The results of SDS-PAGE and densitometry indicated that a significant amount of myofibrillar proteins was lost during surimi processing. Microfiltration (MF) was utilized to recover insoluble particulate. The MF-recovered proteins showed highly functional properties in gel hardness, cohesiveness, color, and water retention ability. The soluble proteins concentrated by ultrafiltration (UF) possessed dark colors and strong odors. However, the use of UF demonstrated the possibility of recycling water in leaching systems.

To reduce the loss of myofibrillar proteins during processing, the factors causing solubilization of myofibrillar proteins were investigated. Myosin and actin were highly soluble when their ionic strengths were substantially reduced. Salt concentrations of 0.25%, 0.5%, and 1.0% NaCl reduced the solubility of myosin and actin but did not remove sarcoplasmic proteins effectively. At 2.0% NaCl, severe loss of myosin, actin, \(\alpha\)-tropomyosin, \(\beta\)-tropomyosin, and troponin-T was observed. At low water/meat ratio (2:1) with increased washing cycles and washing time, more sarcoplasmic proteins per
unit of water were removed without a noticeable loss of myosin or actin. Myosin heavy chain (MHC) content, water retention ability, and whiteness of the washed mince were comparable to that at high water/meat ratio (4:1). Prolonged storage and elevated temperatures caused a severe proteolysis of myofibrillar proteins. The degraded proteins had higher solubility than their native myofibrillar proteins. MHC and actin degradation both showed a good correlation to protein solubility.

The relationship between conformational changes and solubility of myofibrillar proteins was investigated using myosin as a model system. The results showed that adding salt or shifting pH from the isoelectric point of myosin caused an increased surface hydrophobicity and a decreased helix structure. A slightly increased sulfhydryl content was also observed. These conformational changes resulted in an increased solubility. At high salt concentration (>1.0 M), myosin regained its helix structure with a concomitant loss of solubility. The salting out effect was probably due to the dominant hydrophobic interaction among nonpolar amino acids residues.
Solubility and Structure of Fish Myofibrillar Proteins as Affected by Processing Parameters

by

Tein Min Lin

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APPROVED:

Major Professor, representing Food Science and Technology

Head of Department of Food Science and Technology

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Tein Min Lin, author
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CONTRIBUTION OF AUTHORS

Dr. Jae W. Park was involved in the design, analysis, and writing of each manuscript. Dr. Michael T. Morrissey was involved in the design of this study.
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SOLUBILITY AND STRUCTURE OF FISH MYOFIBRILLAR PROTEINS AS AFFECTED BY PROCESSING PARAMETERS

I. INTRODUCTION

GENERAL CONSIDERATION

One of the most critical steps in surimi processing is the washing of minced fish flesh with large amounts of water to remove sarcoplasmic protein, blood, fat, and other nitrogenous compounds. Reduction of sarcoplasmic proteins, in turn, concentrates myofibrillar proteins which are the major components responsible for formation of surimi gels (Niwa, 1992). Texture, as well as color and odor, of the final product is greatly improved by washing out these impurities. The degree of washing required to produce good quality surimi depends upon the type, composition, and freshness of the fish.

The number of washing cycles and water/meat ratios employed for washing varies among processors. Generally, the overall water/meat ratio used for washing ranges from 3:1 to 6:1 for at-sea processors and from 12:1 to 24:1 for on-shore processors. It was estimated that 50% of total proteins were lost during washing and dewatering processes (Adu et al., 1983; Pacheco-Aguilar et al., 1989; Yang and Froning, 1992), and 30 liters of waste water per one kg of surimi produced were generated from on-shore processing plants (Lin et al., 1995). Therefore, maximization of processing parameters to reduce water usage, solid loss, and waste disposal have recently become a major consideration for surimi manufacturers. Myofibrillar proteins constitute approximately 66-77% of total proteins in fish flesh (Suzuki, 1981). Therefore, recovery of myofibrillar proteins is expected to be in this range during surimi manufacturing. However, only about 50% of
total proteins were recovered as myofibrillar proteins in surimi. Subsequently, the loss of myofibrillar proteins in commercial surimi operation has been reported (Adu et al., 1983; Pacheco-Aguilar et al., 1989; Yang and Froning, 1992, Lin et al., 1995).

During surimi processing, minced fish flesh is subjected to pH (Hennigar et al., 1988; Lin et al., 1996) and ionic strength (Sonu, 1986; Trevino et al., 1990; Wu et al., 1991; Lin and Park., 1996) changes, proteolysis (Schwartz and Bird, 1977; Patashnik et al., 1982; Xiong and Brekke, 1989; Chang-Lee et al., 1990), and possible temperature abuse. These processing factors might change the protein structure in primary sequence, conformation, molecular size and shape, charge distribution, intra- and inter-molecular bondings. These changes might, in various degrees, affect protein solubility during processing. The reduction of particle size (Acton, 1972) and involvement of mechanical forces, such as screening and screw pressing, might also cause the loss of proteins in the form of particulates. Therefore, the myofibrillar proteins lost in surimi waste streams could be soluble proteins or insoluble particulates.

Some of the proteins, lost in wash water, could be highly functional myofibrillar proteins. Therefore, it may be beneficial to recover, characterize, and utilize such proteins for food use to increase value and lessen cost of waste treatment. Furthermore, if the processing parameters such as washing condition, ionic strengths, pH, and proteolysis, are properly controlled, the loss of valuable myofibrillar proteins can be minimized. Thus, the processing yield can be increased.
WASHING CONDITIONS

Gel strength of surimi increases with an increase in the concentration of myofibrillar proteins, especially actomyosin, but decreases in the presence of sarcoplasmic proteins (Lee, 1984). Sarcoplasmic proteins bind with actomyosin and impede the cross-linking process which is crucial for the formation of gel network. This emphasizes the importance of washing, which removes water-soluble sarcoplasmic proteins and increases the level of functional actomyosin.

In early studies, it was shown that with an increase in the number of washing cycles, more sarcoplasmic proteins were removed and the gel strength of surimi was improved (Lee, 1984; Nishioka, 1984). However, a recent report (Lin and Park, 1996) indicated that the major portion of sarcoplasmic proteins are fairly soluble and removed during the initial washing steps. Subsequent washing removes the residual sarcoplasmic proteins along with a small amount of myofibrillar proteins. After sarcoplasmic proteins are completely removed, further washing causes a severe loss of myofibrillar proteins. A similar result had been previously reported. Matsumoto (1959) indicated that when salt soluble proteins, i.e., myofibrillar proteins, were repeatedly washed with water, most of the protein was solubilized.

Pacheco-Aguilar et al. (1989) indicated that single washing with one pressing produced superior protein recovery (83.4%) to those observed for two washing cycles (70.1%) and three washing cycles (68.5%), using the same total amount of water. This indicated that the washing efficiency per unit volume of water decreased as the total water volume increased. Increased washing time (Wu and Smith, 1987) and washing
temperature (Lin and Chen, 1989) also resulted in high loss of myofibrillar proteins during leaching.

EFFECTS OF IONIC STRENGTHS

Myofibrillar proteins have been generally classified as salt-soluble proteins. However, solubility of myofibrillar proteins in water has been shown by several researchers. Wu et al. (1991) indicated that after washing the minced muscle tissue of 5 species of white-fleshed fish twice with water and a third time with 0.15% (26 mM) NaCl solution, a considerable portion of the remaining protein was soluble when extracted with water at a 1:20 ratio. The amount of protein extracted was 20-36% of the protein extracted by a buffer of 1 M LiCl solution. The amount of protein indicated that the extracted proteins were primarily myofibrillar proteins. Hennigar et al. (1988) also showed that gels could be prepared by fish muscle without NaCl suggesting that fish muscle myofibrillar proteins could have sizable amount of solubility in water.

The minimal loss of myofibrillar proteins during extensive washing was found at salt concentrations of 0.5 - 1.0% (Wu et al., 1991; Lin and Park, 1996). The solubility of myofibrillar proteins increased as salt concentration decreased or increased. The authors explained that salt water fish muscle contains a variety of salts, roughly equivalent to a solution with an ionic strength of 0.145. This ionic strength corresponds to 0.85% NaCl. This level of salt could inhibit solubilization of the muscle proteins in the fish muscle. This is confirmed by Sonu (1986) showing that a minimum hydrophilic condition for washed mince is at an ionic strength between 0.005 and 0.1 which corresponds to the salt concentration of 0.03 to 0.6%. A linear relationship of moisture content of the fish
paste to the log of the water-soluble protein concentration was also observed by Wu et al (1991) suggesting that factors which improve hydration also improve solubility. Similar results were also reported by Trevino et al. (1990) showing that the solubility of protein in sardine surimi was high in water, rapidly decreased at low salt concentrations, and increased again as the salt concentration was further increased. These results suggested the possibility of using very low concentration of salt to prevent the solubilization of fish myofibrillar proteins during washing process.

Severe loss of protein due to high ionic strength in washing solution has been reported. Lin and Chen (1989) indicated that mechanically deboned poultry meat (MDPM) washed with 1.0% or 1.5 % NaCl contained less protein compared to 0.5% or tap water solutions. Lee (1984) reported that solubilization of myofibrillar protein occurred when too much salt was used during surimi manufacturing. Foegeding (1987) indicated that the solubility of salt-soluble protein increases as the concentration of NaCl increases from 0.25 M to 0.5 M at pH 6.0. However, at pH 5 and 7 there are no major effects of NaCl on solubility of salt-soluble protein (SSP). It was also shown by Wu and Smith (1987) that increasing ionic strength (from 0.10 to 0.35 M) increased myofibrillar solubilization.

Many functional properties of muscle proteins have also been related to the solubilization of myofibrillar proteins. One important example is the formation of surimi gel. Studies have shown that 1.7-3.5 % NaCl (\(=0.29-0.60 \mu\)) is required for surimi to form an adequate gel (Roussel and Cheftel, 1990). Without adding salt, surimi shows poor gel-forming ability due to the insolubility of myofibrillar proteins. In commercial
practice, 1.2-2.5% NaCl (=0.21-0.43 μ) is commonly used to solubilize myofibrillar proteins, especially myosin which is the major component responsible for gelation in surimi. At this salt concentration range, myosin molecules are released from thick filaments of the myofibrils and disperse in the solution as monomers (Ishioroshi et al., 1983). An organized three dimensional gel network is formed from these solubilized myofibrillar proteins upon heating (Niwa, 1992; Lanier, 1986). However, when the salt concentration is too high (>1M) the gel-forming ability of myosin gradually decreases (Suzuki, 1981). This might be due to the decreased solubility of myosin caused by the salting out effect (Regenstein, 1984).

**EFFECTS OF pH**

In addition to ionic strengths, pH is another important factor affecting the solubility of myofibrillar proteins. As the pH approaches the isoelectric point of fish flesh (=pH 5.5), the negative and positive charges among protein molecules are about equal. Therefore, protein molecules are strongly associated with each other through ionic linkages. Myofibrillar proteins are insoluble at this pH because protein-water interaction is replaced by protein-protein interaction. It is a logical assumption that using washing solutions with pH near or at the isoelectric point of fish muscle proteins would reduce the loss of proteins and improve yield. Pacheco-Aguilar et al. (1989) showed that at pH 5.0-5.3, water requirement was reduced by 80%, and yield was improved by up to 34% over conventional processing at pH 6.5. However, the texture of gels was poor when prepared from surimi produced under acidic conditions. Foegeding (1987) also indicated that salt soluble proteins have the least solubility at pH 5.0. The relative amount of myosin heavy
chain extracted with 0.25M NaCl solution, was decreased from 52.0% to 1.6% as pH decreased from 7.0 to 5.0.

At pH above or below the isoelectric point, the protein acquires an increasing amount of net negative or positive charges. The increased net charge offers more binding sites for water and causes the repulsion among protein molecules to increase their surface area for hydration, thus increasing protein solubility. Therefore, gelation of myosin can be expected by shifting pH without adding salt. It has been shown that the gel-forming ability of surimi drastically increases as pH is increased or decreased from the isoelectric point in the absence of salt (Torley and Lanier, 1992; Nishino et al., 1991). Chung et al. (1993) further indicated that at pH above 8 in the absence of salt, surimi gel had higher shear stress values and nearly the same shear strain values as compared to the gel made with 2.5% NaCl at pH 7. However, when the pH approached the isoelectric point, no measurable gels were formed at any salt levels (0-2.5%). Presumably, myosin was not solubilized at this pH regardless of salt concentrations.

**PROTEOLYSIS OF MYOFIBRILLAR PROTEINS**

The loss of myofibrillar proteins might be due in part to proteolysis before and during processing. Evidence has been shown by several researchers that proteolytic degradation of myofibrils resulted in a low yield of whiting surimi (Patashnik et al., 1982; Chang-Lee et al., 1989). Native myosin is in filamentous form and insoluble (Schwartz and Bird, 1977). The increase in protein solubility of myofibrils is due to the dissociation of myofibrils; once the myofibrils are disassembled, the proteins can be readily solubilized (Xiong and Brekke, 1989).
In commercial surimi operations, whiting are usually delivered to processing plants within 6-12 hr after harvest. Due to the limited capacity of processing facilities, fish are often kept in holding tanks with ice water (=0°C) for up to another 6-14 hr. Thus, fish are usually 6-26 hr post-harvest before they are subjected to surimi processing. During this holding period, the temperature can rise if fish are not handled properly. Prolonged holding time and elevated temperatures can cause severe proteolysis of myofibrillar proteins and consequently affect the processing yield and gelation properties of surimi.

Proteolytic activity in Pacific whiting has been related to infection of Myxosporian parasites, *Kudoa paniformis* and *K. thyrsitis* (Kudo et al., 1987; Patashnik et al., 1982). At the early stage, infected muscle fibers appear to be opaque-white and can not be identified without the aid of a microscope, while at the later stage, the infected fibers are readily visible as a dark black streak on the muscle. The former are called white psuedocysts and the latter are called black psuedocysts (Morrissey et al., 1996). A hidden stage, the initial stage of infection, might also exist (Kabata and Whitaker, 1985). By surveying 1,500 fish in 35 plants, Morrissey et al. (1992) indicated that 4-5% of Pacific whiting harvested from the Northwest coast contained black psuedocysts. The overall prevalence of infection, including white and black psuedocysts, was estimated to be about 90-95% of the fish population (Kabata and Whitaker, 1981, 1985). The presence of black psuedocysts can be easily identified and classified into three categories: slight, moderate, and heavy stages. However, a recent report (Morrissey et al., 1996) indicated that the proteolytic activity is mainly related to the presence and intensity of white
psuedocysts ($R^2=0.81$, $P<0.05$) and is not correlated to the degree of black psuedocysts ($R^2=0.23$, $p>0.05$).

An et al. (1994) indicated that in whiting fillets, cathepsin B exhibited the highest peak activity, followed by cathepsins L and H. Cathepsin L and H in fish extract showed their highest activity at 55 and 20°C, respectively, while the maximum activity of cathepsin B was between 20 and 37°C. The total cumulative activity of the three cysteine cathepsins (L, B, and H) was found to be highest at 20°C in fish fillets. In the temperature range for surimi processing (0-5°C), the activity of cathepsin L is insignificant, while cathepsin B still exhibited half of its maximal activity and cathepsin H retained about one fifth of its maximal activity. In the study of hydrolysis of whiting myofibrils by the protease, the authors indicated that MHC was hydrolyzed to smaller molecular weights ranging from 67 to 145 kDa. These bands were also detected in surimi samples suggesting that a large portion of myosin was hydrolyzed during surimi processing.

After extensive washing and dewatering, the activity of cathepsin B and H is substantially reduced, while cathepsin L still remains active in surimi (An et al., 1994). A gradual decrease in protease activity by washing was also reported by Chang-Lee et al. (1989). These previous studies proposed that Cathepsin B and H might contribute to the degradation of myofibrillar protein during surimi processing (0 - 5°C).
RESEARCH OBJECTIVES

The goal of this research was to reduce the loss of myofibrillar proteins and waste disposal during surimi processing. This goal was achieved by identifying the following objectives:

1) To recover and characterize the proteins lost in waste water during washing and dewatering processes;

2) To reduce waste disposal by minimizing water usage for leaching and by reconditioning waste water using ultrafiltration technology;

3) To reduce the loss of proteins by investigating the effects of ionic strengths, proteolysis, and various washing conditions on the solubility of myofibrillar proteins;

4) To investigate the relationship between conformational changes and the solubility of myofibrillar proteins using myosin as a model system.

REFERENCES


Chapter 2

Recovered Protein and Reconditioned Water from Surimi Processing Waste

Tein M. Lin, Jae W. Park, and Michael T. Morrissey

ABSTRACT

Micro- and ultrafiltration were utilized for recovery of proteins and for water recycling from commercial surimi processing waste water. The proteins recovered by microfiltration showed highly functional properties and composition comparable with proteins in regular surimi. Surimi with a substitution (10%) of recovered proteins did not diminish in gel hardness, elasticity, color, or water retention. Aerobic plate count, chemical oxygen demand, protease activity, and turbidity in the surimi waste waters were reduced by ultrafiltration. Results indicated a potential for recovering proteins to extend yield and recycling water.
INTRODUCTION

Production of surimi involves extensive washing of minced fish muscle to remove fat and water soluble substances such as sarcoplasmic proteins, pigments, amines, vitamins, and enzymes. Reduction of sarcoplasmic proteins, in turn, concentrates myofibrillar proteins which are the major components responsible for formation of surimi gels. Minced fish solids (=40-50%) are lost during washing and dewatering (Adu et al., 1983; Pacheco-Aguilar et al., 1989; Yang and Froning, 1992). Wu et al. (1991) reported that myofibrillar proteins of white fleshed fish were considerably soluble in water. Some of the soluble solids, lost in wash water, could be highly functional myofibrillar proteins. Therefore, it may be beneficial to recover, characterize, and utilize such proteins for food use to increase value and lessen cost of waste water treatment.

Several methods of concentrating soluble proteins from surimi wash water have been investigated. Ultrafiltration is the primary commercial method used for dairy whey recovery and has been studied by the National Food Processors Association and others as a means of recovering protein from surimi wash water (Nishioka and Shimizu, 1983; Green et al., 1984; Swafford, 1987; Pedersen et al., 1987, 1989; French and Pedersen, 1990). Nishioka and Shimizu (1983) developed a means of precipitating proteins from surimi waste water by shifting the pH. This method is simple and low cost but results in complete denaturation of the proteins. Use of an ion exchange (IE) resin-binding approach in the dairy whey industry showed promise in producing a very clean product with superior functionality compared to proteins recovered by ultrafiltration (Honer, 1986). A comparison of the three methods (Korhonen and Lanier, 1991) indicated that
the composition of proteins recovered by these methods was nearly identical; however, the yield was lowest with the IE method.

Recycling of processing water is gaining in importance to surimi processors due to rising utility costs, limited water resources, and pollution problems associated with disposal. Considerable research has been reported concerning water reuse and conservation for poultry processing (Sheldon and Brown, 1986; Chang et al., 1989; Chang and Sheldon, 1989a,b; Chang and Toledo, 1989). However, little information is available for surimi processors who need to reduce waste loads and recycle processing water.

Our objectives were to recover and characterize the proteins lost with waste water during surimi processing, as well as to recondition waste water by reducing chemical and microbiological loads.

**MATERIALS AND METHODS**

**Sample collection**

Sample collection was from a commercial surimi processing plant during July through September, 1993 (Fig. 2.1). Whole Pacific whiting (*Merluccius productus*) was headed, gutted, and minced through a screen (5 mm). It was then washed slightly in the first washing tank and more extensively in subsequent tanks (II & III). Screeners were used for both washing and dewatering. Proteins lost in the waste discharged from the screw press (W6) were collected by a rotary screen (100μ) and subjected to microfiltration (MF) with combined screen sizes of 50μ and 30μ (Model MM2, Pure-Grade, Inc., Burbank, CA). The water discharged from the rotary screen and MF unit was
Fig. 2.1. Surimi processing flow and sample collection. W1 through W8 indicate different waste streams. UF: ultrafiltration unit. MF: microfiltration unit.
labeled as W7 and W8, respectively. All waste water discharged from dehydrators (W1, W2, W4), screeners (W3, W5), rotary screen (W7), and MF unit (W8) were collected for further analysis and for reconditioning of waste water using an ultrafiltration (UF) unit. The UF unit was equipped with a membrane of 30,000 molecular weight cut-off (CUNO Separation Corp., Norwood, MA), selected based on characterization of the molecular weight of whiting protease (Seymour et al., 1994). The quality of recovered protein from the MF unit was evaluated by torsion tests, color measurements, and expressible moisture. The water quality of waste waters and their ultrafiltration permeates were evaluated by measuring the values of aerobic plate count (APC), chemical oxygen demand (COD), clarity (%T), and protease activity. The concentrated proteins collected from ultrafiltration were also characterized.

**Surimi gel preparation**

To evaluate the functional properties of gels made of MF recovered protein, 4 treatments were used: 1) 100% surimi (S) control; 2) 100% MF recovered protein (RP); 3) 10% MF recovered protein/90% regular surimi (10RP/90S); 4) 20% MF recovered protein/80% regular surimi (20RP/80S). All treatments were adjusted to 78% moisture and 2% NaCl. Beef plasma protein (1%, AMP 600N, AMP Inc., Ames, IA) was added as a protease inhibitor. Salt and ice were mixed with surimi and/or MF recovered protein and chopped for 5 min at low speed (1,500 rpm) in a vacuum chopper (Model 5289, Stephan Machinery Corp., Columbus, OH). The temperature during chopping was maintained at 3-5°C using a refrigerated circulator (RTE-100LP, Neslab Instruments, Inc., Newington, NH). The paste was vacuum-packed in a polyethylene bag to eliminate potential air
pockets and extruded into stainless steel cooking tubes (i.d. = 19 mm) through a sausage stuffer (Model 14208, The Sausage Maker, Buffalo, NY). Three heating treatments were applied. The paste was heated at 90°C for 15 min in a water bath. In addition, the effect of protease on gel quality was assessed by incubating pastes at 60°C for 30 min prior to heating at 90°C for 15 min. The setting effect was evaluated by setting pastes at room temperature (25°C) for 3 hr prior to cooking at 90°C for 15 min. Gels were immediately chilled in ice water before storing in a refrigerator overnight.

**Torsion test**

Torsion tests were performed as described by NFI (1991). Gels were allowed to reach room temperature and were made into hour-glass shapes by a sample milling machine (Gel Consultants, Model 91, Raleigh, NC). Samples were subjected to torsional shear in a modified Brookfield viscometer (Gel Consultants, Inc, Raleigh, NC). Shear stress and shear strain, at failure, were calculated using the equations of Hamann (1983). Failure shear stress indicated strength of gels, while failure shear strain denoted deformability of gels.

**Color measurement**

Color measurements were made by a Chroma Meter (Model CR-310, Minolta Corp., Ramsey, NJ). The instrument, equipped with a D65 illuminant and 2° observer optical position, was standardized for surimi gel measurement by using a Minolta calibration plate (No. 18133009 Y\textsubscript{CIE}=94.5, x\textsubscript{CIE}=0.3160, y\textsubscript{CIE}=0.3330) and a Hunterlab standard plate (Standard No. D44C-1618, L\*= 82.13, a\* = -5.24, b\* = -0.55). Color
values were expressed using the International Commission on Illumination L*, a*, and b* values (CIE, 1978). Gels were warmed to room temperature before measurements were made (Park, 1994).

**Expressible moisture measurement**

The percentage of free water was measured as described by Tsai and Ockerman (1981). Gels (=0.5 g) were placed on a filter paper (Whatman No. 1) which was set between two pieces of plexiglass and pressed at 35 kg/cm$^2$ by a Carver Laboratory Press (Fred S. Carver Inc., NY) for 1 min. The percentage of free water was calculated by the following equation:

\[
\text{% Free water} = \frac{(\text{Total area} \text{ (cm}^2\text{)} - \text{meat film area (cm}^2\text{)}) \times 9.47 \text{ mg water/cm}^2}{\text{Total moisture (mg) of gel sample}} \times 100\%
\]

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

Preparation of samples for SDS-PAGE was performed by the methods of Morrissey et al. (1993) with slight modification. Sample (3g) were homogenized with 27 mL of 5% (w/v) sodium dodecylsulfate (SDS) solution for 2 min at a speed of 3-4 using a Polytron (Brinkmann Instruments, Westbury, NY). The homogenates were incubated in an 80°C water bath for 1 hr to dissolve all myofibrillar proteins and centrifuged at 8,000 x g for 20 min to remove undissolved debris which was mainly undissolved connective tissue and fish skin. Electrophoresis was conducted according to the procedures of Laemml (1970). Extracted proteins were boiled for 90 sec in a SDS-PAGE treatment
buffer (1:1, v/v), and 40 µg protein, measured by the method of Lowry et al. (1951), was applied on a 4% stacking and 10% running polyacrylamide gel. A high molecular weight standard mixture (Stock No. SDS-6H, Sigma Chemical Company, St. Louis, MO) including myosin of rabbit muscle (205 kDa), β-galactosidase of *E. coli* (116 kDa), phosphorylase b of rabbit muscle (97 kDa), albumin of bovine (66 kDa), albumin of egg (45 kDa), and carbonic anhydrase of bovine erythrocytes (29 kDa) was used. Protein bands were stained with 0.125% Coomassie blue and destained in a 50% methanol and 10% acetic acid solution followed by a 5% methanol and 7% acetic acid solution. Quantitative analysis was performed using a laser scanning densitometer (Biomed Instruments, Inc., Fullerton, CA). The relative amount of myosin heavy chain in each sample was expressed as percentage compared to control surimi.

**Analyses of proximate composition**

Moisture, protein (total N x 6.25 - nonprotein nitrogen), fat, and ash were determined according to AOAC (1990) procedures. Nonprotein nitrogen was measured by the method of Woyewoda et al. (1986).

**Chemical, microbiological, and enzymatic analyses**

The quality of waste waters and the UF permeates were evaluated by measuring COD, %T, APC, and protease activity. The COD was measured using the procedures described by APHA (1980). Water clarity (absorbance) was monitored at 500 nm using a spectrophotometer (Model DU-640, Beckman Instruments, Inc., Fullerton, CA) and converted to %T. For APC, waste waters and filtrates were serially diluted in 1%
peptone water. Total aerobes were enumerated, in duplicate, using 3M aerobic count plates (Petrifilm, Inc., St. Paul, MN). Plates were incubated at 32°C for 48 hrs. Protease activity was assessed by the method of An et al. (1994a) using azocasein (Sigma Chem. Co., St. Louis, MO) as a substrate. Absorbance of the reaction mixture was measured at 450 nm by a spectrophotometer (Model DU-640, Beckman Instruments, Inc. Fullerton, CA). Results were expressed as $A_{450}$.

**Statistical analysis**

Statistical analysis of data was carried out using one way analysis of variance. Differences among mean values were established using the least significance difference (LSD) multiple range test (Steel and Torrie, 1980). Mean values were considered significant when $p < 0.05$.

**RESULTS AND DISCUSSION**

**Compositional profile of Pacific whiting surimi wastes**

The amount of discharged waste and compositional properties of the waste water were compared (Table 2.1). In producing 1 kg of surimi, 29.1±3.5 L (mean ± S.D.) of waste water were discharged from the processing line (W1 through W6). The major portion (≈75%) of the waste water was discharged from screeners (W3 and W5) while the waste water released from dehydrators (W1, W2, W4) and screw press (W6) comprised a relatively minor amount. The processing water discharged from the first dehydrator (W1) contained the highest level of protein, non-protein nitrogen, fat, and ash. The waste water in successive discharge points revealed decreasing protein, nonprotein
nitrogen, fat, and ash. However, W1 constituted only 1.5% of the total processing water and had a strong fishy smell. The high protein content (Table 2.1), presence of low molecular weight protein bands (29 - 45 kDa), and insignificant amount of myosin heavy chain (Fig. 2.2 & 2.3) in the first waste water suggested that the first washing removed mainly sarcoplasmic proteins and did not impart notable losses of myosin. The removal of sarcoplasmic proteins is desirable for surimi processing since they interfere with heat-induced gelation of myofibrillar proteins (Yang and Froning, 1992). For this processing line, screen size 5 mm diameter was used for mincing fish muscle. Particle size of muscle was gradually reduced and sarcoplasmic proteins were gradually released from muscle compartments, dissolved in water, and removed from insoluble portions throughout successive washing and dewatering steps. This was evident from SDS-PAGE (Fig. 2.2) in which presence of low molecular weight bands occurred throughout the waste waters collected from different dewatering steps.

Results from SDS-PAGE (Fig. 2.2) showed that a considerable amount of myofibrillar proteins, myosin and actin, was lost in the waste water during washing and dewatering. Quantitative analysis, using a densitometer, showed that loss of myosin was small in the first waste water but increased substantially in the second waste water and then remained nearly constant throughout the rest of processing (Fig. 2.3). Generally the washing of minced flesh with water has been believed to remove sarcoplasmic proteins which in turn concentrates myofibrillar proteins. However, high losses of myofibrillar proteins during washing and dewatering were observed in our results. Extensive washing, which might cause myofibrillar proteins to be dissolved in water to some extent, and
Table 2.1. Rate and composition of waste waters collected at different points on surimi processing line

<table>
<thead>
<tr>
<th>Waste stream</th>
<th>Waste / surimi*</th>
<th>Moisture (%)</th>
<th>Protein (%)</th>
<th>Non-protein nitrogen (%)</th>
<th>Fat (%)</th>
<th>Ash (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1</td>
<td>0.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.41&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(0.08)</td>
<td>(0.04)</td>
<td>(0.25)</td>
<td>(0.01)</td>
<td>(0.01)</td>
<td>(0.01)</td>
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<tr>
<td>W2</td>
<td>3.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.97&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.38&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td>(0.14)</td>
<td>(0.05)</td>
<td>(0.05)</td>
<td>(0.00)</td>
<td>(0.01)</td>
<td>(0.02)</td>
</tr>
<tr>
<td>W3</td>
<td>10.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>98.87&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.99&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.06&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>0.17&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(1.44)</td>
<td>(0.01)</td>
<td>(0.14)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.01)</td>
</tr>
<tr>
<td>W4</td>
<td>1.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.98&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.21&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(0.49)</td>
<td>(0.03)</td>
<td>(0.01)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.03)</td>
</tr>
<tr>
<td>W5</td>
<td>11.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98.85&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.09&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(6.86)</td>
<td>(0.03)</td>
<td>(0.01)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.01)</td>
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<tr>
<td>W6</td>
<td>2.40&lt;sup&gt;e&lt;/sup&gt;</td>
<td>99.20&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.08&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.14&lt;sup&gt;be&lt;/sup&gt;</td>
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<td></td>
<td>(1.42)</td>
<td>(0.03)</td>
<td>(0.01)</td>
<td>(0.00)</td>
<td>(0.01)</td>
<td>(0.00)</td>
</tr>
<tr>
<td>W7</td>
<td>2.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>99.20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.10&lt;sup&gt;be&lt;/sup&gt;</td>
<td>0.12&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(0.88)</td>
<td>(0.04)</td>
<td>(0.06)</td>
<td>(0.00)</td>
<td>(0.01)</td>
<td>(0.01)</td>
</tr>
<tr>
<td>W8</td>
<td>0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.09&lt;sup&gt;be&lt;/sup&gt;</td>
<td>0.11&lt;sup&gt;de&lt;/sup&gt;</td>
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<td>(0.01)</td>
<td>(0.04)</td>
<td>(0.02)</td>
<td>(0.00)</td>
<td>(0.01)</td>
<td>(0.01)</td>
</tr>
</tbody>
</table>

*Unit: L/kg
<sup>1</sup> and <sup>2</sup> denote mean and standard deviation respectively.
<sup>abcde</sup>. Different letters in the same column indicate significant difference (p < 0.05).
W1-W8 are sources of waste water as illustrated in Fig. 2.1.
Fig. 2.2. SDS-PAGE of regular surimi, recovered proteins, and waste water concentrates. S: high molecular weight standard mixture; lane 1 = regular surimi; lane 2 = the proteins collected by rotary screen; lane 3 = the recovered proteins by microfiltration; lane 4-10: the UF concentrated proteins from waste streams W1, W2, W3, W4, W5, W7, W8, respectively.
Fig. 2.3. Relative amount of myosin heavy chain in regular surimi, recovered proteins, and the concentrates of waste waters. RS: proteins collected by rotary screen; RP: proteins recovered by microfiltration. UF1 through UF 8: the UF concentrates from different sources of waste streams as illustrated in Fig. 2.1.
mechanical force during dewatering, which resulted in the loss of myofibrillar protein in the form of particulates, could be the major procedures responsible for the losses of the functional myofibrillar proteins in surimi processing. This confirmed the studies of Lin and Park (1994) in which the solubility of myofibrillar proteins increased as number of washing cycles increased. Since sarcoplasmic proteins and undesirable non-protein nitrogen compounds, i.e., trimethylamine, dimethylamine, are fairly soluble in water, removal of these components could be accomplished in the first washing step by using enough water to solubilize and remove them. Use of continuous washing cycles to remove all undesirable compounds and reduce losses of solids was suggested by Babbitt (1990).

**Recovery of protein from surimi waste**

When washed mince was subjected to the final dewatering, some protein, in the form of particulates, was lost through the orifice due to the mechanical force of the screw press. These insoluble particulates were recovered by a rotary screen and further concentrated by microfiltration (MF). The moisture of the discharged water (99.0%) was reduced to 93.3% by the rotary screen and then to 84.5% by the MF unit; the solids were increased by 6.7 fold and 15.5 fold, respectively. The solids in the MF recovered protein were concentrated to 16.3%. However, that was slightly lower than regular surimi (19.6%)(Table 2.2).

In this process, the surimi production yield, per total whole whiting processed, was $15.5 \pm 1.5 \%$ (mean ± S.D.). For every 100 Kg of surimi produced, $1.7 \pm 0.2$ (mean ± S.D.) Kg protein was recovered by the MF unit. The surimi production yield could be
Table 2.2. Proximate composition of surimi and MF recovered protein

<table>
<thead>
<tr>
<th>Sample</th>
<th>Moisture (%)</th>
<th>Protein (%)</th>
<th>Fat (%)</th>
<th>Ash (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular surimi</td>
<td>80.6 ± 0.4^a</td>
<td>18.3 ± 0.3^a</td>
<td>0.9 ± 0.1^a</td>
<td>0.39 ± 0.05^a</td>
</tr>
<tr>
<td>MF recovered protein</td>
<td>84.5 ± 0.7^b</td>
<td>15.0 ± 0.3^b</td>
<td>0.8 ± 0.1^a</td>
<td>0.48 ± 0.04^b</td>
</tr>
</tbody>
</table>

^1 and ^2 denote mean and standard deviation respectively.
^a, ^b: Different letters in the same column indicate significant difference (p < 0.05).
increased by $1.7 \pm 0.2\%$ (by weight) and the change in total solids level would be insignificant if the MF recovered protein was added back to the regular processing line.

The low level of myosin in UF7 and the high myosin content in RS (Fig. 2.3) indicated that most of the myosin lost in the form of particulate from the screw press was recovered by the rotary screen (Fig. 2.1). The myosin heavy chain in the protein recovered by rotary screen was subsequently increased by 2-3 fold when microfiltration was applied. Due to high pressure created by microfiltration, some myosin was lost in the water portion (UF8)(Fig. 2.3).

Approximate composition of MF recovered proteins was compared with regular surimi sampled from the same processing line (Table 2.2). Results revealed that the MF recovered proteins contained less protein but higher ash than regular surimi ($p < 0.05$). No significant difference was observed in fat between regular surimi and MF recovered protein. As shown (Table 2.1), ash (minerals) was continuously lost during washing and dewatering. The higher ash in the MF recovered protein indicated that minerals lost in the wastes could be partially recovered by microfiltration (Table 2.2).

Microfiltration was not enough to recover proteins lost in waste water discharged from dehydrators (W1, W2, W4) or screeners (W3, W5) due to the fine particle sizes. However, moisture content in these streams was reduced to a minimal level of 91.3% (Table 2.3) using ultrafiltration (UF) with a membrane of 30,000 molecular weight cut-off. Proteins concentrated by ultrafiltration had considerably darker color and strong odor. These results confirmed the work of French and Pedersen (1990) who observed that
Table 2.3. Water content of ultrafiltration (UF) concentrates

<table>
<thead>
<tr>
<th></th>
<th>UF1</th>
<th>UF2</th>
<th>UF3</th>
<th>UF4</th>
<th>UF5</th>
<th>UF7</th>
<th>UF8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>91.75(^{a1})</td>
<td>96.83(^{b})</td>
<td>93.30(^{c})</td>
<td>96.80(^{b})</td>
<td>91.30(^{a})</td>
<td>93.35(^{c})</td>
<td>93.55(^{c})</td>
</tr>
<tr>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>0.77(^{2})</td>
<td>0.04</td>
<td>0.01</td>
<td>0.14</td>
<td>0.28</td>
<td>0.07</td>
<td>0.49</td>
<td></td>
</tr>
</tbody>
</table>

UF1-UF8 are ultrafiltration concentrates of different waste waters as illustrated in Fig. 2.1.

\(^{1}\) and \(^{2}\) denote mean and standard deviation respectively.

\(^{a,b,c}\): Different letters in the same row indicate significant difference (p < 0.05).
surimi containing 10% UF concentrate and 90% regular surimi had a slight brownish hue and a distinct fishy odor.

**Characterization of recovered protein**

Gel functionality of recovered protein from MF was evaluated vs controls (regular surimi from the same process). Recovered protein revealed a lower stress but the same strain values as regular surimi, regardless of cooking method (Figs. 2.4 & 2.5). Gels made with 10% MF recovered protein and 90% regular surimi had no significant difference in stress or strain values compared to controls. When gels were made by replacing regular surimi with 20% MF recovered protein and preincubated at 25°C or 60°C, a significant reduction (p < 0.05) in stress value was noted. However, Korhonen and Lanier (1991) reported that substitution of regular surimi with 10% UF concentrate resulted in various degrees of weaker gels. The difference in gel quality between proteins recovered by MF and UF could be due to different levels of myosin (Fig. 2.2 and 2.3). The myosin heavy chain content in MF recovered proteins was five times higher than that of the UF concentrate. This was because the MF unit with screen sizes 50μ and 30μ, allowed some soluble proteins to pass through and concentrated the insoluble particulate which might have higher myosin content.

Gels of MF recovered protein, regardless of cooking method, had a higher loss of free water than regular surimi gel when subjected to mechanical force (Fig. 2.6). However, by substituting 10% or 20% regular surimi with the recovered protein, the gel had the same expressible moisture as the regular surimi gel.
Fig. 2.4. Shear stress of gels. S: regular surimi; RP: MF recovered protein; 10RP/90S: 10% RP and 90% S; 20RP/80S: 20% RP and 80% S; 90°C: cooked at 90°C for 15 min; 60°C/90°C: 60°C incubation for 30 min followed by cooking at 90°C for 15 min; 25°C/90°C: setting at 25°C for 3 hrs before cooking at 90°C for 15 min. Different letters in the same group of bars indicate a significant difference (p<0.05).
Fig. 2.5. Shear strain of gels. S: regular surimi; RP: MF recovered protein; 10RP/90S: 10% RP and 90% S; 20RP/80S: 20% RP and 80% S; 90°C: cooked at 90°C for 15 min; 60°C/90°C: 60°C incubation for 30 min followed by cooking at 90°C for 15 min; 25°C/90°C: setting at 25°C for 3 hrs before cooking at 90°C for 15 min. Different letters in the same group of bars indicate a significant difference (p<0.05).
Fig. 2.6. Loss of expressible free water (%) of gels S: regular surimi; RP: MF recovered protein; 10RP/90S: 10% RP and 90% S; 20RP/80S: 20% RP and 80% S; 90°C: cooked at 90°C for 15 min; 60°C/90°C: 60°C incubation for 30 min followed by cooking at 90°C for 15 min; 25°C/90°C: setting at 25°C for 3 hrs before cooking at 90°C for 15 min. Different letters in the same group of bars indicate a significant difference (p<0.05).
Gels of MF recovered protein, except those made by 25°C/90°C heating method, had better color, i.e., higher lightness (L*) and lower greenness (a*), than regular surimi gels (Table 2.4). When 10 or 20% of recovered protein was blended with regular surimi, the gels maintained better a* values and also showed lower b* values. This corresponded with results of Korhonen and Lanier (1991) showing that substitution of surimi with recovered proteins did not deteriorate surimi gel color. The effects of different cooking methods on gel color were not significant (p < 0.05). This also confirmed previous work of Park (1994).

Characterization of waste waters and UF permeates

APC was relatively high in the first discharged water and substantially reduced in successive waste water streams (Fig. 2.7). The increased APC in W7 and W8 were probably due to washing of the microbial load from the meat into the waste stream as a result of the elevated pressures created by the screw press and microfiltration. Regardless of point of sampling, APC levels in every UF permeate were insignificant (< 100 cfu/mL).

The extensive washing of minced flesh caused a high loss of organic substances which resulted in extremely high COD (6,000 to 27,000 mg/L) in the waste waters (Fig. 2.8) compared to 900-1,200 mg/L from poultry processing waste (Chang and Sheldon, 1989b; Chang et al., 1989). However, the COD levels of the wastes were comparable to study of Swafford (1987) in which the COD values were 9,700 to 50,000 mg/L for surimi waste of Alaska pollock. This high level was especially noted in the first discharged waste (Fig. 2.8) in which organic substances were relatively high (Table 2.1). By applying
<table>
<thead>
<tr>
<th>Section</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1) 90°C</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Surimi (S)</td>
<td>80.92±0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-3.77±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.43±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10RP/90S</td>
<td>79.41±0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-3.73±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.88±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20RP/80S</td>
<td>81.22±0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-3.64±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.25±0.21&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Recovered protein (RP)</td>
<td>81.62±0.34&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-3.39±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.42±0.24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>2) 60°C/90°C</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surimi (S)</td>
<td>81.40±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-3.83±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.50±0.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10RP/90S</td>
<td>80.45±0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-3.66±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.89±0.29&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20RP/80S</td>
<td>82.04±0.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-3.68±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.13±0.14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Recovered protein (RP)</td>
<td>82.65±0.35&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>-3.39±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.99±0.22&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>3) 25°C/90°C</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surimi (S)</td>
<td>81.80±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-3.75±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.43±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10RP/90S</td>
<td>80.68±0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-3.72±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.02±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20RP/80S</td>
<td>81.48±0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-3.65±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.91±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Recovered protein (RP)</td>
<td>81.67±0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-3.43±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.45±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>: Values are mean ± S.D.

<sup>abed</sup>: Different letters in the same column within the same section (i.e. heating method) indicate significant difference.

S: surimi; RP: MF recovered protein; 10RP/90S: 10% MF recovered protein with 90% surimi; 20RP/80S: 20% MF recovered protein with 80% regular surimi; 1) 90°C: heating at 90°C for 15 min; 2) 60°C/90°C: 60°C setting for 30 min followed by heating at 90°C for 15 min; 3) 25°C/90°C: 25°C setting for 3 hr followed by 90°C heating for 15 min.
Fig. 2.7. Effect of ultrafiltration on aerobic plate count (APC) of surimi waste waters. W1 through W8 indicate different waste water streams as illustrated in Fig. 2.1.
Fig. 2.8. Effect of ultrafiltration on chemical oxygen demand (COD) of surimi waste waters. W1 through W8 indicate different waste water streams as illustrated in Fig. 2.1.
UF, an 89-94% reduction in COD value was achieved. However, the UF permeate of the first waste water still had a high COD value.

The protease activity in waste waters did not decrease with successive washing processes (Fig. 2.9). The solubility of Pacific whiting proteases during washing was not complete. During postmortem, part of the whiting proteases might associate with myofibrils (An et al., 1994b). This supports our observation that the waste water discharged from dehydrators and the screw press, in which some particulates were squeezed through the orifice into the water, had relatively high (P <0.05) protease activity. Our results were in agreement with Seymour et al. (1994) in which hydrophobic characteristics of cathepsin L were reported. Cathepsin L, a primary protease, could associate with myofibrils through hydrophobic interaction making it less soluble in water. Thus, whiting proteases were only partially removed by each washing and dewatering step.

Our observation confirmed that whiting protease was highly associated with protein particles. With application of UF, using a membrane of 30,000 MWCO to recondition water, a substantial reduction of protease activity in permeate was noted (Fig. 2.9). These results suggest that whiting protease (with 28 kDa, Seymour et al.,1994), due to its high affinity with protein, could be separated from water with a higher molecular weight cut-off membrane.

Clarity of waste water increased in successive discharge points (Fig. 2.10) but decreased in waste water from the screw press through which more solids were liberated into the waste water. The clarity of surimi wastes (0.35 to 25.57% T) were relatively
Fig. 2.9. Effect of ultrafiltration on protease activity of surimi waste waters. W1 through W8 indicate different waste water streams as illustrated in Fig. 2.1.
Fig. 2.10. Effect of ultrafiltration on clarity (%T) of surimi waste waters. W1 through W8 indicate different waste water streams as illustrated in Fig. 2.1.
lower than that of poultry chiller water (13 to 27% T, Chang and Sheldon, 1989a). However, UF permeates of all surimi waste waters were very clear with average percent transmittance 97.8. This was nearly identical to that of the tap water (98.4%T) used for processing. The clarity of UF permeates indicated the potential for water recycling in surimi operation.

CONCLUSIONS

The particulate protein lost in the final dewatering process (screw press) was successfully recovered and concentrated using a rotary screen and microfiltration. Surimi with 10% replacement of MF recovered protein had the same gel quality as regular surimi with respect to gel hardness, elasticity, water retention, and color. The gel made with MF recovered protein had better color quality than regular surimi gels. The surimi production rate could be increased by 1.7% by adding the MF recovered protein back to the production line without diminishing surimi functionality. The APC, COD, turbidity, and protease levels of waste waters were substantially reduced by ultrafiltration. Use of UF equipment provides the possibility of recycling processing water in the leaching system. However, proteins concentrated by ultrafiltration had considerable dark colors and strong odors.

REFERENCES


Chapter 3

Extraction of Proteins from Pacific Whiting Mince at Various Washing Conditions

Tein M. Lin and Jae W. Park

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April 1996, 8 pages, in press.
III. EXTRACTION OF PROTEINS FROM PACIFIC WHITING MINCE AT VARIOUS WASHING CONDITIONS

ABSTRACT

Effects of salt concentrations and washing cycles on the extraction of proteins were evaluated. Sarcoplasmic proteins were readily soluble in water (0% NaCl) and removed in the initial washing steps. Myofibrillar proteins became relatively soluble and were lost during extensive washing. Control of water/meat ratio, washing time, and washing cycles was critical in reducing the loss of myofibrillar proteins. Washing with 0.25%, 0.5%, or 1.0% NaCl solutions reduced the loss of myofibrillar proteins. However, these solutions were not very effective in removing sarcoplasmic proteins even with increased washing cycles. High salt (2.0% NaCl) washing resulted in low removal of sarcoplasmic proteins and severe loss of myofibrillar proteins.
INTRODUCTION

In surimi processing, extensive washing is utilized to remove water soluble substances, mainly sarcoplasmic proteins. The removal of sarcoplasmic proteins concentrates myofibrillar proteins responsible for the gel-forming ability of surimi (Okada, 1964). Myofibrillar proteins are generally classified as salt-soluble proteins. However, our previous study (Lin et al., 1995) revealed that a considerable amount of myofibrillar proteins was lost in surimi waste streams. The loss of myofibrillar proteins during surimi processing could be in part due to the nature of their water solubility. The solubility of myofibrillar proteins in water and low ionic strength solutions was also reported by Stefansson and Hultin (1994) and Wu et al. (1991). Hennigar et al. (1988) reported that gels could be prepared using fish muscle without NaCl, suggesting that fish muscle myofibrillar proteins could be quite soluble in water or very low ionic strength solutions.

Muscle of salt-water fish contains a variety of salts, about equivalent to a solution with ionic strength 0.145 (Wu et al., 1991). This ionic strength, corresponding to 0.85% (0.145 M) NaCl, might inhibit solubilization of the proteins of fish muscle (Sonu, 1986; Trevino et al., 1990; Wu et al., 1991). Extensive washing of muscle might cause decreased salt concentration in the tissue and allow myofibrillar proteins to become highly associated with water. Matsumoto (1959) reported that when the protein extracted by salt solution was repeatedly washed with water, most of the protein was solubilized. Also increased washing time (Lee, 1986) or washing cycles using the same amount of water (Pacheco-Aguilar et al., 1989) resulted in more proteins, possibly myofibrillar proteins, being solubilized. Presumably, these two factors are crucial in changing the ionic strength
of fish muscle during washing. The loss of myofibrillar proteins during surimi processing might be affected by other processing factors, such as pH changes (Foegeding, 1987; Pacheco-Aguilar et al., 1989), particle size (Acton, 1972), rigor state (Suzuki, 1981; Park et al., 1987, 1990), temperature (Lin and Chen, 1989), and proteases (Schwartz and Bird 1977; Patashnik et al., 1982; Xiong and Brekke, 1989; Chang-Lee et al., 1990).

In order to remove sarcoplasmic proteins and preserve myofibrillar proteins during surimi processing, a better understanding of the removal of sarcoplasmic and the loss of myofibrillar proteins at different washing conditions was needed. Our objective was to investigate the effects of salt concentrations and washing cycles on the extraction of sarcoplasmic and myofibrillar proteins of Pacific whiting mince during washing and dewatering.

MATERIALS AND METHODS

Sample preparation and experimental design

Pacific whiting (Merluccius productus) mince, made by a mechanical deboner with a screen size of 5.5 mm perforation, was collected from a local surimi processing plant within 16-24 hr after harvest. Samples (100g) were subjected to 1, 2, 3, or 4 washing cycles with an overall water/meat ratio at 20:1 (V/W) and an overall washing time of 20 min regardless of the number of washing cycles. Five different washing solutions (0%, 0.25%, 0.5%, 1.0%, and 2.0% NaCl) were prepared using cold distilled water. Following each washing step, centrifugation was used as a means of dewatering.
Fig. 3.1. Experimental outline.
(1,300 x g, 5 min). Temperature was maintained at about 5°C throughout all experiments (Fig. 3.1). All experiments were performed in duplicate.

The supernatant of each washing step was considered to be soluble proteins and the washed mince (final precipitate) was considered to be insoluble proteins. Both soluble proteins and washed mince were collected for further analyses.

**Protein analyses**

Mince (3g) or washed mince (3g) was homogenized with 5% (W/V) sodium dodecylsulfate (SDS) solution, to a final volume of 30 mL, for 2 min at a speed of 3-4 using a Polytron (Brinkmann Instruments, Westbury, NY). Due to its low protein concentration, soluble proteins (supernatant) were prepared by mixing the sample solution with 5% SDS (W/V) to a final volume of 30 mL. Homogenizing procedures were the same as for mince. The homogenates were incubated in an 80°C water bath for 1 hr to dissolve all sarcoplasmic and myofibrillar proteins. Samples were then centrifuged (8,000 x g, 20 min) to remove undissolved debris (mainly connective tissues such as fish skin).

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Absorbance was measured at 750 nm. The amount of recovered and soluble proteins was expressed as a percentage of total proteins in the unwashed mince.

Electrophoresis was conducted according to the procedures of Laemmli (1970). Protein (40μg), measured by the method of Lowry et al. (1951), was applied on a 4% stacking and 10% running polyacrylamide gel. A high molecular weight standard mixture (Stock No. SDS-6H, Sigma Chemical Company, St. Louis, MO) including myosin of
rabbit muscle (205 kDa), β-galactosidase of *E.coli* (116 kDa), phosphorylase b of rabbit muscle (97 kDa), albumin of bovine (66 kDa), albumin of egg (45 kDa), and carbonic anhydrase of bovine erythrocytes (29 kDa) was used. Protein bands were stained with 0.125% Coomassie blue and destained in a 50% methanol and 10% acetic acid solution followed by a 5% methanol and 7% acetic acid solution. Quantitative analysis was performed using an HP DeskScan II (Hewlett-Packard Co., Minneapolis, MN) with NIH Image 1.54 software (written by Wayne Rasband at the U.S. National Institute of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, part number PB93-504868). Equal dye-binding by the various muscle proteins was assumed. The fraction of total area was considered to be the same as the fraction of total proteins loaded onto the gel.

**Conductivity measurement**

The ionic strengths of mince and washed mince were estimated by the method of Stefansson and Hultin (1994). Samples were appropriately diluted and their ionic strengths were measured with a conductivity meter (TDSTestr 2, Whatman, Hillsboro, OR) and compared to a standard curve prepared with sodium chloride solutions.

**Water retention (WR) and total moisture measurements**

Water retention of mince and washed mince was measured by a microcentrifuge-based method (Kocher and Foegeding, 1993). The sample (≈0.4 g) was placed in the inner tube of a Micropure-0.22 Separator (Amicon, Inc., Beverly, MA) and centrifuged
at 2451 x g for 10 min by an Eppendorf microcentrifuge 5415 (Brinkmann Co., Westbury, NY). Water retention was determined as:

\[
\text{WR} = \frac{(\text{total g water in sample}) - (\text{g water released})}{\text{total g protein in sample}}
\]

Total moisture of the samples was determined according to AOAC (1990) procedures.

Statistical analysis

Statistical analysis of the data was carried out by ANOVA. Differences among mean values were established using the least significant difference (LSD) multiple range test (Steel and Torrie, 1980). Mean values were considered significant when p < 0.05.

RESULTS & DISCUSSION

Protein patterns of soluble fractions

A high loss of proteins was found when the mince was washed with distilled water (0% NaCl) at a high water/meat ratio (20:1) and long washing time (20 min)(Fig. 3.2). The loss of proteins increased as the number of washing cycles increased. After 4 cycles, > 40% proteins were lost. The loss of proteins decreased and was less affected by the number of washing cycles when a relatively small amount of salt (0.25%, 0.5%, and 1.0% NaCl) was added to the washing solutions. However, as the salt concentration increased to 2%, the loss of proteins increased again and the effects of the washing cycle became significant (p < 0.05). When 2, 3, or 4 washing cycles were applied for the
Fig. 3.2. Effects of salt concentration and washing cycle on the loss of protein during various washings. 1-4 WC: 1-4 washing cycle(s); 1-4 WS: 1-4 washing step(s).
0.25%, 0.5%, and 1.0% NaCl solutions, more proteins were removed in the first washing step and less proteins were removed in subsequent washing steps. On the other hand the proteins were fairly soluble in every washing step with the 2.0% NaCl solution, resulting in a high loss of proteins. An interesting result was that in the 3 and 4 washing cycles with water, a relatively large amount of protein was removed in the first washing step. Only a small amount of protein was removed in the second washing step. However, in further washing steps, the loss of protein gradually increased. These results could be explained through the results of SDS-PAGE (Fig. 3.3).

Distilled water (0% NaCl). In the 4 washing cycles of water washing, more unidentified proteins (possibly, sarcoplasmic proteins) were solubilized in the initial washing steps (Fig 3.3, lane 7 and 8), while more myofibrillar proteins were present in later washing steps (Fig. 3.3, lane 9 and 10). These data suggested that most of the sarcoplasmic proteins were readily solubilized and removed in the first washing step (Fig. 3.3, lane 7). In the second washing step, the residual sarcoplasmic proteins were continuously removed along with a relatively small amount of myosin heavy chain (MHC), actin, and β-tropomyosin/troponin-T (Fig. 3.3, lane 8). In further washing steps, no notable bands representing sarcoplasmic proteins were found, while the density of MHC, actin, β-tropomyosin/troponin-T, and α-tropomyosin bands increased greatly (Fig. 3.3, lane 9 and 10). A similar trend was observed in the samples of the 3 washing cycles (Fig. 3.3, lane 4-6). The high loss of myofibrillar proteins occurred after the 2nd washing step of 3 or 4 washing cycles. Based on these results, it is suggested that 2 washing steps, with water/meat ratio 5:1 and 6.7:1 and washing time at 5 and 6.7 min for each
Fig 3.3. SDS-PAGE profiles of soluble proteins from whiting mince at 0% NaCl. S: high molecular weight standard; lane 1: 1st washing step (WS) of the 1 washing cycle (WC); lane 2-3: the 1st and 2nd WS of the 2 WC, respectively; lane 4-6: the 1st, 2nd, and 3rd WS of the 3 WC, respectively; lane 7-10: 1st, 2nd, 3rd, and 4th WS of the 4 WC, respectively. MHC: myosin heavy chain; ATN: actin; β-TPM/TNT: β-tropomyosin/troponin-T; α-TPM: α-tropomyosin.
washing step, should be adequate for surimi processing to remove sarcoplasmic proteins without a high loss of myofibrillar proteins. The amount of MHC lost with these washing conditions was estimated to be 6.2-7.7% of the total MHC (Fig. 3.8, 3WC and 4WC) and the loss of actin appeared to be negligible (Fig. 3.3, lane 4, 5, 7, and 8).

It was previously proposed to use one washing cycle with more water, instead of the conventional 3 washing steps using less water in each step, to reduce the loss of solids during washing (French and Babbitt, 1990). Our results showed that at a high water/meat ratio (20:1) and long washing time (20 min), the increased washing cycles resulted in a high loss of myofibrillar proteins in later washing steps (Fig. 3.3). However, in comparing the 4 washing cycle and the 2 washing cycle treatments, the first 2 washing steps of the 4 washing cycle were equivalent to the first washing step of the 2 washing cycle in total water/meat ratio (10:1) and washing time (10 min). The proteins of molecular weight between 41.4 k and 39.5 k (possibly, sarcoplasmic proteins) were present during the 2nd washing of the 2 washing cycle (Fig. 3.3, lane 3) but not in the 3rd or 4th washing of the 4 washing cycles (Fig. 3.3, lane 9 & 10). A relatively smaller amount of MHC was lost during the first 2 washings of the 4 washing cycles (7.7%) than during the first washing of the 2 washing cycles (11.9%)(Fig. 3.8). These results suggested that at a low water/meat ratio (10:1) with a short washing time (10 min), the increased washing cycles enhanced removal of sarcoplasmic proteins. However, at a high water/meat ratio (20:1) with a long washing time (20 min), sarcoplasmic proteins were readily soluble in water and removed in the initial washing. Further washing cycles caused a high loss of myofibrillar proteins.
0.25% NaCl solution. As shown previously, the presence of a relatively small amount of salt (0.25%, 0.5%, and 1.0% NaCl) reduced the loss of proteins during washing (Fig. 3.2). However, the question arose whether these salt solutions could remove all sarcoplasmic proteins. The amount of sarcoplasmic proteins was estimated to be 23.5% of total proteins by subtracting the relative amount of all myofibrillar proteins (76.5%) from the total proteins (100%)(Table 3.1). The relative amount of sarcoplasmic proteins in whiting mince was comparable to that of cod (21%) and carp (23-25%)(Suzuki, 1981). In the 0.25% NaCl washing, only 10.7%, 15.7%, and 12.4% of the total proteins were removed during initial washing of the 2, 3, and 4 washing cycles, respectively (Fig. 3.2). This was lower than the estimated sarcoplasmic proteins (23.5%). The proteins contained in these soluble portions appeared to be MHC, actin, β-tropomyosin/troponin-T, α-tropomyosin, and other unidentifiable proteins with a wide range of molecular weights, possibly sarcoplasmic proteins (Fig. 3.4, lane 2, 4, 5, 7, and 8). In subsequent washing steps, the proteins in the soluble fractions were mainly MHC, actin, and β-tropomyosin/troponin-T (Fig. 3.4, lane 3, 6, 9, and 10). This implied that a larger amount of sarcoplasmic proteins remained in the insoluble fraction (washed mince). Subsequent washings, regardless of number of washing cycles, did not remove the sarcoplasmic proteins completely but continue to reduce the amount of myofibrillar proteins.

Less proteins were solubilized in the 0.25% NaCl solution than in the water during extensive washing steps (Fig. 3.2). According to results of SDS-PAGE, the density of myofibrillar proteins in the soluble fraction remained about the same or decreased in the 0.25% NaCl solution (Fig. 3.4) but sharply increased in water (Fig. 3.3) when subsequent
Table 3.1. Protein pattern of whiting mince

<table>
<thead>
<tr>
<th>Molecular mass(kD)</th>
<th>Protein</th>
<th>Relative amount of protein(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>205</td>
<td>myosin heavy chain</td>
<td>35.4</td>
</tr>
<tr>
<td>180</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>135.6</td>
<td>C-protein</td>
<td>2.0</td>
</tr>
<tr>
<td>108.4</td>
<td>α-actinin</td>
<td>2.5</td>
</tr>
<tr>
<td>103.9</td>
<td></td>
<td>1.6</td>
</tr>
<tr>
<td>96.9</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>91.6</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>80.8</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>75.3</td>
<td></td>
<td>1.6</td>
</tr>
<tr>
<td>56.4</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>44.5</td>
<td>actin</td>
<td>11.9</td>
</tr>
<tr>
<td>41.6</td>
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<td>4.5</td>
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<tr>
<td>40.7</td>
<td></td>
<td>1.7</td>
</tr>
<tr>
<td>35.9</td>
<td>β-tropomyosin/troponin T</td>
<td>2.2</td>
</tr>
<tr>
<td>31.9</td>
<td>α-tropomyosin</td>
<td>5.1</td>
</tr>
<tr>
<td>30.5</td>
<td></td>
<td>1.8</td>
</tr>
<tr>
<td>29.3</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>21</td>
<td>myosin light chain 1</td>
<td>5.3</td>
</tr>
<tr>
<td>20.8</td>
<td>troponin-I</td>
<td>1.8</td>
</tr>
<tr>
<td>20.1</td>
<td>myosin light chain 2 &amp; 3</td>
<td>10.3</td>
</tr>
<tr>
<td>18.2</td>
<td></td>
<td>7.7</td>
</tr>
</tbody>
</table>

* Tentative identification.
Fig. 3.4. SDS-PAGE profiles of soluble proteins from whiting mince at 0.25% NaCl. The lane assignment and legend are the same as Fig. 3.3.
washings were applied. This indicated that the 0.25% NaCl solution prevented myofibrillar proteins from being lost during later extensive washing steps. However, it appeared that distilled water was more effective in removing sarcoplasmic proteins. This was evidenced by the large amount of total proteins removed during the initial washing of the 0% NaCl washing (Fig. 3.2) and the relatively small amount of myofibrillar proteins in these soluble fractions (Fig. 3.3, lane 2, 4, and 7).

0.5% NaCl solution. In the 4 washing cycles using 0.5% NaCl solution, neither the MHC band nor the actin band was observed in the soluble fraction of the first or second washing steps (Fig. 3.5, lane 7 and 8). Proteins removed during these washings were mainly sarcoplasmic or low molecular weight myofibrillar proteins. In subsequent washing steps, no significant bands representing sarcoplasmic proteins were present (Fig. 3.5, lane 9 and 10). The amount of proteins removed during the first 2 washing steps constituted only 10.3% of the total proteins (Fig. 3.2). This indicated that the 0.5% NaCl washing did not remove all sarcoplasmic proteins. Similar results had been observed in the 2 and 3 washing cycles (Fig. 3.5).

These results were similar to those seen in the 0.25% NaCl washing. However, it appeared that less MHC and actin were solubilized in the 0.5% NaCl solution than in the 0.25% NaCl solution, especially in the initial washing steps (Fig. 3.4, lane 7 and 8; Fig. 3.5, lane 7 and 8). The low molecular weight myofibrillar proteins, such as β-tropomyosin/troponin-T, were somewhat solubilized in both solutions during initial washing steps (Fig. 3.4, lane 2, 4, and 7; Fig. 3.5, lane 2, 4, and 7).
Fig. 3.5. SDS-PAGE profiles of soluble proteins from whiting mince at 0.5% NaCl. The lane assignment and legend are the same as Fig. 3.3.
1.0% NaCl solution. The protein patterns for the soluble fraction in the 1.0% NaCl solution (Fig. 3.6) were similar to those in the 0.5% NaCl solution (Fig. 3.5): more low molecular weight proteins (<45 kDa) were solubilized in the initial washing while more MHC and actin were solubilized in subsequent washings. The total amounts of proteins solubilized in the two solutions were very similar (Fig. 3.2). This indicated that both the 0.5% and 1.0% NaCl solutions had similar effects preventing the loss of myofibrillar proteins but did not remove sarcoplasmic proteins completely.

2.0% NaCl solution. At a low water/meat ratio (5:1 and 6.7:1) and a short washing time (5 and 6.7 min), neither MHC nor actin was solubilized in the 2.0% NaCl solution (Fig. 3.7, lane 4 and 7), even though MHC and actin are generally recognized as salt-soluble proteins at this salt concentration (Rodger, 1990). However, β-tropomyosin/troponin-T and α-tropomyosin were consistently soluble in every washing step regardless of water/meat ratio and washing time (Fig. 3.7, lane 1-10). This was probably because the low molecular weight myofibrillar proteins along with some salt-soluble sarcoplasmic proteins were more soluble at this salt concentration. They would compete with myosin and actin for available water molecules and therefore, there would not be enough water molecules for myosin and actin solvation. As low molecular myofibrillar proteins were gradually removed, myosin and actin were solubilized in subsequent washing steps (Fig. 3.7, lane, 6, 9, and 10). When a larger amount of water molecules was present and with a longer washing time, myosin, actin, and other proteins were all solubilized (Fig. 3.7, lane 1). The increased water/meat ratio, washing time, or
Fig. 3.6. SDS-PAGE profiles of soluble proteins from whiting mince at 1.0% NaCl. The lane assignment and legend are the same as Fig. 3.3.
Fig. 3.7. SDS-PAGE profiles of soluble proteins from whiting mince at 2.0% NaCl. The lane assignment and legend are the same as Fig. 3.3.
washing cycles using this high salt concentration caused a high loss of myofibrillar proteins.

With the 2.0% NaCl solution, unidentified proteins were solubilized in later washing steps of 2 (Fig. 3.7, lane 3), 3 (Fig. 3.7, lane 5 and 6), and 4 (Fig. 3.7, lane 9 and 10) washing cycles. Similar results, but to a lesser extent, were also observed in the 1.0% NaCl washing. These proteins might be degraded myofibrillar proteins and/or sarcoplasmic proteins. Sarcoplasmic proteins are known as water or low ionic strength soluble proteins and were not solubilized in the later steps of the low salt (0.25% or 0.5% NaCl) washing. Thus, it is unlikely they would be solubilized in the higher salt solution. Degradation of myofibrillar proteins was likely to occur since they could be unfolded and denatured in the high salt concentration, making them more susceptible to proteolytic degradation.

**Loss of myosin heavy chain (MHC)**

It is very difficult to identify and quantitate all myofibrillar proteins in the soluble fraction when a large amount of various sarcoplasmic proteins are present. Since MHC is the major component of the myofibrillar proteins, it would be a good indicator for estimating the loss of myofibrillar proteins. The amount of MHC in the soluble fraction was expressed as a percentage of the total MHC in the unwashed mince (Fig. 3.8).

The minimal loss of MHC was found at salt concentrations of 0.5% and 1.0%. This loss was increased when the salt concentration either decreased or increased regardless of number of washing cycles. Wu et al. (1991) also reported that washed red hake mince (mainly myofibrillar proteins) had the least solubility at the 0.5% NaCl
Fig. 3.8. The effects of salt concentration and washing cycle on the loss of myosin heavy chain during the various washings. 1-4 WC: 1-4 washing cycle(s); 1-4 WS: 1-4 washing step(s).
solution. The solubility of MHC during the washing could be explained by the following hypothesis. When the fish mince was washed with a large amount of water (0% NaCl) and a long washing time, parts of the water-soluble salt were released from the meat portion into the water portion (Fig. 3.9). This might cause the myofibrillar proteins to have more available sites for water to hydrate and thus increase their solubility. The ionic strength of whiting mince was estimated to be equivalent to 0.65% NaCl (Fig. 3.9). Thus, when the 0.5% or 1.0% NaCl solutions, close to the physiological ionic strength of fish mince, were used for washing, the salt level in the proteins was fairly well maintained (Fig. 3.9) and less changes in protein solubility occurred. As salt level in the washing solution was further increased to 2.0% NaCl, the salt ions might bind to the oppositely charged amino acid residues, resulting in rupture of intra-and/or inter-molecular ionic bondings. Therefore, the solubility of the proteins increased due to their increased affinity for water. This was evidenced by the higher loss of MHC in the 2.0% salt solution (Fig. 3.8).

The loss of MHC increased as the number of washing cycles increased. This was especially noted in the 0% and 2.0% NaCl solutions. In the 0.5% or 1.0% NaCl samples, the loss of MHC during washing remained relatively low even with higher numbers of washing cycles.

The highest loss of MHC (42.9%) occurred when the fish mince was washed by water (0% NaCl) for 4 cycles. Among these 4 washing steps, only 1.1% MHC was lost in the first washing step but 21.8% MHC was lost in the last washing step. This result might be explained by the following. At the beginning of washing, the water-soluble
Fig. 3.9. Effects of salt concentration and washing cycle on the ionic strengths of washed mince. 1-4 WC: 1-4 washing cycle(s). Different letters in the same group of bars indicate a significant difference (p < 0.05).
sarcoplasmic proteins were readily solubilized and made the compact and high-molecular-weight MHC less likely to hydrate. At later washing steps, less sarcoplasmic proteins remained in the mince and more water-soluble salts were removed. Therefore, more water became relatively available for MHC solubilization and more charged amino acids in the MHC became available for hydration. The higher loss of MHC in the 0% NaCl washing coincided with the higher loss of salt (Fig. 3.9). However, less MHC was solubilized in the salt solutions than in the water, even when the same amount of water and washing time was allowed. This suggested that the release of water-soluble salt from myofibrillar proteins was more important than the availability of water molecules in determining the solubility of MHC.

**MHC and actin in washed mince**

MHC and actin comprised about half (47.3%) the total proteins of the mince (Table 3.1). After 1 washing cycle with distilled water (0% NaCl), the relative amount of MHC in the washed mince increased from 35.4% to 51.0% while the actin remained about the same (11.9% to 12.4%) (Table 3.2). As the number of washing cycles increased to 2, both the relative amounts of MHC and actin were further increased without diminishing yield (Fig. 3.10). However, when the 3 washing cycles were applied, the relative amount of MHC and actin remained about the same but the yield decreased (Fig. 3.10). This indicated that the number of washing cycles was important in controlling the quality and yield during surimi processing.

Nishioka (1984) indicated that when a greater number of washing cycles was applied, the surimi exhibited stronger gel-forming ability. Since myosin is the protein
Table 3.2. Relative amount of MHC and actin in the washed mince

<table>
<thead>
<tr>
<th>Protein</th>
<th>NaCl&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1WC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>2WC</th>
<th>3WC</th>
<th>4WC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC</td>
<td>0%</td>
<td>51.0</td>
<td>63.5</td>
<td>64.8</td>
<td>72.7</td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td>12.4</td>
<td>17.6</td>
<td>17.2</td>
<td>13.7</td>
</tr>
<tr>
<td>MHC</td>
<td>0.25%</td>
<td>44.7</td>
<td>47.2</td>
<td>49.1</td>
<td>56.4</td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td>19.2</td>
<td>20.4</td>
<td>22.8</td>
<td>16.7</td>
</tr>
<tr>
<td>MHC</td>
<td>0.5%</td>
<td>46.0</td>
<td>53.0</td>
<td>55.5</td>
<td>46.2</td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td>20.9</td>
<td>17.8</td>
<td>16.3</td>
<td>13.5</td>
</tr>
<tr>
<td>MHC</td>
<td>1.0%</td>
<td>47.5</td>
<td>51.7</td>
<td>43.4</td>
<td>40.5</td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td>19.8</td>
<td>23.2</td>
<td>23.6</td>
<td>21.9</td>
</tr>
<tr>
<td>MHC</td>
<td>2.0%</td>
<td>47.2</td>
<td>47.5</td>
<td>43.9</td>
<td>40.3</td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td>18.6</td>
<td>19.1</td>
<td>16.5</td>
<td>18.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>NaCl: the salt concentration of washing solution.
<sup>b</sup>WC: the number of washing cycles.
Fig. 3.10. Effects of salt concentration and washing cycle on the total protein recovery. 1-4 WC: 1-4 washing cycle(s). Different letters in the same group of bars indicate a significant difference (p < 0.05)
primarily responsible for gelation in the meat system (Hung and Smith, 1993), our results support that observation by showing that the relative amount of MHC increased with increased numbers of washing cycles.

Although the salt solutions (0.25%, 0.5% and 1.0% NaCl) were more effective than distilled water in preventing loss of MHC during washing (Fig. 3.8), the salt-washed minces contained relatively less MHC. Using a low salt solution for surimi processing might produce a higher yield (Fig. 3.10) but a lower quality surimi, due to its lower MHC content, than conventional washing (0% NaCl).

**Moisture content and water retention of washed mince**

After washing with a large amount of water (0% NaCl), the moisture content of washed mince substantially increased (Fig. 3.11). The mince washed with 2, 3, or 4 washing cycles exhibited a higher moisture content than that with 1 washing cycle. Adding salt in the washing solution reduced the moisture content of the washed mince. The moisture content increased again when a high concentration of salt (2.0%) was added. The effect of washing cycle on moisture content was not significant in the presence of salt (Fig. 3.11).

The removal of water-soluble salt during washing causes the hydrophilicity to increase (Okada, 1981). Consequently, the washed mince tends to hydrate and swell. To facilitate removal of water from the washed mince, it is common to add 0.1-0.3% salt in the final washing step (Lee, 1984; Sonu, 1986; Park, 1995). Since there was no difference in moisture content among the 0.25%, 0.5%, and 1.0% NaCl washed mince, 0.25% NaCl might be sufficient for adequate dewatering. At a higher salt concentration
Fig. 3.11. Effects of salt concentration and washing cycle on the moisture content of the washed mince. 1-4 WC: 1-4 washing cycle(s). Different letters in the same group of bars indicate a significant difference (p < 0.05).
(2.0% NaCl), the hydrophilicity of the washed mince increased. High concentrations of NaCl caused depolymerization of myofibrillar proteins because the ions compete for electrostatic interactions. This depolymerization process would of course increase the surface area, which then increases protein hydration. It has been reported that Cl\(^-\) ions have a stronger binding affinity to myofibrillar proteins than Na\(^+\) ions (Hamm, 1960; Offer and Trinick, 1983). When a substantial number of Cl\(^-\) ions was bound to the myofibrillar proteins at a high NaCl concentration, the total negative charge on the myofibrillar proteins would increase. Therefore, the myofibrillar proteins would repel one another, leading to swelling and increased hydrophilicity.

As with results for moisture content, the water retention of the washed mince was higher at the very low (0% NaCl) or very high (2.0%) ionic strengths (Fig. 3.12). The effect of washing cycles was not significant except for those of 2.0% NaCl washing.

**CONCLUSIONS**

Extensive washing with water caused high loss of myofibrillar proteins and high moisture content in the washed mince. Two washing cycles are suggested for surimi processing. A water/meat ratio at 5:1 and 6.7:1 and washing time at 5 and 6.7 min for each step would be sufficient to remove sarcoplasmic proteins without loss of myofibrillar proteins. Washing with low salt solutions reduced loss of myofibrillar proteins. However, salt solutions would not be recommended for surimi processing due to their low effectiveness for removing sarcoplasmic proteins.
Fig. 3.12. Effects of salt concentration and washing cycle on the water-retention ability (WRA) of washed mince. 1-4 WC: 1-4 washing cycle(s). Different letters in the same group of bars indicate a significant difference (p < 0.05).
REFERENCES


Chapter 4

Effective Washing Conditions Reduce Water Usage for Surimi Processing

Tein M. Lin and Jae W. Park

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1996, in review
IV. EFFECTIVE WASHING CONDITIONS REDUCE WATER USAGE FOR SURIMI PROCESSING

ABSTRACT

Possible minimization of water usage for leaching was investigated by reduced water/meat (W/M) ratio with increased washing cycles (WC) and washing time (WT). Increased WT did not enhance removal of sarcoplasmic proteins once equilibrium was reached, while the increased WC continuously removed residual sarcoplasmic proteins from the mince. No significant loss of myofibrillar proteins occurred at the low W/M ratio (2:1 or 1:1) regardless of WC and WT. Myosin heavy chain content, water retention, and whiteness of washed mince decreased when W/M ratio was reduced. Increasing WC and/or WT enhanced these properties but also resulted in a higher moisture content.
INTRODUCTION

One of the most critical steps in surimi manufacturing is the washing of minced fish flesh with large amounts of water to remove sarcoplasmic proteins, blood, fat, and other nitrogenous compounds. Texture, color, and odor of the final product is greatly improved by washing out these impurities. However, due to rising utility costs, limited water sources, and pollution problems, minimization of water usage for leaching and reduction of waste water disposal have recently become a major consideration for surimi manufacturers.

The degree of washing required to produce good quality surimi depends upon the type, composition, and freshness of the fish. The number of washing cycles and water/meat ratios employed for washing vary among surimi processors. A water/meat ratio ranging from 4:1 to 8:1 is often employed by on-shore processors. This washing process is often repeated 3 to 4 times to ensure sufficient removal of sarcoplasmic proteins. On the other hand, at-sea processors use a lower water/meat ratio (1:1 to 3:1) with only one or two washing cycles due to their limited access to fresh water. Generally, the overall water/meat ratio used for the washing ranges from 3:1 to 24:1. Increased water usage for washing usually resulted in more protein loss and waste water disposal (Lin and Park, 1995). It was estimated that ≈ 50% of total proteins were lost during washing (Adu et al., 1983; Pacheco-Aguilar et al., 1989; Yang and Froning, 1992), and 30 L of waste water per one kg of surimi produced were generated from on-shore processing plants (Lin et al., 1995). The question has been raised as to whether more water usage guarantees better surimi quality or if it is unnecessary and wasteful. In early
studies, it was shown that the gel strength of surimi continued to increase as the number of washing cycles increased (Nishioka, 1984). However, a recent report (Lin and Park, 1995) indicated that the major portion of sarcoplasmic proteins are fairly soluble and removed during the initial washing steps. Subsequent washing removes the residual sarcoplasmic proteins along with a small amount of myofibrillar proteins. After sarcoplasmic proteins are completely removed, further washing causes a severe loss of myofibrillar proteins. Therefore, excessive washing not only increases the cost for water usage and waste water treatment, but also results in a yield loss. Thus, a careful control of water/meat ratio, washing cycles, and washing time is important for surimi processors to maximize quality and yield, as well as to minimize water usage and waste water disposal.

A logical approach to achieve the same washing effect with less water would be to increase washing time and number of washing cycles with a lower water/meat ratio for each washing step. Theoretically, at a constant water/meat ratio, more extractable proteins will dissolve in water when a longer washing time is allowed until the equilibrium stage is reached. To increase washing efficiency, sufficient washing time should be allowed. Increased washing cycles, at a constant overall water/meat ratio, are likely to have a higher dilution factor, i.e., more extractable proteins will be removed per unit of water used. The objective of this study was to investigate the possible minimization of water usage for surimi processing by maximizing washing efficiency through increased washing cycles and washing time.
MATERIALS AND METHODS

Pacific whiting (*Merluccius productus*) was collected from commercial vessels operating off the Oregon coast within 16-24 hr after harvest. The fish samples were headed, gutted, and minced through a mechanical deboner with a screen size of 5.5 mm perforation. For the control group, three washing cycles with a water/meat ratio at 4:1 and a washing time of 5 min for each washing step were used. To reduce water usage, a lower water/meat ratio (2:1 or 1:1) with increased washing cycles (3 or 4) and/or washing time (5 or 10 min) was applied. Detailed washing procedures are summarized in Table 4.1. Centrifugation (2,600 x g, 5 min) was used as a means for dewatering between washings. Following the final washing step, a higher centrifugal force (6,800 x g, 10 min) was applied to reduce moisture content of washed mince. The extractable protein (supernatant) of each washing step and the washed mince (final precipitate) were analyzed for protein concentration and protein patterns. The physicochemical properties (moisture content, water retention ability, and color) of various washed minces were compared. All analyses were performed in duplicate. The temperature was maintained at ~ 5°C throughout all experiments, unless otherwise specified.

Protein analyses

Three grams of samples (supernatant or washed mince) were homogenized with 5% (W/V) sodium dodecylsulfate (SDS) solution to a final volume of 30 mL. The homogenates were incubated in an 80°C water bath for 1 hr, cooled to room temperature, and then centrifuged (8,000 x g, 20 min) to remove undissolved debris. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum
Table 4.1. Experimental outline

<table>
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<tr>
<th></th>
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<th>WC</th>
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<td>6:1</td>
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<tr>
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<td>2:1</td>
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<td>10</td>
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<td>8:1</td>
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<tr>
<td>T4</td>
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W/M: water/meat ratio; WC: washing cycles; WT: washing time.
albumin as a standard. Absorbance was measured at 750 nm. The amount of proteins lost in each washing step was expressed as a percentage of the total proteins in the unwashed mince.

Protein patterns of supernatant and washed mince were analyzed by SDS-PAGE method (Laemmli, 1970). Twenty μg protein, measured by the method of Lowry et al. (1951), was applied on a 4% stacking and 10% running polyacrylamide gel. A high molecular weight standard mixture (Stock No. SDS-6H, Sigma Chemical Company, St. Louis, MO) including myosin of rabbit muscle (205 kDa), β-galactosidase of *E. coli* (116 kDa), phosphorylase b of rabbit muscle (97 kDa), albumin of bovine (66 kDa), albumin of egg (45 kDa), and carbonic anhydrase of bovine erythrocytes (29 kDa) was used. Protein bands were stained with 0.125% Coomassie blue and destained in a 25% methanol and 10% acetic acid solution. Quantitative analysis for myosin heavy chain (MHC) was performed using an HP DeskScan II (Hewlett-Packard CO., Minneapolis, MN) with an NIH image 1.54 software (NIH, Washington, DC). The relative amount of MHC in various washed minces was expressed as a percentage compared to the control.

**Measurement of physicochemical properties**

The moisture content of washed mince was measured by a microwave procedure (Morrissey et al., 1993). To mimic surimi manufacturing, washed mince was mixed with additives (4% sucrose, 4% sorbitol, 1% beef plasma protein, and 0.3% sodium tripolyphosphate) and kept in a freezer (-20°C) overnight. Surimi gels were made according to the method of Park (1995) with a slight modification. Frozen samples were thawed at room temperature for 2 hr and cut into 3 cm cubes. Samples were chopped in
a Stephan vacuum cutter (Stephan Machinery, Columbus, OH) for 1.5 min at low speed. Salt (2%) was added and chopping was continued at low speed for 1.5 min. Then ice/water was added to adjust the final moisture to a constant (81.5%) and the samples were further chopped at high speed under vacuum for another 3 min. The temperature during chopping was maintained at 3-5°C using a refrigerated circulator (RTE-100LP, NesLab Instruments, Inc., Newington, NH). The paste was vacuum-packed in a polyethylene bag, extruded into stainless steel cooking tubes (i.d. = 19 mm), and cooked in a water bath (90°C, 15 min). Gels were chilled in ice water before storing in a refrigerator overnight and then analyzed for color and water retention.

The color of gels was measured by a Minolta Chroma Meter (Model CR-310, Minolta Corp., Ramsey, NJ). Gels were warmed to room temperature before measurements were made (Park, 1994). Two whiteness indices (I & II), as described by Park (1994, 1995), were used to compare different washing effects on color.

\[
\text{Whiteness I} = 100 - \left[ (100 - L^*)^2 + a^*^2 + b^*^2 \right]^{0.5}
\]

\[
\text{Whiteness II} = L^* - 3b^*
\]

Water retention ability (WRA) of gels was measured by a microcentrifuge method (Kocher and Foegeding, 1993). The sample (=0.4 g) was placed in the inner tube of a Micropure-0.22 Separator (Amicon, Inc., Beverly, MA) and centrifuged at 2451 x g for
10 min by an Eppendorf microcentrifuge 5415 (Brinkmann Co., Westbury, NY). WRA was determined as:

\[
\text{WRA} = \frac{\text{total g water in sample} - \text{g water released}}{\text{total g protein in sample}}
\]

**Statistical analysis**

Statistical analysis of the data was carried out by ANOVA. Differences among mean values were established using the least significant difference (LSD) multiple range test (Steel and Torrie, 1980). Mean value was considered significant when \( p < 0.05 \).

**RESULTS AND DISCUSSION**

**Extraction of proteins by various washings**

The concentration of extractable proteins increased when the water/meat (W/M) ratio was reduced from 4:1 to 2:1 or 1:1 (Fig. 4.1). This was most noticeable during the initial washing steps. In the later washing steps, the difference in protein concentration was less affected by W/M ratio. This data showed that more proteins per unit of water were removed when W/M ratio was reduced. At the W/M ratio of 2:1, increasing washing time from 5 to 10 min increased the concentration of extractable proteins. However, effects of washing time were not significant at the W/M ratio of 1:1 suggesting that at this W/M ratio extractable proteins reached equilibrium at or before the 5 min washing time. Therefore, additional washing time did not enhance removal of extractable
Fig. 4.1. Protein concentration of supernatant at various washing conditions. 5m & 10m: 5 and 10 min of washing time, respectively; 4W:1M, 2W:1M, and 1W:1M: a water/meat ratio at 4:1, 2:1, and 1:1, respectively; 1-4 WS: 1-4 washing step.
proteins. It appeared that when a higher W/M ratio was applied, a longer time was required to reach equilibrium.

Although more proteins per unit of water were extracted at a low W/M ratio (2:1 and 1:1), less total proteins were removed by these washings as compared to the control (Fig. 4.2). When the W/M ratio was reduced from 4:1 to 2:1, with 3 washing cycles and 5 min of washing time, the total extractable protein decreased from 25.5% to 19.5%. The extraction of proteins was enhanced when washing cycles and/or washing time increased. As W/M ratio was further reduced to 1:1, increased washing time did not enhance the removal of extractable proteins. However, when an additional washing cycle was applied, residual extractable proteins were continuously removed.

The results of SDS-PAGE showed that the extractable proteins in the control were mainly sarcoplasmic proteins with a negligible amount of myosin heavy chain and actin (Fig. 4.3a). The patterns of the extractable proteins remained about the same at the lower W/M ratios (2:1 or 1:1), regardless of washing cycles or washing time (Fig. 4.3b and 3c). Lin and Park (1995) indicated that at a higher W/M ratio (5:1), increasing washing cycles or washing time resulted in a high loss of myofibrillar proteins. However, the present study showed that at a reduced water/meat ratio (2:1 or 1:1), increased washing cycles or washing time did not cause a higher loss of MHC or actin (Fig. 4.3a and 4.3b).

The amount of proteins removed by the washing with W/M ratio at 2:1, 4 washing cycles, and 10 min of washing time was 24.1% which was comparable to the control (25.5%)(Fig. 4.2). It was estimated that sarcoplasmic proteins constitute about 23.5% of total proteins in whiting flesh (Lin and Park, 1995). Therefore, 4 washing cycles with
Fig. 4.2. Effects of washing conditions on total extractable proteins. Refer to Fig. 4.1 for legend labels.
Fig. 4.3a. SDS-PAGE profile of extractable proteins at water/meat ratios of 4:1. S: high molecular weight standard; lane 1-3: 1-3 washing steps (WS) of 3 washing cycles (WC) with 5 min of washing time (WT).
Fig. 4.3b. SDS-PAGE profile of extractable proteins at water/meat ratios of 2:1. S: high molecular weight standard; lane 1-3: 1-3 washing steps (WS) of 3 washing cycles (WC) with 5 min of washing time (WT); lane 4-6: 1-3 WS of 3 WC with 10 min of WT; lane 7-10: 1-4 WS of 4 WC with 5 min of WT; lane 11-14: 1-4 WS of 4 WC with 10 min of WT.
Fig. 4.3c. SDS-PAGE profile of extractable proteins at water/meat ratios of 1:1. The lane assignment is the same as Fig. 4.3b.
W/M ratio 2:1 and 10 min of washing time for each washing step appeared to be sufficient for leaching process. Thus, the overall water usage can be reduced from 12:1 (W/M) to 8:1. To further reduce water usage, 3 washing cycles with a W/M ratio of 2:1 and 10 min of washing time, or 4 washing cycles with a W/M ratio of 1:1 and 5 min of washing time could be applied for the leaching process. The amount of proteins removed by these washings (22.1% and 22.9%, respectively) was close to the total amount of sarcoplasmic proteins (23.5%). Therefore, the overall water usage can be further reduced to 6:1 (W/M) and 4:1 (W/M), respectively.

Myosin heavy chain (MHC) content and physicochemical properties of washed mince

MHC. With 3 washing cycles and 5 min of washing time, the MHC content in the washed mince decreased when W/M ratio was reduced from 4:1 to 2:1 or 1:1 (Fig. 4.4). The reduced MHC content in the washed mince was probably due to insufficient removal of sarcoplasmic proteins. At W/M ratio of 2:1, increased washing cycles and/or washing time resulted in an increased MHC content. The MHC contents in these washed minces were comparable to the control. As the W/M ratio was reduced further to 1:1, increased WC enhanced MHC content in the washed mince. However, increased washing time did not show any significant effects on MHC content.

 Moisture content. W/M ratio, washing cycle, and washing time all significantly affected the moisture content of washed mince (Fig. 4.5). When W/M ratio reduced from 4:1 to 2:1 or 1:1, with 3 washing cycles and 5 min of washing time, the moisture content of washed mince decreased from 88.8% to 87.3% and 85.1%, respectively. However, at the low W/M ratios (2:1 and 1:1), prolonged washing time and/or additional washing
Fig. 4.4. Effects of washing conditions on relative amount of myosin heavy chain (MHC) in the washed minces. 3 and 4 WC: 3 and 4 washing cycles, respectively. Refer to Fig. 4.1 for other legend labels.
Fig. 4.5. Effects of washing conditions on moisture content of washed mince. 3 and 4 WC: 3 and 4 washing cycles, respectively. Refer to Fig. 4.1 for other legend labels.
cycles resulted in an increased moisture content. With a combination of more washing cycles (4) and longer washing time (10 min), the washed mince had higher moisture content than the control even though a lower W/M ratio (2:1 or 1:1) was applied. At the W/M ratio of 1:1, increased washing time did not enhance the removal of sarcoplasmic proteins (Fig. 4.2 and 3c) or increase the MHC content in their washed mince (Fig. 4.4), but caused higher moisture content (Fig. 4.5). This data indicated that once extractable proteins reach equilibrium, excessive washing time should be avoided since controlled moisture content is critical in surimi processing.

**Water retention ability.** With 3 washing cycles and 5 min of washing time, water retention ability decreased as W/M ratio was reduced (Fig. 4.6). While increased washing cycles resulted in higher water retention, increased washing time did not show any significant effects. A slightly higher water retention than the control was observed at the W/M ratio of 2:1 with 4 washing cycles. This may be due to their high level of MHC (Fig. 4.4) and moisture content (Fig. 4.5). Since it is known that myofibrillar proteins are responsible for water retention in meat (Offer and Trinick, 1983), the relatively high MHC content in these washed minces might attribute to their increased water retention. It was also reported that muscle proteins with high moisture content tend to have high water retention ability (Lin and Park, 1995; Swift and Berman, 1959; Wu et al., 1991).

When the W/M ratio was reduced further to 1:1, increased washing cycles only slightly improved water retention ability (Fig. 4.6). This might be due to their insufficient removal of sarcoplasmic proteins (Fig. 4.2 and 4.3c) and low MHC content (Fig. 4.4).
Fig. 4.6. Effects of washing conditions on water retention ability (WRA) of washed mince.
3 and 4 WC: 3 and 4 washing cycles, respectively. Refer to Fig. 4.1 for other legend labels.
Fig. 4.7. Effects of washing conditions on whiteness I and II of washed mince. 3 and 4 WC: 3 and 4 washing cycles, respectively. Refer to Fig. 4.1 for other legend labels.
Color. Different color indices have been used by the surimi industry to evaluate the color quality of surimi. Among them, whiteness I & II (as described in Materials and Methods) are the two most widely used methods. Whiteness I did not change, compared to the control, when the W/M ratio was reduced to 2:1, but slightly decreased when W/M ratio was further reduced to 1:1 (Fig. 4.7). Washing cycles and washing time did not exhibit significant effects on whiteness I. On the other hand, whiteness II revealed a significant decrease when W/M ratio was reduced to 2:1 or 1:1. Washing cycles and washing time again did not show significant effects on whiteness II. Visually, the control was slightly whiter than other samples. These results are in agreement with the study of Park (1994) showing that whiteness II is more effective in evaluating surimi color.

CONCLUSIONS

Usage of leaching water could be reduced from 12:1 (overall W/M ratio) to 8:1 by using a lower W/M ratio (2:1) with increased washing cycles (4) and washing time (10 min). This washing was sufficient to remove sarcoplasmic proteins but resulted in a higher moisture content. When the total water used was further reduced to 4:1, MHC content, WRA, and color of the washed mince were compromised.

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Protein Solubility in Pacific Whiting Affected by Proteolysis during Storage

Tein M. Lin and Jae W. Park

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1996, in press
V. PROTEIN SOLUBILITY IN PACIFIC WHITING AFFECTED BY PROTEOLYSIS DURING STORAGE

ABSTRACT

The effects of various post-harvest storage temperatures and times on the proteolysis of Pacific whiting (*Merluccius productus*) and its relationship to changes in protein solubility were evaluated. Myosin heavy chain (MHC) degraded rapidly during post-harvest storage even at low temperatures (0-5°C). Greater degradation of MHC occurred at elevated temperatures. The trend of actin degradation was similar to that of MHC but to a lesser degree. Both log-percent MHC and actin degradation showed a good correlation ($R^2 = 0.88$ and $0.74$) to protein solubility. Degraded proteins, however, were not completely removed by washing, resulting in a lower MHC content in washed mince.
INTRODUCTION

Pacific whiting (*Merluccius productus*) is the most abundant fish resource off the Northwest coast of the United States. A major part of the harvest has been successfully used for surimi manufacturing due to its high volume and low price. There is significant proteolysis of myofibrillar proteins in Pacific whiting (An et al., 1994; Chang-Lee et al., 1989; Erickson et al., 1983; Kabata and Whitaker, 1985). Much research has focused on textural deterioration upon heating (Chang-Lee et al., 1989; Kudo et al., 1987; Morrissey et al., 1993; Patashnik et al., 1982; Yongsawatdigul et al., 1995). Nevertheless, little information is available regarding the effects of proteolysis on the loss of myofibrillar proteins during surimi manufacturing.

Myofibrillar proteins constitute approximately 76.5% of total proteins in whiting flesh (Lin and Park, 1996). Therefore, recovery of myofibrillar proteins is expected to be in this range during surimi manufacturing. However, only 54% of total proteins were recovered as myofibrillar proteins in Pacific whiting surimi (Chang-Lee et al., 1990). Previous studies showed that surimi waste streams contained a significant amount of myofibrillar proteins (Lin et al., 1995). In surimi processing, minced fish flesh is subjected to particle-size reduction, ionic strength changes (Lin et al., 1996; Hennigar et al., 1988), pH changes (Lin and Park, 1996), mechanical forces, such as screening and screw pressing, and possibly temperature abuse. All these factors could contribute to the loss of myofibrillar proteins in various degrees. Therefore, the myofibrillar proteins lost in the surimi waste streams could be soluble proteins or insoluble particulates.
The loss of myofibrillar proteins might be due in part to proteolysis before and during processing, causing more proteins to be dissolved in the water soluble portion (Patashnik et al., 1982; Suzuki, 1981; Xiong and Brekke, 1989). In commercial surimi operations, whiting are usually delivered to processing plants within 6-12 hr after harvest. Due to the limited capacity of processing facilities, fish are often kept in holding tanks with ice water (±0°C) for up to another 6-14 hr. Thus, fish are usually 6-26 hr post-harvest before they are subjected to surimi processing. During this holding period, the temperature can rise if fish are not handled properly. Prolonged holding time and elevated temperatures might cause severe proteolysis of myofibrillar proteins and consequently affect the processing yield and gelation properties of surimi. The objectives of this research were to investigate the effects of post-harvest storage times and temperatures on degradation of myofibrillar proteins and further clarify the relationship between proteolysis and solubility of proteins during washing.

**MATERIALS AND METHODS**

**Materials**

Pacific whiting (*Merluccius productus*) was collected from commercial vessels operating off the Oregon coast. Fish samples were about 6 hr post-harvest and kept in aerated ice water (±0°C) before landing. To minimize the variation in parasite infection among fish, the fish (± 150 Kg) were randomly sampled from the same lot (± 20,000 Kg). Upon receiving, the samples were stored at 4 different temperatures (0, 5, 10, and 20°C) for up to 72 hr, except for those at 10°C (up to 48 hr) and 20°C (up to 24 hr) due
to microbial deterioration. Samples (10 fish) were randomly taken at 0, 2, 6, 14, 24, 48, and 72 hr during storage to evaluate degradation of myofibrillar proteins and loss of proteins during washing. All experiments were performed in duplicate.

**Analysis for myosin heavy chain (MHC) and actin degradation**

MHC and actin degradation were examined by SDS-PAGE method. Fish samples were filleted, skinned, and chopped (5°C). Three gram samples were homogenized with 5% (w/v) sodium dodecylsulfate (SDS) solution to a final volume of 30 mL. To prevent the occurrence of proteolysis during sample preparation, the SDS solution was preheated to 95°C before mixing with the samples. The homogenates were incubated in an 80°C water bath for 1 hr to dissolve all proteins. Centrifugation (8,000 x g, 20 min, 5°C) was conducted to remove undissolved debris (mainly connective tissue and fish skin).

Electrophoresis was carried out according to the procedure of Laemmli (1970). Samples of 30 μg protein, measured by the method of Lowry et al. (1951), were applied on a 4% stacking and 10% running polyacrylamide gel. A high molecular weight standard mixture (Stock No. SDS-6H, Sigma Chemical Company, St. Louis, MO) including myosin of rabbit muscle (205 kDa), β-galactosidase of *E.coli* (116 kDa), phosphorylase b of rabbit muscle (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase of bovine erythrocytes (29 kDa) was used. Protein bands were stained with 0.125% Coomassie blue and destained in a 25% methanol and 10% acetic acid solution. Quantitative analysis for MHC and actin degradation was performed using a HP DeskScan
II (Hewlett-Packard Co., Minneapolis, MN) with an NIH Image 1.54 software (NIH, Washington, DC). MHC and actin degradation were calculated by the following equation:

\[
\% \text{ MHC (actin) degradation} = \frac{Ac - As}{Ac}
\]

where Ac is the area of MHC (actin) peak in the control (0°C, 0 hr) and As is the area of MHC (actin) peak in the sample.

**Analysis for solubility of proteins during washing**

The procedures for evaluating solubility of degraded proteins during washing were designed to mimic surimi processing. Based on our previous study (Lin and Park, 1996), two washing cycles with a meat/water ratio at 1:5 and 5 min washing time for each washing cycle was the optimal leaching condition to remove sarcoplasmic proteins without a significant loss of myofibrillar proteins. This washing condition was applied to the current study. Fish samples were filleted, skinned, and chopped (5°C). The minced sample (100 g) was subjected to two washing cycles with 500 mL prechilled distilled water (5°C) for each washing step. In each washing step, the mixture was stirred at a constant rate for 5 min and then centrifuged at 1,700 x g for 5 min (5°C). Samples were taken from the mince before washing for the measurement of total proteins and from the supernatant for the measurement of soluble proteins. To dissolve all the proteins for protein content determination, 3 g of samples were homogenized with 5% (W/V) SDS solution to a final volume of 30 mL and incubated in a water bath (80°C) for 1 hr. Protein concentration was measured by the method of Lowry et al. (1951) at 750 nm.
using bovine serum albumin as a standard. Protein solubility was defined as the fraction of total proteins remaining soluble after centrifugation.

Analysis for washed mince

The relative amounts of MHC and actin remaining in washed mince were examined by SDS-PAGE method and quantitated by a densitometry method as described above. Equal dye-binding by various muscle proteins was assumed. The fraction of total area was considered to be the same as the fraction of total proteins loaded on the gel.

Statistical analysis

Statistical analysis of data for effects of times and temperatures on MHC/actin degradation and protein solubility was carried out by ANOVA. Differences among mean values were established using the least significant difference (LSD) multiple range test (Steel and Torrie, 1980). Mean value was considered significantly different when \( p < 0.05 \). Data analysis for correlation of MHC/actin degradation to protein solubility was performed by a simple linear regression analysis using Statgraphic Version 6.0 (Manugistics Inc., Rockville, MD).

RESULTS AND DISCUSSION

Degradation of myosin heavy chain (MHC) and actin

Degradation of MHC increased rapidly during the post-harvest storage period (Fig. 5.1). This degradation occurred even though the temperature was maintained at 0°C. In commercial operations, Pacific whiting is commonly kept in holding tanks (≈0°C) for up
Fig. 5.1. Effects of storage times and temperatures on myosin heavy chain (MHC) degradation.
to 14 hr before fish are subjected to processing. This study showed that 23.5% of MHC degradation occurred at this storage condition (0°C, 14 hr). Greater degradation of MHC was observed with prolonged storage time. More than 70% MHC was degraded when fish were stored at 0°C for 72 hr. MHC degradation was significantly affected by storage temperatures. Fish kept at 5°C showed higher degradation than those at 0°C suggesting that ice water was more efficient than refrigeration in controlling proteolysis of Pacific whiting. When temperatures increased further, degradation occurred more rapidly. Within a 14 hr storage period, degradation almost doubled when temperatures increased from 0 to 10°C. After holding at 20°C for 2 hr, 31.6% MHC was degraded which was equivalent to the degradation that occurred when fish were stored at 0°C for 24 hr. This data indicated both time and temperature were critical to MHC degradation. Low temperatures slow down proteolysis. With prolonged storage time, however severe degradation occurred even though the storage temperature was maintained at 0°C. According to An et al. (1994), in the temperature range of 0-5°C, the activity of cathepsin L is insignificant, while cathepsin B exhibited one half of its maximal activity, and cathepsin H retained about one fifth of its maximal activity. Therefore, cathepsins B and H might contribute to the degradation occurring at low temperature storage. To minimize proteolysis, fish should be processed promptly upon landing or kept at 0°C if a holding period is necessary.

The trend of actin degradation was similar to that of MHC but to a lesser extent. Actin degradation increased as storage time or temperature increased (Fig. 5.2). At 0°C, degradation of actin was not significant in the first 6 hr. When prolonged storage time
Fig. 5.2. Effects of storage times and temperatures on actin degradation.
was allowed, degradation increased substantially. Actin degraded about 20% after 14 hr at 0°C. Similar to MHC degradation, actin degraded more rapidly at 5°C than at 0°C. As temperatures increased further, degradation of actin occurred more rapidly.

**Solubility of proteins during washing**

The solubility of proteins during washing increased when fish were held for a longer time and/or at a higher temperature (Fig. 5.3). When fish were kept at the condition similar to commercial operations (0°C, 14 hr) prior to washing, total protein loss increased from 22.8% (0°C, 0 hr) to 33.82% (0°C, 14 hr). The loss of proteins increased slightly after 14 hr and reached a maximum loss of 35% at 72 hr. As storage temperatures increased, more proteins were solubilized during washing. The temperature effect was significant only for storage time up to 14 hr. After 24 hr storage, no difference (p < 0.05) in total protein loss was observed among various temperatures. The solubility of proteins was maximized and maintained at around 35% regardless of storage time and temperature. It appeared that solubility of proteins was limited to a certain level when the water/meat ratio, washing cycle, and washing time were kept constant.

**Correlation of proteolytic degradation to solubility of proteins**

In surimi processing, washing is utilized to remove water soluble sarcoplasmic proteins and to concentrate myofibrillar proteins. When proteolytic degradation occurs, the compact, large molecular weight myofibrillar proteins are hydrolyzed to small molecular weight proteins or peptides. These degraded proteins might have relatively higher solubility in water than their native myofibrillar proteins, resulting in a loss of
Fig. 5.3. Effects of storage times and temperatures on solubility of proteins during washing
yield. When solubility of total proteins was plotted against the log-percent of MHC and actin degradation, a straight line relationship was observed (Fig. 5.4, 5.5). Both log-percent of MHC and actin degradation had a good correlation ($R^2 = 0.88$ and 0.74) to the solubility of proteins during washing. These results indicated that proteolysis prior to processing could cause lower recovery of myofibrillar proteins. However, since there was a considerable amount of sarcoplasmic proteins present in the water soluble portion (Lin and Park, 1996), this data cannot solely represent solubility of MHC or actin for their degradation.

**MHC and actin content of washed mince**

While the MHC content in washed mince decreased as storage time or temperature increased, actin content was less affected (Fig. 5.6). The relative amount of MHC decreased from 55.3±0.4% to 48.5±2.1% after 14 hr storage at 0°C. Less MHC remained in washed mince as storage time increased. The presence and concentration of intact myosin determines the gel strength of surimi (Niwa, 1992). A good correlation between myosin degradation and decreased gel strength has been demonstrated in whiting surimi (Yongsawatdigul et al., 1995; Morrissey et al., 1993; Chang-Lee et al., 1989) as well as in surimi made from other species (Saeki et al., 1995; Haruhiko et al., 1990). According to Peters et al. (1995), gel strength of whiting surimi decreased rapidly as the post-harvest storage time or temperature increased. The present data agrees with Peters et al. (1995) by showing that MHC content of washed mince decreased with increased storage time or temperature.
Fig. 5.4. Relationship between myosin heavy chain (MHC) degradation and solubility of proteins during washing.

\[ R^2 = 0.88 \]
Fig. 5.5. Relationship between actin degradation and solubility of proteins during washing.
Fig. 5.6. Effects of storage times and temperatures on relative amount of myosin heavy chain (MHC) and actin in washed mince.
The reduced MHC content in washed mince with prolonged storage times or elevated temperatures indicated that degraded components were not completely removed by washing. The presence of degraded MHC components (67-145 kDa) in surimi samples was also reported by An et al. (1994). Increasing the water/meat ratio, washing time, or washing cycles might help to remove more degraded proteins and produce surimi with higher MHC content. However, the yield of surimi will be compromised.

CONCLUSIONS

Degradation of MHC and actin increased rapidly at longer storage times and/or elevated temperatures. Degradation produced a higher loss of total proteins during washing and a lower amount of MHC in the washed mince. Low temperatures reduced, but did not completely inhibit, proteolysis. To minimize proteolysis and maximize processing yield, Pacific whiting should be processed promptly upon landing or kept at 0°C if a holding period is necessary.

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Chapter 6

Solubility of Fish Myosin as Affected by Conformational Changes at Various Ionic Strengths and pH

Tein M. Lin and Jae W. Park

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VI. SOLUBILITY OF FISH MYOSIN AS AFFECTED BY CONFORMATIONAL CHANGES AT VARIOUS IONIC STRENGTHS AND pH

ABSTRACT

The effects of ionic strengths and pH on the solubility of myosin were studied in relation to its surface hydrophobicity, reactive sulfhydryl content, and α-helicity. Adding salt or shifting pH away from the isoelectric point of myosin increased surface hydrophobicity and decreased α-helicity. A slight increase in sulfhydryl content was also observed. These results indicated unfolding of protein structure resulted in an increased solubility. At salt concentrations above >1.0 M, myosin regained its helix structure with a concomitant loss of solubility.
INTRODUCTION

Many functional properties of muscle proteins have been related to the solubilization of myosin. One important example is the formation of surimi gel. Gelation of proteins involves the necessity of solubilization and unfolding prior to the orderly protein-protein interaction (Connell, 1960; Busk, 1984; Gossett, 1984; Ziegler and Acton, 1984; Xiong and Brekke, 1989; Chan and Gill, 1994; Sano et al., 1994). Muscle of salt-water fish contains a variety of salts which are equivalent to a solution with an ionic strength ($\mu$) 0.11-0.15 (Wu et al., 1991; Stefansson and Hultin, 1994; Lin and Park, 1995; ). After extensive washing with water to remove sarcoplasmic proteins, some of the water soluble salts were lost concomitantly (Lin et al., 1995). Therefore, the ionic strength of the concentrated myofibrillar proteins (surimi) was greatly reduced to about 0.01 (Lin and Park, 1995). Without adding salt, surimi does not have gel-forming ability due to the insolubility of myofibrillar proteins at this ionic strength (Wu and Smith, 1987; Stefansson and Hultin, 1994). Studies have shown that 1.7-3.5 % NaCl (≈0.29-0.60$\mu$) is required for surimi to form an adequate gel (Suzuki, 1981; Lee, 1984, 1986; Simizu, 1985; Roussel and Cheftel, 1990). In commercial practice, 1.2-2.5% NaCl (≈0.21-0.43$\mu$) is commonly used to solubilize myofibrillar proteins, especially myosin which is the major component responsible for gelation in surimi (Samejima et al., 1981; Yasui et al., 1982; Wang and Smith, 1995). At this salt concentration range, myosin molecules are released from thick filaments of the myofibrils and disperse in the solution as monomers (Ishioroshi et al., 1983). An organized three dimensional gel network is formed from these solubilized myofibrillar proteins upon heating (Niwa, 1992; Lanier, 1986).
Park and Lanier (1989) showed that addition of salt shifted the denaturation transitions to lower temperatures and decreased enthalpies of heat denaturation. These results suggested that addition of salt might cause a partial unfolding state of proteins that eases the latter heat denaturation. When the salt concentration is too high (>1M), the gel-forming ability of myosin gradually decreases (Suzuki, 1981). This might be due to the decreased solubility of myosin caused by the salting out effect (Regenstein, 1984; Stefansson and Hultin, 1994).

In addition to ionic strengths, pH is another important factor affecting protein solubility (Yang and Froning, 1990; Turgeon et al., 1992; Monahan et al., 1995). As the pH approaches the isoelectric point, the negative and positive charges among the protein molecules are about equal. Therefore, protein molecules are strongly associated with each other through ionic linkages (Kinsella, 1984). Protein is insoluble at this pH because protein-water interaction is replaced by protein-protein interaction. At pH above or below the isoelectric point, protein acquires an increasing amount of net negative or positive charges. These net charges offer more binding sites for water and cause the repulsion among protein molecules to increase their surface for hydration, thus increasing protein solubility (Hamm 1960). Therefore, gelation of myosin could be expected by shifting pH without adding salt.

It has been shown that the gel-forming ability of surimi drastically increases as pH is increased or decreased from the isoelectric point in the absence of salt (Torley and Lanier, 1992; Nishino et al., 1991). Chung et al. (1993) further indicated that at pH above 8 in the absence of salt, surimi gel had higher shear stress values and fairly the
same shear strain values as compared to the gel made with 2.5% NaCl at pH 7. However, when the pH approached the isoelectric point, no measurable gels were formed at any salt level (0-2.5%). Presumably, myosin was not solubilized at this pH regardless of salt concentrations.

Since solubilization of protein is the first necessary step for surimi gelation, further study is needed to illustrate how salt and pH affect the conformation of fish myosin in relation to its solubility. Our objectives were to investigate the effects of salting in, salting out, and pH on the changes of surface hydrophobicity, reactive sulphydryl content, and α-helicity; and to further elucidate the relationship between these conformational changes and the solubility of fish myosin.

**MATERIALS AND METHODS**

**Myosin preparation and solubility determination**

Chinook salmon (*Oncorhynchus tshawytscha*), approximately 1 year old, was obtained from Clatsop Economic Development Council Fisheries Project (Astoria, OR). The live samples were sacrificed just prior to experimentation. Myosin was prepared according to the method of Martone et al. (1986) and subsequently suspended in three volumes of 0.5M KCl (10mM Tris, pH 7.0) solution. The purified myosin was kept at 5° C throughout all experiments unless otherwise specified.

For evaluating ionic strength and pH effects on solubility and conformation changes, 2 ml of myosin was mixed with 18 ml of the following solutions: (1) 0, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 3.0, 3.5, or 3.8 M KCl (10mM Tris, pH 7.0); (2) pH 2, 3, 4, 5, 6,
7, 8, 9, or 10 (30 mM citric acid, 30 mM potassium diphosphate, and 40 mM boric acid). The homogenates were then set in a cold room (5°C) for 6 hr and centrifuged at 17,540 x g for 20 min. Precaution was taken to avoid foam formation throughout all the processes, since air-liquid interphases might cause protein denaturation (Pollard, 1982).

Myosin remaining in the supernatant after centrifugation was defined as soluble myosin. Samples were taken from the homogenate before centrifugation for the measurement of total myosin and from the supernatant after centrifugation for the measurement of soluble myosin. Protein concentration was determined by a Bio-Rad Protein Assay method (Bio-Rad Laboratories, INC., Hercules, CA) at 595 nm using bovine serum albumin as a standard. Myosin solubility was defined as the fraction of total myosin remaining soluble after centrifugation. Soluble myosin was further analyzed for surface hydrophobicity, sulfhydryl content, and α-helicity in relation to its solubility.

To evaluate the combination effect of salt with urea on solubility and surface hydrophobicity, 2 ml of myosin was mixed with 18 ml of 0, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 3.0, 3.5, or 3.8M KCl (10mM Tris, pH 7.0) solution in the presence of 6 M urea. The mixtures were incubated in a water bath (40°C) for 1 hr, set in a cold room (5°C) for 6 hr, and then centrifuged at 17,540 x g for 20 min. The protein concentration and surface hydrophobicity of the supernatant were measured.

**Surface hydrophobicity**

Surface hydrophobicity of soluble myosin was determined using hydrophobic fluorescence probes, 1-anilino-8-naphthalenesulfonate (ANS)(Roura et al., 1992) and cis-parinaric acid (CPA)(Hayakawa and Nakai, 1985). Soluble myosin (supernatant) was
serially diluted with its own buffering solution to a final volume of 2 ml with protein concentration ranging from 0.01-0.1%. After stabilizing at 20°C, 10 μL ANS or CPA was added to sample solutions. The relative fluorescence intensity (RFI) of ANS-protein and CPA-protein conjugates was measured with an Aminco SPF-125 spectrophotofluorometer at wavelength (λ_ex, λ_em) of (374, 485) and (325, 420) for ANS and CPA, respectively. The RFI was standardized by adjusting the reading to 80% full scale for ANS in methanol and 70% full scale for CPA in decane. The net RFI was obtained by subtracting the RFI of each sample measured without a probe from that with a probe. The initial slope (S_0) of the RFI vs protein concentration (%), calculated by linear regression analysis, was used as an index of protein surface hydrophobicity.

**Sulphhydryl content**

The SH group exposed on the surface of the protein molecule was defined as a reactive SH group (R-SH). The R-SH content of soluble myosin was determined by the method of Ellman(1959). An aliquot (15 μL) of Ellman's reagent (10 mM 5,5'-dithiobis(2-nitrobenzoic acid)) was added to 2.25 ml of soluble myosin. The mixture was then set at cold room (5°C) for 1 hr. The amount of R-SH was measured at 420 nm using a molar extinction coefficient of 13,600 M⁻¹cm⁻¹. The total content of SH (T-SH) was determined by the method of Hamada et al. (1994). A sample solution with a final concentration of 6 M urea was made by adding 1.875 ml of 8 M urea to 0.625 ml of soluble myosin. The sample solution was then mixed with 15 μL of Ellman's reagent and subsequently set in a water bath (40°C) for 15 min. The T-SH was determined spectrophotometrically as previously described.
**α-Helicity**

α-Helicity of soluble myosin was determined by circular dichroism (CD) according to the method of Ogawa et al. (1995). The CD measurement was carried out with a Jasco J-720 spectropolarimeter (Jasco Inc., Tokyo, Japan). Ellipticities at 222 nm, [θ]_{222}, were measured in a 0.1 cm path length cell. α-Helicity (%) was estimated by following equation:

\[
\% \text{ } \alpha\text{-Helicity} = 100 \times \frac{[\theta]_{222}}{-40,000}
\]

**RESULTS AND DISCUSSION**

**Solubility**

The solubility of fish myosin increased with increased KCl concentrations up to 0.5 M and remained about the same from 0.5 M to 1.0 M (Fig. 6.1). Further increasing salt concentration caused the salting out effect and myosin gradually lost its solubility. The major increase in solubility, from 3.6% to 60.5%, occurred at KCl concentrations between 0.2 M and 0.5 M. The salting out effect, on the other hand, was a much slower process; the solubility decreased from 59.5% to 34.9% over a wider range of KCl concentrations from 1 M to 3.8 M.

Without adding salt, myosin was insoluble at pH 4-5 and slightly soluble at pH 5-7 (Fig. 6.2). A dramatic increase in solubility occurred at pH above 7 or below 4 reaching a maximum value around 60%. Further increasing pH from 9 to 10 did not enhance the solubility of myosin. These data showed that shifting pH away from the
Fig. 6.1. Solubility of myosin at various KCl concentrations with (+) or without (-) urea.
Fig. 6.2. Solubility of myosin at various pH.
The isoelectric point of myosin, pH 4.8-6.2 (Malamud and Drysdale, 1978; Kinsella, 1981), resulted in an increase in solubility.

**Hydrophobicity**

Results of ANS hydrophobicity (ANS $S_0$) showed that the surface hydrophobicity of myosin increased as salt was added (Fig. 6.3). The continuous increase in salt concentrations caused a concomitantly increased surface hydrophobicity.

About half of the amino acids that constitute myosin are hydrophilic, basic or acidic, amino acids (Tsuchiya and Matsumoto, 1975). At the pH of post-rigor fish flesh or surimi (pH 6-7), the carboxyl groups (COO$^-$) of aspartic acid and glutamic acid are negatively charged, while the amino groups (NH$_3^+$) of lysine, arginine, and histidine are positively charged. Therefore, myosin monomers are aggregated due to the formation of multiple intra- and inter-molecule ionic linkages between positive and negative charged amino acids (Niwa, 1992). The myosin aggregates have strong protein-protein interaction with a small surface area/volume ratio, thus are insoluble. When salt is introduced, the positive (K$^+$) and negative (Cl$^-$) charged ions bind to the oppositely charged amino acids and rupture the ionic linkages. Myosin monomer might be released from the aggregates and partially unfolded. The secondary and tertiary structures of myosin are stabilized by multiple intra molecule bondings, including ionic linkages. Rupture of intra molecule ionic linkages might cause the protein to partially unfold and expose its hydrophobic groups. When too many hydrophobic groups (ANS $S_0 > 800$) are exposed to water, myosin molecules might refold and reaggregate again due to the dominant hydrophobic
Fig. 6.3. The effect of KCl concentrations, with (+) or without (-) urea, on the ANS hydrophobicity of myosin.
interaction among nonpolar amino acid residues. Subsequently, proteins lose their solubility.

Another possible reason for the reduced solubility at high salt concentrations (>1.0M) is the lack of water availability. When most of the water is tied up with ionized K⁺ and Cl⁻, there are not enough water molecules for protein solvation. If this hypothesis is true, then adding urea to these salt solutions should further reduce the solubility of myosin since urea would reduce water availability by binding with water via hydrogen bondings. However, this study showed that when 6 M urea was introduced, the solubility of myosin drastically increased at all salt levels (Fig. 6.1).

It is known that urea acts as a denaturant by breaking hydrogen bonds and reducing the free energy of exposed nonpolar amino acid residues in an aqueous environment (Greighton, 1993). When urea was added to high salt solutions, the protein structure was further disrupted. The hydrophobic interactions among the exposed nonpolar amino acid residues, caused by rupture of ionic linkages and hydrogen bonds, were suppressed by urea (Fig. 6.3, 6.4). Therefore, when urea was added, the myosin showed a high solubility in the salt concentrations where the salting out effect occurred. These results suggested that the reduced solubility of myosin at high salt concentrations was due to the effect of hydrophobic interactions rather than the availability of water.

The CPA hydrophobicity (CPA Sₒ) did not show a notable trend along with the increased KCl concentrations (Fig. 6.4). Similar results were reported by Hayakawa and Nakai (1985) showing that CPA Sₒ did not correlate to the solubility of milk and soy proteins. The surface hydrophobicity of proteins can be classified into aliphatic
Fig. 6.4. The effect of KCl concentrations, with (+) or without (-) urea, on the CPA hydrophobicity of myosin.
hydrophobicity due to the exposure of aliphatic amino acid residues and aromatic hydrophobicity due to the exposure of aromatic amino acids (Hofstee and Otillio, 1978; Hayakawa and Nakai 1985). CPA $S_o$ is useful for determining aliphatic hydrophobicity while ANS $S_o$ is useful for determining aromatic hydrophobicity (Kato and Nakai, 1980; Hayaka and Nakai, 1985). It appeared that the exposed aromatic amino acids were more important in determining the solubility of myosin.

Without adding salt, the surface hydrophobicity of myosin at pH 4-7 can not be measured due to the insolubility of myosin at these conditions. When pH was decreased from 3 to 2 or increased from 8 to 10, an increased ANS $S_o$ occurred (Table 6.1). These data revealed that shifting pH away from isoelectric point caused a partial exposure of protein, thus increasing the solubility (Fig. 6.2). Similar to the salt effect, the CPA hydrophobicity did not show a notable trend in relation to the solubility at various pH levels (Table 6.1, Fig. 6.2).

Myosin molecules had higher surface hydrophobicity under acidic conditions compared to alkaline conditions (Table 6.1). Similar results were also shown in whey proteins (Monahan et al., 1995) and β-lactoglobulin (Das and Kinsella, 1989; Shimizu et al., 1985). These authors suggested that the high surface hydrophobicity at acidic pHs was attributed to noncovalent monomer-dimer transitions rather than to substantial changes in protein secondary structure. Our data supported those previous studies showing that protein molecules had higher α-helix structure under acidic conditions than under alkaline conditions (Table 6.1).
Table 6.1. Effects of pH on the surface hydrophobicity and α-helicity of myosin

<table>
<thead>
<tr>
<th>pH</th>
<th>ANS $S_o$</th>
<th>CPA $S_o$</th>
<th>α-Helicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>514.4 ± 19.9</td>
<td>161.2 ± 9.8</td>
<td>14.9 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>430.0 ± 3.3</td>
<td>134.1 ± 1.1</td>
<td>28.2 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>152.8 ± 16.5</td>
<td>52.5 ± 2.5</td>
<td>19.4 ± 0.3</td>
</tr>
<tr>
<td>9</td>
<td>200.0 ± 11.2</td>
<td>56.7 ± 3.2</td>
<td>11.9 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>208.6 ± 13.6</td>
<td>55.5 ± 6.6</td>
<td>12.0 ± 0.1</td>
</tr>
</tbody>
</table>

ND: not detectable due to the insolubility of myosin at these conditions.
SH Content.

The total SH content of salmon myosin (6.5 mole/10⁵ g protein) (Fig. 6.5) was comparable to that of carp myosin (6.0 mole/10⁵ g protein) but was slightly lower than that of rabbit myosin (8.0 mole/10⁵ g protein) (Tsuchiya and Matsumoto, 1975). While the total SH content was not affected by salt concentration, the reactive SH content was slightly increased along with the increased salt concentrations. Some SH groups in the native myosin are located on the protein surface and some of them are buried within the protein structure. A portion of sulphydryl groups is oxidized and form disulfide bonds to stabilize the protein structure. The constant total SH content along with KCl concentrations suggested that salt, at any level, did not cause disruption or formation of disulfide bonds. However, when the ionic linkages of protein structure were ruptured by salt, the protein was then partially unfolded. Subsequently, those buried SH groups were exposed to the protein surface causing an increased R-SH content. The pH effect on the SH content of myosin could not be evaluated because the reaction of sulphydryl groups with Ellman's reagent was highly pH dependent (Hofmann and Hamm, 1978).

α-Helicity.

The trend of α-helicity (Fig. 6.6, Table 6.1) appeared to be reversely correlated to the solubility of myosin along with salt concentrations and pH (Fig. 6.1, 6.2). The α-helicity decreased as the solubility of protein increased at salt concentrations of 0.3 M - 0.5 M. When salt concentrations were higher than 1.0 M, the myosin regained its helix structure with a concomitantly decreased solubility. The regained helix structure might be due to the refolding of proteins caused by hydrophobic interaction (Fig. 6.3). These
Fig. 6.5. The effect of KCl concentrations on the total and reactive sulfhydryl contents of myosin
6.6. The effect of KCl concentrations on the helical structure of myosin
data also suggested that the unfolding of the helical tail portion of myosin molecules may play an important role in myosin solubility.

**CONCLUSIONS**

The maximum solubility of myosin occurred at 0.5 - 1.0 M KCl and at pH 2, 9, and 10. The increased solubility of myosin was correlated to the increased surface hydrophobicity and the decreased α-helicity. The salting out effect (>1.0M) was probably due to the dominant hydrophobicity interaction among nonpolar amino acid residues.

**REFERENCES**


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VII. SUMMARY

Surimi waste streams contain a considerable amount of myofibrillar proteins. The particulate protein lost in the final dewatering process (screw press) was successfully recovered and concentrated using a rotary screen and microfiltration (MF). The proteins recovered by microfiltration showed highly functional properties and composition which were comparable to proteins in regular surimi. Surimi with 10% replacement of MF recovered protein had the same gel quality as regular surimi with respect to gel hardness, cohesiveness, water retention, and color. The gel made with MF recovered protein had better color quality than regular surimi gels. The surimi production rate could be increased by 1.7% by adding the MF recovered protein back to the production line without diminishing surimi functionality. The total aerobic plate count, COD, turbidity, and protease levels of waste waters were substantially reduced by ultrafiltration (UF). Use of UF equipment provides the possibility of recycling processing water in the leaching system. However, proteins concentrated by ultrafiltration had considerably dark colors and strong odors.

To minimize the loss of valuable myofibrillar proteins, the effects of various processing parameters, such as ionic strengths, washing cycles, washing time, water/meat ratio, and proteolysis, on the solubility and physical properties of myofibrillar proteins were investigated. Results showed that severe loss of myofibrillar proteins occurred when washing conditions were not properly controlled. During washing, sarcoplasmic proteins were readily soluble in water (0% NaCl) and removed in the initial washing steps. Myofibrillar proteins became relatively soluble in the subsequent washings. During
extensive washings, parts of water-soluble salt were released from the meat portion into
the water portion. This caused the myofibrillar proteins to have more available sites for
water to hydrate, thus increasing their solubility. When a small amount of salt (0.25%,
0.5%, and 1.0% NaCl) was introduced, less MHC and actin were solubilized. However,
these solutions were not very effective in removing sarcoplasmic proteins. The proteins
from these washings also had lower moisture content and lower water retention ability.
High salt (2.0% NaCl) washing, on the other hand, resulted in low removal of
sarcoplasmic proteins and severe loss of myosin, actin, α-tropomyosin, β-tropomyosin,
and troponin-T. Two washing cycles were suggested for surimi processing. A water/meat
ratio at 5:1 and 6.7:1 and washing time at 5 and 6.7 min for each step were sufficient to
remove sarcoplasmic proteins without notable loss of myosin or actin.

The use of water for surimi processing can be minimized by increasing washing
time and the number of washing cycles with a low water/meat ratio for each washing
step. More sarcoplasmic proteins were extracted when a longer washing time was
allowed until equilibrium was reached. Additional washing cycles continuously removed
residual sarcoplasmic proteins from the mince. The usage of leaching water could be
reduced from 12:1 (overall W/M ratio) to 8:1 by using a lower W/M ratio (2:1) with
increased washing cycles (4) and washing time (10 min). This washing was sufficient to
remove sarcoplasmic proteins but resulted in a higher moisture content. When the total
water used was further reduced to 4:1, MHC content, water retention ability, and color
of the washed mince were compromised. No significant loss of myosin or actin occurred
at the low W/M ratio (2:1 or 1:1) regardless of washing time or washing cycles.
Myosin heavy chain (MHC) degraded rapidly during post-harvest storage even at low temperatures (0-5°C). The trend of actin degradation was similar to that of MHC but to a lesser degree. Degradation of MHC and actin increased rapidly at longer storage times and/or elevated temperatures. Proteolysis of myofibrillar proteins resulted in a higher loss of proteins during washing. Both log-percent MHC and actin degradation showed a good correlation ($R^2 = 0.88$ and 0.74) to protein solubility. However, degraded proteins were not completely removed by washing, resulting in a lower MHC content in washed mince. Low temperatures reduced, but did not completely inhibit, proteolysis. To minimize proteolysis and maximize processing yield, Pacific whiting should be processed promptly upon landing or kept at 0°C if a holding period is necessary.

Myosin was used as a model system to investigate the effects of ionic strengths and pH on the solubility and structure of myofibrillar proteins. The solubility of myosin increased as salt was added. When salt concentrations were > 1.0 M, myosin gradually lost its solubility. Without adding salt, myosin was insoluble at pH 4-7. A dramatic increase in solubility occurred at pH above 7 or below 2.

The increased solubility was due to the unfolding of protein as indicated by the increased surface hydrophobicity and the decreased helix structure. When too much salt was introduced, myosin regained its helix structure and lost its solubility. This might be due to the dominant hydrophobic interaction among nonpolar amino acids.
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