

AN ABSTRACT OF THE DISSERTATION OF

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Title: Biochemical and Gelation Properties of Fish Protein Isolate Prepared under Various pH and Ionic Strength Conditions

Abstract approved:

Jae W. Park

A novel method for isolating fish proteins by shifting pH to high acid or high alkali pH was the focus of the study. Biochemical and physicochemical properties of various pH-treated soluble fish proteins as a function of ionic strength were determined. Effect of ionic strength and various storage conditions on gelation properties and stabilization of fish protein isolate (FPI) were also elucidated.

At low ionic strength (IS 10 mM NaCl), the solubility of Pacific whiting (PW) proteins was low between pH 5 and 10, but increased significantly as the pH was shifted to either acidic or alkaline pH. The isoelectric point (pI) shifted toward acidic direction as IS increased to 600 mM. High IS (600 mM NaCl) resulted in protein aggregation at low pH but improved myosin heavy chain (MHC) solubility at pH 6 - 10. Changes in total sulfhydryl (SH) content and surface hydrophobicity (S_o) were associated with the different molecular weight distributions of the soluble

proteins. At pH 4 and IS 10-100 mM, MHC was soluble but degraded. At pH 10, the formation of high MW polymers was observed at IS $\geq$ 150 mM.

Gels obtained from FPI prepared at pH11/IS150 and conventional surimi (CS) were superior to FPI prepared at pH 3 and/or other IS levels. There was no correlation between protein solubility and gel properties of FPI. Gelation mechanisms of acid- and alkali-treated FPI were identical under the same IS condition. FPI prepared at pH 3 or 11 could be partly refolded at pH 7.

No significant difference in texture was observed between alkali-treated protein isolates (AKPI, pH 11) kept frozen at pH 5.5 and 7.0. Strongest gel was found for AKPI with cryoprotectants (C) and without freeze/thaw (FT) cycles at both pH storage (5C & 7C), while poor gel was obtained from AKPI without cryoprotectants (NC) and with FT (5NC-F & 7NC-F). 5NC-F & 7NC-F demonstrated the lowest S_0 and total SH probably suggesting that proteins were more aggregated as a result of hydrophobic interactions and disulfide bonds.

Scanning electron microscope (SEM) revealed the most discontinuity of gels from AKPI without cryoprotectants and with FT and showed less protein stability when stored at pH 5.5 than at neutral pH. Raman spectral analysis demonstrated that refolding of AKPI by pH adjustment to 7.0 was achieved, but not identical to the native protein. CS contained higher α -helix content (~50%) than AKPI (~20-30%). Frozen storage induced a decrease and an increase in the α -helix of CS and AKPI samples, respectively. Alkali-treated proteins were slightly less stable than CS during frozen storage.

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Biochemical and Gelation Properties of Fish Protein Isolate Prepared Under
Various pH and Ionic Strength Conditions

by
Supawan Thawornchinsombut

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

Supawan Thawornchinsombut, Author

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BIOCHEMICAL AND GELATION PROPERTIES OF FISH PROTEIN ISOLATE PREPARED UNDER VARIOUS pH AND IONIC STRENGTH CONDITIONS

CHAPTER 1

INTRODUCTION

Surimi is washed minced fish muscle mixed with cryoprotectants such as sucrose and sorbitol. The fish muscle consists of salt soluble myofibrillar proteins and has unique gelling properties that make it useful as a food base in seafood analogues. The surimi industry has changed dramatically over the past decade. A sharp decrease in surimi resources since the year 2000 has opened the door for the utilization of new species and the discovery of new technology in the surimi industry. The white fish harvest including Alaska pollock, Pacific whiting, blue whiting, and hoki has encountered a steady decline from 1995 to 2002 from 9 million metric tons (MT) to around 6 million MT. This was due to the collapse of the Russian pollock industry in the Bering Sea while the cod fishery in the North Atlantic and the South American hake fisheries also descended.

It can be expected that good quality white fish should be primarily utilized in fillet processing while surimi will be made from filleting by-products, and from small fish that can not be processed into fillets. Novel technologies have allowed new resources to be used as raw material for surimi (Guenneuges and Morissey 2004). The new pH-shift method, where fish proteins are solubilized by shifting the pH from neutrality and subsequently precipitated at the isoelectric point (pI), has the potential for better utilization of small fish and pelagic species as well as improve recovery for surimi made from traditional species (Hultin and Kelleher 2000). Given the high potential of this new method for application in the surimi industry, a better understanding of protein characteristics, functional properties, and stability must first be undertaken.

Subjecting proteins to low or high pH is known to denature them. This condition is typically avoided in the conventional surimi process since it is a common belief that good surimi gel quality can only be achieved from fresh fish without temperature abuse. When these chemically unfolded proteins are neutralized, good gel functionality, however, can be attained (Cortes-Ruiz and others 2001; Undeland and others 2002; Kim and others 2003; Yongsawatdigul and Park 2004). The modification of protein molecules during acid/alkaline process could potentially be directed into different functional forms. It is therefore of much interest to investigate and understand the biochemical properties of proteins and also protein structural changes at the molecular level as the environment is changed.

Since ionic strength and pH affect protein functionality, changes in structure and function imposed as ionic strength is altered are of additional interest. Salt concentration changes the protein conformation through electrostatic and hydrophobic forces (Nakai and Li-Chan 1988), whereas pH affects the net charges of protein molecules (Kinsella 1984).

Furthermore, storage stability of this modified protein is of significant importance. It has been recognized that at the pI, proteins are more stable than at other pHs (Damodaran 1996). However, pH-shifted protein isolate recovered at the pI perhaps behaves differently from conventional proteins. Deterioration of muscle protein functionality associated with frozen storage is inevitable. The loss of protein functionality and, in particular, the loss of gel-forming ability in frozen protein is due to freeze-denaturation and aggregation of myofibrillar proteins (Sikorski and others 1976; Matsumoto 1980; Suzuki 1981). Nevertheless, pH-shift protein isolate is chemically unfolded; its alternative behaviors during frozen storage could be different from traditional surimi. Various storage conditions affecting the conformation and functionality of pH-shifted proteins are, therefore, the focus of this study.

The hypothesis of this study were 1) in addition to pH, ionic strength would also modulate protein property and functionality of pH-shift fish protein isolate; 2) proteins recovered at the pI would be stable under frozen storage without adjusting to a neutrality; and 3) since proteins are already chemically unfolded by pH adjustment,

cryoprotectant may not be necessary to preserve FPI under frozen storage. The experiments were conducted based on these following goals: 1) to evaluate biochemical properties of various pH-treated soluble fish proteins as a function of ionic strength; 2) to elucidate effect of IS on biochemical, physicochemical and gelation properties of fish protein isolate; and 3) to determine stability of fish protein isolate as affected by various storage conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 CONVENTIONAL SURIMI

Surimi is stabilized myofibrillar proteins obtained from mechanically deboned fish flesh that is washed with water and blended with cryoprotectants. Surimi is an intermediate product used in a variety of products, ranging from the traditional kamaboko product of Japan to surimi seafood, otherwise known as shellfish substitutes (Park and Morissey 2000). Japan has been improving surimi technology for several hundred years.

The first deliveries of Alaska pollock (*Theragra chalcogramma*) for making surimi were landed in the US (Kodiak, Alaska) in January 1985 (Gwinn 1992). The surimi industry in the US mainly uses Alaska pollock, which covers 50-70% of total surimi, but its proportion has been continuously reduced. Pacific whiting (*Merluccius productus*) is one of many whiting harvested throughout the world. Use of Pacific whiting from the Pacific coast for surimi manufacturing showed a rapid growth in the 1990s and at one time represented approximately 20% of surimi production in the United States.

The presence of high levels of protease enzymes within fish muscle tissue makes controlling quality difficult in surimi products. Beef plasma protein was found to be the most active protease inhibitor for Pacific whiting surimi. However, there is resistance in many countries associated with beef plasma in fish, primarily because of the outbreak of mad cow disease; therefore use of beef plasma has become problematic. Other protease inhibitors such as egg white and whey protein are therefore used in the current production of whiting surimi (Guenneuges and Morissey 2004).

Because of the instability of fish muscle protein during frozen storage, fish is usually processed into surimi by a series of washing processes before being processed into various secondary products such as kamaboko, chikuwa, imitation crab, hanpen,

etc. The processing of surimi involves leaching in order to remove undesirable compounds such as pigments, enzymes, and fat, and to concentrate the myofibrillar proteins, which can be further processed into secondary products. The concentrated proteins (surimi) are usually stabilized by mixing with cryoprotectants, which can minimize denaturation and loss of functionality during subsequent frozen storage. The basic steps include: 1) preparation of raw materials including heading, gutting, and filleting; 2) mechanical deboning to separate bones, hard cartilage, and skin and give mince meat simultaneously; 3) washing (the number of washing cycles and the volume of water varies with fish species, freshness of fish, type of washing unit, and desired quality of the surimi, however two or three washing cycles are usually applied. It is also common to use a 0.3 % salt mixture of NaCl and CaCl_2 to facilitate the removal of water); 4) refining to remove small skin, bone fragments, connective tissue and other debris; 5) dewatering to remove excess water by either pressing or centrifugation; 6) mixing with cryoprotectants such as sucrose and sorbitol (alone or mixed at ~ 9% w/w of dewatered fish meat) as well as a mixture (1:1) of sodium tripolyphosphate and tetrasodium pyrophosphate (0.2-0.3%) ; 7) forming and panning; 8) freezing to obtain a block center temperature of -25 °C (contact plate freezer or air blast freezer); 9) depanning and packing; and 10) frozen storage at -25°C (Jiang and Chen 1999; Park and Morrissey 2000).

2.2 FISH PROTEIN ISOLATE PREPARED USING pH-shift

The process for isolating the protein composition from a muscle source and recovering the protein composition using pH-shift was first patented by Hultin and Kelleher (1999). The effort was designed to better utilize low value raw materials including fatty, pelagic fish and deboned muscle tissue from fish and poultry processing. Considerable effort has also been made to produce a protein concentrate from underutilized fish species. One process that has had some success in stabilizing protein food has been the process for producing “surimi.” In producing surimi, the muscle is ground and extensively washed and dewatered. During the washing process 20-30% of the fish muscle proteins, especially sarcoplasmic proteins, are solubilized

and generally not recovered from the wash water. A significant amount of myosin heavy chain was also soluble when mince was washed with fresh water during surimi manufacturing (Lin and Park 1996).

These novel processes have been developed in which proteins are isolated from animal muscle using acid (pH 2-3) or alkaline (pH 10.5-11) solubilization of the proteins. The soluble proteins are subsequently recovered by isoelectric precipitation (pI ~ 5.5) (Hultin and Kelleher 1999; Hultin and others 2000; Choi and Park 2002; Kim and others 2003; Yongsawatdigul and Park 2004). Muscle tissue is ground and homogenized with enough water. pH of the homogenate is adjusted to solubilize a major proportion, preferably substantially all of the available protein and to reduce the viscosity to allow easy separation of insoluble materials from the solubilized composition. Typically the ratio of volume of aqueous liquid to weight of tissue is 5:1 to 9:1. This process differs from the conventional process in that major myofibrillar proteins are much less solubilized in the conventional process but water-soluble materials that lead to loss of quality of the product are removed. After the muscle proteins are solubilized, they are centrifuged at an adequate force (greater than 5000xg) to separate the membrane lipid and other insoluble fractions from neutral lipids and soluble proteins. The neutral lipids can be skimmed off and the soluble supernatant, protein-rich fraction is recovered by decanting.

The protein in the aqueous phase can be precipitated by raising/decreasing its pH to about 5.0 or 5.5. The salt concentration can be optionally adjusted to aid precipitation. The total precipitated protein comprised of myofibrillar proteins and sarcoplasmic proteins can be collected by centrifugation. At least 70% yield of the total animal muscle protein can be obtained as precipitated protein. The protein isolate can be adjusted to a neutral pH with the addition of bicarbonate or other base. Cryoprotectants can be added to the precipitated protein to preserve the product during freezing and storage (Hultin and Kelleher 1999; Hultin and others 2000).

This new method offers several advantages over the traditional surimi process. The new process gives improved processing yield. Greater than 90% yields are generally obtained from fillets compared with 55-70% from the conventional process

(Hultin and Kelleher 1999; Kim 2002; Undeland and others 2002). The improved yield results in less protein in the waste water, therefore environmental pollution is decreased. It is also clear that most of the sarcoplasmic proteins are recovered by this process as well. Furthermore, there was no indication that these sarcoplasmic proteins interfered with the gel formation of myofibrillar proteins and there is evidence that sarcoplasmic proteins may actually enhance myofibrillar protein gelation (Morioka and Shimizu 1990; Ko and Hwang 1995; Nowsad and others 1995).

A functional protein isolate can be produced with low lipid content since this process can remove the oxidation-prone membrane lipids by centrifugation, thus stabilizing the proteins against oxidation. One advantage to the elimination of lipid from the proteins is that lipid-soluble toxins i.e. polychlorinated biphenyls and polyaromatic hydrocarbons can be reduced in the final product.

The new process also increases productivity since the extensive washing and refining steps can be excluded. Undesired materials such as scale, skin, and bone would also be removed in the sediment of the first centrifugation. Furthermore, unlike the conventional method, this method does not require fresh or lean animal muscle as a starting material. In addition, frozen or slightly damaged muscle tissue is still capable of producing a gel with excellent quality. On the other hand, in the manufacture of gels from surimi using the conventional method, high quality fish should be used to obtain high quality products (Hultin and Kelleher 1999; Hultin and others 2000; Hultin and Kelleher 2000).

2.3 pH AND IONIC STRENGTH EFFECTS ON PROTEIN FUNCTIONALITIES

Acid or alkaline processing of muscle protein is expected to cause dramatic changes of protein conformation and function. According to conventional muscle chemistry, it is generally known that denatured muscle proteins contribute negative protein functionalities. Conversely, it has been shown that the acid- or alkali-treated muscle proteins exhibited either comparative or improved gel qualities compared to proteins from conventional processes (Kim 2002, Underland and others 2002,

Yongsawatdigul and Park 2004). Challenges for the unexplained evidence are to find out which mechanisms help maintain or improve their properties through severe pH conditions. Effects of pH and ionic strength on protein functionalities, particularly solubility, protein conformation, and gelation properties will be discussed.

2.3.1 Solubility

Among the functional properties of proteins, solubility is of primary importance due to its significant influence on the texture of protein gels. Many researchers use protein solubility as a secondary category of test under the thermodynamic index of protein structure. In general, proteins used for functionality are required to have high solubility, in order to provide good emulsion, foam, gelation, and whipping properties (Vojdani 1996).

Solubility of proteins relates to surface hydrophobic (protein-protein) and hydrophilic (protein-solvent) interactions with water. The relative proportion of surface hydrophobic and hydrophilic groups dictates the degree of solvation by water (Damodaran 1989). Protein solubility is a function of many factors, such as the amino acid and non-amino acid composition of proteins, native or denatured state, and environmental factors (i.e. pH, temperature, nature and concentration of salts (ionic strength), pressure, and concentration of protein). Solubility can be defined as the amount of the total protein (%) that goes into solution under specified conditions and is nonsedimentable by a specified centrifugal force. Unfortunately, there exists no standardized, universally accepted method for determination of protein solubility (Xiong 1997).

It is well known that protein solubility depends on the pH of the solution. A protein in an aqueous system has a zero net charge at its pI and usually has the least solubility. Protein-protein interaction increases because the electrostatic repulsive forces of the molecules are at a minimum and less water interacts with the protein molecules. At pH values above and below the pI, where a protein has a net negative or positive charge, more water interacts with the protein charges. Net charges and charge repulsion contribute to greater protein solubility and the protein may stay in the

solution. Soy isolate in 0.2 M NaCl is only 50% soluble at pH 10, while at pH 11 and 12 it is 70% and 90% soluble, respectively. Fish protein concentrate produced by organic solvent, which is almost insoluble at pH 2 to 11, is soluble at pH 12 (Vojdani 1996). Alkaline pH has also been used in the processing of fish muscle and muscle from other sources. One common use of high pH is to recover protein from deboned meat (McCurdy and others 1986; Opiacha and others 1994).

At extreme acidic or basic pH values, strong electrostatic repulsions of ionized groups occur, which can cause protein unfolding, and exposing more hydrophobic groups. Unfolding at low or high pH values results from a decrease in electrostatic bonds. In some cases, the protein may recover its native structure when the pH is readjusted to the initial stable range. Sometimes, at high or low pH values, due to a decrease in electrostatic bonds, the protein undergoes denaturation followed by aggregation and precipitation. Some proteins that are unfolded at extreme pH values, under conditions of low ionic strength, may refold and stabilize when the concentration of salts is increased (Hamaguchi 1992).

Like pH, ionic strength (IS) influences protein solubility as well as protein-protein and protein-solvent interactions. For some proteins such as globulin, the solubility increases as the concentration of neutral salts increases. But the solubility will reach a maximum and then decrease with further increase in salt content. At this point there is competition between protein and salt ions for binding water. The phase where solubility increases is called “salting-in” and the removal phase is called “salting-out”.

Neutral salts, at a concentration in the order of 0.1-1 M may increase protein solubility depending on the protein, salt, pH, and temperature. The salt ions interact with oppositely charged groups on the protein to form a double layer of ionic groups, which decreases electrostatic interaction between protein molecules and causes more protein solvation, thereby increasing protein solubility. At higher concentrations of salt ions, most of the water molecules are strongly bound to the salt ions, while there is some reorganization of the water molecules around the protein molecule. This could result in stronger protein-protein interactions through hydrophobic interactions rather

than water-protein interactions. This results in association, followed by precipitation (Vojdani 1996).

Muscle proteins have been traditionally grouped into one of the three categories based on their solubility after sequential extractions varying in salt concentrations, the sarcoplasmic proteins (myogen), the myofibrillar proteins (salt-soluble proteins) and the proteins of the stromal fraction. Most proteins in the “sarcoplasmic protein” family are of a globular structure and relatively small in size (most have molecular weight (MW) between 30,000 and 65,000 daltons). The surface of protein molecules is made up of charged and uncharged polar groups. Such distribution of amino acids and tertiary structural organization permit the sarcoplasmic proteins to freely interact with the surrounding water, therefore, the proteins are highly hydrophilic and soluble in water or dilute salt solutions. On the other hand, myofibrillar proteins in muscle are associated with one another in a highly organized, integral structure unit of a myofibril. Thus, although most myofibrillar proteins have a balanced amino acid composition, their pI are mostly falling in the pH 5-6 range (King and MacFarlane 1987; Pearson and Young 1989). The stromal proteins include connective tissue. The major fraction of connective tissue is collagen. For most practical purposes collagen is insoluble. However, extremes of pH (below 3, above 9) can result in extensive swelling or even dissolution of collagen. This dissolution/swelling at pH 3 is reduced at high salt concentrations (Rodger 1990).

The low pI is related to protein structural arrangement and the tendency to interact between different segments of polypeptides. Thus, the solubility of myofibrillar proteins under the physiological condition or low ionic strengths is negligible (Xiong 1997). However, recent studies have shown that these proteins can be dissociated at low IS (Stefansson and Hultin 1994; Feng and Hultin 1997; Krishnamurty and others 1996). The intracellular myofibrillar proteins of chicken breast muscle were solubilized at IS of 0.001 (Stanley and Hultin 1968; Comissiong and others 1971; Comissiong and Hultin 1976).

2.3.2 Protein Conformation

Proteins are polypeptide chains existing at four different structural levels: primary, secondary, tertiary, and quaternary levels. Under normal conditions of pH and temperature, each polypeptide assumes one specific conformation, called “native” (Boye and others 1997). The term “protein folding” refers to the process involved in the conversion of an ensemble of newly synthesized (or denatured) polypeptide chain conformations to the unique three-dimensional conformation of native protein. When present, disulfide bonds serve to stabilize the native conformation by reducing the structural fluctuations of alternative (denatured) forms. In some cases, the attainment of the final native conformation depends on the presence of specific ligands, frequently metal ions or prosthetic groups.

It is believed that the native protein in a given environment (solvent, pH, ionic strength, presence of other components, temperature, etc.) is the one in which the Gibbs free energy of the whole system is a minimum with respect to all degrees of freedom, i.e., that the native conformation is determined by the various interatomic interactions and hence by the amino acid sequence, in a given environment (Anfinsen and Scheraga 1975). Thus, in an aqueous environment, this causes the peptide chain to orient its hydrophilic amino acids toward the outside of the molecule and to bury the hydrophobic amino acids in the interior of the molecule with the exclusion of water from this core. Conformational entropy is the major destabilized force of the protein structure, and the difference between the native and denatured states is usually less than 10 kcal/mol. Protein unfolding is therefore easily accomplished by changing the solvent conditions disrupting the forces stabilizing the protein and bringing about conditions favoring the extended configuration of the protein (Price 2000).

Proteins can undergo reversible denaturation. When the myosin molecule is completely dissociated in 5 M guanidine hydrochloride solution, removal of the denaturant by dialysis at neutral pH leads to the regeneration of an entity having the hydrodynamic properties of the original myosin (Godfrey and Harrington 1970b). Enzyme activity is not recovered after dialysis, possibly because of a lack of proper incorporation of the light chains.

It is well known that changes of the pH of the protein environment lead to protein unfolding (Goto and Fink 1994). Proteins are stable against denaturation at their isoelectric point than at any other pH. At neutral pH, most proteins are negatively charged, and a few positively charged (Damodaran 1996). At extreme pH, the electrostatic free energy of the charged protein is reduced on unfolding since charge repulsion is reduced as charges are distributed over a larger volume of the unfolded protein (Dill and Shortle 1991). During pH adjustment, amino acid residues may change conformational orientation. When the pH is readjusted, all of the original structures may not be regained, leading to a mixture of structure. Some peptides bonds (particularly aspartyl bonds) may be hydrolyzed when a protein is held at an acidic pH for long periods of time; deamidation of asparagine 21 of insulin also occurs (Schein 1990).

Recent studies have shown the existence of intermediate conformational states for several proteins. Such intermediates are commonly called “molten globule states”, which may be induced by lowering the pH and involve the partial denaturation of the proteins into intermediates (Poklar and others 1997). Until recently, the molten globule state was considered to be a partly folded state having a stable native-like secondary structure but lacking a specific tertiary structure. However, in several studies it has been shown that a great variety of molten globule states do exist, ranging from those having specific tertiary interactions or native-like topology to those resembling the unfolded state of a protein. Evidence has been provided that the molten globule states of some proteins share conformational features with those transiently observed in kinetic experiments of protein folding (Laureto and others 2002). In the presence of salts, conformation transitions at acidic or alkaline pH from a largely unfolded state to an intermediate conformational state have been reported for several small globular and even multimeric proteins.

Goto and Fink (1989) reported that unique conformational states of β -lactamase at both acidic and alkaline pH regions were found at high salt concentrations, which showed the characteristic properties of the molten-globule state. They also indicated that hydrophobic interactions play an important role in the

formation of intermediate conformational states during folding.

2.3.3 Gelation Properties

Heat-induced gelation of fish proteins such as surimi is a complex physicochemical process involving structural and functional changes of myofibrillar proteins. The interactions involved in gelation have been shown to be dependent upon the species of fish employed (Taguchi and others 1987, Sano and others 1990a, Chan and others 1993). The formation of myofibrillar networks can be represented by three stages, that is, dissociation, thermal denaturation, and aggregation (Roussel and Cheftel 1990). In the presence of salts, muscle proteins undergo major structural changes, leading to the solubilization of myosin, actin, and a number of other myofibrillar constituents (Parsons and Knight 1990). Partial unfolding of the protein conformation is accelerated by an increase in temperature, resulting in the aggregation of unfolded regions between protein molecules to form a three-dimensional network.

Four main types of chemical bonds can link proteins: hydrogen bonds, ion linkages, hydrophobic interactions, and covalent bonds. Hydrogen bonds are important in the stabilization of bound water in the hydrogel and increase when the gel is colder. Electrostatic interactions can be altered by the manipulation of pH and/or ionic strength. NaCl is typically added to break the ionic linkages and assist in the dispersion of the proteins during gel preparation. In contrast to hydrogen bonds, which dissipate on heating, hydrophobic interactions are strengthened by raising the temperature (at least to near 60 °C). Hydrophobic interactions are presently thought to be a primary mechanism for surimi gel formation during heating. Disulfide bonding (S-S) is the predominant covalent bond thought to contribute to gel formation of proteins at high temperature (> 40 °C). An intermolecular disulfide bond is formed by the oxidation of two cysteine molecules on neighboring protein chains that have reactive SH groups (Lanier 2000).

Gel formation is generally influenced by several parameters (i.e. pH, ionic type and strength, protein concentration, heating time/temperature, and interaction with other components). Salt concentration and pH particularly affect protein solubility

and chemical bonds of heat-induced protein gels. As previously described, salt (2-3%) must be added to extract and destabilize the native structures of myofibrillar proteins in order to form heat-induced gels.

The mechanisms of gel formation at high salt concentration have been extensively investigated. The tail and head of myosin molecules play different roles in gel formation. The head portion undergoes irreversible aggregation involving hydrophobic interactions and disulfide bond formation. A partially irreversible helix-to-coil transition takes place in the tail portion, which subsequently participate in the gel structure through hydrophobic residues (Samejima and others 1976, 1981; Sharp and Offer 1992).

Gelation of myofibrillar proteins at low salt concentrations has been recently explored. Several studies have reported that gels of myofibrils formed at low IS (0.3 M) were weak (Samejima and others 1985; Foegeding 1987; Xiong and others 1993). However, stronger gels were obtained when IS was lowered from 0.6 to 0.2 M KCl (Boyer and others 1996). The rate of decrease in IS affects the formation of filaments resulting in different gel structures. A slow rate of IS reduction (0.6 to 0.2 M KCl) by dialysis at pH 6.0 contributes filament formation and a strand-type of gel structure resulting in better gel quality.

The ability to form gel without salt is species-dependent. Excellent to adequate gel qualities could be achieved from red hake, cod and winter flounder without adding salt when the mince tissue was washed prior to gel formation (Hennigar and others 1989; Stefansson and Hultin 1994). However, dark fleshed fatty species, beef, and chicken breast muscle do not exhibit this property (Bakir and others 1994; Hennigar and others 1989; Chang 1997). In addition, at high protein concentration (>14%), better gel qualities are attained at neutral pH and high salt concentration (0.4-0.6 M NaCl), and at pH $\geq$ 7.0 and low ionic strength (0.1-0.3 M NaCl) (Chung and others 1993; Barbut 1997; Chang 1997).

2.4 ANALYTICAL METHODS TO DETERMINE PROTEIN FUNCTIONALITY

2.4.1 Sulfhydryl Content

The formation of disulfide bonds between protein molecules during food processing contributes to the functionality of the protein in different systems. Treatments that destabilize native protein structure and promote unfolding can facilitate increased protein-protein interactions between proteins in solution and lead to intra- or intermolecular thiol/disulfide (SH/S-S) interchange or thiol/thiol (SH/SH) oxidation reactions. Disulfide-mediated polymerization of whey proteins has been shown to occur in response to heating, increasing pH, and treatment with urea and reducing agents (Monahan and others 1995). Heat-induced gelation of the protein involves unfolding of proteins followed by orientation of unfolded molecules during aggregation, which influences the development of the gel network.

Disulfide bonding can contribute to the gel formation of actomyosin upon heating and is also responsible for the polymerization of the protein (Runglerdkriangkrai and others 1999a, 1999b). Controlling the extent of disulfide-mediated polymerization reactions by varying conditions under which polymerization can proceed may be an effective way of manufacturing gels with different rheological properties and food applications (Monahan and others 1995).

The estimation of free thiol groups in native and denatured proteins using the Ellman reagent, 5,5'-dithiobis(2-nitrobenzoic acid)(DTNB) has been used extensively. This compound is very useful as a free sulfhydryl (SH) assay reagent due to its high specificity for SH groups at neutral pH values, its high molar extinction coefficient, and its short reaction times (Anonymous 2001). This procedure is based on the reaction of the free thiol groups with DTNB to form a mixed disulfide of protein and one mole of 2-nitro-5-thiobenzoate (TNB) per mole of protein sulfhydryl group (Fig. 2.1), which is quantified by the absorbance of the dianion (TNB^{2-}) at 412 nm (Riddles and others 1979). The target of DTNB in this reaction is the conjugate base (R-S-) of a free sulfhydryl group. Therefore, the rate of this reaction is dependent on several

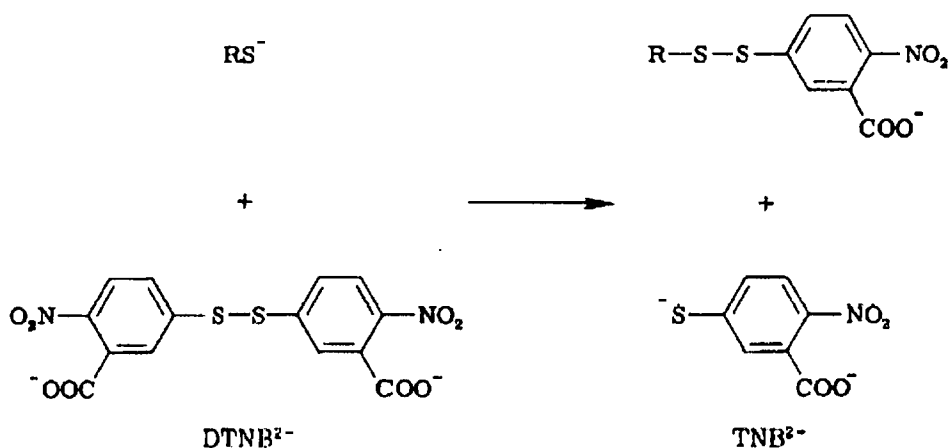


Figure 2.1: The reaction of Ellman reagent with thiol group.

factors: 1) the reaction pH, 2) the pKa' of the SH, and 3) steric and electrostatic effects (Riddles and others 1983). The determination of disulfide bonds in protein solutions has been indirectly observed by monitoring a decrease in SH content after treating proteins in Ellman reagent. In addition, several studies have been researched the contribution of disulfide bonds to protein gel by combining results of SH group content and polymerization of myosin heavy chain (MHC) through disulfide bonding using SDS-PAGE with and without reducing agents (β -mercaptoethanol) (Sompongse and others 1996a; Runglerdkriangkrai and others 1999a, 1999b; Hossain and others 2001). Since disulfide bonds are formed through the oxidation of SH groups, the application of reducing agents (DDT, dithiothreitol), blocking agents (NEM, *N*-ethylmaleimide), or chelating agents (EDTA, ethylenediaminetetraacetic acid) have been conducted to investigate the significance of disulfide bonds in protein functionality (Sompongse and others 1996b; Smyth and others 1998; Runglerdkriangkrai and others 1999a, 1999b,)

2.4.2 Surface Hydrophobicity

The importance of hydrophobicity interactions for the stability, conformation, and function of proteins is well recognized. Due to the macromolecular structure of proteins, surface or effective hydrophobicity is more influential for functionality than

total hydrophobicity. Therefore information on surface hydrophobicity is essential to understand protein functionality (Haskard and Li-Chan 1998).

The term “lyophobic” is used to describe a solute that has little or no affinity for the solvent medium in which it is placed. When the medium is composed of water or an aqueous solution, the more specific term “hydrophobic” is used as the descriptor. Similarly, “lyophilic” or hydrophilic” is used to indicate the affinity of a solute for solvent or water.

According to Edsall and McKenzie (1983) the hydrophobic interaction was defined by Kauzmann (1959) with a concept of entropy driven forces. The theory is based on hypothesis that the hydrophobic effect is purely the result of the phobia of water from contact with hydrocarbons. The tendency of non-polar solutes to adhere to one another in an aqueous environment is also defined by Cardamone and Puri (1992). 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.

To achieve the minimum free energy in folding of macromolecules, non-polar or hydrophobic groups should be restricted to the interior of folded molecules, thus not being exposed to 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).

Even though many recent investigations have demonstrated the important roles of surface hydrophobicity in the mechanisms of protein functionality, measurement of surface hydrophobicity is still quite controversial so that no standard method has been established. Several methods though have been used to achieve protein surface hydrophobicity such as partition in aqueous two-phase systems, HPLC (high performance liquid chromatography), binding methods, contact angle measurement, intrinsic fluorescence, derivative spectrophotometry, or probe spectrofluorometry (Nakai and others 1996). However, only the last method, which was applied in this research, is discussed. The method using fluorescent probes has proved most popular

due to its simplicity, speed, and ability to predict functionality, and use of small quantities of purified protein for analysis.

Probe spectrofluorometry

The hydrophobic nature of proteins can be assessed using compounds for which quantum yields of fluorescence and wavelength of maximum emission depend on the polarity of the environment. Several types of probes have been applied, such as the amphiphilic 1-anilinonaphthalene-8-sulfonate (ANS) or its dimeric form bis-ANS, the anionic probes of the aromatic sulfonic acid class. These probes have low quantum yield of fluorescence in aqueous solutions, thus they fluoresce when bound to membranes or relatively hydrophobic cavities in many proteins (Nakai and others 1996).

Another group of anionic fluorescence probes is of the fatty acid analog type, including *cis*-parinaric acid (CPA) and *trans*-parinaric acid, which have been used as a probe for proteins and biological membranes in particular. The advantages of these probes are their similarity to native fatty acids and non fluorescence in water, which may be important in protein-lipid interactions in food systems. Good correlations between the relative hydrophobicity values of proteins determined by CPA and properties related to protein-lipid interactions such as interfacial tension and emulsifying activity were obtained (Kato and Nakai 1980). Alizadeh-Pasdar and Li-Chan (2000) reported that the fluorescence of CPA bound to β -lactoglobulin is three-times lower at pH 3.0 than at pH 7.0, indicating that CPA may lose binding to β -lactoglobulin at the acidic pH.

The drawback of using these anionic probes includes the possible contribution of both electrostatic and hydrophobic interactions to the binding of these anionic probes and protein. The influence of electrostatic forces between proteins and charged probes was determined by Greene (1984). Significant ionic interferences were observed. Nevertheless, the majority of binding energy was derived from hydrophobic interactions. It has been suggested that, in general, electrostatic effects are more

likely to enhance protein binding of anions rather than cations. Thus, the use of anionic probes may lead to an overestimation of the hydrophobicity value.

Uncharged probes may be used to overcome this problem. The most commonly used neutral lipophilic fluorescent probe is diphenyl-hexatriene (DPH), which labels the interior of membranes. Fluorescence of DPH outside membranes or non-polar solvents is low. Unfortunately, the non-polar nature of DPH restricts its solubility in aqueous systems and thus, limits its use as a probe in protein solution (Nakai and others 1996). PRODAN (6-propionyl-2-(dimethylamino)-naphthalene) is a solvent-sensitive probe and has no charge (Weber and Farris 1979). Hence, it will eliminate potential inclusion of electrostatic interaction. This electrically neutral fluorescent probe exhibits shifts in both excitation and emission spectra with changes in solvent polarity (Slavik 1994). The nondissociable nature of the PRODAN probe is an advantage in enabling investigation of the effects of changes in protein surface hydrophobicity when various pH conditions are applied (Alizadeh-Pasdar and Li-Chan 2000).

2.4.3 Rheological Properties

Rheology is defined as the study of material deformation and flow. With regard to the fluid properties of protein paste or solution, viscosity, which may be time- or shear-dependent, is the main rheological property of interest. Myofibrillar proteins exhibit a behavior between that of a purely viscous fluid and a purely elastic solid (Rao 1999). The term “viscoelastic” is used to describe this intermediate behavior. Viscoelastic material, therefore, exhibits both an elastic and viscous response to deformation.

With regard to the formation of heat-set gels by surimi or surimi-containing pastes, rheological study would include both what is termed “small strain testing” and “large-strain testing.” The former is called in case that a small percentage of a deformation required to break a sample such as dynamic test, the latter is a deformation to the point of permanent structural change such as fracture analysis. Both large- and small-strain testing may yield important information regarding the

structure of the gel formed, which in turn is determined by the chemical bonding mechanisms at work in forming the gel. Thus, rheological measurements can yield information relating to both the acceptability of product texture and the chemical mechanisms operating under the various processing conditions whereby gels are formed (Hamann and MacDonald 1992).

Rheological scanning during thermal processing has developed with the availability of the programmable thermal control. Most gelation research has been conducted using time as a continuous, independent variable while the temperature changes. Rheological characteristics of protein gels obtained from small-strain testing are described by three parameters, the loss modulus (G''), the storage modulus (G'), and the loss tangent ($\tan \delta$, the ratio of G''/G'), which are obtained from small-amplitude (strain) oscillatory shear or dynamic testing with holding frequency and strain amplitude constant while varying temperature through a gel-forming history. This measurement does not directly produce food texture data, but instead monitors the physical properties of gels at the molecular level.

Changes in gel rigidity or elasticity are circumstantial evidence that something occurred in the material over a recorded time and/or temperature increment. This change may relate to protein unfolding, bonding of molecules, gelatinization of starch when present, etc. (Hamann and MacDonald 1992). As the gel is deformed by an imposed strain, a part of the energy input is stored and recovered in each shear cycle due to the elastic response of the gel. A part of the energy is also dissipated as heat due to internal friction or viscous flow of the sample. Thus G' and G'' are measures of elasticity and viscosity of the gel samples, respectively, and $\tan \delta$ reflects the relative contribution of each to the overall rheological characteristics (Xiong 1997). A perfectly elastic material would exhibit $\delta = 0$, whereas for a perfectly viscous material $\delta = 90^\circ$. Protein gels are normally quite elastic so values of δ are near 10° . The transition from sol to gel is evident from changes in δ .

It has been generally observed that the structures that changed the material from viscous to elastic formed almost instantaneously and the modulus increased with a function of time. A muscle sol prior to cooking exhibits some elasticity but is

viscous, so a typical δ would be 45° . However, when fish myosin solution was subjected to a dynamic test, the δ was about 70° (Kim and Park 2000). Surimi pastes with low protein concentration show a cross over point at which G' equals to G'' , indicating a gel point. Surimi gels cooked at 90°C showed the same proportional change for G' and G'' , in relation to frequency, over a wide range of frequencies (Kim and Park 2000).

2.4.4 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) has emerged as the technique of choice for the study of thermal transitions in foods. Studies of protein structure and conformation by DSC can provide insight into the controlling mechanisms of protein functionality and its manipulation by changing formulation or processing conditions. The behavior of the proteins can be studied *in situ* by solubilizing the sample. This allows direct analysis of proteins in slurries and semisolid or solid states, and relatively crude sample can be examined (Ma and Harwalkar 1991).

DSC is a technique in which the difference of energy input in to a substance and a reference material, an inert material to compensate for the thermal effect linked to the sample's heating up, is measured as a function of time or temperature when the temperature of the sample is scanned in a controlled atmosphere (MacKenzie 1978). Any thermally induced changes occurring in the sample are then recorded as a different heat flow, displayed normally as a peak on a thermogram.

Parameters obtained from the DSC thermogram include the enthalpy of denaturation (ΔH), the peak of denaturation temperature (T_d), and width of the calorimetric transition at half peak height (ΔT_w). ΔH is an estimate of the thermal energy required to denature the protein and is measured from the peak area of the thermogram. T_d is a measure of the thermal stability of the proteins, and at this temperature the change in Gibbs free energy (ΔG) between the folded and unfolded protein structure is zero (Boye and others 1997).

Micro DSC is a type of high sensitivity DSC such as the Tian-Calvet type micro calorimeter. The main difference in comparison to the micro DSC technique

with a flat plate probe is that the Tian-Calvet type fluxmetric probe envelopes the sample and is therefore capable of measuring almost all the energy exchanges between the sample vessel and the calorimetric unit, a characteristic that gives this technique a clear metrological advantage in terms of both the quantity of the measurements and their sensitivity (MicroDSC III, Setaram Inc., Lyon, France).

Several researchers have studied the denaturation mechanisms and conformation changes of protein components. For example the DSC thermogram demonstrated that myosin is made up of six cooperative domains with transition temperatures ranging from 43-61°C and enthalpy values from 440-820 kJ/mol (Potekhin and others 1979). Tropomyosin exhibited a complex melting process and smaller enthalpies than myosin, but the transition was extended over a wider range of temperatures (Potekhin and Privalov 1982).

Studies on thermal behavior of proteins in different media have also been investigated. Atha and Ackers (1971) determined the enthalpy changes associated with the acid denaturation of lysozyme at a number of concentrations of guanidine hydrochloride. They demonstrated that the unfolding reaction of lysozyme was of the two-state type. In general, T_d and ΔH values decreased at extreme pH. In muscle proteins, only myosin is strongly pH dependent. As pH increases, T_d decreases (Samejima and others 1983). The T_d of actin slightly decreased at pH > 6.5 (Stabursvik and Martens 1980).

The effect of salt on the thermal behavior of proteins has been extensively studied as well. The destabilization of myosin and actin were noted when salt concentrations increased. The number of endotherms for myosin varied from one at 0.1 M KCl (Samejima and others 1983), two at an ionic strength of 0.05 M KCl (Wright and others 1977), to three at 0.6-1.0 M KCl (Wright and others 1977, Samejima and others 1983). The enthalpies and denaturation temperatures of actin and myosin extracted from chicken (Kijowski and Mast 1988) and fish (Park and Lanier 1989) decreased as a result of the addition of 1-4% salt.

2.4.5 Raman Spectroscopy

Raman spectroscopy is an old analytical technique that is recently undergoing a tremendous revival due to technological advances in lasers, detectors, and spectroscopic optical system. The 1930 Nobel Physics Laureate *Sir Chandrasekhara Venkata Raman* first demonstrated inelastic scattering of light in 1928.

Raman spectroscopy is a branch of vibrational spectroscopy that can give useful information on the vibrational motions of molecules. This technique is based on the shifts in wavelength or frequency of the exciting incident beam resulting from inelastic collisions with the sample molecules (Carey 1982). The Raman scattering effect arises from the interaction of incident light with the electrons in the illuminated molecule. In nonresonance Raman scattering, the energy of the incident light is not sufficient to excite the molecule to higher electronic level. Instead, Raman scattering results in changing the molecule from its initial vibrational state to a different vibrational state. In order for a molecule to exhibit the Raman effect, the incident light must induce a dipole-moment change or a change in molecular polarizability. An electron with certain vibrational modes can couple with the incident-light photon and lead to scattered photons with altered frequency (Tu 1982).

Of other available techniques, circular dichroism is another powerful physical method for the study of protein structure, especially for the peptide backbone. However, this technique does not provide much structural information and is not suitable for direct spectroscopy measurements of samples in the solid state (Compton and Johnson 1986). Fluorescence spectroscopy is another analytical tool for studying proteins, but this method is incapable of determining peptide backbone structure and is also limited to the study of the environments of tryptophan and tyrosine side chains (Mantulin and Pownall 1986). By contrast, Raman spectroscopy can be a useful tool to probe protein structure in solid and liquid food systems.

Bands in Raman arising from amide I, amide III, and skeletal stretching modes of peptides and proteins are useful for characterizing backbone conformation, including the estimation of secondary structure fractions. Bands attributed to various stretching or bending of vibrational modes of functional groups of amino acid residues

can be used to monitor the environment around these side chains. In particular, valuable information may be obtained on SS or SH groups of cystinyl or cysteinyl residues, CH groups of aliphatic residues, and aromatic rings of tryptophanyl, tyrosinyl, and phenylalanyl residues.

One important parameter distinguishing Raman spectroscopy from many other spectroscopic methods is its applicability to systems containing high concentrations of protein, which is critical for the investigation of structural changes during processes such as coagulum or gel formation. Thus, changes in both intramolecular and intermolecular interactions can be studied. Raman spectroscopy can also be applied to investigate protein structure as a function of processing, such as pH change, salt concentration, heating, or drying, which is important to correlate with protein functionality in food systems (Li-Chan and others 1994).

Several studies have been conducted using the Raman spectroscopic technique to elucidate protein structures in muscle systems. Barrett and others (1978) reported that the Ca^{2+} ion affects an α -helix to β -sheet transition in myosin, while LiBr appeared to denature the protein, resulting in increased random coil structure. MgCl_2 had an effect intermediate between the other two salts. They speculated that an increase in β -structure would mean an increase in interchain hydrogen bonding, which is compatible with the observation that 0.1 mM Ca^{2+} induced reversible aggregation of myosin heads.

Denaturation of bovine serum albumin (BSA) by heating, acid, and alkali treatment was investigated by Raman spectroscopy (Lin and Koenig 1976). Heating to 70 °C, or changes in the pH of the solution to below pH 5 or above pH 10, caused gradual intensification of the 1246 cm^{-1} band relative to 1337 cm^{-1} . The Raman spectrum of BSA gel held at 90 °C for 30 min showed an intense line at 1240 cm^{-1} . These results were interpreted as unfolding of α -helices upon denaturation, which were observed as decreased intensity at 938 cm^{-1} , and accompanied by an increase in disordered structure reflected in the 1246 cm^{-1} band. These changes were reversible at temperature up to about 50 °C as well as for acid or alkali induced denaturation between pH 1.72 and 10.9. At high temperature that led to aggregation and gel

formation, β -structures were formed as reflected by the appearance of a band at 1240 cm^{-1} .

CHAPTER 3

ROLE OF pH IN SOLUBILITY AND CONFORMATIONAL CHANGES OF PACIFIC WHITING MUSCLE PROTEINS

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3.1 ABSTRACT

This study was conducted to better understand biochemical changes of fish muscle proteins as affected by novel surimi process, acid- or alkali-aided solubilization. At 10 mM NaCl, between pH 5 and 10, the solubility of Pacific whiting muscle proteins was low but increased dramatically as the pH was shifted to either acidic or alkaline pH. At 600 mM NaCl, the isoelectric point was shifted to the acidic direction by about 2 pH units, resulting in aggregation of proteins at low pH, but improving the solubility of MHC (myosin heavy chain) between pH 6 and 10. ANS surface hydrophobicity (ANS- S_o) showed much greater values than PRODAN surface hydrophobicity (PRODAN- S_o) for samples treated at pH 2 - 4 perhaps due to an enhancement of the electrostatic interactions between the ANS probe and proteins. At very high pH, according to hydrophobicity results, proteins were partially refolded when the ionic strength increased. Under acidic conditions, SDS-PAGE demonstrated the degradation of MHC at 10 mM NaCl. The formation of MHC polymers was observed under alkaline treatment with a concomitant decrease of SH content.

Keywords: Fish protein, pH, solubility, surface hydrophobicity, SDS-PAGE

3.2 INTRODUCTION

Pacific whiting (*Merluccius productus*) is a major fishery resource of the Pacific Northwest and is primarily used for surimi manufacturing due to its abundance and competitive price (Lin and Park 1996). Conventional surimi processing, which requires extensive washing and dewatering, yields around 25% of whole fish. However, acid-aided processing, which solubilizes all proteins and subsequently recovers them, could give a theoretically achievable maximum yield. The acid solubilization process with Pacific whiting yielded 20% more than the conventional 3-washing process (Choi and Park 2002). However, low breaking force and deformation values of the gels were reported, probably due to acid-activated proteolytic enzymes presenting in Pacific whiting. Yongsawatdigul and Park (2004) investigated the recovery of rockfish proteins at various pH conditions. They found that gels prepared from rockfish proteins solubilized at alkaline pH (10-11) and subsequently recovered at pH 5.0, exhibited higher gel quality than those prepared from acid solubilization and the conventional washing process, respectively.

In general, the functionality of proteins is highly dependent on protein solubility, in order to provide good emulsion, foam, gelation, and whipping properties (Vojdani 1996). Several studies have been recently conducted regarding the significant effect of pH on protein solubility (Steffansson and Hultin 1994; Monahan and others 1995; Dagher and others 2000; Yongsawatdigul and Park 2004). In addition, the ionic strength of the environment of the proteins also has considerable influence on protein solubility. Based on conventional muscle chemistry, the formation of fish gels requires about 1.7-3.5% sodium chloride to solubilize the myofibrillar proteins (Suzuki 1981; Lee 1984). However, Steffansson and Hultin (1994) reported that cod myofibrillar proteins could be solubilized at low ionic strength at both neutral and acidic pH. The results showed that the reduction of ionic strength from 0.001 to 0.0002 induced a significant increase in protein solubility from 15% to greater than 90%, while the reduction of pH from neutral to 5.5 decreased protein solubility. In addition, the study of Dagher and others (2000) indicated that at low ionic strength (10 mM), the solubility of cod muscle proteins increased

considerably between pH 8.9 and 9.2, while at ionic strength of 430 mM, the solubility was high and remained unchanged over the pH range from 7 to 9.5.

Feng and Hultin (1997) observed that greater than 90% of mackerel proteins were soluble at physiological ionic strength or lower. They also found that it was necessary to remove certain proteins (M-protein, α -actinin, and desmin), which are known to be possible solubility inhibiting (PSI) polymers at moderate ionic strength and neutral pH, before extracting the remaining myofibrillar and cytoskeletal proteins in water.

Since the solubilization of myofibrillar proteins is known to be a prerequisite for gel formation, it is necessary to determine how pH and/or ionic strength affect the solubility and functional properties of the proteins. In response to the novel surimi process, where functional protein isolates are obtained using acid or alkaline solubilization of the proteins followed by isoelectric precipitation, a better understanding of the solubility of Pacific whiting muscle proteins prepared from various pH and ionic strengths including their biochemical properties and gelation properties is required. As a result, a new process for better utilization of enzyme-rich Pacific whiting could be developed. Our objectives were to evaluate the biochemical characteristics of Pacific whiting muscle proteins soluble at various pH conditions and at low and high ionic strength levels, as well as to further investigate the conformational changes and protein patterns.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Pacific whiting (*Merluccius productus*) was obtained from Pacific Surimi (Warrenton, OR). Fresh whole fish, less than 24 h post-harvest, were transported on ice to the OSU Seafood Laboratory within 30 min. Fish were then filleted, ground using a grinder (Hobart, The Hobart Manufacturing Co., Troy, OH), mixed thoroughly with sorbitol (Neosorb 20/60, Roquette American Inc., Gurnee, IL) at 10%

concentration, vacuum-packed in small portions (~ 50 g), and stored at -80°C until used.

3.3.2 Sample Preparation

Frozen Pacific whiting (PW) mince was thawed at 5°C for 2 h and washed (mince:water = 1:5) twice with cold (5°C) deionized water to remove sorbitol. Mince was then homogenized (Power Gen 700, GLH 115, Fisher Scientific Inc., Pittsburgh, PA) with 5 mM histidine buffer for 1 min at speed level 3. The mixing ratios of mince to buffer, which were determined based on the preliminary experiments to maintain both low and high ionic strength conditions as described below, were 1: 19 for pH 5, 6, 7, 8, and 9; 1:39 for pH 4 and 10; 1:59 for pH 11; and 1:149 (w/v) for pH 2, 3, and 12. The pH of the homogenized samples was adjusted using 0.5 and/or 1 N cold HCl and NaOH. The pH measurement was conducted using a pH meter (Accumet Research AR 15, Fisher Scientific, Inc., Pittsburgh, PA).

The effects of various pH were determined at two levels of ionic strengths (IS): 10 mM and 600 mM NaCl. The former is the minimum concentration, which could be controlled after pH adjustment of twice-washed Pacific whiting mince and the latter is equivalent to salt concentration typically used for myosin extraction. Once the pH was fixed, the desired ionic strength was adjusted by adding sodium chloride granules. The change of pH was noted as NaCl was added to the suspensions. At 600 mM, the final pH levels after adjustment of ionic strength were 1.85, 2.91, 4.5, 5.3, 6.02, 6.96, 7.85, 8.8, 9.85, 10.77, and 11.91, which corresponded to pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 in this study, respectively. However, at 10 mM, the final pH levels at 4, 5, 6, 7, 8, 9, 10, and 11 were insignificantly changed with the following exceptions. At extreme pH conditions (2, 3, and 12), it was impossible to control IS at 10 mM due to the arising of IS by the addition of HCl or NaOH. Therefore, the corresponding IS was finalized at 72, 12.5, and 32 mM, respectively. Protein suspensions were kept at low temperature ($4 - 5^{\circ}\text{C}$) while pH and IS were adjusted.

The ionic strength of the protein suspensions was measured using a conductivity meter (YSI 3100, YSI Inc., Yellow Spring, OH) equipped with a

conductivity cell (cell constant 1.0 cm^{-1}). Conductivity readings were obtained based on the standard curve prepared using sodium chloride (reagent grade, Sigma Chemical, Co., St. Louis, MO) in a concentration range between 0 and 600 mM.

The homogenate samples prepared above were centrifuged at $19,950 \times g$ for 30 min at less than 5°C (Sorvall RC-5B, DuPont Co., Newtown, CT). The supernatants were analyzed for protein content, total sulfhydryl (SH) content, surface hydrophobicity (S_o), and changes of protein patterns by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

3.3.3 Protein Solubility

The supernatant was diluted appropriately using 5 mM histidine buffer. The buffer was adjusted to each pH and IS treatment prior to mixing with the sample. No significant change of pH was observed when the sample was mixed with buffer. Protein concentration was determined using the Bradford dye binding method (Bradford 1976). The absorbance was measured at 595 nm (UV-VIS Spectrophotometer; UV 2401PC, Shimadzu Corp., Kyoto, Japan) using bovine serum albumin as the standard.

3.3.4 Surface Hydrophobicity

Protein surface hydrophobicity of the supernatant was determined using two common probes, ANS (1-anilinonaphthalene-8-sulfonic acid) and PRODAN (6-propionyl-2-(dimethylamino)-naphthalene), according to the method of Alizadeh-Pasdar and Li-Chan (2000). ANS and PRODAN probes were used to analyze the conformational changes of the hydrophobic regions on the protein molecules. ANS is a charged hydrophobic dye with negative sulfonate anions at $\text{pH} \leq 3.6$ and can interact through hydrophobic as well as electrostatic interactions. In contrast to ANS, PRODAN is an uncharged hydrophobic probe, which will eliminate possible electrostatic interactions contributed by the environment (Alizadeh-Pasdar and Li-Chan 2000).

Stock solution of 8×10^{-3} M ANS was prepared in 0.1 M phosphate buffer (pH 7.4), while 1.40×10^{-3} M PRODAN solution was prepared in methanol. The excitation/emission slits were set at 5 nm each for both measurements using Luminescence Spectrometer LS 50 B (Perkin Elmer, Norwalk, CT). The excitation/emission wavelengths of 390 nm/470 nm and 365 nm/465 nm were used for ANS and PRODAN, respectively. The protein concentration of the supernatant was adjusted to 0.063, 0.125, 0.25, and 0.50 mg/mL using 5 mM histidine buffer. The buffer was adjusted to each pH and IS treatment before making the serial dilution. However samples at pH 2, 3, and 4 were diluted at lower concentrations (≤ 0.10 mg/mL) to obtain a linear initial slope with the correlation coefficient ($r^2 \geq 0.99$). Four milliliters of each sample were added into 20 μ L of ANS stock solution and 10 μ L of PRODAN stock solution, respectively. After holding for 15 min for PRODAN (in the dark) and 10 min for ANS, the relative fluorescence intensity (RFI) of each solution was measured. The initial slope (S_0) of the net RFI versus protein concentration was calculated by linear regression analysis and used as an index of the protein surface hydrophobicity.

3.3.5 Total Sulphydryl Content

Total sulphydryl group content was determined using Ellman's reagent (5, 5'-dithiobis (2-nitrobenzoic acid); DTNB) as described by Hamada and others (1994) with a slight modification. The supernatant was diluted to 0.5 mg/mL protein concentration with 5 mM histidine buffer. The pH and IS of histidine buffer were adjusted to the desired values for each treatment before mixing with the sample. The diluted sample (0.5 mL) was mixed with 2 mL of 8 M urea in 0.2 M Tris-HCl buffer (pH 7.0) and then with 50 μ L of 10 mM DTNB solution. The sample was incubated at 40 °C for 15 min before measuring the absorbance at 412 nm. The SH content was calculated based on absorbance using the molar extinction coefficient of $13,600 \text{ M}^{-1} \text{ cm}^{-1}$ (Riddles and others 1979).

3.3.6 SDS-PAGE

The supernatants prepared from the various treatments were subjected to SDS-PAGE to compare protein patterns according to the method of Laemmli (1970). The protein concentration was determined using the method of Lowry and others (1951). The supernatant (500 μ L) was mixed with 125 μ L of 5x sample buffer containing 60 mM Tris-HCl buffer (pH 6.8), 25% glycerol, 2% SDS, 10% β -mercaptoethanol, and 0.1% bromophenol blue. Samples were then heated in boiling water for 5 min and centrifuged at 8,000 x g for 5 min. SDS-PAGE was performed in 4% (stacking) and 10% (separating) polyacrylamide gels. The gel was then stained in a staining solution containing 0.1% Coomassie blue R-250, 45% methanol, and 10% acetic acid. Destaining was conducted using destaining solution (methanol: glacial acetic acid: water = 1:1:8 (v/v/v)).

To determine the polymerization of myosin heavy chain (MHC), samples prepared with 5x sample buffer with and without β -mercaptoethanol were compared. Molecular masses were estimated using high molecular weight markers ranging from 29-205 kDa and wide range MW markers ranging from 6.5–205 kDa (Sigma Chemicals Co., St. Louis, MO). SigmaGel™ software (SPSS Science, Chicago, IL) was used to estimate the molecular weights of protein bands.

3.3.7 Statistical Analysis

Data were analyzed using an analysis of variance (ANOVA) procedure. A general linear model was applied with further analysis using Tukey's test to determine differences between treatment means (SPSS for Windows, version 10.0, SPSS Inc., Chicago, IL). Due to the range in magnitude of data obtained, logarithmic transformation of the surface hydrophobicity (S_o) values was performed prior to statistical analysis.

3.4 RESULTS AND DISCUSSION

3.4.1 Protein Solubility

At 10 mM NaCl, Pacific whiting muscle protein was less soluble between pH 5 and 10 (Fig. 3.1). However, muscle proteins were highly soluble at either extremely acidic or alkaline pH. Solubility increased rapidly as the pH shifted either from 5 to 4 (4.3 to 99.5 mg/g) or from 10 to 11 (41.3 to 99.7 mg/g). Protein solubility reached a maximum value at pH 2 and 12 with corresponding IS at 72 and 32 mM NaCl, respectively. Similar results were also reported at various pH conditions without controlling IS for other fish species: cod myofibrillar proteins (Steffansson and Hultin 1994), salmon myosin (Lin and Park 1998), rockfish (Yongsawatdigul and Park 2004), Pacific whiting (Choi and Park 2002), and Alaska pollock and mackerel (Song and others 2002).

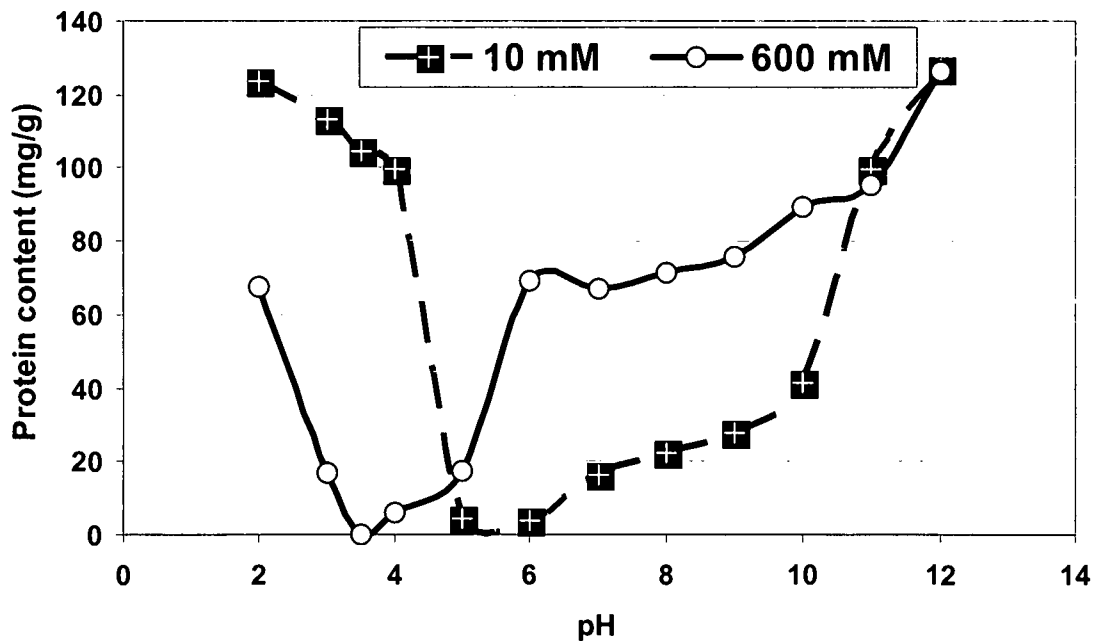


Figure 3.1 Solubility of Pacific whiting muscle protein prepared at various pH and two IS levels. For pH 2, 3, and 12, IS was adjusted to 72, 12.5 and 32 mM NaCl instead of 10 mM due to technical difficulties, respectively.

At pH values far from the isoelectric point (pI around pH 5.5), proteins carry a higher net positive or negative charge, resulting in electrostatic repulsion and hydration (Hamm 1960). In addition, low salt concentration (10 mM) can enhance the solubility by a salting-in effect. The salt ions interact with oppositely charged groups on the protein to form a double layer of ionic groups, which decreases electrostatic interactions between protein molecules and thereby causes more protein solubilization (Vojdani 1996). The solubility at alkaline pH gradually increased as the pH shifted up to 10 and then a dramatic increase was observed from pH 10 to 11 (Fig. 3.1).

At high IS (600 mM) and neutral pH, protein solubility significantly improved, compared to low IS (10 mM) (Fig. 3.1). The effect of ionic strength on protein solubility was clearly differentiated between pH 6 and 10. Solubility increased around 17 times at pH 6 while IS increased from 10 to 600 mM NaCl. The rate of increase in solubility gradually decreased to 2 times as pH increased to 10. However, for samples treated at pH 11 and 12, salt addition did not enhance solubility. At 600 mM NaCl, shifting the pH from 6 to the acidic side dramatically decreased solubility. But when the pH decreased beyond 3.5, solubility increased. In contrast, as the pH was shifted from 7 to the alkaline side, solubility increased gradually and reached a maximum at pH 12.

High salt concentration caused a shift of pI of the Pacific whiting proteins by about 2 pH units towards the acidic side. Sakar (1950) also reported that at 430 mM of potassium chloride, the pI of rabbit myosin shifted to pH 4. At 2 M NaCl concentration, cowpea protein isolate showed a decrease of protein solubility when the pH decreased from 8 to 3 (Aluko and Yada 1997). Ockerman (1980) proposed that when NaCl is added, the chloride ion binds with the positively charged amino acids to a stronger degree than the sodium ion reacts with the negative charges, which resulted in more hydrogen ions and thereby decreased the pH.

As salt concentration increased to 600 mM, some proteins started to aggregate even between pH 2 and 4. This was thought possibly due to a salting-out effect. The high concentration of cations and anions contributed by salt may reduce the effect of protein charges and destabilize the hydrophilic surface, causing the exposed

hydrophobic areas of the proteins to interact leading to aggregation (von Hippel and Schleich 1969). These results were in accordance with those obtained from SDS-PAGE analysis (Fig. 3.5). No myosin heavy chain (MHC) bands appeared at low pH (2 to 5) and 600 mM NaCl.

3.4.2 Surface Hydrophobicity (S_o)

The binding of uncharged PRODAN or ANS anions to PW proteins between pH 5 and 12 was enhanced at the higher IS (Figs. 3.2 and 3.3). As a result, hydrophobic interactions were strengthened through hydrophobic and/or van der Waals interactions (von Hippel and Schleich 1969; Nakai and Li-Chan 1988). In Figure 3.2, the ANS results showed that, at low IS, S_o values at pH 2 to 5 were higher than those at neutral and alkaline pH. This was probably due to the fact that below the isoelectric point of the fish protein, the protein molecules have a net positive charge while ANS has a net negative charge. Therefore, the binding of ANS to the protein may not only reflect hydrophobic interactions but also binding in a hydrophobic site that is strengthened by electrostatic interactions between the probe and protein (Haskard and Li-Chan 1998).

According to Matulis and Lovrien (1998), the ANS anion can bind dominantly and strongly to cationic groups of water-soluble proteins and polyamino acids through ion-pair formation. If the hydrophobic regions of a protein are adjacent to charged side chain residues, the binding of ionic probes with the protein may be perturbed by electrostatic effects in addition to hydrophobic interactions (Greene 1984). Folawiyo and Owusu Apenten (1997) reported that exposure of rapeseed albumin to low pH solvents (pH 1-2) led to around 10-fold increase in protein surface hydrophobicity (ANS- S_o) compared to native protein. Our result also showed that the same degree of the increase in ANS- S_o value was observed when pH decreased from 7 to 2. Alizadeh-Pasdar and Li-Chan (2000) observed higher ANS- S_o values for whey protein isolate, β -lactoglobulin, and bovine serum albumin at pH 3 than at other pH. The highest ANS- S_o values of β -lactoglobulin at pH 3 and 2.8 were also reported by Shimizu and others (1985) and by Das and Kinsella (1989), respectively.

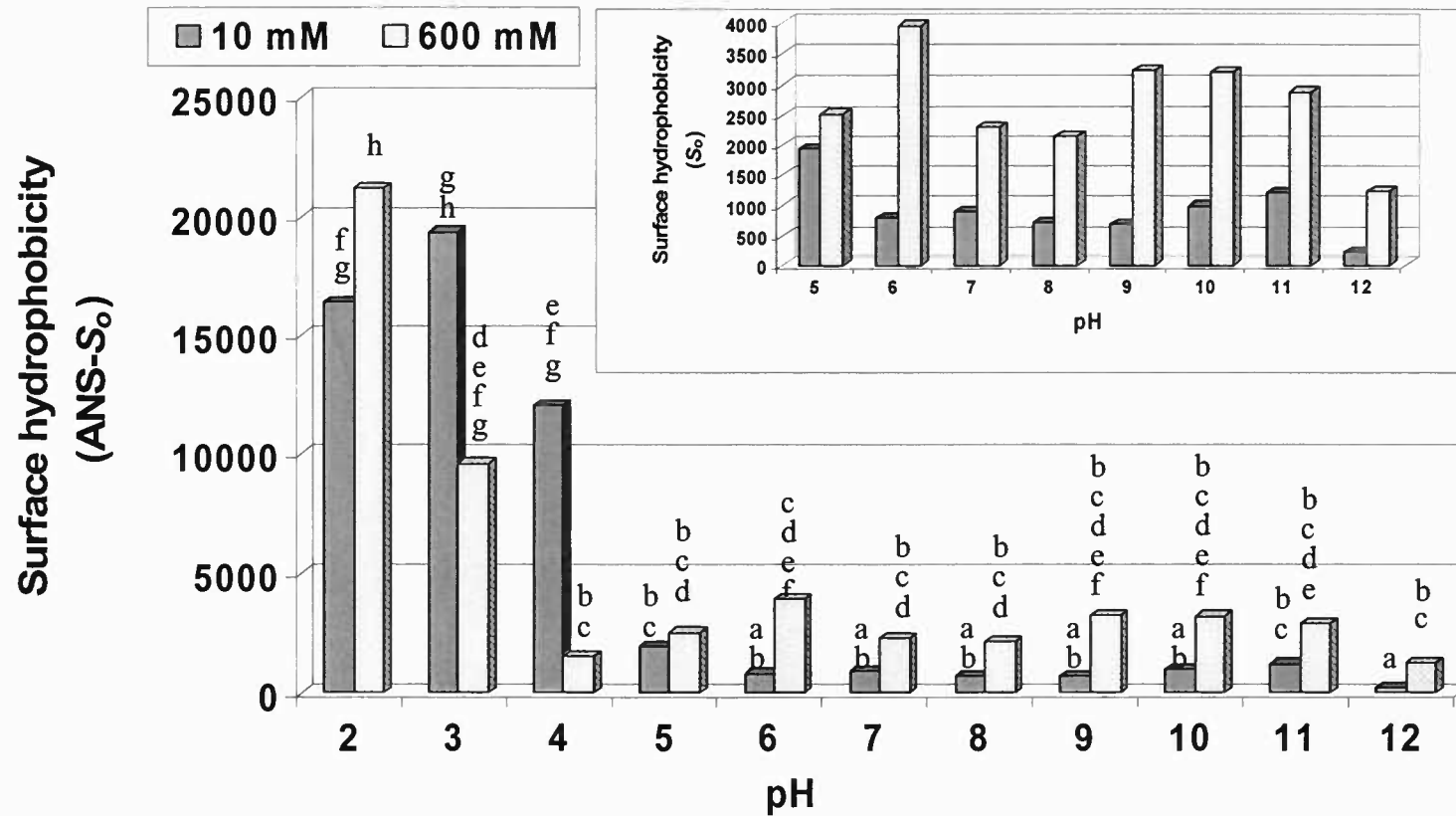


Figure 3.2 Surface hydrophobicity of Pacific whiting muscle proteins using ANS probe analysis. An insert enlarges S_0 between pH 5 to 12. Different letters on each bar represent significant differences ($p \leq 0.05$). For pH 2, 3, and 12, IS was adjusted to 72, 12.5 and 32 mM NaCl instead of 10 mM due to technical difficulties, respectively.

Lower S_o values were obtained using ANS than PRODAN when low IS and high pH (greater than 6) were applied (Figs. 3.2 and 3.3), suggesting that the negative charges on PW muscle proteins and the ANS anion may have resulted in a repulsive force between them. Conversely, an increase in IS to the higher IS level (600 mM NaCl) demonstrated a greater value for ANS- S_o than PRODAN- S_o . The salt ions probably enhanced the binding of ANS anion and proteins. ANS and PRODAN probes yielded the same trend of S_o values for samples treated at pH between 6 and 12. However, the opposite results were observed for samples at pH 2 and 3 (Figs. 3.2 and 3.3). Our results were in agreement with the study of Alizadeh-Pasdar and Li-Chan (2000) for whey protein isolate. Li-Chan (2002) has also noted that the fluorescent intensity of PRODAN is somewhat lower at acidic pH than at neutral or alkaline pH: this effect is usually in the order of two folds or so. Therefore, the changes in measured intensity greater than 2- or 3-fold might be attributed to the changes in the protein itself, not the inherent, lower fluorescence of PRODAN at acidic pH.

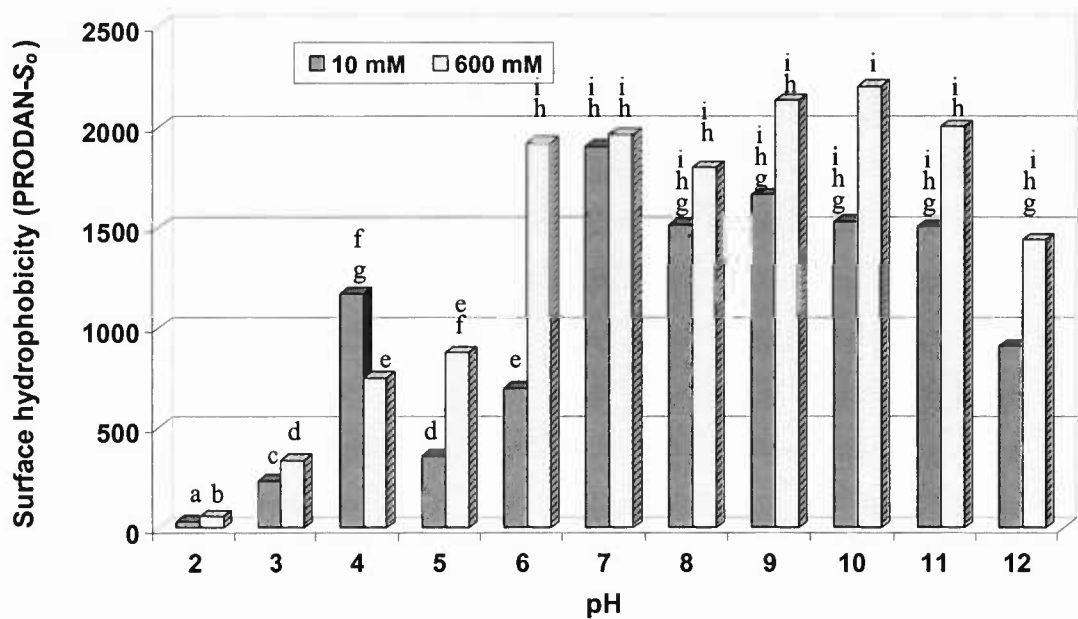


Figure 3.3 Surface hydrophobicity of Pacific whiting muscle proteins using PRODAN probe analysis. Different letters on each bar represent significant differences ($p \leq 0.05$). For pH 2, 3, and 12, IS was adjusted to 72, 12.5 and 32 mM NaCl instead of 10 mM due to technical difficulties, respectively.

Several studies have been reported that salt can induce the transition from an acid-unfolded protein to a molten globule state (partially folded form) for several types of proteins (Arakawa and Timasheff 1982a; Goto and Fink 1989; Goto and others 1993; Makhataze and others 1998). At extremely low or high pH, the presence of counter ions around the charged groups can weaken these repulsions and thus, permit other forces including hydrophobic interactions favoring folding to become relatively strengthened (Goto and Fink 1989, Goto and others 1993). This probably explains the lower values of PRODAN- S_o at pH 2 and 3 or both ANS- and PRODAN- S_o at pH 12.

At extreme alkali condition (especially pH 12) and low IS, the S_o values were significantly low ($p \leq 0.05$) for both measurements. Under these conditions, the molecule unfolds due to electrostatic repulsions and, therefore, S_o should have increased. When adding salt, however, alkali-induced unfolding might have been followed by refolding into a molten globule state (Goto and Fink 1989). In addition, hydrophobic interactions and/or SH/SS interchange reactions probably played an important role for the transition from the unfolded to the folded state as evidenced by S_o and SH content. In Figure 3.4, the results showed that at pH 12, the SH contents were lowest at both IS levels (32 and 600 mM). It is speculated that SH groups on the protein molecules underwent an oxidation reaction, resulting in protein cross-linking via disulfide bonds. The depletion of SH content was also possibly due to lysinoalanine (LAL) cross-links, which were discussed more in SH content result. In contrast to S_o values and SH content, the solubility of proteins at pH 12 was very high (Fig. 3.1). Despite the lower S_o values and SH content, the dissociation of myosin into monomers under extremely alkaline pH and/or high IS probably resulted in high protein solubility. According to the literature (Godfrey and Harrington 1970a, b; Weeds and Pope 1977; Kaminer and Bell 1966), at a concentration of KCl lower than 0.3 M, myosin exists primarily as filaments. When the KCl concentration increased over 0.3 M and the pH value was greater than 7.0, dissociation of myosin occurred. In addition, the high electrostatic repulsions enhanced the solubility while the pH was far away from the pI and/or salt was applied.

3.4.3 SH Content

Between pH 5 and 7, the SH content at 10 mM was significantly higher than at 600 mM (Fig. 3.4). This is probably due to the fact that the protein compositions in supernatant fractions, especially MHC, were significantly different between the two IS levels (Figs. 3.5 and 3.6). In addition, there was no influence of acidic or alkaline environment on the reaction of the SH groups on the protein molecules.

Under alkaline conditions at both IS levels, the SH content significantly decreased as pH increased. This result perhaps indicated that SH groups became more susceptible to oxidation at alkaline pH resulting in the formation of disulfide bonds. In addition, lysinoalanine (LAL) cross-links might have taken into account in decreased SH content. Cysteine was found to be the most sensitive to alkaline and contributed mostly to the formation of LAL (Friedman and Masters 1982; Chang and others 1999). LAL is a major cross-link commonly presenting in alkali- and/or heat-treated proteins. The higher the pH, the greater is the extent of LAL formation (Maga 1984; Damodaran 1996). The decrease in SH content in alkaline conditions

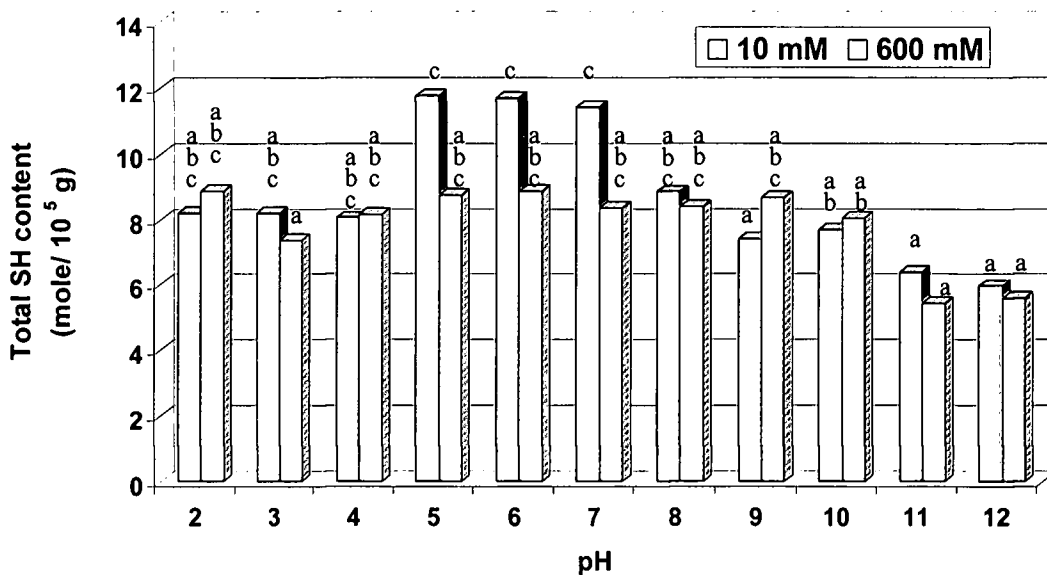


Figure 3.4 Total SH content of Pacific whiting muscle proteins. Different letters on each bar represent significant differences ($p \leq 0.05$). For pH 2, 3, and 12, IS was adjusted to 72, 12.5 and 32 mM NaCl instead of 10 mM due to technical difficulties, respectively.

was also in accordance with the formation of high molecular polymers as illustrated by SDS-PAGE (Fig. 3.7).

At pH 9 and 11, polymerization of whey proteins by intermolecular disulfide bonding occurred at room temperature, while at pH 3, 5, and 7, polymerization was observed only at higher temperatures (Monahan and others 1995). At the higher pH values, thiol oxidation reactions between exposed $-SH$ or S^- groups can occur in addition to disulfide interchange reaction (Watanabe and Klostemeyer 1976). Alkaline treatment may, therefore, be an effective way to enhance disulfide-mediated polymerization reactions for manufacturing protein gels.

3.4.4 SDS-PAGE

The protein compositions in supernatant fractions were determined using SDS-PAGE. Figure 3.5 illustrates the protein patterns of samples treated at low pH. SDS-PAGE at 10 mM NaCl showed the degradation of myosin heavy chain (MHC) at acidic pH, especially at pH 3.5 and 4. This result might be due to acid hydrolysis and/or proteolytic enzyme activity. An and others (1994) reported that 97% of the proteolytic activity in Pacific whiting was due to cysteine proteases. The optimum pH for cathepsin B, D, H, and L are 5.0-6.0, 3.0-4.5, 5.0, and 3.0-6.5, respectively (Kang and Lanier 2000). Myosin heavy chain was most extensively hydrolyzed at 55 °C resulting in low MW hydrolysis products between 67 – 145 kDa, as well as molecules with 29, 36, and 45 kDa (An and others 1994). Actin was degraded very slowly compared to MHC.

At pH 5 and 6, which are close to the pI, only small molecular weight polypeptides were solubilized at 10 mM NaCl. From pH 2 to 5, when the IS increased to 600 mM, no MHC bands appeared in the samples probably due to the salting-out effect resulting in protein precipitation. Between pH 6 and 10, proteins were more soluble at 600 mM than at 10 mM coinciding with more intensive MHC bands (Fig. 3.1, 3.5, and 3.6). It indicated that more MHC, compared to other smaller molecular weight (MW) proteins, was soluble at 600 mM NaCl than at low IS condition. On the other hand, at 10 mM NaCl, more small MW proteins (33-<45 kDa and 55-100 kDa)

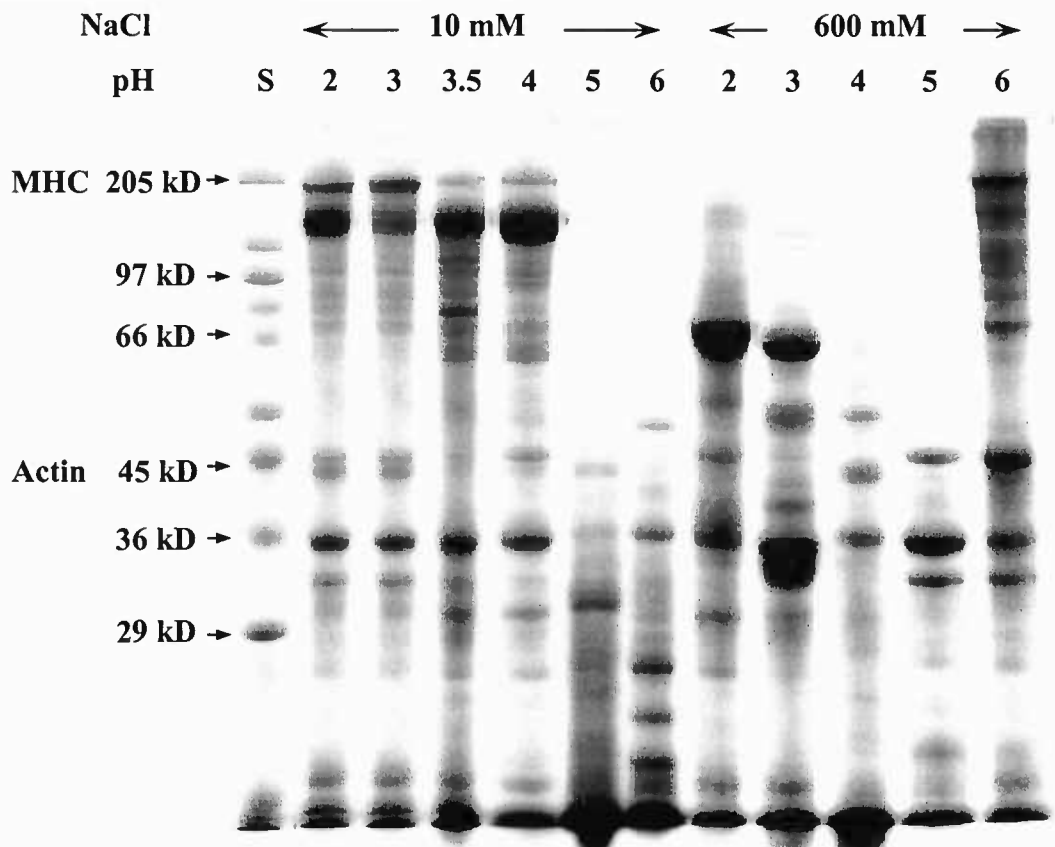


Figure 3.5 SDS-PAGE patterns of Pacific whiting muscle proteins at pH 2 to 6. An equal quantity (25 μ g of proteins) was applied per each well on the 10% acrylamide separating gel. S: standard wide range MW markers ; MHC: myosin heavy chain. For pH 2, 3, and 12, IS was adjusted to 72, 12.5 and 32 mM NaCl instead of 10 mM due to technical difficulties, respectively.

were soluble than at 600 mM NaCl.

The formation of the polymers, as evidenced by the presence of larger molecules (>205 kDa), was observed in samples treated under neutral and alkaline conditions. At 10 mM NaCl, polymer bands were clearly observed only at pH 11 and 12, however, there was no MHC solubilized in samples treated at pH 7 to 9 (Fig. 3.6). At 600 mM NaCl, high molecular weight bands (>205 kDa) and no MHC bands appeared in samples treated without β -mercaptoethanol at pH 7 to 12 (Fig. 3.7). However, samples with β -mercaptoethanol clearly showed the presence of MHC. This indicated that MHC formed these high molecular weight polymers via disulfide

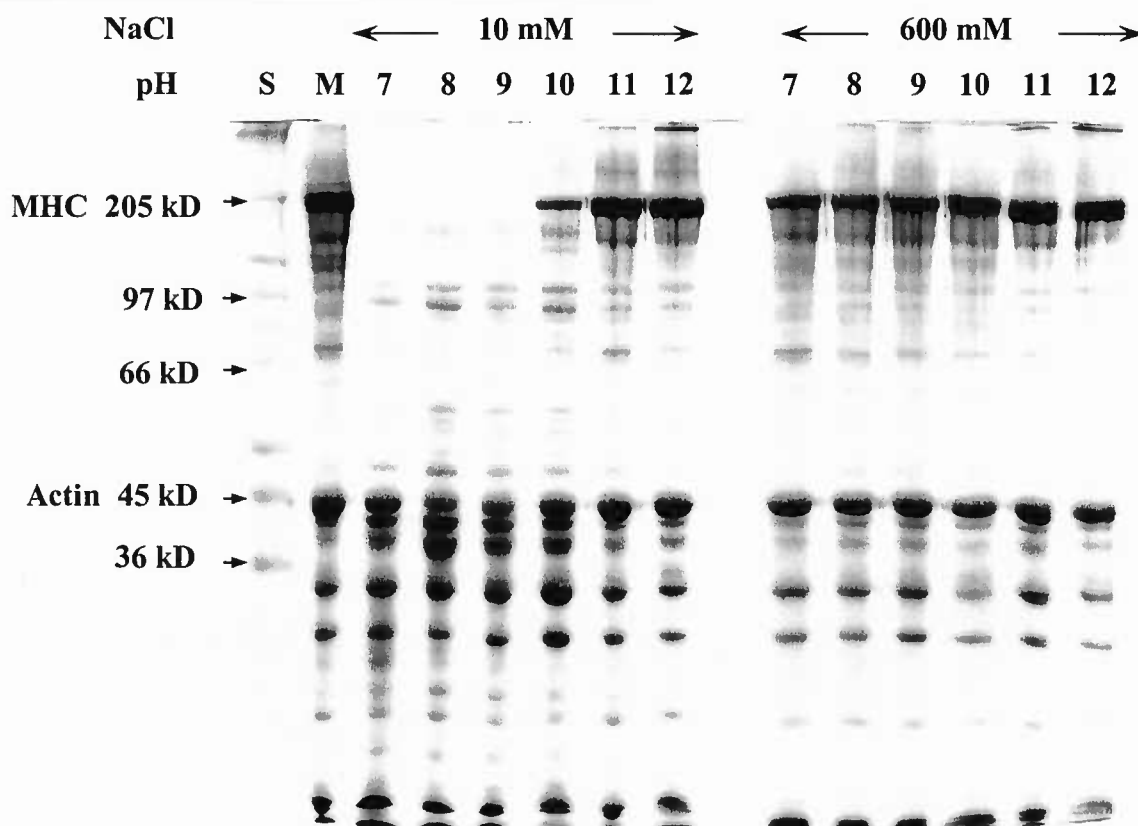


Figure 3.6 SDS-PAGE patterns of Pacific whiting protein at pH 7 to 12. An equal quantity (20 μ g of proteins) was applied per each well on the 10% acrylamide separating gel. S: standard high MW markers; M: whole PW muscle proteins; MHC: myosin heavy chain. For pH 2, 3, and 12, IS was adjusted to 72, 12.5 and 32 mM NaCl instead of 10 mM due to technical difficulties, respectively.

bonds. The presence of polymers even after treating the sample with β -mercaptoethanol in Figure 3.7 was possibly due to the fact that Pacific whiting muscle proteins contain transglutaminase (TGase) enzyme (Peters and others 1995), which catalyzes the polymerization and cross-linking of proteins through the formation of nondisulfide covalent bonds (epsilon-(gamma-Glu) Lys linkages) between protein molecules. Lysinoalanine (LAL) cross-links may also be considered to attribute to these polymers. Sodium dodecyl sulfate gel electrophoresis of the alkali-treated lysozyme indicated intermolecular cross-linking as well as the hydrolysis of one bond

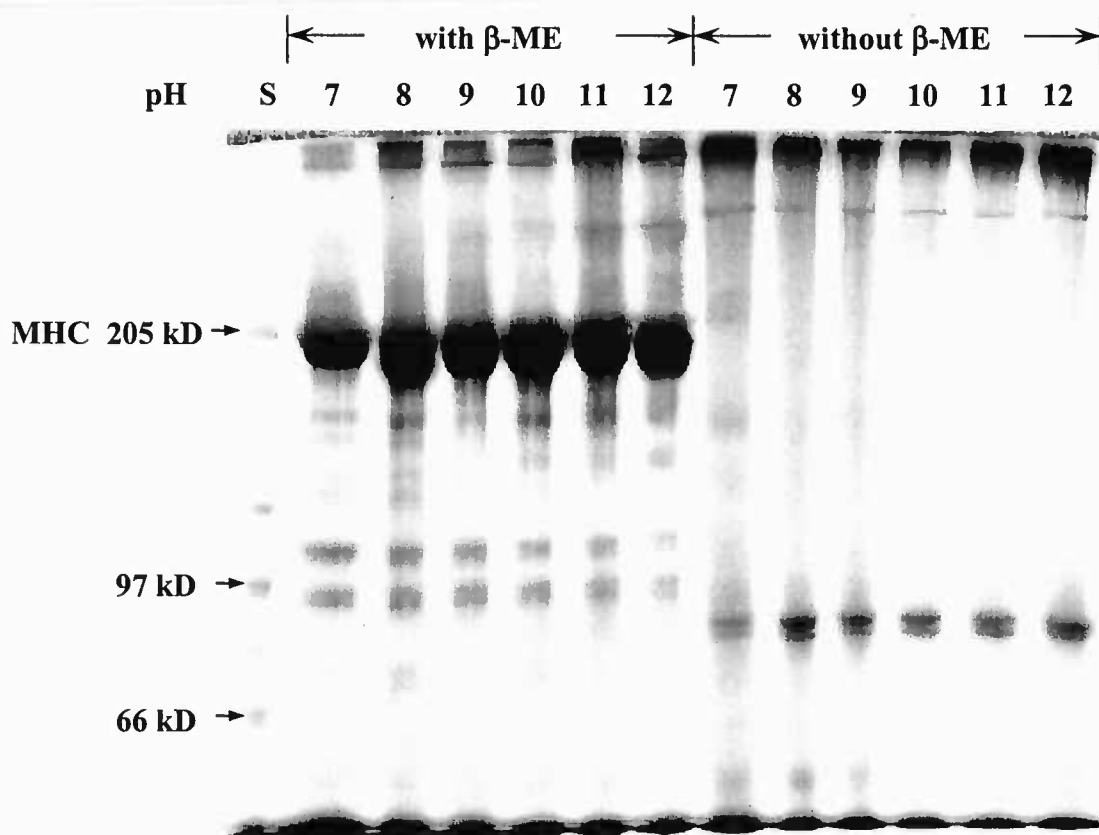


Figure 3.7 SDS-PAGE patterns of Pacific whiting protein at pH 7 to 12 and 600 mM NaCl, with and without β -ME. An equal quantity (25 μ g of proteins) was applied per each well on the 6 % acrylamide separating gel. S: standard high MW markers; MHC: myosin heavy chain.

resulting in a small peptide (Nashef and others 1977). However, Pellegrino and others (1998) have shown that LAL is responsible for intramolecular but not intermolecular cross-linking in casein. Thus, further studies would be needed to investigate roles of LAL in alkali-treated protein polymerization.

When pH increased, the concentration of high molecular weight polymer, as evidenced by the intensity, also increased. These results also correspond to the total SH content analysis as previously described. Similar results were observed in whey proteins treated at various pH conditions (Monahan and others 1995). The high molecular weight protein bands appeared in unheated solutions at pH 11, but at lower

pH they showed up only when solutions were heated. The propensity of whey proteins to form polymers was in the descending order of pH: 11 > 9 > 7 > 5 > 3.

3.5 CONCLUSION

In conclusion , the pH and ionic strength have a significant effect on protein solubility and protein conformation, respectively, particularly MHC. At 10 mM NaCl, shifting the pH away from the pI to either acidic or alkaline side increased solubility. Addition of NaCl to obtain IS 600 mM shifted the pI of fish muscle proteins by about 2 pH units towards the acidic side. In addition, an increase of IS induced the insoluble protein aggregates at low pH, and partial refolding of the protein at very high pH. Different protein patterns were obtained between acid and alkali-treated proteins. The formation of high molecular weight polymers from MHC in the alkali-treated proteins was thought due to disulfide interactions. However, degradation of MHC was noticed under acidic conditions. It is, therefore, possible that this information could help us to better understand the protein characteristics underlying fish proteins in the novel surimi process using the pH shift method.

CHAPTER 4

ROLE OF IONIC STRENGTH IN BIOCHEMICAL PROPERTIES OF SOLUBLE FISH PROTEINS ISOLATED FROM CRYOPROTECTED PACIFIC WHITING MINCE

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4.1 ABSTRACT

Biochemical characteristics of Pacific whiting muscle proteins extracted at acidic, neutral, and alkaline conditions were investigated as affected by various ionic strength levels. The protein solubility at pH 4 declined, as NaCl was added up to 200 mM, due to protein aggregation through hydrophobic interactions. In contrast, at pH 7 and 10, solubility increased as NaCl was added up to 400 mM after which it remained constant. Changes in total SH content and S_0 were highly related to the different molecular weight distributions of the soluble proteins. At pH 4, myosin heavy chain (MHC) was soluble as evidenced by the presence of MHC in the soluble fraction, even though degraded molecules were shown at IS 10-100 mM, and became completely insoluble at IS ≥ 150 mM. At pH 10, the density of the MHC band gradually increased as IS increased and the formation of high MW polymers was observed at IS ≥ 150 mM.

Keywords: Fish protein, ionic strength, solubility, surface hydrophobicity, SDS-PAGE

4.2 INTRODUCTION

Protein solubility has been the subject of various studies due to its correlation to protein functionality, such as gelation, foaming ability, emulsification, binding ability, and whipping properties (Vojdani 1996). Ionic strength (IS) and pH of the protein environment are the two most important factors affecting protein solubility, conformation, and functional properties. A new fish protein recovery method involving pH shifting (Hultin and Kelleher 1999 and 2000; Choi and Park 2002; Kim and others 2003; Yongsawatdigul and Park 2004) utilizes the pH-dependent solubility properties of muscle proteins for maximum solubilization with recovery by isoelectric precipitation. Acidic (pH 2-3.5) or alkaline conditions (pH 10.5-11.5) are used for maximum solubilization (Hultin and Kelleher 1999; Cortes-Ruiz and others 2001; Underland and others 2002; Choi and Park 2002; Kim and others 2003).

Although pH control is important for this process, ionic strength, which also affects the solubility and functionality of muscle proteins (Wu and others 1991; Chung and others 1993; Stefansson and Hultin 1994; Lin and Park 1998; Chang and others 2001), should not be ignored. Foegeding and others (1996) reported that myofibrillar proteins are soluble at high salt concentration (300 to 600 mM) at neutral or slightly alkaline or slightly acidic conditions. Solubilization of muscle proteins under high IS involves two events: the depolymerization of the thick filament backbone, and the subsequent dissociation of the myosin heads from the actin filaments (Parsons and Knight 1990).

Stefansson and Hultin (1994), however, explored solubilization of myofibrillar proteins at extremely low salt concentration (<0.3 mM). Lin and Park (1996) also reported a significant amount of myosin heavy chain was soluble when mince was washed with fresh water during surimi manufacturing. The water solubility of myofibrillar proteins is highly sensitive to ionic strength and pH, and also varies with species and extraction methods.

Myofibrillar proteins washed with 150 mM salt solution at neutral pH were found to be soluble in water (Krishnamurthy and others 1996; Feng and Hultin 1997). The study of Dagher and others (2000) also indicated that at low ionic strength (10

mM), the solubility of cod muscle proteins increased considerably between pH 8.9 and 9.2. At an ionic strength of 430 mM, the solubility was high and remained unchanged over the pH range from 7 to 9.5.

Thawornchinsombut and Park (2004d) showed that protein solubility, conformation, and molecular weight composition of Pacific whiting muscle proteins changed significantly over the pH range 2-12 and change in ionic strength (10 and 600 mM NaCl). The present study is a continuation of this work which investigates the effects of a wide range of ionic strengths on protein solubility, conformational changes, and the molecular weight composition of myofibrillar proteins from Pacific whiting at three (acidic, alkaline, and neutral) pH levels.

4.3 MATERIALS AND METHODS

4.3.1 Materials

Pacific whiting (*Merluccius productus*) was obtained from Point Adams Packing Co. (Hammond, OR). Fresh whole fish, less than 24 h post-harvest, were transported on ice to the OSU Seafood Laboratory within 30 min. Fish were then filleted, ground using a grinder (Hobart, The Hobart Manufacturing Co., Troy, OH), mixed thoroughly with sorbitol (NeoSorb 20/60, Roquette America Inc., Gurnee, IL) at 10 % (w/w) concentration, vacuum-packed in small portions (~ 50 g), and stored at – 80 °C until used.

4.3.2 Sample Preparation

Cryoprotected Pacific whiting (PW) mince was thawed at 5 °C for 1 h and washed (mince:water = 1:5) twice with cold (5 °C) deionized water to remove sorbitol. Four layers of cheese clothes were used to remove water from washed mince with manually squeezing. Mince was then homogenized (Power Gen 700, GLH 115, Fisher Scientific Inc., Pittsburgh, PA) in 5 mM histidine buffer for 1 min at speed level 3. The mixing ratios of mince to buffer, which were determined based on the preliminary experiments to maintain all ionic strength (IS) levels as described below, were 1:19 for

pH 7 and 1:39 (w/v) for pH 4 and 10, respectively. The pH of the homogenized samples was adjusted using 0.5 and/or 1 N cold HCl and NaOH using a pH meter (Accumet Research AR 15, Fisher Scientific Inc, Pittsburgh, PA).

Once the pH was fixed at pH 4, 7, or 10, the desired ionic strength (10, 25, 50, 75, 100, 150, 200, 300, 400, or 600 mM NaCl) was adjusted by adding sodium chloride granules. A change in pH was noted as NaCl was added to the suspensions. For proteins treated at pH 4, the pH readings after adjusting ionic strength (IS) were 4.05, 4.17, 4.24, 4.28, 4.30, 4.36, 4.42, 4.44, 4.48, and 4.50, which corresponded to IS 10, 25, 50, 75, 100, 150, 200, 300, 400, and 600 mM NaCl, respectively. However, the change of pH was in a very narrow range for pH 7 (7.00 to 6.96) and pH 10 (9.99 to 9.85) when the IS was adjusted from 10 to 600 mM NaCl. Protein suspensions were kept at low temperature (4 – 5 °C) while pH and IS were adjusted.

The ionic strength of the protein suspensions was measured using a conductivity meter (YSI 3100, YSI Inc., Yellow Spring, OH) equipped with a conductivity cell (cell constant 1.0 cm⁻¹). Conductivity readings were obtained based on the standard curve prepared using reagent grade sodium chloride (Sigma Chemical, Co., St. Louis, MO) in a concentration range between 0 and 600 mM.

The homogenate samples prepared above were centrifuged at 19,950 x g for 30 min at less than 5 °C (Sorvall RC-5B, DuPont Co., Newtown, CT). The supernatants were analyzed for protein content, total sulfhydryl (SH) content, surface hydrophobicity (S_o), and changes of protein patterns by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

4.3.3 Protein Solubility

Protein concentration in the supernatant was determined according to the method of Bradford (1976). Prior to the determination of protein content, the supernatant was appropriately diluted using 5 mM histidine buffer. The buffer was adjusted to each pH and IS treatment, respectively, before mixing with the sample. Consequently, the pH of the samples was insignificantly altered after adding the buffer. The absorbance was measured at 595 nm (UV-VIS Spectrophotometer; UV

2401PC, Shimadzu Corp., Kyoto, Japan) using bovine serum albumin as a standard. Protein content was calculated as mg protein in the supernatant per gram of dried fish muscle.

4.3.4 Surface Hydrophobicity

ANS (1-anilinonaphthalene-8-sulfonic acid) and PRODAN (6-propionyl-2-(dimethylamino)-naphthalene) fluorescent probes were used to investigate protein surface hydrophobicity of the supernatant fraction according to the method of Alizadeh-Pasdar and Li-Chan (2000). ANS is a charged hydrophobic dye with negative sulfonate anions at $\text{pH} \leq 3.6$ and can interact through hydrophobic as well as electrostatic interactions. In contrast to ANS, PRODAN is an uncharged hydrophobic probe, which will not be affected by possible electrostatic interactions contributed by the environment (Alizadeh-Pasdar and Li-Chan 2000).

The supernatant was serially diluted with 5 mM histidine buffer to obtain protein concentrations ranging from 0.063 to 0.50 mg/mL. The buffer was adjusted per each pH and IS treatment before making the serial dilution. However, samples at pH 4 were diluted at lower concentrations (≤ 0.10 mg/mL) to obtain a linear initial slope with the correlation coefficient (r^2) ≥ 0.99 . Samples (4 mL) were mixed with 20 μL of ANS stock solution (8×10^{-3} M in 0.1 M phosphate buffer, pH 7.4) or 10 μL of PRODAN stock solution (1.40×10^{-3} M in methanol), respectively.

After holding for 15 min for PRODAN (in the dark) and 10 min for ANS-treated samples, the relative fluorescence intensity (RFI) of each solution was measured. The excitation/emission slits were set at 5 nm each for both measurements using a Luminescence Spectrometer LS 50 B (Perkin Elmer, Norwalk, CT). Excitation/emission wavelengths of 390 nm/470 nm and 365 nm/465 nm were used for ANS and PRODAN, respectively. The initial slope (S_0) of the net RFI versus protein concentration was calculated by linear regression analysis and used as an index of the protein surface hydrophobicity.

4.3.5 Total Sulfhydryl Content

Total sulfhydryl group content was assayed according to the method of Hamada and others (1994) with a slight modification. The protein concentration in the supernatants was diluted to 0.5 mg/mL using 5 mM histidine buffer. The pH and IS of the histidine buffer were adjusted to the desired values for each treatment before mixing with the sample. An aliquot (0.5 mL) of the sample was mixed with 2 mL of 8 M urea in 0.2 M Tris-HCl buffer (pH 7.0) and then with 50 μ L of 10 mM DTNB (Ellman's reagent; 5, 5'-dithiobis (2-nitrobenzoic acid)) in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.2 mM EDTA. After incubating at 40 °C for 15 min, the absorbance was measured at 412 nm. The SH content was then calculated based on the absorbance using a molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ (Riddles and others 1979).

4.3.6 SDS-PAGE

The supernatants prepared from the various treatments were evaluated for molecular weight distribution using SDS-PAGE, according to the method of Laemmli (1970). Protein concentration was measured using the method of Lowry and others (1951). An aliquot (500 μ L) of the supernatant was added with 125 μ L of 5x sample buffer containing 60 mM Tris-HCl buffer (pH 6.8), 25% glycerol, 2% SDS, 10% β -mercaptoethanol, and 0.1% bromophenol blue. Samples were then heated in boiling water for 5 min and centrifuged at 8,000 x g for 5 min. SDS-PAGE was performed using 4% (stacking) and 10% (separating) polyacrylamide gels, except samples treated at pH 10, which were applied on 3.5 % and 6 % of stacking and separating gels, respectively, due to the presence of high MW protein components. Gels were stained in a staining solution containing 0.1% Coomassie blue R-250, 45% methanol, and 10% acetic acid. Destaining was conducted using a destaining solution (methanol: glacial acetic acid: water = 1:1:8 (v/v/v)).

Molecular masses were estimated using the wide range MW marker ranging from 6.5–205 kDa (Sigma Chemicals Co., St. Louis, MO). SigmaGelTM software

(SPSS Science, Chicago, IL) was used to estimate the molecular weights and the intensity of the protein bands.

4.3.7 Statistical Analysis

Data were analyzed using SPSS for Windows, version 10.0.1 (SPSS Inc., Chicago, IL). Analysis of Variance (ANOVA) was made with the General Linear Models (GLM) with a significant level of $p \leq 0.05$. The Tukey's test with significant difference at $p \leq 0.05$ was used to compare sample means. Due to the range in magnitude of data obtained, logarithmic transformation of the surface hydrophobicity (S_o) values was performed prior to statistical analysis.

4.4 RESULTS AND DISCUSSION

4.4.1 Protein Solubility

The solubility of protein treated at both pH 7 and 10 exhibited similar trends (Fig. 4.1). At these pH levels more proteins were solubilized as IS increased to 300-400 mM NaCl. However, protein solubility was higher at pH 10 than pH 7 for all IS levels. Greater net negative charge of proteins is associated with the higher pH. The enhancement of protein solubility by salt has been widely described as the salting-in phenomenon. This contributes to the electrostatic repulsive forces among protein molecules, which results in higher solubility (von Hippel and Schleich 1969; Underland and others 2002).

The IS at which protein solubility increased significantly was 200 mM NaCl for pH 7 and 75 mM NaCl for pH 10. Maximum solubility was observed at IS 400-600 mM NaCl at both pH levels. Parsons and Knight (1990) demonstrated that 0.4 to 0.6 M salt concentration would be required to maximally solubilize myofibrillar proteins at neutral or slightly acid or alkaline pH.

At pH 4, protein solubility was greater at the lowest IS (10 and 25 mM) (Fig. 4.1). Solubility, sharply decreased at IS 75 to 150, remaining constant as IS was increased from 200 to 600 mM NaCl. Mireles Dewitt and others (2002) also showed

that protein solubility of acid-treated protein from beef heart was adversely affected by the addition of NaCl. At low pH where the protein has a net positive charge, negatively charged chloride ions from NaCl bind with protein, thereby decreasing the electrostatic repulsions as well as leading to protein precipitation (Kellerher and Hultin 1999). Chloride ion interacts with the positively charged amino acids to a stronger degree than sodium ion binds with the negative charges, resulting in more hydrogen ions and thus causing a shift of the pI to lower pH (Ockerman 1980). Several studies have reported the similar phenomenon (Feng 2000; Mireles Dewitt and others 2002; Thawornchinsombut and Park 2004d).

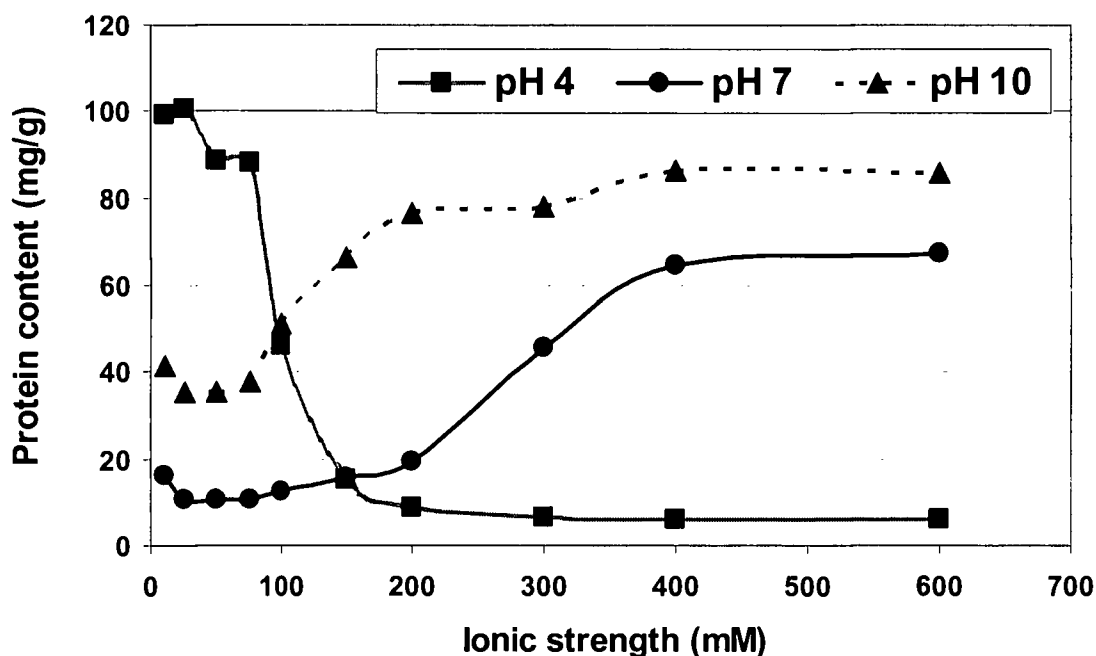


Figure 4.1 Solubility of Pacific whiting muscle proteins prepared at various IS and three pH levels.

4.4.2 Surface Hydrophobicity (S_o)

Fish proteins treated at pH 4 exhibited extremely high hydrophobicity at 10 mM NaCl as indicated by the ANS probe (ANS- S_o), which was 10 times larger than PRODAN- S_o (Figs. 4.2 and 4.3). ANS- S_o values were also generally higher at acidic

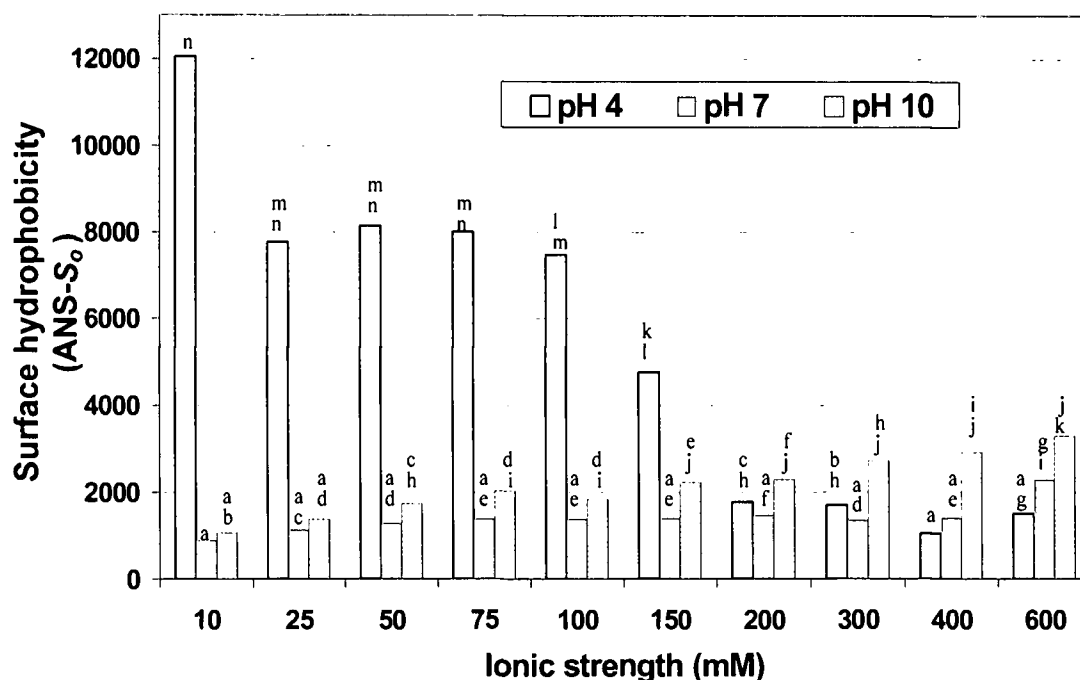


Figure 4.2 Surface hydrophobicity of Pacific whiting soluble proteins prepared at various IS and three pH levels using ANS probe analysis. Two letters on each bar indicate only the first and the last letters of a series of letters due to the space limitation. For example, “ch” denotes “c d e f g h”. Different letters on each bar represent significant differences ($p \leq 0.05$).

pH compared to neutral or alkaline pH (Alizadeh-Pasdar and Li-Chan 2000). Since at acidic pH (pH 4) the ANS probe yield negative sulfonate anions ($pK_a \sim 3.6$) while proteins have a net positive charge, the ANS anion can bind prominently to the cationic group of proteins. If the hydrophobic regions of the protein are adjacent to charged side chain residues, electrostatic interactions of the anionic ANS and the protein charges possibly strengthen the hydrophobic interactions between ANS and proteins (Greene 1984; Haskard and Li-Chan 1998)

Our previous results (Thawornchinsombut and Park 2004d) also overestimated ANS- S_0 values at low pH (pH 2-4) possibly due to the perturbation of electrostatic interactions between anionic ANS and protein in addition to their hydrophobic interactions. Several additional studies (Shimizu and others 1985; Das and Kinsella 1989; Alizadeh-Pasdar and Li-Chan 2000) have indicated that when salt was

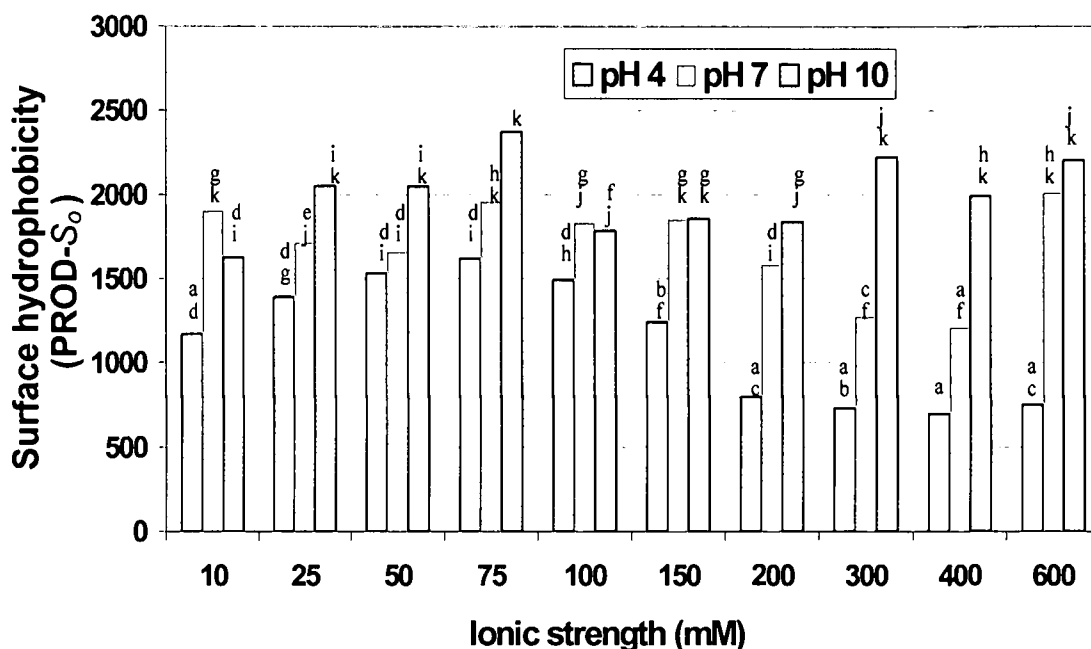


Figure 4.3 Surface hydrophobicity of Pacific whiting soluble proteins prepared at various IS and three pH levels using PRODAN probe analysis. Two letters on each bar indicate only the first and the last letters of a series of letters due to the space limitation. For example, “ch” denotes “c d e f g h”. Different letters on each bar represent significant differences ($p \leq 0.05$).

introduced into the system at acidic pH, ANS- S_0 reduced (particularly with IS at 10 to 25 mM in this study). The addition of salt probably interrupted the electrostatic interactions between the anion probe and the proteins, thus reducing the strength of hydrophobic binding between the protein and ANS probe. Although salt addition might suppress the enhancement of hydrophobicity from electrostatic interactions between ANS and the protein molecules at pH 4, ANS- S_0 still showed 4.6 to 5.6-fold greater values than PRODAN- S_0 as the IS decreased from 150 to 25 mM (Figs. 4.2 and 4.3). When IS was increased from 10 to 75 mM (pH 4), a slight increase in PRODAN- S_0 was seen (Fig. 4.3). It is noteworthy that we observed quite similar patterns of S_0 of acid-treated proteins from both probes even though their magnitudes were considerably different. A significant decrease ($p \leq 0.05$) in ANS- S_0 and PRODAN- S_0 was noticed while the IS increased from 100 to 150, and further to 200 mM NaCl. Substantial changes of hydrophobicities upon changes in IS at pH 4 were

probably associated with the differences of MW distributions in soluble proteins between low IS (10-100 mM) and high IS (150-600 mM). MHC was shown at low IS although degraded into smaller molecules, while no MHC was shown at high IS (Fig. 4.5).

Based on these present results, the use of an anionic ANS fluorescent probe needs to be more carefully considered for measuring protein surface hydrophobicity under acidic conditions. In addition, Weber and others (1979) suggested careful interpretation of fluorescence changes, which may not result from a hydrophobic site on the protein due to the interaction between ANS and macromolecules.

In a neutral environment, $ANS-S_0$ insignificantly changed as the salt content was increased, except at 600 mM NaCl ($p \leq 0.05$). Aluko and Yada (1997) reported that the presence of high NaCl concentration (0.5-2 M) in cowpea isoelectric protein isolate dispersed in buffers pH 3-8 did not seem to have significant effect on the interaction between the probe and protein molecules for each pH level. Salmon myosin, however, exhibited an increase in surface hydrophobicity with the addition of KCl up to 3.8 M at pH 7.0 (Lin and Park 1998). This discrepancy was possibly due to the difference of protein types and salt concentration between the studies.

The $ANS-S_0$ of alkaline samples represented more protein unfolding at all IS levels compared to those at neutral pH (Fig. 4.2). This difference due to pH became more obvious ($p \leq 0.05$) at IS between 300-600 mM. This result demonstrated the interactive effects of pH and IS on protein unfolding.

All samples regardless of pH showed similar trends of IS effects on $PRODAN-S_0$ except at IS 10 mM (Fig. 4.3). The highest value was found at pH 10 while the lowest value at pH 4. However, $PRODAN-S_0$ of acid-treated proteins was lower than that of neutral and alkali-treated samples at all IS levels, and most predominantly at IS ≥ 200 mM, which coincides with lower protein solubility (Fig. 4.1). At pH 7, $PRODAN-S_0$ measurements were relatively stable in the range of 10 to 200 mM NaCl, and then decreased as IS increased to 300 and 400 mM. A significant increase ($p \leq 0.05$) of $PRODAN-S_0$ was found, though, as IS changed from 400 to 600 mM NaCl. This trend was in accordance with the $ANS-S_0$ data (Fig. 4.2). The comparative study

by Alizadeh-Pasdar and Li-Chan (2000) using three different probes with whey protein isolate, β -lactoglobulin and bovine serum albumin treated at pH 3-9 showed that PRODAN probe yielded the lowest S_o at pH 3 than at other pH values for all protein types whereas ANS probe revealed the opposite trend. Yang and others (2002) compared hydrophobic probe binding of β -lactoglobulin at pH from 2.5 to 10.5 using ANS (an anionic molecule with an aromatic structure) and retinol (a molecule with an aromatic and an aliphatic moiety). A substantially lower relative fluorescence at acidic pH than at alkaline pH was observed in the retinol probe. This was explained due to the conformation changes of β -lactoglobulin at pH less than 6.0 resulting in reducing the accessible open calyx binding site of the protein molecule. However, in our study, due to the differences in the nature of protein tested as well as protein compositions of the soluble fractions from the three pH levels, we may not be able to draw a conclusion. The subsequent study is required to clarify these data.

A dramatic increase in hydrophobicity at 600 mM NaCl was likely due to the fact that myosin can dissociate into monomers under harsh conditions (IS > 0.3 mM KCl and/or pH $\geq$ 7) (Godfrey and Harrington 1970 a,b; Weeds and Pope 1977; Kaminer and Bell 1996). This would have created more available hydrophobic patches on the protein molecules for probe bindings. For pH 10-treated proteins, the lowest surface hydrophobicity was noted at the minimal salt concentration for both PRODAN- S_o and ANS- S_o . However, no significant ($p>0.05$) changes of PRODAN- S_o were attained when IS was further increased (Fig. 4.3).

4.4.3 Total SH Content

Results clearly showed that acidic conditions significantly affected total SH group content in the protein solutions. At pH 4 with IS 10 - 100, 200, and 600 mM NaCl, lower SH contents were noted when compared to those at IS 150, 300, and 400 mM (Fig. 4.4). Our previous research focusing on the effect of various pH levels at IS 10 and 600 mM revealed that the SH contents of pH 4-treated samples at both IS levels were comparative although their protein patterns were obviously different (Thawornchinsombut and Park 2004d). Therefore, the alteration of SH contents of pH

4-treated proteins under various IS conditions particularly between 150 and 600 mM was most likely due to the inconsistency of MW distributions in protein supernatants (Fig. 4.5). Low SH values were also observed at pH 10 at most IS levels compared to those at pH 7. Yongsawatdigul and Park (2004) and Kim and others (2003) observed lower SH group contents as well in acid- and alkali-aided fish protein isolate compared to washed mince and conventional surimi (pH 7), respectively. A notable decrease in SH contents was also reported when threadfin bream protein was treated

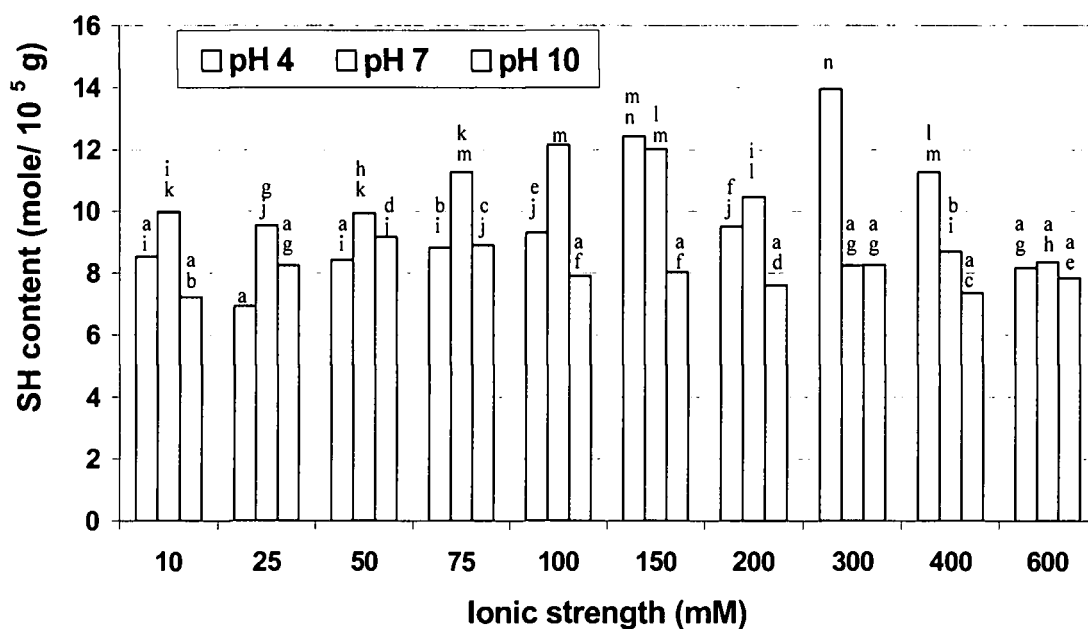


Figure 4.4 Total SH content of Pacific whiting soluble proteins prepared at various IS and three pH levels. Two letters on each bar indicate only the first and the last letters of a series of letters due to the space limitation. For example, “ch” denotes “c d e f g h”. Different letters on each bar represent significant differences ($p \leq 0.05$).

with acetic acid compared to untreated samples (Chawla and others 1996).

These results perhaps indicated that SH groups were sensitive to oxidation at both acidic and alkaline pH, resulting in the formation of SS bonds. At acidic pH, oxidation of cysteine and cystine yields the formation of several intermediate oxidation products (Damodaran 1996). At higher pH values, thiol oxidation reactions between exposed $-SH$ or S^- groups can occur in addition to SS interchange reactions,

which lead to SH content reduction (Tanford 1968; Watanabe and Klostemeyer 1976). We suspected that the decline of total SH content might have also been caused by lysinoalanine (LAL) cross-links. LAL is a major cross-link commonly present in alkali- and/or heat-treated proteins (Maga 1984; Damodaran 1996) and primarily contributed by cysteine (Friedman and Masters 1982; Chang and others 1999). Specific studies relative to LAL formation in fish protein processed under alkali condition are very limited. According to Lawrence and Jelen (1982) who reported the alkaline extraction of residual meat from poultry bone, small amounts of LAL were formed at 22 °C at pH 10.7 and 11.5 after holding for 16 h. Thus, based on the alkali condition and holding time used in this study, it would be very unlikely to form a discernable amount of LAL.

The molecular weight distributions of the soluble proteins revealed by SDS-PAGE were somewhat associated with an alteration of SH content. At pH 4, the density of MHC gradually reduced as IS increased from 10 to 100 mM NaCl (Fig. 4.5), which was related to a gradual increase in SH content (Fig. 4.4). At IS greater than 100 mM and pH 4, the major protein components in the soluble portions did not migrate at the same location as the myofibrillar proteins (Fig. 4.5). Changes in SH content while IS increased from 150 mM would possibly correspond to the differences in protein molecular weight distribution as previously described. A correlation between MHC and SH content was also noted in samples treated with salt at pH 7. At high IS levels (300-600 mM NaCl) of pH 7-treated proteins, where MHC bands and high MW polymers were observed (Fig. 4.6), the total SH contents were low (Fig. 4.4). In contrast, the SH contents were greater at $IS \leq 200$ mM where no MHC bands were shown. Le Blanc and Le Blanc (1992) reported that disulfide bond contents in sarcoplasmic and myosin-rich fractions of cod fillet after storage at -12 °C for about 300 d had increased from 0.10 and 1.4 μ moles/g proteins to 0.34 and 2.8 μ moles/g proteins, respectively. Their study and our data probably suggested that the change of SH content would primarily be contributed by the myosin fraction. At pH 10 regardless of IS level, all soluble fractions contained MHC (Fig. 4.7) and

demonstrated relatively lower SH contents than those at pH 7.0 and IS 10-200 (no MHC) (Fig. 4.4).

4.4.4 SDS-PAGE

At IS between 150 and 600 mM, only small MW proteins (MW 33-97 kD and < 24 kD) were observed at pH 4 (Fig. 4.5). It is noteworthy that there were no actin bands at all IS levels, except at IS 10, where a very thin line of the band is identifiable.

MHC were more extractable at IS 10-100 mM. However, due to the degradation through acid hydrolysis and/or proteolytic enzymes, MHC was degraded to a smaller molecular weight protein with 116-160 kD. Similar observations were reported with 1% acetic acid-treated muscle proteins from threadfin bream (Chawla

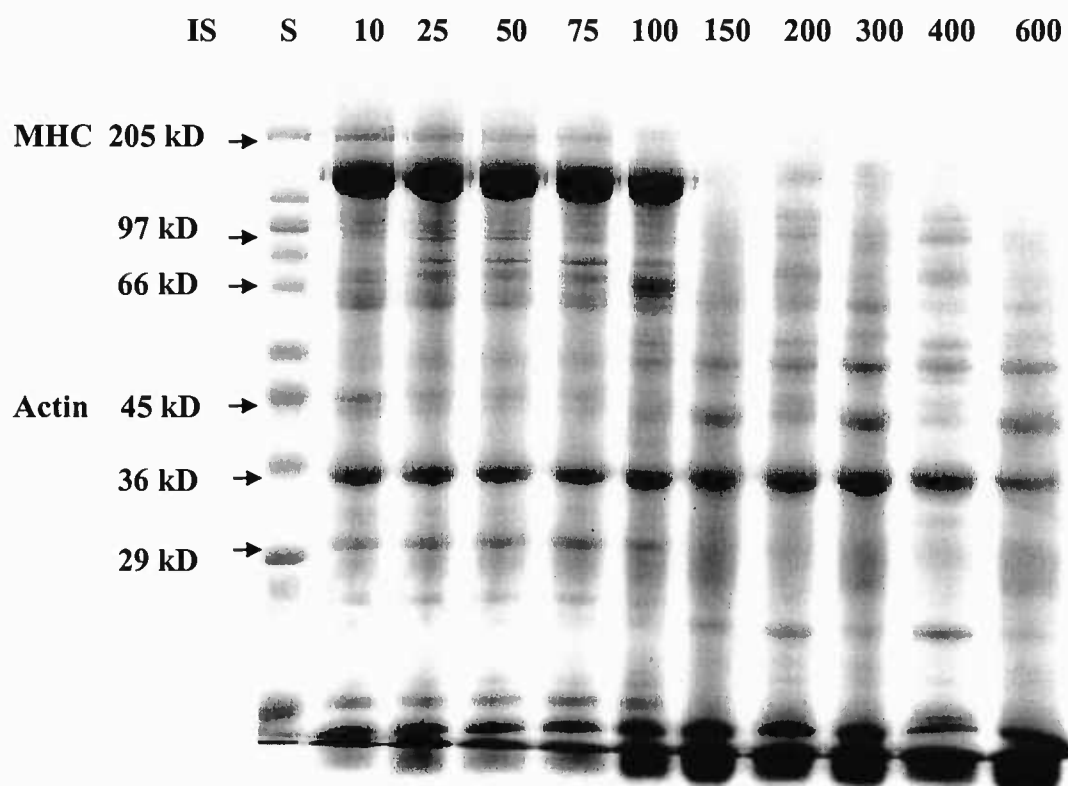


Figure 4.5 SDS-PAGE patterns of Pacific whiting soluble proteins at pH 4 and various IS conditions. An equal quantity (25 µg of proteins) was applied per each well on the 10 % acrylamide separating gel (S: wide range mw standard; MHC: myosin heavy chain).

and others 1996), pH 2.7-treated herring proteins (Underland and others 2002), and pH 2.5-treated rockfish proteins (Yongsawatdigul and Park 2004).

Saunders (1994) found that the degradation of acidified (pH 3.0) myofibrillar proteins from mutton, beef, and chicken could be inhibited by protease inhibitors. The presence of endogenous proteases in PW at acidic pH has also been previously reported (An and others 1994). Cysteine proteases such as cathepsin L, B, and H are the major enzymes related to muscle softening in PW. The total accumulative activity of the three cysteine cathepsins was highest at 20 °C in fish fillets. MHC was most extensively hydrolyzed, followed by troponin-T, and α - and β -tropomyosin, while both actin and myosin light chain were least affected (An and others 1994). The optimum pH for cathepsin B, D, H, and L are 5.0-6.0, 3.0-4.5, 5.0, and 3.0-6.5, respectively (Kang and Lanier 2000).

In mackerel, the hydrolytic rates of cathepsin B, L, and L-like proteinases were faster at pH 5.0 than at pH 6.0 (Jiang and others 1996). Gomez-Guillen and Batista (1997) reported that cathepsin D-like enzyme in sardine (*Sardina pilchardus*) had an optimum pH at 3.2 and its activity was strongly inhibited by 6 % NaCl (~1 M). These literatures indicated that low salt concentration (10-100 mM) has no inhibitory effect on the degradation of PW muscle proteins treated at pH 4.

When PW muscle proteins were treated at pH 7, the major protein bands appearing in the pattern were 35, 40, 43, and 45 kD particularly for IS 10-200 mM (Fig. 4.6). The intensity of the bands around 52, 61, 94, 104, and 167 kD increased as IS increased. A similar result was also seen in the electropherogram of the supernatant extracted from washed mackerel light muscle at 25 and 150 mM NaCl at neutral pH (Feng and Hultin, 1997). They reported that some of these proteins such as 166, 95, and 56 kD, which could be M-protein, α -actinin, and desmin, respectively, might prevent the solubilization of other myofibrillar proteins in water. However, at higher IS levels in this study (300-600 mM NaCl), high MW polymers, MHC and actin comprised the majority of the extracts.

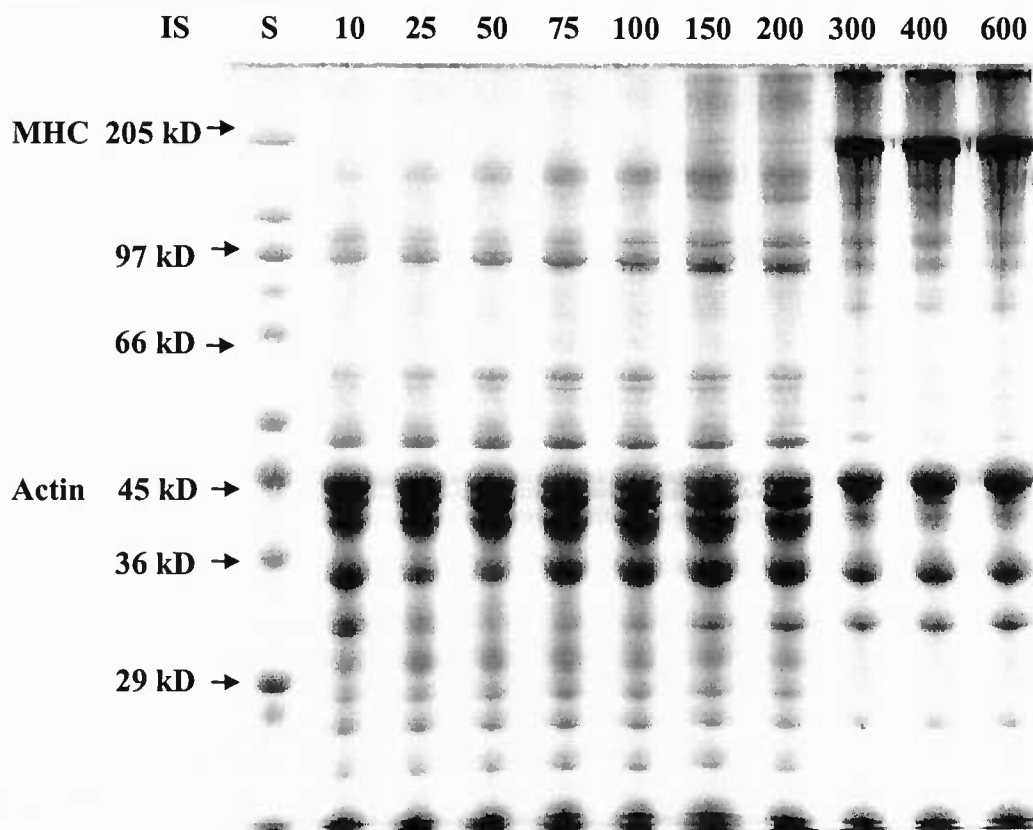


Figure 4.6 SDS-PAGE patterns of Pacific whiting soluble proteins at pH 7 and various IS conditions. An equal quantity (25 μ g of proteins) was applied per each well on the 10 % acrylamide separating gel (S: wide range MW standard; MHC: myosin heavy chain).

Under alkaline conditions, MHC was solubilized at all IS treatments (Fig. 4.7). However, the intensity of MHC significantly increased as IS increased from 10 to 600 mM NaCl. At the same time, low MW proteins bands (33, 38, and 42 kD) became less soluble as IS increased (data not shown). For IS at 150 to 600 mM, SDS-PAGE on 6 % separating gel clearly showed the polymer bands above MHC (Fig. 4.7). This may suggest that the polymerization of alkali-treated proteins could only occur when subjected to suitable IS conditions.

According to our previous work (Thawornchinsombut and Park 2004d) and Yongsawatdigul and Park (2004), disulfide bonds most likely played an important role in the polymerization of PW proteins under alkaline conditions (pH 10 - 12). In

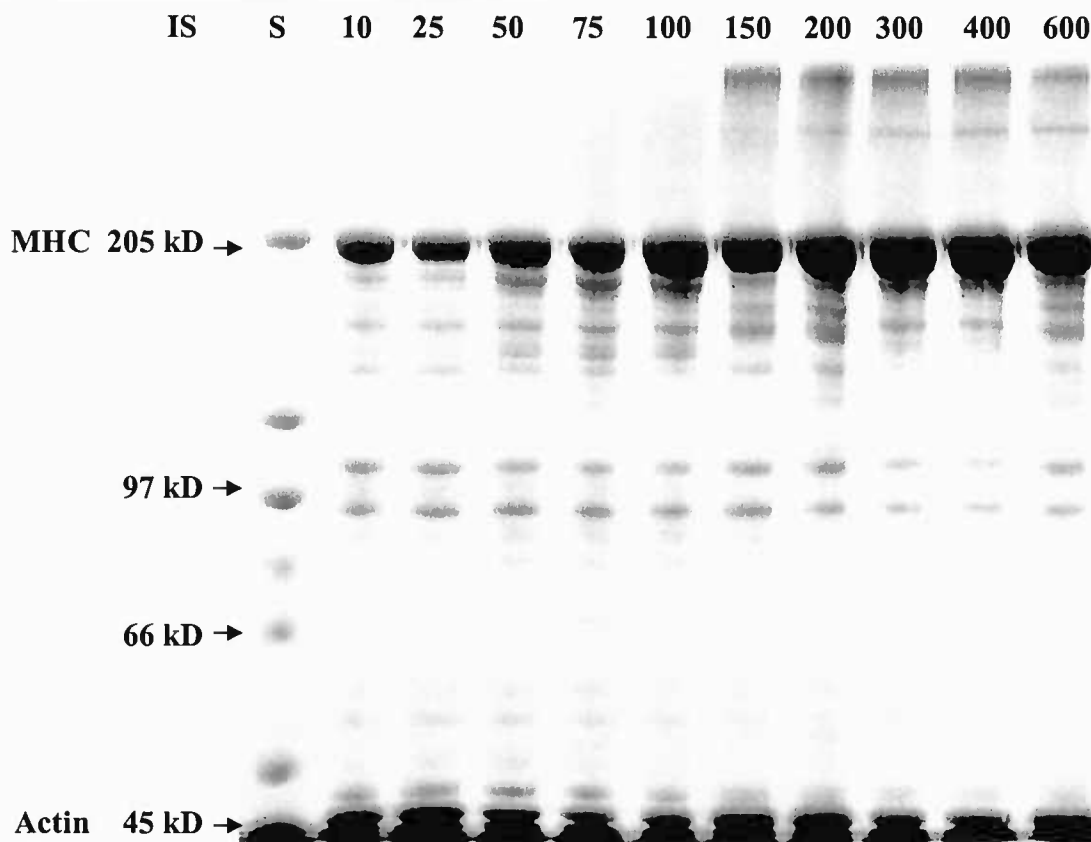


Figure 4.7 SDS-PAGE patterns of Pacific whiting soluble proteins at **pH 10** and various IS conditions. An equal quantity (25 μ g of proteins) was applied per each well on the 6 % acrylamide separating gel (S: wide range MW standard; MHC: myosin heavy chain).

addition, Pacific whiting muscle proteins contain endogenous transglutaminase (TGase) (Peters and others 1995), which catalyzes cross-linking reactions between the glutamine and lysine residues (epsilon-(gamma-Glu) Lys linkages) in the protein molecules (Folk 1980). Although its activity is lower than in Alaska pollock or threadfin bream (Soeda and others 1996), it would also be partly involved in the formation of high MW protein polymers. Nowsad and others (1994) discovered the combined contribution of non-disulfide covalent bonds by TGase and SS bonds to the formation of cross-linked MHC in suwari gel from Alaska pollock surimi.

4.5 CONCLUSION

Present findings suggest that varying IS conditions in an acidic, neutral, or alkaline environment have a significant impact on changes of the solubility, surface hydrophobicity, and protein compositions of soluble PW proteins. Effects of IS on protein solubility at pH 4 were opposite to those at pH 7 and 10. The interference of electrostatic interactions to hydrophobic binding between the ANS probe and protein should also be highlighted when the surface hydrophobicity analysis was performed under acidic conditions. The changes of total SH content and surface hydrophobicity were greatly related to the presence of MHC in the samples, which corresponded to both IS and pH conditions. MHC was not stable to protein degradation by proteolytic enzymes and/or acid hydrolysis under acidic pH. In addition, MHC precipitated more as the salt content increased and completely disappeared from the protein patterns at $IS \geq 150$ mM. On the other hand, under alkaline pH, MHC was more stable and the high pH might have partly contributed to some or partial polymerization at higher IS (> 150 mM NaCl).

CHAPTER 5

EFFECT OF IONIC STRENGTH ON GELATION CHARACTERISTICS OF ACID- AND ALKALI-TREATED PACIFIC WHITING FISH PROTEIN ISOLATES

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5.1 ABSTRACT

The physicochemical properties of gels prepared from Pacific whiting protein isolated at low (3) and high pH (11) with three ionic strength levels (IS 10, 150, and 400 mM NaCl) were characterized after pH readjustment to 7.0. The strongest gels were obtained from fish protein isolate (FPI) prepared at pH11/IS150 and conventional surimi. There was no correlation between protein solubility and gel properties of FPI. Disulfide bonds seem to play an important role on gel quality of FPI samples. Surface hydrophobicity and DSC thermograms demonstrated that, in addition to adjusting the pH to 3 or 11, salt addition during protein solubilization and subsequent recovery at the pI led to protein denaturation. Rheological study indicated that gelation mechanisms of isolated fish proteins were identical under the same IS condition regardless of pH level. FPI prepared at pH 3 or 11 could be partly refolded at pH 7. Nevertheless, some myosin fragments and actin were not recovered.

Key words: pH, ionic strength, surface hydrophobicity, SDS-PAGE, dynamic test, DSC.

5.2 INTRODUCTION

Conventional surimi processed from white flesh fish, such as Pacific whiting (PW) and Alaska pollock, which requires extensive washing and dewatering, yields around 25% of whole fish (Park and others 1997). New protein recovery processes have been developed by solubilizing proteins at acid (pH 2-3) or alkaline (pH 10.5-11.5) conditions followed by isoelectric precipitation (Hultin and Kelleher 1999; Hultin and others 2000). This new technology is considered to give the maximally achievable yield.

Hultin and Kelleher (1999) have patented the acid-aided process of cod and mackerel, which demonstrated excellent gel-forming ability as well as higher yield. Undeland and others (2002) extracted proteins from herring light muscle using acid or alkaline solubilization at pH 2.7 and 10.8, respectively. They reported that gels prepared from acid- and alkali-aided proteins exhibited equal gel qualities. The acid solubilization process with Pacific whiting yielded 20% more than the conventional process (Choi and Park 2002). Kim and others (2003) conducted a comparative study of acid- and alkaline-aided methods and revealed that gels prepared at pH 11 exhibited superior gel quality, followed by gels prepared at pH 2. All fish protein isolates prepared using pH-shift yielded better gel quality than conventional surimi. However, higher cathepsin activities were noted in the pH-shifted samples.

The importance of ionic strength as a factor in gel development and final structure is widely recognized. A change of ionic strength influences muscle characteristics including heat-induced gelation, thermal properties, water binding capacity, and emulsification properties (Stanley and others 1994). The structure of a protein and its functionality can also be modulated by environmental factors, especially pH and ionic strength (Myers 1988). By controlling environmental conditions, it is possible to gain more insight into how the structure of a protein dictates its function.

There are reports of changes in physicochemical and functional properties of several proteins as a result of varying pH and ionic strength of the medium. For example, emulsifying, foaming, and heat coagulability properties of cowpea isolate

were greatly modulated by changes in pH and ionic strength (Aluko and Yada 1997). Molecular structural changes of soy glycinin were shown to be a function of pH and ionic strength (Lakemond and others 2000). Previous studies in our lab also demonstrated that pH and ionic strength play an important role on PW muscle proteins, including protein solubility, biochemical properties, and molecular distribution of proteins using SDS-PAGE (Thawornchinsombut 2004 c,d). However, few studies have employed both pH and ionic strength in fish protein isolates using the pH-shift method. Interest in incorporating ionic strength effect into acid- or alkali-aided method led us to conduct this research.

Our overall objectives were to evaluate the combined effect of pH and ionic strength on acid- and alkali-treated fish protein isolates from enzyme-rich Pacific whiting and to compare it with conventional surimi. Additional efforts were also made to assess their physicochemical properties, gelation properties, and protein compositions.

5.3 MATERIALS AND METHODS

5.3.1 Materials

Pacific whiting (*Merluccius productus*) was obtained from Point Adams Packing Co. (Hammond, OR). Fresh whole fish were transported on ice to the OSU Seafood Laboratory within 30 min. Fish were then filleted, ground to improve homogeneity of samples, mixed with sorbitol (powder, Neosorb 20/60, Roquette American Inc., Gurnee, IL) at a 9:1 (mince:sorbitol) ratio, vacuum-packed in small portions (~ 350 g), and stored at – 80 °C until used. Due to the limited availability of fresh PW while conducting this research, cryoprotected mince (5 months old) was used.

5.3.2 Sample Preparation

Frozen Pacific whiting (PW) minces were thawed at 5 °C for 2 h and then subjected to conventional surimi (CS) and various pH and IS controlled processes.

For the conventional method, mince was washed 3 times using chilled (4-6 °C) distilled water at a 1 to 3 ratio (mince:water). The final washing was conducted using 0.3% NaCl solution to facilitate the dewatering step. The homogenate was centrifuged at 6,000 x g for 20 min at 4 °C (Sorvall RC-5B, DuPont Co., Newtown, CT) for each washing step. Washed mince was then mixed with cryoprotectants (5% sucrose, 4% sorbitol, and 0.3 % sodium tripolyphosphate). Surimi was adjusted to pH 7.0 and 80% moisture, and then was vacuum-packed and stored at -80°C until analyzed.

According to Kim and others (2003), two pH levels (3 and 11) were selected for fish protein isolation. At each pH, three levels of ionic strength (IS) were employed: 10, 150, and 400 mM NaCl. Ten mM was the lowest IS that can be obtained after pH adjustment of twice washed mince. IS 150 mM is the physiological IS of PW muscle and is also the point where a significant change in protein patterns by SDS-PAGE at pH 4, 7, and 10 was observed (Thawornchinsombut and Park 2004c). The final IS, 400 mM, was selected based on the salt concentration (2%) used for gel preparation of conventional surimi.

Thawed mince was washed twice with cold deionized (DI) water at 1 to 5 and 1 to 4 ratios, respectively, to remove sorbitol and reduce ionic strength. The homogenate was centrifuged at 6,000 x g for 15 min for each washing step. Washed mince was then homogenized (Power Gen 700, GLH 115, Fisher Scientific Inc., Pittsburgh, PA) with chilled DI water (1:8 ratio) for 2 min at speed level 2. The pH of homogenates was adjusted using 1 and/or 2 N cold HCl and NaOH. The pH measurement was done using a pH meter (HI 9025 microcomputer pH meter, Hanna Instruments, Inc., Woonsocket, RI) with Spear Gel Combo pH probe (Corning Incorporated Life Sciences, Acton, MA). Once the pH was fixed, the desired ionic strength was adjusted by adding sodium chloride granules. The ionic strength of the homogenates was measured using a conductivity meter (YSI 3100, YSI Inc., Yellow Spring, OH) equipped with conductivity cell (cell constant 1.0 cm⁻¹). Conductivity readings were obtained based on a standard curve prepared using sodium chloride in the concentration range between 0 and 600 mM. The change of pH was noted as NaCl was added to the suspensions. At 10, 150, and 400 mM, the final pH after

adjustment of ionic strength was 3.03, 3.25, and 3.47; and 10.94, 10.69, and 10.57, which corresponded to pH 3 and 11, respectively. After pH and IS adjustment, samples were centrifuged at 7,000 x g for 20 min at 4°C to separate insoluble parts. Proteins were subsequently recovered at pH 5.5 for samples treated at IS 10 and 150 mM, however, pH 4.5 was used for samples treated at IS 400 mM due to pI shifting caused by high salt concentration (Thawornchinsombut and Park, 2004c). Protein precipitates were then collected by centrifugation (8,000 x g, 25 min, at 4°C). Precipitates were then mixed with cryoprotectants (5% sucrose, 4% sorbitol, and 0.3 % sodium tripolyphosphate). The final pH and moisture content of all treatments were adjusted to 7.0 and 80%, respectively. The change of IS of samples after adjusting to pH 7.0 was recorded (Table 5.1). Samples were vacuum-packed and stored at -80°C until tested. All treatments were prepared in a walk-in cold room (5 - 6 °C) to control the process at low temperature.

5.3.3 Protein Solubility

Three and one gram of sample was homogenized with 27 mL of chilled, double deionized (DDI) water and 19 mL of 0.6 M NaCl in 20 mM Tris-HCl buffer (pH 7.0), respectively. The homogenization was set at speed level 3 for 1 min. After centrifugation at 20,000 x g, 4 °C, for 25 min, the supernatant was diluted appropriately before determining the protein concentration (Bradford 1976). The absorbance was measured at 595 nm (UV-VIS Spectrophotometer; UV 2401PC, Shimadzu Corp., Kyoto, Japan) using bovine serum albumin (Bio-Rad Laboratories, Hercules, CA) as the standard.

5.3.4 Gel Preparation

Frozen sample was partially thawed, chopped in a food processor (702 R, Hamilton Beach/Proctor-Silex Inc., Washington, NC) with 1.5 % beef plasma protein (BPP) as an enzyme inhibitor, and adjusted to 80 % moisture content (A&D Infrared Moisture Determination Balance AD 4714A, A&D Co., Ltd., Tokyo, Japan). All treatments were prepared without salt except CS, which contained 2 % salt. This

process was conducted in a walk-in cold room (5-6 °C). A small amount of paste was saved for analysis of surface hydrophobicity, total sulfhydryl (SH) content, dynamic test, and thermodynamic properties. The remaining paste was then stuffed into stainless steel tubes (20 mm I.D.), cooked at 90 °C for 15 min in a circulating water bath and then chilled in ice/water for 15 min. The chilled gels were set at room temperature for 2 h before fracture analysis.

5.3.5 Fracture Analysis

Gels were cut into 30 mm lengths and then applied to the punch test using a Texture Analyzer (TA.XT.plus, Texture Technologies Corp., New York). A 5 mm diameter spherical probe was used with a test speed set at 1 mm/sec. Breaking force (g) and deformation (mm) were recorded to determine the strength and cohesiveness of the gel, respectively.

5.3.6 Surface Hydrophobicity

Protein surface hydrophobicity of the paste was determined using an ANS (1-anilinonaphthalene-8-sulfonic acid) probe according to the method of Alizadeh-Pasdar and Li-Chan (2000).

Stock solution of 8×10^{-3} M ANS was prepared in 0.1 M phosphate buffer (pH 7.4). The excitation/emission slits were set at 5 nm each and the excitation/emission wavelengths were 390 nm / 470 nm (Luminescence Spectrometer LS 50 B, Perkin Elmer, Nor Walk, CT). Three grams of paste was homogenized with 27 mL of 0.6 M KCl in 20 mM Tris-HCl buffer (pH 7.0). After centrifugation at 20,000 x g for 30 min, 4 °C, protein concentration of the supernatant was adjusted to 0.1, 0.2, 0.3, and 0.4 mg/mL, respectively. Four milliliters of each sample were mixed with 20 μ L of ANS stock solution. After holding for 10 min, the relative fluorescence intensity (RFI) of each solution was measured. The initial slope (S_0) of the net RFI versus protein concentration was calculated by linear regression analysis and used as an index of the protein surface hydrophobicity.

5.3.7 Total Sulfhydryl Contents

Total sulfhydryl group content was determined using Ellman's reagent (5, 5'-dinitrobis (2-nitrobenzoic acid); DTNB) as described by Hamada and others (1994) with slight modification. Paste was solubilized in 0.6 M KCl in 20 mM Tris-HCl buffer (pH 7.0). Protein content of the supernatant was adjusted to 1.0 mg/mL using the same buffer. The diluted sample (0.5 mL) was mixed with 2 mL of 8 M urea in 0.2 M Tris-HCl buffer (pH 7.0) and then with 50 μ L of 10 mM DTNB solution. The sample was incubated at 40°C for 15 min before measuring absorbance at 412 nm. The SH content was calculated based on the absorbance using the molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ (Riddles and others 1979).

5.3.8 SDS-PAGE

Fish protein gels were subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) according to the method of Laemmli (1970). Two grams of gel was homogenized with 5 mL of 8 M urea and 13 mL of 5% SDS solution. Samples were shaken for 2 hrs at 150 rpm (Lab-Line Orbit Environ-Shaker, Lab-Line Instruments, Inc., Melrose Park, IL) and at ambient temperature then heated in water-bath at 90 °C for 1 h. After centrifugation at 20,000 x g for 30 min, the protein concentration of the supernatant was determined using the method of Lowry and others (1951). To determine disulfide bonds in the protein polymers, samples containing sample buffer with and without β -mercaptoethanol (β -ME) were compared. The supernatant (500 μ L) was mixed with 125 μ L of sample buffer containing 60 mM Tris-HCl buffer (pH 6.8), 25% glycerol, 2% SDS, 10% β -ME (0% β -ME for non-reduced samples), and 0.1% bromophenol blue (BPB). Samples were then heated in boiling water for 5 min and centrifuged at 10,000 x g for 10 min (Eppendorf Centrifuge 5415 C, Brinkmann Instruments, Inc., Westbury, NY). SDS-PAGE was performed in 3.5% (stacking) and 7.5% (separating) polyacrylamide gels. An equal quantity (30 μ g) of proteins was applied per each well. Gels were stained in a staining solution containing 0.1% Coomassie blue R-250, 45% methanol, and 10%

acetic acid. Destaining was conducted using destaining solution (methanol: glacial acetic acid: water = 1:1:8 (v/v/v)).

Molecular masses were estimated using wide range MW markers ranging from 6.5–205 kDa (Sigma Chemicals Co., St. Louis, MO). SigmaGel™ software (SPSS Science, Chicago, IL) was used to estimate the molecular weights of protein bands.

5.3.9 Rheological Measurements

The rheological properties of sample pastes were monitored with temperature using dynamic oscillation, CS-50 Rheometer (Bohlin Instruments, Inc., East Brunswick, NJ). A 4/4 cone (4 cm diameter, 4 ° angle) and plate arrangement were used for all experiments. Storage modulus (G') was measured at the test temperature ranges of 20-90 °C and at a heating rate of 1 °C/min. A solvent trap with moistened sponge inside was used to prevent moisture evaporation during measurement. Based on our preliminary calibration to determine the linear viscoelastic region, 1 Pa torque value and 0.1 Hz frequency were selected.

5.3.10 Thermodynamic Properties

Differential scanning calorimetry (DSC) was performed on a Micro DSC III (Setaram Inc., Lyon, France). The instrument was calibrated for temperature accuracy using DDI water and naphthalene. Sample pastes weighing around 480 ± 5 mg were sealed in a hastelloy sample vessel. Another calibration with samples was performed along with an empty reference vessel to determine the amount of DDI water required as a reference. Samples were scanned with a reference vessel containing DDI water at a heating rate of 1.0 °C/min over a temperature range of 20-90 °C.

5.3.11 Statistical Analysis

At least two replications of all treatments were performed. Data were analyzed using an analysis of variance (ANOVA) procedure. General linear model was applied with further analysis using Tukey's test to determine differences ($p < 0.05$) between treatment means (SPSS for Windows, version 10.0, SPSS Inc., Chicago, IL). Due to

the range in magnitude of data obtained (the difference between highest and lowest values was more than 10 times), logarithmic transformation of the surface hydrophobicity (S_o) values was performed prior to statistical analysis.

5.4 RESULTS AND DISCUSSION

5.4.1 Protein Solubility

Salt (0.6 M NaCl) substantially enhanced protein solubility of conventional surimi (CS) (Table 5.1). However, this observation was not found in FPI (fish protein isolates prepared using pH-shift) at all IS levels. The data revealed that sample treated at pH 11/IS 10 showed the highest protein solubility in water and no significant difference from CS. The reduced solubility in both water and salt solution at IS 150 and 400 mM compared to 10 mM was noted. Since twice washed mince was used to prepare FPI, a certain amount of sarcoplasmic proteins was already leached out. Thus, the major proteins involved in this experiment were myofibrillar proteins.

At neutral pH, an intermolecular salt linkage is likely to be formed between acidic (i.e. glutamic and aspartic acid residues) and basic (i.e. lysine and arginine residues) amino acids resulting in a water insoluble aggregate of myofibrillar proteins. When salt is applied, the salt ions will bind to the oppositely charged groups exposed on the protein surface and rupture the intermolecular salt linkages among the proteins, thus increasing the affinity for water (Niwa and others 1986).

However, the salt also destabilizes the protein molecular structure to thermal denaturation (Wu and others 1985; Park and Lanier 1990). Kim (2002) reported that NaCl did not significantly enhance the solubility of acid-treated myofibrillar protein isolate compared to its solubility in buffer, pH 7 without NaCl. Isolation of rockfish muscle proteins at either pH 2.5 or 11 induced denaturation and aggregation of both sarcoplasmic and myofibrillar proteins (Yongsawatdigul and Park 2004). The results of total SH content (Fig. 5.3) and SDS-PAGE (Figs. 5.4a and 5.4b) possibly imply that disulfide linkages stabilized the protein aggregates in pH-shifted protein isolates

Table 5.1: Protein solubility of PW protein isolates prepared at various pH and IS conditions and conventional surimi (CS). All treatments were adjusted to pH 7.0 before analysis.

Conditions at protein solubilization step		Protein recovered at the pI	IS of gel at pH 7.0** (mM)	Solubility (mg/g dry basis)	
pH*	IS (mM)			DDI water	0.6 M NaCl
3	10	5.5	44	56.26 ± 4.62	67.71 ± 5.40
	150	5.5	154	39.25 ± 3.28	34.04 ± 3.45
	400	4.5	380	41.14 ± 3.27	40.99 ± 3.87
11	10	5.5	49	107.01 ± 5.63	79.04 ± 2.03
	150	5.5	172	37.45 ± 0.66	35.18 ± 1.52
	400	4.5	302	26.86 ± 0.25	37.61 ± 3.49
CS	38	-	425	94.62 ± 2.52	309.18 ± 11.08

Note: * All treatments were adjusted to pH 7.0 during mixing with cryoprotectants.

** All gels were prepared without salt except the CS (2% NaCl).

exhibiting lower protein extractability. When NaCl was added, the balance of a net negative or positive charge on the protein molecules was changed depending on the concentration of Na^+ and Cl^- . An increase in charge density on the proteins would strive to separate them, allowing water molecule to bind with the proteins (Kristinsson and Hultin 2003c). However, if proteins themselves possess very low IS (low charge density) and exposed hydrophobic groups (higher S_o , Fig. 5.2) due to the chemical denaturation, the excess of Na^+ or Cl^- perhaps causes the electronegative interactions among the protein molecules enhancing the hydrophobic interactions. As a result, lower protein solubility was detected.

Wu and others (1991) reported a decrease in protein extractability from washed fish mince as the concentrations of salt increased up to 26 mM. Chicken-breast muscle with very low IS (washed twice with 10 parts of water) exhibited lower water holding capacity when salt was added to control IS at physiological condition (150 mM) (Kristinsson and Hultin 2003c).

5.4.2 Fracture Analysis

Texture properties of gels are presented in Figure 5.1. It is well known that protein solubilization is a prerequisite step to protein gelation, however, solubility may not be a sufficient indicator of gel forming ability of low and high pH-treated protein isolates (Kim 2002). This phenomenon was also recognized in our results. At both pH levels, the better gel quality was obtained at IS 150. At each IS level, texture qualities of gel made from FPI prepared at pH 11 were superior to those of gel obtained from pH 3-treated FPI. Gel strength of treatment at pH 11/IS 150 was comparable to CS ($p \geq 0.05$), but its gel strain was slightly lower than CS. A study by Kim (2002) on the effect of salt addition during gel preparation on gel qualities of acid- and alkali-treated FPI showed that better gels were obtained without salt. On the other hand, gel rigidity and gel strength of conventional Alaska pollock surimi increased as the IS increased (0 to 2 %). However, the opposite trend was found when the IS was increased up to 3%.

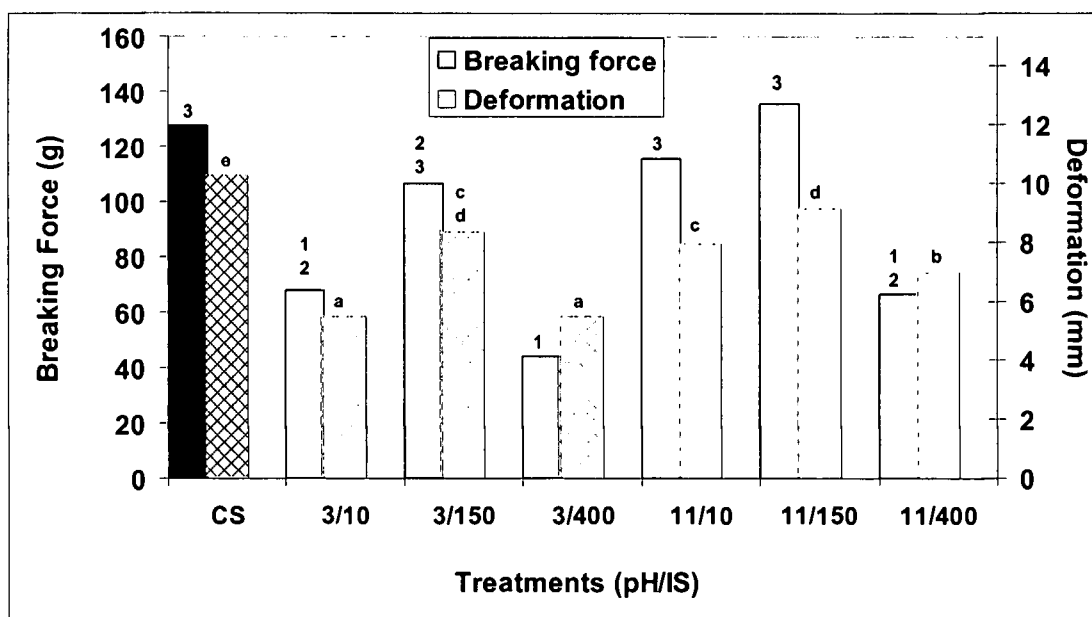


Figure 5.1: Texture properties of gels prepared from PW protein isolated at various pH and IS conditions and conventional surimi. All treatments were adjusted to pH 7.0 before analysis. Different numbers (breaking force) or letters (deformation) on each bar represent significant differences ($p \leq 0.05$).

5.4.3 Surface hydrophobicity (S_o)

It was interesting to see that CS demonstrated the lowest S_o value ($p < 0.05$) although its IS was comparable to the refolded acid- or alkali-treated FPI at IS 400 (adjusted to pH 7.0) (Fig. 5.2). This indicated that chemical solubilization and precipitation of the proteins, even at very low IS (10 mM), caused more protein unfolding than CS. Similar findings have been reported for cod myosin (Kristinsson and Hultin 2003b) and rockfish muscle proteins treated at low and high pH regardless of IS (Yongsawatdigul and Park 2004). Changed protein conformation induced by chemical denaturation might not be fully refolded to its native form. Our study using Raman spectroscopy revealed that the Raman spectra of pH 11-treated rockfish protein isolates were significantly different from the CS even though the pH was adjusted to 7.0 before analysis (Thawornchinsombut and others 2004). More exposed tyrosine residues and peptide backbone stretching were proposed for alkali-treated FPI. An increase in IS from 10 to 150 mM NaCl dramatically increased ($p \leq 0.05$) protein

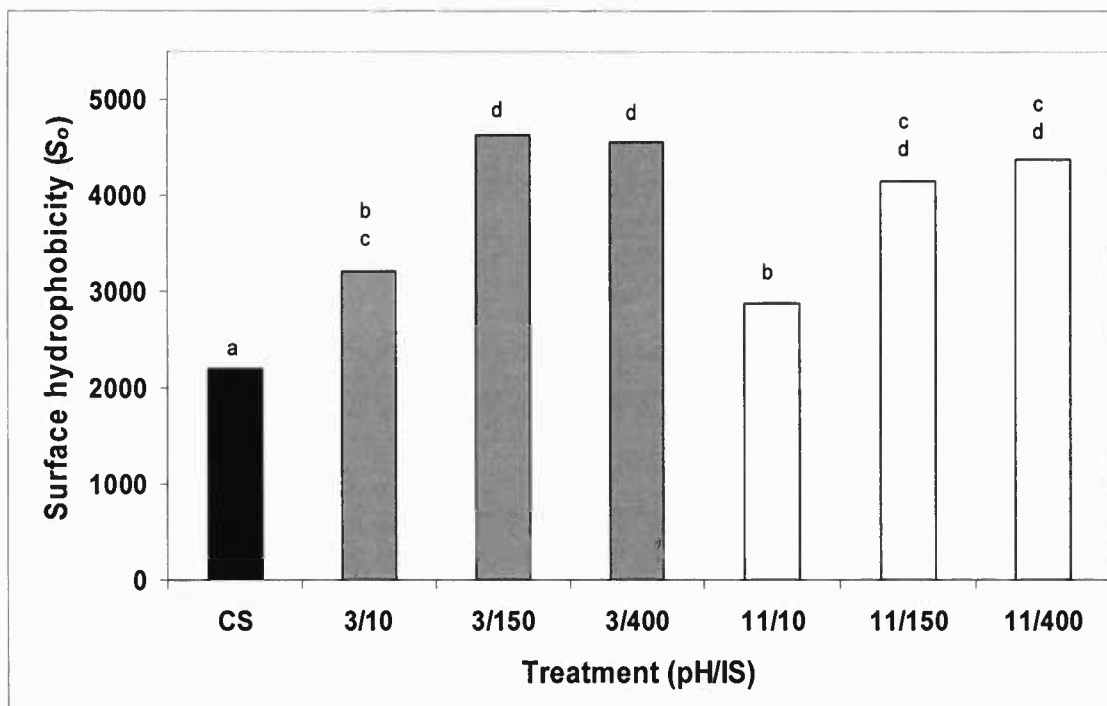


Figure 5.2: Surface hydrophobicity of PW protein isolates prepared at various pH and IS conditions and conventional surimi. All treatments were adjusted to pH 7.0 before analysis. Different letters on each bar represent significant differences ($p \leq 0.05$).

unfolding. However, when the IS was increased to 400 mM, no significant change of S_o was noticed (Fig 5.2). In addition, no significant difference was found between acidic and alkaline treatments at the same IS levels.

Under high IS (>0.3 M NaCl) and high pH (>7) conditions, dissociation of myosin occurred (Kaminer and Bell 1996; Godfrey and Harrington 1970a, b; Weeds and Pope 1977). Under the pH-shift treatment the conformation of the myosin head did not revert to its native state upon refolding (Kristinsson and Hultin 2003a). This might have contributed more available hydrophobic patches on the protein molecules for probe bindings. Moreover, the greater ability of PW muscle proteins to bind ANS at neutral and alkaline conditions could be promoted by salt ions (Thawornchinsombut and Park 2004c,d). Electrostatic interactions possibly strengthen the hydrophobic interactions between ANS and proteins (Haskard and Li-Chan 1998).

5.4.4 Total SH Content

Figure 5.3 illustrates total SH contents analyzed from paste samples. Under alkaline conditions and all IS levels, the total SH content was significantly lower than that of CS. It probably indicated that SH groups in these samples became more susceptible to oxidation resulting in disulfide bond formation in the gel network. The lower SH content of alkali-treated FPI corresponded to their higher gel qualities (Fig. 5.1) compared to those of acid treatments. Kim and others (2003) and Yongsawatdigul and Park (2004) also found a correlation between gel qualities and SH content and concluded that disulfide linkages play a more important role in gelation of alkali-treated FPI than acid-treated FPI. Changes of IS had no significant effect on SH content. These results were concomitant with protein solubility in solvent containing urea and SDS, regarding the presence of β -ME, as described in the SDS-PAGE results.

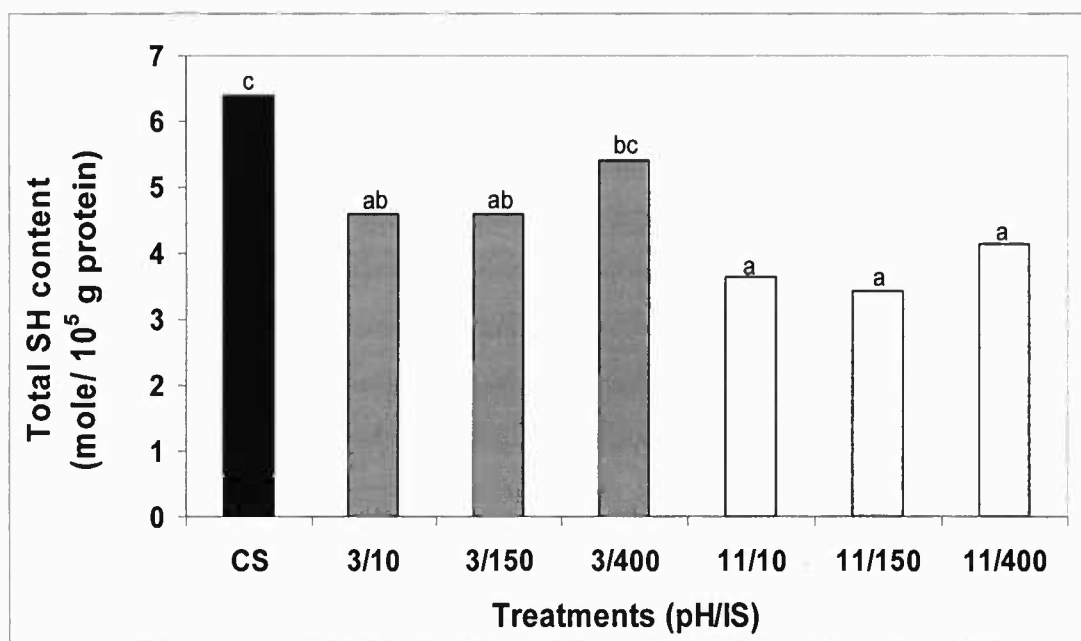


Figure 5.3: Total SH content of PW protein isolates prepared at various pH and IS conditions and conventional surimi. All treatments were adjusted to pH 7.0 before analysis. Different letters on each bar represent significant differences ($p \leq 0.05$).

Similar findings of PW muscle proteins were reported in our previous research (Thawornchinsombut and Park 2004 c,d). At the higher pH values, thiol oxidation reactions between exposed $-SH$ or S^- groups can occur in addition to disulfide interchange reaction (Watanabe and Klostermeyer 1976). At pH 9 and 11, polymerization of whey proteins by intermolecular disulfide bonding occurred at room temperature, while at pH 3, 5, and 7, polymerization was observed only at higher temperatures (Monahan and others 1995).

5.4.5 SDS-PAGE

For the SDS-PAGE study, after solubilizing with SDS containing urea solution, samples were then treated with and without β -mercaptoethanol (β -ME) to investigate disulfide bond interactions in the gel polymers. To confirm the presence of disulfide bond-forming polymers, samples were homogenized with SDS solution containing urea with and without β -ME. In the solvent without β -ME, less protein content was noted in pH 11-treated samples and/or higher IS (data not shown), while in solvent with β -ME, less undissolved gel particles were observed in all treatments. In both solvents, CS exhibited the highest protein solubility.

No MHC band was visualized in all non-reduced treatments except CS. High MW bands (> 205 kD) were seen in all samples (Fig. 5.4a). These high MW proteins possibly resulted from both pH/IS treatments and frozen stored raw materials. Several studies have demonstrated that protein denaturation during frozen storage resulted in lower extracted proteins, especially myosin, with a decrease in SH groups and an increase in surface hydrophobicity (Owusu-Ansah and Hultin 1987; LeBlanc and LeBlanc 1992; del Mazo and others 1999; Sultanbawa and Li-Chan 2001). Nevertheless, non-disulfide covalent bonds catalyzed by the transglutaminase enzyme might have been involved. In FPI samples, less protein polymers were extracted resulting in smaller high MW polymer bands predominantly for alkali-treated treatments (Fig 5.4a).

After reduction with β -ME, MHC bands appeared in all treatments with the > 205 kD MW bands right above MHC (except the CS) suggesting that the protein

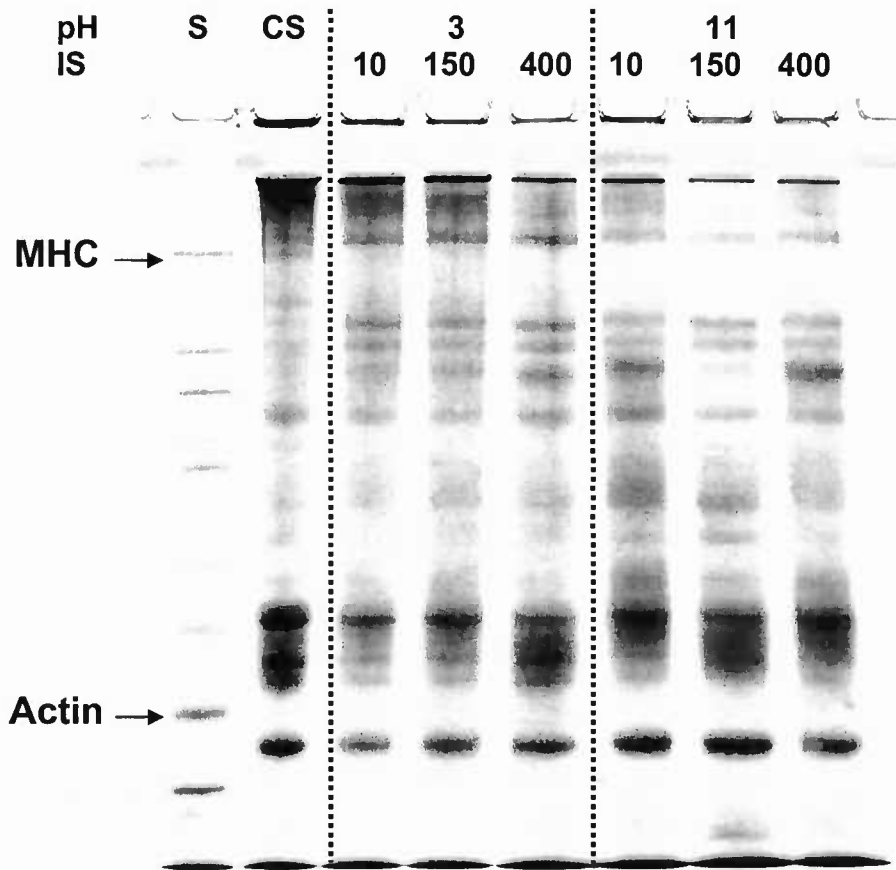


Figure 5.4a: SDS-PAGE patterns of gels made from PW protein isolates prepared at various pH and IS conditions and conventional surimi solubilized in buffer *without* β -ME (3.5/7.5% stacking/separating gel, 30 ug protein/well). All treatments were adjusted to pH 7.0 before analysis. S: standard marker proteins; MHC: myosin heavy chain.

polymer in pH and IS controlled treatments were more stable. In pH/IS-treated samples, disulfide linkages possibly enhance gel strength but reduce gel strain compared to CS. It has been noted that disulfide bonds reduce the flexibility of a protein. In the case of soy protein, disulfide bonds not only limit molecular flexibility but also restrict foaming (Kinsella and others 1994). McGuffey and Foegeding (2001) investigated the physical properties of particulate whey protein isolate gels under varying electrostatic conditions and proposed that disulfide bond formation affected strain values of gels.

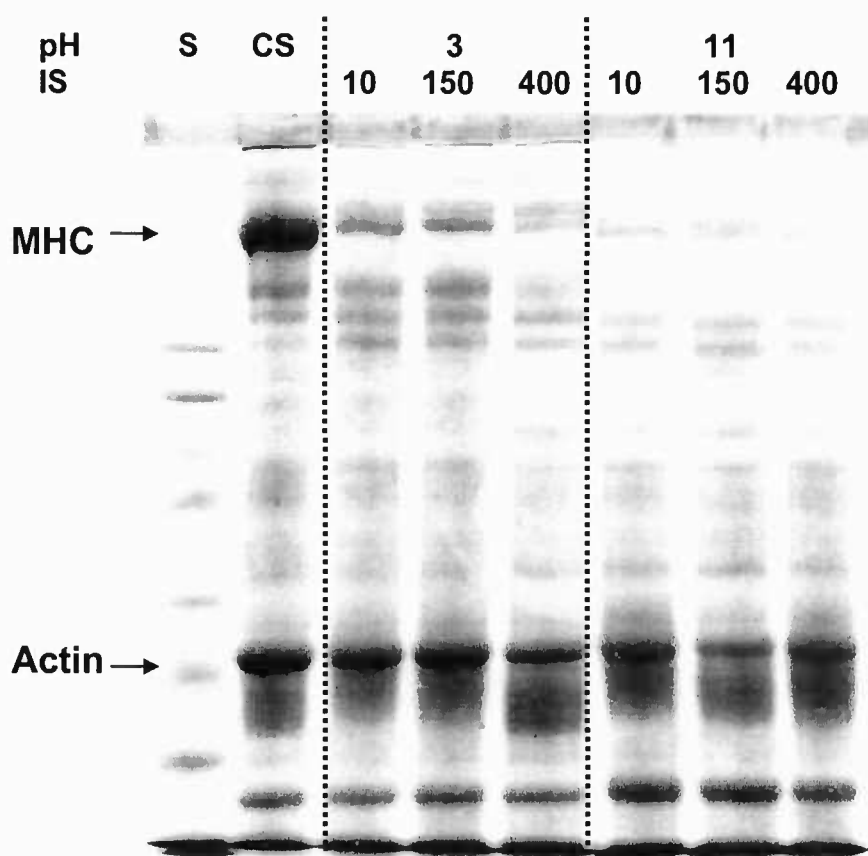


Figure 5.4b: SDS-PAGE patterns of gels made from PW protein isolates prepared at various pH and IS conditions and conventional surimi solubilized in buffer *with* β -ME (3.5/7.5% stacking/separating gel, 30 ug protein/well). All treatments were adjusted to pH 7.0 before analysis. S: standard marker proteins; MHC: myosin heavy chain.

In acid- and alkaline-aided processes, myofibrillar protein degradation was significantly more pronounced after acidification than alkalization (Undeland and others 2002; Kim and others 2003). Cortes-Ruiz and others (2001) also reported proteolytic activity in acid produced proteins from Bristly sardine. Cathepsins L and B were found to be active in whiting fish. However, only cathepsin L, which still remains after the washing steps of the conventional surimi process, causes the gel-weakening phenomenon (An and others 1994). Choi and Park (2002) discovered that cathepsins were retained along with the acid-treated PW myofibrillar proteins. Alkali-treated PW proteins prepared at pH 10.5 showed the highest activities of cathepsin L-like enzymes, while cathepsin B-like enzymes appeared to be highly

activated during acid treatment (Kim and others 2003). Nevertheless, in the present study, no MHC degradation was found in all treatments as evidenced by SDS-PAGE of reduced sample at 10% gel (data not shown). This might result from twice washing the fish mince before acidification or alkalization.

5.4.6 Rheological Properties

Storage (G') and loss (G'') modulus achieved by dynamic viscoelasticity measurements represent the elasticity and viscosity, respectively, of a viscoelastic body (Nakagawa 1978; Sano and others 1988). The loss tangent ($\tan \delta$; G''/G') reflects the relative contribution of each to the overall rheological characteristics. In this research we discussed the results focusing on G' more than G'' and loss tangent due to its greater accuracy (Egelandsdal and others 1986a).

In Figure 5.5, the rheogram of CS during gelation is represented. Several studies have reported a similar pattern of rheograms from fish myosin, actomyosin, and surimi (Wu and others 1985; Visessanguan and others 2000; Benjakul and others 2001; Esturk 2003; Kim and others 2003). The rheograms of pH-treated proteins were uniquely different compared to CS although the final pH of all samples was set at 7.0. The conformations of acid- or alkali-treated proteins were partially unfolded and then were refolded by pH adjustment to neutrality (Kristinnsson and Hultin 2003a; Thawornchinsombut and others 2004). However, these proteins seemed to exhibit a rheological behavior resembling the native protein under various IS conditions (Sano and others 1990a). At each IS level, the G' thermograms of pH 11-treated samples were relatively consistent to that of pH 3-treated samples with slightly to moderately higher magnitude over the temperature range. Nevertheless, the transition temperatures of the samples between 20 and 55 °C appear to be different when the IS was changed.

At 20 °C, the storage modulus was highest at IS 10 mM (Fig. 5.5). In the low IS condition, the refolded protein molecules assemble and form filaments as a result of the polar bindings among the tail portions with the head portions. This probably led to

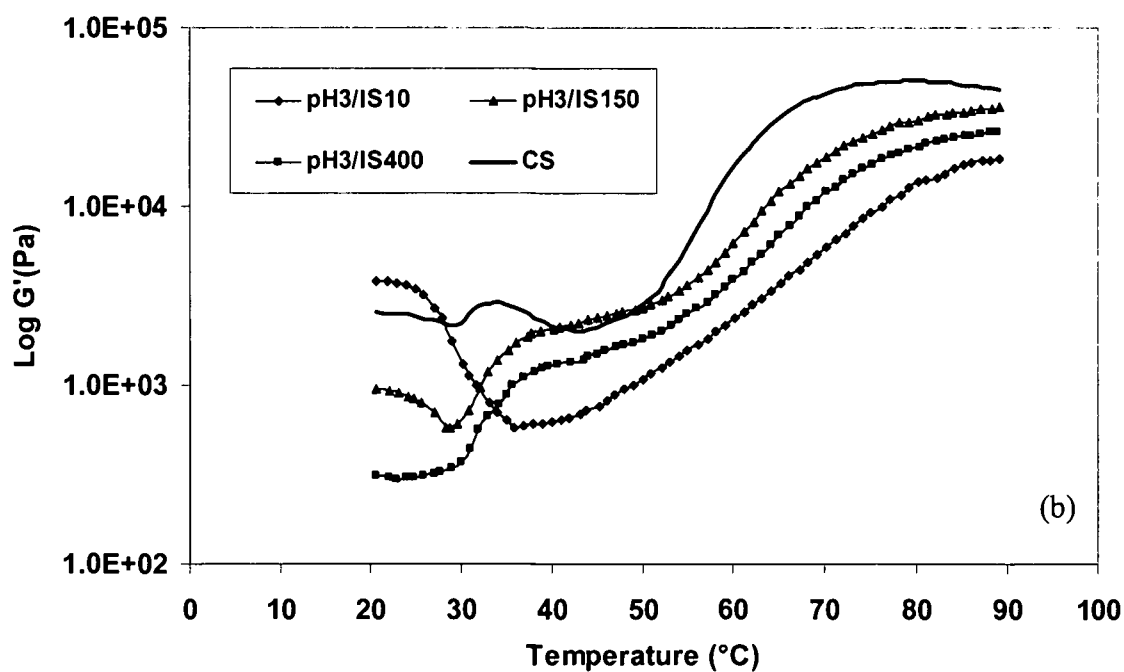
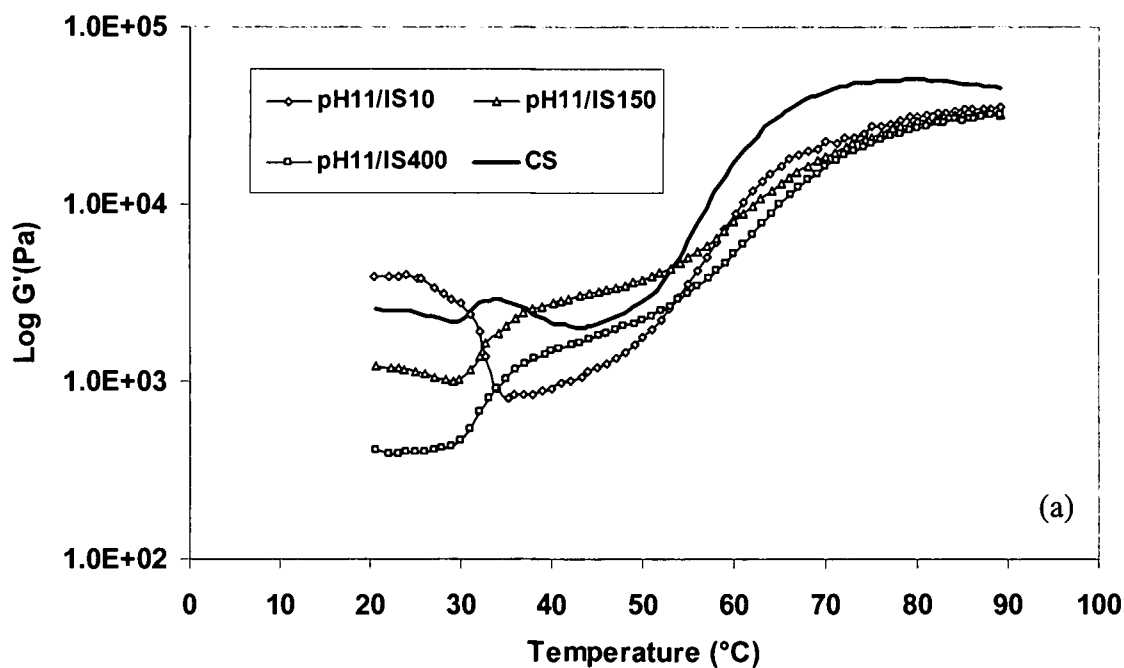


Figure 5.5: Changes in storage moduli ($\log G'$) as a function of temperature of PW protein isolates prepared at various pH and IS conditions: (a) pH 3, (b) pH 11, and conventional surimi. All treatments were adjusted to pH 7.0 before analysis.

the myosin molecules being resistant against rheological changes (Sano and others 1990a). G' decreased as the temperature reached around 36 and 34 °C for acid- and alkali-treated samples, respectively. As the temperature increased, the polar bindings among the tail portions became unstable (Tanford, 1980) yielding a decrease in G' due to an increase in the mobility of the molecule. After reaching the onset point, G' showed a considerable increase as the system takes on a more elastic structure. The alkali-treated samples possessed a higher G' at most temperatures in the range of this study than the acidic treatments corresponded to higher gel quality.

As IS increased, the G' at 20 °C decreased. When IS is raised, especially more than 0.3 M, myosin molecules disperse individually and exist as monomers contributing lower intrinsic viscosity (Dreizen and Gershman 1970; Gershman and Dreizen 1970). This phenomenon is probably also applied to the unfolded/refolded proteins.

The G' thermogram for T range of 20 to 34 °C of proteins prepared at IS 150 mM resembled the thermogram obtained from CS. Nevertheless, the thermograms from 34 to ~55 °C showed different patterns from CS probably due to the fact that their protein conformations were altered by pH adjustments (unfolding and refolding).

A sharp increase in the storage modulus upon increasing the temperature around 29-38 °C was noticed in both IS 150 and 400 treatments. This change of G' has been proposed to result from aggregation of the unfolding head and hinge portions (Ishioroshi and others 1982). G' continuously increases and shows another onset temperature around 55 °C.

As shown in Fig. 5.5, the second decline in G' was not observed in pH-shifted treatments as found in CS (34-43 °C). This weakening G' value was postulated to be due to the dissociation of the actin-myosin complex (Egelandsdal and others 1986b; Sano and others 1994) and helix-to-coil transformation of the myosin tail (Sano and others 1988). The disintegration of actomyosin during the pH and IS alteration in FPI processes was probably irreversible.

Egelandsdal and others (1986b) observed that there was no decrease in G' at 50-55 °C in ATP (adenosine triphosphate) added beef myosin compared to the control.

ATP has been reported to increase the solubility of myosin in meat by dissociating the myosin-actin complex (Hamm 1970; Bagshaw 1982). Furthermore, there were no measurable ATPase activities of PW proteins isolated at acidic pH (Choi and Park 2002). Kristinsson and Hultin (2003a) discovered the dissociation of myosin light chain in acid-treated cod myosin after refolding at neutrality while only half of the light chains were dissociated in alkali-treated samples. The ATPase activity begins to decrease at approximately pH 9 as a result of the denaturation of the myosin molecule as well as loss of the alkali light chain (Pearson and Young 1989).

Above 55 °C, a steady increase of G' was noted in all treatments (Fig. 5.5). The rigidity of the previously formed elastic network is enhanced as aggregation continues (Sano and others 1990 a,b). Whereas tail-tail interactions through cross-linking had been predominantly involved at lower temperatures, the globular head portion (head region of heavy-meromyosin, HMM-S1) of myosin assumedly plays a role above 60-70 °C (Taguchi and others 1987; Sano and others 1990 a,b). However, the controversy of this mechanism had been reported by Samejima and others (1981), Ishioroshi and others (1982), Egelandstad and others (1986a), and Sharp and Offer (1992).

The thermograms at high temperature range were less dependent on pH and IS (Figs. 5.5a and 5.5b). This finding might be supported by Samejima and others (1981) and Ishioroshi and others (1982). They found that heat-induced gelation of isolated S-1 was independent of pH and IS.

5.4.6 Thermodynamic Properties

In general, it is known that differential scanning calorimetry (DSC) is a useful technique to determine the thermodynamic data of protein denaturation temperature and energy under various circumstances. Fig. 5.6 shows the DSC thermograms of CS and pH/IS treated samples in the sol state. Only CS was added 2% NaCl while DSC analysis was performed. The DSC thermogram of CS demonstrated 5 endothermic peaks. According to several literatures (Hastings and others 1985; Beas and others 1990; Ogawa and others 1993; Fernandez-Martin and others 1998; Herrera and others

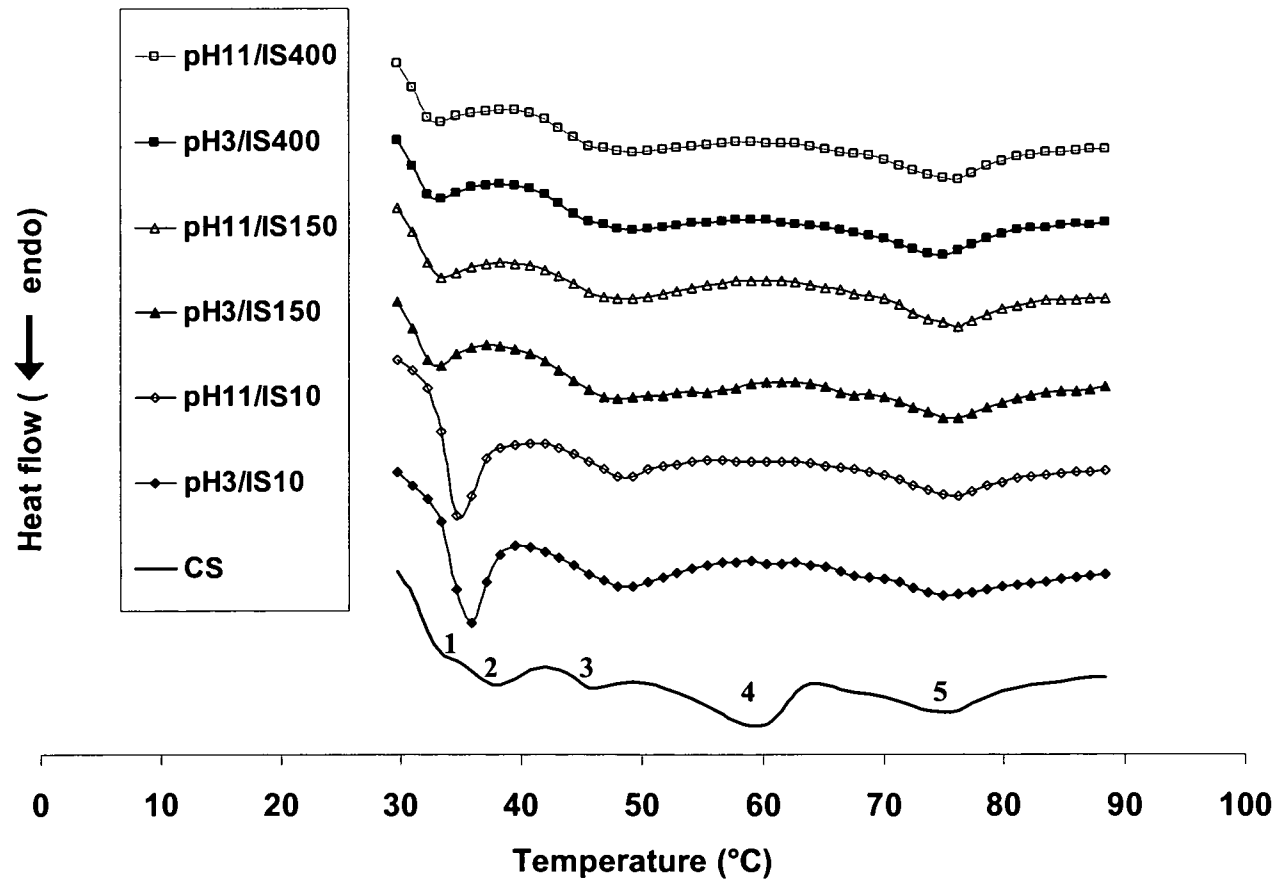


Figure 5.6: DSC thermograms of PW protein isolates prepared at various pH and IS conditions and conventional surimi. All treatments were adjusted to pH 7.0 before analysis.

2001; Badii and Howell 2003), we presumably assigned those peaks corresponding to different protein compositions as follows: (1) “32.4°C” combined peak of first myosin and collagen transitions, (2) “37.8 °C” second myosin transition, (3) “45.7 °C” third myosin transition, (4) “60.2 °C” actin transition, and (5) “75.7 °C” supposed sarcoplasmic proteins or protein polymers through frozen storage. A similar pattern was obtained from PW surimi (2% NaCl) with only 4 transition temperatures (T_m) at 33, 38, 46.3, and 62.7 °C, respectively (Esturk 2003).

Different T_m values have been reported by other investigators. Hsu and others (1993) reported that the DSC thermogram for whole muscle of PW revealed two endothermic transitions, with T_m values of approximately 45.5 and 75.0 °C, respectively. T_m of myosin and actin of fresh hake (*Merluccius hubbis*) (Beas and others 1990), blue whiting (*Micromesistius poutassou* Risso) (Fernandez-Martin and others 1998), and cod (*Gadus morhua*) (Hastings and others 1985) muscles were 46.5 and 75.3, 45 and 75, 44.5 and 74.7 °C, respectively. Those T_m values were not consistent with Esturk (2003), who reported T_m values at 41.2 and 68.8°C for PW surimi without salt analyzed using the micro DSC III, Setaram, in our lab. This discrepancy was because of the differences in heating rates (10°C/min vs 1 °C/min) applied to DSC analysis. Ogawa and others (1993) compared the DSC thermograms of myosin from different species and found that rabbit and horse mackerel myosin showed only one major peak. The DSC curves of myosin of sardine, stone flounder, walleye pollock, sea bream, and carp had two peaks, whereas those of rainbow trout, greenling, bigeye tuna, and yellow tail showed three peaks.

All pH-shift with IS controlled treatments exhibited only 3 endothermic peaks at temperatures around 32.6-35.5, 47.5-48.3, and 75.5 °C (Fig. 5.6). The second transition possibly included two myosin domains. However, the actin peak completely disappeared. A similar trend was also observed in our another research (Thawornchinsombut and Park 2004b). The increase in IS of protein homogenate followed by protein precipitation at the pI probably play an important role on the irreversible denaturation of these two peaks, since they also disappeared in sample prepared at pH 7/IS 400 (data not shown). Nevertheless, actin seemed to be more

stable than the second myosin transition peak because for the pH 7/IS 10 sample (precipitated at pH 5.5) the actin peak was seen but merged with the last peak (data not shown).

The thermogram of acid-treated herring muscle (pH 4) showed that the myosin and actin transitions were almost completely lost but only one low broad peak of possible myosin was seen when herring was soaked in 14% salt (~1.5 M) and pH 4. Myosin and actin were recovered with higher T_m after dialysis (Hastings and others 1985). However, in the present study, it should be noted that only parts of myosin were recovered but not actin. The presence of the peak at 75.7 °C in all samples was probably derived from protein aggregates or irremovable sarcoplasmic proteins due to frozen storage of the raw material (PW mince mixed with 10% sorbitol, stored at -80 °C for ~ 5 months) since this T_m was unnoticed in pH-shifted samples regardless of IS control prepared from fresh PW (Thawornchinsombut and Park 2004b) or commercial PW surimi (Esturk 2003). This protein peak seemed not to be susceptible to denaturation by pH-shift and salt.

Freeze-dried sarcoplasmic proteins extracted with deionized water from rockfish showed a major endothermic peak at 74.2°C and at around 70°C when 3 and 5 % was added into Alaska pollock surimi, respectively (Kim 2002). Badii and Howell (2003) determined the changes of cod collagen in the presence of fish oil under frozen storage. The T_m did not significantly change, however, the enthalpy increased by 8.7% and an extra peak was observed. There have been numerous reports of a relationship of lean species of the gadiform order (whiting, hake, cod, pollock, etc.) between changes in muscle texture and myofibrillar protein extractability and the formation of formaldehyde and dimethylamine from trimethylamine oxide (TMAO) during frozen storage (Haard 1992). Depending on species, conditions, and time of storage these proteins will be less extractable in sodium dodecyl sulfate (SDS) or SDS plus mercaptoethanol, and eventually, a residue nonextractable in these solutions can be obtained (Tejada and others 1996; Careche and others 1998; del Mazo 1999).

In contrast to the last peak, the 1st myosin peak of FPI samples was sensitive to a change of environment. Both enthalpy and T_m of this peak decreased when IS of the protein environment increased (Fig. 5.6).

5.5 CONCLUSIONS

Based on the results from SDS-PAGE, rheological measurements, and DSC, new information of the influence of ionic strength on the functional properties of FPI was obtained. Protein solubility did not contribute any significance to the gelation properties of FPI prepared by pH-shift. Better gel quality was achieved at alkaline pH and near physiological IS (150 mM). Increasing or decreasing IS from this level lowered gel quality. Gels of FPI prepared particularly at high pH contained more disulfide bonds than that of CS, probably resulting in reduced elasticity. The refolding step by adjusting pH of FPI to neutral did not fully convert unfolded proteins to the refolded stages. More protein unfolding was observed in FPI than CS even at lower IS. Dynamic tests and DSC analysis revealed that heat-induced gelation mechanisms of various pH/IS treated FPI were similar under the same IS conditions regardless of pH level. The significant differences of protein characteristics were more influenced by IS than pH as evidenced by rheological and thermodynamic data.

CHAPTER 6

FROZEN STABILITY OF FISH PROTEIN ISOLATE

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6.1 ABSTRACT

To characterize the stability of alkali-treated Pacific whiting protein isolate (AKPI) during frozen storage as affected by cryoprotectants and pH with regards to gelation properties, salt extractable protein (SEP), surface hydrophobicity (S_o), and total sulfhydryl content, and differential scanning calorimetry. No significant difference in gel texture was observed between AKPI stored at pH 5.5 and 7.0 during frozen storage. The highest gel texture was found for samples frozen at pH 5.5 (5) and 7.0 (7) with cryoprotectants (C) and without freeze/thaw (F), while lowest gel texture was obtained from frozen/thawed samples without cryoprotectants (NC). 5NC-F and 7NC-F demonstrated the lowest S_o and total SH perhaps suggesting more protein aggregation through hydrophobic interactions and disulfide bonds. Slightly lower T_m of the peaks of AKPI kept at pH 5.5 than at pH 7 was noticed in DSC thermograms. AKPI, whether kept at pH 5.5 or 7.0, requires cryoprotectants to maintain frozen stability.

Keywords: Alkali-treated protein isolate, pH, frozen storage, surface hydrophobicity, DSC

6.2 INTRODUCTION

Pacific whiting is primarily used for surimi manufacturing in the Pacific Northwest. Conventional surimi is prepared with water leaching of mechanically separated fish muscle followed by the addition of cryoprotectants to improve protein stability during frozen storage (Lee 1984). The leaching process removes unnecessary components such as sarcoplasmic proteins and fat. In accordance with conventional muscle chemistry, denaturation during surimi processing must be avoided.

A novel process for fish protein isolate has been investigated to improve gelation properties and yield using acid- or alkali-aided treatment. This new method unfolds fish proteins using pH-shift to maximize solubility. Recovery of all proteins is subsequently obtained at the isoelectric point (pI) by centrifugation. Unlike the conventional process, sarcoplasmic proteins remain along with the myofibrillar proteins. It was noted that the acid or alkaline solubilization process with Pacific whiting yielded 20% more than the conventional 3-washing process (Choi and Park 2002). Fish proteins solubilized at alkaline pH (10-11) and subsequently recovered at the pI, exhibited better gel quality than those prepared from acid solubilization and the conventional process, respectively (Kim and others 2003; Yongsawatdigul and Park 2004).

Structural changes occurring during frozen storage of surimi lead to protein denaturation and subsequent loss of gelling capacity. Therefore, the inclusion of cryoprotectants is required to ensure long-term frozen stability of conventional surimi (Lee 1984). Sucrose and sorbitol have been reported to protect against freeze-denaturation of Alaska pollock surimi (Park and others 1988). Sucrose is usually combined with sorbitol to reduce sweetness. The cryoprotective effect of sugar is enhanced by adding polyphosphates, perhaps by the buffering effect of polyphosphates on muscle pH and/or the chelation of metal ions (Matsumoto and Noguchi 1992). Cryoprotectant increases the surface tension of water as well as the binding energy, preventing withdrawal of water molecules from the protein, thus stabilizing the protein (Arakawa and Timasheff 1982b).

The new process for fish protein isolate, where protein structures are intentionally unfolded/refolded by pH-shift, is nearly ready for commercialization. However, it is unknown whether cryoprotectant would be required or not. Our hypothesis to verify the need for cryoprotectant would, therefore, be critical. Moreover, it has been known that most proteins are more stable at the pI than any other pHs (Damodaran 1996). Therefore, it is also of much interest to investigate a stability of fish protein isolate stored at the pI. Our overall goal was to investigate whether new fish proteins, which were recovered as chemically unfolded/refolded proteins, were affected by freezing and frozen storage. Detailed objectives were 1) to determine the effect of pH on fish protein isolate during frozen storage (pH 5.5 (pI) and pH 7.0); 2) to determine the effect of cryoprotectant on the functional properties of the fish protein isolate.

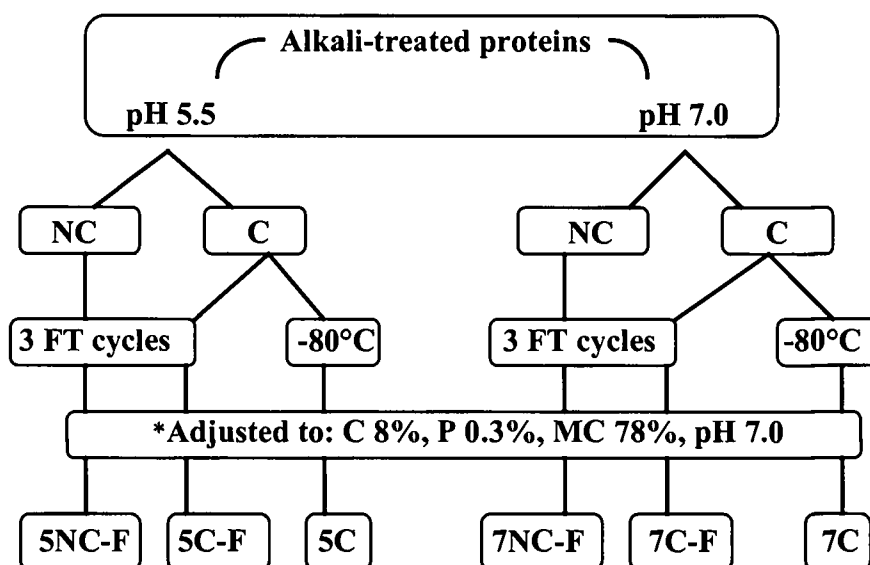
6.3 MATERIALS AND METHODS

6.3.1 Materials

Fresh whole Pacific whiting fish (PW) were transported on ice to the OSU Seafood Laboratory within 30 min. Fish were then filleted and ground into mince. Mince was used as the raw material for alkali-treated proteins.

6.3.2 Sample Preparations

Alkali-treated PW proteins (pH 11) were prepared as detailed in Kim (2002). Mince was homogenized with cold deionized (DI) water at a 1 to 9 ratio using a Power Gen 700 homogenizer (GLH 115, Fisher Scientific Inc., Pittsburgh, PA) for 2 min at speed level 2. The pH of the homogenates was adjusted using 1 and/or 2 N cold HCl and NaOH to pH 11.0. The pH measurement was conducted using a pH meter (HI 9025 microcomputer pH meter, Hanna Instruments, Inc., Woonsocket, RI) with Spear Gel Combo pH probe (Corning Incorporated Life Sciences, Acton, MA). After pH adjustment, samples were centrifuged (Sorvall RC-5B, DuPont Co., Newtown, CT) at 8,000 x g for 25 min at 4°C to separate the insoluble parts. Soluble proteins were



C : 8 % cryoprotectants (sucrose + sorbitol = 1+1)

FT cycle : freezing at -18 ± 2 °C, 18 h; thawing at 4 ± 2 °C, 6 h

P : 0.3 % sodium tripolyphosphate

MC : Moisture content

* All samples were thawed at 5 °C in order to adjust those parameters

Abbreviations of treatments:

5 = frozen storage at pH 5.5

7 = frozen storage at pH 7.0

C = with cryoprotectants, NC = without cryoprotectants

F = with 3 freeze/thaw cycles

Figure 6.1: Diagram of experimental design.

Table 6.1: The compositions of experimental treatments before subjected to freeze-thaw cycles

Treatment	Crude proteins (%)	Moisture content (%)	Sucrose + sorbitol = 1:1 (%)	Ratio of Water/Protein	Ratio of Protein/ Cryoprotectant
With C	14.7	77.0	8	5.24	1.84
Without C	16.0	83.7	0	5.24	0

Note: C is 8 % cryoprotectants (a mixture (1:1) of sucrose and sorbitol).

subsequently recovered at pH 5.5. Protein precipitates were then collected by centrifugation (4,000 x g, 20 min) at 4°C. The pH and cryoprotectant content of the experimental treatments was designed as shown in Figure 6.1. One half portion of the pellet was adjusted to pH 7.0. At both pH levels, the pellet portions were subdivided into 2 sets and mixed with 0% and 8% cryoprotectants (sucrose:sorbitol = 1:1), respectively. The ratio of water to protein of the samples with and without cryoprotectants before subjecting to freeze-thaw cycles was controlled (Table 6.1). Samples were stored at -80°C for 4 weeks before analysis. After 4 weeks, four treatments were subjected to 3 freeze-thaw cycles at -18°C and 4°C. All samples were thawed at 5 °C for 30 min before adjusting to maintain equal pH (7.0) and cryoprotectant prior to making gels. Gels were prepared with 1.5 % beef plasma protein (enzyme inhibitor). Final moisture content was controlled at 78%. Protein isolate and gel preparation were conducted in a walk-in cold room (6 °C).

Pastes were analyzed for salt extractable proteins, surface hydrophobicity, total sulfhydryl content, and thermal denaturation (differential scanning calorimeter, DSC). Gels were evaluated for textural properties.

6.3.3 Salt Extractable Proteins

A total protein extraction of protein paste was carried out in duplicate using 0.6 M KCl, buffered at pH 7.0 with 20 mM Tris-HCl. Samples were centrifuged for 10 min at 10,000 x g and the Bio-Rad protein assay with bovine serum albumin as a standard (Bradford 1976) was performed to estimate protein concentration in the supernatant (Sych and others 1990). Salt extractable protein (SEP) was reported as protein content, estimated by Bio-Rad analyses, per g (dried) protein.

6.3.4 Surface Hydrophobicity

Three grams of paste was homogenized with 27 mL of 20 mM Tris-HCl, pH 7.0, containing 0.6 M KCl. After centrifugation, protein surface hydrophobicity of the supernatant was determined using an ANS (1-anilinonaphthalene-8-sulfonic acid) probe according to the method of Alizadeh-Pasdar and Li-Chan (2000). Stock

solution of 8×10^{-3} M ANS was prepared in 0.1 M phosphate buffer (pH 7.4). Excitation/emission wavelengths of 390 nm/470 nm were used. The supernatant was serially diluted with 20 mM Tris-HCl, pH 7.0, containing 0.6 M KCl from 0.1 to 1.0 mg/mL. Four milliliters of each sample were added into 20 μ L of ANS stock solution. After 10 min, the relative fluorescence intensity (RFI) of each solution was measured. The initial slope (S_0) of the net RFI versus protein concentration was calculated by linear regression analysis and used as an index of the protein surface hydrophobicity.

6.3.5 Total Sulfhydryl Content

Total sulfhydryl group content was determined using Ellman's reagent (5, 5'-dinitrobis (2-nitrobenzoic acid); DTNB) as described by Hamada and others (1994) with slight modification. Paste was solubilized in a solution of 0.6 M KCl in 20 mM Tris-HCl buffer (pH 7.0). Protein content of the supernatant was adjusted to 1.0 mg/mL using the same buffer. The diluted sample (0.5 mL) was mixed with 2 mL of 8 M urea in 0.2 M Tris-HCl buffer (pH 7.0) and then with 50 μ L of 10 mM DTNB solution. Samples were then incubated at 40°C for 15 min before measuring absorbance at 412 nm. The SH content was calculated based on the absorbance using the molar extinction coefficient of $13,600 \text{ M}^{-1} \text{ cm}^{-1}$ (Riddles and others 1979).

6.3.6 Thermodynamic Properties

Alkali-treated protein pastes obtained from various storage conditions were subjected to a micro differential scanning calorimeter (Micro DSC III, Setaram Inc., Lyon, France). The instrument was calibrated for temperature accuracy using double deionized water (DDI) water and naphthalene. The reference calibration for all samples was performed to calculate the amount of DDI water required in the reference vessel. Sample pastes weighing around 480 ± 5 mg were sealed in a hastelloy sample vessel. Samples were then heated over a temperature range of 20-90 °C at 1 °C/min. Transition temperatures and enthalpy measurements were compared among sample treatments.

6.3.7 Gel Analysis

The sample pastes were stuffed into stainless tubes (20 mm I.D.) and then cooked at 90°C for 15 min. Cooked gels were cooled in iced water for 15 min and then refrigerated overnight. The chilled gels were set at room temperature for 2 h before fracture analysis. Gels were cut into 30 mm length and then subjected to the punch test using a Texture Analyzer (TA.XT.plus, Texture Technologies Corp., New York). A 5 mm diameter spherical probe was used as a measuring tool and the test speed was set at 1 mm/sec. Breaking force (g) and deformation (mm) required to penetrate the surface of the gel were recorded.

6.3.8 Statistical Analysis

At least two replications of all treatments were performed. Data were analyzed using an analysis of variance (ANOVA) procedure. General linear model was applied with further analysis using Tukey's test to determine differences ($p \leq 0.05$) between treatment means (SPSS for Windows, version 10.0, SPSS Inc., Chicago, IL).

6.4 RESULTS AND DISCUSSION

6.4.1 Salt Extractable Protein

Salt extractable protein (SEP) of the alkali-treated protein isolates (AKPI) kept frozen at pH 7 was 43 – 45 mg/g dry basis regardless of cryoprotectants and freeze/thaw cycles. In our preliminary study, conventional surimi showed approximately 7 times higher solubility in 0.6 M NaCl than the AKPI (data not shown). We also reported that the protein solubility of PW protein isolate at pH 11 and IS 150 mM and conventional surimi in 0.6 M NaCl were 35 and 309 mg/g dry basis, respectively (Thawornchinsombut and Park 2004a). Kim (2002) also revealed substantially low solubility of Alaska pollock proteins isolated at pH 11 in Tris buffer containing 0.6 M NaCl. A similar trend was also observed in rockfish by Yongsawatdigul and Park (2004).

Protein denaturation during frozen storage typically causes a decrease in protein solubility due to intermolecular hydrogen or hydrophobic bonds, as well as disulfide bonds and ionic interactions among protein molecules (Matsumoto 1980; Akahane 1982). It has also been recognized that gadoid species (i.e. hake, cod, whiting) tend to be more susceptible to protein denaturation during frozen storage (Haard 1992). However, in this study it should be noted that no significant difference ($p > 0.05$) was observed when severe conditions (without cryoprotectants addition and/or FT cycling) were applied (Fig. 6.2). The molecular structure of AKPI, which were chemically unfolded and refolded, might be reoriented. Thus, they no longer behave like a native protein.

Kristinsson and Hultin (2003a) reported that acid and alkali conditions each partially unfolded myosin in a different manner. These treatments led to the almost complete loss of myosin ATPase activity and exposure of more reactive sulfhydryl groups. Hydrophobicity of the acid- and alkali-treated cod myosin (Kristinsson and Hultin 2003b), and PW muscle proteins (Thawornchinsombut and Park 2004d)

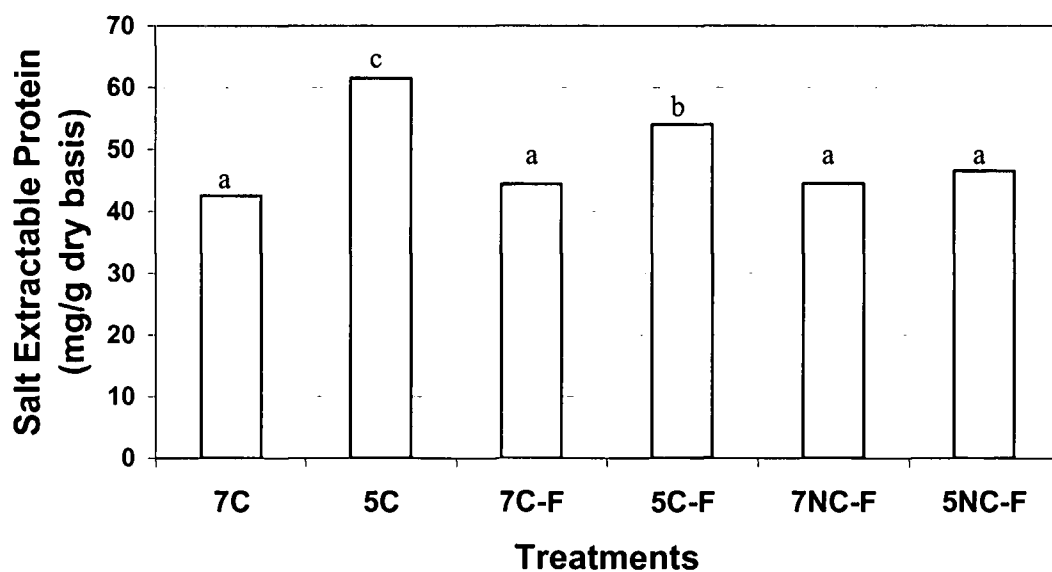


Figure 6.2: Salt extractable protein of alkali-treated PW protein isolates at various storage conditions. Different letters denote significant differences among treatments ($p \leq 0.05$). All treatments were adjusted to an equal cryoprotectant content and pH 7.0 before analysis.

refolded to a neutral pH were greater than those treated at pH 7.5 and the conventional surimi, respectively. As a result, when fish protein isolates were exposed to freezing conditions, its structure probably became more susceptible to freeze-denaturation even at -80 °C and short periods of time (4 weeks for 7C and 5C treatments). This led to more protein aggregation, which resulted in less SEP.

Altering the pH of samples kept frozen at pH 5.5 to 7 considerably increased its SEP ($p \leq 0.05$), except the 5NC-F sample. This was probably due to an increase in protein charges resulting from NaOH and sodium tripolyphosphate addition during pH adjustment. However, this effect was less predominant in 5C-F and 5NC-F treatments (Fig. 6.2). This may suggest that the freeze/thaw process (5C-F) and/or the absence of cryoprotectants (5NC-F) cause a significant degree of protein denaturation for samples kept frozen at the pI. Rabbit myosin in a glycerol-treated fiber bundle, in which the morphological features are maintained as they were in the intact muscle, when placed in medium at 3 °C and pH 5.5, showed little change in either ATPase activity or extractability, whereas isolated myosin under the same conditions underwent denaturation (Yasui and others 1973).

6.4.2 Surface Hydrophobicity

Extrinsic fluorescence ANS probe has been commonly used to study surface hydrophobicity in fish proteins. An increase in fluorescence is an indication of denaturation of a protein, with the exposure at the surface of hydrophobic groups (Mackie 1993). After FT cycles, the surface hydrophobicity (S_o) of sample kept at pH 7 with cryoprotectants (7C) increased (Fig. 6.3). It has been suggested that the initial increase of surface hydrophobicity of frozen actomyosin is due to a greater exposition of hydrophobic groups when actomyosin is denatured by freezing (Niwa and others 1986). Subsequent evolution depends on the balance between denaturation and aggregation of the proteins.

del Mazo and others (1994) reported a slight increase in S_o of natural actomyosin from hake upon freezing, which subsequently tended to diminish slightly

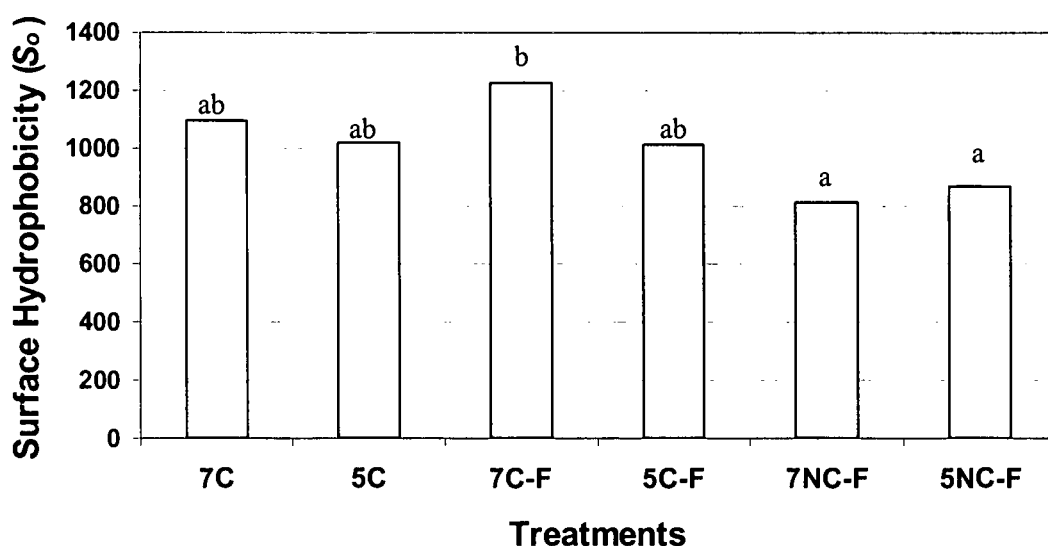


Figure 6.3: Surface hydrophobicity of alkali-treated PW protein isolates at various storage conditions. Different letters denote significant differences among treatments ($p \leq 0.05$). All treatments were adjusted to an equal cryoprotectant content and pH 7.0 before analysis.

during frozen storage. Denaturation and aggregation of hake 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). The partly unfolded tertiary structure, probably from the head region but a native secondary structure of acid- and alkali-treated proteins as evidenced by an increase in surface hydrophobicity (Kristinsson and Hultin 2003b) and the circular dichroism study (Kristinsson and Hultin 2003a), may have facilitated the intermolecular interactions leading to protein aggregation during frozen storage. Formation of formaldehyde in certain gadoid species, including whiting, is known to increase the rate of protein denaturation during frozen storage (Haard 1992; Tejada and others 1996; Careche and Li-Chan 1997). The denaturation would be mainly due to increased exposure of hydrophobic groups and this would lead to subsequent aggregation by non-covalent forces (Ang and Hultin 1989). The alkali-treated process may have greatly accelerated the formation of formaldehyde compared to that of the process at neutrality.

The pH of the storage conditions did not seem to significantly affect ($p > 0.05$) the S_o regardless of FT cycle and cryoprotectant inclusion (Fig. 6.3). In the absence of cryoprotectants, samples at both pH storage conditions (7NC-F & 5NC-F) represented the lowest S_o perhaps suggesting greater protein aggregation. It is well established that the inclusion of cryoprotectant is required to improve long-term stability of fish myofibrillar proteins during frozen storage (Takayoshi 1977; Yoon and Lee 1990; MacDonald and Lanier 1991). The data suggested that the refolded proteins obtained through the pH-shift also need cryoprotectants to preserve their molecular structures during frozen storage.

6.4.3 Total Sulphydryl Content

Another way to elucidate protein aggregation is to monitor changes in sulphydryl (SH) groups (LeBlanc and LeBlanc 1992). A change in total SH is attributed to oxidation of SH, reduction of disulfide bonds (S-S), and S-S/SH interchange reactions. Several studies provided evidence for the loss of SH groups and for the formation of S-S bonds during frozen storage (Lim and Haard 1984; LeBlanc and LeBlanc 1992; Sultanbawa and Li-Chan 2001). Furthermore, a number of studies have reported a decrease in SH content of alkali-treated proteins (Monahan and others 1995; Kim and others 2003; Thawornchinsombut and Park 2004a; Yongsawatdigul and Park 2004) and proposed that disulfide linkages play an important role in gelation of alkali-treated proteins.

In this study, total SH contents relatively corresponded to S_o values (Figs. 6.3 and 6.4). The highest and lowest SH contents were found in the 7C-F and 7 NC-F treatments, respectively ($p > 0.05$). S-S formation has been implicated as a causative agent in protein aggregation during frozen storage (Buttkus 1970; Matsumoto 1980; LeBlanc and LeBlanc 1989). It is possible to hypothesize that S-S formation also plays a meaningful role in protein aggregation of alkali-treated PW protein isolate during FT treatment when proteins were not preserved by cryoprotectants.

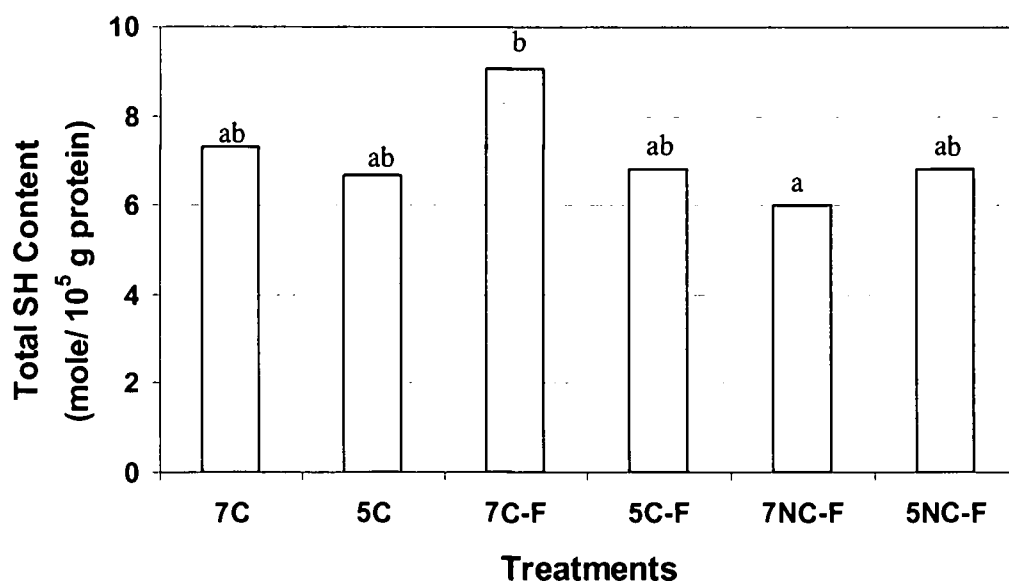


Figure 6.4: Total SH content of alkali-treated PW protein isolates at various storage conditions. Different letters denote significant differences among treatments ($p \leq 0.05$). All treatments were adjusted to equal cryoprotectant content and pH 7.0 before analysis.

6.4.4 Thermodynamic Properties

The thermogram of PW conventional surimi contained four endothermic transitions. The temperatures at which maximum heat input occurred (T_m) were 35.4, 41.2, 51.1, and 68.8 °C, respectively. The first three and the last transition were assumedly assigned to myosin domains and actin denaturation, respectively (Esturk 2003). Depending on species (Howell and others 1991; Ogawa and others 1993), the environment (pH, ionic strength) (Wright and Wilding 1984; Howell and others 1991), as well as scanning rate (Thawornchinsombut and Park 2004a), isolated myosin may show various peaks at different T_m .

AKPI, which were determined after adjusting to an equal amount of cryoprotectants and pH (at 7.0), showed different thermograms as influenced by the various storage conditions (Fig. 6.5). All samples with cryoprotectants with and without freeze/thaw treatments showed three endothermic peaks with T_m around 33.5-34.7 (P1), 46.2-47.8 (P2+P3), and 66.5-68 °C (P4), respectively (Table 6.2 and Fig.

6.5). The first peak perhaps consisted of the myosin domain and sarcoplasmic proteins. Collagens and connective tissues would also be exhibited in the first peak (Hastings and others 1985) if they were not removed through centrifugation. Badii and Howell (2003) observed the transition temperature of cod connective tissue at 34.9 °C. Peak 2 actually resulted from two endothermic transitions, as clearly visualized in treatments without cryoprotectants (7NC-F and 5NC-F) at $T_m \sim 47$ -48 and 53-54 °C, respectively (Fig. 6.5).

The T_m of P1 and P2 of fish protein isolates stored at pH 5.5 were slightly lower than those of samples kept at pH 7.0. The pH at storage, however, seemed to insignificantly affect the heat input for protein denaturation except treatments without cryoprotectants. A lower endothermic transition of P 2 for 5NC-F compared to 7NC-F

Table 6.2: Peak maximum temperatures and enthalpies for denaturation of protein components in experimental samples.

Treatments*	Parameters	P1		P2 & P3	
		Means	SD	Means	SD
7C	T_m^a	34.4	0.4	47.4	0.4
	ΔH^b	0.180	0.008	0.200	0.013
5C	T_m	33.5	0.1	46.2	0.1
	ΔH	0.189	0.005	0.180	0.005
7C-F	T_m	34.7	0.1	47.8	0.2
	ΔH	0.185	0.002	0.212	0.019
5C-F	T_m	33.9	0.2	46.3	0.7
	ΔH	0.185	0.018	0.194	0.039
7NC-F	T_m	35.2	0.3	48.2 53.2	0.3 0.4
	ΔH	0.204	0.006	0.303	0.005
5NC-F	T_m	33.9	0.1	46.7 54.0	0.3 0.4
	ΔH	0.188	0.008	0.231	0.002

Means and standard deviations (SD) were calculated based on at least 2 replicates.

* All treatments were adjusted to equal cryoprotectant content and pH 7.0 before analysis.

^a T_m = Peak maximum temperature.

^b ΔH = Enthalpy in Joules/g total protein

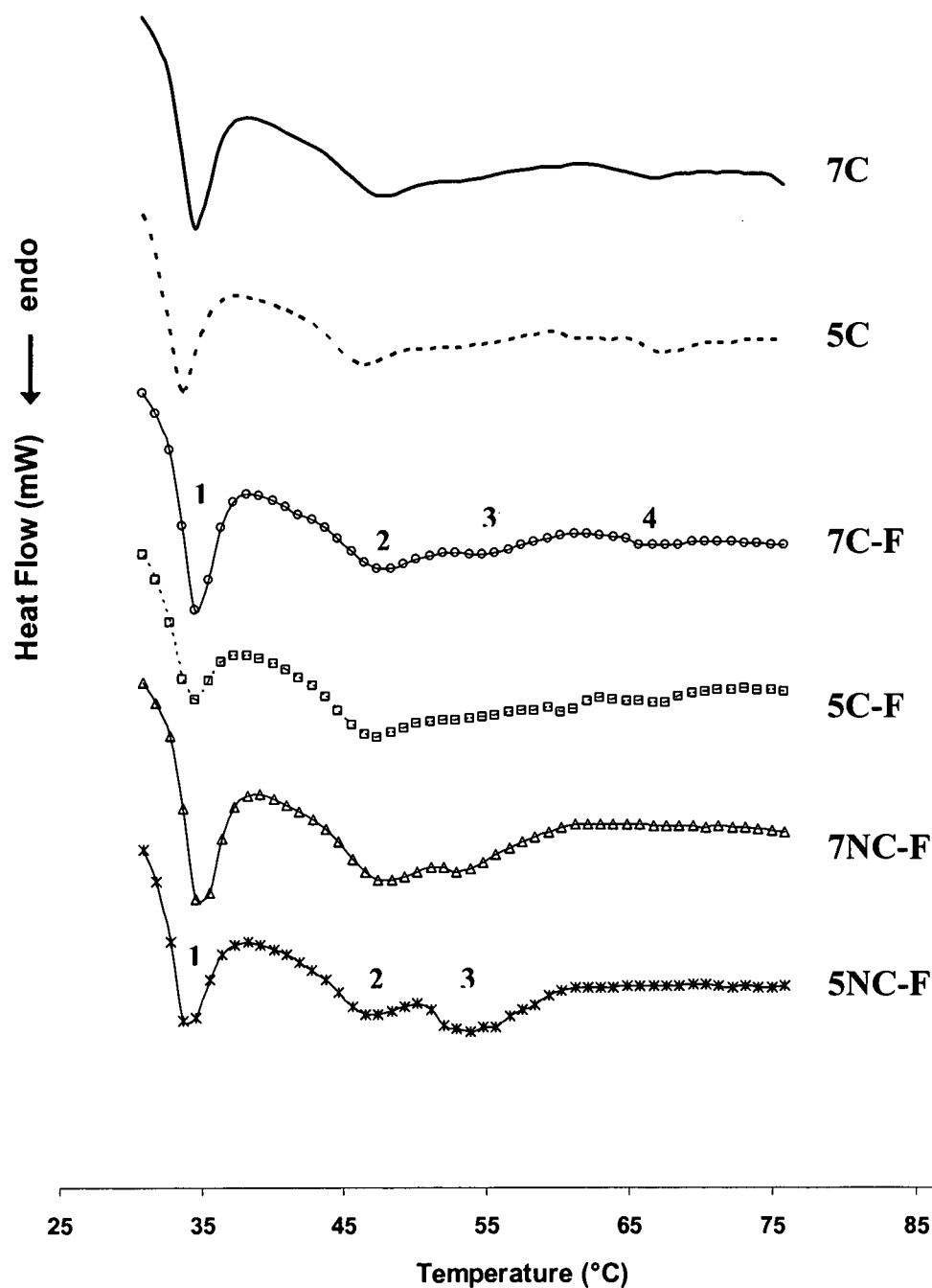


Figure 6.5: DSC thermograms of alkali-treated PW protein isolates at various storage conditions. All treatments were adjusted to equal cryoprotectant content and pH 7.0 before analysis.

was noticeable (Table 6.2). Generally, a decrease in T_m or ΔH is interpreted as a decrease in protein stability. Our results suggested that regardless of cryoprotectant inclusion and/or FT cycling factors, AKPI kept frozen at pH 5.5 were relatively less stable than those stored at pH 7.0. Interestingly, the thermogram of 7NC-F samples demonstrated slightly greater T_m and enthalpy than 7C. Moreover, a third myosin transition was distinguishable. Davies and others (1994) studied changes of the total area under the myosin peaks of red snapper and cod muscles (a warm and a cold water species, respectively). The area under the red snapper thermogram decreased with frozen storage time, while the area of the cod thermogram appeared to increase to a value similar to that of unfrozen sample after storing between 10 and 30 weeks at -20 °C. The total area of the myosin peak for cod muscle continued to increase during 30 and 54 weeks of frozen storage.

Actin was highly sensitive to the pH-shift method particularly samples without cryoprotectants. Thermograms of actin (Peak 4 in Fig. 6.5) appeared at $T_m \sim 66-68$ °C with very small endothermic transition (< 0.04 J/g) in the samples with cryoprotectants and disappeared in samples without cryoprotectants. This perhaps suggests that cryoprotectants certainly minimize the freeze-induced denaturation of actin. Howell and others (1991) reported that T_m of actin was similar in all fish species, regardless of habitat temperature, and was destabilized by increasing both pH and ionic strength.

6.4.5 Gel Texture

Texture properties of AKPI with cryoprotectants (7C, 5C) exhibited superior quality followed by samples with cryoprotectants and FT cycling (7C-F, 5C-F), and sample without cryoprotectants with FT cycling (7NC-F, 5NC-F), respectively (Fig. 6.6). Nevertheless, the pH of the storage condition did not affect the fracture properties of gel texture. The lowest breaking forces were noted in treatments without cryoprotectants with FT cycling (7NC-F & 5NC-F). These results were perhaps associated with low surface hydrophobicity and the appearance of P 3 at 53.2 (7NC-F) and 54.0 °C (5NC-F) (Table 6.2 and Fig. 6.5). Gel elasticity decreased significantly

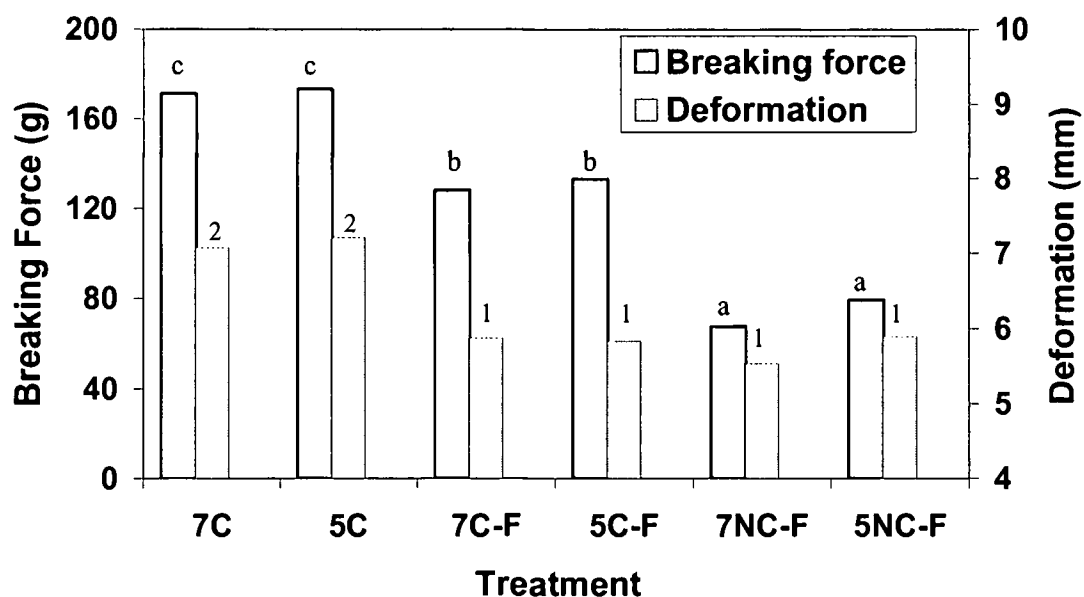


Figure 6.6: Texture properties of alkali-treated PW protein isolates at various storage conditions. Different letters and numbers denote significant differences of breaking force and deformation, respectively ($p \leq 0.05$). All treatments were adjusted to an equal cryoprotectant content and pH 7.0 before analysis.

after FT cycling was applied ($p \leq 0.05$). Freeze-induced denaturation followed by aggregation probably contributed to the considerable decrease in gel quality. However, no significant difference of deformation values was found between FT treatment with and without cryoprotectants.

6.5 CONCLUSION

Unlike conventional surimi, salt-soluble proteins extracted from fish protein isolates prepared using the pH-shift method were very low. When pH of the AKPI stored at pH 5.5 was adjusted to neutral pH, more proteins were extracted due to the change of the net protein charge. However, salt extractable protein did not seem to be related to others protein functionalities observed in this study. Surface hydrophobicities of AKPI corresponded with total SH content, suggesting that freeze-induced aggregation of protein molecules during frozen storage was facilitated by hydrophobic interactions and/or disulfide bonds, particularly samples without cryoprotectants. The factors responsible for decreased texture quality were freeze/thaw

cycles and/or the absence of cryoprotectants. Like conventional surimi, alkali-treated protein isolate also needs cryoprotectants to prevent freeze-induced aggregation during frozen storage. In the present study, DSC analysis detected that alkali-treated protein isolate kept frozen at pH 5.5 was less stable than at pH 7.0. Further finding is required to elucidate changes of these pH-shift proteins under various storage conditions at the molecular level.

CHAPTER 7**RAMAN SPECTROSCOPY DETERMINES STRUCTURAL CHANGES OF
FISH PROTEINS RECOVERED AT ALKALINE pH****S. THAWORNCHINSOMBUT, J.W. PARK, G. MENG, and E.C.Y. LI-CHAN**

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The American Chemical Society, Washington, DC, USA

7.1 ABSTRACT

Structural changes of alkali-treated rockfish protein isolate (AKPI) during frozen storage were elucidated using a Raman spectrometer and scanning electron microscope (SEM). The results were compared to conventional surimi (CS). No significant textural difference was noted between AKPI stored at pH 5.5 and 7.0. Strongest texture was found for AKPI frozen with cryoprotectants and CS, while the weakest texture was observed in AKPI frozen without cryoprotectants. SEM revealed the most discontinuity of gels for AKPI with no cryoprotectants. Storage at pH 5.5 caused more aggregated microstructure than storage at neutral pH. Raman spectral analysis demonstrated that refolding of AKPI by pH adjustment to 7.0 was achieved, but not identical to the native protein. CS showed higher α -helix content (~50%) than AKPI (~20-30%). Frozen storage induced a decrease and an increase in the α -helix of CS and AKPI samples, respectively. Alkali-treated protein isolate were slightly less stable than CS during frozen storage.

Keywords: Alkali-treated protein isolate, frozen storage, texture properties, Raman spectroscopy, SEM

7.2 INTRODUCTION

Alternative methodologies to conventional surimi processing to produce a functional protein concentrate from small pelagic fish species and other surimi resources in the US have been developed. In general, this novel procedure features a method for isolating edible protein from animal muscle by decreasing or increasing the pH of the protein slurry to a level sufficient to solubilize the protein more than 75%. Membrane lipids and other insoluble parts are removed by centrifugation. Protein is precipitated from the aqueous phase by adjusting to the isoelectric pH (Kelleher and Hultin 1999; Hultin and Kelleher 1999; Choi and Park 2002; Kim and others 2003; Yongsawatdigul and Park 2004). Although this protein isolate is refolded by re-adjusting pH to neutrality, the proteins which have undergone pH-induced treatments may have alternative structures and stability while kept under frozen condition.

Our previous study (Thawornchinsombut and Park 2004b) to investigate changes of protein functionality, conformation, and stability as a function of various frozen storage conditions indicated that protein isolate, obtained by the pH-shift method, substantially lost its gelation properties and critically required cryoprotectants to retard freeze-denaturation and aggregation. DSC thermograms showed a slight difference when proteins were stored at neutral pH and at the isoelectric pH (pI). However, there was no significant difference detected when analyzed by other methods, including total sulfhydryl content, surface hydrophobicity, and gel qualities by the punch test (Thawornchinsombut and Park 2004b). More sensitive protein analysis methods are required to monitor the changes of the alkali-treated protein isolate (AKPI).

The structural changes of protein at the molecular level can be examined by several methods such as circular dichroism, fluorescence spectroscopy, nuclear magnetic resonance (NMR), infrared absorption, and Raman spectroscopy. Raman spectroscopy can be used advantageously to determine the molecular structure of food proteins in liquid or solid form without the need to extract a purified component. This

technique yields information on the secondary structure fractions, disulfide bond conformation, and/or the aromatic side chain environment (Li-Chan and others 1994).

Due to the opaque or solid nature of many foods, changes in protein functionalities that cannot be differentiated by other methods are amenable to Raman spectroscopy. Proteins have been studied extensively using this technique. The reversible denaturation of bovine serum albumin solutions by heat, acid, and alkali were studied using the Raman spectroscopic technique (Lin and Koenig 1976). In 1997, Raman spectroscopy was used to study the *in situ* protein structure in raw, salted surimi, gel setting, and cooking (Bouraoui and others 1997).

Careche and others (2002) assessed the structural properties of aggregates formed during frozen storage of hake muscle using Raman spectroscopy and found the protein backbone adopted a conformational structure rich in β -sheets. Protein structural changes of hake muscle kept frozen at different temperature (-10 and -30 °C) were monitored using Raman spectrometer. An increase of β -sheet at the expense of α -helix structure was revealed. Raman data also indicated protein denaturation through the exposure of aliphatic hydrophobic groups to the solvent (Herrero and others 2004).

The primary objective of this study was to investigate the stability of alkali-treated rockfish protein isolate at various frozen storage conditions with or without cryoprotectants using the Raman technique. In addition, the gel properties, microstructure, and molecular structure of the alkali-treated rockfish protein isolate were compared to conventional surimi (CS).

7.3 MATERIALS AND METHODS

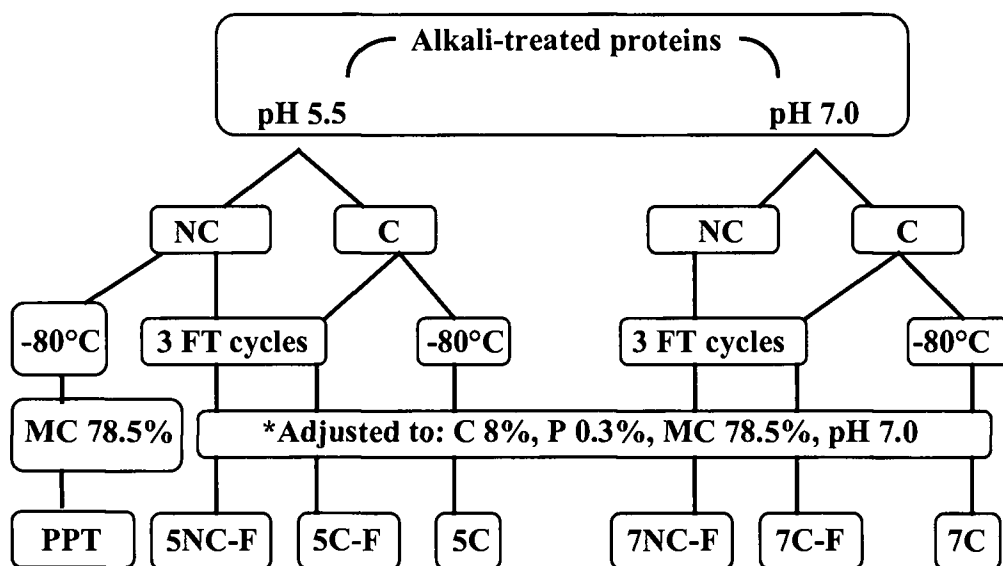
7.3.1 Materials

Fresh whole rockfish (*Sebastes flavidus*) were transferred on ice from a local processor to the OSU Seafood Lab within 30 min. Fish were then filleted and ground into mince. Mince was used as the raw material for AKPI.

7.3.2 Sample Preparations

For the conventional method, mince was washed 3 times at a 1 to 3 ratio (mince to water). Final washing was conducted using 0.3% NaCl solution to facilitate the dewatering step. The homogenate was centrifuged at 6,000 x g for 20 min at 4 °C for each washing step. Washed mince was then mixed with cryoprotectants (4% sucrose, 4% sorbitol, and 0.3 % sodium tripolyphosphate). Surimi was adjusted to pH 7.0 and 78.5 % moisture, and then vacuum-packed and stored at -80°C.

Alkali-treated rockfish proteins (pH 11) were prepared as detailed in Kim



C : 8 % cryoprotectants (sucrose + sorbitol = 1+1)

FT cycle : freezing at -18 ± 2 °C, 18 h; thawing at 4 ± 2 °C, 6 h

P : 0.3 % sodium tripolyphosphate

MC : Moisture content

Abbreviations of treatments:

5 = Frozen storage at pH 5.5

7 = Frozen storage at pH 7.0

C = With cryoprotectants, NC = without cryoprotectants

F = With 3 freeze/thaw cycles

PPT = Protein precipitate (pH 5.5) was not adjusted to pH 7.0 and no cryoprotectants

* All samples were thawed at 5 °C in order to adjust all parameters equally before testing.

Figure 7.1: Experimental design.

Table 7.1: Compositions of experimental treatments before adjusted to equal pH, moisture and cryoprotectants.

Treatment	Crude proteins (%)	Moisture content (%)	Sucrose + sorbitol = 1:1 (%)	Ratio of Water/Protein	Ratio of Protein/Cryoprotectant
With C	14.7	77.0	8	5.24	1.84
Without C	16.0	83.7	0	5.24	0

Note: C = cryoprotectants (a mixture (1:1) of sucrose and sorbitol).

(2002). Mince was homogenized with cold deionized (DI) water at a 1 to 9 ratio using a Power Gen 700 homogenizer (GLH 115, Fisher Scientific Inc., Pittsburgh, PA) for 2 min at speed level 2. The pH of homogenates was adjusted using 1 and/or 2 N cold HCl and NaOH to pH 11.0. The pH measurements were conducted using a pH meter (HI 9025 microcomputer pH meter, Hanna Instruments, Inc., Woonsocket, RI) with a Spear Gel Combo pH probe (Corning Incorporated Life Sciences, Acton, MA). After pH adjustment, samples were centrifuged (Sorvall RC-5B, DuPont Co., Newtown, CT) at 8,000 x g for 25 min at 4°C to separate the insoluble parts. Soluble proteins were subsequently recovered at pH 5.5. Protein precipitates were then collected by centrifugation (4,000 x g, 20 min, at 4°C).

The pH and cryoprotectant content of the experimental treatments is diagrammed in Figure 7.1. Two pH (5.5 and 7.0) were applied to the recovered pellet, which were further subdivided into 2 sets of cryoprotectants: 0% and 8% (sucrose:sorbitol = 1:1), respectively. Samples with no freeze/thaw treatment were stored at -80°C for 2 weeks before analysis. After 11 days, one set of each treatment was subjected to 3 freeze-thaw cycles at -18°C (18 h) freezing and 4°C (6 h) thawing, respectively, which were completed in 3 days. Before evaluation, the ratio of water to protein of the samples with and without cryoprotectants was controlled (Table 7.1). Prior to protein analysis or gel preparation, all samples were thawed at 5 °C for 30 min before adjusting to maintain equal pH (7.0), cryoprotectants, and moisture content (78.5%). The precipitate, pellets recovered at the pI after alkaline solubilization, was adjusted for equal moisture content (pH = 5.5) and subjected only to Raman analysis.

Gels were prepared without salt except CS, which was chopped with 2% NaCl and the process was conducted in a walk-in cold room (5-6 °C).

7.3.3 Texture Analysis

Paste (21.5 g) was stuffed into a plastic mold (30 mm I.D. and 25 mm height), vacuum-packed in a plastic bag, and then cooked at 90°C for 15 min. Cooked gels were cooled in iced water for 15 min and refrigerated overnight. The chilled gels were set at room temperature for 2 h before fracture analysis. Gels were subjected to the punch test using a texture analyzer (TA.XT.plus, Texture Technologies Corp., New York). A spherical probe (5 mm diameter) was used as a measuring tool and the penetration speed was set at 1 mm/sec. Breaking force (g) and deformation (mm) required to fracture the surface of the gel were recorded.

The folding test is another indicator measuring gel cohesiveness. It was conducted by slowly folding a 3 mm thick slice of gel (diameter = 30 mm) in half lengthwise and then in half again while examining it for structural failure. A folding score from 1 to 5 was given based on the rating system of Kudo and others (1973): 1 = broke under finger pressure; 2 = broke immediately when folded in half; 3 = broke gradually when folded in half; 4 = did not break when folded in half, but break when folded in quarter; and 5 = folded in quarter without breaking.

7.3.4 Scanning Electron Microscope (SEM)

Sample preparation for SEM was described by Feng (2000) with slight modification. Small samples of heated gels were cut (5x5x5 mm³) with a razor blade and fixed with 2.0 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2 at ambient temperature for 1 h. Samples were then washed twice with 0.1 M sodium phosphate buffer, pH 7.2. Fixed samples were dehydrated through a series of ethanol solutions of increasing concentration (25, 50, 75, 95, and 100% v/v) for 30 min each. Dehydrated samples were stored in 100% ethanol at 4 °C until dried. The samples were dried by the hexamethyldisilazane (HMDS) method (Nation 1983). Dried samples were mounted on aluminum specimen stubs and coated with gold:polonium

(60:40 w%) using a Sputter Coater (Edwards High Vacuum S 150 B, West Sussex, England). The samples were examined at 7,500X using a scanning electron microscope (AmRay 3300 FE, Bedford, MA) at an accelerated voltage of 10 kV.

7.3.5 Raman Spectroscopy

Raw frozen samples were packed in an ice box and transferred within 8 h to the Food Science building at the University of British Columbia (Vancouver, Canada). The temperature was controlled using iced jelly bags. After delivery, the samples were stored at -35 °C until tested.

Samples were thawed at 4 °C for 10 min before examining by a Laser Raman Spectrometer (JASCO model NR-1100, Japan Spectroscopic Co. Ltd., Tokyo, Japan). Samples were placed in a capillary tube (Nichiden-Rika Glass Co. Ltd., Tokyo, Japan) and held horizontally in the spectrometer, thermostated at 4.0 ± 0.1 °C using a JASCO temperature controller model RT-1C (Japan Spectroscopic Co. Ltd., Tokyo, Japan). The incident laser beam was vertical, i.e., perpendicular to the capillary axis. Raman spectral data were collected on the Raman spectrometer with 488 nm excitation from an argon ion laser (Coherent Innova 70C series, Coherent Laser Group, Santa Clara, CA), cooled with the Coherent Laser Pure heat exchanger system. The conditions used were as follows: incident laser power 100 mW, slit height 4 mm, spectral resolution 5.0 cm^{-1} at 19000 cm^{-1} , sampling speed $120 \text{ cm}^{-1} \text{ min}^{-1}$ with data collected every cm^{-1} . Frequency calibration of the instrument was performed daily using the 1050 cm^{-1} band of a 1 M KNO_3 standard solution. Optical alignment was also monitored by checking the intensity of this standard solution. To increase the signal to noise ratio, at least 6 scans of each sample were collected to obtain averaged spectral data. Triplicate samples of each treatment were scanned.

Spectral were smoothed, baseline corrected, and normalized against the phenylalanine band at 1007 cm^{-1} by GRAMS/32[®] Spectral Notebase[™] version 4.14 level II (Galactic Industries Corp., Salem, NH). Estimation of the secondary structure composition of the proteins based on the Raman spectra in the amide I region was carried out using the Raman Spectral Analysis Package (RSAP version 2.1) of

Przybycien and Bailey (1989). Assignments of peaks in the Raman spectra to specific vibrational modes of amino acid side chains or the polypeptide backbone were made according to published literatures (Bouraoui and others 1997; Careche and Li-Chan 1997; Careche and others 1999; Ogawa and others 1999; Sultanbawa and Li-Chan 2001; Badii and Howell 2002b; Careche and others 2002).

7.3.6 Statistical Analysis

At least two replicates were performed for texture analysis of all treatments. Data were analyzed using an analysis of variance (ANOVA) procedure. General linear model was applied with further analysis using Tukey's test to determine differences ($p < 0.05$) between treatment means (SPSS for Windows, version 10.0, SPSS Inc., Chicago, IL).

7.4 RESULTS AND DISCUSSION

7.4.1 Texture Analysis

Regardless of pH at frozen storage, the texture properties of alkali-treated protein isolates (AKPI) with cryoprotectants (5C & 7C) exhibited superior quality followed by samples with cryoprotectants and FT cycling (5C-F & 7C-F), and sample without cryoprotectants with FT cycling (5NC-F & 7NC-F), respectively (Fig. 7.2).

Unlike AKPI from rockfish, our previous study using Pacific whiting (PW) as a raw material demonstrated a detrimental decrease of gel elasticity when AKPI was treated with freeze/thaw cycles regardless of inclusion of cryoprotectants (Thawornchinsombut and Park 2004b). This probably was due to the fact that Pacific whiting is a gadoid species, which tends to have more denaturation during frozen storage. Formaldehyde produced from the enzyme trimethylamineoxide (TMAO) in this species triggers protein denaturation and subsequent aggregation resulting in changes in texture and functionalities (Matsumoto 1980; Careche and Li-Chan 1997; Badii and Howell 2002a). There was no significant difference of gel quality between the AKPI kept frozen at pH 7.0 and 5.5. Nevertheless, the texture qualities of 5NC-F

seemed to show slightly higher breaking force than 7NC-F. We actually observed slightly poorer quality in 5NC-F, which represented more brittle and discontinuous texture than 7NC-F. This observation was also supported by SEM analysis (Fig. 7.3).

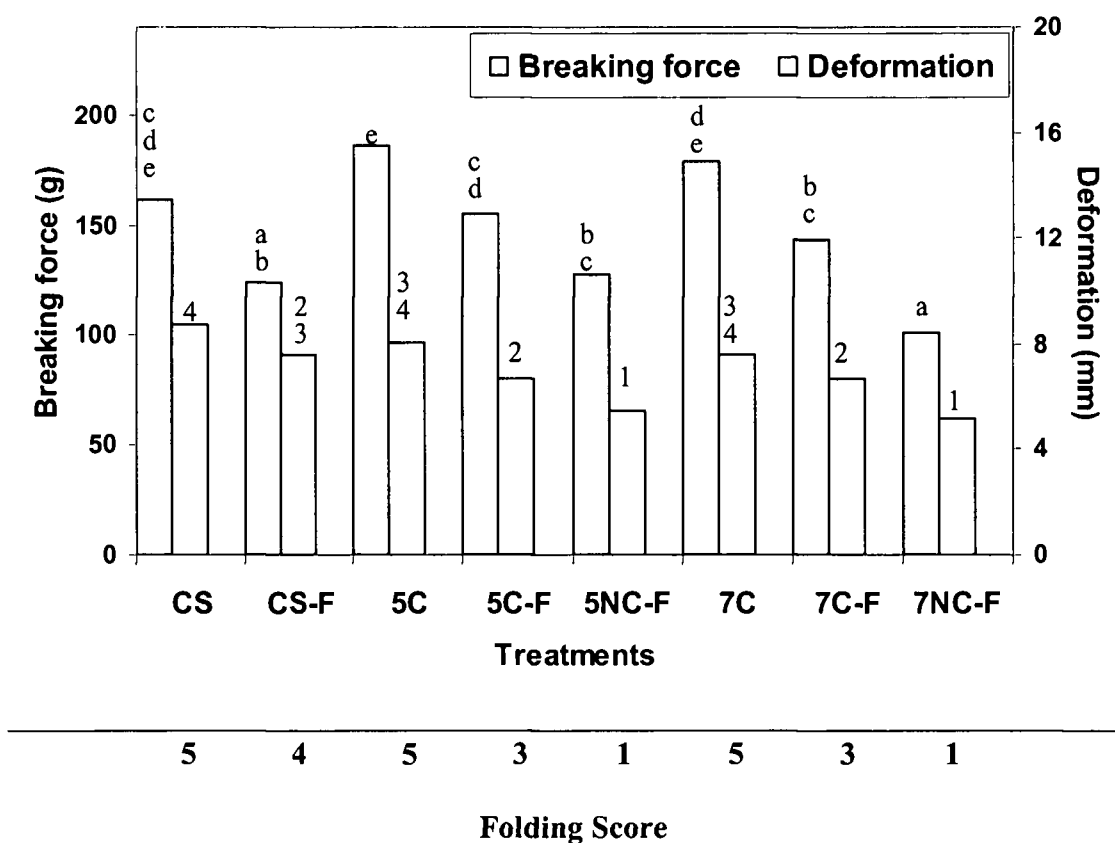


Figure 7.2: Texture properties of gels prepared from rockfish surimi and alkali-treated protein isolates at various storage conditions. All treatments were equally adjusted to the same cryoprotectant concentration and pH 7.0 before gel preparation. Abbreviations for treatments are as in Fig. 7.1, and CS = conventional surimi.

In this study, the penetration test might not be sensitive enough when proteins showed poor elasticity due to aggregation. The results suggested that cryoprotectants are needed to preserve AKPI during frozen storage. Gel strength of CS was slightly lower than AKPI, however, after frozen/thawed, CS exhibited slightly better gel quality regarding elasticity, as evidenced by deformation values and folding score.

7.4.3 SEM

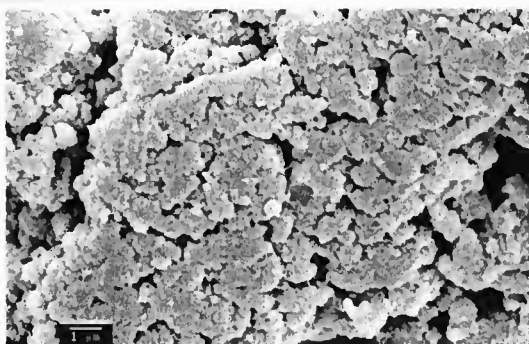
Gel microstructure of CS and AKPI with 7C treatment showed a relatively smooth and continuous structure while that with 5C treatment appeared discontinuous with several voids (Fig. 7.3). This difference, however, could not be differentiated using the texture analyzer (Fig 7.2). When AKPI with added cryoprotectants in the 5C-F treatment were subjected to freeze-thaw cycles, they exhibited more discontinuous and aggregated structure. When samples without cryoprotectants were treated with FT cycles, the gels were more aggregated exhibiting less continuous structures with several big voids and interconnecting grooves. Samples stored at pH 5.5 also showed more discontinuity and were grainier than samples at neutrality. With regards to pH at frozen storage, the SEM images were in accordance with texture qualities. The higher the continuous gel microstructure, the better the gel quality obtained. These results strongly indicated that AKPI require cryoprotectants to prevent protein aggregation when frozen, and also suggested that protein quality could be better maintained when kept frozen at neutral pH.

7.4.3 Raman Spectroscopy

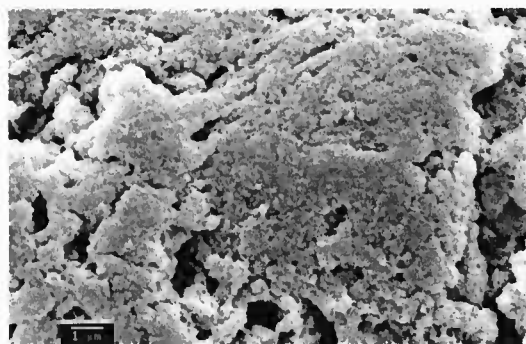
Secondary structure estimation from the amide I and III bands

Figure 7.4 shows the Raman spectra of all treatments in the 400-1800 cm^{-1} range. Table 7.2 shows the assignment of major bands to amino acid side chains and peptide backbones. The most outstanding band, center near 1655 cm^{-1} for CS, has been assigned to the amide I vibration mode, which mainly involves C=O stretching and, to a lesser degree, C-N stretching, $\text{C}_\alpha\text{-C-N}$ bending, and N-H in-plane bending of peptide groups. In general, the amide I band consists of overlapped band components falling in the 1658-1650, 1680-1665, and 1665-1660 cm^{-1} , which are attributable to α -helices, β -sheets, and random coil structures, respectively (Frushour and Koenig 1975; Tu 1982).

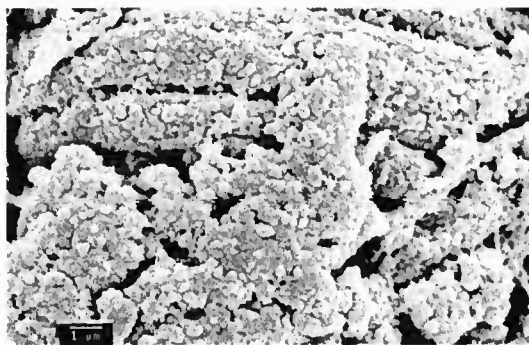
For CS, the amide I region slightly shifted toward higher frequency when FT cycling was applied (1657 to 1661 cm^{-1}) (Fig. 7.4), which indicated a decrease in α -helical structure and an increase in undefined or random coil structure. A similar trend



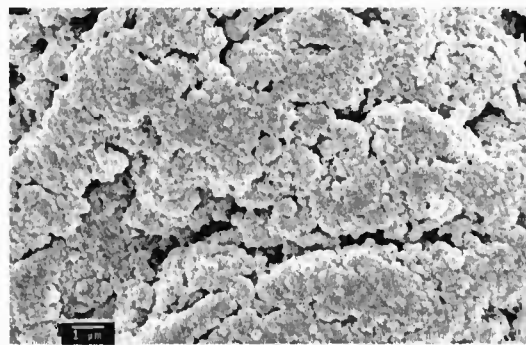
(a) 5C



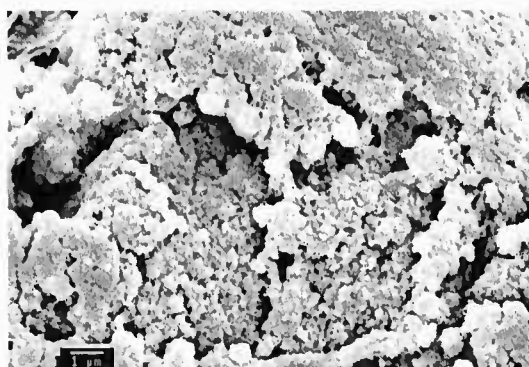
(b) 7C



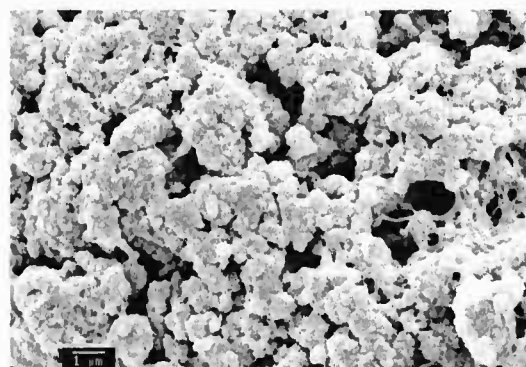
(c) 5C-F



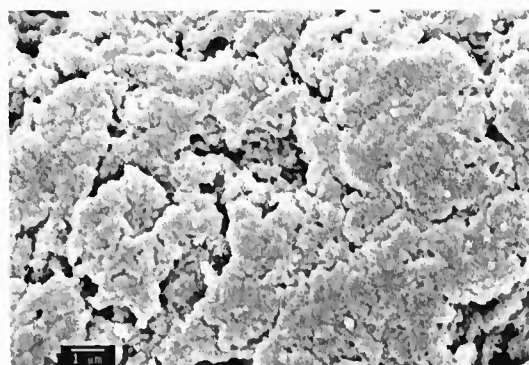
(d) 7C-F



(e) 5NC-F



(f) 7NC-F



(g) CS

Figure 7.3: Microstructures of gels prepared from rockfish surimi and alkali-treated protein isolates at various storage conditions. All treatments were equally adjusted to the same cryoprotectant concentration and pH 7.0 before gel preparation (*Magnification 7500X, bar = 1 μ m*). Abbreviations for treatments are as in Fig. 7.1, and CS = conventional surimi.

was found in hake muscle (Careche and others 1999). In addition, we observed that the intensity of a shoulder at $\sim 1614\text{ cm}^{-1}$ was considerably reduced; this Raman band is assigned to the aromatic ring vibrational modes of phenylalanine and tyrosine residues.

In AKPI samples, the amide I band was shown around $1661\text{--}1663\text{ cm}^{-1}$ for PPT, 5C, and 7C treatments, which indicates a random coil structure. The peak intensity (I) of this band of all AKPI treatments was significantly lower than CS. When pH 5.5 treatments with and without cryoprotectants were treated with FT cycling, the band shifted to a lower frequency; 1659 cm^{-1} for 5C-F, and 1655 cm^{-1} for 5NC-F, reflecting a recovery of helical structure. However, no significant change was noted in pH 7.0 treatments. Unlike CS, a rising intensity of the peak shoulder (1614 cm^{-1}) assigned to aromatic ring vibrations was shown when harsh conditions were applied to AKPI.

Another way of looking at these Raman spectra is the use of the C-C stretching vibrations near 940 and 990 & 1239 cm^{-1} , which are characteristics of α -helices and β -sheets, respectively. Generally, gradual loss of these structures leads to broadening and weakening in intensity (Frushour and Koenig 1975; Barret and others 1978; Tu 1982; Badii and Howell 2002a). A marked decrease in I_{941} was evident in CS after FT cycling. A significantly lower I_{941} was recorded in all AKPI treatments when compared with that of CS.

The amide III regions around $1220\text{--}1275\text{ cm}^{-1}$ were not easily distinguishable (Fig. 7.4). This is because of an overlap of the β -structure and random coil bands as well as the low S/N ratio. The amide III mode involved N-H in plane bending and C-N stretching as well as contributions from C_{α} -C stretching and C=O in plane bending. The amide III band was located at 1307 and 1295 cm^{-1} in the Raman spectrum of hake myosin (Careche and Li-Chan 1997). In the present study, the amide III bands were observed in two regions around 1248 cm^{-1} and 1304 cm^{-1} , characteristic of β -sheet and α -helical structures, respectively (Fig 7.4). The AKPI samples showed more balance between those two peaks while CS had a more prominent feature at 1304 cm^{-1} .

corresponding to higher α -helical structure (Table 7.3). Careche and Li- Chan (1997) also found similar results for cod myosin.

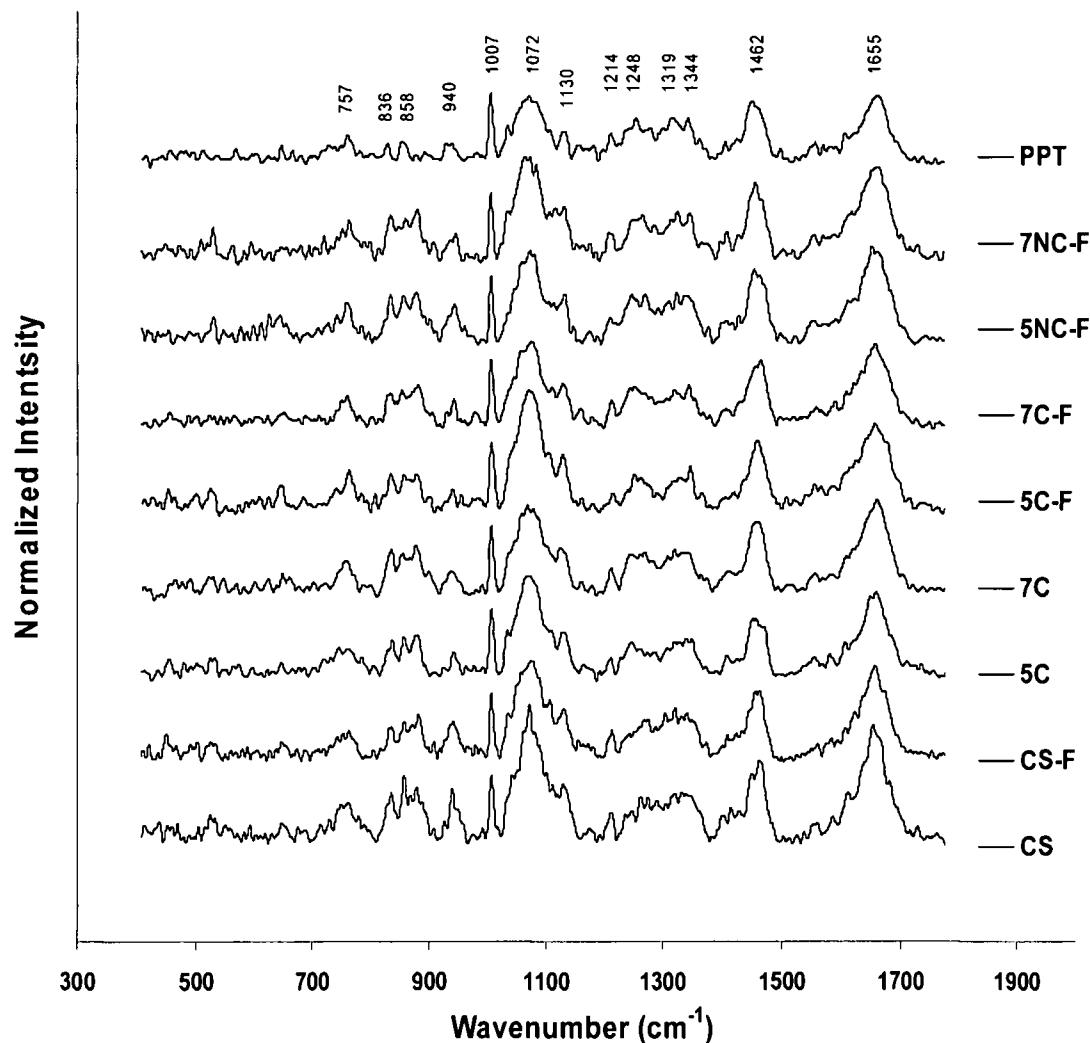


Figure 7.4: Raman spectra in the 400-1800 cm^{-1} region of rockfish surimi and alkali-treated protein isolates at various storage conditions. All treatments were equally adjusted to the same cryoprotectant concentration and pH 7.0 except the PPT (recovered proteins, pH 5.5, without cryoprotectants). Abbreviations for legend are as in Fig. 7.1, and CS = conventional surimi.

Table 7.2: Tentative assignment of some bands in the Raman spectrum of rockfish surimi.

Wavenumber (cm ⁻¹)	Tentative assignment
524	Cystine S-S stretch or aliphatic C-C-C deformation
757	Tryptophan
836, 858	Tyrosine
880	Tryptophan
941	α -helix
1007	Phenylalanine
1072	Backbone C-C, C-N stretch
1130	Backbone C-N stretch
1214	Tyrosine or phenylalanine
1248	Amide III polypeptide backbone
1304	CH bending or amide III (α -helix)
1320, 1339	Tryptophan or aliphatic C-H bending
1400	C=O stretch of Asp, Glu, CoO ⁻
1462	CH ₂ bending
1655	Amide I polypeptide backbone
2938	Aliphatic (alkyl) C-H stretching
3068	Aromatic or unsaturated C-H stretching
3236	Water O-H stretching

Carew and others (1975) proposed that the two bands (1250 and 1304 cm⁻¹) were assigned to the globular and fibrous α -helical conformations of myosin, respectively. The band at 1248 cm⁻¹ was observed at a higher wave number after CS was frozen/thawed, suggesting more disordered or random coil structures (Fig 7.4). Sultanbawa and Li-Chan (2001) revealed typical α -helical bands at 1269 and 1302 cm⁻¹ for unfrozen surimi. After frozen storage or freeze/thaw treatment, these bands were observed at 1276 and 1304 cm⁻¹.

Table 7.3: Secondary structure fractions estimated from the amide I band.

Treatment*	Total α -helix	Total β -sheet	Total random coil
CS	0.53	0.24	0.23
CS-F	0.15	0.49	0.35
PPT	0.23	0.47	0.30
5C	0.21	0.45	0.34
5C-F	0.42	0.35	0.23
5NC-F	0.47	0.29	0.23
7C	0.34	0.38	0.28
7C-F	0.46	0.33	0.22
7NC-F	0.36	0.43	0.21

* All treatments were equally adjusted to the same cryoprotectant concentration and pH 7.0 except the PPT (recovered proteins, pH 5.5, without cryoprotectants). Abbreviations for treatments are as in Fig. 7.1, and CS = conventional surimi.

More detailed evaluation of changes of the protein secondary structures was achieved by quantitative estimation using RSAP version 2.1 software for least square analysis of the amide I band (Table 7.3). The results demonstrated that CS predominantly contained α -helical structure (53%) and an approximately equal amount of β -sheet and random coil (23 and 24%, respectively). Pacific whiting surimi consisted of 44, 24, and 32% of α -helical, β -sheet, and random coil structures, respectively (Bouraoui and others 1997). Freezing and thawing resulted in a substantial diminution of α -helix and an elevation of β -sheet content and random coil. Other researchers have reported concurrent results in hake fillet (Carache and others 1999), ling cod actomyosin (Sultanbawa and Li-Chan 2001), and cod fillet (Badii and Howell 2002a).

Generally, gelation of CS is accomplished by first grinding surimi with salt to increase the solubility or the extractability of the myofibrillar proteins. The resulting paste is then cooked at high temperature (Lee 1984). From the study in our lab by Kim (2002) and the present case, AKPI, however, can form a gel without adding salt at pH 7.0. It is perhaps due to the reorganization of protein molecules as a result of

pH adjustments. Thus, protein structures particularly the secondary fractions of CS and AKPI examined by Raman spectroscopy without salt addition were discrepant.

A study using Raman spectroscopy with globular protein found that when a gel is formed, there is a tendency for the β -sheet content to increase with a simultaneous decrease in a α -helical content (Clark and others 1981). Similar results were also discovered in heat-induced Pacific whiting surimi gel (Bouraoui and others 1997). Boye and others (1996) suggested that increasing pH from 3.0 to 9.0 as well as heating (26-97 °C) of β -lactoglobulin increased the formation of β -sheet structure. Casal and others (1988) found that the intermediate stages of alkaline and thermal denaturation were suggested to be similar, but the final stage of thermal denaturation is completely different from alkaline denaturation because protein unfolding is more affected by high pH than by heating due to repelling forces of the negatively charged functional groups. The lower α -helical and greater β -sheet content of AKPI than CS may explain its ability to form gels at a suitable pH of neutrality (7C) (Table 7.3). Interestingly, too much β -sheet structure, such as observed in the PPT or 5C samples, did not favor the ability to form a good gel. Furthermore, freezing of AKPI resulted in secondary structure fractions more similar to CS than AKPI before freezing, but these changes did not correlate with good gelling ability. This suggests additional subtle structural differences upon freezing that are not reflected by merely estimating the fractions of the secondary structures.

The pellets recovered from alkali-treated proteins at the pI (PPT) and 5C (with cryoprotectants and final pH = 7.0) exhibited similar proportions of the secondary fractions, which contained only ~ 50% of the α -helix content of CS and the highest proportion of β -sheet. A decrease in α -helix and an increase in β -sheet are indicative of protein-protein interactions, which have also been reported for heat-denatured proteins (Howell and Li-Chan 1996) and freeze-denatured fish proteins (Careche and others 1999; Sultanbawa and Li-Chan 2001).

After the denaturation process, produced by frozen storage, one can obtain some protein aggregates that are not extractable by detergents that break hydrophobic interactions (Tejada and others 1996; Careche and others 1998). Protein aggregates

from other sources that were found to be water insoluble and insoluble in the presence of sodium dodecyl sulfate (SDS) showed high proportions of β -sheet structures (Jaenicke 1987; Carmona and others 1988). These changes could be due to an unfolding of the helical structures (during frozen storage for CS and/or chemical treatment during the process for AKPI), followed by the formation of sheet structures, possibly through intermolecular interactions between exposed hydrophobic residues (Bouraoui and others 1997).

It was noteworthy that a contrasting trend to CS was observed when AKPI were subjected to FT cycling. There was an increase in α -helix and slight decline of β -sheet and random coil after FT cycling for AKPI at both storage pH. After FT cycles, samples without cryoprotectants stored at the pI (5NC-F) showed relatively similar composition to 5C-F (greater α -helix and less β -sheet content), while those stored at pH 7.0 contained less α -helix but higher β -sheet structures. Similar increases in helical structure were reported after freezing ling cod without cryoprotectants (Sultanbawa and Li-Chan 2001), and upon aquacade concentration of cod myosin (Careche and Li-Chan 1997). It was suggested that aggregation of myosin heads and arrangement of the helical tails in a daisy wheel configuration (Yamamoto 1990) may lead to increased Raman signal intensity characteristic of helical structures, and that these changes can be induced by solute concentration, including that which occurs during freezing.

Changes of tryptophan residue bands

Many Raman bands such as those at 760, 879, 1336, 1359, 1363, and 1557 cm^{-1} display information about the microenvironment of the tryptophan (Trp) residues. Among these many bands, the 1363 cm^{-1} is influenced by the environment; therefore, it is useful to use this band to monitor whether the Trp side chain is buried or exposed. A sharp and intense band is an indicator of a buried Trp (Tu 1986). However, in this work, we did not see a clear trend of the band at 1363 cm^{-1} when different storage conditions were applied to the samples. The band intensity, particularly at 757, 1339, and 1557 cm^{-1} , slightly decreased for the CS sample treated with FT cycles (Fig. 7.5 a

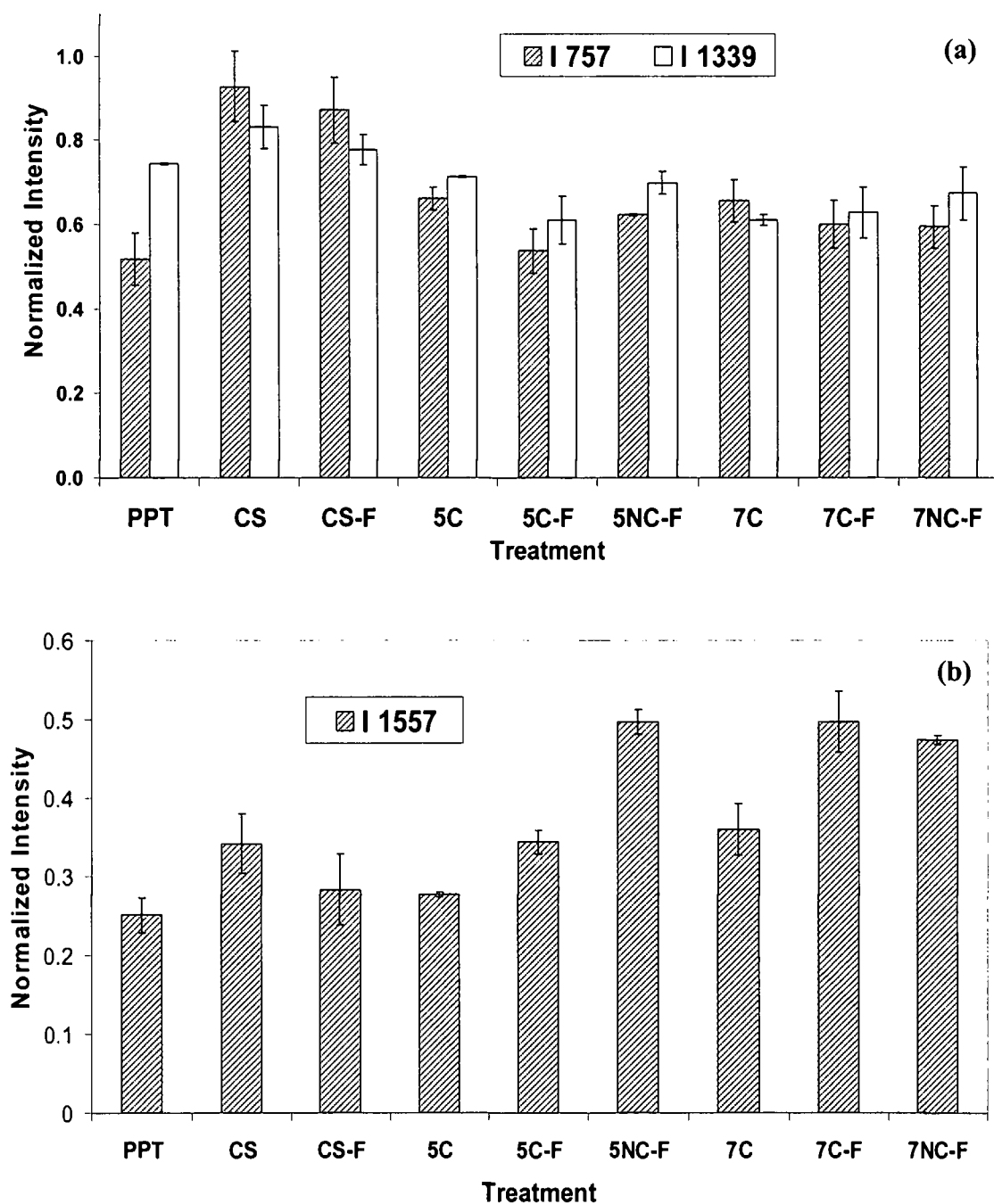


Figure 7.5: Raman intensity at the 757 & 1339 cm⁻¹ (a) and at the 1557 cm⁻¹ (b) of rockfish surimi and alkali-treated protein isolates at various storage conditions. All treatments were equally adjusted to the same cryoprotectant concentration and pH 7.0 except the PPT (recovered proteins, pH 5.5, without cryoprotectants). Abbreviations for treatments are as in Fig. 7.1, and CS = conventional surimi.

and b), which perhaps indicated the exposure of Trp residues. Similar findings were reported by Careche and others (1999) and Badii and Howell (2002b).

The I_{757} and I_{1339} of all AKPI treatments were significantly lower than CS, however, no significant change was noticed when FT and/or no cryoprotectant treatments were applied regardless of pH during storage (Fig. 7.5 a). It should be noted that there is some overlap of the Trp band at 757 cm^{-1} with aliphatic residues at 755 cm^{-1} for cod myosin. Similarly, the 1339 cm^{-1} band can also be assigned to aliphatic CH bending (Careche and Li-Chan 1997). Hence, changes of bands at 757 and 1339 cm^{-1} may have resulted from changes in the aliphatic side chains and CH bending, respectively.

The opposite trend to the CS sample was found in the I_{1557} of the AKPI samples (Fig. 7.5 b). The intensity considerably increased in samples with and without cryoprotectants treated with FT cycling at both storage pH. This probably suggested that the Trp residues were buried in the hydrophobic environment. The aggregation of proteins following freeze-denaturation perhaps led to hydrophobic interactions, resulting in the buried Trp residue. These data possibly confirmed that rockfish proteins underwent different freeze-denaturation mechanisms when treated by the pH-shift method.

The Tyrosine doublet bands

The intensity ratio I_{836}/I_{858} corresponds to the tyrosine (Tyr) doublet at 836 and 858 cm^{-1} . It was suggested by Yu and others (1973) that the ratio of the tyrosine ring vibrations at 850 and 830 cm^{-1} (R_{Tyr}) reflects “buried” and “exposed” tyrosine groups, respectively. $R_{\text{Tyr}} \geq 1$ in the myosin Raman spectrum was indicative of Tyr residues relatively exposed on the protein surface, which could interact with solvent water molecules as a hydrogen bond donor and acceptor. If R_{Tyr} falls between 0.7 and 1.0 , the Tyr residues may be considered buried. If the ratio is as low as 0.3 , strong H-bonding to a negative acceptor is indicated (Tu 1986).

Precipitate from alkali-treated proteins had the highest intensity ratio (1.4) (Fig 7.6). A significant decrease was attained when PPT was adjusted to neutrality even

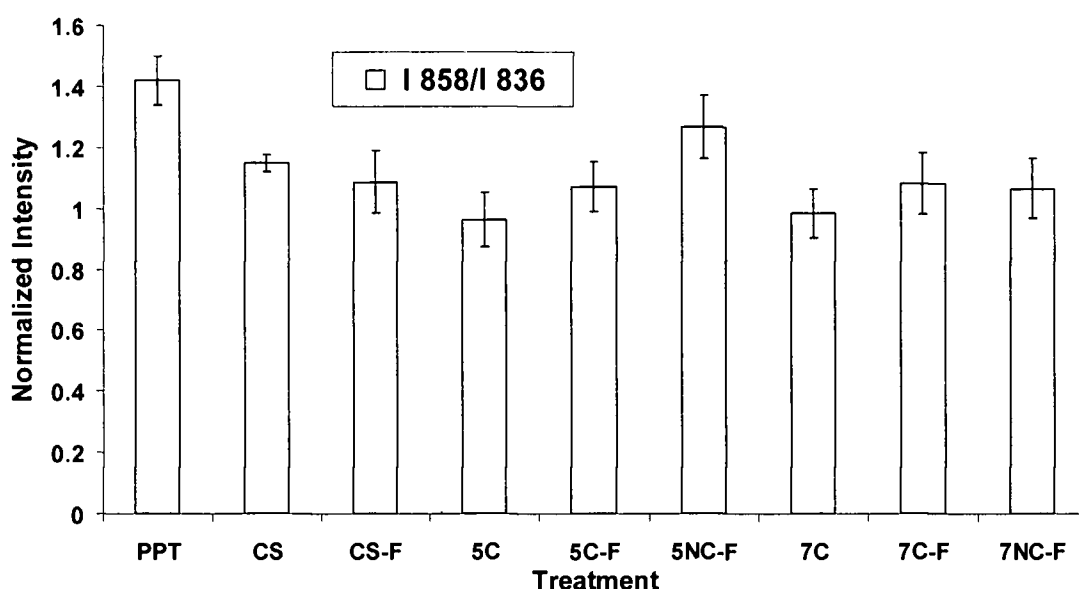


Figure 7.6: Raman intensity ratio of tyrosine doublet of rockfish surimi and alkali-treated protein isolates at various storage conditions. All treatments were equally adjusted to the same cryoprotectant concentration and pH 7.0 except the PPT (recovered proteins, pH 5.5, without cryoprotectants). Abbreviations for treatments are as in Fig. 7.1, and CS = conventional surimi.

after frozen storage at pH 5.5 for 2 weeks (5C and 7C), suggesting protein refolding. The intensity ratio of 5NC-F demonstrated a high value (1.3), thus representing the exposed Tyr residues. This may indicate that the proteins could not be refolded after altering the pH to neutral due to lacking of preservative effect of cryoprotectants. Nevertheless, an insignificant different intensity ratio among the AKPI samples stored at pH 7.0 was evident. These results correlated to the microstructure obtained by SEM that the AKPI without cryoprotectants stored at the pI underwent greater detrimental protein quality changes than those kept frozen at pH 7.0.

Polypeptide backbone stretching at 1072 cm^{-1}

Raman intensity at $\sim 1072\text{ cm}^{-1}$ of the CS contained conformationally sensitive skeletal vibrations (mostly C-N and C-C stretch modes) (Carew and others 1975), decreased after FT cycling, with a slight shift to a higher wave number at 1078 cm^{-1}

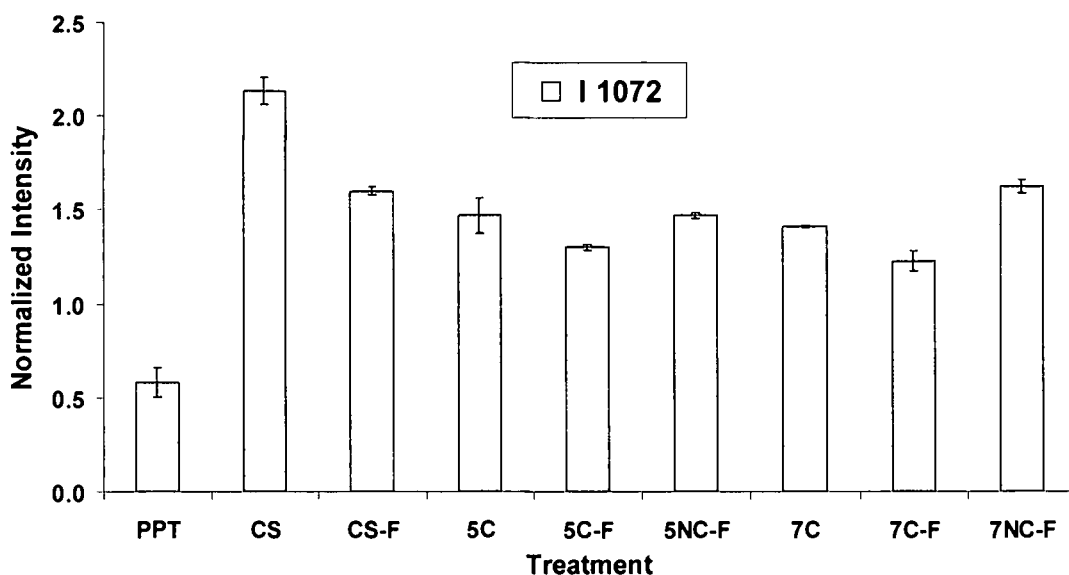


Figure 7.7: Raman intensity at 1072 cm^{-1} of rockfish surimi and alkali-treated protein isolates at various storage conditions. All treatments were equally adjusted to the same cryoprotectant concentration and pH 7.0 except the PPT (recovered proteins, pH 5.5, without cryoprotectants). Abbreviations for treatments are as in Fig. 7.1, and CS = conventional surimi.

(Fig. 7.7). Ogawa and others (1999) reported a diminution of Raman intensity in the region of $1050\text{--}1100\text{ cm}^{-1}$ upon heating (setting at $40\text{ }^{\circ}\text{C}$) of rockfish actomyosin. On the other hand, an increase in peak intensity around $1060\text{--}1080\text{ cm}^{-1}$ was reported when lysozyme was heated. This contradiction perhaps resulted from the different protein forms during evaluation. The former protein was studied in a semisolid state (paste), while in the latter lysozyme solution was employed.

Cod collagen showed considerably lower peak intensity at 1096 cm^{-1} , which also corresponds to backbone stretching, when stored at higher freezing temperatures. Several studies have reported a reduction in collagen solubility because of frozen storage, particularly at higher storage temperature (Badii and Howell 2003). In the present study, a predominantly low intensity of this band (I_{1072}) for the PPT sample was detected. This information probably suggested that a significant decrease in this band intensity involves intermolecular interactions of protein molecules at the pI to form aggregates. The intensity subsequently increased to approximately the same

extent as the CS-F sample after cryoprotectant addition and/or pH adjustment. However, no distinct trend could be made when AKPI samples were treated under different storage conditions. In addition, published data are limited for this polypeptide backbone stretching band in protein spectra.

The aliphatic bending vibrations

Most C-H deformations appear in the region of 1400-1500 cm^{-1} ; that is, roughly half of the C-H stretching vibrations (Tu 1986). A band observed at 1462 cm^{-1} for the methylene (CH_2) bending in the CS sample showed a slight decline in its peak intensity (1.2 to 1.0) after FT cycling. A slight shift of the band toward lower frequency with lower intensity of its shoulder ($\sim 1400\text{-}1440$) for AKPI stored at the pI compared to those at pH 7.0 was illustrated (Fig 7.4). Ling cod natural actomyosin showed a band at 1460 and at 1446 cm^{-1} after freezing without cryoprotectants with a shoulder at 1464 cm^{-1} (Sultanbawa and Li-Chan 2001). Such decrease has been observed for myosin isolated from cod, particularly after frozen storage in the presence of formaldehyde (Careche and Li-Chan 1997), and hake fillet (Careche and others 1999). Changes of this band may have resulted from hydrophobic interactions of the aliphatic residues (Lippert and others 1976).

C-H stretching vibrations in the 2500-3400 cm^{-1} region

Hydrophobic groups of amino acids, peptides, and proteins exhibit C-H stretching vibrational bands in the 2800-3100 cm^{-1} region. Bands found near 2874-2879 cm^{-1} are assigned to CH_3 symmetrical stretching and $\text{R}_3\text{C-H}$ stretching bands of aliphatic amino acids, whereas the C-H stretching bands of aromatic amino acids can be found near 3061-3068 cm^{-1} (Howell and others 1999). The CS sample showed two major bands at 3236 and 2938 (with a shoulder at 2900) cm^{-1} . CS-F treatment demonstrated a slight decrease of I_{2938} with a more distinct shoulder around 2897 cm^{-1} and a small shoulder appeared at 2977 cm^{-1} (Fig. 7.8). The shoulders at wavenumber around 2903 and 2976 cm^{-1} were clearly distinguishable in the AKPI samples. The

PPT treatment exhibited both shoulders with substantially lower I_{2938} (2.8 compared to 3.5 and 3.0 for the CS and CS-F, respectively).

Sultanbawa and Li-Chan (2001) and Badii and Howell (2002b) reported a similar trend in ling cod actomyosin and cod fillets, respectively, under various frozen storage conditions. When fish proteins were stored under harsher conditions (i.e. without cryoprotectant, higher frozen storage temperature and/or freeze-thaw treatment), the greater evidence of a peak shoulder of a band at 2938 cm^{-1} was illustrated.

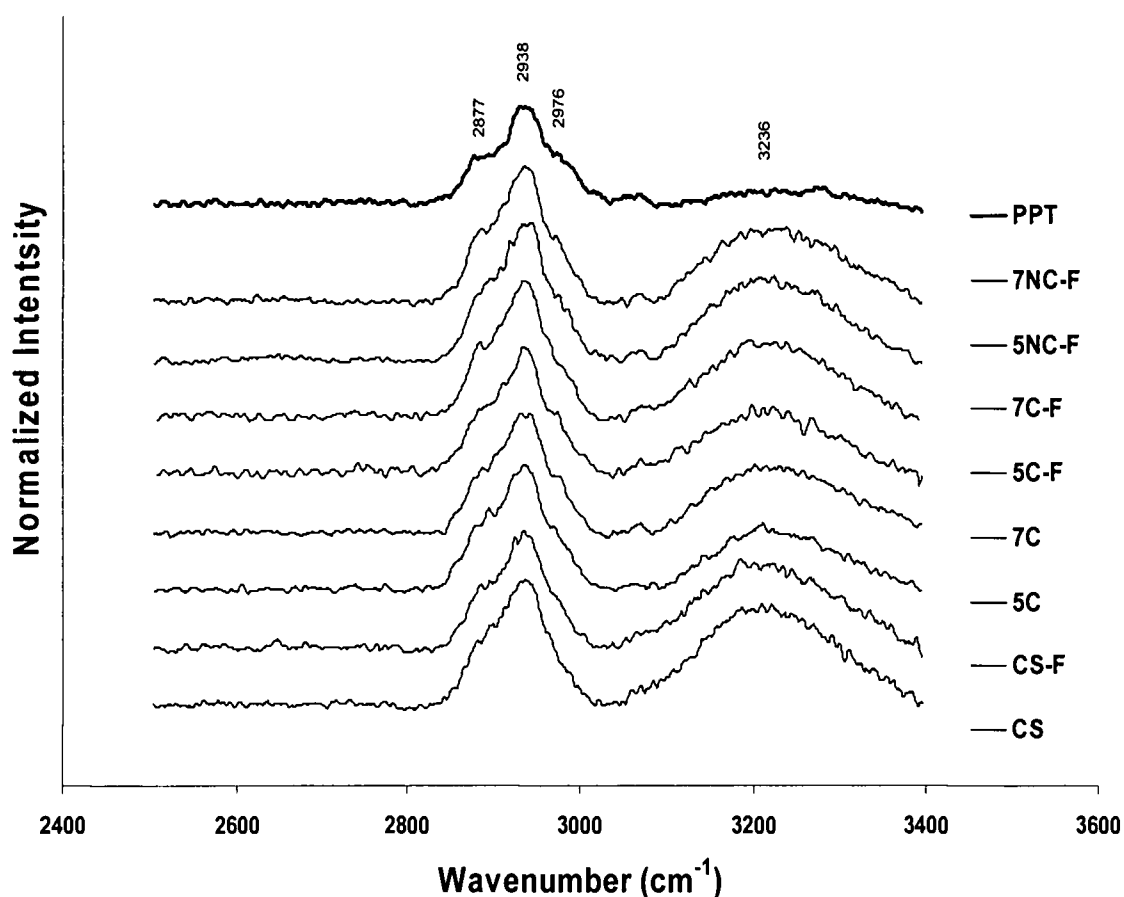


Figure 7.8: Raman spectra in the $2500\text{--}3400\text{ cm}^{-1}$ region of rockfish surimi and alkali-treated protein isolates at various storage conditions. All treatments were equally adjusted to the same cryoprotectant concentration and pH 7.0 except the PPT (recovered proteins, pH 5.5, without cryoprotectants). Abbreviations for treatments are as in Fig. 7.1, and CS = conventional surimi.

The band at 3236 cm^{-1} , which reflects the OH stretch, indicated lower intensity for all AKPI treatments, particularly the PPT in comparison to CS (Figs. 7.8 and 7.9). This probably suggested that the AKPI underwent a higher rate of dehydration due to protein-protein interactions through aggregation mechanisms during the protein recovery process and/or frozen storage. Storage pH and cryoprotectants demonstrated a marked influence on this band. At the pI storage condition, protein molecules lost more water after frozen/thawed. However, when cryoprotectants and pH were adjusted (5NC-F), rehydration could be observed. This observation was less prominent in samples stored at pH 7.0.

A similar finding was reported by Sultanbawa and Li-Chan (2001). The OH stretch band intensity decreased for the frozen natural actomyosin and surimi without cryoprotectant compared to the fresh samples. They proposed that such decreases

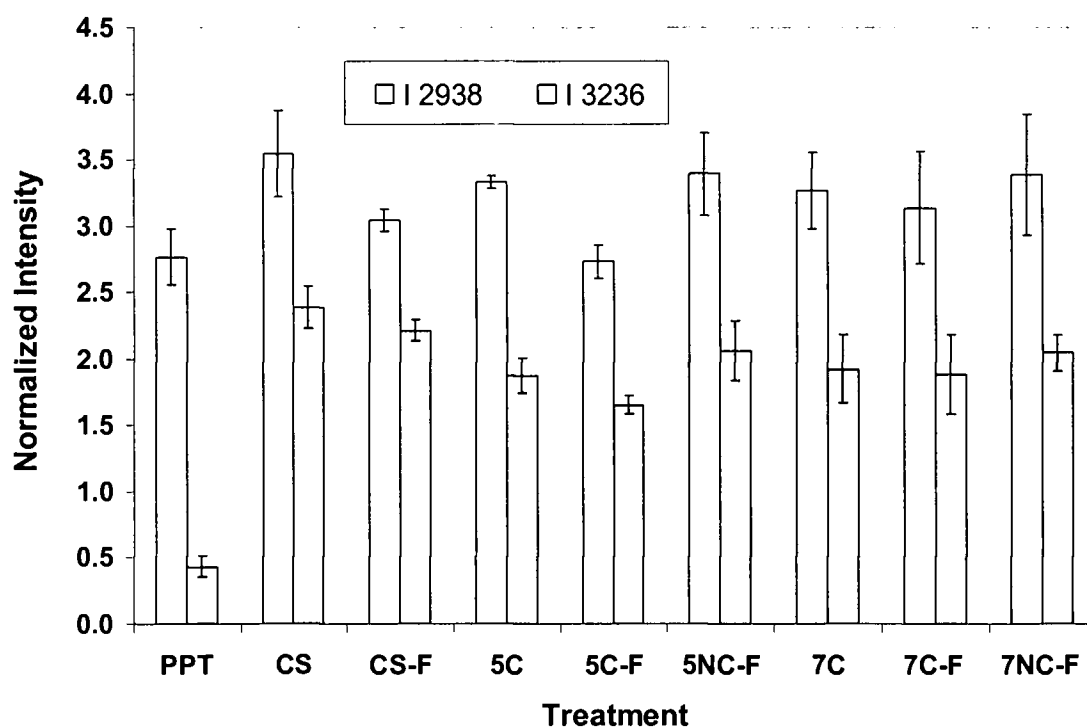


Figure 7.9: Raman intensity ratio of C-H and water O-H stretching of rockfish surimi and alkali-treated protein isolates at various storage conditions. All treatments were equally adjusted to the same cryoprotectant concentration and pH 7.0 except the PPT (recovered proteins, pH 5.5, without cryoprotectants). Abbreviations for treatments are as in Fig. 7.1, and CS = conventional surimi.

may be related to dehydration as a result of the formation of ice crystals during frozen storage. Careche and others (1999) reported a decrease in the intensity ratio of O-H/C-H bands, which correlated with harshness of the condition of freezing or frozen storage of the hake fillets. This intensity ratio was suggested to be of use for monitoring quality change due to freezing and frozen storage. Nevertheless, our results (the intensity ratio) did not clearly reveal this phenomenon. No significant difference was discernable among AKPI treatments (data not shown).

7.5 CONCLUSION

Texture qualities of AKPI decreased in descending order of samples added with cryoprotectants, samples added with cryoprotectants and subjected to FT cycles, and samples without cryoprotectants and subjected to FT cycles. No distinct difference of gel texture was detected between AKPI stored at neutral pH and at the pI. Nevertheless, a greater reduction in protein qualities was observed by SEM and Raman analysis when AKPI were kept frozen at pH 5.5 than at pH 7.0. The greater the harsh storage condition, the more the discontinuous the microstructure of the gel.

The Raman spectral data showed that the protein structure in the pellet recovered from alkali-treated proteins (PPT) and frozen without cryoprotectant could be partially refolded by adjusting to neutral pH. However, this reorganized structure was not identical to conventional surimi. There was a decrease in the α -helical content from 53 to 15 % when the conventional surimi was frozen/thawed, while the β -structure and random coil content increased. In contrast, for the AKPI stored at pH 5.5 (5C), the α -helical fraction increased (21 to 42%) with a diminution of β -structure and random coil. The AKPI stored at neutrality (7C) contained a higher content of α -helix than the pI-stored samples, but less than conventional surimi. Such changes of Raman bands led to the conclusion that several amino acid side chains and peptide backbones of AKPI samples were more exposed during frozen storage in comparison to CS. As a result, protein-protein interactions (aggregation) were induced. In addition, this deteriorated mechanism under frozen storage seemed to take place differently compared to the traditional sample.

Cryoprotectants exclusively played an important role to stabilize the unfolded/refolded AKPI. When the protein isolate was kept frozen at the pI, a decrease of protein functionalities and structural changes were more intense. The results suggest that alkali-treated protein isolate is slightly less stable than conventional surimi under frozen storage conditions.

CHAPTER 8

GENERAL CONCLUSION

Ionic strength (IS) and pH are two of the most important parameters driving the changes of protein functionality of fish proteins. A typical U-shape of protein solubility was attained when muscle protein suspensions were treated over a pH range of 2 to 12 at IS 10 mM. An increase in ionic strength to 600 mM enhanced protein solubility at pH between 6 and 10 and caused the pI shift of Pacific whiting proteins toward the acidic side by about two pH units. When the pH was further reduced to lower than the pI (pH 2-4), protein aggregation was induced at high IS and myosin heavy chain (MHC) degradation was discernable at low IS (10 mM). Under high alkaline conditions (pH 11 and 12) a possible molten globule state of the protein was obtained at IS 600 mM and polymerization occurred at both IS levels. Disulfide bonds may account for the high molecular weight polymer formation of MHC.

An increase in IS of acidified muscle proteins (pH 4) reduced the solubility to a minimum when the IS reached 200 mM due to protein aggregation, particularly MHC, through hydrophobic interactions. On the other hand, protein solubility increased to a maximum while IS increased to IS 400 mM for the neutral and alkali-treated proteins (pH 10) and then remained constant. There was a correlation between changes of total SH as well as surface hydrophobicity (S_o) and molecular weight distribution of soluble proteins as pH and IS were altered.

S_o measured by ANS probe represented an overestimated number for acid-treated proteins due to the interference of electrostatic interactions on hydrophobic binding between the probe and protein. Protein surface hydrophobicity determined using PRODAN probe demonstrated greater protein unfolding when proteins were treated at pH 10 > pH 7 > pH 4 at most IS levels. At pH 4, degraded MHC bands were observed in soluble proteins at IS between 10 and 100 mM. However, MHC was insoluble as IS increased to 150 mM or higher. The high MW polymers could be formed when the environment of the protein was controlled at pH 10 and IS equal to

or higher than 150 mM.

Ionic strength significantly affects the gelation properties of pH-shifted protein isolate. Superior gel quality was obtained from alkali-treated protein isolate (pH 11) at physiological IS (150 mM). Although their pH were readjusted to neutrality, pH-shifted protein isolates revealed a higher degree of denaturation than conventional surimi at all IS treatments (10, 150 and 400 mM). Rheological measurement showed that heat-induced gelation mechanisms of either acid- or alkali-treated protein isolates were identical when the same IS was applied. Differential scanning calorimeter (DSC) demonstrated that some myosin fragments and actin of pH-shifted fish protein isolates were not recovered.

The frozen stability of alkali-treated protein isolates (AKPI) was investigated using biochemical analysis, DSC, SEM, and Raman spectroscopic technique. The AKPI was less extractable in 0.6 M KCl than the conventional surimi. No correlation between salt extractable protein and gelation quality was noted. The AKPI underwent freeze-induced aggregation through hydrophobic interactions and disulfide bonds. Texture analysis indicated that AKPI was slightly less stable than the CS after being frozen/thawed. Nearly identical texture qualities were achieved from AKPI stored at pH 5.5 and 7.0. DSC results suggested that regardless of cryoprotectant inclusion and/or FT cycling, AKPI kept frozen at pH 5.5 were relatively less stable than those stored at pH 7.0. The most disintegrated and aggregated microstructure was observed in AKPI kept frozen at the pI without cryoprotectants, with FT cycles.

Raman technique is a potential tool to study changes of protein structures in fish proteins as a semisolid state. The significantly different protein structures between AKPI and CS was confirmed. AKPI, which can form gel without salt, contained less content of α -helical structure but higher β -sheet structure than CS. Raman data demonstrated the different structures between AKPI stored at different pH. The exposures of amino acid side chains and peptide backbone were greater in AKPI kept at pH 5.5 than pH 7.0. According to the Raman data, the freeze-induced aggregation process of AKPI did not resemble CS. Alkali-treated protein isolate requires cryoprotectant to stabilize protein quality during frozen storage.

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