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ARTICLE

Protein Isolation from Gutted Herring (*Clupea harengus*) Using pH-Shift Processes

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Herring (*Clupea harengus*) and other pelagic fish species are mainly used for fish meal and oil production and not for human consumption. In this study, acid pH-shift processing and alkaline pH-shift processing were used to isolate proteins from whole gutted herring with the aim to investigate the potential use of herring proteins as a food ingredient. The acid and alkaline processes gave rise to similar protein yields, 59.3 and 57.3%. The protein isolates from both processes had a significantly ($p < 0.05$) whiter color and higher protein and lower lipid contents than the starting material. The removal of ash was >80% for both processes, with a trend ($p = 0.07$) toward higher removal during the alkaline process. Also, Ca and Mg removal was significantly ($p < 0.05$) higher during the alkaline process. The isolated proteins from the acid process contained myosin degradation products and had a lower salt solubility than proteins from the alkaline process. Both protein isolates had an amino acid profile meeting the recommendations for adults according to FAO/WHO/UNU and could produce a surimi gel of medium strength. The results show that pH-shift processing could be a valuable method for the production of functional food proteins from gutted herring.

KEYWORDS: Herring; acid; alkaline; solubilization; precipitation; protein functionality

INTRODUCTION

Fish is a valuable resource containing highly nutritious proteins and n-3 fatty acids. The catches have leveled out around 90 million tonnes per year, and the level of exploitation of the fish stocks indicates that no increase could be seen in the near future (*1*). The fish caught, or farmed, should thus be used as sustainably as possible. Of the top 10 species contributing to the world catches, 5 are small pelagic fish species (*1*). A large part of these fishes is not used directly for human consumption, but for production of fish meal and fish oil, and some are even discarded (*2*). Herring (*Clupea harengus*) is one of these fish species, in 2006 yielding the fourth highest catch of all species (*1*). The exact proportion of herring used for human consumption differs between different fisheries. As an example, around 50% of the herring catch from Swedish fisheries in 2008 was directed toward human consumption (*3*). In roe fisheries, the on-shore discards of herring males can be high, and spent females are largely used for fish meal and oil production (*2*).

There are many arguments for an increased use of herring for human consumption. Herring, and other small pelagic fishes, can be caught with very little by-catch (*2*), and many of the herring stocks are stable (*1*). Studies have shown herring to have health-promoting effects (*4*, *5*). However, small pelagic fish species are hard to process due to their high content of dark muscle with heme proteins, bones, proteolytic enzymes, and fat. Previous attempts to increase the usage of herring for human consumption have been based on either mechanical separation of the meat or enzymatic hydrolysis (*6*−*8*). A new technology, called pH-shift processing or acid/alkaline/isoelectric solubilization and precipitation, has, however, yielded the possibility to isolate protein from complex muscle materials without using any of the techniques above. The process is performed cold and has the big advantage over, for example, hydrolysis in that whole proteins with retained functionality are isolated (*9*−*11*). In short, the process uses the feature that muscle proteins in water can be solubilized at high or low pH. When soluble, the proteins can be separated from insoluble matter as bones, scales, and, to some degree, lipids. The purified proteins can then be isolated by precipitation at their isoelectric point (p*I*) (*9*). Herring light muscle (*9*) and herring fillets (*12*, *13*) have previously been studied using these methods. It is, however, hypothesized that the most promising use of the pH-shift processes is on more unrefined materials that currently have a low value (*14*). A few other studies have shown that the pH-shift process can be applied on various complex materials such as whole Antarctic krill (*15*), rainbow trout (*Oncorhynchus mykiss*) byproducts (*16*), and whole gutted silver carp (*Hypophthalmichthys molitrix*) (*17*). Our research group has also shown that the processes can be used to remove up to 80% of toxins from blue mussels (*Mytilis edilus*) (*18*) and herring (*19*). To make the pH-shift process a successful strategy for increasing the direct use of underutilized marine materials such as herring for food production, a broad investigation of the produced isolates is required, taking into account also protein yield, nutritional properties, protein functionality, visual appearance, and stability.

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The aim of this study was to investigate the quantity and quality of proteins isolated from whole gutted herring with the acid and alkaline versions of the pH-shift process. Qualitative measures include crude composition, polypeptide profile, amino acid profile, minerals (Ca, Mg, Fe, Cu, and Zn), salt solubility, color, and gelation ability.

MATERIALS AND METHODS

Materials. Freshly caught Baltic herring was kindly provided by Gävlefisk AB (Gävle, Sweden). The herring was kept on ice until it reached the laboratory 48 h after catch.

Metal ion standards (AAS or IC) of 1000 ppm were obtained from Fluka (Buchs, Switzerland). All other reagents were purchased from commercial sources and were of at least reagent grade.

Methods. *Sample Preparation*. The herring was manually gutted and cut into smaller pieces. Herring mince was then produced in a cold room (4 °C) using a food grinder (KitchenAid, St. Joseph, MI), first using a grid with holes of 10 mm ∅ and thereafter using a grid with holes of 5 mm ∅. The minced herring was then stored at −80 °C in plastic LDPE zip-lock bags (VWR, Stockholm, Sweden) until use.

Acid and Alkaline pH-Shift Processing. The herring mince was thawed under running cold water. The herring, typically 100−130 g, was mixed with 9 times ice-cold distilled and deionized water and homogenized 2 × 30 s using an Ultra Turrax T18 Basic homogenizer (IKA, Taquara, RJ, Brazil). To choose solubilization pH, the protein solubility as a function of pH was investigated in the pH ranges of 1.5−4.0 and 10.0−12.5. Within these pH ranges, sampling was performed at pH intervals of 0.5. On the basis of the results from this preliminary study (see Results), the pH of the homogenate was adjusted to either 11.2 (alkaline pH-shift method) or 2.7 (acid pH-shift method) using 2 M NaOH or 2 M HCl during constant manual stirring until the pH was stable. The pH was monitored with a calibrated Hamilton double-pore electrode (Bonaduz, Switzerland) coupled with a pH-meter (MeterLab PHM210, Radiometer Analytical S.A., Villeurbanne Cedex, France). The pH-adjusted muscle homogenates were centrifuged at 8000*g* in an Avanti centrifuge J-20 XP (Beckman Coulter, Fullerton, CA) at 4 °C for 20 min. The solubilized proteins in the supernatant were collected and separated from the pellet and the floating fat layer by filtering through three layers of cotton gauze (AKLA AB, Danderyd, Sweden). The floating fat layer and pellet were separately weighed. To choose a pH for protein precipitation, the protein solubility as a function of pH within the pH range of 5−6.3 was tested. On the basis of this trial, pH 5.4 (alkaline pH-shift method) and 6.1 (acid pH-shift method) were chosen for precipitation of the proteins. The pH was adjusted using 2 M HCl or 2 M NaOH. A second centrifugation was performed, and the pellet, referred to as protein isolate, was collected and weighed. Analysis of color, salt solubility, gel making ability, and electrophoresis pattern was made on freshly produced protein isolate. For other analyses the protein isolate was stored at −80 °C in plastic zip-lock bags. Also, the supernatant of the second centrifugation was weighed. During all processing steps the material was kept in a beaker on crushed ice.

The protein solubility at pH 2.7 and 11.2, as well as at the various precipitation pH values tested, was investigated following centrifugation at 8000*g* by analyzing proteins in both the noncentrifuged homogenate and the supernatant. Calculations were then done according to the following formula:

$$\frac{[\text{protein}]_{\text{supernatant}}}{[\text{protein}]_{\text{before centrifugation}}} \times 100$$

The protein yields in the two centrifugation steps were calculated according to

$$\frac{[\text{protein}]_{\text{supernatant}} \times \text{mL}_{\text{supernatant}}}{[\text{protein}]_{\text{before centrifugation}} \times \text{mL}_{\text{before centrifugation}}} \times 100$$

Protein Analysis. The protein content was measured according to the method of Lowry et al. (*20*) as modified by Markwell et al. (*21*). A Cary50 BIO UV−vis spectrophotometer (Varian Australia Pty Ltd., Victoria, Australia) was used for absorbance measurements, and the standard curve was based on bovine serum albumin using a concentration range of 10−100 μg of protein/mL.

Total Fat Content. The total fat content was analyzed using the method of Lee et al. (*22*) as modified by Undeland et al. (*23*). For the herring mince, a chloroform to methanol ratio of 2:1 (v/v) was used, whereas for the protein isolates the ratio 1:1 (v/v) was used.

Moisture Content. The moisture content was determined by drying 2 g samples at 105 °C until constant weight (overnight) using an Electrolux 939 oven (Electrolux, Stockholm, Sweden). The balance used was a Precisa XR 405A-FR (Precisa Gravimetrics AG, Dietikon, Switzerland). During surimi production, when faster results were needed, the moisture content was measured on a moisture balance Precisa HA 300 (Precisa Gravimetrics AG).

Ash. Dried samples were transferred to Pyrex glas beakers and heated at 400 °C in an Lenton furnace, Eurotherm 2416 (Lenton, U.K.) overnight. Drops of concentrated nitric acid were then added and the samples heated at 400 °C for an additional 4 h or overnight. If the samples contained black spots, that is, carbon, the procedure was repeated until the samples were completely white and the weight was stable.

Mineral Analysis. Dried and ashed samples were prepared as described above, with the addition that all glassware used was previously washed in 0.1 M HCl to remove mineral residues. The ash was dissolved in 5 mL of 0.2 M HCl and diluted to 0.1 M by the addition of distilled water. For analysis of Cu, Zn, and Fe, 0.9 mL was mixed with 0.1 mL of ascorbic acid (20 mg/mL). The samples were then analyzed according to the method of Fredriksson et al. (*24*) using a Waters HPLC system (Milford, MA). The system consisted of a Spark Endurance autosampler (Emmen, Netherlands) coupled to a Waters 626 quaternary pump. The column was a Dionex CS5a (Dionex Corp., Sunnyvale, CA), the detector a Waters 2487 dual absorbance detector, and the postcolumn pump a Knauer K-500 (Berlin, Germany). The chromatographic evaluation system was Waters Empower Pro. Cu, Zn, and Fe in the samples were compared to standards of up to 10 ppm. Ca and Mg were analyzed according to the same method, with a modification based on the addition of Zn-EDTA (1 mM) to the postcolumn reagent (PAR). This addition allows detection of alkaline earth metals (*25*). The samples were diluted 10−5000 times using distilled water and hydrochloric acid to a final concentration of 0.1 M HCl. The Ca and Mg in the samples were compared to standards of up to 10 ppm and 0.1 M HCl.

Color. The color of herring mince, freshly produced protein isolate, and surimi gels was measured using a Minolta colorimeter. From the *L*, *a**, and *b** values obtained, the whiteness (W) value was calculated with the following formula:

$$\text{whiteness} = 100 - \sqrt{(100 - L)^2 + a^2 + b^2}$$

Salt Solubility. The protein salt solubility was measured as described earlier (*26*) with some modifications. One gram of freshly produced sample was homogenized with 10 times distilled water for 50 s at 10000 rpm with an Ultra Turrax T18 Basic homogenizer (IKA) while kept on ice. From the samples four aliquots of 2 mL were taken. To two of them was added 2 mL of 2 M NaOH, and to the other two was added an equal amount LiCl buffer (8.4% LiCl and 0.04 M Li_2CO_3, pH 7.2). The samples were stored on ice overnight and then centrifuged at 12000*g* for 20 min at 4 °C. The protein content in the supernatant was then measured as described above. Salt solubility was calculated as amount of protein in the LiCl buffer over the amount in NaOH and expressed as percentage.

SDS-PAGE. Protein samples were taken from both the acid and alkaline versions of the process at the following times: directly after homogenization with water, after pH adjustment, after the first centrifugation (supernatant), and after the second pH adjustment and centrifugation (supernatant and protein isolate). The proteins were analyzed using SDS-PAGE according to the procedure of Laemmli (*27*). All of the samples were stepwise diluted to 2 μg/μL and mixed with an equal amount of Laemmli buffer (Bio-Rad, Hercules, CA), in which 5% β-mercaptoethanol was freshly added. The supernatants after the second centrifugation were not diluted, but directly mixed with Laemmli buffer. The samples were then boiled for 5 min, cooled, centrifuged at 5000*g* for 5 min using an Eppendorf centrifuge 5415 (Eppendorf, Hamburg, Germany), and kept at −20 °C until analysis. The gel used was PageR Gold 4−20% (Lonza, Switzerland) with 12 wells, and it was run according to instructions, at 125 V for 80 min. The ladder used was Protein marker

III (VWR International, West Chester, PA) with a range of 6.6−200 kDa. Ten microliters of ladder was used and 15 μg of protein of each sample, except for the samples from the supernatants after the second centrifugation, for which 10 μg of proteins was used. After running, the gel was rinsed in water and fixed in 10% acetic acid/10% methanol for 2 × 15 min, stained for 60 min in Coomassie G-250 (Bio-Rad), and destained at three times for 10 min in 40% acetic acid. The gel was scanned in a GS-800 Calibrated Densitometer (Bio-Rad) and the picture analyzed with the program QuantityOne 4.5.1 (Bio-Rad) to achieve molecular weights and relative amounts of the proteins present.

Amino Acid Composition. Gutted herring was freeze-dried and ground, whereas the protein isolates were processed frozen. All amino acids except tryptophan were analyzed using an amino acid analyzer according to SS-EN ISO 13903:2005. The tryptophan was analyzed using HPLC according to SS-EN ISO 13904:2005. The analytical margin of the analysis was 8%.

Surimi Production and Analysis of Gel Characteristics. About 90−100 g of freshly produced protein isolate was processed into surimi in accordance with Undeland et al. (*9*) with the exception that the pH was adjusted before freezing. The samples were frozen at −80 °C until gel production. Paste was produced by the addition of 2% salt and chopping in an Ideline miniprocessor (Adexi AB, Göteborg Sweden). The paste was stuffed in 29 mm ∅ polyamide casings (Atlantis-pak, Lenin, Russian Federation) and heated to produce a gel in an M20 Lauda water bath (Lauda, Lauda-Königshofen, Germany) at 90 °C for 30 min, cooled in ice water for 15 min, and stored at 4 °C for 24 h. The gels were equilibrated to room temperature for 2 h. Gel strength (*g*) and deformation (mm) were measured on 25 mm gel pieces according to the method of Park (*28*). For this purpose, a texture analyzer (Stable Micro Systems, Godalming, U.K.) was used, equipped with a stainless steel 1/4 in. ∅ spherical probe (Stable Micro Systems). Running speed was 1 mm/s. Three readings were made on each side of the gel piece. A folding test was performed according to the method of Park (*28*). Color and water content were measured as described above.

Statistics. The program PASW Statistics 18 (SPSS, Chicago, IL) was used for all statistical analysis. When two groups were to be compared, a *t* test was used. When three groups were to be compared, one-way ANOVA followed by Tukey's test was used. The number of replicates (*n*) presented in the figures and tables refers to the number of batches of protein isolates that were produced and analyzed. At least duplicate samples from each batch were taken for each analysis. To calculate the variation among the different *n* values, an average value of these replicate analyses was used. An exception was the amino acid composition analyses, for which only one batch and one sample were analyzed.

RESULTS AND DISCUSSION

In this study, the acid and alkaline versions of the pH-shift process were evaluated for application on gutted herring. Below, quantitative and qualitative results from this evaluation are given, together with data from the optimization of solubility and precipitation pH values.

Protein Solubility. The protein solubility as a function of pH showed a typical profile (*13*, *29*, *30*), with low protein solubility at pH values around 5.5 and drastically increased solubility in the pH ranges of 10−11 and 3−4. The large solubility changes then leveled out at pH ≤3 and ≥11, respectively. Lowering the pH to values <2.5 led to somewhat decreased solubility, which was the basis for selecting pH 2.7 (solubility = 77.4%) as solubilization point in the acid process. On the alkaline side, a continued increase in protein solubility was seen when the pH was increased to values >11.5. However, pH 11.2 (solubility = 78.8%) was chosen to avoid possible amino acid changes and excess use of base. To select proper precipitation pH, solubility tests between pH 5 and 6.3 were carried out. This showed that the lowest solubility occurred at pH 6.1 and 5.4 for the acid and alkaline pH-shift processes, respectively (9.3 and 11.0%). Solubilization of herring proteins at pH 2.7 has been applied in previous studies on herring light muscle (*9*) and herring fillets (*12*). The solubilization pH used on the alkaline side was, however, higher than previously used for herring light muscle (pH 10.8) (*9*), but in line with other studies on more complex materials, such as whole fish and byproduct (*15*−*17*). For the acid process, the precipitation pH selected was comparatively high (pH 6.1) in relation to other studies (*9*, *16*). It is possible that some nonmuscle compound present in gutted whole herring affected the optimal precipitation pH in our study. In addition, it has been shown that conformational changes can take place in cod myosin following extreme acidification that affects the refolding pattern (*31*, *32*). On the alkaline side, the precipitation pH used (pH 5.4) was in line with previous studies (*9*, *16*, *17*)

Table 1. Protein Yield and Solubility in the Different Steps of the pH-Shift Processes[a]

		alkaline process	acid process
centrifugation 1	solubility (%)	78.8 ± 5.5 (*n* = 10) a	77.4 ± 2.8 (*n* = 7) a
	yield (%)	64.5 ± 2.3 (*n* = 9) a	64.7 ± 2.8 (*n* = 6) a
centrifugation 2	solubility (%)	11.0 ± 2.8 (*n* = 7) a	9.3 ± 1.8 (*n* = 5) a
	yield (%)	90.1 ± 2.6 (*n* = 7) a	91.7 ± 1.7 (*n* = 5) a
total	yield (%)	57.3 ± 2.5 (*n* = 6) a	59.3 ± 3.0 (*n* = 5) a

[a] Letters within a row show that no significant differences are determined between the acid and alkaline versions of the pH-shift methods ($p > 0.05$) according to *t* test. Values are presented as mean values ± standard deviation.

Precipitation pH. In **Table 1** it is shown that protein solubilities and protein yields in the two centrifugation steps were very similar between the acid and alkaline processes. This also resulted in very similar total protein yields, 57.3 and 59.3%, with the alkaline and acid processes, respectively.

Both protein solubility and yield from gutted herring following solubilization at pH 2.7 were lower than those reported for herring fillets (84.3 and 72.1%, respectively, centrifugation at 10000*g*) (*12*) and herring light muscle (92.1 and 77%, centrifugation at 18000*g*) (*9*). The lower solubility was probably due to, for example, more collagen present in the gutted herring. The protein solubility for gutted herring at pH 11.2 was also lower than for herring light muscle at pH 10.8 (88.6%) (*9*), probably for the same reason as suggested for the acid process. During the protein precipitations at pH 6.1 and 5.4, more proteins (9−11%) remained soluble with gutted herring than during precipitation of herring light muscle proteins, for which 4−6% remained soluble (*9*). This could be due to a higher amount of small proteins and polypeptides for the gutted herring, possibly due to more proteolysis.

Protein Yields. With regard to the total protein yield obtained here (57−59%). it was lower than for herring light muscle and herring fillets (*9*, *12*). Compared to more complex materials, the yield for the process was higher than that reported for alkaline processing of whole Antarctic krill (45−50%) (*33*), similar to that reported for whole gutted silver carp (60%) (*17*), and lower than that reported for trout byproducts (80−90%) (*16*).

Although there were great similarities in solubilities and yields between the acid and alkaline processes, there were significant differences in the distribution of sediment and floating layer in the first centrifugation (**Figure 1**). For the acid process, the floating layer was small, but the pellet contained a large loosely structured gel holding a large fraction of the supernatant and, thus, the solubilized proteins. For the alkaline process, the pellet was harder, but the floating layer was larger and more gel-like. Although the same amounts of supernatant were obtained after acid and alkaline solubilizations, these differences may play a role if a separation strategy other than centrifugation is applied.

Proximate Composition. The proximate compositions of the acid- and alkali-made protein isolates did not differ from one

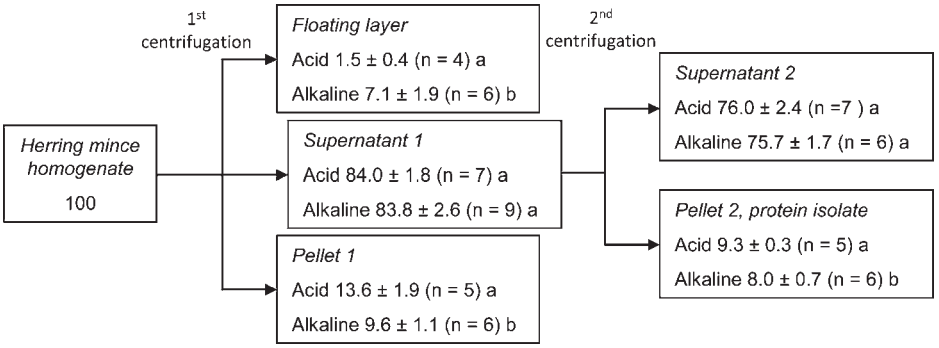

Figure 1. Percent weight distribution of the fractions produced during pH-shift processing. Different letters, within one box, indicate significant differences ($p < 0.05$) according to *t* test. Values are presented as mean ± standard deviation.

Table 2. Proximate Composition of Herring Mince and Protein Isolates[a]

	herring mince	alkali-made isolate	acid-made isolate
water (%)	78.0 ± 0.4 (n = 3) a	89.3 ± 0.9 (n = 8) b	89.8 ± 0.7 (n = 6) b
lipids (%, dry weight)	35.9 ± 1.5 (n = 3) a	17.7 ± 2.2 (n = 7) b	22.2 ± 6.9 (n = 5) b
protein (%, dry weight)	56.5 ± 3.2 (n = 3) a	81.0 ± 6.4 (n = 5) b	81.3 ± 6.5 (n = 6) b
ash (%, dry weight)	8.8 ± 1.0 (n = 5) a	1.1 ± 0.3 (n = 5) b	1.5 ± 0.4 (n = 5) b

[a] Different letters within a row indicate significant differences ($p < 0.05$) according to ANOVA followed by Tukey's test. Values (%) are presented as mean ± standard deviation.

another, but were significantly different from that of the herring mince (**Table 2**). The isolates had higher water content than the herring mince, and the protein content was increased by about 40% on a dry weight basis. The lipid content, on the other hand, was lowered by ~50% in the isolates compared to the herring mince. In herring light muscle a greater lipid reduction (~60−70%) has been reported (*9*). Taskaya et al. (*34*) reported a reduction in lipid content >85% for gutted silver carp. One reason for this difference might be the fatty acids present. The silver carp had a relatively high content of saturated fatty acids (30−40% of total fat) (*34*), whereas herring generally contains only around 20% saturated fatty acids (*35*). Also, differences in centrifugation conditions (our 8000*g* vs their 10000*g*) could have affected the results.

The ash content of whole fish and fish byproduct is very high due to its content of bones, skin, and cartilage. The amount of ash should be seen as a measure of impurities, and it is of great importance to reduce it to concentrate the proteins. The ash content in the protein isolates was drastically lowered to <20% of the ash content in the herring mince (**Table 2**). There was also a trend ($p = 0.074$, *t* test) toward lower ash content in the alkali-produced isolate compared to in the acid-made isolate. The final ash content found here (1−1.5%) was similar to what has been found in protein isolates from trout byproducts (1.4−2.1%) (*36*) and lower than what has been found in protein isolates from whole krill (3.7−5.6%) (*33*) and gutted silver carp (3.8−5.8%) (*34*). The study on silver carp also showed that the alkaline process removed significantly more ash than the acid process.

Minerals. The ash content was investigated further in that some selected minerals (Ca, Mg, Fe, Cu, and Zn) were analyzed (**Table 3**). The lowered ash content in the protein isolates compared to the herring mince was mirrored in the lowered calcium and magnesium contents. However, the copper and zinc levels of the isolates were higher than in the herring mince. For iron, this was also true for the acid-made isolate. Significantly lower levels of calcium, magnesium, copper, and zinc were found in the alkali-made isolate compared to the acid-made one. The removal of calcium and magnesium after pH-shift processing, with the alkaline process being most effective, has also been seen

Table 3. Content of Selected Minerals Found in Herring Mince and Protein Isolates[a]

	herring mince (μg/g, dry weight)	alkali-made isolate (μg/g, dry weight)	acid-made isolate (μg/g, dry weight)
copper	1.6 ± 0.1 a	5.0 ± 0.9 b	7.3 ± 2.2 b
zinc	46 ± 6 a	93 ± 12 b	165 ± 32 c
iron	26 ± 1 a	43 ± 12 ab	46 ± 4 b
calcium	(12 ± 0.6) × 10^3 a	(0.15 ± 0.03) × 10^3 b	(1.6 ± 0.54) × 10^3 c
magnesium	960 ± 58 a	85 ± 11 b	170 ± 46 c

[a] Different letters within a row indicate significant differences ($p < 0.05$) according to ANOVA followed by Tukey's test. Herring mince (n = 3) and isolates (n = 4). Values are presented as mean ± standard deviation.

for whole krill (*33*), rainbow trout byproducts (*36*), and whole gutted silver carp (*34*). In our study, the increase seen in copper and zinc content was too large to originate only from the endogenous amounts in the herring mince. Some of the zinc and copper was therefore a contamination, for example, from equipment used, and this contamination was higher for the acid than for the alkaline process. The levels of zinc and copper in the protein isolates would, however, not be any concern for a normal consumer, because the levels are low in relation to tolerable intake levels (*37*).

Amino Acids. As shown in **Table 4**, there were some differences in the amino acid profiles between the herring mince and the two isolates. However, because no replicates were made in the amino acid analyses, only differences that exceeded 30% are discussed. From this standpoint, the isolates had lower levels of hydroxyproline and glycine. Because hydroxyproline is almost only found in collagen, the very low hydroxyproline levels in the isolates indicate that collagen was effectively removed during both acid and alkaline pH-shift processing. The lower glycine levels could be due to that part of this amino acid is in the free form and, thus, that it is removed in the second supernatant. Previous studies have shown glycine to be one of the most abundant free amino acids in herring (*38*). The lower levels could also be a result of removal of specifically glycine-rich protein(s). Overall, the amino acid composition of the produced protein isolates showed that they are a highly nutritious protein source, with a content of essential amino acids well above recommendations for adults (*39*).

The tryptophan levels are, however, too low for the isolates to be used as the only protein source in an infant formula (*39*). It should be stressed in this context that the amino acid composition is not the only factor determining the nutritional value. Bioavailability is also an important factor for protein quality. The one study available on this topic for pH-shift-isolated proteins showed that the bioavailability of a krill protein isolate was very similar to that of casein (*40*).

Color. The color (**Table 5**) of the protein isolates is of great importance from a consumer perspective and determines what products the isolates can be incorporated into. The pH-shift protein isolation processes both increased the lightness (L^*) and reduced the redness (a^*) of the proteins compared to the herring mince. Thus, the total whiteness value was also increased. The acid-produced isolates were lighter and whiter than the alkali-made ones. A whiter color could be due to greater removal of pigments such as melanin. In contrast to L^* and a^* values, the yellowness (b^*) was not reduced during pH-shift processing. In comparison with protein isolates produced with the acid pH-shift method from herring fillets (*12*), the b^* and a^* values of acid-made isolates from whole gutted herring were substantially higher.

Protein salt solubility (**Table 5**) is considered to be an indicator of protein functionality (*41*). The salt solubility was drastically changed after the pH-shift processes, and there were significant differences between all three sample types. The alkaline process lowered the protein salt solubility from about 70 to 50%, whereas the acid process decreased it even further, to about 24%. Removal of sarcoplasmic proteins and increased hydrophobicity, protein oxidation, and protein cross-linking have been associated with decreased salt solubility (*42*, *43*) and may have contributed to the present results. During pH-shift processing, the myosin head has been known to refold into a conformation with increased hydrophobicity (*31*, *32*), which could have influenced the protein solubility negatively. As recently reviewed, the acid process has most often led to higher lipid oxidation than the alkaline process (*14*). Lipid and protein oxidations are closely linked (*44*), and pronounced lipid oxidation may therefore be an indirect reason for the difference between the two protein isolates.

SDS-PAGE. The proteins were followed throughout the acid and alkaline pH-shift processing with SDS-PAGE, as shown in **Figure 2**. SDS-PAGE shows that in the acid process, there was degradation of a ~600 kDa protein, myosin heavy chain (MHC, 190 kDa), and a 37 kDa protein tentatively identified as tropomyosin α. The latter two produced degradation products of ~130−150 and ~35−37 kDa. There was also a trend toward lower relative amounts of myosin and tropomyosin α in the acid-processed samples (lanes 4 and 5) compared to the herring mince homogenate (lane 2). During the acid process, the protein profile also changed somewhat in other areas. Most notably, the herring mince homogenate and acid-made protein isolate (lane 5) differed in their polypeptide profiles between 68 and 116 kDa. It is possible that these changes were also a result of proteolytic degradation. In supernatant 2 from the acid process (lane 6) several

Table 4. Amino Acid Composition of Herring Mince and Protein Isolates

	herring mince (mg/g of protein)	alkali-made isolate (mg/g of protein)	acid-made isolate (mg/g of protein)
cysteine	10	11	10
methionine	31	36	34
aspartic acid and asparagine	89	102	98
threonine	40	46	44
serine	41	42	39
glutamic acid and glutamine	130	128	143
proline	43	33	32
hydroxyproline	11	1	1
glycine	65	35	38
alanine	61	53	58
valine	51	56	56
isoleucine	43	51	50
leucine	70	82	81
tyrosine	32	33	38
phenylalanine	39	40	40
tryptophan	11	14	12
histidine	25	26	27
lysine	83	86	95
arginine	59	64	60
total amino acids	935	939	958

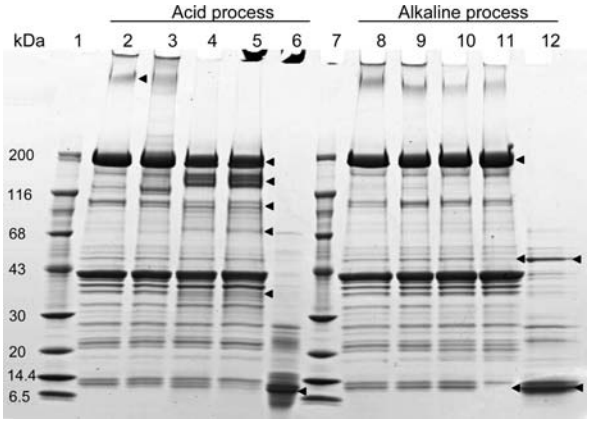

Figure 2. SDS-PAGE (4−20%, linear gradient) of gutted herring at selected stages of acid and alkaline pH-shift processing. Lanes: 1, ladder (marker III, VWR); 2, herring mince homogenate; 3, homogenate after adjustment to pH 2.7; 4, supernatant 1 of the acid process; 5, protein isolate of the acid process; 6, supernatant 2 of the acid process; 7, ladder; 8, herring mince homogenate; 9, homogenate after adjustment to pH 11.2; 10, supernatant 1 of the alkaline process; 11, protein isolate of the alkaline process; 12, supernatant 2 of the alkaline process. For the ladders, 10 μL is loaded in each well. For the samples, 15 μg is loaded in each well, except for supernatant 2 (lanes 6 and 12) for which 10 μg is used. Polypeptides marked with an arrowhead are mentioned under Results and Discussion.

Table 5. Color and Protein Salt Solubility Values for Herring Mince and Protein Isolates[a]

	herring mince	alkali-made isolate	acid-made isolate
L^*, lightness	43.2 ± 2.0 (n = 7) a	57.9 ± 3.9 (n = 7) b	61.7 ± 1.5 (n = 6) c
a^*, redness	4.8 ± 0.6 (n = 7) a	1.9 ± 0.9 (n = 7) b	1.7 ± 0.6 (n = 6) b
b^*, yellowness	6.9 ± 0.6 (n = 7) a	6.3 ± 1.1 (n = 7) a	6.9 ± 1.4 (n = 6) a
whiteness	42.5 ± 1.9 (n = 7) a	57.4 ± 3.7 (n = 7) b	61.0 ± 1.4 (n = 6) c
salt solubility (%)	71.8 ± 1.5 (n = 3) a	51.5 ± 5.3 (n = 3) b	24.3 ± 3.2 (n = 2) c

[a] Different letters within a row indicate significant differences (for color, $p < 0.05$; for salt solubility, $p < 0.01$) according to one-way ANOVA followed by Tukey's test. Values are presented as mean ± standard deviation, except for when n = 2, and then values are presented as mean ± (maximum value − minimum value)/2.

Table 6. Characteristics of Surimi Gels Produced from Protein Isolates[a]

	alkali-made isolate ($n = 2$)	acid-made isolate ($n = 2$)
gel strength (g)	810 ± 28	827 ± 52
deformation (mm)	9.8 ± 0.0	10.7 ± 0.1
folding	5 ± 0.0	5 ± 0.0
water content (%)	70.8 ± 0.2	71.0 ± 0.3
L^*, lightness	52.1 ± 0.0	53.1 ± 2.4
a^*, redness	1.3 ± 0.6	1.6 ± 0.6
b^*, yellowness	8.6 ± 0.0	9.1 ± 1.8
whiteness	51.4 ± 0.0	52.2 ± 2.0

[a] Values are presented as mean ± (maxium value − minimum value)/2.

diffuse bands of proteolysis products were found < 30 kDa, with a clear dominance of a band of 9 kDa. During the alkaline process, proteolytic degradation of MHC was not seen, and the relative amount of MHC even appears to be increased in the protein isolate (lane 11) compared to the herring mince homogenate (lane 8). From a comparison of the protein profiles of the herring mince homogenate (lane 8) and the alkali-made protein isolate (lane 11), notable changes were also that the isolate lacked a protein of 11 kDa and had less of a protein of 51 kDa, tentatively identified as desmin. Both of these proteins were found in the second supernatant of the alkaline process (lane 12). Desmin is an intermediate filament protein, which maintains the structural integrity of the muscle cells, and it has been suggested to prevent solubilization of muscle proteins (*45*). The retention of desmin in the acid-made isolate as compared to the alkali-made one could thus be one more reason for the difference in protein salt solubility.

Gel Characteristics. The possibility to form a gel is regarded as a very important functional property for a protein isolate. The gels made of acid- and alkali-made protein isolates (**Table 6**) were of similar quality. Thus, the difference in protein salt solubility was not mirrored in these heat-induced gels. The folding test resulted in the highest test score for both gel types. The elasticity was high and the gel strength medium. The gels are of similar strength as has previously been reported for herring light muscle (*9*). The whiteness of the gels was lower than for surimi gels prepared from herring light muscle (63−65.5) (*9*) and was, in fact, too low to be graded according to the normal surimi standard, which is based on surimi from white-fleshed fish species. That the whiteness of the gels was lower than for the protein isolates was probably due to the lower water content in the gels and, thus, enrichment of the pigments present. Future studies to selectively remove pigments such as melanin and heme during pH-shift processing would thus be of great interest to increase the possibilities to utilize protein isolates originating from dark-muscle fish species.

Conclusions. Our investigation of pH-shift processing as a method to isolate proteins from gutted herring can be regarded as successful. Considering protein yield, the acid and alkaline versions of the pH-shift process performed equally well. Also, the crude compositions of the isolated proteins were similar, with a trend toward lower ash in the alkali-made isolate compared to the acid-made isolate as the only difference. In more detail, the acid process resulted in a protein isolate that was lighter and whiter but had lower salt solubility and higher mineral content than the alkali-made protein isolate. The acid process also promoted myosin degradation. Thus, the process version to prefer may depend on the intended use for the protein isolate. Both protein isolates had a nutritious amino acid composition and would therefore be interesting as food ingredients. The results clearly show that the isolated proteins have gel-forming abilities and may be used as a functional ingredient in food in which gelling ability is desirable.

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