990) stated that with the removal of the water-soluble substrate and the enzyme system responsible for the degradation of TMAO, the undesirable protein changes can be effectively reduced. On the other hand, the decrease in the concentration of TMAO in fish meat may by itself contribute to freeze denaturation, as the TMAO may serve in the fish muscle as a protective osmolyte.

At present, there is nothing that can be done to eliminate deterioration of fish, but there are ways to decrease FA-induced denaturation. The removal of dark muscle, kidney and other internal organs, and blood before deboning and mincing help to reduce the rate of DMA and FA formation (Castell and others 1971). It is also beneficial to maintain lower storage temperatures, such as -18°C.

Physical properties

Texture

Surimi is highly concentrated myofibrillar protein, primarily actomyosin, which is solubilized by salt during comminution and forms an irreversible gel upon heating. The most distinctive characteristic of surimi is rubbery texture. It is the gel structure that accounts for the textural strength and elasticity of the food, as well as, the entrapment of water, fat, and other food constituents (Lanier 1986). Freshness, fishing methods, season, size, and species are some of the factors that determine the gel-forming ability of fish muscle proteins (Shimizu 1985).

Several studies have shown that myosin is the most important protein affecting the gel-forming ability of fish during low temperature setting (Gill and Conway 1989; Numakura and others 1987). A three dimensional network is formed during the setting process through linkages in the tail portion of the myosin molecule via hydrophobic interactions (Stone and Stanley 1992). Setting at between 5-10°C or 40°C prior to cooking at 90°C results in a stronger gel than cooking alone (Okada 1959; Hashimoto and others 1986; Stone and Stanley 1992). The setting process allows more protein-protein interactions to occur and form a more ordered and stronger three dimentional gel (Foegeding and others 1986), which contributes to greater elasticity and higher water holding capacity of surimi gels (Kimura and others 1991).

Adding salt during the comminution of fish mince increases the water binding capacity of myofibrillar proteins by increasing the negative charges, which attracts water molecules (Chung and Lee 1991). Phosphates with their anionic groups also increase the water binding capacity of proteins. This is essential because the grinding and freezing processes rupture cells and can result in a loss of fluid (Pigott 1986).

The salt concentration used for gel preparation ranges from 2-3% of the weight of the surimi. Gel strength reaches a maximum at 1 M NaCl (5.8%) and gradually decreases with increased salt concentration as salting out occurs (Shimizu and Simidu 1955). When salt concentration increases, the salt ions attract some of the water molecules which decreases the number of water molecules available to interact with the charged part of the protein. As a result of the increased demand for solvent molecules, the protein-protein interactions are stronger than the solvent-solute interactions. Protein molecules, therefore, coagulate by forming hydrophobic interactions with each other and decrease the solubility of myosin.

Solubilization of actomyosin increases with extended comminution of surimi and reaches a maximum within 15-20 min of chopping. Beyond this chopping period, the temperature of the meat rises, resulting in protein-protein interactions, which cause a decrease in gel-forming ability (Lee and Toledo 1976). The final paste temperature at the completion of chopping, in industrial practices, is generally near 8°C. It has been suggested that the temperature of the paste must be kept at or less than the temperature above which fish actomyosin becomes unstable (Lee 1984). More recently Park (2005b) reported that the old chopping principle – "the colder the

chopping temperature, the better the gel texture" – is no longer acceptable. The optimum chopping temperature must instead be determined based on the habitat temperature of the species that is used.

In order to make surimi seafood products that require a high elastic and resilient texture it is important to use top quality surimi. Surimi of good quality can be readily recognizable as its paste becomes tacky, glossy, and translucent upon chopping with salt and is extruded smoothly. Surimi of poor quality, in contrast, produces a dull opaque and less tacky paste that breaks easily when extruded (Lee 1984).

The quality of surimi is graded on the basis of the chemical and visual conditions of raw surimi and its gel-forming ability. The texture of gels prepared from a given surimi system is affected by the moisture content of the surimi, levels of added salt and polyphosphates, extent of actomyosin solubilization (chopping time), pH, and heating parameters. Generally, the gel weakens as the moisture content increases (Lee and Toledo 1976; Lippincott and Lee 1983).

Most methods utilized for the determination of quality deterioration in frozen fish meats have been based on chemical assays. But these methods provide little information about physical and thermal properties of the fish meats. Physical properties are closely related to the functional properties of proteins and it is of considerable importance that these properties affect consumers' acceptance of the products through the sensory properties of texture (Hsu 1990).

The punch test is the most popular gel measurement technique used in the surimi industry for evaluating gel strength and texture. The punch test imitates the

large deformations to failure involved in mastication. The test was initially developed by Matsumoto and Arai (1952) and later modified by Okada and Yamazaki (1958). The okada gelometer became the standard instrumental method used in the Japanese surimi industry. In this test, a punch probe of a specific diameter (3.0 mm) and length (25 mm) is used to compress the surface of gel specimen at a constant deformation rate (10 to 60 mm/min) until puncture occurs. Many of the modern penetrometers used in industry operate at a fixed 60 mm/min and 5.0 mm probe is commonly used (Kim and others 2005).

The recorded peak force at break and the depth of penetration are used to describe the gel properties. Often these two values are multiplied together to give the jelly strength which is the value that is used in Japanese grading standards. This type of measurement has been frequently used for surimi gel samples and offers good correlation with attributes such as first-bite hardness (Kim and others 2005).

Water retention ability (WRA)

The water retention ability of food is often one of the more important properties to be measured. It is a common belief that proper gelation can only be achieved under optimum salt conditions where myofibrillar proteins become soluble. However, contradictorily, it has been reported that strong gels with good water-holding capacity can be formed under low ionic strength conditions where the

myofibrillar proteins are essentially insoluble (Chang and others 2001a, b; Feng and Hultin 2001). Stefansson and Hultin (1994) reported the nearly perfect solubility of cod myofibrillar proteins when ionic strength reached near zero. Choi and others (2002) presented that elastic gels were prepared at very low ionic strength (0.63 mM) when the pH was adjusted to 11.0-11.4.

It was further demonstrated that gelation and water-holding capacity were considerably greater at pH 7.0 than at pH 6.4 at low ionic strength (Feng and Hultin 2001). This suggested that electrostatic repulsion of muscle proteins is a major driving force behind gel formation and water-holding capacity. Proteins are polyelectrolytes, which under physiological pH conditions give rise to a gel pressure resulting from electrostatic repulsion between the electrical double layers at their surfaces (Elliott and Hodson 1998; Regini and Elliott 2001). This makes the protein gel matrix swell by the absorption of additional fluids because of the negative charges generated by their fixed charge (Kristinsson and Hultin 2003).

Increasing pH from the pI increases negative charges on muscle protein and thus increases charge repulsion between the proteins in the gel network. This repulsion is important in evenly distributing the proteins in the gel matrix, which is believed to be the basis for a strong elastic gel (Feng and Hultin 2001). This type of gel structure is expected to have a greater ability to hold water via capillary forces, through direct binding of water with protein charges and via the strong tendency of the matrix charges to repel each other. A strong electrostatic repulsion between the muscle proteins would create a need for the protein matrix to expand giving rise to the gel.

According to this model, water flow into protein gels and water retention is expected to increase with increased charge on the proteins since electrostatic repulsion between the proteins would increase. On the other hand, increasing ionic strength would be expected to decrease the electrostatic repulsive forces in part via charge screening at a given pH (Kristinsson and Hultin 2003).

Color

Color, like texture and flavor, is another important quality factor of surimi and its related products (Park 1995). Many different commercial surimi seafoods have unique color and appearance based on its gel and applied pigments. Surimi gels are generally opaque to translucent.

Surimi processing requires extensive washing to remove fat and other undesirable materials, such as pigments. Therefore, raw surimi is naturally mild in odor and translucent in appearance. Myoglobin and hemoglobin, responsible for the yellow and red hue of fish meat, respectively, are thoroughly removed during the dewatering step of surimi production (Park 1995). Color of surimi gels is typically evaluated using the CIE Lab color scale (Commission International de I'Eclairage). Commercial surimi has four to five different grades based on the L* and b* values of cooked gels (Park and Morrissey 1994).

Park and Morrissey (1994) reported that, for measuring color, the world surimi industry uses three instruments: Hunter, Minolta, and Nippon Denshoku. All are tristimulus filter colorimeters based on the same technological principles. However, the instruments do not provide similar color values for a given sample. Measurements vary between instruments based on their ability to consistently match light source-filter-detector responses. However, repetitive results can be obtained as long as a specified instrument is used.

In addition to affecting gel strength, moisture will also affect the color of the product. The addition of water affects both the L*and b* values. The higher the moisture contents the whiter the gel appears. Also, it has been shown that freezing results in darker colored gels (Park 1995).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

An electrophoretic method commonly employed for estimation of purity and molecular weight uses detergent sodium dodecyl sulfate (SDS). SDS binds to most proteins, by hydrophobic interactions, in amounts roughly proportional to the molecular weight of the protein, about one molecule of SDS for every two amino acid residues. The bound SDS contributes a large net negative charge, rendering the intrinsic charge of the protein insignificant and conferring on each protein a similar charge-to-mass ratio. In addition, the native conformation of a protein is altered when

SDS is bound, and most proteins assume a similar shape. Electrophoresis in the presence of SDS therefore separates proteins almost exclusively on the basis of mass (molecular weight), with smaller polypeptides migrating more rapidly. After electrophoresis, the proteins are visualized by adding dye, which binds to proteins but not to the gel itself. Thus it is possible to monitor the progress of a protein purification procedure because the number of protein bands visible on the gel should decrease after each new fractionation step. When compared with the positions to which proteins of known molecular weight migrate in the gel, the position of an unidentified protein can provide an excellent measure of its molecular weight (Nelson and Cox 2004).

SDS-PAGE systems are used to characterize the number and size of protein chains or protein subunits. SDS-PAGE systems are a simple and useful qualitative and quantitative gel-electrophoresis tool. Purified protein preparations can be readily analyzed for their homogeneity (Clark and Switzer 1964). Most electrophoresis studies of fish muscles after frozen storage have not shown any major differences among the muscle proteins, except for a general loss in solubility (Ragnarsson and Regenstein 1989).

CHAPTER 3

EFFECTS OF SALINITY ON PHYSICOCHEMICAL PROPERTIES OF ALASK POLLOCK SURIMI AFTER REPEATED FREEZE-THAW CYCLES

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Abstract

The effects of the residual salt of surimi on biochemical and physical properties as affected by various freeze and thaw cycles were examined. Fresh Alaska pollock (*Theragra chalcogramma*) surimi was mixed with 4.0% sugar, and 5.0% sorbitol, along with eight combinations of salt (0.4, 0.6, 0.8, and 1.0 % NaCl) and sodium polyphosphate (0.25 and 0.5%). Surimi was then vacuum packed and stored at -18°C until used. Freeze-thaw (FT) cycles (0, 3, 6, and 9) were used to mimic long term frozen storage. At the time of gel preparation, each treatment was adjusted to maintain 2% salt and 78% moisture. The pH decreased as the residual salt increased during frozen storage. Salt extractable protein (SEP) and Ca^{2+}-ATPase activity decreased as FT cycles extended. Regardless of residual salt and phosphate during frozen storage, whiteness value (L*-3b*) decreased as FT cycles extended. Water retention ability (WRA) and texture significantly decreased at higher salt content (0.8 and 1.0 %) after 9 FT cycles.

Keywords: Alaska pollock surimi, salinity, freeze-thaw cycles, frozen storage

Introduction

Alaska pollock (*Theragra chalcogramma*) is an important commercial species caught off the Alaska coast and Bering Sea. Alaska pollock has been a good source of surimi, producing strong and cohesive gels (Reynolds and others 2002). Surimi is concentrated myofibrillar protein obtained from fish flesh, which has been mechanically deboned, washed, and mixed with cryoprotectants, such as sugar and sorbitol. Surimi, depending upon fish species and grade, can be stored up to 24 months and still produce commercially acceptable gels for surimi-based analog products (Iwata and others 1971; Lee 1984; MacDonald 1992).

Due to seasonal availability, Alaska pollock production is limited to 5-6 months per year. Therefore, frozen storage has proven to be an important long-term storage method. Frozen storage prevents microbial spoilage and minimizes the rate of biochemical reactions in muscle. Nevertheless, inevitably there is some deterioration of muscle protein functionality associated with frozen storage (Powrie 1973; Matsumoto 1979, 1980; Park and Lanier 1987). Extended frozen storage can produce profound effects on the structural and chemical properties of muscle proteins, which can, in turn, significantly influence the quality attributes of muscle food products (Park and Lanier 1987).

High quality surimi can only be made from fish with myofibrillar proteins that have not been denatured during frozen storage (Iwata and others 1971; Matsumoto 1979; Suzuki 1981; Acton and others 1983; MacDonald and others 1990). The loss of

protein functionality and, in particular, the gel-forming ability in frozen fish is due to freeze denaturation and aggregation of myofibrillar proteins (Grabowska and Sikorski 1976; Sikorski and others 1976; Matsumoto 1980; Suzuki 1981). Factors influencing protein denaturation during freezing and frozen storage include salt concentration, pH, ionic strength, surface tension, and mechanical effects of ice and dehydration (Park 1994). Freezing and frozen storage also cause textural changes that often decrease the water retention of muscle proteins, particularly for fish of the gadidae family (gadoids). This is probably due to the enzymatic breakdown of trimethylamine oxide (TMAO) to dimethylamine (DMA) and formaldehyde (FA). FA is believed to react with the fish proteins to accelerate undesirable textural changes (Hebard and others 1982; Regenstein and others 1982). Additionally, Childs (1973) reported that cross-linking by FA caused texture toughening and loss of water holding capacity.

The discovery of cryoprotectants in 1960, which minimized changes of functional properties during frozen storage, has revolutionized the world surimi industry by enabling the stabilization of frozen surimi (Matsumoto 1978). Sucrose and sorbitol combined mixed at approximately 9% (w/w) serve as the primary cryoprotectants for surimi. It has been known that when sucrose and glycerol are present at high concentration (>0.5 M) enzyme activity is stabilized in solution and during freeze-thawing (Carvajal and others 2005).

In addition a mixture of sodium tripolyphosphate and tetrasodium pyrophosphate at 0.2-0.3% is used as a fish protein cryoprotectant. Phosphate works on actomyosin as a metal chelator, resulting in antioxidant properties. Even though

surimi goes through several washing and dewatering processes, small quantities of metal ions remain in the dewatered meat causing oxidation reactions that can accelerate the denaturation of myofibrillar proteins during frozen storage. Phosphate also cryoprotects fish proteins by neutralizing the pH of the washed fish meat. This pH rise increases the water binding of the gels and extracts more salt soluble proteins.

However, cryoprotectants can not completely eliminate the denaturation of surimi during the frozen storage. Cryoprotected surimi still undergoes severe physicochemical changes during long term frozen storage depending on various factors, such as mixing procedures, freezing methods, and the ingredients of cryoprotectants.

During the last 15 years, surimi production yield increased from 12-15% to 26-30% (Park and Lin 2005). The objective of surimi processing is to remove all unnecessary compounds, including salt. When salt is not properly removed during washing, it can act as a denaturing agent against myofibrillar proteins. However, today's production-driven process often employs sea water and/or adds salt to facilitate the water removal process at the expense of maintaining surimi quality. As a result, rapidly decreased surimi gel texture has been reported during frozen storage.

Our objective was to investigate the effects of residual salt and phosphate concentrations on the physicochemical properties of surimi during 9 freeze-thaw (FT) cycles. The results of this study will contribute to improved conditions for maintaining surimi quality during frozen storage.

Material and Methods

Materials

Fresh Alaska pollock (*Theragra chalcogramma*) surimi (grade AA) was provided by American Seafoods Company (Seattle, WA, USA). Surimi manufactured on the vessel was super-chilled (frozen outer layer, with core temperature at 3-4ºC) before transporting by air to the Oregon State University Seafood Laboratory (OSU-SFL) in an ice box packed with blue ice. Upon arrival, the surface was found partially frozen and the core temperature was around 2-3ºC. Surimi was placed in the 4ºC cooler for two days before sample preparation.

Upon arrival to the OSU-SFL, the salinity of the fresh surimi was first measured using a conductivity meter (YSI 3100, YSI Inc., Yellow Spring, OH, USA) equipped with conductivity cell (cell constant 1.0 cm^{-1}). Surimi sample (10 g) was homogenized with 190 mL of distilled water. Salinity readings were obtained based on a standard curve prepared using 0 to 0.15% salt solutions. As shown in Table 1, the salinity concentration of fresh surimi was 0.4%, which was used as a control (A1).

Additional salt and phosphate were properly added according to Table 1 at low speed for 2 min using a Hobart silent cutter (Model VCM-40, Hobart Manufacturing Co., Troy, OH, USA). Then the paste was filled into 10 kg trays and placed on a plate freezer for about 2 hr to obtain a core temperature at -18°C. The samples were

Table 1. Experimental treatments for surimi samples.

Sample Name	Addition of phosphate (g)* / Addition of Salt (g)	Total % of phosphate / Total % of salt
A1	0	0.25
	0	0.40**
A2	18.80	0.50
	0.08	0.40
B1	0.04	0.25
	15.06	0.60
B2	18.83	0.50
	15.14	0.60
C1	0.08	0.25
	30.12	0.80
C2	18.87	0.50
	30.20	0.80
D1	0.11	0.25
	45.18	1.00
D2	18.91	0.50
	45.26	1.00

* Phosphate was a mixture (1:1) of tetrasodium pyrophosphate and sodium tripolyphosphate (Brifisol S-1) from BK-Ladenburg Corp. (Cresskill, NJ, USA).
** Control (0.4% NaCl) was determined for fresh surimi using a conductivity meter as described above.

then cut into 500 g blocks, vacuum packed, and stored at -18°C until used.

Determination of pH

Ten grams of surimi sample were homogenized with 90 mL of deionized water (PowerGen 700, GLH 115, Fisher Scientific, Pittsburg, PA, USA) for 1 min at speed setting 3.5. The pH was measured with an Accumet® research pH meter (AR 15, Fisher Scientific, Pittsburg, PA, USA) at room temperature (20.0 ± 5°C). The pH measurements were performed at least in duplicate.

Salt extractable protein (SEP)

Salt soluble proteins were extracted from surimi sample as described by Noguchi and Matsumoto (1970) with slight modification. Surimi sample (5 g) was homogenized in 100 mL of chilled 0.6 M KCl solution using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburg, PA, USA) at setting 3.5 for 1 min. The homogenized samples were placed on a stirring plate for 30 min in the cold room and then centrifuged at 5000 x g at 4°C for 20 min. The supernatant was collected and the protein concentration was determined using the method outlined by Lowry (Lowry and others 1951). Bovine serum albumin (BSA, Sigma A 39120, St. Louis, MO, USA)

was used as a standard. The concentration of SEP was expressed as mg of proteins per g of sample.

Assay of Ca^{2+}-ATPase activity

Actomyosin (AM) extraction was done using the method by MacDonald and Lanier (1994) with slight modifications. Surimi (5 g) was homogenized in 100 mL of 0.6 M KCl (pH 7.0) using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburg, PA, USA) at speed 3.5 at 4°C for 1 min . The homogenate was extracted in a 4°C cold room for 30 min and centrifuged at 5000 x g for 20 min. Supernatant was used as the AM solution. AM extraction was prepared in at least duplicate.

A mixture of 0.25 mL of AM solution, 0.125 mL of 0.5 M Tris-maleate buffer (pH 7.0), 0.125 mL of 0.1 M $CaCl_2$, and 1.875 mL of deionized water was incubated at 25°C for 5 min. After the addition of 0.125 mL of 20 mM ATP solution, the reaction was carried out for 8 min at 25°C. By adding 1.25 mL of chilled 15% trichloroacetic acid (TCA) the reaction was stopped. The mixture was centrifuged at 3000 x g in an Eppendorf tube for 5 min and the supernatant was analyzed for liberated inorganic phosphate by the method described by MacDonald and Lanier (1994). The Ca^{2+}-ATPase activity was defined as micromoles of inorganic phosphate liberated per milligram protein at 25°C. Phosphate determinations were performed at least in duplicate.

Surface hydrophobicity (S_o)

A stock solution of 8 x 10^{-3} M 1-anilinaonaphthalene-8-sulfonic acid (ANS) was prepared in 0.1 M phosphate buffer (pH 7.4). Measurements were performed according to the method of Alizadeh-Pasdar and Li-Chan (2000), with modifications as described below.

Surimi sample (5 g) was homogenized with 100mL of chilled reagent (0.6 M KCl in 20 mM Tris-HCl buffer, pH 7.0) using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburg, PA, USA). The Lowry method (Lowry and others 1951) was used to measure protein content. Then the sample was serially diluted to achieve protein concentrations of 0, 0.1, 0.2, 0.3, and 0.5 mg of protein per mL, respectively. Twenty micro liters of ANS solution were added to 4 mL of each of the sample solutions, respectively.

The relative fluorescence intensity (RFI) of the ANS-protein conjugates was measured with a luminescence spectrophotometer (Perkin-Elmer LS-50B, Norwalk, CT, USA) at excitation and emission wavelengths of 390 nm and 470 nm, respectively, and slit widths of 5 nm. Surface hydrophobicity of the protein was calculated from the initial slope of the net RFI and the protein concentration. Each sample was run at least in duplicate.

Formaldehyde (FA) measurements

In preparation for the formaldehyde (FA) analyses, 10 g of each surimi sample, after thawing, was homogenized for 90 sec in 90 mL of 6% trichloroacetic acid (TCA). This extract was filtered and aliquots were used for the FA measurement by the procedure of Nash (1953)

Gel preparation

Surimi blocks were removed from frozen storage and the moisture content of the samples was determined, in triplicate, with a gravity oven (1330GM; VWR Scientific, Buffalo Grove, IL, USA) set at 105°C for 16 hr. Samples (~ 4.0 g) were spread into pre-weighed aluminum dishes that were re-weighed after drying to calculate the percentage of moisture loss. All surimi samples were then adjusted to 78% moisture during chopping using ice/water based on the measured moisture content. In addition, added salt was adjusted during chopping, taking into account the different initial salinity contents of the frozen samples, so the final salt content for each sample respectively would be 2%.

Surimi cubes were cut into approximately 5 cm cubes. Surimi cubes were placed in a Stephan vacuum cutter UM-5 (Stephan Machinery Corp., Columbus, OH, USA). Frozen surimi cubes were chopped at low speed for 1 min. Salt was sprinkled

and chopping continued at low speed for 1 min. Ice/water was added and the samples were chopped at low speed for 1 min. During the final 3 min, the mixture was chopped under vacuum (0.4-0.6 bar) to a final temperature below 5-7ºC. The paste was vacuum packed in a plastic bag to eliminate air bubbles prior to putting into a sausage stuffer. Then the paste was extruded into stainless steel tubes (inner diameter, 1.9 cm; length, 17.5 cm) with stainless steel screw caps. The interior wall of the tubes was sprayed with PAM cooking spray (Boyle-Midway Inc., NY, USA) and cooked in a 90°C water bath for 15 min. After chilling in ice water the surimi gels were removed from the tubes, sealed in plastic bags, and kept at 4°C for testing within 24 hr.

Gel texture measurement

Gels were equilibrated to room temperature at 25ºC and cut into 2.9 cm long. Gel samples were measured for breaking force (g) and deformation (mm) to determine the strength and cohesiveness of gels, respectively, using a Texture Analyzer (Ta-XT plus, Texture Technologies Corp., NY, USA), equipped with a 5 mm spherical probe at a test speed of 1.1 mm/s.

Water retention ability

Water retention ability (WRA) was measured according to the method developed by Kocher and others (1993). A micro centrifuge filtration unit consisted of a 2.0 mL microcentrifuge tube, which collected released fluid, and a filter insert (inner tube), which held the sample. The insert had a nylon screen with 0.45 µm pore size. Cooked gel (0.4 ± 0.05 g) was placed in the inner tube and the microcentrifuge filtration unit was spun in a microcentrifuge (Model: 5415C, Eppendorf, Hamburg, Germany). Triplicate measurements were centrifuged for 10 min at 5000 x g for each gel sample. Water retention ability was determined as:

WRA = (total g water in surimi gel – g water released)/ total g surimi gel where, total g water = % moisture of surimi gel x surimi gel weight, g water released = (microcentrifuge tube weight + g of water) – microcentrifuge tube weight.

Color measurements

Five samples (2.9 cm) cooked at 90ºC for 15min were subjected to a Minolta Chroma Meter (Minolta USA, Ramsey, NJ, USA). CIE (Commission Internationale de l'Eclairage) L* (lightness), a*(red to green), and b*(yellow to blue) were measured and the whiteness was calculated as suggested by Park (1995) using the equation L*-

3b*. All samples were kept at room temperature in a plastic bag for >2 hr to eliminate the effects of gel temperatures at measurement.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the procedure developed by Laemmli (1970) and outlined by Bollag and others (1996). Surimi gel (3 g) was homogenized for 1 min 30 sec at a speed setting of 3.5 with 27 mL 5% SDS buffer solution and then heated at 90ºC for 60 min. The samples were centrifuged at 17,000 x g for 20 min and the supernatant was used to determine the protein concentration using the Lowry method (Lowry and others 1951). The samples were diluted to obtain a final concentration of 1 mg protein/mL in the sample when mixed with 5 x sampler buffer (1 M Tris-hydrochloric acid- pH 6.8, 50% glycerol, 10% SDS, ß-mercaptoethanol, 1% bromophenol blue, and water) at a 1:4 ratio. After the samples were mixed with 5 x sample buffer, they were heated at 90ºC for 3 min and used for running gels electrophoresis. Samples that were not used immediately were stored frozen at -80ºC.

All electrophoresis was carried out with a Mini-Protean 3 Electrophoresis cell unit (Bio-Rad, Hercules, CA, USA). Slab gels consisted of a 10% separating gel and 4% stacking gel. SDS protein sample (10 µL) was loaded into each well. The protein samples were run at a constant current of 200 volts. Following electrophoresis, the proteins were stained with 0.125% (w/v) Coomassie brilliant blue R-250 in 45% (v/v)

methanol, and 10% (v/v) acetic acid in water solution overnight. The gels were then destained with 50% methanol and 10% acetic acid in water for 1 hr 30 min followed by 10% methanol and 10% acetic acid in water for 30 additional min. Gels were scanned using an Epson Stylus scanner (CX3810, Seiko Epson Corp., Long Beach, CA, USA). Molecular weights of the proteins in the sample were estimated by comparing them with a molecular weight (MW) marker ranging from 6.5 to 205 kD (Sigma Chemicals Co., St. Louis, MO, USA)(205 kD = myosin; 116 kD = β-galactosidase; 97 kD = phosphorylase b; 66 kD = albumin; 55 kD = glutamic dehydrogenase; 45 kD = ovalbumin; 36 kD = glyceraldehyde-3-phosphate dehydrogenase; 29 kD = carbonic anhydrase; 24 kD = trypsinogen; 20 kD = trypsin inhibitor; 14.2 kD = α-lactalbumin; 6.5 kD = aprotinin).

Statistical analysis

Analysis of variance (ANOVA) (Stat Package, SPSS ver 13.0, SPSS Inc., Chicago, IL, USA) was conducted on all experiment data in order to determine the significance of the freeze and thaw cycles. Tukey's least significant difference (LSD) at $p<0.05$ was used to determine significant differences between sample mean values.

Results and Discussion

pH

In general pH decreased as the concentration of residual salt increased. The pH of the samples frozen with 0.4 and 0.6% salt content decreased as FT cycles were extended (Figure 1). However, an almost opposite trend was observed with samples frozen with 0.8 and 1.0% salt. The reduction of pH might have been due to the deploymerization of myofibril thick filament (weakening interactions between myosin tails), releasing more hydrogen ions (Offer and Knight 1988). A study done by Puolanne and others (2001) showed the pH values for pork and beef batter and cooked sausage tended to decrease by about 0.1 pH-units per % unit of salt. The addition of salt probably exposed the buried charged and/or hydrophilic groups supposedly revealing new groups having pKa values in the pH range of 6.0-6.9. In our case it would be revealing new groups having pKa values in the pH range of 7.0-7.5. Effect of phosphate on pH was clearly noted regardless of residual salt content showing a higher pH value throughout the FT cycles when more phosphate (0.5%) was included.

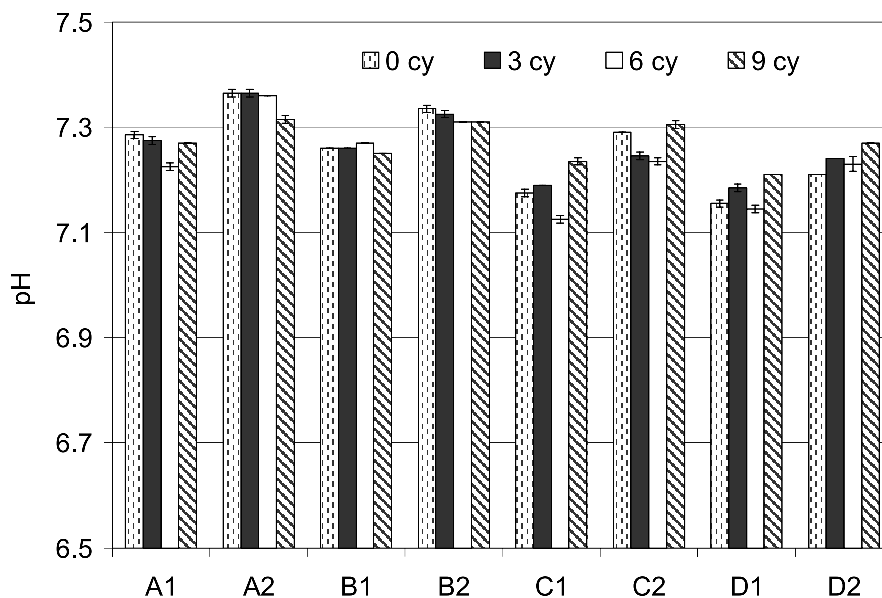

Figure 1. Changes of pH as affected by freeze thaw (FT) cycles.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.

Salt extractable protein (SEP)

In general, more SEP was obtained as the concentration of residual salt increased with freshly frozen surimi (0 FT cycle). However, as FT cycle extended, the concentration of SEP decreased, indicating a negative function of residual salt in the muscle tissue during frozen storage (Figure 2). A positive effect of phosphate on SEP was clearly noted regardless of residual salt content during 9 FT cycles. Especially, after 0 and 3 FT cycles the positive effect of phosphate was shown very well. At 6 FT

cycles, samples frozen with 0.4 and 0.6% salt still showed the positive effect of more phosphate addition while samples frozen with 0.8 and 1.0% salt did not show a significant effect by increased phosphate addition. At 9 FT cycles, only A1 and A2 (0.4% salt, 0.25% phosphate; 0.4% salt and 0.5% phosphate) showed the positive effect of higher phosphate concentration while the rest of the samples showed a minimal effect of increased phosphate addition. This indicates that during 9 FT cycles more proteins were cross-linked through freeze-induced denaturation, resulting in the least amount of extractable protein. Additional phosphate was no longer effective in extracting salt soluble protein once protein denaturation reached a certain level.

According to Matsumoto (1980), solubility data does not tell precisely how much protein is denatured or how much is native; rather, it provides a relative measure of denaturation. The loss of protein solubility is used as one of the many indicators of protein denaturation, and solubility loss is a well known result of protein aggregation (Ohnishi and Rodger 1979). Solubility reduction can be found with either intact muscle, protein solutions or with suspensions of isolated actomyosin in frozen storage experiments (Matsumoto 1980).

At freshly frozen conditions (0 cycle), overall, the samples frozen with higher salt content showed higher SEP. However, after 3 FT cycles there was a dramatic change particularly for those samples frozen with a larger amount of salt. Surimi frozen with 0.8 and 1.0% salt decreased by more than 50% while the sample frozen with 0.4 and 0.6% salt had no significant reduction, except for B1 (0.6% salt and 0.25% phosphate). After 9 FT cycles the surimi frozen with 0.8 and 1.0% salt had

lower protein solubility compared to that of 0.4 and 0.6% salt. This clearly indicates that higher residual salt content and long term frozen storage would cause a reduction of salt extractable protein and lower gel functionality.

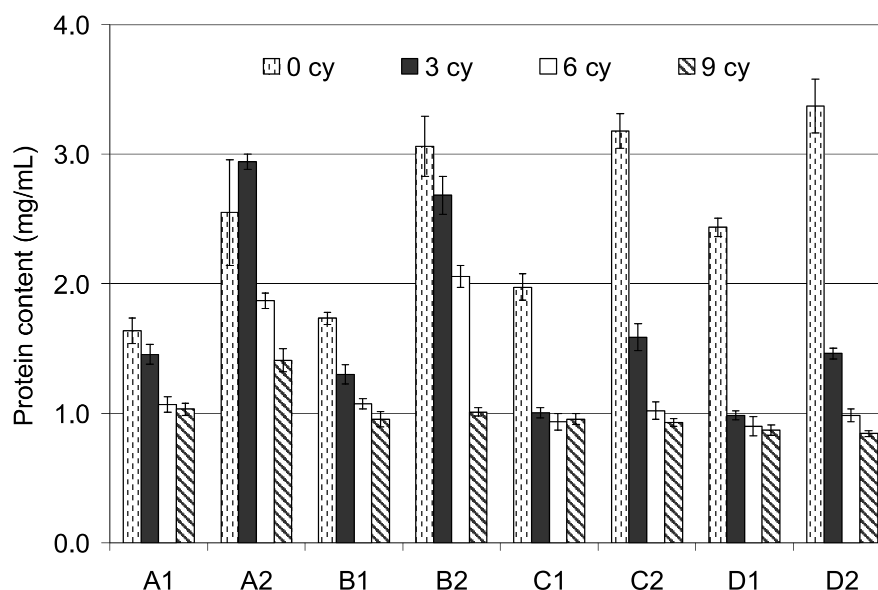

Figure 2. Changes of SEP as affected by freeze thaw (FT) cycles.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.

The effect of phosphate on SEP was very positive. SEP content increased as phosphate concentration increased. However the effectiveness of phosphate decreased as FT cycle extended and/or the concentration of residual salt increased. Park and others (1988), likewise reported a 43% reduction in SEP concentration when Alaska pollock surimi was treated with 0.5% sodium tirpolyphosphate and stored at -20°C from 1 to 3 months.

Changes in Ca^{2+}-ATPase activities

Ca^{2+}-ATPase activity decreased significantly as FT cycles extended (Figure 3). This reduction rate, however, decreased significantly when more phosphate (0.5%) was added, indicating phosphate may delay freeze-induced protein denaturation. It was interesting that a significantly positive effect by 0.5% phosphate was found for lower residual salt concentrations (0.4 and 0.6%), but not noticed when residual salt concentration was higher (0.8 and 1.0%). Surimi frozen with 1.0% salt had the most dramatic decrease in ATPase activity after 9 FT cycles. When the salt content was the same, surimi containing less phosphate showed a greater decrease in ATPase activity values, indicating more phosphate can be added to minimize denaturation of ATPase. However, it was not clear whether that the added phosphate was measured as increased ATPase activity since it was liberated phosphate measured in the test. Further clarification research is needed.

The decreasing rate of Ca^{2+}-ATPase activity varied during frozen storage depending on the amount of salt and phosphate that was added. Samples containing 0.5% phosphate demonstrated a lower reduction rate of ATPase activity after 9 FT cycles except at highest residual salt content (1.0%), where, the greatest reduction of activity was obtained regardless of phosphate concentration.

Ca^{2+}-ATPase activity can be used as an indicator for the integrity of myosin molecules (Benjakul and others 1997). From the result, it was noted that myosin underwent denaturation during extended frozen storage. Based on the decrease in

Ca^{2+}-ATPase activity, myosin underwent denaturation during frozen storage to a greater extent when there was more residual salt.

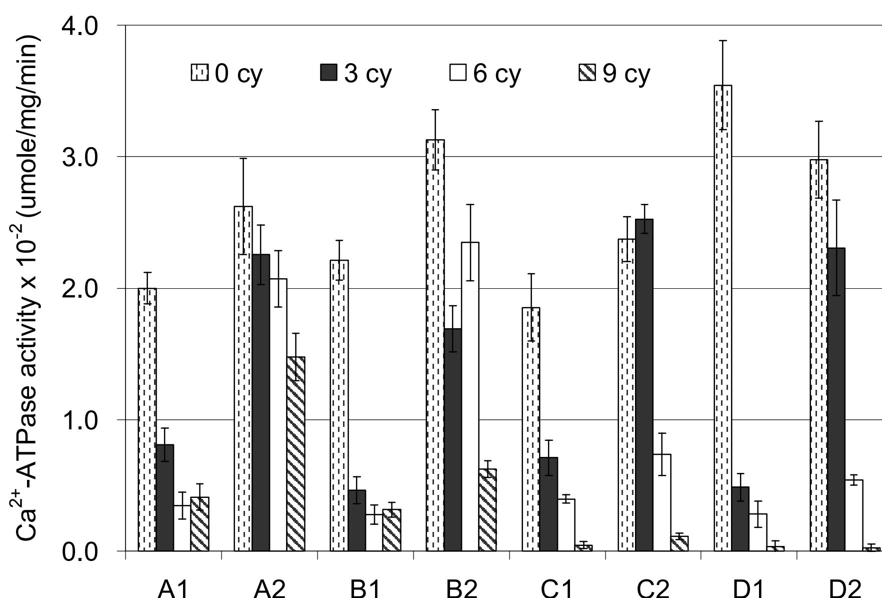

Figure 3. Changes of Ca^{2+}-ATPase as affected by freeze thaw (FT) cycles.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.

Nambudiri and Gopakumar (1992) found a decrease in the ATPase activity of fresh water and brackish fish by 70-90% after 6 months of storage at -20ºC. The 24% decrease in Ca^{2+}-ATPase activity in Alaska pollock was observed after 226 days of frozen storage at -29ºC (Scott and others 1988). The loss in ATPase activity was due to tertiary structural changes caused by ice crystals and an increase in the ionic strength of the system (Benjakul and Bauer 2000). They also assumed that

rearrangement of proteins via protein-protein interactions contributed to the loss in ATPase activity.

Surface hydrophobicity (S_o)

Extrinsic fluorescence 1-anilinonaphthalene-8-sulfonic acid (ANS) probe is a common method to measure surface hydrophobocity of fish proteins (Thawornchinsombut and Park 2006). An increase in fluorescence indicates the exposure of the surface of hydrophobic groups showing denaturation of the protein (Mackie 1993).

After 9 FT cycles, all samples showed a maximum S_o regardless of salt and phosphate concentrations (Figure 4). This observation clearly indicates 9 FT cycles caused nearly all hydrophobic groups to be exposed, resulting in freeze-induced denaturation. Low S_o observed for freshly frozen (0 FT cycle) samples A1 and B1 (0.4 and 0.6% salt, respectively with 0.25% phosphate, each) indicates that less hydrophobic groups were exposed at the lower phosphate concentration.

However, this trend was not observed in samples frozen with higher contents of residual salt (0.8 and 1.0% salt) during frozen storage. As FT cycles extended, S_o increased for surimi frozen with 0.4% or 0.6% salt, respectively with 0.25% phosphate. Surimi frozen with 1.0% salt showed a slight increase after 3 FT cycles but remained unchanged throughout 9 FT cycles.

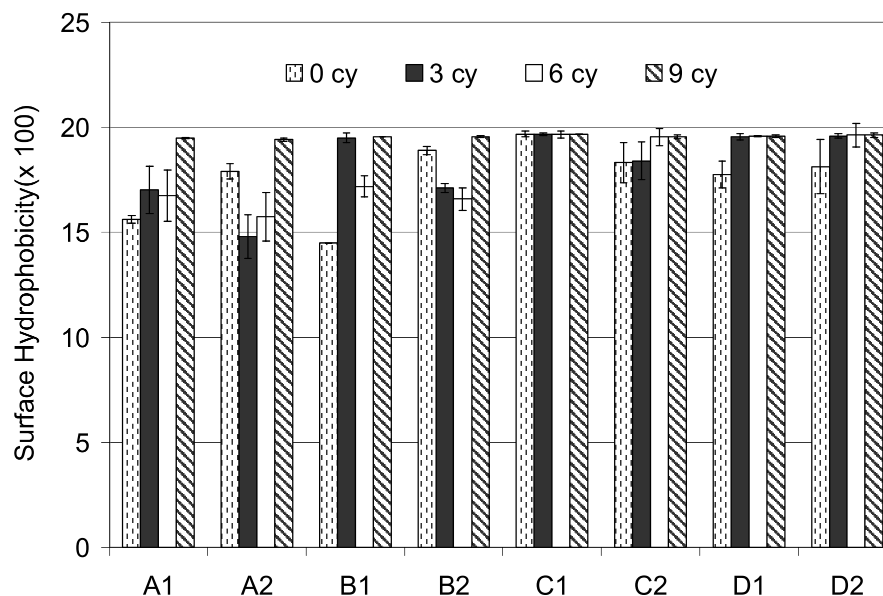

Figure 4. Changes of S_o as affected by freeze thaw (FT) cycles.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.

According to Niwa and others (1986) the initial increase of surface hydrophobicity of frozen actomyosin is due to a greater exposition of hydrophobic groups when actomyosin is denatured by freezing and subsequent evolution depends on the balance between denaturation and aggregation of the proteins.

After 3 and 6 FT cycles, a slight decrease for samples A2 and B2 was observed. A similar trend was shown in a study by Del Mazo and others (1994), where a slight increase in S_o of natural actomyosin from hake upon freezing was followed by diminishing slightly during frozen storage. Denaturation and aggregation of actomyosin, as a consequence of freezing and frozen storage, occurred essentially through direct aggregation of actomyosin molecules without dissociating into actin

and myosin (Cofrades and others 1996). It has been shown in previous studies (Aluko and Yada 1997; Thawornchinsombut and Park 2005) that in a neutral environment, the presence of salt did not significantly interfer with the interaction between the probe and protein molecules.

Formaldehyde (FA) content

FA formation during frozen storage at -18°C was monitored at 0, 3, 6, and 9 FT cycles, respectively (Figure 5). In general, FA concentration increased as residual salt concentration increased and/or FT cycles extended. Effect of FT cycles was more clearly noticeable at higher residual salt contents (0.8 and 1.0%), indicating biochemical reactions of FA formation is enhanced when more salt is present during frozen storage.

FA is produced mostly in gadoids during frozen storage by degrading trimethylamine oxide (TMAO) to dimethylamine (DMA) and FA in equimolar amounts (Amano and Yamada 1965) caused by trimethylamine oxide demethylase. Parking and Hultin (1982) clarified the role of FA in denaturation of fish proteins during frozen storage by showing the reaction rate of FA changes depending on the storage temperature, species, muscle integrity and reducing conditions. FA has been known to react with different functional group of protein side chains and form intra-

and intermolecular methylene bridges. Pérez-Villarreal and Howgate (1991) detected an increase of FA in hake fillet during frozen storage as well.

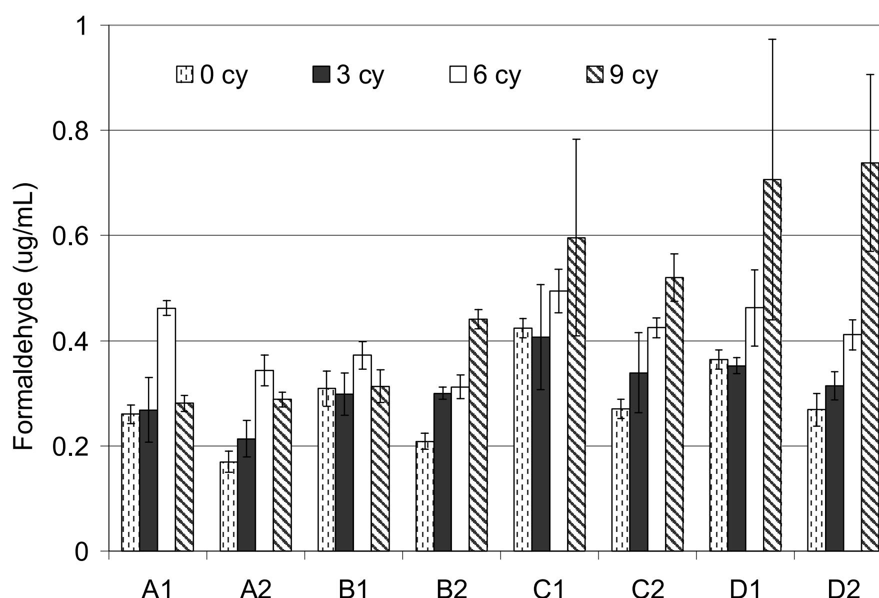

Figure 5. Changes of FA as affected by freeze thaw (FT) cycles.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.

As FA levels increased SEP levels decreased (Figure 1 and 4). Tokunga (1978) also found a decrease in SEP as there was a proportional increase in DMA and FA in Alaska pollock. In our study, the FA content increased significantly for the surimi with higher residual salt content (0.8 and 1.0%) as FT cycles extended. The increased formaldehyde, indicating a sign of frozen denaturation, was coincidental with the decrease in Ca^{2+}-ATPase activity. Cross-linking induced by formaldehyde formation possibly changed the conformation of myosin, particularly in the head region, leading

to a decrease in Ca^{2+}-ATPase activity. This observation was also in agreement with Benjakul and others (2005).

Texture

Adverse effects of freezing and frozen storage on muscle proteins include denaturation, intermolecular aggregation or changes in intramolecular conformation (MacDonald and Lanier 1991). Factors influencing protein denaturation during freezing and frozen storage include salt concentration, pH, ionic strength, surface tension, mechanical effects of ice and dehydration (Simpson and others 1994).

The strength of gels, as denoted by breaking force (g), was reasonably consistent for all samples at 0 FT cycles except for C2. Effect of FT cycle was highlighted more at higher concentrations of residual salt (0.8% and 1.0%) (Figure 6). At lower salt concentrations (0.4 and 0.6%), all samples showed similar gel strength. All gels at 3 FT cycles appeared somewhat out of the trend line compared to the other measurements obtained during 0-9 FT cycles. The testing series was then repeated and similar results were again obtained, showing an increase of gel strength after 3 FT cycles. Hsu and others (1993) also had a similar trend with their results. After 12 weeks of storage with two FT cycles twice per week, Pacific whiting surimi sample showed a sudden increase in shear stress due to the loss of water holding capacity the

protein gels had become relatively tough. This could possibly explain the increase of gel strength in our samples as well after 3 FT.

Effect of phosphate on enhancing gel strength was also observed at lower concentrations of residual salt (0.4 and 0.6%). However, the effect of phosphate was not noticeable as FT cycle and salt concentration increased. Thawornchinsombut and Park (2006) showed that gel elasticity increased significantly after FT cycling was applied, which is similar to our results at 3 FT cycle. The cohesive nature of surimi gels as indicated by deformation (mm) was within the range of 10-14 mm at 0 FT cycle. Most significant reduction was observed at higher residual salt concentrations (0.8 and 1.0%) (Figure 7).

Effect of phosphate was also noted, in agreement with Figure 2 where more extractable protein was obtained at higher phosphate concentration. Similar to the data presented above, deformation values at 3 FT cycle also appeared to be out of the trend line. Deformation of gels made from samples containing higher residual salt concentrations (0.8 and 1.0%) was much lower than those with lower residual salt concentration concentrations (0.4 and 0.6%), when FT cycles reached 3 or higher. FT effect was reduced significantly at high content of residual salt (0.8 and 1.0%). Figure 7 shows that gels made from surimi frozen with higher residual salt concentration (0.8 and 1.0%) can be denatured easily during 9 FT cycles (long term frozen storage).

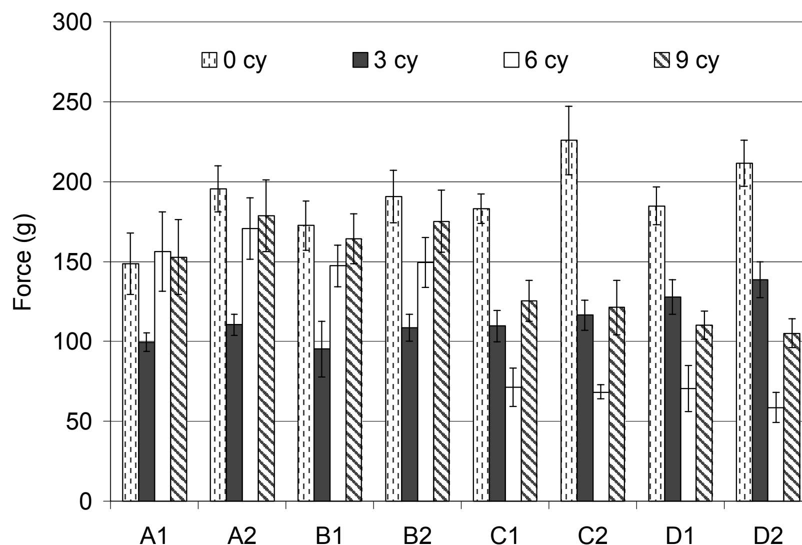

Figure 6. Changes of force as affected by freeze thaw (FT) cycles.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.

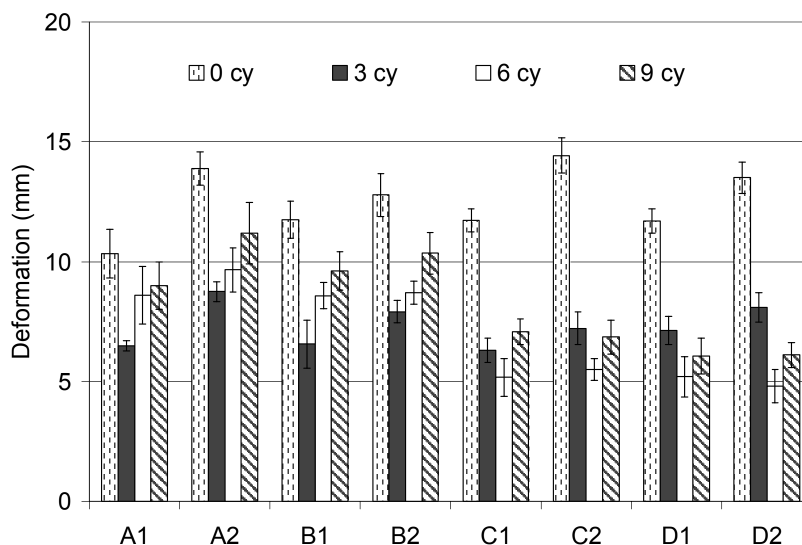

Figure 7. Changes of gel deformation as affected by freeze thaw (FT) cycles.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.

Water retention ability (WRA)

Freshly frozen samples (0 FT cycle) showed similar water retention ability (WRA) regardless of the residual salt and phosphate contents (Figure 8). Overall, WRA gradually decreased as FT cycles extended. Especially after 9 FT cycles, the samples with relatively higher residual salt content (0.8 and 1.0%) showed a bigger decrease rate of 26%, compared to that of the samples with lower residual salt content (0.4 and 0.6%) with a decrease rate of 11%. Krivchenia and Fennema (1988) found significantly less centrifugal drip loss and about one-third less firmness in 28 wk-frozen white fish (*Coregonous cupleaformis*) fillets with the addition of sodium tripolyphosphate at 11.8%. This may be due to the ability of phosphate to split the actomyosin complex into myosin and actin, improving water binding capacity and protein solubility (Shimp 1987; Gard and others 1992).

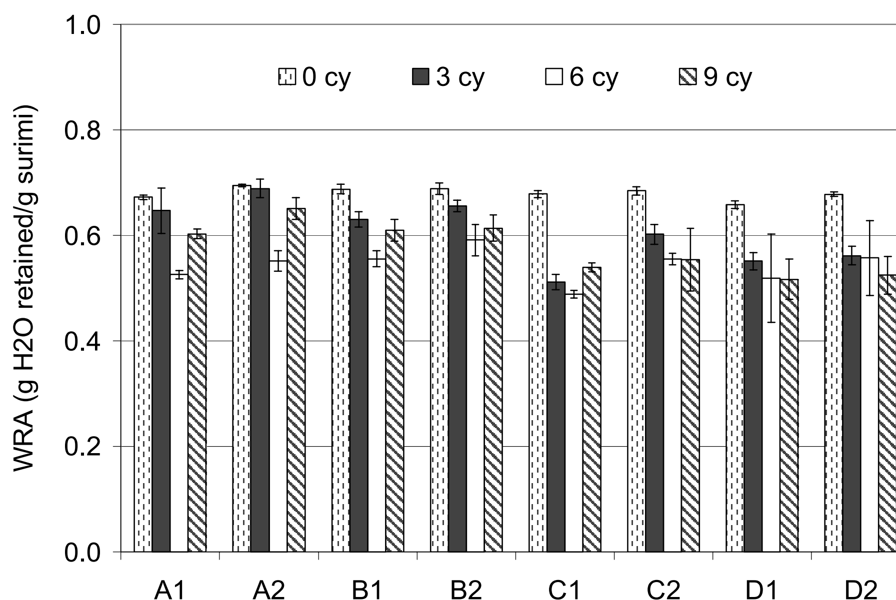

Figure 8. Changes of Water retention ability as affected by freeze thaw (FT) cycles.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.

Color

Lightness (L*) values increased as FT cycles extended. However the effect of FT was minimized significantly when residual salt reached 1.0% (Figure 9). Effect of phosphate was not noticed on the L* value. As the number of FT cycles increased, a* and b* values increased (Figure 10 and 11, respectively). Their values remained very constant among all samples. However the yellow hue increased and the green hue decreased as FT cycles extended. The calculated whiteness value, L*-3b*, gradually

decreased as FT cycle extended (Figure 12). For surimi frozen with 0.4 and 0.6% salt, no significant decrease ($p>0.05$) was found after 3 FT cycles except for B1. However, the whiteness (L*-3b*) for surimi frozen with 0.8 and 1.0% salt showed a significant decrease in whiteness after 3 FT cycle and remained stable throughout the 9 FT cycles.

The reason for some color changes during FT cycles might be due to lipid oxidation. The conventional method of surimi production goes through several washing cycles to remove blood, myoglobin, and fat. Even though the majority of undesirable residues are removed, certain undesirable materials such as membrane lipid still remain during conventional surimi processing. Therefore these compounds might undergo oxidation after several FT cycles, resulting in increased b* values (Reynolds and others 2002). A pH-shift process for fish protein isolates (Hultin and Kelleher 2000) may help stabilize the b* values, which would result in higher whiteness values (Reynolds and others 2002).

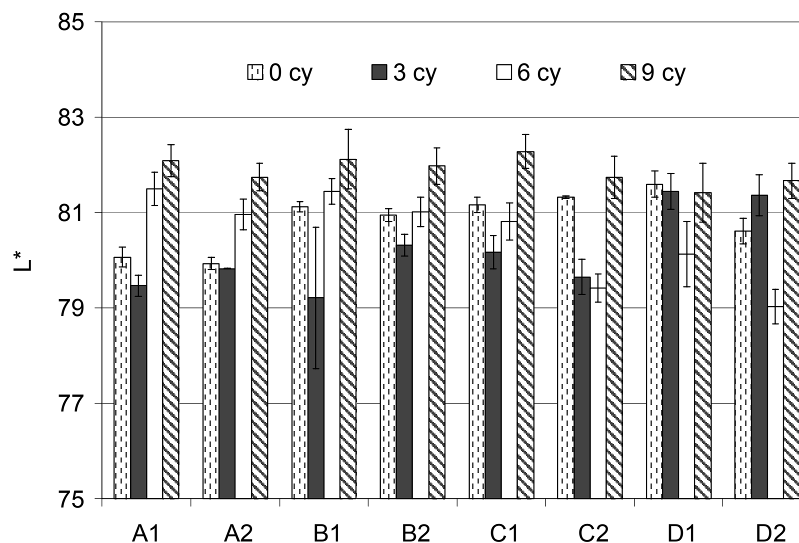

Figure 9. Changes of lightness (L*) as affected by freeze thaw (FT) cycles.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.

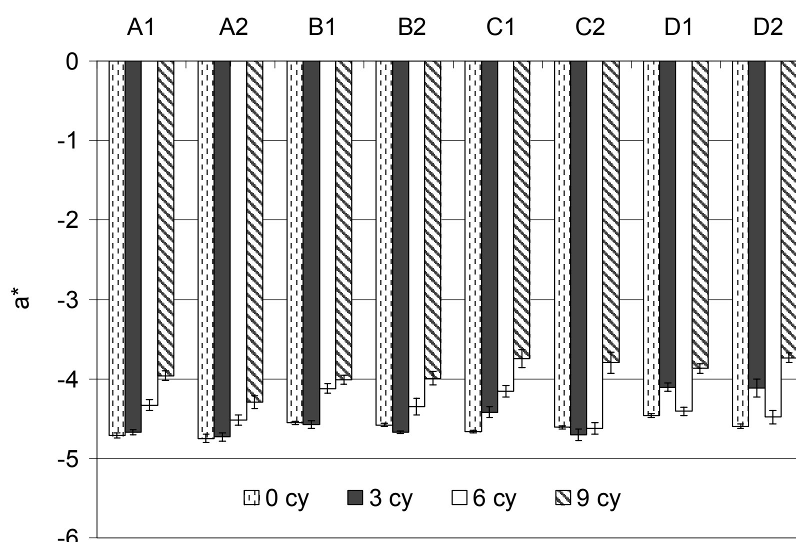

Figure 10. Changes of a*[green (-) to red (+) hue] as affected by freeze thaw (FT) cycles.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.

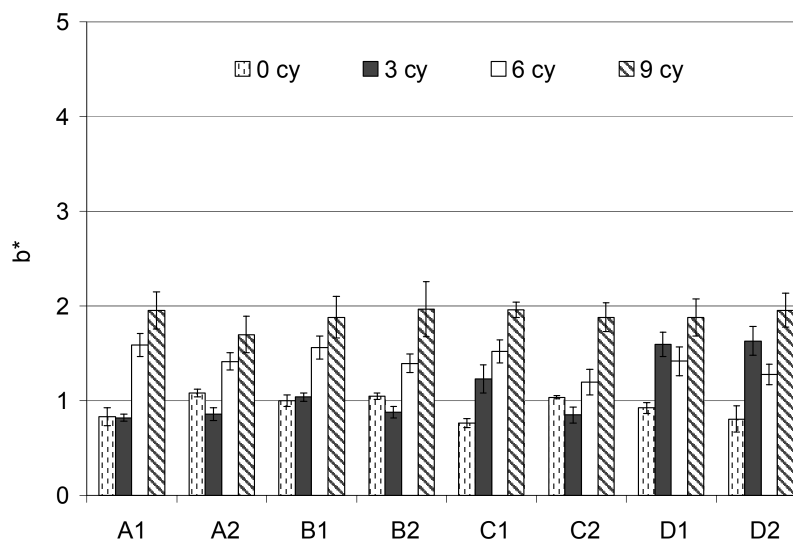

Figure 11. Changes of b*[blue (-) to yellow (+) hue] as affected by freeze thaw (FT) cycles.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.

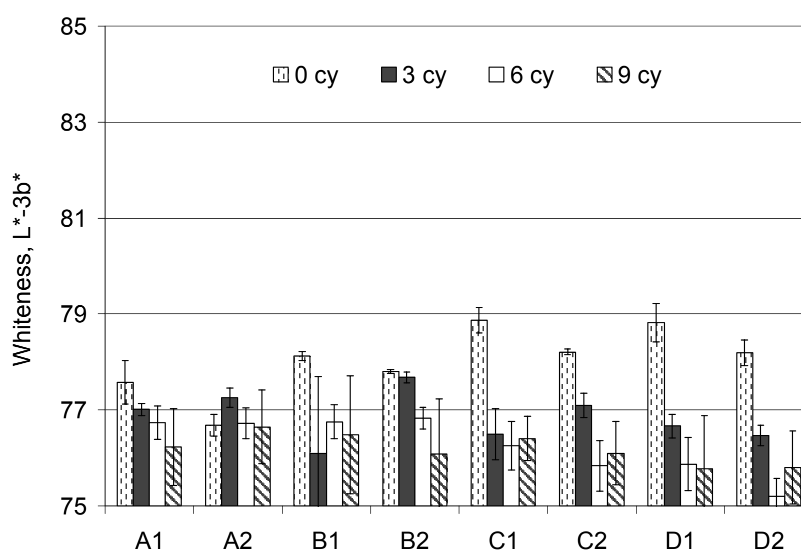

Figure 12. Changes of whiteness as affected by freeze thaw (FT) cycles.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using cooked gels made from surimi after 0, 3, 6, and 9 FT cycles, respectively. The electrophoresis profile reflects changes in the functionality and extractability of proteins during frozen storage (Chang and Regenstein 1997; Huidobro and others 1998; Lian and others 2000). The present study employed electrophoresis to examine the effect of various salt and phosphate contents on intermolecular interactions and to help verify the results obtained from textural measurements and protein extraction.

Freshly frozen samples (0 FT cycle) are shown in Figure 13. A clear effect of phosphate can be seen by looking at the density of the MHC (205 kD) band. A slightly darker band was found for gels with 0.5% phosphate. According to Chang and Regenstein (1997), the addition of polyphosphates increased the intensity of the MHC band due to the increased water uptake ability. By comparing SDS-PAGE gels, it was interesting that MHC in SDS-PAGE gel with 3 cycles (Figure 14) was slightly lighter than that with 6 FT cycles (Figure 15). The SDS-PAGE results for 3 and 6 FT cycles, however, were in agreement with their corresponding gel texture measurement (Figure 6 and 7). Although the cause of the rapid reduction of texture and reduced MHC band observed for gels at 3 FT cycle was unclear.

It was noted that at 9 FT cycles the density of MHC band was noticeably reduced as salt content increased (Figure 16). Cross-linking might have resulted from repeated FT and/or increased concentrations of residual salt in the surimi. This cross-

linking would have been formed between peptide chains (Sikorski and others 1976) and/or by the function of FA formation. When comparing two SDS-PAGE samples (Figures 13 and 16), darker bands above 205 kD are clearly shown after 9 cycles, indicating the formation of freeze induced crosslinking. The addition of salt and phosphate into the surimi before freezing tissue may have reduced large ice crystal formation and increased water uptake ability, thus increasing the salt concentrations, which are known to induce intermolecular cross-linking (Sikorski and others 1976; Matsumoto 1980).

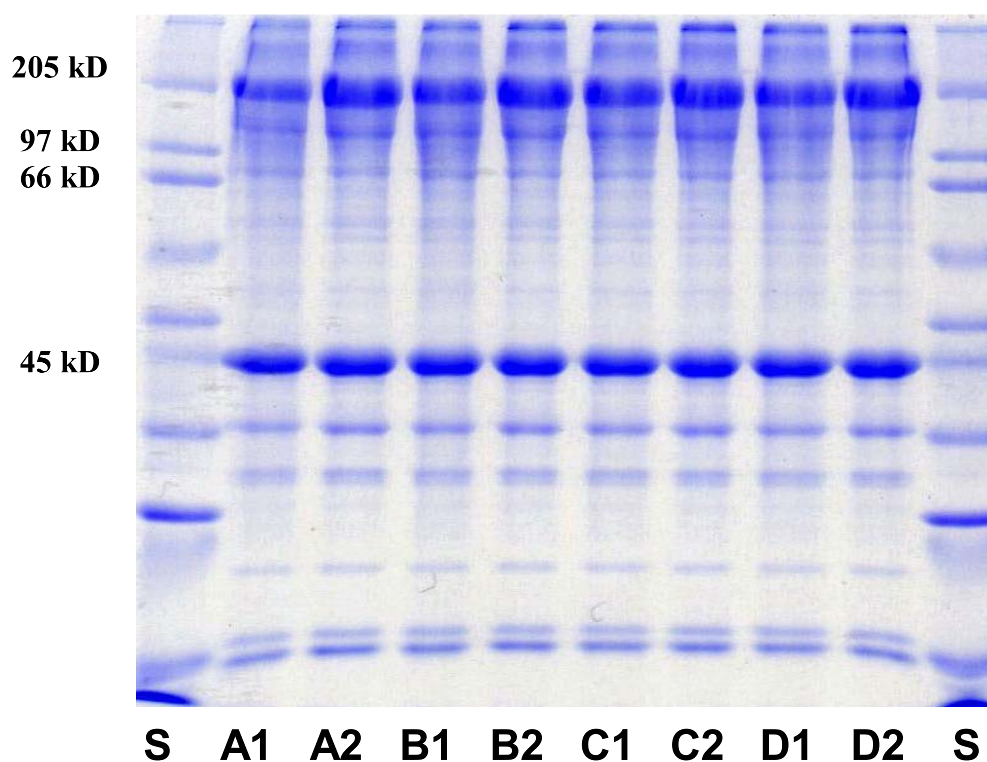

Figure 13. SDS-PAGE patterns of proteins as affected by 0 FT cycle.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.
S = molecular weight standard.

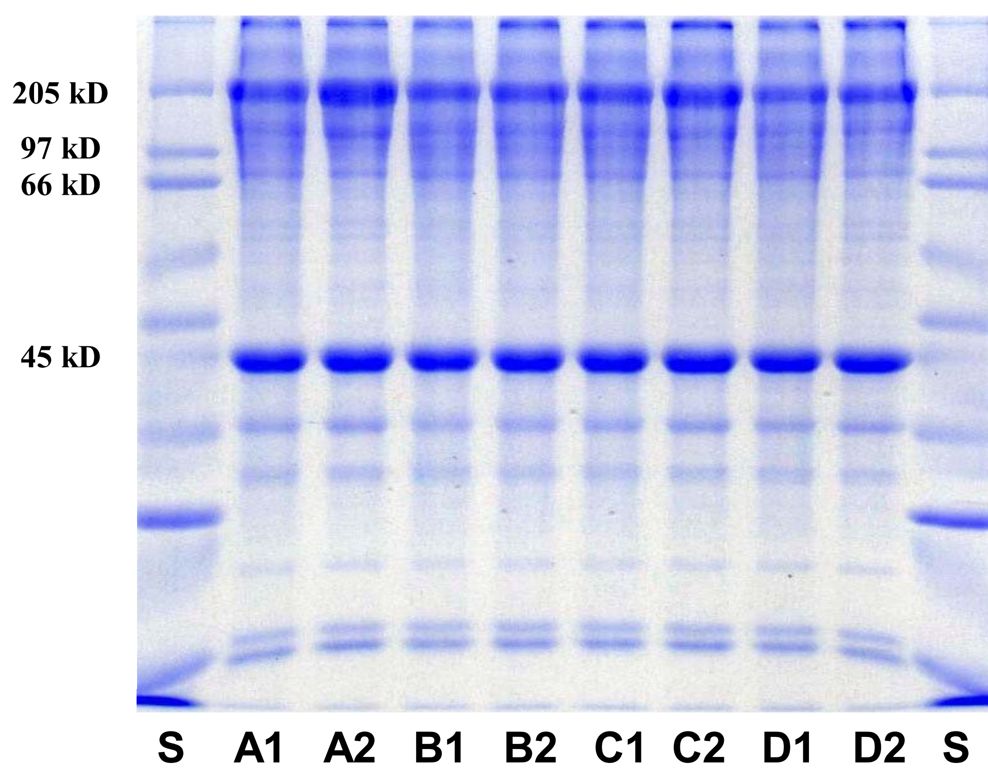

Figure 14. SDS-PAGE patterns of proteins as affected by 3 FT cycle.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.
S = molecular weight standard.

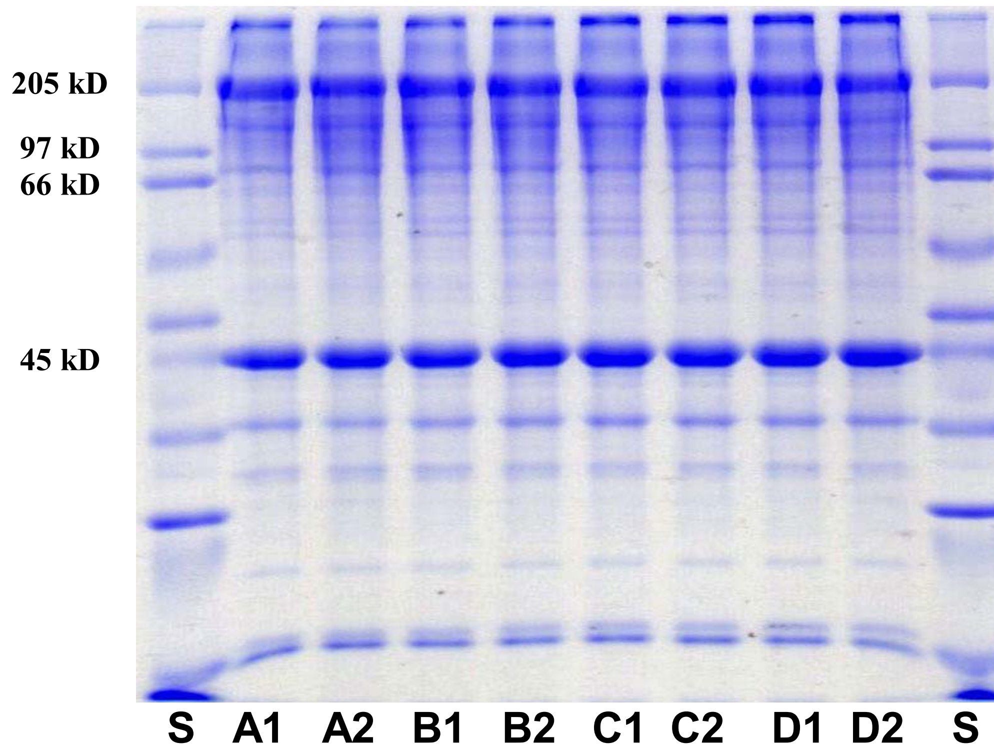

Figure 15. SDS-PAGE patterns of proteins as affected by 6 FT cycle.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.
S = molecular weight standard.

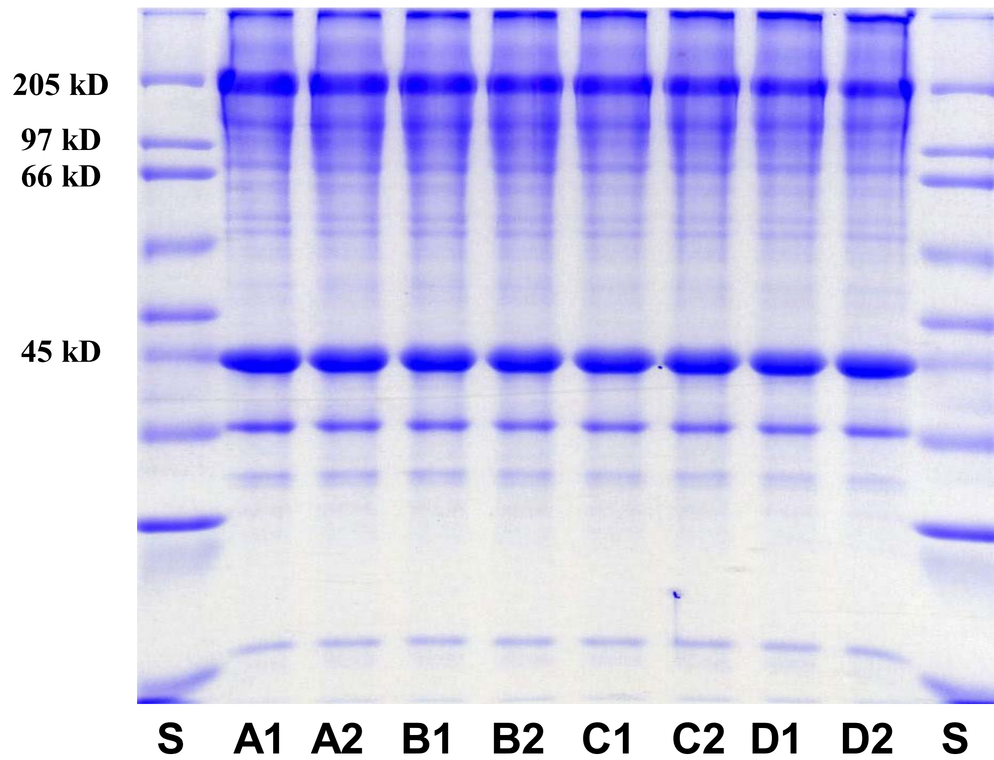

Figure 16. SDS-PAGE patterns of proteins as affected by 9 FT cycle.

A1 = 0.4% salt, 0.25% phosphate; A2 = 0.4% salt, 0.5% phosphate;
B1 = 0.6% salt, 0.25% phosphate; B2 = 0.6% salt, 0.5% phosphate;
C1 = 0.8% salt, 0.25% phosphate; C2 = 0.8% salt, 0.5% phosphate;
D1 = 1.0% salt, 0.25% phosphate; D2 = 1.0% salt, 0.5% phosphate.
S = molecular weight standard.

Conclusion

The pH was clearly affected by the presence of higher phosphate content. The effect of residual salt in frozen surimi was noted when salt concentration increased to 0.8% or higher, possibly contributing to protein denaturation and exposing amino acids with a lower pKa value. In all samples SEP decreased while FA increased, indicating FA formation plays an important role in freeze induced denaturation. The increase of surface hydrophobicity was thought due to the exposure of hydrophobic groups, which would cause subsequent aggregation

If the surimi is going to be used fresh or after short frozen storage the amount of residual salt is not likely to give a negative effect on the gel-forming ability based on the SEP results. However, as frozen storage is extended, the gel-forming ability would decrease at a higher rate when more residual salt is retained during frozen storage. Ca^{2+}-ATPase activity showed a similar trend with the SEP results. The increase in S_o after FT cycles during frozen storage indicated denaturation of the protein structure, revealing the hydrophobic sites.

The MHC bands on the SDS-PAGE gel showed similar trends with gel deformation. The elasticity of surimi-based gels was influenced by both the quality and quantity of salt soluble proteins (Kawashima and others 1973; and Nishioka and others 1983). However, it is possible that the gel-forming properties of surimi may be affected by factors other than protein denaturation. Indeed, a truer indication of salt and phosphate as an ingredient against protein denaturation is to monitor the

extractability of salt-soluble proteins during frozen storage (Park and others 1988). Therefore, based on the SEP, FA, and Ca^{2+}-ATPase activity results, an optimum concentration for salt and phosphate could be suggested as 0.4% or lower of salt and 0.5% phosphate in order to maintain surimi quality during long term frozen storage.

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