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

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ABSTRACT

The physicochemical properties of gels prepared from Pacific whiting protein isolated at pH 3 and 11 with three concentrations of NaCl were characterized after a pH readjustment to 7.0. The strongest gels were obtained from fish protein isolate (FPI) prepared at pH 11/150-mM NaCl and conventional surimi. The protein solubilities of FPI treatments did not contribute significantly to their gel properties. Surface hydrophobicity and differential scanning calorimetry demonstrated that, in addition to adjusting the pH to 3 or 11, salt addition during protein solubilization and subsequent recovery at the isoelectric point (pI) led to protein denaturation. Rheological study indicated that gelation mechanisms of FPI were identical with the same NaCl concentration regardless of pH. The FPI prepared at pH 3 or 11 with NaCl could be partly refolded at pH 7. Nevertheless, some myosin fragments and actin did not refold.

PRACTICAL APPLICATIONS

Fish protein isolate (FPI) processing is a new concept for protein recovery using a pH shift. While conventional surimi processing is performed by avoiding protein denaturation, this FPI process is performed chemically by unfolding proteins and subsequent refolding. Since FPI is made with the inclusion of sarcoplasmic proteins, which are removed at the conventional surimi process, it guarantees a significant yield increase. This study revealed that the strongest gels were made from conventional surimi and FPI prepared at pH 11 and 15-mM NaCl. It also confirmed that protein solubility does not support the

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gelation properties of FPI. Refolding of FPI prepared at extreme pHs (3 or 11) was noted, but in a full scale.

INTRODUCTION

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

Hultin and Kelleher (1999) have patented the acid-aided process using cod and mackerel, which demonstrated excellent gel-forming ability as well as higher yield. Underland *et al.* (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 had equal gel qualities. The acid solubilization process with PW yielded 20% more protein than the conventional process (Choi and Park 2002; Kim *et al.* 2003) conducted as a comparative study of acid- and alkaline-aided methods and showed that gels prepared at pH 11 had superior gel quality, followed by gels prepared at pH 2. All fish protein isolates (FPI) prepared using a pH shift yielded better quality gels than CS. 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 *et al.* 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 *et al.* 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

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Thawornchinsombut and Park 2004, 2005). However, few studies have employed both pH and ionic strength changes with the FPI prepared using the pH-shift method. Interest in incorporating ionic strength effects with acid- or alkali-aided methods led us to conduct this research.

Our overall objectives were to evaluate the combined effect of pH and NaCl on acid- and alkali-treated FPI from proteinase-rich PW and to compare it with CS. Additional efforts were also made to assess the physicochemical properties, gelation properties and protein compositions.

MATERIALS AND METHODS

Materials

Pacific whiting (PW) (*Merluccius productus*) was obtained from Point Adams Packing Co. (Hammond, OR). Fresh whole fish (25–30 cm long) less than 24 h after harvest was transported on ice to the Oregon State University Seafood Lab within 30 min. The fish was filleted, ground using a grinder (Hobart, The Hobart Manufacturing Co., Troy, OH), and mixed with sorbitol (powder, Neosorb 20/60; Roquette American Inc., Gurnee, IL) at a 9:1 ratio (mince : sorbitol). The samples were then vacuum-packed in polyethylene bags with an approximate weight of 350 g before being frozen and stored in a −80C chest freezer (Forma Scientific −86C ULT Freezer, Thermo Electron Corporation, Waltham, MA). Because of the limited availability of fresh PW while conducting this research, cryoprotected mince (5 months old) was used.

Sample Preparation

Frozen PW minces were fully thawed at 5C for 2 h and then subjected to conventional surimi (CS) and various pH and NaCl-controlled processes. For the conventional method, mince was washed three times using chilled (4–6C) distilled water at a 1:3 ratio (mince : water). The final washing was carried out using 0.3% NaCl solution to facilitate the dewatering step. The suspension was centrifuged at $6{,}000 \times g$ for 20 min at 4C (Sorvall RC-5B, DuPont Co., Newtown, CT) for each washing step. The washed mince was then mixed with cryoprotectants (5% sucrose, 4% sorbitol and 0.3% sodium tripolyphosphate). Surimi was adjusted to pH 7.0 using 0.5-N NaOH and/or 0.5-N HCl and 80% moisture, and then was vacuum-packed and stored at −80C until analyzed.

According to Kim *et al.* (2003), two pH levels (3 and 11) were selected for fish protein isolation (FPI). At each pH, three concentrations of NaCl were employed: 10, 150 and 400 mM. Ten millimolar was the lowest NaCl concentration that could be obtained after pH adjustment of twice-washed mince

based on a preliminary experiment. A NaCl concentration of 150 mM is the physiological ionic strength 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 2004). The final concentration, 400 mM, was selected based on the salt concentration (2% NaCl) used for gel preparation of CS.

Thawed mince was washed twice with cold (6C) deionized (DI) water at 1:5 and 1:4 ratios, respectively, to remove sorbitol and reduce ionic strength (IS). The homogenate was centrifuged at $6{,}000 \times g$ 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 measurements were carried out 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). Once the pH was fixed, the desired NaCl concentration was obtained by adding NaCl granules (reagent grade). The salt concentration of the homogenates was measured using a conductivity meter (YSI 3100; YSI Inc., Yellow Spring, OH) equipped with a conductivity cell (cell constant 1.0/cm). Conductivity readings were obtained based on a standard curve prepared using NaCl 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 NaCl, the final pH after adjustment of NaCl concentration was 3.03 ± 0.03, 3.25 ± 0.04 and 3.47 ± 0.06 for pH 3, respectively, and 10.94 ± 0.05, 10.69 ± 0.03 and 10.57 ± 0.03 for pH 11, respectively. After pH and salt adjustment, the samples were centrifuged at $7{,}000 \times g$ for 20 min at 4C to separate insoluble parts. Proteins were subsequently recovered at pH 5.5 for samples treated at 10- and 150-mM NaCl; however, pH 4.5 was used for the samples treated at 400-mM NaCl because of the isoelectric point (pI) shifting caused by the high salt concentration (Thawornchinsombut and Park 2004). When NaCl is added, the chloride ion preferentially binds with the positively charged amino acids to a stronger degree than the sodium ion, resulting in more negatively charged amino acids (Ockerman 1980). Thus, the system requires more H^+ ion to reach the approximately zero net charge needed to precipitate proteins. Protein precipitates were collected by centrifugation ($8{,}000 \times g$ for 25 min at 4C). The precipitates were then mixed with the 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 salt concentration of samples after adjusting to pH 7.0 was recorded (Table 1). The samples were vacuum-packed and stored at −80C until tested. All treatments were prepared in a walk-in cold room (5–6C) to keep the processing at low temperature.

TABLE 1.
PROTEIN SOLUBILITY OF PACIFIC WHITING PROTEIN ISOLATES PREPARED AT VARIOUS pH AND NaCl CONDITIONS AND CONVENTIONAL SURIMI (CS). ALL TREATMENTS WERE ADJUSTED TO PH 7.0 BEFORE ANALYSIS

Conditions at protein solubilization step		Isoelectric point (pI) used for protein recovery	NaCl concentration of gel after adjusting pH to 7.0† (mM)	Solubility (mg/g dry basis)‡	
pH*	mM NaCl			Double deionized water	0.6 M NaCl
3	10	5.5	44	56.3 ± 4.6^{c}	67.7 ± 5.4^{b}
	150	5.5	154	39.3 ± 3.3^{b}	34.0 ± 3.5^{a}
	400	4.5	380	41.1 ± 3.3^{b}	41.0 ± 3.9^{a}
11	10	5.5	49	107.0 ± 5.6^{d}	79.0 ± 2.0^{b}
	150	5.5	172	37.5 ± 0.7^{ab}	35.2 ± 1.5^{a}
	400	4.5	302	26.9 ± 0.3^{a}	37.6 ± 3.5^{a}
CS	38	–	425	94.6 ± 2.5^{d}	309.2 ± 11.1^{e}

* All treatments were adjusted to pH 7.0 during mixing with cryoprotectants.
† All gels were prepared without salt except the CS (2% NaCl), because better gel is obtained without salt for fish protein isolate treatments (Kim 2002).
‡ Values are the means of three replicates ± SD. Values within a column with different superscripts differ significantly ($P < 0.05$).

Protein Solubility

Three grams of sample were homogenized with 27 mL of chilled double deionized (DDI) water and one gram of sample was homogenized with 0.6-M NaCl in 20 mM Tris-HC1 buffer (pH 7.0). The homogenization was set at speed level 3 for 1 min (Ultra-Turrax T25; IKA Working Inc., Willington, NC). After centrifugation at 20,000 × g for 25 min at 4C, the Bio-Rad protein assay with bovine serum albumin (BSA) (Bio-Rad Laboratories, Hercules, CA) as a standard was performed to estimate protein concentration of the supernatant (Bradford 1976). The absorbance was measured at 595 nm (UV-VIS Spectrophotometer, UV 2401PC; Shimadzu Corp., Kyoto, Japan). The supernatant was diluted appropriately using the same buffer for each analysis to obtain an absorbance in the linear range of the BSA calibration curve.

Gel Preparation

Frozen samples were partially thawed under ambient temperature for 1 h, and 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 (Morrissey *et al.* 1993). Its moisture content was adjusted to 80%. All treatments were prepared without salt except CS, which contained 2% salt. This process was conducted in a walk-in cold room (5–6C). A small amount of paste (uncooked gel) was saved for analysis of surface hydrophobicity, total sulfhydryl (SH) content, dynamic testing and thermodynamic properties. The remaining paste was then stuffed into stainless steel tubes (2.0-cm I.D., 20.0-cm long), cooked at 90C 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.

Fracture Analysis

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

Surface Hydrophobicity

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

A 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, Norwalk, 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 × g for 30 min at 4C, 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 μL of ANS stock solution. After holding for 10 min, the relative fluorescence intensity (RFI) of each solution was measured. The initial slope (S_o) of the net RFI versus protein concentration was calculated by linear regression analysis and was used as an index of the protein surface hydrophobicity.

Total SH Contents

Total SH group content was determined using Ellman's reagent (5, 5′-dinitrobis[2-nitrobenzoic acid]; DTNB) as described by Hamada *et al.* (1994) with slight modification. The paste (uncooked gel) 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 μL of 10-mM DTNB in 0.1-M sodium phosphate buffer, pH 7.2, containing 0.2-mM ethylenediaminetetraacetic acid (EDTA). The sample was incubated at 40C 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/cm (Riddles *et al.* 1979).

SDS-PAGE

Fish protein gels (cooked) were subjected to SDS-PAGE according to the method of Laemmli (1970). Two grams of sample was homogenized with 5 mL of 8-M urea and 13 mL of 5% SDS solution. Samples were shaken for 2 h at 150 rpm (Lab-Line Orbit Environ-Shaker; Lab-Line Instruments, Inc., Melrose Park, IL) at ambient temperature and then were heated in a water bath at 90C for 1 h according to the method of Yongsawatdigul and Park (2004) with slight modifications. It should be noted that the FPI gels were not completely dissolved by the mixed solvent (8-M urea and 5% SDS). Thus, insoluble proteins were not subjected to SDS-PAGE analysis. After centrifugation at 20,000 × g for 30 min, the protein concentration of the supernatant was determined using the method of Lowry *et al.* (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 μL) was mixed with 125 μL of sample buffer containing 60-mM Tris-HCl buffer (pH 6.8), 25% glycerol, 2% SDS, 10% β-ME (0% β-ME for nonreduced samples) and 0.1% bromophenol blue. Samples were then heated in boiling

water for 5 min and centrifuged at 10,000 × 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. Thirty micrograms of protein was placed into each well. Electrophoresis was performed using a Fisher FB-VE 16-1 unit (Fisher Biotech; Fisher Scientific, Pittsburgh, PA) at 200 volts/gel and ambient temperature. The 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 a set of wide range molecular weight (MW) markers (Sigma Chemicals Co., St. Louis, MO) containing the following 13 components: rabbit muscle myosin (205 kDa), *Escherichia coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97 kDa), rabbit muscle fructose-6-phosphate kinase (84 kDa), bovine albumin (66 kDa), bovine liver glutamic dehydrogenase (55 kDa), egg ovalbumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine erythrocytes carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa), bovine milk α-lactalbumin (14.2 kDa) and bovine lung aprotinin (6.5 kDa). SigmaGel software (SPSS Science, Chicago, IL) was used to estimate the molecular weights of protein bands.

Rheological Measurements

The rheological properties of sample pastes (uncooked gel) were monitored as a function of temperature using dynamic oscillation with a 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 for temperatures from 20 to 90C at a heating rate of 1C/min. A solvent trap with a moistened sponge inside was used to prevent moisture evaporation during measurements. Based on a preliminary calibration to determine the linear viscoelastic region, a 1-Pa torque value and 0.1-Hz frequency were selected.

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 (1 cm^3). Another calibration with the samples was performed along with an empty reference vessel to determine the amount of DDI water required as a reference. The

samples were scanned along with a reference vessel containing DDI water at a heating rate of 1.0C/min from 20 to 90C.

Statistical Analysis

All the tests were run in triplicate. Data were analyzed using an analysis of variance (ANOVA) procedure. The 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 Science, Chicago, IL). Because of the magnitude range of data obtained (the difference between the highest and the lowest values was more than 10 times), logarithmic transformation of the surface hydrophobicity (S_o) values was performed prior to statistical analysis.

RESULTS AND DISCUSSION

Protein Solubility

Because 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 with some connective tissue proteins.

Salt (0.6-M NaCl) substantially enhanced the protein solubility of CS (Table 1). However, this high salt solubility was not observed with FPI at all NaCl levels. The data showed that samples treated at pH 11 and 10-mM NaCl showed the highest protein solubility in water, which was not significantly different from CS ($P \geq 0.05$). The reduced solubility in both water and salt solution at 150- and 400-mM NaCl compared with 10-mM NaCl was noted.

The solubility of acid- and alkaline-treated FPI was lower than CS in both water (except pH 11 and 10-mM NaCl) and 0.6-M NaCl ($P < 0.05$). Our previous study also showed that the solubility of alkali PW protein isolate (pH 11) in 0.6-M NaCl and 20-mM Tris-HCl solution was lower than CS by ~7 times (Thawornchinsombut and Park 2006). Isolation of rockfish muscle proteins after solubilizing at either pH 2.5 or 11 induced denaturation and aggregation of both sarcoplasmic and myofibrillar proteins (Yongsawatdigul and Park 2004). Protein denaturation (either from frozen storage or chemical change) 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; Badii and Howell 2002; Thawornchinsombut and Park 2006). The results of total SH content (Fig. 1) and SDS-PAGE (Fig. 2a,b) possibly imply that disulfide linkages stabilized the protein aggregates in FPI, resulting in lower protein extract-

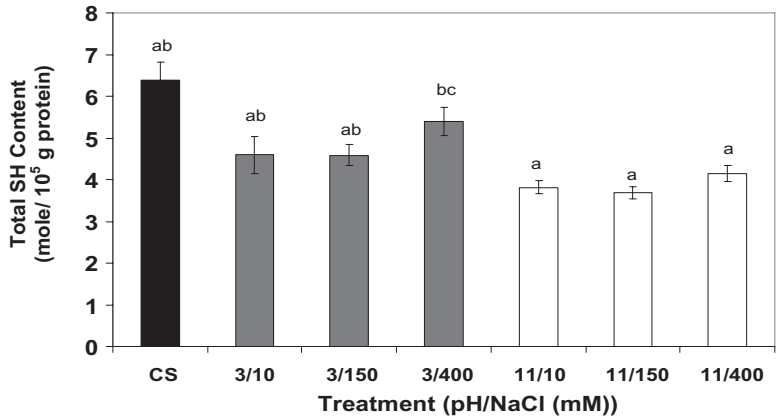

FIG. 1. TOTAL SULFHYDRYL (SH) CONTENT OF PACIFIC WHITING CONVENTIONAL SURIMI AND PROTEIN ISOLATED AT VARIOUS pH AND NaCl CONDITIONS
All treatments were adjusted to pH 7.0 before analysis. The pH/NaCl corresponds the pH and NaCl concentration (mM) treatment of fish protein isolate solubilizing process. Different letters on each bar represent significant differences ($P < 0.05$).

ability than CS. In addition, electronegative interactions due to an effect of salt on chemically denatured proteins may also contribute to less protein solubility of FPI. When NaCl was added, the net balance of negative or positive charge on the protein molecules was changed depending on the concentration of NaCl. An increase in protein charge density would lead to increased separation, allowing more water molecules to bind with the proteins (salting-in effect) (Kristinsson and Hultin 2003c). However, if hydrophobic groups of protein molecules are already exposed because of chemical denaturation, the excess of Na^+ or Cl^- (at 150- and 400-mM NaCl) perhaps causes the negative interactions among the protein molecules enhancing the hydrophobic interactions. As a result, protein solubility might have decreased. Kim (2002) reported that NaCl did not significantly enhance the solubility of acid-treated myofibrillar protein isolate compared to its solubility in pH 7 buffer without NaCl. Kristinsson and Hultin (2004) found that inclusion of NaCl to pH 3-treated trout hemoglobin led to less solubility. The dramatic loss in solubility after acid unfolding in the presence of salt corresponded to less refolding compared with the control (no salt).

Fracture Analysis

Some textural properties of gels are presented in Fig. 3. It is well known that protein solubilization of myofibrillar proteins in salt solutions is related to

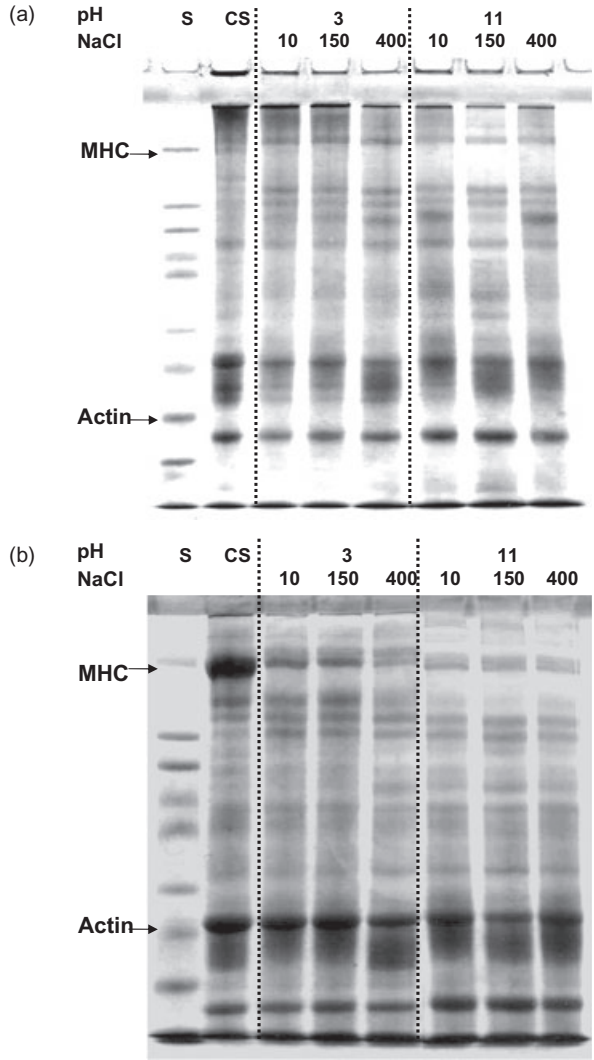

FIG. 2. SDS-PAGE OF VARIOUS PROTEIN GELS MADE WITHOUT (a) AND WITH (b) β-ME (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of gels made from Pacific whiting (PW) conventional surimi (CS) and protein isolated at various pH and NaCl conditions solubilized in buffer *without* β-mercaptoethanol (β-ME) (3.5/7.5% stacking/separating gel, 30 μg protein/well). (b) SDS-PAGE patterns of gels made from PW CS and protein isolated at various pH and NaCl conditions solubilized in buffer *with* β-ME (3.5/7.5% stacking/separating gel, 30 μg protein/well).

All treatments were adjusted to pH 7.0 before analysis. The pH/NaCl corresponds the pH and NaCl concentration (mM) treatment of fish protein isolate solubilizing process. S, standard marker proteins; MHC, myosin heavy chain.

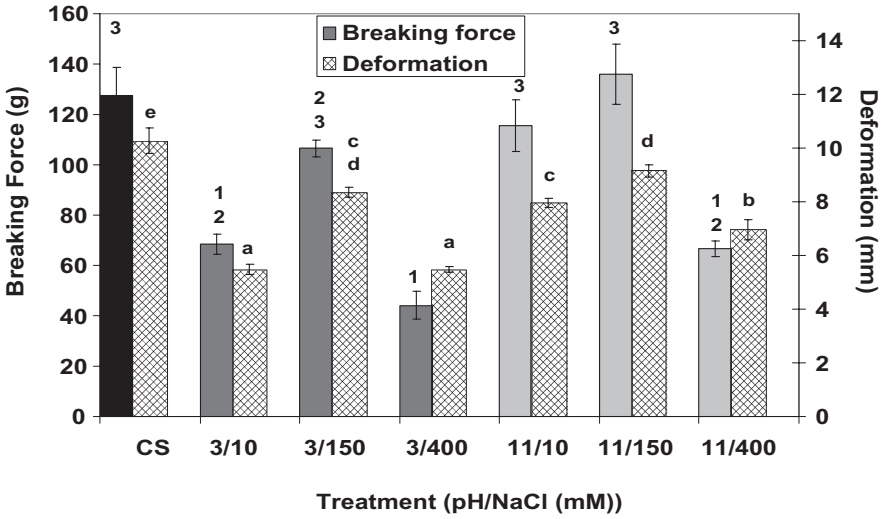

FIG. 3. TEXTURE PROPERTIES OF GELS PREPARED FROM PACIFIC WHITING CONVENTIONAL SURIMI AND PROTEIN ISOLATED AT VARIOUS pH AND NaCl CONDITIONS

All treatments were adjusted to pH 7.0 before analysis. The pH/NaCl corresponds the pH and NaCl concentration (mM) treatment of fish protein isolate solubilizing process. Different numbers (breaking force) or letters (deformation) on each bar represent significant differences ($P < 0.05$).

functional properties of fish meat such as gel-forming ability, emulsifying property and water-holding ability (Suzuki 1981; Regenstein *et al.* 1983; Akahane *et al.* 1984). 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 observed in this work. At both acidic and alkaline pH, gel texture was better at 150-mM NaCl. At each salt level, texture qualities of gels made from FPI prepared at pH 11 were superior to those of gel obtained from pH 3-treated FPI. Kristinsson and Hultin (2004) reported that acid-treated trout hemoglobin underwent protein denaturation at a faster rate than alkali treatments. Inclusion of 500-mM NaCl at low pH considerably increased the rate of unfolding. These probably explain why gels from acid treatments in this study showed lower texture qualities than alkali treatments. Gel strength of treatments at pH 11/150-mM NaCl was comparable with CS ($P \geq 0.05$), but its gel strain was slightly lower than CS. According to our previous study (Thawornchinsombut and Park 2005), the polymerization of alkali-treated proteins could occur more effectively when subjected to suitable salt levels (150–600 mM NaCl). However, salt at high level (400-mM NaCl) might result in a stiff gel due to precipitation of chemically denatured protein induced by ionic and hydrophobic interactions as well as disulfide bonding. 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.

Surface Hydrophobicity (S_o)

It was interesting to see that CS showed the lowest S_o value ($P < 0.05$) although its salt content was comparable with the refolded acid- or alkali-treated FPI at 400-mM NaCl (adjusted to pH 7.0) (Fig. 4). This indicated that chemical solubilization and precipitation of the proteins, even at very low salt concentration (10-mM NaCl), 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 salt content (Yongsawatdigul and Park 2004). The changed protein conformation induced by chemical denaturation might not be fully restored when pH is moderated. Our Raman spectroscopy study showed that the Raman spectra of pH 11-treated rockfish protein isolates were significantly different from CS (the control in that study) even though the pH was adjusted to 7.0 before analysis (Thawornchinsombut *et al.* 2006). More exposed tyrosine residues and peptide backbone stretching were proposed for the alkali-treated FPI. An increase in salt concentration from 10- to 150-mM NaCl dramatically

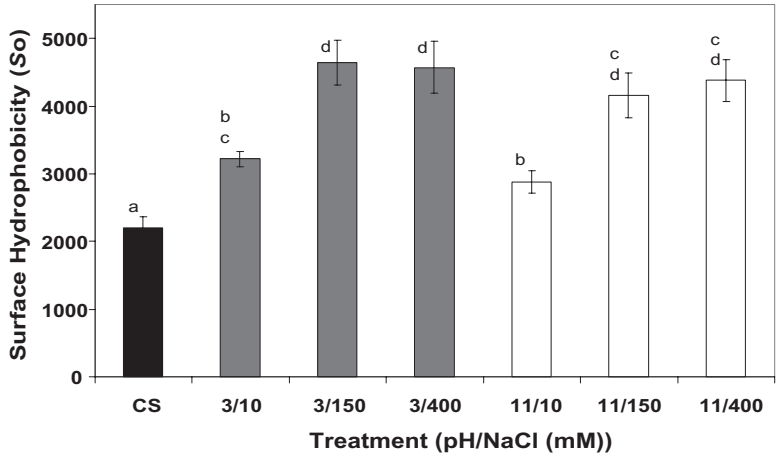

FIG. 4. SURFACE HYDROPHOBICITY OF PACIFIC WHITING CONVENTIONAL SURIMI AND PROTEIN ISOLATED AT VARIOUS pH AND NaCl CONDITIONS
All treatments were adjusted to pH 7.0 before analysis. The pH/NaCl corresponds the pH and NaCl concentration (mM) treatment of fish protein isolate solubilizing process. Different letters on each bar represent significant differences ($P < 0.05$).

increased ($P \leq 0.05$) protein unfolding. However, when the salt content was increased to 400 mM, no significant change of S_o was observed (Fig. 4). In addition, no significant difference was found between acidic and alkaline treatments at the same NaCl levels.

With high salt (>0.3-M NaCl) and high pH (>7) conditions, dissociation of myosin occurred (Godfrey and Harrington 1970a,b; Weeds and Pope 1977). With 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 the probe to bind. 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 2004, 2005). Electrostatic interactions possibly strengthen the hydrophobic interactions between ANS and proteins (Haskard and Li-Chan 1998).

Total SH Content

Figure 1 illustrates the total SH contents of samples. Changes of NaCl level had no significant effect on SH content. Under alkaline conditions and at all NaCl 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. These results are supported by the protein solubility results in a solvent-containing urea and SDS, regarding the presence of β-ME, as described in the SDS-PAGE results (see later discussion).

In general, at each NaCl level, alkali-treated FPI contained lower SH content and showed somewhat higher gel qualities (Fig. 3) compared with those of acid treatments. Kim *et al.* (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. Similar findings with PW muscle proteins were reported in our previous research (Thawornchinsombut and Park 2004, 2005). At higher pH, thiol oxidation reactions between exposed –SH or S^- groups can occur in addition to disulfide interchange reactions (Watanabe and Klostemeyer 1976). The study of biodegradable films prepared from myofibrillar proteins of blue marlin flesh showed that the important chemical bondings involved in the protein–protein interactions were hydrophobic interactions for acidic conditions, hydrogen bondings for neutral conditions, and hydrophobic interactions and disulfide bondings for alkaline conditions (Shiku *et al.* 2005). 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 *et al.*

1995). The reduction of total SH content with alkaline conditions might have also been caused by lysinoalanine (LAL) cross-links, commonly presenting in alkali- and/or heat-treated proteins (Maga 1984; Damodaran 1996) and primarily contributed by cysteine (Friedman and Masters 1982; Chang *et al.* 1999). However, specific studies involving LAL formation in fish protein processed under alkali condition are very limited. Fujimaki *et al.* (1980) reported that the optimum pH for forming LAL was >13 in fish protein concentrate. LAL formation was maximized at 90C and continued to increase over a longer period of heating.

SDS-PAGE

FPI samples (cooked gel) were not completely soluble in the SDS-PAGE solvent after shaking and heating. Alkali-treated FPI retained more particles than acid-treated FPI. More undissolved particles were found in FPI with higher salt content than with lower salt content. After centrifugation, only the supernatant was mixed with sample buffers with and without β-ME. No myosin heavy chain (MHC) band was seen in all nonreduced treatments except possibly CS. High MW bands (>205 kD) were seen in all samples especially on the top of the polyacrylamide gel (Fig. 2a). These high MW proteins possibly resulted from both the pH/NaCl treatments and frozen storage of raw materials. Several studies have demonstrated that protein denaturation during frozen storage resulted in lower extractable 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 *et al.* 1999; Sultanbawa and Li-Chan 2001). In FPI samples, fewer protein polymers were extracted (more insoluble gel particles than CS), resulting in a smaller high MW polymer bands, especially for the alkali-treated samples (Fig. 2a). In addition, PW muscle proteins contain endogenous transglutaminase (TGase) (Peters *et al.* 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 *et al.* 1996), it would also be partly involved in the formation of high MW protein polymers.

After reduction with β-ME, bands on the top of the gel (loading well) were not observed. MHC bands appeared in all treatments with the >205-kD MW bands right above the MHC (except for CS), perhaps suggesting that protein polymers in the pH and NaCl-controlled treatments were more stable than those in CS. Although the MHC band appearing for CS showed more recovery than all FPI treatments under reducing condition, it should be noticed that more insoluble gel particles of FPI could not be subjected to SDS-PAGE. To confirm the presence of disulfide bond-forming polymers, protein solubility

(Bradford 1976) using different compositions of solvents was determined. Cooked gels 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 NaCl, while in solvent with β-ME, no protein particles were observed in all treatments. In both solvents, CS showed the highest protein solubility (data not shown). In pH/NaCl-treated samples, the disulfide linkages possibly enhanced the gel strength but reduced the gel strain compared with CS as seen from the texture measurements (Fig. 3). It has been noted that disulfide bonds reduce the flexibility of a protein. In the case of soy protein, the disulfide bonds not only limited molecular flexibility but also restricted foaming (Kinsella *et al.* 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 the strain values of gels.

In acid- and alkaline-aided processes, myofibrillar protein degradation was significantly more pronounced after acidification than alkalinization (Underland *et al.* 2002; Kim *et al.* 2003). Cortes-Ruiz *et al.* (2001) also reported proteolytic activity in acid-produced proteins from sardines. 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 CS process, cause the gel-weakening phenomenon (An *et al.* 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 *et al.* 2003). Nevertheless, in the present study, no MHC degradation was found in all treatments as evidenced by SDS-PAGE of the reduced sample with 10% separating gel (data not shown). This might have resulted from twice washing the fish mince before acidification or alkalinization.

Rheological Properties

Storage (G′) and loss (G″) modulus obtained with dynamic viscoelasticity measurements represent the elasticity and viscosity, respectively, of a viscoelastic body (Nakagawa 1978; Sano *et al.* 1988). The loss tangent (tan δ; G″/G′) reflects the relative contribution of each factor to the overall rheological characteristics. Egelansdal *et al.* (1986a) studied the reproducibility of the rheological thermograms of myosin suspensions and reported that G′ showed the least relative error compared with G″ and tan δ. The transitions of G″ and tan δ were somewhat obscured by the standard errors of the mean. Thus, in this study, we will discuss the results of rheological property studies focusing on G′.

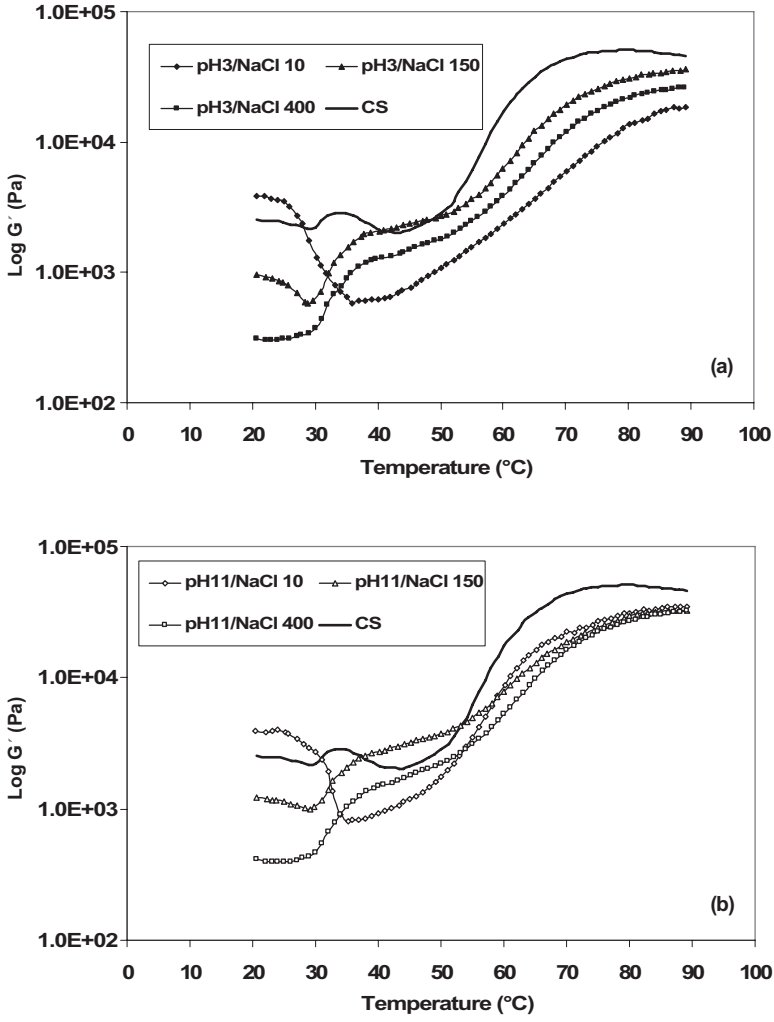

FIG. 5. CHANGES IN STORAGE MODULI (LOG G′) AS A FUNCTION OF TEMPERATURE OF PW PROTEIN ISOLATES PREPARED AT VARIOUS pH AND NaCl CONDITIONS
(a) pH 3, (b) pH 11 and conventional surimi (CS).
All treatments were adjusted to pH 7.0 before analysis. The pH/NaCl corresponds to the pH and NaCl concentration (mM) treatment of fish protein isolate solubilizing process.

In Fig. 5, the rheogram of CS during gelation is presented. Several studies have reported a similar rheogram pattern for fish myosin, actomyosin and surimi (Wu *et al.* 1985; Visessanguan *et al.* 2000; Benjakul *et al.* 2001; Esturk 2003; Kim *et al.* 2003). The rheograms of pH-treated proteins were different

compared with CS, although the final pH of all samples was set at 7.0. The conformations of the acid- or alkali-treated proteins were partially unfolded and then were refolded by pH adjustment to neutrality (Kristinsson and Hultin 2003a; Thawornchinsombut *et al.* 2006). However, these proteins seemed to exhibit a rheological behavior resembling the native protein under various NaCl contents (Sano *et al.* 1990a). At each NaCl level, the G′ thermograms of pH 11-treated samples were relatively similar to that of the pH 3-treated samples with slight to moderately higher magnitude over the temperature range. Nevertheless, the transition temperatures of the samples between 20 and 55C appear to be different when the NaCl concentration was changed.

At 20C, the storage modulus was highest at 10-mM NaCl (Fig. 5). With low NaCl condition, the refolded protein molecules assemble and form filaments as a result of the polar bindings of the tail portion with the head portion (Sano *et al.* 1990a). This probably led to the myosin molecules being resistant to rheological changes. G′ decreased as the temperature reached around 36 and 34C for the acid- and alkali-treated samples, respectively. As the temperature increased, the polar bindings of 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 gel onset point, G′ showed a considerable increase as the system takes on a more elastic structure. The alkali-treated samples had a higher G′ at most temperatures in the range of this study than the acidic treatments, corresponding to higher gel quality.

As NaCl content increased, the G′ at 20C decreased. Egelansdal *et al.* (1986b) reported that the initial increase of G′ was attributed to the cross-linking of myosin, while the decline of G′ resulted from denaturation of light meromyosin, leading to increased fluidity. Because in the present work, the FPI treatments were formerly chemically denatured (pH-shifted and NaCl effects), we only observed the initial reduction of G′. The G′ thermogram for the temperature range of 20–34C of proteins prepared at 150-mM NaCl resembled the thermogram obtained from CS. Nevertheless, the thermograms from 34 to ~55C showed different patterns from CS.

A sharp increase in the storage modulus upon increasing the temperature from 29 to 38C was observed with both 150- and 400-mM NaCl treatments. This change of G′ has been proposed to be the result of aggregation of the unfolding head and hinge portions (Ishioroshi *et al.* 1982). As temperature continuously increases, unfolded actomyosin entangled and formed gel networks, which was evident by an increase of G′ at temperature around 55C.

As shown in Fig. 5, the second decline in G′ as was found in CS (34–43C) was not observed in pH-shifted treatments. This weakening of the G′ value was postulated to be due to the dissociation of the actin–myosin complex (Egelansdal *et al.* 1986b; Sano *et al.* 1994) and to helix-to-coil transformation

of the myosin tail (Sano *et al.* 1988). The disintegration of actomyosin during the pH and NaCl alteration in the FPI processes was probably irreversible.

Egelansdal *et al.* (1986b) observed that there was no decrease in G′ at 50–55C in adenosine triphosphate (ATP)-added beef myosin compared with 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 Ca^{2+}- and Mg^{2+}-ATPase activities of PW proteins isolated at acidic pH (Choi and Park 2002). Kristinsson and Hultin (2003a) discovered that the myosin light chain was dissociated in acid-treated cod myosin after refolding at neutrality, while only a 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 55C, a steady increase in G′ was noted for all treatments (Fig. 5). The rigidity of the previously formed elastic network is enhanced as aggregation continues (Sano *et al.* 1990a,b). Whereas, tail–tail interactions through cross-linking had been predominantly involved at lower temperatures, the globular head portion (head region of heavy meromysin) of myosin assumedly plays a role above 60–70C (Taguchi *et al.* 1987; Sano *et al.* 1990a,b). However, controversy with respect to acceptance of this mechanism had been suggested by Samejima *et al.* (1981), Ishioroshi *et al.* (1982), Egelansdal *et al.* (1986a), and Sharp and Offer (1992).

The thermograms at the higher temperature range were less dependent on pH and NaCl level (Fig. 5a,b). This finding might be supported by Samejima *et al.* (1981) and Ishioroshi *et al.* (1982). They found that heat-induced gelation of isolated S-1 was independent of pH and salt concentration.

Thermodynamic Properties

In general, it is known that DSC is a useful technique to determine thermodynamic data for protein denaturation temperatures and energy under various circumstances. Figure 6 shows the DSC thermograms of CS and pH/NaCl-treated samples. Only CS was mixed with 2% NaCl for the DSC analysis. The DSC thermogram of CS showed five endothermic peaks. According to several literature reports (Hastings *et al.* 1985; Beas *et al.* 1990; Ogawa *et al.* 1993; Fernandez-Martin *et al.* 1998; Herrera *et al.* 2001; Badii and Howell 2003), we can presumably assign those peaks to the following protein transitions: (P1) “32.4C” is the combined peak of the first myosin and collagen transitions, (P2) “37.8C” is the second myosin transition, (P3) “45.7C” is the third myosin transition, (P4) “60.2C” is the actin transition and (P5) “75.7C” is supposed to be the sarcoplasmic proteins transition or a

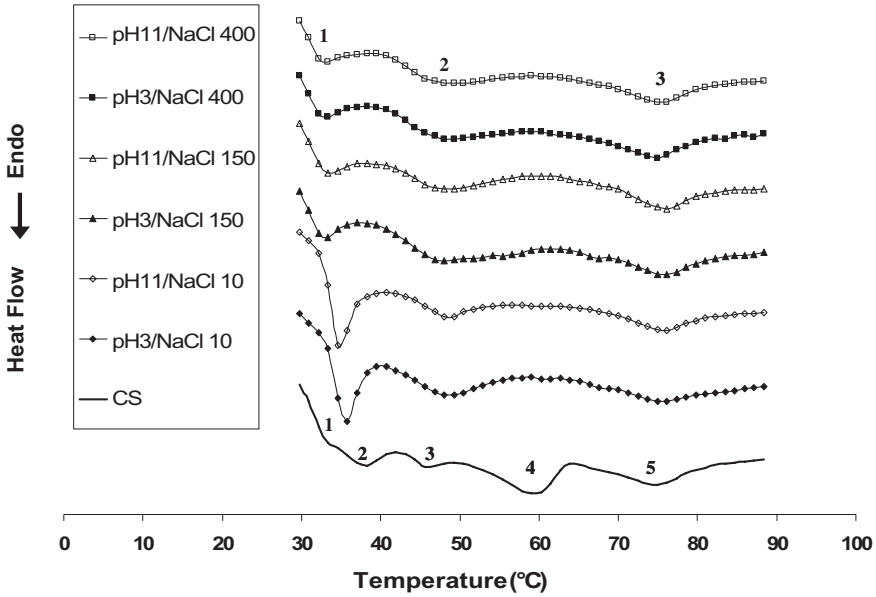

FIG. 6. DIFFERENTIAL SCANNING CALORIMETRY (DSC) THERMOGRAMS OF PACIFIC WHITING PROTEIN ISOLATES PREPARED AT VARIOUS pH AND NaCl CONDITIONS AND CONVENTIONAL SURIMI (CS)
All treatments were adjusted to pH 7.0 before analysis. The pH/NaCl corresponds to the pH and NaCl concentration (mM) treatment of fish protein isolate solubilizing process.

transition of protein polymers formed during frozen storage. A similar pattern was obtained from PW surimi (2% NaCl) with only four transition temperatures (T_m) at 33, 38, 46.3 and 62.7C, respectively (Esturk 2003).

Different T_m values have been reported by other investigators. Hsu *et al.* (1993) reported that the DSC thermogram for whole muscle of PW showed two endothermic transitions, with T_m values of approximately 45.5 and 75.0C, respectively. T_m of myosin and actin of fresh hake (*Merluccius hubbis*) (Beas *et al.* 1990), blue whiting (*Micromesistius poutassou Risso*) (Fernandez-Martin *et al.* 1998) and cod (*Gadus morhua*) (Hastings *et al.* 1985) muscles were 46.5 and 75.3, 45 and 75, and 44.5 and 74.7C, respectively. Those T_m values were not consistent with Esturk (2003) who reported T_m values of 41.2 and 68.8C for PW surimi without salt analyzed using the same micro DSC III calorimeter. This discrepancy may be due to different heating rates (10C/min versus 1C/min). Ogawa *et al.* (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-shifted FPI with NaCl-controlled treatments exhibited only three endothermic peaks at temperatures around 32.6–35.5, 47.5–48.3 and 75.5C (Fig. 6). The second transition possibly included two myosin domains. However, the actin peak completely disappeared. The increase in NaCl content of the protein homogenate followed by protein precipitation at the approximate pI probably played an important role in the irreversible denaturation of these two peaks (P2 and P4 in CS), because they also disappeared in sample prepared at pH 7/400-mM NaCl (data not shown). Howell *et al.* (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. In our previous study, we observed three endothermic peaks with T_m around 33.5–34.7, 46.2–47.8 and 66.5–68C (possibly actin), respectively (Thawornchinsombut and Park 2006). It should be noted that PW mince were not frozen and NaCl was not included in those alkali-FPI samples. Consequently, the peak at ~75C was not observed.

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 a possible myosin transition was seen when herring was soaked in 14% salt (~1.5 M) and pH 4. Myosin and actin were recovered with a higher T_m after dialysis (Hastings *et al.* 1985). However, in the present study, it should be noted that only parts of the myosin transition were renatured but not that of actin. It is known that refolding a denatured protein from a low or high pH is sometimes accompanied by transient associations of partly folded intermediates (Tanford 1968; Fink *et al.* 1994; Ptitsyn 1995) The conformational changes as affected by pH readjustment of trout hemoglobin were studied (Kristinsson and Hultin 2004). When acid (pH 1.5–3.5) or alkali (pH 10–12) treatments were adjusted to neutrality, the recovery of native structure on refolding was proportional to the extent of unfolding prior to pH readjustment: the more unfolded the protein, the less was the recovery of native structure. In addition, the presence of salt led to a smaller recovery of native structure. In the structural changes study of the alkali-FPI, the Raman spectral data showed that the protein structure in the pellet recovered from alkali-treated proteins could be partially refolded by adjusting to neutral pH (Thawornchinsombut *et al.* 2006).

The presence of the peak at 75.7C 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 –80C for ~5 months), because this T_m was not noticed in the pH-shifted samples prepared from fresh PW without adding salt (Thawornchinsombut and

Park 2006) 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 74.2C (Kim *et al.* 2005). Badii and Howell 2003 determined the changes of cod collagen in the presence of fish oil with 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 during frozen storage (Haard 1992). Depending on species, conditions and time of storage, these proteins will be less extractable in SDS or SDS plus mercaptoethanol, and eventually, a nonextractable residue can be obtained (Tejada *et al.* 1996; Careche *et al.* 1998; Del Mazo *et al.* 1999). In contrast to the last peak, the first myosin peak of FPI samples was sensitive to a change of environment. Both enthalpy and T_m of this peak decreased when NaCl content of the protein environment increased (Fig. 6).

CONCLUSIONS

Based on the results from SDS-PAGE, rheological measurements and DSC, the influence of NaCl 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 quality gels were obtained from FPI prepared at alkaline pH (11) and near-physiological salt concentration (150-mM NaCl). Increasing or decreasing NaCl from this level lowered gel quality. FPI gels, particularly those prepared at high pH, underwent greater denaturation and contained less SH groups than CS, probably resulting in protein precipitation with less elasticity. The refolding step, which was carried out by adjusting the 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 NaCl levels. Dynamic tests and DSC analysis showed that heat-induced gelation mechanisms of various pH/NaCl-treated FPI samples were similar at the same NaCl levels regardless of pH. The significant differences of protein characteristics were more influenced by salt concentration than pH, as evidenced by the rheological and thermodynamic data.

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