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Protein and Lipid Oxidation in Parma Ham during Production

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ABSTRACT: Lipid oxidation adds to the characteristic flavor of dry-cured products, but the role of protein oxidation in the production of such meats is unknown. Lipid and protein oxidations in biceps femoris (BF) and semimembranosus (SM) muscles were evaluated throughout the production period of Parma ham (0−13 months). Lipid oxidation, determined as primary and secondary lipid oxidation products (POV and TBARS), and protein thiol loss commenced immediately without any lag phase and preceded the initiation of protein radicals and protein carbonyls, which increased after a lag phase of 3 months. TBARS reached a maximum value of 2.5 mg MDA/kg dry matter in SM after 1 month and leveled off at 1 mg MDA/kg dry matter in both SM and BF between 6 and 12 months. Loss in protein thiols proceeded similarly for SM and BF from initial ∼50 to ∼27 nmol/mg protein after 6 months and stayed constant. Gel electrophoresis showed that myosin was lost primarily by proteolytic degradation and not by polymerization through protein disulfides. Overall, oxidation accelerated during the first stages of production but stabilized toward the final stages of maturation.

KEYWORDS: *protein oxidation, lipid oxidation, Parma ham, protein radicals*

INTRODUCTION

Parma ham, the traditional dry-cured product from Italy, is made entirely from pork legs and salt from the Mediterranean Sea without any addition of nitrite or nitrate. The typical procedure for the production of Parma ham consists of three main steps: salting, resting, and maturation for at least one year.[1] This long maturation time is the key to the formation of the characteristic flavor and texture of Parma ham, which is developed through numerous biochemical reactions.[2] Several studies describe the proteolysis, lipolysis, and lipid oxidation taking place during the maturation of different types of dry-cured ham including Parma ham.[3−7] However, protein oxidation has been only sparsely explored and especially not for Parma ham.

Oxidation of lipids and proteins is considered a main cause for the limited shelf life of fresh meat and meat products due to the generation of off-flavors causing rancidity by lipid oxidation[8] and altered textural properties resulting from protein oxidation.[9] In dry-cured ham, like in Parma ham, lipid oxidation plays a considerable role in the development of the desirable flavor.[10] Buscailhon et al.[11] reported that the aroma of dry-cured ham was closely connected to the formation of alcohols and ketones, which are products of lipid oxidation. As a consequence, a large number of studies deal with the effect of lipid oxidation in the maturation of dry-cured hams,[12−14] but the direct effect of protein oxidation in dry-cured hams has hardly been described. Accordingly, it is necessary to investigate the progression of protein oxidation processes throughout the production of Parma ham as an important example of dry-cured hams.

Protein oxidation can be initiated by metal ion catalysis, by proteins reacting with oxidizing lipids, or by photosensitizers generating reactive oxygen species during the processing of meat.[15] Ventanas et al.[3] reported that during the ripening of dry-cured loins from Iberian pigs, oxidation processes influenced not only lipids but also proteins. Protein oxidation has been found to affect the water-holding capacity and texture of meat,[9] and the formation of protein carbonyls is commonly used as an overall marker of protein oxidation.[16] Loss of thiol groups is another consequence of protein oxidation, and one of the reaction products, protein disulfides, may have a strong influence on the texture development of dry-cured ham. Wang et al.[6] found that the concentration of protein carbonyls increased during the salting period of dry-cured Xuanwei ham, indicating the relevance of investigating protein oxidation in dry-cured ham during processing. In fresh meat such oxidative protein modifications may change the susceptibility of proteins to the proteolytic enzymes[17] and may be of relevance in the production of Parma ham. The precursors of protein oxidation products are protein radicals, and it is highly relevant to consider the formation of such reactive species to understand their effect as initiators of oxidation during the production of dry-cured hams.

Preservation by high-pressure (HP) treatment is a common procedure for Parma ham before distribution in some nations (due to state regulations). HP treatment at 600 MPa has been found to enhance lipid and protein oxidation in vacuum-packed Iberian dry-cured ham,[7] and the effect on Parma ham of this preservation technique of increasing importance was included in the present study.

The primary aim of the present study was to gain knowledge on the progression of oxidation during the production of Parma ham to obtain insight into the interplay between different oxidation substrates and their oxidation products. Accordingly, the progression of protein oxidation in Parma ham during the production from "green" ham (fresh meat) to fully matured Parma ham was investigated and correlated to the progression of lipid oxidation and the overall oxidative stability of the product. The current interest in the development of meat

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products using fewer and lower levels of additives encourages such studies.

MATERIALS AND METHODS

Chemicals. Reagent grade chemicals and distilled−deionized (Milli-Q) water were used throughout.

Sampling of Parma ham. Two muscles, an external semimembranosus (SM) and an internal biceps femoris (BF), were collected from "green" ham (GH), ham at the end of salting (ES), ham at the end of resting (ER), half-matured ham (HM), matured ham (M), and fully matured Parma ham (FM) (Table 1). The first three sampling times are related to hams in their cold-processing stage (1−4 °C), and the last three are related to their maturation stage (15−18 °C). The hams were obtained from local producers in Parma (Italy) by Stazione Sperimentale per I'Industria delle conserve Alimentari, and the muscles were collected in replicates from three different hams. The different stages of production (GH, ES, ER, HM, M, and FM) correspond to 0, 1, 3, 6, 9, and 13 months, respectively. The samples were packed in vacuum and sent refrigerated to Denmark for oxidation analyses. Upon arrival, all of the samples were cut into smaller pieces, vacuum packed, and stored frozen (−80 °C) until analysis.

Table 1. Sampling of Biceps Femoris (BF) or Semimembranosus (SM) during the Production of Parma Ham[a]

sampling time (months)	description of production stage	abbreviation
0	"green" ham	GH
1	end of salting	ES
3	end of resting	ER
6	half-matured ham	HM
9	matured ham	M
13	fully matured	FM

[a]Three replicates were obtained of each muscle for each stage during production.

Oxygen Consumption. An aliquot of 2.0 g of meat was homogenized using an Ultra Turrax T-25 homogenizer (Bie and Berntsen A/S, Denmark) with 20 mL of air-saturated 0.050 M MES (*Z*-[*N*-morpholino]ethanesulfonic acid hydrate) buffer (pH 5.6) for 20 s. The suspension was filtered through a colander, and 2.60 mL was immediately transferred to a glass chamber with no headspace. The meat homogenate was kept under constant stirring, and the oxygen concentration was measured with a Unisence Picoammeter (PA 2000 A/S, Aarhus, Denmark) at 25 °C every 10 min by an oxygen electrode (Unisense OXMR-10534, A/S, Aarhus, Denmark). A two-point calibration was used to calibrate the electrode and oxygen analyzer before use. A solution of 0.10 M sodium ascorbate and 0.10 M NaOH or N_2-saturated MES buffer was used for the 0% oxygen reading, and air-saturated MES buffer was used for the 100% oxygen reading. The slope of a linear fit between 80 and 40% oxygen concentration was used to calculate the rate of oxygen consumption, which is presented relative to the concentration of dry matter (DM) in the sample (% O_2/DM/min). Each sample consisted of two independent replicates, and data are presented as the mean ± standard deviation (SD). For presentation, the oxygen consumption curves were smoothed by Savitzky−Golay processing.

High-Pressure Processing. Vacuum-packed fully matured (FM) Parma hams from BF and SM muscle were used for the high-pressure processing by QUINTUS Food Processing Cold Isostatic Press QFP-6 (ABB Pressure Systems, Västerås, Sweden). Approximately 10 g of intact meat was exposed to hydrostatic pressure at 300, 600, or 800 MPa for 6 min with water as pressurization fluid, and the initial temperature was set to 20 °C. The temperature of the pressurization fluid after 6 min of 300, 600, or 800 MPa hydrostatic pressure was 32, 40, or 54 °C, respectively. Subsequently, all samples were stored frozen (−20 °C) until determination of the oxygen consumption rate was carried out as described above.

Peroxide Value (POV). POV was determined according to IDF standard method 74A[18] and expressed as millimoles equivalent peroxide per kilogram lipid. Before the determination of POV, the total fat of the hams was extracted according to the method of Folch et al.[19] Data are presented as the mean ± SD of three independent replicates unless otherwise stated in the figure caption.

TBARS value. The TBARS values were determined by TBARS analysis according to the method of Sørensen and Jørgensen and Vyncke.[20,21] An aliquot of 10.0 g of meat was homogenized in 30 mL of 7.5% trichloroacetic acid (TCA) with 0.10% propyl gallate and 0.10% EDTA for 45 s at 13500 rpm using an Ultra Turrax T-25 homogenizer (Bie and Berntsen A/S, Denmark). Afterward, the sample was filtered (80 g/m^2, 589/3, Schleicher & Schuell, Germany), and 5.0 mL of the filtrate was mixed with 5.0 mL of 20 mM thiobarbituric acid (TBA) and incubated in a 100 °C water bath for 40 min. The absorbance was measured at 532 and 600 nm at room temperature. Results are expressed as 2-thiobarbituric reactive substances (TBARS) in milligrams malondialdehyde (MDA) equivalent per kilogram dry matter using a standard curve and are presented as the mean ± SD of three independent replicates unless otherwise stated in the figure caption.

Isolation of Myofibrillar Proteins. Myofibrillar proteins (MPI) were isolated from the hams according to the method of Park et al.[22] with slight modifications. After removal of visible fat and connective tissue, an aliquot of 4.0 g of lean meat was cut into small pieces and homogenized in 20 mL of isolation buffer (10 mM sodium phosphate, 0.10 M NaCl, 2.0 mM $MgCl_2$, 1.0 mM EDTA, pH 7.0) in a centrifuge plastic tube using an Ultra Turrax T-25 homogenizer. Samples and buffers were kept on ice as often as possible. The homogenate was centrifuged at 4 °C for 15 min at 2600*g* (Sigma Laborzentrifugen 3k15, Bie and Berntsen A/S, Denmark), and the supernatant was discarded. The pellet was resuspended in 10 mL of isolation buffer using the homogenization and centrifugation as described above. This wash procedure was conducted three times in total per sample. After the last wash, the pellet was resuspended in 10 mL of 0.10 M NaCl, homogenized, and centrifuged as above. This procedure was also conducted three times. Before the final centrifugation, the suspension was filtered through a colander and subsequently centrifuged as above. Finally, the supernatant was discarded, and the pellet was frozen to −80 °C overnight and lyophilized. The samples were weighed and stored frozen (−20 °C) until use.

Protein Radical Formation by ESR Spectroscopy. Lyophilized MPI were transferred to fused quartz ESR tubes (inner diameter = 4 mm, wall = 0.5 mm; Wilmad, Buena, NJ, USA) to reach >5 cm filling of the tube. The tubes were placed in the cavity of a JEOL JES-FR30X ESR spectrometer (JEOL Ltd., Tokyo, Japan) with the following settings: microwave power, 4 mV; center field, 336 mT; sweep width, 5 mT; sweep time, 2 min; modulation width, 0.2 mT; amplitude, 320; time constant, 0.3 s; accumulations, 4. The *g* value and the radical signal intensity of the ESR spectra were determined relative to the internal standard, Mn(II). The radical signal intensity was furthermore related to the density of the sample measured as grams per centimeter in the ESR tube [radical signal intensity = (signal area sample/signal area Mn(II))/density (g/cm)]. All samples (independent triplicates) were measured twice and presented as the mean ± SD. Characterization of the radical species in the MPI of the SM muscle from fully matured Parma ham was conducted by mounting the ESR tube in the cavity (ER 4103 TM) of an ECS 106 electron spin resonance spectrometer (Bruker, Rheinstetten, Germany) equipped with a Dewar flask filled with liquid nitrogen. The magnetic field was modulated with a microwave frequency of 100 GHz, and ESR spectra were recorded using the following settings: gain, 1.6×10^5; modulation amplitude, 0.5 G; time constant, 20.48 ms; sweep time, 20.97 s; center field, 3382 G; field sweep width, 15 G; and microwave power, 2.0 mW, with 128 scans accumulated. Spectral manipulations were performed by using WINEPR software version 2.11.

Protein Thiol Groups. Protein thiol groups were determined by derivatization with 5,5 dithiobis(2-nitrobenzoic acid) (DTNB).[23] An aliquot of 10.0 mg lyophilized MPI was dissolved in 1.0 mL of 5.0% sodium dodecyl sulfate (SDS) buffer in 0.10 M tris(hydroxymethyl)-

aminomethane) (Tris) buffer (pH 8.0) for 2 h in an 80 °C water bath and subsequently centrifuged at 10000*g* for 10 min (Microcentrifuge 154, Ole Dich Instrumentmakers ApS, Hvidovre, Denmark). The supernatant was subjected to protein determination by measuring the absorbance at 280 nm (Cary UV−visible spectrophotometer, Varian, Herlev, Denmark) using a standard curve prepared from bovine serum albumin (BSA). Furthermore, the protein thiol concentration was determined by mixing 0.50 mL of supernatant, 2.0 mL of 5.0% SDS/Tris buffer, and 0.50 mL of 10 mM DTNB in 5.0% SDS/Tris buffer. The absorbance was measured at 412 nm before the addition of DTNB ($Abs_{412\text{-before}}$) and after exactly 30 min of reaction in the dark with DTNB ($Abs_{412\text{-after}}$). A solution containing 2.5 mL of 5.0% SDS/TriS buffer and 0.50 mL 10 mM DTNB in 5.0% SDS/Tris buffer was used as a blank sample ($Abs_{412\text{-blank}}$). Hence, the absorbance corresponding to the thiol concentration in the sample was $Corr_{Abs412} = Abs_{412\text{-after}} - Abs_{412\text{-before}} - Abs_{412\text{-blank}}$. The final thiol concentration was calculated on the basis of a five-point standard curve ranging from 5 to 200 μM thiols prepared from L-cysteine diluted in 5.0% SDS/Tris buffer (pH 8.0). The thiol concentrations in nanomoles thiol per milligram protein are presented as the mean ± SD of three independent replicates.

Protein Carbonyl Content. The concentration of protein carbonyls was determined by derivatization with dinitrophenylhydrazine (DNPH) as described by Levine et al.[16] with some modifications. An aliquot of approximately 2 mg of lyophilized MPI was dissolved in 500 μL of 6.0 M quanidine hydrochloride in 20 mM potassium dihydrogenphosphate (pH 2.3) for 1 h in a 50 °C water bath. Dissolved samples (S) were incubated with 500 μL of 10 mM 2.4 DNPH dissolved in 2.0 M HCl, and dissolved blanks (B) were incubated with 500 μL of 2.0 M HCl instead of DNPH solution. The samples were placed in a 37 °C water bath for 1 h, and all samples and blanks were vortexed every 10 min. To the samples and blanks was added 325 μL of 50% TCA, and the mixtures were vortexed for 30 s and placed on ice for 10 min before centrifugation at 11000*g* for 10 min (Microcentrifuge 154, Ole Dich Instrumentmakers ApS), after which the supernatant was discarded. Excess DNPH was removed by washing with 1.0 mL of ethanol/ethyl acetate (1:1) with 10 mM HCl, vortexing, leaving to react for 10 min, and then centrifugation at 11000*g* for 10 min. This washing procedure was repeated three times, and after every wash, the supernatant was discarded. Following the final wash excess solvent was removed by gently purging air over the pellets, which subsequently were dissolved in 1.0 mL of 6.0 M quanidine hydrochloride. Hereafter, the samples were placed in a water bath at 37 °C for 30 min. The final solution was centrifuged at 11000*g* for 10 min to remove insoluble material. The carbonyl concentration (nmol/mg protein) was calculated from the absorbance at 280 and 370 nm of the samples by using an absorption coefficient at 370 nm of 22000 M^{-1} cm^{-1} for the formed hydrazones.[16,24] The contribution obtained from the blanks (B) was subtracted from the contribution obtained from the corresponding sample (S). Data are presented as the mean ± SD of three independent replicates unless otherwise stated in the figure caption.

SDS-PAGE. Lyophilized MPI were analyzed by gel electrophoresis using NuPAGE Novex 3−8% Tris−acetate gels according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Before that, 10.0 mg of lyophilized MPI was dissolved in 1.0 mL of 5.0% SDS in 0.10 M Tris buffer (pH 8.0) for 2 h in an 80 °C water bath, following centrifugation at 10000*g* for 10 min. The supernatant was used for protein determination as described above for the thiol analysis and for SDS-PAGE. For analysis by SDS-PAGE, the samples were diluted in 5.0% SDS/Tris buffer to obtain 1.6 mg/mL protein in each sample. Reduced samples were prepared by mixing 1.6 μL of diluted sample and 14.4 μL of loading solution, which were prepared from 60 μL of LDS (lithium dodecyl sulfate) sample buffer (Invitrogen), 24 μL of 1.0 M DTT (dithiothreitol), and 132 μL of Milli-Q water. The nonreduced samples were prepared by mixing 1.6 μL of diluted sample and 14.4 μL of loading solution, which were prepared from 60 μL of LDS sample buffer (Invitrogen) and 156 μL of Milli-Q water. Aliquots of 10 μL reduced or nonreduced samples, as well as 3 μL of Precision Plus Protein Standard All Blue marker (Bio-Rad Laboratories, Inc., Hercules, CA, USA), were loaded to the wells of the gels. Electrophoresis was run for 80 min at 150 V in cassettes containing ice-cold SDS Tris−acetate running buffer (Invitrogen). Subsequent to electrophoresis, the gels were fixed overnight in fixation solution (50% ethanol, 7% acetic acid, and 43% Milli-Q water) at room temperature on a rocking table. Following staining overnight by the fluorescence SYPRO Ruby Protein Gel Stain (Invitrogen), the gels were photographed by a charge-coupled device (CCD) camera (Raytest, Camilla II, Straubenhardt, Germany). The pixel intensity of the myosin bands, determined by the peak height after subtraction of background, was quantified using Phoretix 1D software, version 2003.02. Each sample consisted of three independent replicates, and data are presented as the mean ± SD.

Statistical Analysis. Data were analyzed using the one-way ANOVA procedure of the SPSS statistical software (SPSS 19.0, 2010). When a significant probability was distinguished ($P < 0.05$), paired comparisons between means for each sampling time were carried out using Tukey's test. Pearson's correlation coefficients were calculated between loss of thiol groups and formation of carbonyl and between the radical signal intensity of ESR and oxygen consumption using the correlation procedure of SPSS.

RESULTS AND DISCUSSION

Oxidative Stability. The overall oxidative stability of Parma ham was determined by measuring the oxygen consumption in meat homogenates from different stages of the production. Figure 1 shows the oxygen consumption in the biceps femoris (BF) and semimembranosus (SM) muscles after homogenization in buffer at physiological pH. No oxidation initiator was added to the samples, and the pH was kept constant (deviation was < ±0.1 between samples). To compensate for variations in dry matter (DM) content between samples, the oxygen consumption rates (Figure 1, insets) are presented relative to the DM content. Figure 1 shows that the rate of oxygen consumption increased for samples collected during the processing of Parma ham, indicating an increased oxidation potential in the ham during maturation. This is consistent with the increased oxidation−reduction potential (ORP) during the processing of Parma ham previously reported by Wakamatsu et al.[25] The final oxygen consumption rates were found to be higher in the SM than in the BF muscle. However, the rates increased more rapidly in the BF muscle compared to the SM muscle during the production. During the production of Parma ham, BF and SM muscles are exposed to different processing conditions. SM is positioned directly on the cutting surface and is exposed to instant penetration of salt and following dehydration. In contrast, BF is positioned distal to the cutting surface, and the salt concentration will only slowly increase and dehydration will progress more slowly compared to the SM muscle.[26] These factors may influence the progressions of oxidation in the two muscles and accordingly the rate of oxygen consumption in the homogenates. The natural occurrence of hydrogen peroxide in meat from biological processes and from microbiological growth may also affect oxidation rates and may explain the progression of oxidation in the internal BF muscle. With regard to the BF muscle, the samples collected from the initial stages of the Parma ham production, GH and ES (months 0 and 1, respectively) showed significantly lower oxygen consumption rates than the remaining samples ER, HM, M, and FM (months 3, 6, 9, and 13, respectively), which showed comparable rates (Figure 1, upper panel). For the SM, it was only at the final stages of production (M and FM) that the oxygen consumption rates in the homogenized samples seemed to stabilize. Approaching the completion of maturation, the oxidation processes are decelerating, which may be ascribed

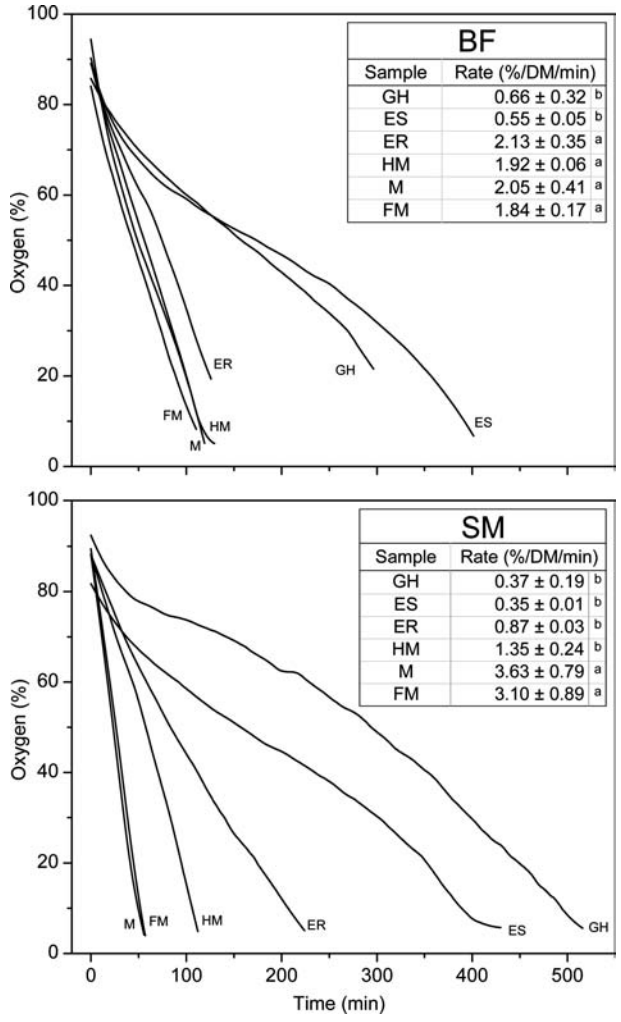

Figure 1. Oxygen consumption in meat homogenates prepared from Parma ham, BF (upper panel) or SM (lower panel), during all stages of production: GH, green ham (month 0); ES, end salting (month 1); ER, end resting (month 3); HM, half-matured (month 6); M, matured (month 9); FM, full matured (month 13). Results are given as the mean ± SD of two independent replicates. (Insets) Rate of oxygen consumption (%/DM/min) are given as the mean ± SD. Means with different letters differ significantly ($P < 0.05$).

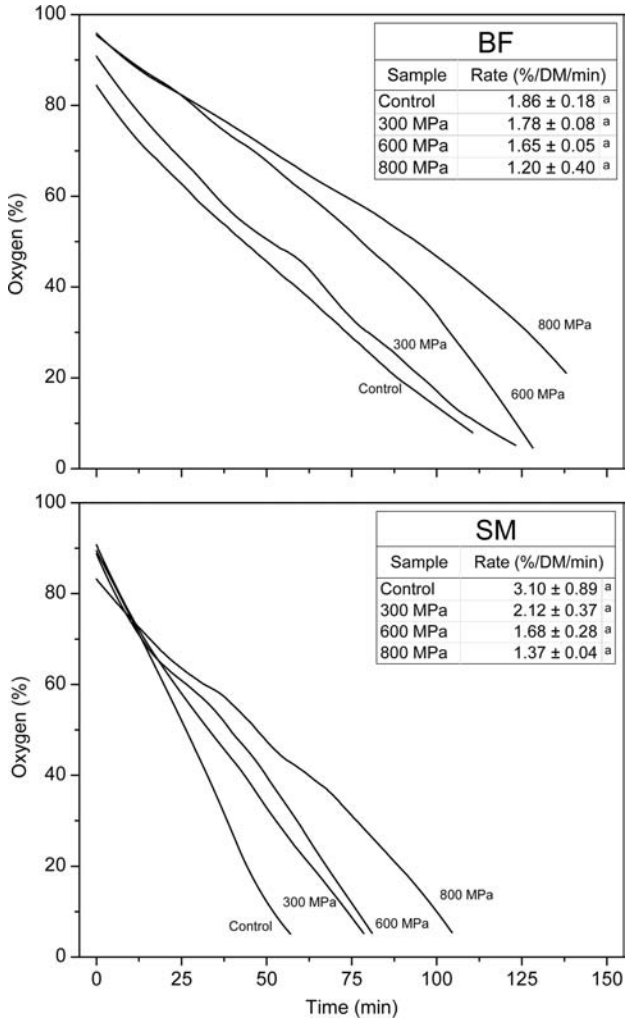

Figure 2. Oxygen consumption in meat homogenates from high-pressure-treated fully matured Parma ham: BF (upper panel) or SM (lower panel) following high-pressure processing at 300, 600, or 800 MPa and a nonpressurized control. Results are given as the mean ± SD of the same sample measured twice. (Insets) Rate of oxygen consumption (%/DM/min) given as the mean ± SD. Means with different letters differ significantly ($P < 0.05$).

to the decrease in water activity, lowering the reaction rates and thereby protecting the product from further oxidation. Oxidation of the myofibrillar proteins may even facilitate the dehydration process as studies have shown that oxidation-derived protein aggregation leads to increased gapping between muscle fibers.[27] The expansion of the extracellular spaces may facilitate the extraction of water from muscle cells by salt during Parma ham production.

The effect of HP treatment on oxidative stability was evaluated by the rate of oxygen consumption in homogenized samples of fully matured Parma ham exposed to high hydrostatic pressure. In general, homogenized BF samples showed lower oxidation rates than SM samples (Figure 2), which is consistent with the behavior of the nonpressurized samples (Figure 1). Interestingly, the results showed that the oxygen consumption rate decreased for the homogenized samples from ham exposed to increasing pressure, although the differences between the treatments were not statistically different. The increase in temperature during HP treatment may affect the overall oxidation rates, but did not, in this case, result in elevated oxidation rates. Indeed, the observed decreasing oxidation rates indicate that HP treatment hampers the oxidative processes in Parma ham even when the temperature is temporarily increased. This is consistent with the results of a previous study by Clariana et al.,[28] who found decreased TBARS values following HP treatment (600 MPa) of vacuum-packed dry-cured ham stored at 4 °C for 50 days. However, increased levels of protein carbonyls have been found following HP treatment (600 MPa) of Iberian ham.[7] In fresh meat, HP treatment has been found to increase oxidation as demonstrated by Orlien et al.[29] for fresh chicken breast muscle. The accelerated oxidation in fresh meat subjected to HP treatment was ascribed to the damage of cellular membranes and a resulting disturbance of the redox balance. However, future studies should more specifically focus on the effects of HP treatment on lipid and protein oxidation in Parma ham to understand the effects this preservation technique will have on the quality of dry-cured hams.

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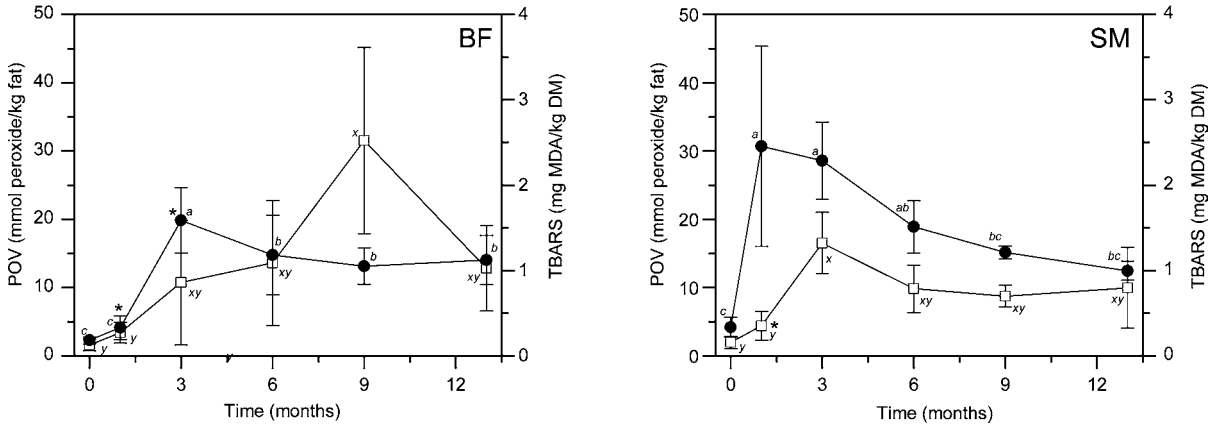

Figure 3. Lipid oxidation in Parma ham: concentration of lipid peroxides (POV) (□) and TBARS (●) in Parma ham from BF (left panel) or SM (right panel) during production (0−13 months). Results are given as the mean ± SD of three independent replicates, except for the marked samples (∗), which were measured in duplicate. Means with different letters differ significantly ($P < 0.05$).

Lipid Oxidation. The progression of lipid oxidation during the production of Parma ham was evaluated by two markers of lipid oxidation, peroxide value (POV) and thiobarbituric acid reactive substances (TBARS), as shown in Figure 3. POV was quantified in the lipid fraction extracted from the hams and corresponds to the concentration of lipid peroxides, normally considered as primary lipid oxidation products. As shown in Figure 3, POV increased during 9 months of maturation for the BF muscle followed by a rapid decrease. In contrast, POV in SM muscle reached a maximum after only 3 months of maturation, indicating that lipid oxidation progresses more rapidly in the external muscle (SM) compared to the internal (BF). This may be explained by the higher availability of oxygen and salt in the external muscle. Similar behaviors were found in Iberian dry-cured ham by Cava et al.,[14] who reported a maximum POV value after 7 months and a subsequent minimum level at the end of the ripening stage (∼24 months), and by Antequera et al.,[13] who demonstrated an increase in peroxide values during the first months of the production of the ham, followed by a fast decrease during the final stages. The decline in both muscles may be explained by the subsequent formation of secondary lipid oxidation products, such as aldehydes or ketones, often referred to as reactive carbonyl species.[13] Moreover, a possible interaction between peroxides and proteins must be taken into consideration. In mixed systems, lipid-derived peroxyl radicals may induce alterations in proteins as they are believed to be involved in both polymerization and fragmentation processes.[30] The high standard deviations for POV seen from Figure 3 are ascribed to meat variations.

TBARS were used as an estimate of secondary lipid oxidation products and are also shown in Figure 3. For both muscles, TBARS increased during the first stages of production, followed by a decrease during the following stages. TBARS increased more rapidly in the external muscle (SM) compared to the internal muscle (BF) in accordance with the formation of the primary lipid oxidation as described above. Increased TBARS values in dry-cured ham during the first stages of production followed by a decrease toward the final stages have previously been reported for Iberian ham.[12,14] The decrease in TBARS values for BF and SM muscle was assigned to the advanced reactions of secondary lipid oxidation products with protein residues, especially for conditions of low water activity to yield oxidatively modified proteins.[31] The results show that TBARS formation peaks before POV, which is in contrast to the common understanding that primary lipid oxidation products are formed prior to the generation of the secondary lipid oxidation products. However, as discussed by Antequera et el.,[13] the rapid breakdown of peroxides makes their detection difficult, and underestimation may distort the relative formation of TBARS and POV.

Protein Radical Formation. The formation of protein radicals was evaluated by subjecting lyophilized MPI extracted from Parma ham to ESR spectroscopy. As seen from Figure 4, the radical signal intensity of the ESR spectra increased with increasing maturation time, indicating an extended formation or accumulation of protein radicals during maturation of the ham. Furthermore, clear indications of a lag phase of 3 months of

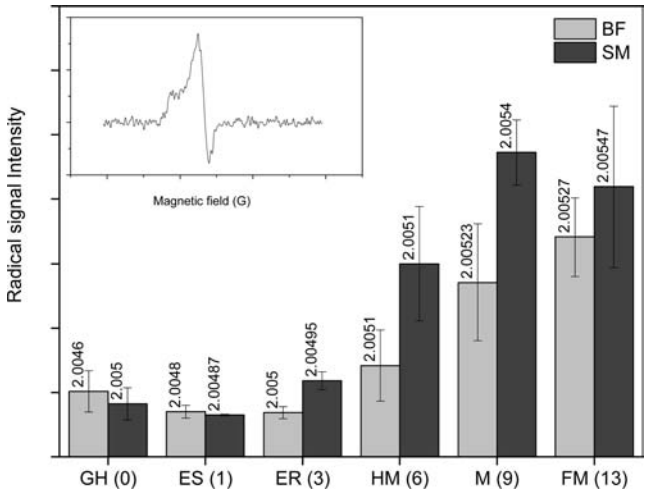

Figure 4. Protein radical formation in Parma ham. Radical signal intensity and g values (numbers above columns) were determined by ESR spectroscopy of myofibrillar protein isolate (MPI) extracted from Parma ham muscles, BF or SM, during all stages of production: GH, green ham (month 0); ES, end salting (month 1); ER, end resting (month 3); HM, half-matured (month 6); M, matured (month 9); FM, full matured (month 13). Results are given as the mean ± SD of three independent replicates. Means with different letters differ significantly ($P < 0.05$). (Inset) ESR spectra of lyophilized MPI extracted from FM Parma (SM) as determined by low-temperature (liquid nitrogen) ESR spectrometry. Means with different letters differ significantly ($P < 0.05$).

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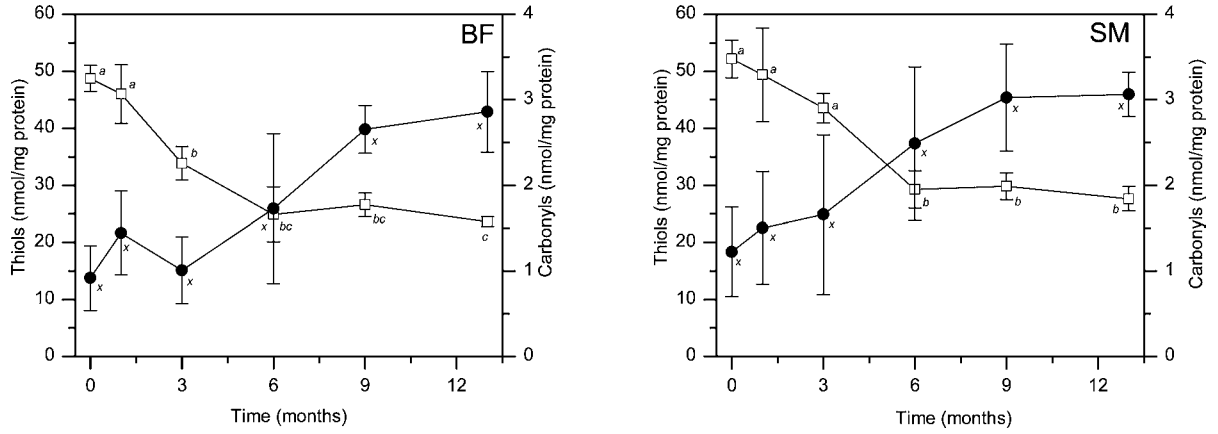

Figure 5. Protein oxidation in Parma ham: concentration of protein thiols (□) and protein carbonyls (●) in myofibrillar protein isolates (MPI) extracted from Parma ham, BF (left panel) or SM (right panel), during production (0−13 months). Results are given as the mean ± SD of three independent replicates, except for the marked samples (∗), which were measured in duplicate. Means with different letters differ significantly ($P < 0.05$).

maturation for the formation of stable radicals were observed for both muscles. The concentration of radicals tended to be higher in the MPI extracted from the SM muscle compared to those extracted from the BF muscle, which is in agreement with the results obtained for lipid oxidation (Figure 3) and for the oxidative stability as evaluated by oxygen consumption in homogenized samples (Figure 1). The protein radical species was characterized by a g value of 2.0051 ± 0.0002, indicating that the radical species may originate from carbon, aminoalkyls, or alkoxyls, whereas possible phosphorus radicals can be excluded.[32] Furthermore, the ESR spectrum obtained for the fully matured (FM) sample from the SM muscle revealed that the spectrum contains features similar to those seen for a tyrosyl radical.[33] It may accordingly be suggested that the protein radicals generated in the Parma ham originate from tyrosine (Figure 4, inset), as tyrosine residues are considered to be a sink of radicals in proteins.[29] In the present study, the radical signal intensity was found to stabilize toward the final production stages of the SM muscle, indicating that the oxidation processes are decelerating or that the protein radicals are reacting further to generate nonradical species. A positive correlation coefficient between the radical signal intensity and the rate of oxygen consumption of homogenates was found for the SM muscle, $R^2 = 0.962$ ($P < 0.0001$), which is consistent with the observations made by Carlsen et al.,[34] who also reported positive correlation between the rate of oxygen consumption and the radical formation in processed pork.

Thiols and Carbonyls. Protein oxidation was evaluated by the formation of carbonyls and the loss of thiol groups in the MPI extracted from Parma ham during the different stages of the production (Figure 5). The results showed that the concentration of protein carbonyls increased, although not significantly, for both the SM and BF muscles and that the concentration of protein thiol groups decreased significantly ($P < 0.05$) for both the SM and BF muscles. Figure 5 shows that during the production of Parma ham, thiol groups were lost presumably due to oxidation resulting in the formation of protein disulfide bonds as described for fresh pork by Lund et al.[35] In the present study, the initial concentration of thiol groups was ∼50 nmol/mg protein, which decreased to 27 nmol/mg protein during the first 6 months of production. Thereafter the concentration of thiol groups stabilized, and no further loss was observed. Similar losses of thiols due to oxidation have been reported for Cantonese sausages,[36] fresh turkey muscles,[37] and fresh longissimus dorsi muscle from pork and beef.[35,38] The protein carbonyl concentration increased from ∼1 to 3 nmol/mg protein and is consistent with previous reports on pig myofibrils (see, e.g., ref 35). Furthermore, increased carbonyl content has been found during the production of Cantonese sausages[36] and dry-cured ham[6,14] and during the storage of fresh meat.[35,38]

Thiol loss and protein carbonyl formation were found to be significantly negatively correlated with correlation coefficients of $R^2 = -0.58$ ($P < 0.05$) for the BF muscle and $R^2 = -0.74$ ($P < 0.05$) for the SM muscle. As shown in Figure 5, the progression of thiol oxidation and the formation of carbonyl groups were slightly faster for the SM muscle than for BF, again ascribed to the increased availability of oxygen and higher salt concentration in the external muscle. The formation of carbonyls accelerated as the oxidation of thiols had stopped after approximately 6 months. A similar tendency was observed during the drying process of sausages[36] and indicates that thiols are oxidized prior to the formation of protein carbonyl groups. At the final stages of Parma ham maturation, the level of protein carbonyls also seemed to stabilize, which may indicate that the oxidation processes are decelerating toward the final production stage as also indicated by the rate of oxygen consumption of homogenized samples and protein radical formation. However, protein carbonyl formation does not require molecular oxygen, but is catalyzed by transition metal ions.[39,40] The release of iron from the heme protein, which is substituted by zinc during the formation of the Parma pigment, the zinc protoporphyrin (ZnPP),[25] may be involved in these oxidation processes. The decelerating carbonyl formation may therefore rather be caused by advanced reactions, such as oxidation of the carbonyls to carboxylic acids or dimerization reactions as recently reviewed by Estévez.[40] The carbonyl groups may even be involved in the formation of Strecker aldehydes by reaction with free amino acids, and generation of such volatile low molecular weight aldehydes may affect flavor development in Parma ham. Hence, the advanced reaction mechanisms of protein carbonyls may be essential for understanding flavor development, and further studies must be conducted to understand the importance of these protein oxidation products in Parma ham.

Protein Cross-Linking and Protein Degradation. The myofibrillar proteins were separated by SDS-PAGE as shown in Figure 6, and myosin heavy chain (MHC) and actin were

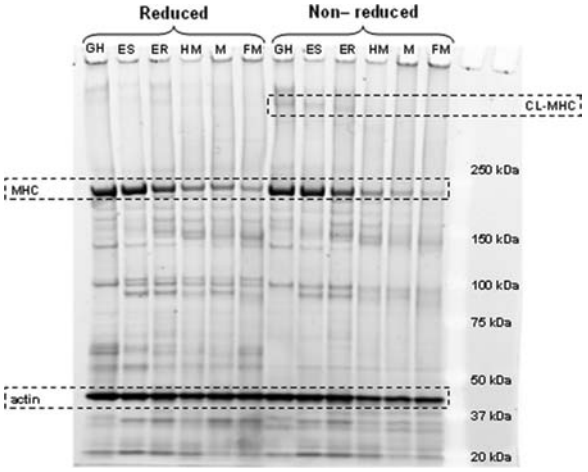

Figure 6. Separation of myofibrillar proteins from Parma ham. SDS-PAGE was performed for reduced and nonreduced myofibrillar protein isolates (MPI) from the SM muscle of Parma ham during all stages of production: GH, green ham (month 0); ES, end salting (month 1); ER, end resting (month 3); HM, half-matured (month 6); M, matured (month 9); FM, full matured (month 13). MHC, myosin heavy chain; CL-MHC, cross-linked myosin heavy chain.

identified by their molecular weights of ~220 and ~45 kDa, respectively. The figure shows that actin and especially MHC were lost during the production of Parma ham. During the processing of dry-cured hams, the muscle proteins are degraded by enzymes, which are naturally present in the muscles.[41] The gradual disappearance of MHC during the production of Parma ham demonstrates the proteolytic degradation of myosin to yield smaller peptides. These structural conformations may impair the protein solubility, which again may affect the amount of protein loaded to the gel. Hence, the protein concentration in each sample was adjusted before loading onto the gel to compensate. However, the relative distribution of myofibrillar proteins could not be controlled and may affect the observed band intensity of MHC and actin, respectively.

Myosin is known to be enzymatically degraded in dry-cured ham.[42−44] Toldra et al.[45] found that cathepsins B, H, and L were still active in Serrano dry-cured hams after 15 months of processing, and the disappearance of MHC seen in the present study may also be explained by enzymatic activity. However, considering the loss of protein thiols observed throughout the production of Parma ham (Figure 5), the disappearance of MHC may also be due to polymerization resulting from oxidatively induced disulfide cross-links. Figure 6 shows weak bands at ~450 kDa for nonreduced GH, ES, and ER, which are expected to be MHC disulfide cross-links (CL-MHC), as the bands were absent under reduced conditions (Figure 6). CL-MHC was not visual in HM, M, or FM Parma ham, indicating that the proteolytic degradation also affects CL-MHC. Quantification of the MHC band intensity before and after reduction makes it possible to estimate the level of protein polymerized through reducible disulfide bonds, and this estimate was used to evaluate the extent of disulfide cross-linking. The estimate includes not only the dimer MHC but also higher levels of polymerization. Accordingly, Figure 7 presents the pixel intensity of the MHC and actin bands from

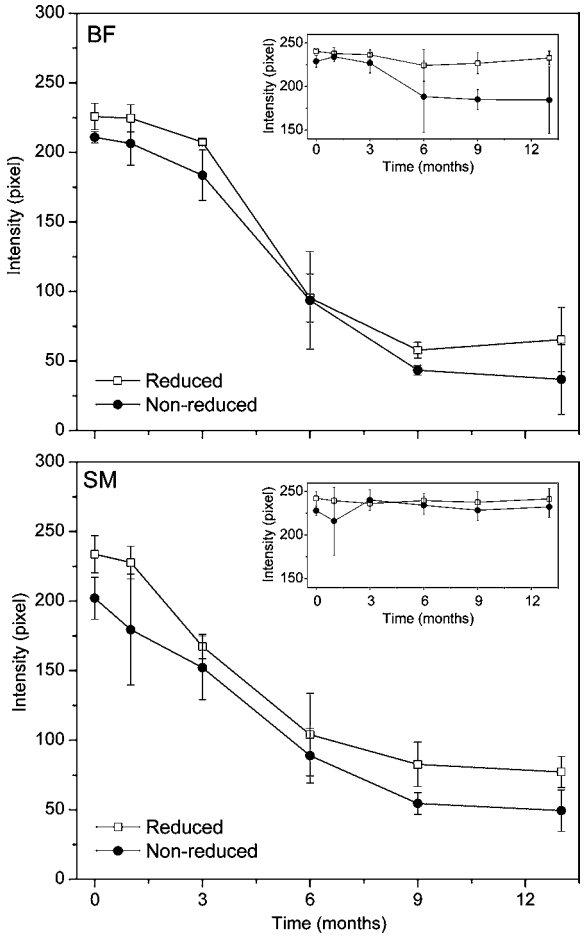

Figure 7. Degradation of myosin and actin in Parma ham. Pixel intensity of myosin heavy chain (MHC) and actin bands (insets) was determined from SDS-PAGE of reduced (●) or nonreduced (□) myofibrillar protein isolates (MPI) from Parma ham BF (upper panel) or SM (lower panel) during all stages of production: GH, green ham (month 0); ES, end salting (month 1); ER, end resting (month 3); HM, half matured (month 6); M, matured (month 9); FM, full matured (month 13). Results are given as the mean ± SD of three independent replicates.

nonreduced or reduced samples separated by SDS-PAGE. The figure shows that MHC was degraded during the production of Parma ham, and not regained by reduction, indicating that the loss in MHC is primarily a result of proteolytic degradation and not caused by formation of reducible bonds generated by oxidative polymerization through disulfide cross-links. For the external SM muscle, a linear loss of MHC was observed starting from month 1 and terminating after month 6, whereas the degradation of MHC in the internal BF muscle commenced after a lag phase, declined linearly after 3 months, and terminated after 9 months. No significant ($P < 0.05$) difference between the BF and SM muscles was detected for the rate of MHC degradation in the linear part of the curves. The difference between the nonreduced myosin and the reduced myosin as seen from Figure 7 corresponds to the amount of cross-linked myosin generated by the formation of reducible disulfide bonds in the Parma ham samples. The results indicate that the level of CL-MHC did not change throughout the production of Parma ham. This observation suggests that oxidatively induced myosin disulfide cross-linking has no effect on the proteolytic degradation of myosin, and furthermore that

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the decrease in protein thiols observed over time may not be directly linked to the formation of reducible protein disulfides in Parma ham.

Actin was evaluated parallel to MHC, and as may be seen from the results presented in Figure 7 (inset), no actin was lost in the SM muscle, whereas actin was degraded in the BF muscle. SDS-PAGE of reduced samples verified that actin was lost entirely due to disulfide cross-link formation, as the protein band was fully regained by reduction. This increased oxidation of thiols to generate disulfides in the BF muscle was unexpected due to the lower availability of oxygen in the internal muscle compared to the external SM muscle. Furthermore, this demonstrates that actin was unaffected by proteolytic degradation during the production of Parma ham, and this is in contrast to what has previously been reported for dry-cured ham[42−44] and for dry-fermented pork sausages by Fernando and Fox,[46] who assigned the loss in actin to proteolytic processes. Larrea et al.[44] showed that actin and myosin were less degraded in SM muscle than in BF at the end of the process, due to less enzymatic activity in the SM muscle in the final stages of the production of the ham. This is in accordance with the observations of the present study, from which it is concluded that the onset of proteolytic degradation is postponed in the BF muscle compared to the SM muscle. In fact, the decelerating oxidation processes toward the final stages of Parma ham production may be influenced by the release of peptides from the proteolytic degradation. Peptides or hydrolysates are known to suppress oxidation in food products, and Park et al.[47] recently suggested that the effect may rather arise from shielding effects resulting from microdistribution of peptides rather than simply by radical scavenging activity.

In conclusion, lipids and protein thiols were oxidized instantly without any lag phase. The level of primary and secondary lipid oxidation products decreased toward full maturation, and the level of protein thiols stabilized after 6 months. After 6 months, stable protein radicals and protein carbonyls emerged, indicating that the lipids and thiols may be oxidized prior to the generation of protein radicals and the formation of protein carbonyls. Myosin heavy chain was found to be predominately lost due to proteolytic degradation, whereas actin was lost due to disulfide cross-link formation. The present work showed that the overall oxidation rate increased throughout the production of Parma ham, but seemed to decelerate toward the final stages of maturation, which is in agreement with expected increased product stability following maturation. The present study forms the basis for understanding the oxidative reactions and interplay between different oxidation substrates in the processing of Parma ham. The findings are valuable for the broader advancement of dry-cured ham production in regions other than Parma, Italy, as understanding of these chemical processes leading to the final Parma ham product may facilitate development of novel dry-cured meat products by use of fewer or lower levels of additives.

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Notes

The authors declare no competing financial interest.

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