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Changes in Salt Solubility and Microstructure of Proteins from Herring (*Clupea harengus*) after pH-Shift Processing

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ABSTRACT: Salt solubility of pH-shift isolated herring (*Clupea harengus*) muscle proteins was studied in relation to pH exposure and microstructure using transmission electron microscopy (TEM). Using protein solubilization at pH 11.2 with subsequent precipitation at pH 5.5, salt solubility of the proteins decreased from 78 to 17%. By precipitating the alkali-solubilized proteins at the pH of native herring muscle, 6.5, salt solubility only decreased to 59%, proving that pH values between 6.5 and 5.5 affected protein salt solubility more than the pH cycle 6.5 → 11.2 → 6.5. Precipitation at pH 5.5 resulted in hydrogen bonds, hydrophobic interactions, and S−S bridges, whereas precipitation at pH 6.5 resulted only in the formation of hydrophobic interactions. The alkaline pH-shift isolation process severely rearranged the protein microstructure, with precipitation at pH 6.5 forming a finer, more homogeneous network than precipitation at pH 5.5. The former protein isolate also contained less lipid oxidation products and formed more deformable gels, without affecting protein yield.

KEYWORDS: *pH-shift, alkaline solubilization, precipitation, salt solubility, protein, herring, transmission electron microscopy*

■ INTRODUCTION

Muscle protein isolation using the alkaline version of the pH-shift method[1] is a promising tool for increased usage of complex raw materials such as whole small fish and byproducts from fish and avian sources.[2−5] The method uses high pH to solubilize proteins in water and then separates them from impurities using centrifugation. No previous mechanical separation of the muscle proteins is thus needed. The proteins are then precipitated at a pH close to the isoelectric point (p*I*), typically pH 5.5, and are collected using a second centrifugation. The process gives high protein yields together with efficient separation of, for example, bones, scales, and lipids.[1,6] An interesting feature is that the isolated proteins in several studies have had a very low salt solubility.[2,7−9] Native myofibrillar proteins are generally soluble in 0.5−1 M salt solution, which is attributed to the "salting-in" effect. Traditionally, salt solubility has been used as an indirect measure of protein functionality.[10] In several studies of, for example, frozen fish muscle,[11] loss of protein salt solubility has been closely linked to decreased protein functionality, oxidation, and freeze-induced protein changes.[11−13] Proteins produced with the alkaline pH-shift process have, despite their low salt solubility, performed similarly to conventionally made surimi in terms of gelation[6] and have in several cases produced even stronger and more deformable gels.[14−16] It is thus intriguing what the low salt solubility of pH-shift-produced proteins actually means and what changes cause this phenomenon.

Some authors[7,9] have discussed the involvement of aggregation or denaturation of proteins due to structural changes induced during the alkaline solubilization. However, we hypothesize that also the precipitation step could be critical for the formation of new interactions because the proteins are then adjusted to a pH near their p*I* (pH ∼5.5), at which a minimum of electrostatic repulsion exists between the proteins and, thus, at which a close proximity is created. Furthermore, the pH span between neutrality and the slightly acidic pH 5.5 is known to be critical from a lipid oxidation perspective.[17] Lipid oxidation products can increase protein hydrophobicity,[18] and oxidation of proteins can induce S−S bridges.[19]

In this study the aim was to test the above hypothesis by distinguishing between the drop in salt solubility induced by the alkaline solubilization step and the lowering of the pH (below neutrality) in the last part of the precipitation step. Comparisons of salt solubility were therefore made between the crude herring mince (pH 6.5), pH-shift-isolated proteins precipitated at pH 5.5 (the traditional precipitation pH), and pH-shift-isolated proteins precipitated at pH 6.5. The two precipitation pH values were further compared with regard to their effects on protein yield, lipid oxidation, and protein gelation capacity. The study also aimed at investigating the relationship between changes in salt solubility and protein microstructure during pH-shift processing. For this, transmission electron microscopy (TEM) was used. This is a valuable method due to its high resolution of protein networks. TEM has previously been used, for example, to determine structural changes in fish muscle[20] and to visualize microstructural differences between myosin gels from cod and burbot.[21]

■ MATERIALS AND METHODS

Materials. Herring (*Clupea harengus*) was caught in the North Atlantic. The herring was mechanically skinned and filleted and

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transported on ice to the laboratory, where it was further processed within 56 h from catch and 4 h from filleting. The fillets were then minced in a food grinder (KitchenAid, St. Joseph, MI, USA) using a hole plate with 5 mm ⌀. During handling the herring and herring mince were kept on ice, and the grinding process was performed in a walk-in cold-room (4 °C). The minced herring was then stored at −80 °C in plastic LDPE zip-lock bags (VWR, Stockholm, Sweden). Reagents were purchased from commercial sources and were of at least reagent grade.

Sample Preparation. Herring mince at original pH (6.5 or 6.8, two batches of herring were used) and protein isolates precipitated at pH 6.5 and 5.5, respectively, were the main samples used in the study.

The pH-shift process was performed as previously described.[22] Approximately 140 g of herring mince was thawed under running cold water and mixed with 9 parts ice-cold distilled water and henceforth kept on ice. It was homogenized 2 times for 30 s at 14000 rpm with intermediate cleaning of the equipment using an Ultra Turrax T18 Basic homogenizer (IKA, Taquara, RJ, Brazil), and the pH was adjusted to 11.2 using 2 M NaOH. The pH was monitored with a calibrated Hamilton double-pore electrode (Bonaduz, Switzerland) coupled to a pH-meter (MeterLab PHM210, Radiometer Analytical S.A., Villeurbanne Cedex, France). The homogenate was centrifuged at 8000*g* in a precooled (4 °C) Avanti centrifuge J-20 XP (Beckman Coulter, Fullerton, CA, USA) for 20 min, and the supernatant was collected from the formed sediment and floating lipid rich layer by filtration through three layers of cotton gauze (AKLA AB, Danderyd, Sweden). The supernatant was first adjusted to pH 6.5 using 2 M HCl, and approximately half of the protein slurry was transferred to centrifuge tubes and stored on ice. The remaining half of the protein slurry was further adjusted to pH 5.5. Both of the protein slurries were then centrifuged at the same conditions as above.

In addition to the above-mentioned samples, four further pH-adjusted samples were made: (i) herring mince adjusted to pH 5.5; (ii) herring mince adjusted to pH 5.5, incubated for 2.5 h, and adjusted back to pH 6.8; (iii) pH-shift protein isolate precipitated at pH 5.5 and then adjusted to pH 6.5; and (iv) pH-shift protein isolate precipitated at pH 6.5 and then adjusted to pH 5.5. Before pH adjustment, the herring mince was homogenized using an Ultra Turrax T18 Basic homogenizer at 10000 rpm for a few seconds. The pH was measured directly in the solid sample and was adjusted by dropwise addition of 0.2 M HCl or 0.2 M NaOH during manual mixing.

Protein Salt Solubility. The protein salt solubility was measured in 1.0 M LiCl (pH 7.2) as described earlier[23] with some modifications.[2] When pH-adjusted samples were analyzed, the samples were incubated for 2.5 h on ice after pH adjustment before analysis to allow for slow pH-induced changes affecting the solubility of myofibrillar protein.[24] Protein salt solubility was calculated as the concentration of protein soluble in the LiCl over the total protein concentration in the sample (solubilized in 3 M NaOH).

Extended Protein Solubility Test. To distinguish between the bonds (ionic, hydrogen, hydrophobic interactions, and S−S bonds) present in the herring mince and the two protein isolates precipitated at pH 6.5 and 5.5, respectively, the method described by Perez-Mateos et al.[25] was used. The bonds were identified by sequential solubilization of the proteins in (1) 0.6 M NaCl, (2) 0.6 M NaCl and 1.5 M urea, (3) 0.6 M NaCl and 8 M urea, and (4) 0.6 M NaCl, 8 M urea, and 0.5 M *β*-mercaptoethanol. All solutions included 0.05 M phosphate buffer, pH 7.2. For each sample, 2 g was homogenized at full speed (24000 rpm) for 1 min in 10 mL of solution 1 using an Ultra Turrax T18 Basic homogenizer. The homogenate was then incubated for 1 h during shaking (300 rpm) at 4 °C. A subsample of 0.5 mL was removed and mixed with 6 M NaOH and used for analysis of total protein concentration. The remaining homogenate was centrifuged for 20 min, at 20000*g* and 4 °C, and the supernatant was then collected and used for determination of the protein concentration. The protein sediment was further solubilized in solution 2, by the same process. The process was then repeated with solutions 3 and 4, but then with incubation and centrifugation at 10 °C instead of 4 °C to avoid precipitation of the urea. To avoid interference from *β*-mercaptoethanol in the protein measurements, the proteins solubilized in solution 4 were dialyzed against distilled water using Spectra/Por Dialysis membrane 7, with a 1 kDa molecular cutoff (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA).

Electrophoresis. The polypeptide pattern was analyzed using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). All of the samples were stepwise diluted to 2 *μ*g of protein/*μ*L and then mixed with an equal amount of Laemlli buffer (Bio-Rad, Hercules, CA, USA). Five percent *β*-mercaptoethanol was freshly added into the buffer before mixing.[26] Mini-protean TGX protein gels (4−20%, Bio-Rad) were used and run according to the manufacturer's instructions. Fifteen micrograms of protein was added to each lane. Also, 10 *μ*L of a 6.6−200 kDa molecular weight standard (Protein marker III, VWR International, West Chester, PA, USA) was used. The gel was stained using Coomassie R-250 and scanned in a GS-800 Calibrated Densitometer (Bio-Rad), and the data were analyzed with the program QuantityOne 4.5.1 (Bio-Rad) to achieve molecular weight and relative quantity of the proteins.

Protein Content. Protein content was measured according to the method of Lowry et al.[27] as modified by Markwell et al.[28] A Cary50 BIO UV−visible 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 0−100 *μ*g of protein/mL. Predilutions were, when needed, made in 0.1 M NaOH. The protein content of the protein isolates is expressed on a dry weight basis. Furthermore, the yield during the second centrifugation was determined in percentage as the amount of protein in the protein isolate after centrifugation over the total amount of protein before centrifugation.

Transmission Electron Microscopy Analysis. Fixation of samples (1 × 1 × 2 mm) was carried out overnight in 2.5% glutaraldehyde in citric acid−Na_2HPO_4 buffer solutions at pH 5.5 or 6.5, depending on the sample pH, at 4 °C. The samples were rinsed in respective buffer before further fixation in 1% osmium in the same buffers for 2 h. Samples were once more rinsed thoroughly in respective buffers and then dehydrated with grading ethanol (30, 50, 70, 95, and 99%), infiltrated and embedded in LR White Resin (TAAB Laboratories, Berks, U.K.) using a Leica EM Tissue Processor (Leica Microsystems, Vienna, Austria). Thin sections were cut (60 nm) on a diamond knife by using a Powertome XL ultramicrotome (RMC Products, Boeckeler Instruments, Tuscon, AZ, USA). The sections were transferred onto Cu grids, mesh 400, and stained with uranyl acetate and lead citrate.[29] The sections were examined in TEM (LEO 906E, Oberkochen, Germany) at 80 kV and at several magnifications.

Moisture Content. The moisture content of herring mince and protein isolates was determined by drying 2 g samples overnight at 105 °C using an Electrolux 939 oven (Electrolux, Stockholm, Sweden). The balance used was a Precisa XR 405A-FR (Precisa Gravimetrics AG, Switzerland). For rapid estimation of moisture content during surimi production, a moisture balance was used (Precisa HA 300, Precisa Gravimetrics AG).

Total Fat Content. The total fat content of herring mince and protein isolates was analyzed using the method of Lee et al.[30] as modified by Undeland et al.[31] using a chloroform to methanol ratio of 1:1 (v/v, plus 0.05% BHT) for the protein isolates and 2:1 for the herring mince. The amount of lipids was measured gravimetrically after evaporation of an aliquot of the chloroform phase under nitrogen gas. Samples were also taken from the chloroform phase and the methanol−water phase and stored at −80 °C for later analyses of peroxide values (PV) and thiobarbituric acid reactive substances (TBARS), respectively.

TBARS. As described above, an aliquot of the methanol−water phase was removed during total fat extraction for TBARS analyses. An amount of 2.5 mL of this phase was mixed with an equal amount of TBA reagent and analyzed as described by Schmedes and Hølmer.[32] For quantification, a standard curve of 0−10 *μ*M MDA in methanol/water (10:8 v/v) was used.

Peroxide Value. Using an aliquot of the chloroform phase obtained during total fat content analysis, PV was analyzed according to the ferrothiocyanate method as described by Undeland et al.[31]

Quantification was done using a standard curve made from cumene hydroperoxide using a concentration range of 0−20 μM.

Color. The color of herring mince, freshly produced protein isolates adjusted to 80% moisture, and surimi gels was measured using a chroma meter CR-400 (Konica Minolta, Västra frölunda, Sweden) using the CIE $L^*a^*b^*$ scale. From the L, a*, and b^* values obtained, the whiteness (W) value was calculated:[33]

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

Protein Gel Production and Evaluation. Surimi and gel thereof were made as previously described.[2] Gel strength (g) and deformation (mm) were measured using a punch test on 25 mm gel pieces according to the method of Park.[33] For this purpose, an Instron 5542 single-column universal materials testing machine (Instron, Norwood, MA, USA) with a 500 N loading cell was used, equipped with a stainless steel $^1/_4$ in. ⌀ spherical probe (Stable Micro Systems). Running speed was 1 mm/s.

A folding test was performed according to the method of Park.[33] Furthermore, the water-holding capacity was measured as described by Kim et al.[34] by placing a 3 mm slice between filter papers under a pressure of a 3 kg weight for 1 min. The weight of the gel slice before and after the applied pressure was recorded and the pressure-induced drip expressed as percent of the total water content. Color and water content were measured as described above.

Statistics. The analyses, unless otherwise noted, were performed in triplicates ($n = 3$). For the salt solubility, at least four different samples ($n \geq 4$) of the three main sample types (herring mince, protein isolate precipitated at pH 5.5, and protein isolate precipitated at pH 6.5) were analyzed, with each analysis in triplicate. Four the four additional samples, $n = 1−4$, where each sample was analyzed in triplicate. The extended solubility test was performed in triplicate, with the protein samples analyzed in duplicates. For TEM analysis duplicates ($n = 2$) of each sample were prepared, and one to five sections from each duplicate were analyzed in the microscope.

The program SPSS Statistics 19 (SPSS, Chicago, IL, USA) was used for all statistical analyses. When two groups were compared, an independent sample t test was used, assuming equal variance. When groups of three or more were compared, one-way ANOVA followed by Tukey's test was used. Values of $p < 0.05$ were considered to be significant.

RESULTS

Protein Salt Solubility. Alkaline pH-shift processing with precipitation at pH 5.5 lowered the solubility of herring proteins in 1 M LiCl (pH 7.2) from 78% in the starting mince to about 20% (Table 1). Alkaline pH-shift processing with precipitation at pH 6.5 gave an intermediate salt solubility, which was significantly different from both the herring mince and the protein isolate precipitated at pH 5.5. Adjustment of herring mince to pH 5.5 and adjustment of protein isolate precipitated at pH 6.5 to pH 5.5 both resulted in a salt solubility drop of about 40 percentage points. Readjustment of the pH in herring mince from pH 5.5 to 6.8 or of protein isolate precipitated at pH 5.5 to pH 6.5 had no marked effect on the salt solubility.

Table 1. Protein Salt Solubility[a]

	protein solubility in 1.0 M LiCl (%)
mince at original pH, average of batches	78 ± 5 a
batch 1 (pH 6.5)	74 ± 1
batch 2 (pH 6.8)	82 ± 1
mince pH 6.8 → pH 5.5	42 ± 3 c
mince pH 6.8 → pH 5.5 → pH 6.8	47[b]
isolate precipitated at pH 5.5	19 ± 2 d
isolate precipitated at pH 6.5	59 ± 7 b
isolate precipitated at pH 5.5 → pH 6.5	17 ± 1 d
isolate precipitated at pH 6.5 → pH 5.5	23 ± 5 d

[a]Data are presented as the mean ± standard deviation. → indicates a pH adjustment. Samples were incubated at adjusted pH for 2.5 h before further adjustment or determination of salt solubility. Different letters indicate significant difference ($p < 0.01$) analyzed with one-way ANOVA followed by Tukey's test. [b]Not included in statistical analysis ($n = 1$).

The polypeptide profile of the proteins remaining soluble in 1 M LiCl, pH 7.2, after centrifugation is presented in Figure 1.

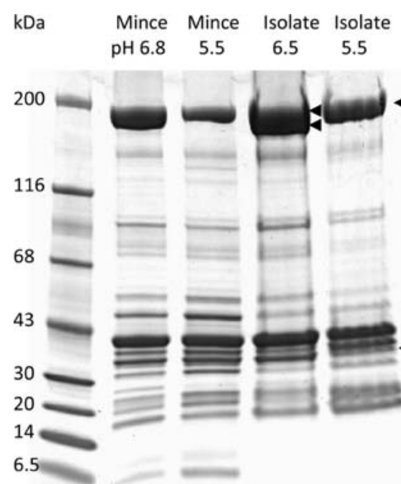

Figure 1. SDS-PAGE analysis of the proteins remaining soluble in 1.0 M LiCl, pH 7.2, following centrifugation. The samples are herring mince at original pH (6.8), herring mince at adjusted pH (5.5), protein isolate precipitated at pH 6.5, and protein isolate precipitated at pH 5.5. Arrowheads show proteins mentioned under Results or Discussion.

The solubilized proteins had a wide range of sizes and were similar for all samples. However, less myosin heavy chain (MHC, about 190 kDa) was solubilized from the two samples subjected to pH 5.5. No difference was seen in the relative abundance of actin (about 41 kDa, the second most abundant band). For the protein isolate precipitated at pH 5.5, there was also a lower relative amount of a 38 kDa protein (arrowhead) in the salt-soluble fraction.

In an attempt to distinguish between the bonds present in the herring mince and those formed after pH-shift processing, an extended solubility test was performed (Figure 2). The protein isolate precipitated at pH 6.5 had a significantly higher ($p < 0.05$) content of hydrophobic bonds and corresponding lower ($p < 0.01$) content of electrostatic bonds than the herring mince, whereas there was no significant difference in the amount of hydrogen bonds or S−S covalent bonds between these samples. The protein isolate precipitated at pH 5.5 had a higher content of hydrogen bonds ($p < 0.01$), hydrophobic interactions ($p < 0.05$), and S−S covalent bonds ($p < 0.01$) and a lower content of electrostatic bonds ($p < 0.01$) than both the herring mince and the protein isolate precipitated at pH 6.5.

Protein Microstructure. The TEM micrographs show that there were large differences between, especially, the protein isolates and the herring mince (Figure 3). In the mince from fresh herring the sarcomere structure was still intact with clearly visible Z lines. In the herring mince that had been frozen and thawed, bundles of myosin filaments were seen, although the sarcomere structure was ruptured. When the frozen/thawed

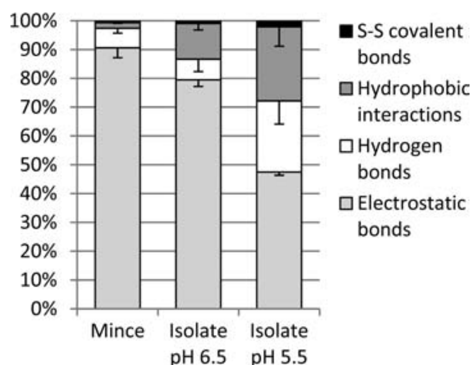

Figure 2. Relative distribution of bonds broken in herring mince at its original pH (6.8) and in protein isolates precipitated at pH 6.5 and 5.5. The bonds were identified by sequential solubilization of proteins in (1) 0.6 M NaCl, (2) 0.6 M NaCl and 1.5 M urea, (3) 0.6 M NaCl and 8 M urea, and (4) 0.6 M NaCl, 8 M urea, and 0.5 M β-mercaptoethanol.

herring mince was further adjusted to pH 5.5, there was only an indication of the former filament structure. The protein isolates were strikingly different from the minces and contained no myofibril structure, but formed a loose evenly distributed network of both very fine and denser fibrous strands as well as aggregated protein structures. Protein isolates precipitated at pH 5.5 formed a coarser and more inhomogeneous network with larger pores than proteins precipitated at pH 6.5. The coarser structure remained after the pH was raised from 5.5 to 6.5. The microstructure in protein isolates precipitated at pH 6.5 was formed of very fine fibrous strands giving rise to a relatively homogeneous network. Furthermore, protein isolates originating from unfrozen herring mince had finer strands in the network than protein isolates originating from frozen herring mince.

Protein Gelation. Both protein isolates formed strong gels, but the gels made from the proteins precipitated at pH 6.5 had a higher strength and deformability compared to the gels made from proteins precipitated at pH 5.5 (Table 2). Already during the production of surimi it was noted that the protein isolates precipitated at pH 6.5 and 5.5 behaved markedly differently. The protein isolate at pH 6.5 was difficult to press to 80% water content. It had a sculptable consistency and tended to stick to the cloth used during dewatering. The protein isolate precipitated at pH 5.5, on the other hand, had a crumbly texture, was easily dewatered, and did not stick to the cloth during the dewatering.

Effects of Precipitation pH on Protein Yield and Protein Isolate Characteristics. Precipitation at pH 6.5 instead of pH 5.5 did not result in any difference in total protein yield (Table 3). Although precipitation at pH 6.5 instead of pH 5.5 corresponded with a higher protein solubility ($14 \pm 1.3\%$ instead of $11 \pm 1.3\%$, $p < 0.05$), the protein isolate precipitated at pH 6.5 had a significantly higher moisture content (Table 3) and thus retained more of the non-precipitated proteins compared to the isolate precipitated at pH 5.5. This fraction of proteins, which remained soluble following precipitation at pH 6.5 and 5.5, respectively, is seen in Figure 4. The gel electrophoresis visualizes three proteins of 20, 23, and 37 kDa that did not precipitate at pH 6.5 but did at pH 5.5. No proteins of >53 kDa were soluble at either pH, but some actin seems to remain in solution at both pH 5.5 and 6.5.

Both protein isolates had a significantly lower lipid content than the herring mince (Table 3). However, the amount of lipid oxidation products increased in the protein isolates, and more so in the isolate precipitated at pH 5.5 than at pH 6.5.

When compared on the basis of the same water content (80%), there was a significant difference in color between the protein isolates (Table 3). The proteins precipitated at pH 6.5 were significantly redder (a^*) and yellower (b^*) and had higher lightness and whiteness values than the proteins precipitated at pH 5.5. These differences remained after production of protein gels, except that the proteins isolated at pH 5.5 produced a yellower gel (Table 2). However, both protein isolates were significantly whiter than the unprocessed herring mince.

DISCUSSION

It is clear from this study that the solubility of muscle proteins in a salt solution (1 M LiCl) is largely affected by alkaline pH-shift protein isolation (Table 1). This has been documented in several other studies.[2,7−9] There are two different parts of the alkaline pH-shift process that may induce this lowered protein salt solubility. First, it is the solubilization of the proteins during alkaline pH and adjustment back to original pH of the herring mince (pH ∼6.5). Second, it is the lowering of the pH to about 5.5 for protein precipitation. The current study shows that the lowering of the pH from pH 6.5 to 5.5 during the precipitation step causes a larger decrease in salt solubility than the solubilization at pH 11.2. A similar difference in salt solubility (about 40 percentage points) was seen between herring mince at its original pH and herring mince adjusted from its original pH to pH 5.5 as between the protein isolates precipitated at pH 6.5 and 5.5. The theory given in some previous studies[2,7−9] that structural changes, for example, denaturation and aggregation, induced during protein solubilization under very alkaline conditions, are the reason for lowered salt solubility in pH-shift-produced protein isolates thus appears to be only partly valid.

The lowering in salt solubility of proteins subjected to solubilization at pH 11.2 and precipitation at pH 6.5 (from 78 to 59%) was largely due to an increase in hydrophobic interactions (Figure 2).

The extended solubility test indicated that the protein isolate precipitated at pH 5.5 had a further increase in hydrophobic interactions as well as an increase in ionic bonds and sulfhydryl bridges compared to both the herring mince and the protein isolate precipitated at pH 6.5. These further changes were probably responsible for the additional drop in salt solubility from 59 to 19%. The microstructure of the proteins as visualized by TEM (Figure 3) also showed a coarser structure in the protein isolates precipitated at pH 5.5 compared to the proteins precipitated at pH 6.5, indicating that the increased hydrophobicity of the proteins led to aggregation. The structure did not change upon readjustment from pH 5.5 to 6.5, which was also in agreement with the results obtained by the salt solubility test (Table 1).

Native myofibrillar proteins can, in addition to being soluble in salt solutions, be soluble in pure water.[12] It has previously been shown that the post-mortem pH of the fish muscle largely affects the protein solubility in pure water.[24] A marked difference in solubility was seen between fillets having a post-mortem pH ≥6.6 and those having a pH ≤6.5. Furthermore, the solubility of fish mince that was adjusted from pH >6.6 to 6.4 or 5.8 had a lowered solubility in water. The authors suggested that the low pH induced changes in certain proteins,

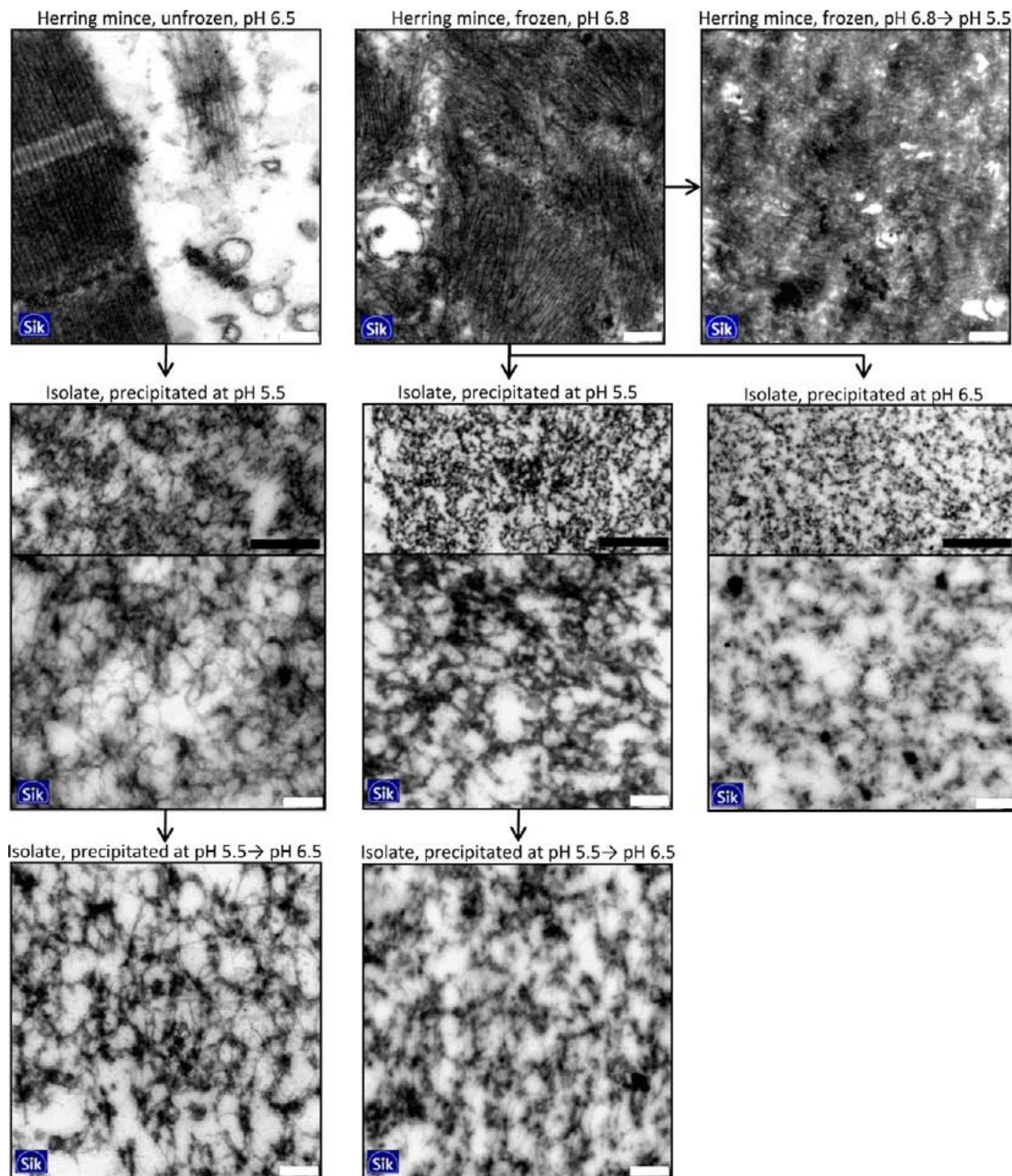

Figure 3. TEM micrographs of herring mince and protein isolates thereof. White scalebar corresponds to 200 nm and black scalebar to 1000 nm. Isolate denotes protein isolate made by the alkaline pH-shift method. → indicates a pH adjustment. Frozen means that the herring mince has been frozen, and later thawed, prior to use. No protein isolates have been frozen.

blocking the extraction of the other myofibrillar proteins. The current results, however, show a similar decrease in salt solubility when a protein mixture with no retained myofibril structure was adjusted from pH 6.5 to 5.5 as when the pH of fish mince was adjusted from pH 6.5 to 5.5. This implies that other solubility-preventing mechanisms interplay in the protein isolate and, possibly, also in the mince. The latter is supported by the fact that the minced herring in the current study had lost a large part of the myofibrillar structure after freezing and adjustment to pH 5.5 (Figure 3).

Most fish and mammalian muscle proteins have their p*I* at approximately pH 5.5, meaning that at this pH there is minimum electrostatic repulsion between oppositely charged amino acids within and between proteins. The interaction with water is thus minimized, resulting in low water solubility. Muscle proteins at this pH also have a low water-holding capacity.[35] Presumably the lowered water−protein interaction in proteins precipitated at pH 5.5 compared to pH 6.5 allows for further protein−protein interactions, both hydrophobic interactions and hydrogen bonds, due to a closer proximity and less repulsion between the proteins. Once formed, neither a neutralization of the pH or subjection to high salt concentration could break these interactions. Thus, the bonds formed restricted the salt and water to interact with the proteins, thereby giving a lowered salt solubility (Table 1).

Several studies have shown that the alkaline version of the pH-shift protein isolation process decreases the total content of sulfhydryl groups, which indicates formation of S−S bonds.[7,14,36] However, the formation of S−S bonds does not seem to be of any importance for the loss of salt solubility caused by the alkaline protein solubilization step, as the amount of proteins being solubilized when sulfhydryl bonds were

Table 2. Characteristics of Gels Made from Proteins Precipitated at pH 5.5 or 6.5 following Alkaline Solubilization[a]

	gel of protein precipitated at	
	pH 6.5	pH 5.5
gel strength (g)	1390 ± 40 a	1170 ± 110 b
deformation (mm)	13.6 ± 0.9 a	11.5 ± 0.9 b
folding (score)	5 ± 0 a	5 ± 0 a
water content (%)	71.2 ± 0.4 a	70.7 ± 0.3 a
expressible moisture (%)	2.8 ± 0.3 a	3.1 ± 0.2 a
whiteness	57.3 ± 0.8 a	63.0 ± 0.1 b
*L**	57.8 ± 0.8 a	63.0 ± 0.1 b
*a**	−1.6 ± 0.1 a	−2.4 ± 0.1 b
*b**	5.7 ± 0.3 a	6.8 ± 0.3 b

[a]Different letters within a row indicate significant differences (unpaired *t* test, $p < 0.05$).

broken did not differ between proteins precipitated at pH 6.5 and the herring mince (Figure 2). However, S−S bonds appeared to significantly contribute to the salt solubility loss taking place during protein precipitation at pH 5.5, although the values were relatively low. The slightly higher level of S−S bridges present in isolates precipitated at pH 5.5 than at pH 6.5 indicates some oxidative modifications of the proteins, possibly caused by pro-oxidative forms of hemoglobin and myoglobin, which are known to form at pH values below neutrality.[17] Furthermore, more lipid oxidation products had accumulated after precipitation at pH 5.5 compared to precipitation at pH 6.5. There were also indications of more heme protein oxidation in the protein isolate precipitated at pH 5.5 compared to that precipitated at pH 6.5 because lower redness values were seen in the former isolate (Table 3).

To further validate the results, two batches of herring were used in the current study. These batches differed in their post-mortem pH (6.5 and 6.8, respectively). In accordance with the previous study on the water solubility of proteins in relation to post-mortem pH,[24] a higher ($p < 0.05$, *t* test) salt solubility was seen for the proteins in the pH 6.8 herring batch (Table 1). Interestingly, there was no notable difference ($p > 0.05$) in salt solubility between the fresh and frozen samples from the same batches, despite the visible rupture of the myofibril structure appearing after freezing and thawing (Figure 3). It is well-known that freezing and thawing damage muscle cells.[37]

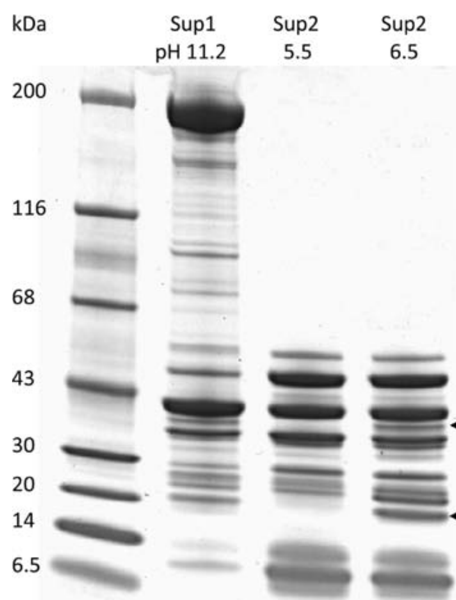

Figure 4. SDS-PAGE of proteins staying soluble in the supernatant after centrifugation 1 and 2 of the pH-shift process. Sup1, supernatant of the first centrifugation; Sup2, supernatant of the second centrifugation. Arrowheads indicate bands mentioned under Results.

Although there were differences ($p < 0.05$) in salt solubility between the two herring batches, no such differences ($p > 0.05$) were seen between the corresponding protein isolates. The starting pH of the herring mince therefore does not seem to influence the protein salt solubility of the protein isolates.

The extended solubility test (Figure 2) also contains information about salt solubility, although based on NaCl (0.6 M), instead of LiCl (1.0M), and also with a rougher homogenization. The trends in the two tests were similar, but higher absolute values were obtained with NaCl, which was in agreement with Munasinghe and Sakai.[38] It is not possible to pinpoint from these data whether higher physical disruption or another ion (Na^+ instead of Li^+) affected the solubility the most.

Although the absolute amount of protein in the isolate precipitated at pH 5.5 that was soluble in salt was low, the whole range of myofibrillar proteins was represented in the soluble fraction (Figure 1). The protein profile was similar to

Table 3. Protein Yield and Characteristics of Protein Isolates Produced by Precipitation at pH 5.5 and 6.5 following Alkaline Solubilization[a]

		protein isolate precipitated at	
	herring mince pH 6.8	pH 6.5	pH 5.5
protein yield, second centrifugation (%)		89 ± 0.9 a	90 ± 1.1 a
water content (%)	71 ± 0.4 a	93 ± 1.2 b	89 ± 1.0 c
protein content (% dw)	53 ± 2 a	102 ± 12 b	96 ± 4 b
lipid content (% dw)	50 ± 4 a	5.2 ± 1.5 b	6.8 ± 2.1 b
PV (μmol/kg (dw))	120 ± 50 a	570 ± 160 b	890 ± 90 c
TBARS (μmol/kg (dw))	4 ± 2 a	40 ± 16 b	70 ± 5 c
whiteness	53 ± 1.8 a	59.0 ± 0.7 b[b]	60.6 ± 0.3 c[b]
*L**	55 ± 1.9 a	59.8 ± 0.8 b[b]	61.6 ± 0.4 b[b]
*a**	7.7 ± 0.4 a	3.4 ± 0.2 b[b]	1.3 ± 0.2 c[b]
*b**	10.9 ± 0.2 a	10.1 ± 0.4 b[b]	8.9 ± 0.3 c[b]

[a]Different letters within a row indicate significant differences ($p < 0.05$). dw, dry weight. Characteristics of the herring mince are included for comparative purpose [b]At 80% water content.

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that obtained for proteins solubilized at pH 11.2 (sup 1, Figure 4). One apparent difference between the salt-soluble fractions from samples subjected to pH 6.5 and 5.5 was that less MHC was salt soluble in samples adjusted to pH 5.5. This was the case for both herring mince and protein isolate. MHC therefore seems to be the protein most involved in the "insolubilization interactions" formed at pH 5.5. Similarly, a decrease in protein salt solubility after long-term storage at −20 and −30 °C of hake (*Merluccius merluccius*) fillets was linked to a lower proportion of MHC being soluble.[11] Furthermore, there are two herring MHC isoforms with different molecular weights, where the larger isoform is found in the dark muscle and the smaller isoform is found in the light muscle.[39] Although these MHC isoforms are not well separated in Figure 1, the densitometer profiles (not shown) revealed that it was mainly the larger isoform that was soluble after exposure to pH 5.5.

The protein profile shown in Figure 1 is very different from what was previously reported by Tadpitchayangkoon and Yonsawatdigul.[7] These authors showed that the protein fraction of striped catfish protein isolate that was soluble in 0.6 M NaCl contained only trace amounts of actin and myosin, but had a large abundance of a 38 kDa protein. It appears that differences in the salt solubility determination method (e.g., Na^+ vs Li^+), between fish species, or in the execution of the pH-shift method can result in a large difference regarding which proteins of a pH-shift-produced protein isolate are salt soluble.

The salt solubility test has been considered an index of general protein functionality.[12] In this study the gelation capacity of herring proteins with different salt solubilities was evaluated. A higher salt solubility indeed indicated improved gelation, with higher gel strength and elasticity in the gels made from proteins precipitated at pH 6.5 (Table 2). When the microstructure of the protein isolates was compared before the production of gels, the proteins precipitated at pH 5.5 contained more aggregates and larger pores, whereas the proteins precipitated at pH 6.5 had a somewhat more homogeneous structure. This more homogeneous network with smaller mesh size (and more strands per volume to take up the force) is suggested to facilitate gel formation, resulting in higher gel strength (Figure 3). It is possible that use of unfrozen herring for pH-shift processing and subsequent surimi and gel production would have given even better gels as fresh herring resulted in protein isolates with finer strands. In addition to the indicated improved gelation capacity, the proteins precipitated at pH 6.5 also retained more water upon centrifugation and manual pressing, suggesting a better water-holding capacity. This might have been a result of the already mentioned smaller pores and mesh size. However, there was no difference in water content or expressible moisture in the final protein gels.

The use of a precipitation pH resulting in as low protein solubility as possible has been an unquestioned standard for the pH-shift method to maximize yields. However, the current study shows that the protein yield is not necessarily affected by using a higher pH value for protein precipitation. In fact, no significant difference in protein yield was seen between precipitation at pH 5.5 and 6.5. The reason for this was that a larger quantity of soluble proteins was retained in the protein isolate at pH 6.5, due to its higher water content (Table 3). If the protein isolate should be further dewatered by pressing, these soluble proteins would be lost and a difference in yield would presumably be seen. However, in applications when the water content of the protein isolate is of low concern, precipitation at pH 6.5 can have several advantages. In addition to the higher solubility of the proteins, better gelation properties, less lipid oxidation, and higher redness (i.e., less metHb/metMb formation) of the isolate, less acid is used to neutralize the proteins after solubilization and less base is also used to neutralize the proteins in a gelation process.

To conclude, the salt solubility of protein isolates made with the pH-shift method was more influenced by the precipitation at pH 5.5 than by the solubilization at pH 11.2. The decrease in pH from the native pH 6.5 to 5.5 had a major influence on the salt solubility of both herring mince and protein isolate made thereof. The lowered salt solubility in protein isolates precipitated at pH 5.5 was mainly due to increased hydrophobicity and formation of hydrogen bonds and was accompanied by altered protein microstructure and gelation properties. However, the microstructure of the proteins was much more influenced by the solubilization process than by the lowering of the pH during precipitation, and myofibrillar structure was lost after the solubilization/precipitation process.

The large changes in salt solubility seen as a function of precipitation pH corresponded to only small differences in gelation properties. The salt solubility test is therefore thought to reflect formation of hydrophobic and hydrogen bonds more than microstructural changes of the proteins, at least in samples that are as heavily restructured as the pH-shift-produced protein isolates.

Protein precipitation at pH 6.5 as a modification of the original alkaline pH-shift method[1] showed great promise, as it did not influence the protein yield, but still gave higher salt solubility, a more homogeneous microstructure, less lipid oxidation, and better gelation properties than proteins precipitated at pH 5.5.

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