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Recovery of Functional Proteins from Herring (*Clupea harengus*) Light Muscle by an Acid or Alkaline Solubilization Process

INGRID UNDELAND,*,† STEPHEN D. KELLEHER,‡,§ AND HERBERT O. HULTIN‡

University of Massachusetts Marine Station, P.O. Box 7128, Gloucester Massachusetts 01930; Department of Food Science, Chalmers University of Technology, and SIK—The Swedish Institute for Food and Biotechnology, P.O. Box 5401, S-402 29 Göteborg, Sweden

Proteins from herring (*Clupea harengus*) light muscle were extracted using acidic or alkaline solubilization; 92 and 89% of the initial muscle proteins were solubilized at pH 2.7 and 10.8, respectively, of which 96 and 94% were recovered during precipitation at pH 5.5. Consistency of the pH-adjusted muscle homogenates increased with increased raw material age and homogenization intensity; it declined following holding on ice. Some hydrolytic myofibrillar protein degradation occurred during cold storage of the acidified (pH 2.7) homogenates. With alkalized homogenates, hydrolysis was negligible. The total lipid content changed from 0.13 g/g of protein in the muscle to 0.04 g/g of protein in both the acid- and alkali-produced protein isolates. Corresponding values for the phospholipid content were from 0.037 to 0.02 g/g of proteins. Acid- and alkali-produced proteins made gels with equal strain and color. Stress values were equal or lower in acid- versus alkali-produced protein gels. When ice-stored raw material was used, strain and stress values of gels were reduced.

KEYWORDS: Herring; *Clupea harengus*; muscle; fish; protein; extraction; acid; alkali; solubility; consistency; pH

INTRODUCTION

Along with a growing interest for muscle proteins to be used as food ingredients, the large stocks of herring (*Clupea harengus*) and other pelagic fish species have received increased attention as potential protein resources. Today, ∼90% of the estimated 2.5 million metric tons of herring that are captured (*1*) are directed toward nonhuman consumption as fishmeal and fertilizers. Isolation of pelagic fish proteins for food production would be a more responsible way of using a nutritious and abundant raw material. The seasonality, small size, and unfavorable composition of herring have made it difficult to extract stable and functional proteins for use in human food products (*2*, *3*).

Processing techniques that have been examined to potentially overcome these problems include mincing in combination with (i) acidification, heating, and solvent extraction (*4*); (ii) washing with water or a slightly alkaline solution as part of a surimi preparation (*5*, *6*); and (iii) washing with a sodium bicarbonate solution and water followed by acidification and spray drying (*7*). Most techniques reduced the amount of pro-oxidants, pigments, and total lipids, the latter primarily by removing neutral lipid deposits. The contribution of neutral lipids to rancidity development in meat (*8*) and fish muscle (*9*) has been questioned, and it has been suggested that the membrane phospholipids are the main substrates in muscle tissue lipid oxidation. Thus, removing/reducing the phospholipids in addition to the neutral lipids is considered an additional step forward in the production of stable pelagic fish protein isolates.

Recently, procedures have been developed for the purpose of recovering stable, functional proteins from low-value muscle sources such as herring. The muscle proteins are first solubilized; this may be done either at low pH (pH ≤3) or at high pH (pH ≥10.5) (*10*−*16*). At this point, insoluble materials may be separated from the soluble myofibrillar, cytoskeletal, and sarcoplasmic proteins by a technique such as centrifugation. This can separate fats/oils by flotation. Materials more dense than the solution such as skin, bones, and, under favorable circumstances, even cellular membranes, are removed in the sediment. The soluble proteins are then precipitated by adjusting the pH and/or ionic strength and recovered by centrifugation. The recovered proteins retain their functionality, including their ability to form a gel. To obtain high yields of good-quality protein, it is necessary to solubilize a large portion of the proteins. A low consistency aids in the removal of cellular membranes by centrifugation. The objective of this study was to determine the yield and quality of the isolated proteins from herring light muscle with emphasis on their gel-making properties.

* Author to whom correspondence should be addressed (telephone +46-31-335 56 00; fax +46-31-83 37 82; e-mail ingrid.undeland@fsc.chalmers.se).
† Chalmers University of Technology and SIK.
‡ University of Massachusetts Marine Station.
§ Current address: Proteus Industries, Inc., 2 North Main Street, Ipswich, MA 01938.

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Table 1. Protein Mass Balance through Selected Steps in the Acid/Alkali-Aided Protein Isolation Technique

	muscle[a]	first sediments[b]	supernatant 1[b]	→	sediment 2[a,b]	supernatant 2[b]
acid process (g/100 g of muscle)[c]	19.7 ± 1.7^1	4.5 ± 1.3^1	15.2 ± 1^1	→	14.5 ± 0.9^1	0.7 ± 0.1^1
	(100 ± 0^1)	(22.7 ± 4.9^1)	(77.4 ± 5.0^1)		(74 ± 4.8^1)	(3.4 ± 0.5^1)
alkaline process (g/100 g of muscle)[c]	19.6 ± 1.7^1	5.5 ± 1.2^1	14.1 ± 1.3^1	→	13.3 ± 1.2^2	0.8 ± 0.1^2
	(100 ± 0^1)	(27.9 ± 4.7^1)	(72.1 ± 4.7^1)		(68 ± 4.4^2)	(4.3 ± 0.5^2)

[a] The ratio between lipids and proteins changed during processing from 0.13 ± 0.02 g/g ($n = 5$) in the initial muscle to 0.044 ± 0.007 and 0.042 ± 0.005 g/g ($n = 5$) in the second acid- and alkali-derived sediments, respectively. [b] First sediments = the nonliquid fractions from the first centrifugation (18000*g*, 20 min). These fractions were a gelatinous soft sediment ("gel-sed"), a harder "bottom sed", and a floating "emulsion layer". Supernatant 1 = the supernatant from the first centrifugation. Sediment 2 = the sediment from the second centrifugation (10000*g*, 20 min). Supernatant 2 = the supernatant from the second centrifugation. [c] Protein data are expressed as mean values ± standard deviation (SD) ($n = 7$). Numbers within parentheses illustrate relative values (mean ± SD) under the assumption that the proteins analyzed in the muscle homogenate are 100%. Comparable values within the same column bearing a different superscript number are significantly different ($p \leq 0.05$) ($n = 7$).

MATERIALS AND METHODS

Materials. Fresh whole herring was obtained from D&B Bait, Gloucester, MA, and transported on ice to the University of Massachusetts Marine Station (~15 min). The post-mortem age upon arrival in the laboratory generally ranged between 6 and 36 h. The fish was used either at once, after storage on ice in a refrigerator (4 °C), or after storage at −20 °C. At the time of use, white muscle was manually excised and ground through a 5 mm plate using a kitchen grinder (KitchenAid Inc., St. Joseph, MI). The ground herring light muscle contained 80 ± 0.9% moisture (mean ± SD, $n = 5$), 19.7 ± 1.7% proteins ($n = 5$), 2.4 ± 0.2% total lipids ($n = 5$), and 0.67 ± 0.03% phospholipids ($n = 2$).

Protein Isolation via the Acid and Alkaline Solubilization Processes. Ground muscle (usually 120−300 g) was homogenized for 1 min (speed 50) with 9 volumes of ice-cold distilled water using a Kinematica GmbH Polytron (Westbury, NY) connected to a variable autotransformer (Staco Energy Products Co., Dayton, OH). The proteins in the homogenate were solubilized by dropwise addition of 2 N HCl or 2 N NaOH until a pH of 2.7 or 10.8 was reached. The protein suspension was centrifuged within 15 min at 18000*g* (20 min), giving rise to four phases: a floating "emulsion layer", a clear supernatant, a soft gellike sediment ("gel-sed"), and a slightly harder bottom sediment ("bottom-sed"). The supernatant was separated from the emulsion layer by filtering these two phases through double cheesecloth. The soluble proteins were precipitated by adjusting the pH to values between pH 4.8 and pH 7 using 2 N NaOH or 2 N HCl. A pH of 5.5 was used in most cases. Precipitated proteins were collected via a second centrifugation at 100000*g* (20 min).

To calculate the protein recovery (percent) in the acid and alkaline processes (**Table 1**), the following formula was used:

[(total muscle proteins − proteins of nonliquid fractions from the first centrifugation − proteins of supernatant from the second centrifugation)/total muscle proteins] × 100

Total muscle proteins refer to the proteins analyzed in the initial 1:9 muscle homogenate. When the term *theoretical* protein recovery (percent) is used, it is assumed that all proteins solubilized by acidification or alkalization could be recovered from the nonliquid fractions, that is, that there was no protein loss into the emulsion layer, gel-sed, and bottom-sed. This gave rise to the following formula:

[(total muscle proteins − proteins insoluble at pH 2.7 or 10.8 − proteins of supernatant from the second centrifugation)/total muscle proteins] × 100

To calculate the relative distribution (percent) of proteins between the different supernatants, sediments, and the floating layer obtained in the processes (**Table 1**), the proteins measured in each of these fractions were divided by the total muscle proteins.

Manufacture of Surimi. Excess water in the acid- and alkali-produced protein precipitates was squeezed out manually or removed via centrifugation (20 min, 18000*g*). This significantly ($p \leq 0.05$) lowered the moisture contents of the acid- and alkaline-produced precipitates from 90 ± 1 to 75 ± 3% ($n = 7$) and from 88 ± 1 to 72 ± 3% ($n = 7$), respectively. Both precipitates were then adjusted to 80% moisture with distilled water and blended with the cryoprotectant mixture (4% sucrose, 4% sorbitol, and 0.3% sodium tripolyphosphate). The final moisture was 73.2 ± 0.5% ($n = 7$). The surimi was frozen in plastic bags at −80 °C.

Manufacture of Surimi Gels. Gels were prepared as described by Kelleher and Hultin (*14*) with the exception that the pH of the surimi was adjusted to 7.1−7.2 using 10% NaOH or 10% HCl *after* the prefreezing, thawing, and mixing with 2% NaCl. Surimi was packed either in cellulose casings (The Sausage Maker Inc., Buffalo, NY) or in metal tubes (diameter = 19 mm), depending on the type of gel measurements to be carried out.

Quality of Gels. Strain and stress (at structural failure) were analyzed using the torsion technique of Wu et al. (*17*) or with a Rheo Tex model AP-83 gelometer (Sun Sciences Co., Seattle, WA). For the latter, gels were cut into 2.5 cm sections, and the deformation (millimeters) and peak force required to penetrate the surface of the gels were measured. Gels were also subjected to the folding test described by Kudo et al. (*18*) by folding a 3 mm slice of the gel once or twice. The Hunter color values, *L*, *a*, and *b*, were also measured on gels.

Proteins and Protein Solubility. Total proteins were measured according to the method of Lowry et al. (*19*) as modified by Markwell et al. (*20*). Protein solubility (percent) was expressed according to the following formula:

(protein concentration in supernatant after centrifugation/protein concentration in homogenate before centrifugation) × 100

Electrophoresis. Polypeptides in the homogenate, first supernatant, second supernatant, and final precipitate were separated according to the electrophoresis procedure described by Laemelli (*21*) using precast mini linear gels 4−20% (OWL Systems, Woburn, MA) on a vertical PAGE Mini device (Daiichi Scientific, Tokyo, Japan) with a constant current of 30 mA per gel. Proteins were solubilized in a sample buffer containing 4% SDS, 40 mM EDTA, 160 mM DTT, 40 mM Tris, 0.06% bromophenol blue, and 40% glycerol. Sample and buffer (1:4) were mixed and heated to 100 °C for 2 min following the procedure of Wang (*22*). Protein bands were fixed using a 1 h incubation in 12% trichloroacetic acid, followed by overnight staining using Pro-Blue (Owl Separation Systems, Portsmouth, NH). Scanning of the stained gels was accomplished using a Hoefer model GS 300 scanning densitometer (Hoefer Sci., San Fransisco, CA) in the transmittance mode with model 365W densitometer analysis software for protein quantification. A standard curve was constructed using high (29−205 kDa) and wide (6.5−205 kDa) molecular weight SDS-PAGE standards (Sigma, St. Louis, MO) on a linear gradient as described by Hames (*23*).

Consistency. Consistency was followed during solubilization and precipitation of proteins. The homogenates were placed into a 600 mL Pyrex beaker on ice. Consistency was measured at 4−6 °C using a "HAT" Brookfield Syncro-lectric viscometer (Stoughton, MA) equipped with a no. 2 spindle at 60 rpm. A manufacture's chart allowed for the readings to be converted to mPa·s.

Analyses of Total Lipids and Phospholipids. Total lipids in the muscle, nonliquid fractions from the first centrifugation and protein isolates were analyzed according to the method of Lee et al. (*24*) using

Table 2. Relative Distribution[a] of the Lipids in the Initial Muscle between the Four Nonliquid Fractions Obtained in the First and Second Centrifugations of the Acid and Alkaline Solubilization Processes

fraction	acid process	alkaline process
muscle	100	100
gel sed	14	18
bottom sed	8	4
emulsion layer	45	37
second sediment	32	40

[a] The amount of lipids in each of the nonliquid fractions of the acid and alkaline processes is given as a percentage of the total lipids in the initial muscle (2.4 ± 0.06 g/100 g, $n = 2$). The data are based on one processing experiment.

chloroform and methanol (1:1) as the extraction solvent. Phospholipids were determined according to the procedure of Kovacs (*25*). To calculate the relative distribution (percent) of lipids between the different nonliquid fractions obtained in the processes (**Table 2**), the lipids measured in each of these fractions were divided by the total muscle lipids.

Analyses of Thiobarbituric Acid Reactive Substances (TBARS). TBARS were analyzed after the method of Lemon (*26*). A modification included using 1 g of sample and extracting with 6 mL of trichloroacetic acid (TCA) by homogenization with a tissue Tearor at high speed (model 986-370, Biospec Products, Racine, WI).

Moisture Content, Ionic Strength, and pH. The moisture content of the sediments, protein isolates, surimi, and surimi gels was measured using a Cenco infrared moisture balance (CSC Scientific Co., Inc.) or by heating the samples at 105 °C overnight. The ionic strength of the homogenate was measured using a conductivity meter (YSI, Inc., Yellow Springs, OH). The value was expressed in equivalent sodium chloride concentration by comparing the conductivity reading to a standard curve prepared with sodium chloride. The pH was recorded with an Orion combination epoxy Ross Sure-Flow electrode (Orion Research Inc., Beverly, MA) in conjunction with a pH-meter (Orion Research Inc., Boston, MA). Solid muscle and protein isolate samples were first stirred with 9 volumes of distilled water.

Statistics. Standard deviations (SD) and Pearson's correlation coefficients were calculated using Excel 2000 (Microsoft Corp., Seattle, WA). Significant means, separated by "paired *t* test", were performed with Sigmaplot (1997). Values are reported as significantly different when $p \leq 0.05$.

RESULTS

Acidification and Alkalization of the Homogenate. To adjust the herring light muscle homogenate from an initial pH of 7 to pH 2.7 or 10.8, 220 ± 40 μL of 2 N HCl or 98 ± 12 μL of 2 N NaOH ($n = 9$, mean ± SD) was needed per gram of muscle. The concentration of HCl and NaOH in the 1:9 muscle homogenates thereby became 43 or 19.4 mM, respectively. This increased the ionic strength from the initial ~0.017 at pH 7 to 0.061 ± 0.008 at pH 2.7 and 0.037± 0.002 ($n = 9$) at 10.8, the latter being significantly lower ($p \leq 0.05$).

During acidification or alkalization, the initial homogenate consistency (44 ± 10 mPa·s) increased dramatically around pH 3.5 and 10, respectively, followed by rapid decreases as the pH was adjusted to more extreme values. At pH 2.7 and 10.8, average consistencies for fresh herring homogenates were 45 ± 11 and 31 ± 5.5 mPa·s ($n = 7$), respectively. When muscle from herring that had been prestored on ice was used, the pH-adjusted homogenates had higher consistency than when muscle from fresh herring was used, particularly at pH 2.7. As an example, muscle from herring stored for 3 days on ice gave consistencies of ~110 and ~65 mPa·s in the homogenates adjusted to pH 2.7 and 10.8, respectively. On the basis of all available data from November 1999−April 2000, the correlation between the time during which the whole herring had been stored on ice and the consistency of the muscle homogenates after they had been adjusted to pH 2.7 and 10.8 was $r = 0.57$ and 0.72, respectively, using Pearson's correlation coefficient.

Holding of the muscle homogenates on ice *after* they had been adjusted to pH 2.7 and 10.8 reduced the consistency, relatively more so when the homogenates were made from aged herring raw material. Extended holding of homogenates on ice therefore minimized the initial consistency differences that were seen as a result of variations in the herring post-mortem age. **Figure 1** shows consistency changes observed during holding of a muscle homogenate on ice at pH 2.7 at 30 min intervals from 5 min up to 125 min. The largest consistency reduction occurred within the first 30 min (~50%); after 125 min, the total consistency drop was 64%. When holding periods of <30 min were considered, it became evident that consistency reductions started appearing after only 5−10 min, usually ~15%.

Soluble Protein Separation. Acidification solubilized slightly higher percentages of the total herring light muscle proteins than did alkalization as determined by high-speed centrifugation, 92.1 ± 3.4% compared to 88.6 ± 3.8% ($n = 5$). Prestorage of the herring raw material on ice increased the solubilities of the muscle proteins somewhat, for example, in one experiment from 87 and 83% at pH 2.7 and 10.8, respectively, to 93 and 88%, respectively, after 5 days. When fresh herring raw material was used, the amount of proteins solubilized at pH 10.8 also increased by extending the holding time between alkalization of the homogenate and centrifugation, for example, from 76 to 98% over a period of 150 min. Using herring that had been stored prior to application of the protein isolation processes, such solubility improvement occurred at both pH 2.7 and 10.8. The changes in protein solubility occurring during holding of the muscle homogenates on ice could be due to a time-dependent diffusion of added acid/base into the protein aggregates of the homogenates or the result of hydrolytic degradation at pH 2.7 and 10.8 (**Figure 2**, lanes 3−5 and 8−10).

Of the proteins that became solubilized at pH 2.7 and 10.8, 84 and 81%, respectively, could be recovered when the supernatant was collected from the first centrifugation. As shown in **Table 1**, this was equal to 77 and 72% of the total initial proteins in the muscle. It is further shown in this table how the total protein recovery became significantly higher ($p \leq 0.05$) with the acid than the alkaline process, 74 ± 4.8 versus 68 ± 4.4%. If all of the proteins that were initially solubilized at pH 2.7 and 10.8 could have been recovered after the high-speed centrifugation, the theoretical maximal yields would have been 88 ± 3.8 and 83.5 ± 3.5%, respectively.

However, after the first centrifugation, the weight of the supernatant contributed only 85 ± 3.1 and 82 ± 2.8% ($n = 7$) of the weight of the initial acid and alkaline homogenates, respectively. The relative weight of the supernatant increased when the holding time was extended between pH adjustment and centrifugation. **Figure 1** illustrates how the weights of the nonliquid fractions were reduced from 17 to 8% (w/w) after 2 h at pH 2.7.

Among the nonliquid fractions from the first centrifugation (the emulsion layer, gel-sed, and bottom-sed), the gel-sed was the largest (at both pH values, 57% of the total weight of the nonliquid fractions). On the acid side, the bottom-sed ranked second (26% w/w) followed by the emulsion layer (18% w/w). This order was reversed on the alkaline side. The moisture content of the gel-sed, bottom-sed, and emulsion layer were

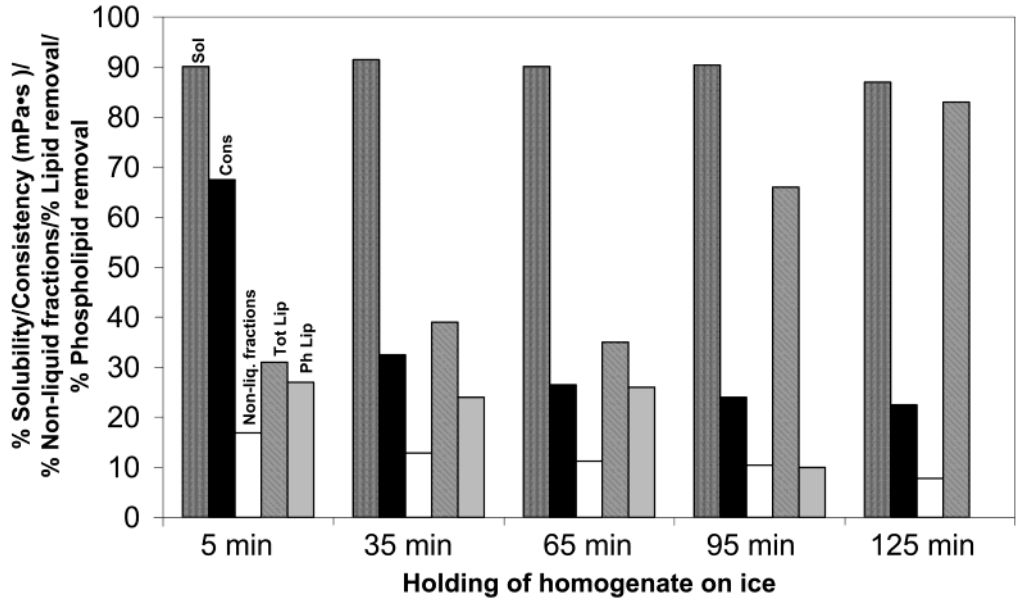

Figure 1. Effect of holding an acidified (pH 2.7) herring light muscle homogenate on ice for various periods of time (5–125 min) prior to high-speed centrifugation (18000*g*, 20 min): effect on protein solubility (percent of the total proteins in the homogenate before centrifugation that were found in the supernatant after centrifugation) (medium gray bar); consistency (mPa•s) (white bar); size of nonliquid fractions (percent of total homogenate, w/w) (dark gray bar); total lipid removal (percent of initial total lipids in the homogenate; 2.4 mg/g homogenate (ww) (light gray bar); and phospholipid removal (percent of initial phospholipids in the homogenate; 0.67 mg/g homogenate) (dotted bar).

97, 95, and 91% at pH 2.7 and, at pH 10.8, 97, 96, and 96% (w/w).

Table 2 shows the percentage of total lipids in the gel-sed, bottom-sed, and emulsion layer based on the contents of lipids in the initial muscle. For both the acid and alkaline processes, the emulsion layer contained the largest amounts of lipids (37 and 45%), followed by the gel-sed (14 and 18%) and then the bottom-sed (8 and 4%). After a homogenate had been held for 2 h at pH 2.7 (**Figure 1**), the percentage of total lipids and phospholipids in the initial homogenate that remained in the supernatant after the first centrifugation changed from 70 to 17% and from 70 to 100%, respectively.

Precipitation of Proteins in the Supernatant. When the pH of the supernatant was adjusted from pH 2.7 and 10.8 to pH 5.5, 17 ± 2 μL of 2 N NaOH and 15 ± 2 μL of 2 N HCl were needed per milliliter of supernatant. Following centrifugation, similar amounts, 95.6 ± 0.8 and 94.3 ± 1.1% (n = 7), respectively, of the proteins in the first supernatant could be recovered in the sediments (**Table 1**). To investigate the flexibility of the precipitation step and to determine whether protein recoveries could be improved by adjusting the precipitation pH away from 5.5, acid and alkaline supernatants were precipitated at different pH values in the ranges of pH 4.8−5.9 and 7−5.1 (**Table 3**). Changes in solubility, recovery, and moisture content were minor at pH >4.8 and pH <7, and because the optimal pH (shown in bold) differed for the different characteristics, pH 5.5 was used in all further experiments in the protein precipitation step.

There were qualitative differences between the polypeptides remaining soluble at pH 5.5 depending on whether the acid or alkaline process had been used (**Figure 3**, lanes 5 and 10). Following acidification, weak bands could be seen up to ~75 kDa, although 8 and 23 kDa polypeptides dominated (black dots in **Figure 3**, lane 5). After alkalization, bands were seen up to ~75 kDa, but in addition to the 8 and 23 kDa polypeptides, the supernatant also contained polypeptides with molecular weights of 29, 38, 41, 43, 54, and 60 kDa (black dots in **Figure 3**, lane 10). On the basis of repeated electrophoretic analyses, four of them were tentatively identified as tropomyosin β (38 kDa), troponin T (41 kDa), actin (43 kDa), and desmin (54 kDa). The 29 and 60 kDa polypeptides remained unidentified. Although 15 μg of proteins was added to both lanes 5 and 10, the total peak area obtained after densitometer scanning of lane 5 (**Figure 3**) accounted for only ~7.5 μg of proteins. It is possible that the acid-derived precipitation supernatant contained low molecular weight hydrolytic fragments that were partly lost in the electrophoresis run or staining procedure. This hypothesis is supported by the presence of <6 kDa polypeptides in this sample. Such polypeptides were also seen in an extensively hydrolyzed homogenate held for 20 h at pH 2.7 (**Figure 2**, lane 5). The hydrolytic fragments could have been formed over the time period between the acidification of the homogenate and collection of the precipitate at pH 5.5.

Characteristics of Protein Precipitates. The moisture content in the acid-derived precipitate, 90 ± 1.3% (n = 7), was significantly higher ($p \leq 0.05$) than in the alkali-derived precipitate, 87.8 ± 1.2% (n = 7). The contents of total lipids were similar in the acid- and alkali-derived precipitates, 0.044 ± 0.007 and 0.042 ± 0.005 g/g of proteins (n = 5), respectively. The same was true for the phospholipids: 0.02 ± 0.0015 g/g (n = 2) for both precipitates.

Hydrolysis during Acid and Alkaline Processing. SDS-PAGE analysis indicated that little apparent hydrolytic degradation took place between the start of the acidification/alkalization (**Figure 3**, lanes 2 and 7) and the first centrifugation (~15 min) (**Figure 3**, lanes 3 and 8). In the pH 2.7 homogenate, some changes became obvious after the first centrifugation, that is, ~30 min later (**Figure 3**, lane 4). These included decreases in the amounts of polypeptides tentatively identified as tropomyosin β (~38 kDa), nebulin (600−800 kDa), and titin (>2000 kDa). Tropomyosin β was identified on the basis of its quantity and molecular weight, whereas nebulin and titin were identified on the basis of their quantity and their location in the electrogram (above the myosin heavy chain). Concurrent with the above-mentioned losses, two polypeptides of ~37 kDa, one polypeptide of ~75 kDa, and a polypeptide triplet from ~150 to 175

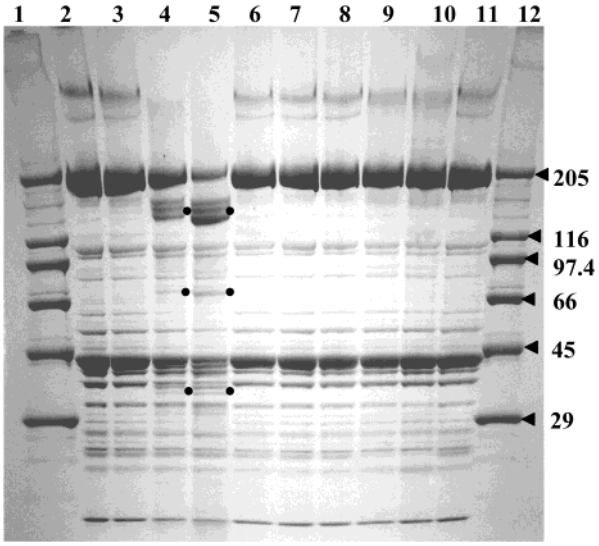

Figure 2. SDS-PAGE (4–20%, linear gradient) of unadjusted and pH-adjusted (pH 2.7 and 10.8) herring light muscle homogenates (1:9 in ice-cold distilled deionized water) after different holding periods on ice: (lane 1) molecular weight standards (29, 45, 66, 97.4, 116, and 205 kDa); (lane 2) proteins from the unadjusted (pH 7) herring light muscle homogenate (1:9 in ice-cold distilled deionized water); (lane 3) proteins from the pH 2.7-adjusted homogenate after 15 min on ice; (lane 4) proteins from the pH 2.7-adjusted homogenate after 4 h on ice (peptides with molecular weights of 37, 75, and 150–175 kDa are marked with dots); (lane 5) proteins from the pH 2.7-adjusted homogenate after 20 h on ice (peptides with molecular weights of 37, 75, and 150–175 kDa are marked with dots); (lane 6) proteins from the unadjusted (pH 7) homogenate after 20 h on ice; (lane 7) proteins from the unadjusted (pH 7) herring light muscle homogenate; (lane 8) proteins from the pH 10.8-adjusted homogenate after 15 min on ice; (lane 9) proteins from the pH 10.8-adjusted homogenate after 4 h on ice; (lane 10) proteins from the pH 10.8-adjusted homogenate after 20 h on ice; (lane 11) proteins from the unadjusted (pH 7) homogenate after 20 h on ice; (lane 12) molecular weight standards (29, 45, 66, 97.4, 116, and 205 kDa). Protein was applied to all lanes at 15 μg/lane.

kDa developed. The newly formed polypeptides are indicated with black dots in lane 4 and 6 of **Figure 3**. In the pH 10.8 homogenate, there was some breakdown of titin into its α and β forms and loss of nebulin during the time needed for centrifugation (**Figure 3**, lane 9). Prolonged holding of the pH 2.7 homogenate on ice for 4 and 20 h intensified the changes above (black dots in **Figure 2**, lanes 4 and 5) but also induced degradation of actin (~43 kDa), desmin (~54 kDa), a 60 kDa polypeptide, and the myosin heavy chain (~200 kDa). Extended holding on ice at pH 10.8 did not cause any additional changes (**Figure 2**, lanes 9 and 10). These findings applied to homogenates from fresh muscle as well as muscle from herring stored on ice for 6 days (data not shown). The non-pH-adjusted homogenate did not undergo any changes within 20 h on ice (**Figure 2**, lanes 7 and 11).

Gelation of Precipitates. Table 4 summarizes data on moisture, lipid content, pH, and gels trength from the acid and alkaline preparations of surimi and surimi gels using fresh muscle and muscle from herring stored for 6 days on ice. With fresh muscle, the elasticities of gels from acid- and alkali-isolated proteins were comparable; both passed the double-folding test (i.e., score 5) (*18*) and gave average deformation values of 9.2 $\pm$ 0.8 mm ($n = 5$). The alkali-produced protein isolate gave significantly ($p \leq 0.05$) harder gels and required a break force of 871 $\pm$ 62 g compared to 566 $\pm$ 36 g ($n = 5$) for the acid-produced protein isolate. In the acid-produced gels, the use of aged fish caused lowering of folding scores (from 5 to 3+), strain values (by 20%), and stress values (by 12%) (**Table 4**). Strain changes were significant ($p \leq 0.05$), but stress changes were not. In the alkali-produced gels, the use of aged fish caused significant ($p \leq 0.05$) lowering of folding scores (from 5 to 3), strain values (by 33%), and stress values (by 47%). With the aged fish, acid-produced gels were significantly more elastic ($p \leq 0.05$) than alkali-produced gels. Stress values were similar.

In another experiment, using the torsion technique for evaluating gel quality (**Table 5**), true strain values (1.8 $\pm$ 0.2 and 1.6 $\pm$ 0.1, respectively) and stress values (58.2 $\pm$ 7.3 and 56.1 $\pm$ 2.4 kPa, respectively) were similar for both acid- and alkali-produced gels.

The Hunter color values were determined for alkali- and acid-produced gels (**Table 5**). *L* values (lightness) were 64 and 65, *a* values (redness) 8 and 8.1, and *b* values (yellowness) −2.4 for both gels, respectively. Whiteness values were 63 and 65.5 for acid- and alkali-produced gels, respectively (*27*). For regular surimi gels produced from water-washed herring light muscle, the following Hunter color values were reported (*5*); $L = 71.8$, $b = 10.4$, and $a = -1.9$. Lower *L* values, 50, were reported for gels made from acid-produced brisling sardine (*Opisthonema liberate)* protein isolates than for gels made from regular brisling sardine surimi, 62 (*28*). This was ascribed to the formation of protein−protein aggregates in the acid process, which adversely affected light reflection (*28*).

DISCUSSION

Solubility and Recovery of Proteins in Acid/Alkaline Processing. Fifty-five to 70% of the initial muscle proteins are generally found in regular fish muscle surimi (*11*). With herring light muscle, 65% (*5*) and 47% (*7*) were reported when three (1:3) washes with water or with water and sodium bicarbonate were used to produce surimi. When acid or alkaline solubilization of fish muscle proteins is combined with high-speed centrifugation and isoelectric precipitation, most sarcoplasmic proteins can be recovered in addition to the myofibrillar proteins. This allows for higher protein recoveries than with regular surimi procedures. The theory behind acid and alkaline protein solubilization is the formation of net positive and net negative charges, respectively, on the proteins. This creates electrostatic repulsive forces, which drive the molecules of the protein aggregates apart. In herring light muscle, protonations of glutamic and aspartic acid side chains (pK_a values at 4.2 and 3.8, respectively) are thought to be largely involved in acid solubilization, whereas deprotonations of lysine, tyrosine, and cysteine side chains (pK_a values at 9.5−10.5, 9.1−10.8, and 9.1−10.8) are thought to play an important role in alkaline solubilization (*29*).

The theoretical maximal protein recovery in the acid and alkaline solubilization procedures is determined by the difference between the total amount of proteins that become solubilized at pH 2.7 or 10.8 and the amount of proteins that remain in solution during precipitation. However, this theoretical recovery would occur only if there were no loss of solubilized proteins into the floating layer and sediments from the first centrifugation. With herring light muscle, these two fractions entrapped 16 and 19% of the total acid- and alkali-solubilized proteins, respectively, which reduced the theoretical protein recoveries in the acid and alkaline procedures from 88 to 74% and from 83.5 to 68%, respectively. These were larger recovery reductions than were obtained during acid-aided isolation of proteins from mackerel and chicken light muscles: from 87.3 to 85% and

Table 3. Consistency, Solubility, Precipitation Recovery, and Precipitate Moisture during Precipitation of Acidified/Alkalized Herring Light Muscle Proteins[a]

	acidified protein solution						alkalized protein solution					
precipitation pH	4.8	5.1	5.4	5.5	5.7	5.9	7.0	6.2	5.8	5.5	5.3	5.1
consistency (mPa·s)	173	85	60	60	**49**	50	243	48	36	30	30	**25**
% protein solubility %	13.4	5.9	4.8	4.6	4.5	**4.3**	15.2	8.4	6.1	**6**	**6**	6.2
% proteins precipitated[b]	89.5	95.2	96	96.1	96.2	**96.3**	88.6	93	94.7	**94.8**	**94.8**	94.6
% moisture in second sediment	93.2	91.9	90.4	**90.1**	**90.1**	90.8	94.9	91.9	**87.3**	87.6	88.2	89.2

[a] The lipid and moisture contents in the muscle used were 2.5% (on dw basis: 12%) and 79.5%, respectively. [b] Expressed as (precipitated proteins/proteins in the supernatant from the first centrifugation) × 100.

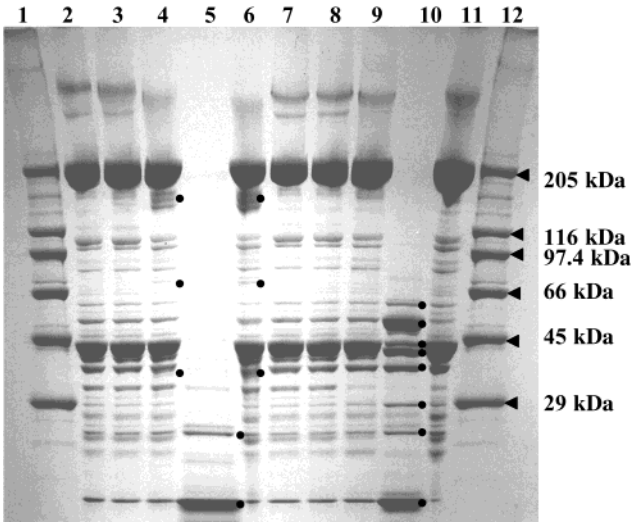

Figure 3. SDS-PAGE (4–20%, linear gradient) of herring light muscle at selected stages of the acid and alkaline solubilization/precipitation techniques: (lane 1) molecular weight standards (29, 45, 66, 97.4, 116, and 205 kDa); (lane 2) proteins from herring light muscle homogenate (1:9 in ice-cold distilled deionized water) at pH 7; (lane 3) homogenate proteins at pH 2.7; (lane 4) proteins from the pH 2.7 supernatant fraction after high-speed centrifugation (18000*g*, 20 min) (peptides with molecular weights of 37, 75, and 150–175 kDa are marked with dots); (lane 5) proteins from the pH 2.7 supernatant fraction after precipitation at pH 5.5 and centrifugation at 10000*g* for 20 min (peptides with molecular weights of 8 and 23 kDa are marked with dots); (lane 6) proteins from the final acid-produced sediment precipitated at pH 5.5 (peptides with molecular weights of 37, 75, and 150–175 kDa are marked with dots); (lane 7) proteins from herring light muscle homogenate (1:9 in ice-cold distilled deionized water) at pH 7 (same as lane 2); (lane 8) homogenate proteins at pH 10.8; (lane 9) proteins from the pH 10.8 supernatant fraction after high-speed centrifugation (18000*g*, 20 min); (lane 10) proteins from the pH 10.8 supernatant fraction after precipitation at pH 5.5 and centrifugation at 10000*g* for 20 min (peptides with molecular weights of 8, 23, 29, 38, 41, 43, 54, and 60 kDa are marked with dots); (lane 11) proteins from the final alkali-produced sediment precipitated at pH 5.5; (lane 12) molecular weight standards (29, 45, 66, 97.4, 116, and 205 kDa). Protein was applied to all lanes at 15 μg/lane.

from 87.8 to 83.5%, respectively (*15*). It was believed that the major feature suppressing recovery of solubilized herring light muscle proteins was the relatively high consistency of the acidified/alkalized herring light muscle homogenates, which created a large floating layer and sediment with a high water content. **Figure 1** shows how the total weight of the nonliquid fractions closely followed the consistency of the pH-adjusted homogenate ($R^2 = 0.92$).

Under the assumption that the aqueous fraction of the sediments and floating layer had the same composition as the supernatant, it was found that the gel-sed was the largest trap for both acid- and alkali-solubilized proteins, 57%. In acid processing of brisling sardine whole muscle, Cortes-Ruiz et al. (*27*) reprocessed the gel-sed, which improved the total protein recovery of the process from 65.8 to 76%. Another strategy for increasing the protein recovery would be to reduce the solid volume of the sediments and floating layer. In alkaline processing of herring light muscle, the floating emulsion layer trapped 7% of the total proteins, resulting in a significantly larger total protein loss in the alkaline than in the acid process. Kelleher (*15*) described how the neutral lipids of mackerel light muscle that had risen to the surface during acid processing emulsified 7.3% of the total proteins. Kristinsson (*30*) recently found that alkaline treatment (pH 11) improved the emulsifying properties of precipitated cod myosin more than did acid treatment (pH 2.5). This could explain why the emulsion layer became larger following alkalization than acidification of herring light muscle proteins. It is also possible that the 25% lower consistency at pH 10.8 than at pH 2.7 reduced the resistance for neutral lipid to rise to the surface during centrifugation.

The results in **Table 3** suggest that changes in the pH used to precipitate the proteins did not appear to significantly affect protein recoveries once pH 4.8 and 7 were reached in the acid and alkaline processes, respectively. It is worth noting, though, that solubility, consistency, and precipitate moisture content

Table 4. Data on Moisture, Lipid Content, pH, and Gel Strength from the Acid and Alkaline Preparations of Surimi and Surimi Gels Using Fresh Muscle and Muscle from Herring Stored for 6 Days on Ice

raw material/surimi characteristics	fresh herring		aged herring	
	pH 2.7	pH 10.8	pH 2.7	pH 10.8
moisture in muscle (%)	79.6		80.6	
lipid muscle	11.1		8.8	
muscle TBARS (μmol MDA/kg)	5		28	
moisture in protein precipitate (%)	89.2	87.3	88.3	87.7
moisture in dewatered protein precipitate (%)	77.4	74.4	77.8	74.5
moisture in surimi with cryoprotectants[a]	73.3	72.5	73.9	73.1
pH in surimi with cryoprotectants[a]	7.66	6.87	6.35	6.42
pH prior to gelation	7.19	7.15	7.11	7.11
gel characteristics[b]				
moisture (%)	70.3	70.7	71.9	69.9
folding test	5^1	5^1	$3+^2$	3^2
break force[c] (g)	566 ± 36^1	871 ± 62^2	$498 \pm 58^{1,3}$	464 ± 11^3
deformation[c] (mm)	9.2 ± 0.9^1	9.2 ± 0.7^1	7.3 ± 0.5^2	6.2 ± 0.3^3

[a] The cryprotectant mixture consisted of 4% sorbitol, 4% sucrose, and 0.3% sodium tripolyphosphate. [b] Gels contained 2% NaCl and were formed at 90 °C for 30 min. [c] Break force and deformation measured with a Rheotex AP-83 (Sun Science Co. Ltd., Nichimo International Inc, Seattle, WA). Values within the same row bearing different superscript numbers are significantly different ($p \leq 0.05$).

Table 5. Data on Moisture, pH, and Gel Strength from Acid/Alkali-Aided Preparations of Surimi and Surimi Gels Using Fresh Herring Light Muscle

raw material/surimi characteristics	pH 2.7	pH 10.8
moisture in muscle (%)	80	
lipid muscle	11.3	
moisture in protein precipitate (%)	89.4	87.5
moisture in dewatered protein precipitate (%)	75	72.8
moisture in surimi with cryoprotectants (%)[a]	74.5	73.6
pH in surimi with cryoprotectants [a]	5.8	6.0
pH prior to gelation	7.1	7.1
gel characteristics[b]		
moisture (%)	72.8	74.1
folding test	5^1	5^1
stress (kPa)[c]	58.2 ± 7.3^1	56.1 ± 2.4^1
strain[c]	1.8 ± 0.2^1	1.6 ± 0.1^2
G'[c]	32.1 ± 4.4^1	35.4 ± 2.1^1
L[d]	64 ± 1.2^1	66.5 ± 0.3^1
a[d]	-2.4 ± 0.4^1	-2.4 ± 0.4^1
b[d]	8.0 ± 0.6^1	8.1 ± 0.9^1
whiteness[e]	63	65.5

[a] The cryprotectant mixture consisted of 4% sorbitol, 4% sucrose, and 0.3% sodium tripolyphosphate. [b] Gels contained 2% NaCl and were formed at 90 °C for 30 min. [c] Stress and strain measured with the torsion technique (*17*) using a Brookfield Digital viscometer (model DV-II, Brookfield Engineering Inc., Stoughton, MA). Results are expressed as mean ± SD ($n = 4$). Values within the same row bearing different superscript numbers are significantly different ($p \leq 0.05$). [d] Color was measured with a Hunter LabScan II colorimeter (Hunter Associates Laboratories, Reston, VA). Data are given as mean ± SD ($n = 5$). [e] Whiteness was calculated according to the following formula: $100 - [(100 - L)^2 + a^2 + b^2)^{0.5}$ (*28*) using the average values of *L*, *a*, and *b*.

differed between the acid- and alkali-treated proteins where the investigated pH spans overlapped, pH 5.1−5.9. Following the acid treatment, the average solubilities were ∼20% lower whereas the consistencies and moisture contents were 100% and ∼3% higher, respectively, as compared to after alkaline treatment. This suggests that different protein conformational changes took place during acid and alkaline treatment, which was further supported by the wider variety of proteins/polypeptides staying soluble in the pH 5.5 supernatant from alkali-produced than from acid-produced proteins (**Figure 3**, lanes 5 and 10). In addition to more sarcoplasmic proteins, the former also contained significant amounts of myofibrillar proteins, particularly actin. The significantly ($p \leq 0.05$) higher ionic strength caused by lowering the pH to 2.7 than by raising the pH to 10.8 may also have contributed to such differences.

Consistency Development during Acidification and Alkalization. The presence of strong positive or negative charges on protein molecules such as under extreme acid or alkaline conditions controls protein solubilization as well as the flow properties of aggregated proteins (*31*, *32*). The increased electrostatic repulsion causes the protein aggregates to swell, which significantly increases their effective volume. The effective volume is directly related to consistency (*33*).

On the basis of the above, the difficulties found in controlling the homogenate consistency during protein solubilization could be explained by the strong effect that changes in the processing conditions could have had on both the exposure and charge of amino acid side chains. Aging of the herring raw material, increased intensity of the homogenization, and holding of the pH-adjusted homogenates all strongly affected the consistency; the two former by increasing it, the latter by reducing it. The effect from aging could possibly be explained by cytoskeleton degradation "opening up" the protein structures, releasing more proteins and allowing for more extensive swelling (*34*). The observed reduction of the herring muscle ionic strength during aging, for example, from 0.0124 to 0.0074 after 8.5 days on ice, may also have contributed (*16*). SDS-PAGE analysis revealed that slight hydrolysis had taken place at pH 2.7 and 10.8 within the holding period where the largest consistency reduction occurred, that is, from 5 to 35 min after acidification and alkalization (**Figures 1**−**3**). The reduction in consistency on holding could also have been the result of a time-dependent dissociation of protein aggregates to produce a lower effective volume and a dissolution of the proteins.

Lipid Removal in Acid/Alkaline Processing. Using a *g* force of 18000*g* (20 min) in the first centrifugation, the lipid content of the herring light muscle was reduced from 0.13 to 0.043 g/g of protein in the final protein isolate. When acid-made surimi was prepared from mackerel light muscle with 0.53 and 0.64 g of lipids/g of protein (*11*, *15*) and from brisling sardine whole muscle with 0.17 g of lipid/g of protein (*28*), the final lipid concentrations obtained ranged from 0.007 to 0.043 g/g of protein after centrifugations at 10000−127000*g*. When regular surimi was produced from herring light muscle and brisling sardine whole muscle with 0.5 and 0.17 g of lipids/g of protein, respectively, final lipid levels either did not change (*7*, *28*) or were reduced to ≥0.17 g of lipid/g of protein (*5*, *7*, *28*).

That lower lipid contents can be reached by solubilizing the proteins as opposed to washing of unsolubilized proteins may result from the lipids becoming "liberated" as the muscle structure disintegrates. As "free" molecules/aggregates, the proteins and lipids can be separated on the basis of their differences in density. In acid processing of brisling sardine whole muscle, lipid removal was improved by increasing the homogenization intensity and the centrifugation forces (*28*). In present study, the efficiency of the density-based lipid removal was related to the holding time that had elapsed between acidification and high-speed centrifugation (**Figure 1**). After 5 min of holding on ice, only ∼30% of the lipids in the initial muscle homogenate were removed into the gel-sed, bottom-sed, and emulsion layer, whereas after 125 min, this removal was almost 85%. Holding also reduced homogenate consistency, and because less resistance during centrifugation theoretically would aid in lipid sedimentation/flotation, it could be expected that consistency and lipid removal would be related. However, the largest consistency drop (5−35 min) did not concur with the largest increase in lipid removal (65−125 min), suggesting the involvement of other mechanisms.

It was interesting to note that when the holding time between acidification and high-speed centrifugation was varied, phospholipid removal was reduced from ∼30% without holding to 0% after 125 min holding.

Hydrolytic Changes in Acid/Alkaline Processing. An important difference between the acid and alkaline processes was that myofibrillar protein degradation was significantly more pronounced after acidification than alkalization of the homogenate (**Figures 2** and **3**). At acid conditions, titin, nebulin, myosin heavy chain, and tropomyosin β started to degrade during the first centrifugation, that is, in the period from 15 to 45 min after the pH had been reduced to 2.7 (**Figure 3**, lanes 3 and 4). After adjustment to pH 10.8, the slight hydrolysis seen in this period targeted only titin and nebulin (**Figure 3**, lanes 8 and 9). The lower hydrolysis at pH 10.8 compared to pH 2.7 was confirmed by holding the homogenates at the acid and alkaline pH values up to 20 h on ice (**Figure 2**). It was not

determined whether the major reason for protein degradation was proteolysis or acid/alkaline hydrolysis. In any case, the results in both **Figures 2** and **3** imply that the homogenates should not be held for longer than necessary at low pH. In acid and alkaline processing of rockfish (*Sebastes flavidus*), myosin degradation occurred at both pH 2.5 and 11, although the former was more pronounced (*35*). Myosin heavy chain degradation in acid was also seen with chicken breast muscle but not with mackerel light muscle (*15*). As part of a regular surimi process, washing Pacific whiting (*Merluccius productus*) three times (1:2) with 0.1% NaCl gave surimi with lower cathepsin B and L activities as compared to surimi produced with the acid solubilization process (pH 3) (*36*). Cathepsin H activity was eliminated with both processes. Ground rockfish muscle washed three times (1:3) with water had been subjected to less proteolytic degradation of myosin and actin as compared to unwashed muscle, acidified (pH 3) unwashed muscle, and alkalized (pH 11) unwashed muscle, respectively (*35*).

Gel Characteristics. The muscle proteins being particularly responsible for gelation are myosin and actomyosin (*37*). Kristinsson (*30*) found that the exposure of cod myosin to pH 2.5/11 followed by adjustment to pH 5.5 reduced the gelation temperature and gave stronger gels as compared to non-pH-adjusted myosin. Along with this it was found that the conformation of the myosin headgroup had been changed into a more unfolded state. When cod myofibrillar proteins were exposed to the same pH conditions, only the gelling temperature was reduced.

The capacity of acid/alkali-produced herring proteins to form gels was tested at ~pH 7.1−7.2. Chang et al. (*38*) showed that neutrality improved gelation compared to an acid pH. This improvement may be due to increasing electrostatic repulsion. To obtain pH 7.1−7.2, variable amounts of base had to be added as the surimi pH values unexpectedly varied extensively between experiments (**Tables 4** and **5**).

According to the folding test scores obtained (5; **Tables 4** and **5**), both the acid and alkaline gels were grade AA. However, according to the strain data as given by the torsion technique (1.6−1.8; **Table 5**), the gels were grade B (*27*). That gelometer stress values were significantly higher ($p \leq 0.05$) for alkali-produced than for acid-produced protein gels could be the retention of more myosin heavy chains in alkaline processing (**Figure 3**). Yongsawatdigul et al. (*35*) obtained stronger and more elastic gels with alkali-produced than with acid-produced rockfish isolates. This was accompanied by less hydrolysis and a larger loss of sulfhydryl groups at pH 11 as compared to pH 2.5. Cortes-Ruiz et al. (*28*) found gels made from acid-produced brisling sardine protein isolates to have equal elasticity and cohesiveness but higher hardness compared to gels made from regular brisling sardine surimi. According to the authors, the 13% higher protein concentration in the acid-produced isolates than the surimi could have favored formation of more protein−protein interactions.

Ice storage of the whole herring lowered both stress and strain values of acid- and alkali-produced gels. One reason could be protein denaturation initiated by lipid-free radicals/lipid oxidation products (*39*). TBARS values increased substantially during the 6 days on ice (from 5 to 28 μmol of TBA/kg of muscle) (**Table 4**). Acid processing of whole muscle from ice stored (5 days) brisling sardine gave rise to gels with the same hardness, but with significantly lower elasticity and cohesiveness as compared to gels made from fresh brisling sardine muscle (*28*). A suggested reason was the proteolytic activity detected during storage.

The results of this paper illustrate that acid and alkaline production of protein isolates to be incorporated into surimi gels is a promising way of increasing the utilization of herring for food production. Protein recoveries could be further improved by reducing the size of the nonliquid fractions from the first centrifugation. The use of a fresh raw material helped to do so and also gave the best gel quality. The hydrolytic changes seen during acid processing could be minimized by avoiding long exposure of the proteins to low pH.

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