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Characterization of Oxidative Changes in Salted Herring (*Clupea harengus*) During Ripening

EVA ANDERSEN,[†] MOGENS L. ANDERSEN,[‡] AND CAROLINE P. BARON*,[†]

Department of Seafood Research, Danish Institute for Fisheries Research, Technical University of Denmark, Building 221, DK-2800 Kgs. Lyngby, Denmark, and Food Chemistry, Department of Food Science, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

Salted herring were prepared in barrels according to a traditional recipe. The biochemical changes in the fish and in the brine were monitored during a prolonged ripening period (12 months). The process was followed by measuring pH, protein, salt, dry matter, free fatty acids, and lipid content in the brine and in the fish according to standard protocols. The results showed that most of the biochemical changes occurred at an early stage in the ripening process. Lipid oxidation was followed in the fish muscle using spectroscopic determination for lipid hydroperoxide (PV) and by GC-MS for determination of secondary oxidation products. Protein oxidation was determined using spectrophotometric determination of protein carbonyl groups. To follow protein degradation (proteolysis) and protein oxidation SDS-PAGE and immunoblotting for protein carbonyl were performed on both brine and fish during the ripening period. Results revealed that no lipid oxidation occured in fish muscle during ripening but a significant level of protein oxidation was detected. Finally, iron α-tocopherol, and 3-methylbutanal levels were also measured. Alpha-tocopherol levels decreased during ripening, further supporting that oxidative reactions took place. Peroxidase activity was demonstrated in the brine, suggesting that hemoglobin might be a crucial parameter, which might trigger protein oxidation. This indicates that protein oxidation might be important for the development of the characteristic organoleptic properties of salted herring.

INTRODUCTION

Salting of fish is a traditional preservation process and a common practice in many European countries. In Northern Europe, herring are commonly salted, whereas in Southern Europe anchovies and sardines are usually salted. The traditional production of salted herring is a process that takes several months, and the specific recipes are often based on a long tradition and a unique "savoir faire". Briefly, whole herrings are placed in barrels with salt, 24 h after a natural brine (or blood brine) is formed. Subsequently, the barrels are filled up with saturated brine and stored for up to 12 months at chilling temperature and the product is allowed to ripe. The blood brine and brine are believed to be important for the development of the characteristic organoleptic properties of salted fish during ripening. During this long ripening period many chemical and biochemical changes take place leading to the development of the characteristic texture and taste of salted herrings.

Today production methods are still based on experience, and as the ripening process is not fully understood, obtaining a high quality product relies on highly trained and qualified staff. Considerable research has been carried out regarding the salting process (*1*, *2*), but little is known about the biochemical changes that take place in the fish muscle (*3*). Understanding of the crucial factors governing the ripening process of salted herrings is still limited. Most of the research has concentrated on protein degradation occurring during ripening and on the activity of proteolytic enzymes (*4*, *5*). It is now generally accepted that both intestinal and muscle proteases are partially responsible for some of the changes in muscle texture observed during the ripening period of salted herrings (*6*). However, other aspects have been overlooked, as some of the changes observed in the fish muscle during ripening can not only be explained by the activity of enzymes. For example the significant toughing observed in fish texture during ripening cannot be associated with proteolysis of the muscle protein. In addition, herring contain high levels of unsaturated fatty acid and are susceptible to severe oxidation, and this aspect deserves further attention. The high concentration of salt has been shown to prevent microbial spoilage in similar products (*7*) but salt has also an impact on muscle tissue structure by inducing swelling and aggregation (*8*). In addition, salt in muscle food has been demonstrated to have either pro- or antioxidative effects (*9*), but the possible role of salt being a pro- or an antioxidant during the ripening process has never been investigated. It has also been documented that only fatty herrings caught at the right season give high quality products (*10*); therefore, it can be expected that some oxidative processes may also play a role in

* Corresponding author phone: (+45) 45 25 49 19; fax: (+45) 45 88 47 74; e-mail: cba@dfu.min.dk.
[†] Technical University of Denmark.
[‡] University of Copenhagen.

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the production of high quality salted products. Furthermore, during the production of salted herring, the fish are not bled and the brine consequently contains a large proportion of blood, which itself contains high level of prooxidants such as metals and hemoglobin (Hb). Iron and heme proteins are known to be strong prooxidants in muscle foods (*11*, *12*). It has been shown that in herring, Hb is a crucial parameter that leads to rapid quality loss (*13*). However, no details about the prooxidative activity of Hb under ripening conditions exist, but it is possible that even under such conditions Hb is able to trigger oxidation and might therefore be involved in the development of the characteristic organoleptic properties of salted fish. A recent report indicates that the prooxidative activity of heme proteins such as Hb is greatly enhanced under denaturing conditions (*14*). Additionally, heme proteins can be activated by hydrogen peroxide to pro-oxidative iron IV ferryl species, which have been shown to be strong prooxidants (*15*, *16*). These activated heme proteins can induce oxidation to a wide range of cellular components and to promote protein oxidation leading to protein cross-linking (*17*). Therefore, the role of Hb in the ripening process deserves further attention, especially due to the fact that no details about the role of Hb in the ripening process exist despite the fact that producers of salted herring recognize that the blood brine is a critical parameter in the ripening process.

This investigation aimed at examining the role of oxidative reactions during ripening of salted herring, and to reveal to which extent they are involved in the development of the characteristic organoleptic properties of salted herrings.

MATERIALS AND METHODS

Materials. All chemicals and reagents were of analytical grade and were purchased from either Merck (Darmstadt, Germany) or Sigma (St Louis, MO). Unstained low molecular weight protein standard, Mark 12 and See-Blue prestained standard were purchased from Invitrogen, (Carlsbad, CA).

Salted Herring Production. Herrings (*Clupea harengus*) caught in the Baltic Sea by commercial vessels were obtained from Lykkeberg A/S (Hørve, Denmark). The fish had an average weight of 230 g (±20) and were processed approximately 24 h *post mortem*. Salted herrings were prepared according to the Lykkeberg A/S production protocol. Briefly, 100 kg of intact and whole herrings were dry salted with 12 kg coarse salt (99% NaCl, Pioner rocksalt 2, (Brøste A/S, Kgs Lyngby, Denmark)) and placed in a 100 L barrel for 24 h at 1 °C. After 24 h, a natural brine, also called blood brine, was formed; subsequently, the fish and the brine were equally divided into 15 small barrels. Each barrel contained 5.8 kg herring, 1.6 kg blood brine, and 0.6 kg fresh saturated brine (total 8 kg). The brine covered the herring completely, and no headspace was present in the barrels. The barrels were stored for up to 371 days at approximately 0 °C and samples of fish and brine were analyzed throughout the entire ripening period by analyzing the brine and fish from a barrel after 2, 17, 32, 45, 85, 151, 207, and 371 days. Due to the length of the ripening period (12 months) the experiment was carried out only once. At each time point, the brine was collected, and the fish (in triplicate) were filleted. Only fish with intact muscle tissue presenting no bruises or cuts were used for the analysis. Fish samples were divided into smaller pieces (equivalent pieces from the same location on each fish were used for the same analysis), vacuum packed, and stored at −80 °C until further analysis.

Biochemical Characterization. *pH.* The pH was determined using 0.5 g of herring muscle minced and suspended in 5 mL of deionized water. The pH of the brine was measured directly without dilution.

Lipid Content. Herring muscle (50 g) was minced and 10 g (in duplicate) were used for lipid content determination. The herring muscle lipids were extracted using a chloroform and methanol mixture (ratio 1:1) according to the protocol of Bligh and Dyer (*18*), but with a reduced amount of solvent (*19*). The lipid content was determined gravimetrically. The lipid extracts were immediately used for subsequent analysis of the peroxide value, the fatty acids, and the antioxidants.

Protein Content. The crude protein content of fish and brine was analyzed using the Kjeldahl nitrogen determination. Samples of approximately 2 g of muscle and 5 g of brine were used and the crude protein content was determined as Kjeldahl-protein using a factor 6.25 following the AOAC standard method (*20*).

Dry Matter. The dry matter of fish and brine was determined by weighing after drying a sample of approximately 2 g at 102–105 °C for 20 to 24 h according to the AOAC standard method (*21*).

Iron. The total iron content of fish and brine was determined using an atom absorption spectrophotometer 3300 (Perkin Elmer, Waltham, MA). Herring muscle or brine sample of 2.0 g were mixed with 5.0 mL concentrated HNO_3 and 2.0 mL H_2O_2 and the samples were destructed using a microwave oven (CEM, MDS 81D, Matthews NC). After destruction, the samples were analyzed according to the protocol from the Nordic Committee on Food Analysis (*22*). Free iron was determined using ferrozine color complex, which bind iron at all valences, and reading the absorbance at 562 nm (*23*). The iron concentrations were determined using a Fe^{2+} standard curve (0–20 μg).

NaCl Content. The salt content was determined in the fish muscle and in the brine by potentiometric titration of chlorine ions using $AgNO_3$ and according to the AOAC standard method (*24*).

Fatty Acid Composition. Fatty acids in the lipid extract were transesterified to methyl ester using a base catalyzed transesterification followed by a boron–trifluoride catalyzed esterification according to the AOCS method (*25*). The methyl esters were dissolved in *n*-heptane to a concentration of about 20 mg/ml. A HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector was used for separation of the fatty acid methyl esters. The column was an Omegawax 320 fused silica capillary (0.32 mm × 30 m × 0.25 μm) (Supelco, Belafonte, PA). The injection volume was 0.2 μL injected in the split mode with a split ratio of 1:50. The injection and detection temperature program were 250 and 240 °C, respectively. The initial oven temperature was 160 °C, immediately raised by 3 °C min^{-1} to 200 °C, held for 1 min, further raised by 3 °C min^{-1} to 220 °C and held for 12 min. The helium carrier gas flow was 21 cm s^{-1}. An internal standard, methyl tricosanoate (C:23), was used to calculate the relative percentage of the different fatty acids in the lipid fraction.

Free Fatty Acid (FFA). FFA content was determined by acidometric titration using NaOH (0.1 M) of the lipid extract. The FFA content was calculated as oleic acid according to the AOCS method (*26*).

Antioxidant. Tocopherol content of the fish muscle was determined on the lipid extract using an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA), equipped with a fluorescence detector. A fraction of the lipid extract was evaporated under nitrogen and redissolved in 2 mL *n*-heptane before injection of an aliquot (40 μL) on a Spherisorb s5w column (250 mm × 4.6 mm) (Phase Separation LtD, Deeside, UK). Elution was performed with an isocratic mixture of *n*-heptane/2-propanol (100: 0.4; v/v) at a flow of 1 mL min^{-1}. Detection was done using a fluorescence detector with excitation at 290 nm and emission at 330 nm and according to the AOCS method (*27*). Only the alpha-tocopherol isomer was detected in the herring samples.

Lipid Oxidation. *Peroxide Values.* Peroxide values were measured on the lipid extract by colorimetric determination using the ferric thiocyanate assay (*28*).

Volatiles. Volatiles were collected from the herring muscle by dynamic headspace sampling. The samples were prepared according to the protocol described by Refsgaard et al. (*29*). Briefly, fish pieces were frozen in liquid nitrogen and blended into a fine powder. The powder (20 g in triplicate) was slurried in water and the sample was purged with nitrogen at 340 mL/min for 10 min at 37 °C. The volatiles were trapped on Tenax-GR traps column (Varian Chrompack International, Bergen op Zoom, The Netherlands) and volatiles were released from the column by thermal desorption (ATD-400, Perkin-Elmer, MA) and analyzed by GC-MS on a 30-m DB 1701 capillary column (J & W Scientific, Folsom, CA). The temperature program used was 40 °C for 10 min, ramping 3 °C min^{-1} to 140 °C, 5 °C min^{-1} to 170 °C and 10 °C min^{-1} to 240 °C final temperature. The ionization energy was

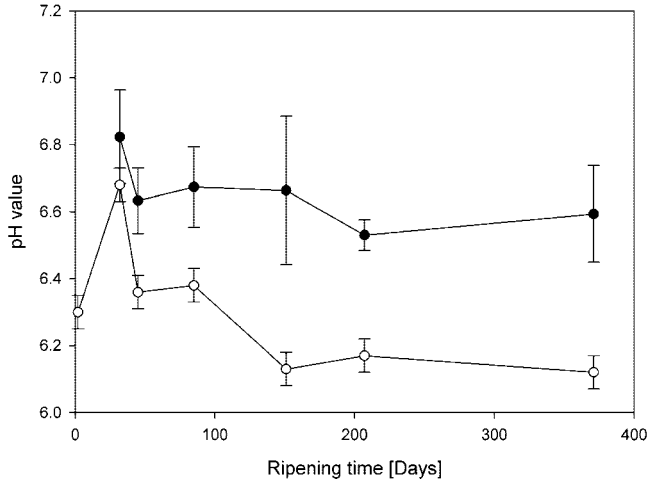

Figure 1. pH value of brine (-○-) and herring (-•-) during the ripening period.

set to 70 eV in the IE and the scan range from 30 to 250 atomic mass units with repetition rate at 3.4 scans per s. For quantification, standards were directly injected to the Tenax-GR traps column, and analyzed as described above for the samples.

Protein Oxidation. *Spectrophotometric.* Protein carbonyl groups were measured as described by Levine et al. (*30*, *31*). A fish sample (0.5 g) was homogenized in 10 mL Tris buffer (pH: 7.4, 50 mM, 1 mM ethylendiaminetetra acetic acid (EDTA)) containing 0.01% butylated hydroxytoluene (BHT). For spectroscopic determination of carbonyl groups 100 μL of the homogenate was precipitated with 10% trichloroacetic acid (TCA) (v:v). After centrifugation (12600*g*, 3 min) the pellet was incubated with 500 μL dinitrophenylhydrazine (DNPH) in 2 M HCl, in the dark for 30 min. For each sample a blank incubated in 500 μL 2 M HCl and without DNPH was run in parallel. The samples were precipitated with 10% TCA (v:v) and the pellets were washed three times with 1 mL ethanol/ethyl acetate 1:1 (v/v). The pellet was redissolved in 6 M guanidine chloride in 20 mM KH_2PO_4. The carbonyl content was calculated using the absorbance measurements at 370 and at 280 nm

SDS-PAGE. Protein concentrations in the fish muscle homogenates prepared above and in the brine were adjusted to 1 mg/mL using the Bradford reagent for protein determination. Thereafter the samples were mixed with Laemmli sample buffer 1:1, (v:v). Fish muscle samples were (20 μL, 10 μg protein) loaded onto a 10% NuPage Bis-Tris gel (Invitrogen A/S, Taastrup, Denmark) and run with MOPS (4-morpholinepropanesulfonic acid) running buffer. Brine samples (10 μL, 5 μg protein) were loaded onto 4–12% NuPage Bis-Tris gel and run with MES (4-morpholineethanesulfonic acid) running buffer. After the run, the gels were stained with coomassie brilliant blue G-250.

Protein Carbonyl Immunoblot. Carbonyl groups on the protein were detected using the protocol described by Levine for immunoblotting (*30*). Prior to DNPH labeling the protein concentration in the fish muscle homogenate was adjusted to 5 mg/mL using the Bradford reagent for protein determination. Subsequently, samples (30 μL) were mixed 1:1 with 12% SDS and further diluted with 60 μl DNPH in 10% trifluoroacetic acid (TFA). The samples were incubated for 15 min in the dark at room temperature. The reaction was stopped by adding 60 μL neutralizing solution (1.85 M Trizma-base, 28% glycerol and 0.1 M DTT). The samples were centrifuged for 3 min at 12600*g* and 20 μL (16.6 μg protein) loaded onto the gel (10% NuPage Bis-tris, Invitrogen A/S). The proteins were transferred to polyvinylidene difluoride (PVDF) membrane 0.2 μ (Millipore, Billerica, MA) using a Mini Cell SureLock, equipped with a XCell II blot module (Invitrogen A/S, Taastrup, Denmark) for 60 min. After transfer the membranes were blocked in 5% skimmed milk in Tris-buffered saline (TBS; 0.137 M NaCl and 20 mM Tris-HCl, pH 8.0), and incubated with a 1:15000 dilution of rabbit anti-DNP (DAKO Denmark AS, Glostrup, Denmark) in 1% skim milk, in TBS for 1 h. The membranes were washed in TBS and incubated in a 1:7500 dilution of the secondary antibody, peroxidase-conjugated swine anti-rabbit (DAKO Denmark A/S, Glostrup, Denmark). After washing in TBS the blot was developed using the ECL+ kit (Invitrogen A/S, Taastrup, Denmark). Chemiluminescence was detected on hyper-film ECL (Amersham Health AS).

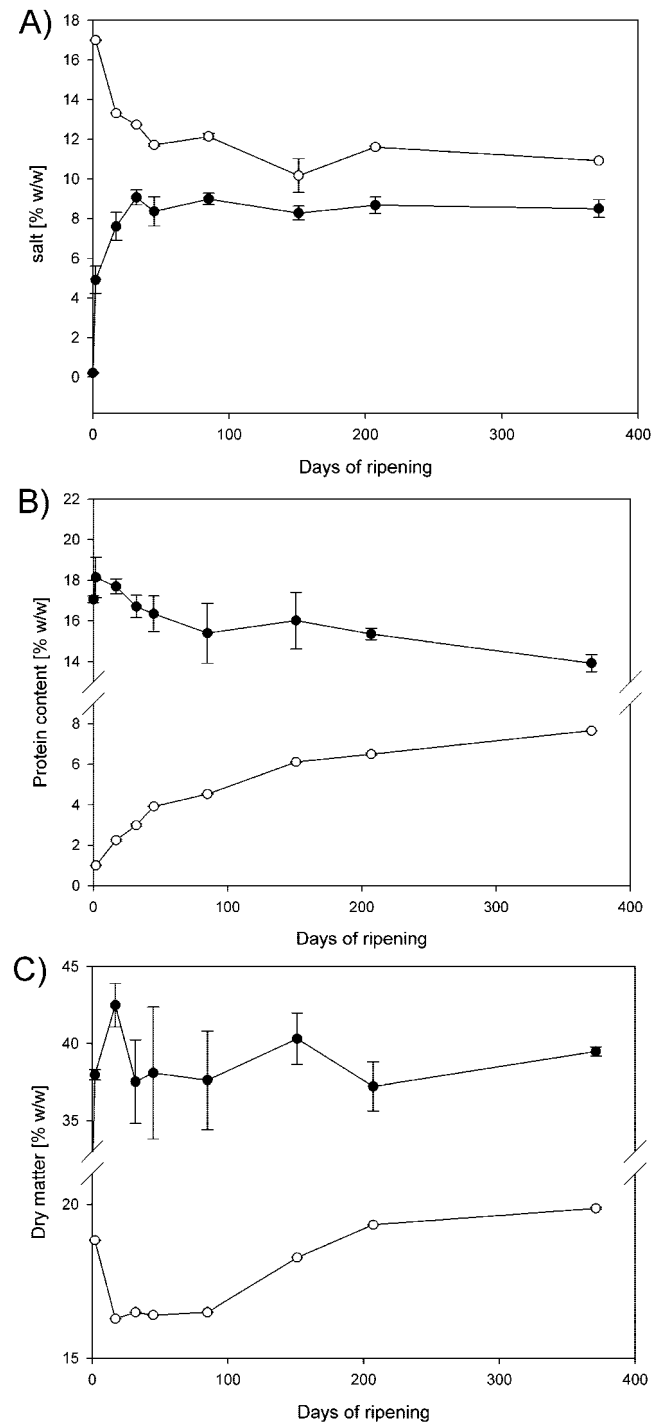

Figure 2. (**A**): Salt (Cl^-) content of brine (-○-) and herring (-•-), (**B**): Protein content (Kjelldahl) of brine (-○-) and herring (-•-), and (**C**): Dry matter of brine (-○-) and herring (-•-) during the ripening period.

Enzymatic Activity. *Proteolytic Activity.* The proteolytic enzymes activity was determined using a method described by Sarath et al. (*32*). Approximately 2.0 g of fish muscle or brine were homogenized in 8.0 mL distilled water, mixed for 30 min on ice and subsequently centrifuged at 5 °C at 16000 *g* for 15 min. The supernatant was collected, filtered on paper, and 200 μL were mixed with 300 μL assay buffer (Tris buffer, 50 mM, pH 8.0, and containing 0.25 g/L chlorohexidine) and with 500 μL substrate (2% azo-casein in assay buffer). The samples were incubated for 24 h exactly and the reaction was stopped using 2 mL TCA (10%). The precipitate was removed by

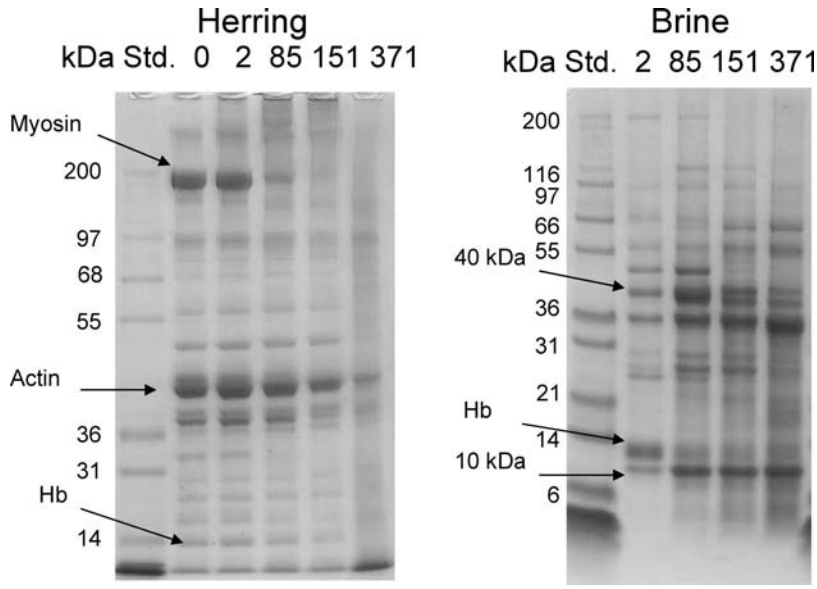

Figure 3. SDS-PAGE. Herring samples (days: 0, 2, 85, 151, 371) and Mw standard (Mark 12) run on 10% Bis-Tris gel with MOPS running buffer. Brine (days; 2, 85, 151, 371) and Mw standard (Mark 12) on 4–12% Bis-Tris gel run with MES running buffer.

centrifugation for 20 min at 4000*g*, proteolytic activity was measured in the supernatant by reading at 450 nm on a HP lambda 2 spectrophotometer (Hewlett Packard, Palo Alto, CA). The activity was defined as $\Delta Abs_{450\ nm}$/mL/hour.

Detection of Peroxidase Activity. The peroxidase activity in the brine was determined. Samples of 2 μL brine were deposited on a nitrocellulose membrane. Thereafter, the membrane was dried at room temperature and subsequently incubated in 1% milk for 1 h and washed three times with TBS. The positive controls were horseradish peroxidase conjugated immunoglobulins (Dako, Denmark A/S, Glostrup, Denmark) diluted 1:5000 in TBS. The peroxidase activity was revealed using 2.7 mM H_2O_2, *p*-coumaric acid (0.07 mM) and luminol (1.25 mM) in Tris-HCl buffer (pH: 8.6; 0.1M). Chemiluminescence was detected on hyperfilm ECL (Amersham Health AS). Approximately 45 min after the first activation cycle of the peroxidase a second activation cycle was performed by applying the reagents (H_2O_2, *p*-coumaric acid and luminol) one more time to the membrane.

Data and Image Analysis. The measurements were carried out at least on triplicate samples (unless otherwise stated). The results are given as mean values of triplicates with indication of the standard deviation. Evaluation of statistical significance of differences was performed using the Student's *t* test. Image analysis was performed using Quantity One from Bio-Rad (Bio-Rad, Herlev, Denmark).

RESULTS

Biochemical Characterization. The pH in the fish muscle was relatively stable throughout the entire ripening period (**Figure 1**). The pH in the brine was lower than in the fish, which is probably due to the difference in ionic strength. A slight decrease in the pH in the brine was observed during the ripening period, and from day 151 and onwards it had stabilized around 6.1. The salt was progressively transferred from the brine, which was initially saturated, to the herrings (**Figure 2A**). The salt content of the herring muscle was equilibrated within the first two months. Thereafter, the salt content in both the herrings and the brine remained constant for the rest of the ripening period. At the same time there was a transport of soluble matter (mainly proteins and peptides) from the herring to the brine (**Figure 2B**). The protein content in the brine increased throughout the entire ripening period i.e. from around 1% at day 2 to 8% at day 371, with a fast initial increase before day 100. The protein content of the herring muscles slowly decreased from 18% at day 0 to 14% at day 371. The dry matter content of the herring muscles was stable during the ripening

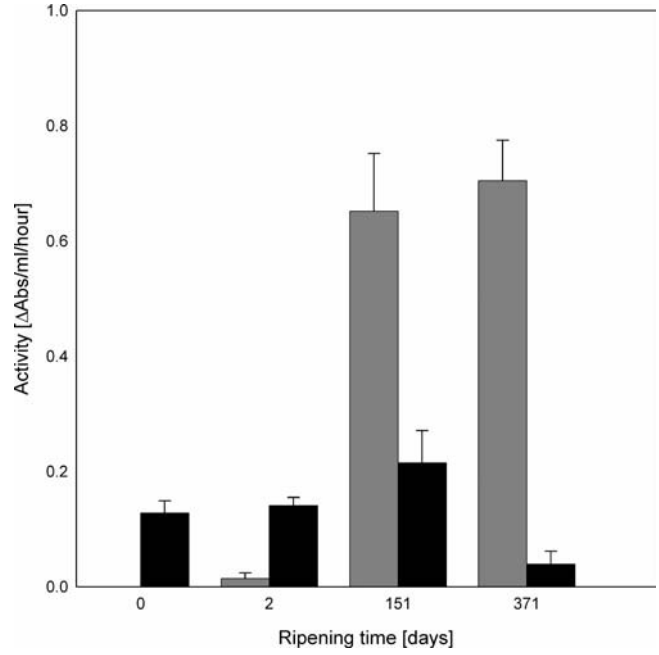

Figure 4. Activity of proteolytic enzymes in brine (gray) and herring (black) at ripening time day 0, day 151, and day 371, measured using azocasein as substrate.

period, albeit with very high standard deviations, which illustrate that herring was a more heterogeneous material compared to brine (**Figure 2C**). Indeed, the lipid content of the herrings, which is a part of the dry matter varied from 8.2 to 19.8% with an average of 14.1% (data not shown). The constant dry matter content in the herring muscle was probably due to the increase in the salt content which counteracted the loss of protein over time. The dry matter content of the brine increased with ripening time from 16 to 20 %.

Protein Degradation. The proteins in herring muscle homogenate and in the brine were analyzed by SDS-PAGE throughout the entire ripening period (**Figure 3**). Structural proteins such as actin (42 kDa) and myosin (200 kDa), which were found in high concentration in the raw fish, almost completely disappeared during ripening. In addition, high molecular weight aggregates appeared as seen at day 85 and

Table 1. Fatty Acid Composition Expressed as % of Total Fatty Acids for the Fresh Herrings ($n = 3$) Used for the Salting Experiment (±SD)

fatty acid	%
C14:0	6.47 (±0.16)
C16:0	11.11 (±0.32)
C18:0	0.94 (±0.02)
Σ saturated	18.52 (±0.88)
C16:1(n-7)	4.68 (±0.93)
C18:1(n-7)	1.32 (±0.11)
C18:1(n-9)	6.94 (±0.37)
C20:1(n-9)	11.37 (±1.15)
C22:1(n-11)	19.50 (±0.97)
Σ MUFA	43.81 (±3.47)
C18:2(n-6)	1.52 (±0.17)
C18:3(n-3)	1.26 (±0.16)
C18:4(n-3)	2.81 (±0.37)
C20:4(n-3)	0.60 (±0.03)
C20:5(n-3)	6.12 (±0.24)
C22:5(n-3)	0.79 (±0.07)
C22:6(n-3)	11.09 (±1.65)
Σ PUFA	24.19 (±1.87)
others[a]	13.48 (±0.82)

[a] Fatty acids that were not identified.

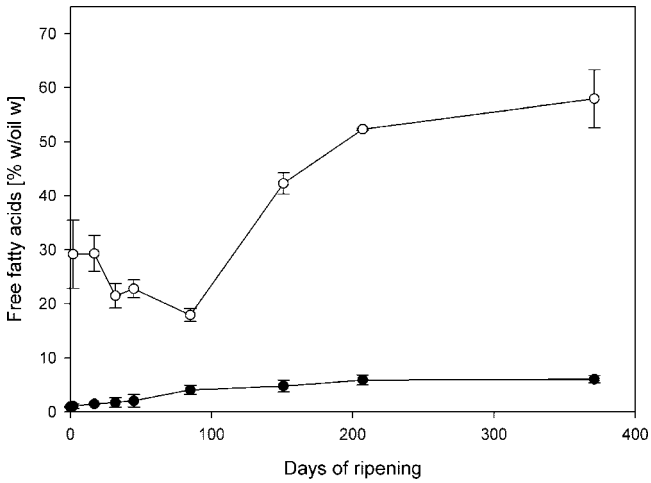

Figure 5. Content of free fatty acids in brine (-○-) and herring (-•-) during the ripening period.

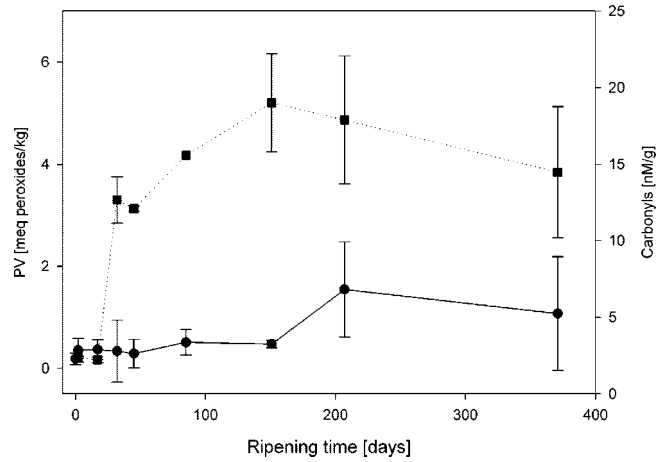

Figure 6. Development of peroxide value, PV, [mequivalent peroxide/kg] (-•-) and of protein carbonyl [nM/g protein] (■) in herring during the ripening period.

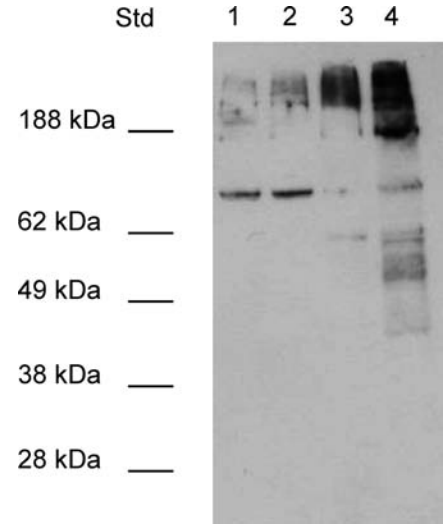

Figure 7. Immunoblot for protein carbonyl groups in salted fish during ripening. With 1) 2 days, 2) 45 days, 3) 151 days and 4) 371 days of ripening. Std: Mw standard, Sea Blue.

151, while the myosin band was missing after 151 days and the actin band was fading with ripening time. After 371 days of ripening the lane was almost a smear of proteins and peptides, with no distinct protein bands. More and more protein/peptides with low molecular weight (below 55 kDa) were found in the brine with increasing ripening time. Band with molecular weight of 40, 36, and 26 kDa appeared and represented 50% of the total protein at day 85. The characteristic band of Hb subunits was very faint in herring muscle and only represented 3–4% of the total protein at day 2. In contrast, it was observed that at day 2, Hb represented approximately 25% of the total protein in the brine, and thereafter the Hb band appeared rather smeared and was difficult to quantify. The proteolytic activity of enzymes in the herring was relatively low and around 0.2 ΔAbs/ml/hour at day 0 and close to 0 ΔAbs/ml/hour at day 371 (**Figure 4**). In contrast, the activity of the proteolytic enzymes in brine was much higher and around 0.6–0.7 ΔAbs/ml/hour at day 151 and 371.

Oxidation. The herrings used in the ripening experiment had the characteristic fatty acid profile with a high level of PUFAs, which are prone for oxidation (**Table 1**). The relative composition of fatty acids did not change during the ripening period (data not shown). The average fat content of the herrings used in the experiment was around 14% and the level did not change significantly during the ripening period. The fat content in the brine was null at the beginning of the ripening period and reached 0.2% at the end. The free fatty acid content increased steadily from 1% in the raw herrings to 6% after 371 days of ripening (**Figure 5**). For the brine the free fatty acid content was relatively constant, 20–30% for the first 100 days and thereafter, an increase was observed and the level reached 57% at day 371.

The lipids in the herrings were not extensively oxidized during the ripening period as judged by the modest increase in the peroxide value from 0.4 to 1.1 meq peroxide/kg at the end of the ripening period (**Figure 6**). The development of secondary lipid oxidation products such as volatiles were studied by dynamic headspace GC, however, none of the volatiles characteristic of oxidation of the unsaturated fatty acids such as propanal, pentanal, and hexanal, were detected at any significant level in herring and in brine during ripening (data not shown).

In contrast to the lipids, measurement of protein carbonyls, which is an indication of protein oxidation, revealed that the carbonyl content in the herrings increased dramatically from day 2 to 17 with a steady but much slower increase up to day 151. The protein carbonyl level decreased thereafter probably

Table 2. Iron and Alpha-Tocopherol Content in Herring Muscle ($n = 3$) and in Brine ($n = 2$) during Ripening Expressed in ppm (±Standard Deviation)

	iron in herring		iron in brine		
day ripening	total [ppm]	free [ppm]	total [ppm]	free [ppm]	α-tocopherol in herring [ppm]
0	8.18 (±2.92)	2.21 (±0.29)	na	na	25.35 (±0.80)
2	8.54 (±4.87)	2.20 (±0.25)	6.41 (±0.07)	0.48 (±0.03)	24.54 (±2.72)
85	na[a]	2.07 (±0.45)	7.97[b] (±0.07)	1.85 (±0.51)	18.52 (±7.71)
151	8.25 (±2.30)	1.52 (±0.33)	10.42[b] (±0.06)	1.67[b] (±0.01)	na
371	8.54 (±0.60)	na	8.63[b] (±0.06)	na	12.98[b] (±2.73)

[a] na, nonanalyzed; [b] $p < 0.01$ comparing with day 0 for the herring samples and with day 2 for the brine.

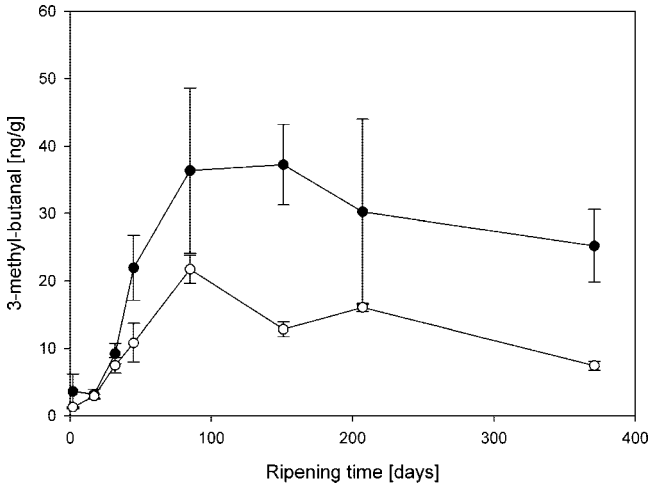

Figure 8. Development of 3-methyl butanal in brine (-○-) and herring (-•-) during the ripening period.

due to extensive breakdown of proteins as seen from the SDS-PAGE and increasing amount of TCA nonprecipitable proteins (**Figure 3**). It was not possible to perform a total protein carbonyl determination in the brine due to the large amount of nonprecipitable proteins and protein fragments. Immunoblot for oxidized proteins in fish during the ripening period indicated that proteins with molecular weight above 40 kDa are oxidized in salted fish during ripening but that proteins or protein aggregates with molecular weight above 188 kDa are more heavily oxidized with prolonged ripening time (**Figure 7**). This also confirmed the data obtained from the spectroscopic determination indicating a significant increase in protein oxidation during ripening (**Figure 6**). The total and free iron contents were steady in the herring muscle during ripening and were around 8–9 ppm and 2 ppm, respectively. In contrast, the iron level in the brine was increased up 10 ppm during the first 151 days, and then leveled off at 9 ppm after 371 days (**Table 2**). Similarly, the free iron level increased significantly in the brine and reached a maximum at 85 days. The concentration of the natural antioxidant, α-tocopherol present at the membrane in the herring muscle decreased from around 25 ppm to 13 ppm after approximately 351 days during ripening (**Table 2**). The decrease was already significant after 207 days of ripening (not showed). Leucine is converted during oxidation to 3-methyl butanal and the levels of this volatile was detected using GC-MS and was found to be increasing during ripening (**Figure 8**) from 1 ng/g sample at the beginning of the ripening period, reaching a maximum at approximately 100 days, and thereafter stabilized at around 30 ng/g for herrings and at 10–15 ng/g for brine. A blot revealing peroxidase activity in the brine during ripening is presented in **Figure 9**. Peroxidase activity is already present at day 2 in the brine. Peroxidase breakdown can occur during activation, therefore a second activation cycle was performed in order to examine if there was any remaining activity after activation. The second activation cycle demonstrated that the peroxidase activity persisted in the brine after 45 days ripening.

DISCUSSION

Most of the studies investigating changes in salted fish during ripening have looked at enzymatic degradation of the fish protein by proteolytic enzymes from the intestine and the muscle tissues (*4*, *5*). However, there is still a significant lack of understanding of the biochemical changes taking place during ripening. The aim of this study was to investigate biochemical and oxidative changes in salted herring during ripening in order to reveal to which extent oxidative reactions are involved in the ripening process.

A large biological variation between individual fish was observed and resulted in large standard deviations when analyzing herring muscle tissue. This was expected since a catch of wild fish were used for this study. In contrast, analysis of the brine, which is more homogenous, gave more reproducible results. A study in farmed salmon indicated that the biological variation could contribute by up to 20% of the observed variation in the data obtained from individuals from the same batch and same family (*33*), this can only be higher in wild fish such as herring. However, the obtained data about the biochemical composition of the fish and brine during the ripening period are in agreement with previous reports (*4*, *5*).

Diffusion of salt from the brine to the fish muscle took place rapidly, and equilibrium was obtained already after a month. Salt induced swelling of the myofibrillar proteins and maximum swelling was reported to be obtained at a level of salt of 5–6% (*8*). The level of salt in the fish after 1 month was around 10% and was stable thereafter. High salt levels can induce structural protein solubilization (salting in) but also denaturation and aggregation (salting out) (*34*). From the gel electrophoresis of the herring muscle, disappearance of the fish muscle structural proteins was observed and the myosin band disappeared totally at 151 days in the fish tissue. This can be attributed to protein solubilization from the herring to the brine. Indeed, small proteins or protein fragments migrated from the fish to the brine but no significant amounts of intact myosin and actin were revealed in the brine (**Figure 3**). This can be explained by the enzymatic degradation (proteolysis) of muscle structural protein as also suggested by others, therefore resulting in fragmentation of the cytockeletal proteins (*35*, *36*). The enzymatic activity in the muscle did not increase drastically in the herring muscle during the ripening period (**Figure 4**) but was higher in the brine. It is possible that after solubilization, the proteins in the brine are subsequently degraded by proteolytic enzymes, and this could explain to some extent why only little intact myosin and actin was observed in the brine. However, the enzymatic activity was expected to be low under the ripening conditions (i.e., high salt, low pH, and low temperature); therefore, the contribution of endogenous enzymes to such extensive protein degradation can be questioned.

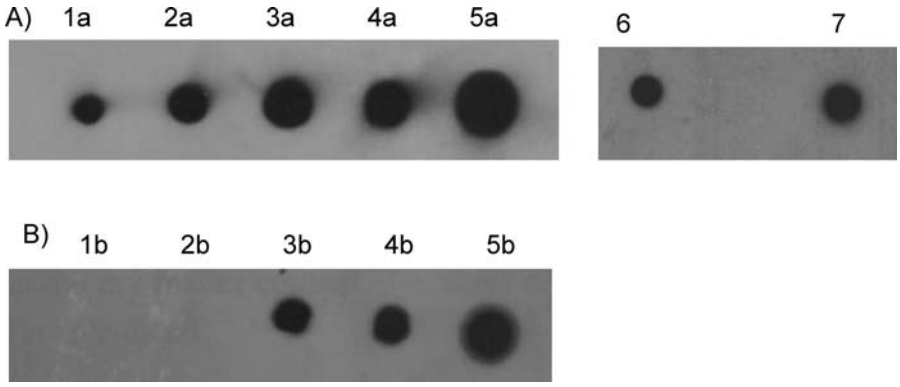

Figure 9. Brine samples (2μl) revealing peroxidase activity upon activation using luminol (1.25 mM), hydrogen peroxide (2.7 mM) and *p*-coumaric acid (0.07 mM) on nitrocellulose membrane and detection on film. With 1): 2 days; 2) 45 days, 3) 85 days, 4) 151, and 5) 371 days old brine and with 6) control: mouse horseradish peroxidase conjugated immunoglobulin 7) control: rabbit horseradish peroxidase conjugated immunoglobulin. With **A**) first activation and **B**) second activation.

The proteins were also found to be highly susceptible to oxidation already after 17 days of ripening and a maximum level of protein carbonyl groups was observed after 151 days. Protein oxidation is believed to result in protein fragmentation, and it is possible that degradation of structural proteins was also due to protein oxidation. In addition, the gel electrophoresis revealed that high molecular weight aggregates were formed and this could be due to protein cross linking. Recent investigation with purified myosin showed that oxidation can lead to degradation and cross-linking (*37*). The decrease in protein carbonyl groups observed at day 371 can be explained by the limitation of the spectroscopic methods, as well as the lack of precipitation by TCA of the small protein fragments obtained after prolonged ripening. From the immunoblot of protein carbonyls (**Figure 7**) it is clear that high molecular weight proteins in the fish tissue are heavily oxidized during ripening, and it is suggested from our data that a combination of both protein oxidation and proteolysis is responsible for the disappearance of the structural proteins during ripening. The increase of oxidized protein in the brine during ripening also suggests extensive protein oxidation. In addition, production of 3-methyl butanal both in brine and in fish, a Strecker-type aldehyde, which is believed to be formed in the interaction between glucose and leucine in the Maillard reaction (*38*), has also been reported to be produced via interaction with leucine and some lipid oxidation products. The pattern of 3-methyl butanal production matched the pattern of protein carbonyls groups formation, and it is possible that interactions between lipids, proteins and sugars contribute to the formation of aggregates with high molecular weight, which give this give dark band in the high molecular region (above 200 kDa, **Figure 7**) when performing the immunoblot. The aldehyde 3-methyl butanal can also be produced by bacteria, and is reported to be responsible for a malty aroma, which is one of the characteristics of salted herring products (*39*). Interestingly, a limited extent of lipid oxidation was detected during the ripening process, and this does not support previous findings indicating that oxidation of lipids contributes to the characteristic flavor and aroma of salted fish products (*39*). However, tocopherols present in situ in the muscle were consumed (**Table 2**). Solubilization of tocopherol from the fish muscle to the brine is rather unlikely; it is therefore most probably consumed via its antioxidant activity. This finding supports the evidence that some free radical reactions took place in salted herring during ripening and that these reactions could possibly target proteins. In a model system tocopherol failed to protect protein from oxidative damage, but it is likely that in muscle tissue, tocopherols are able to scavenge radicals that could otherwise damage protein (*40*). According to Davies, proteins are due to their abundance, the main substrate for oxidative damage in vivo (*41*), and they have been shown to be either pro-oxidant or antioxidant depending on the conditions (*42*). Under the ripening conditions, the proteins might be able to protect the lipid from oxidative damage. Others have also reported an antioxidative activity of proteins and peptides (*43*). Aqueous extracts from fish and chicken muscle have been shown to inhibit lipid oxidation of fish membrane (*44*). The identification of the factors responsible for such antioxidative properties of these aqueous extracts has not been yet fully clarified but it is suggested that proteins and water-soluble antioxidants are involved (*45*). A previous study has shown that protecting proteins from oxidative damage was resulting in the protection of the lipid fraction, indicating that proteins might have an essential role in scavenging free radicals that could otherwise be harmful to lipids (*40*). In the ripening process, the brine itself with its high protein content might inhibit lipid oxidation in herring muscle. However, the high level of Hb, iron and salt also makes it a potentially good pro-oxidant. The role of salt in muscle food is also controversial, and early reports indicated that salt can be either pro-oxidative or antioxidative. Kanner reported that salt pro-oxidative activity towards lipids was due to the displacement of iron ions from binding to macromolecules like proteins (*9*). During ripening, iron (free or protein bound) is released from the muscle to the brine and salt might trigger iron release from proteins. The released iron might attack protein at the site of release rather than inducing lipid oxidation in Fenton type reaction. Standley et al. recently showed that in human atherosclerotic tissue, iron level correlated positively with the extent of protein oxidation but not with lipid oxidation (*46*) further supporting our findings.

The presence of a peroxidase activity in the brine was demonstrated, and it is suggested that Hb is responsible for such activity (**Figure 9**). Indeed heme proteins have pseudo-peroxidase activity and have been shown to be able to efficiently oxidize a variety of cellular components (*17*). It was further demonstrated that brine samples from day 85, 151, and 371 could be reactivated indicating that the peroxidase activity was persistent in the brine after prolonged ripening period (**Figure 9B**). Surprisingly, the Hb protein band on SDS-PAGE fainted in the brine with ripening time (**Figure 3**), in contrast a lower band with a molecular weight of 10 kDa appeared. It is unclear if this 10 kDa band was related to Hb degradation or oxidation at all but this needs to be investigated further. However, it has been shown that cleaved heme proteins were much more pro-oxidative than in their native form (*14*). This can further support

the present findings if the 10 kDa band can be attributed to cleaved or degraded Hb. The ability of heme protein to induce cross-linking is also well documented but little investigation has been performed in food systems. The pseudo-peroxidase activity of Hb might be exacerbated during the ripening process via cleavage of the heme protein and/or release of the heme group and might be responsible for protein oxidation and formation of protein aggregate during ripening. In summary, our findings suggest that Hb might play an important role in the ripening process and in the development of protein oxidation in marinated fish products.

The present study indicates that proteolysis might not be the only factor responsible for the ripening of salted herring. Indeed, protein oxidation was shown to be extensive and resulted in the formation of high molecular oxidized protein aggregates. At the same time the unsaturated lipids, which are very susceptible to oxidation, did not oxidize, and it is evident that the nutritional value of salted herring is optimal, making them a good source of omega-3 fatty acids. The importance of the blood brine and especially the peroxidase activity of Hb, in the progress protein oxidation in salted herring will be investigated further to reveal in more details to which extent it contributes to production of high quality salted herring products.

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