JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Oxidation of Myofibrillar Proteins and Impaired Functionality: Underlying Mechanisms of the Carbonylation Pathway

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ABSTRACT: The potential impact of protein oxidation on the functional properties of myofibrillar proteins (MP) was investigated in the present study. To accomplish this purpose, myofibrillar proteins were oxidized in vitro for 12 days at 37 °C in the presence of Cu^{2+}, Fe^{3+}, and Mb in combination with H_2O_2 and analyzed at sampling times for chemical changes induced by oxidative reactions and functional properties. The oxidation measurements included specific protein carbonyls (α-aminoadipic semialdehyde, AAS), advanced lysine oxidation products (α-aminoadipic acid, AAA, and Schiff bases), and thiobarbituric acid-reactive substances (TBARS). The factors and mechanisms involved in the oxidative degradation of lysine residues through the carbonylation pathway are precisely described. According to the present results, intense lipid and protein carbonylation, principally induced by Cu^{2+}/systems, leads to a fast and severe loss of MP functionality, including impaired water-holding, foaming, and gelling capacities. The implication of Mb in the oxidation events enhances the production of AAA and Schiff bases, compromising to a larger extent the solubility of MP and worsening the aggregation and the gelling capacity. The connection between the oxidation-induced chemical changes and the loss of protein functionality is thoroughly discussed.

KEYWORDS: *myofibrillar proteins, carbonylation, α-aminoadipic semialdehyde, γ-glutamic semialdehyde, α-aminoadipic acid, Schiff bases, functionality*

INTRODUCTION

During several decades, the oxidation of proteins has been relegated to playing a secondary role in food systems while lipid oxidation was exhaustively investigated. Today, the completion of studies on protein oxidation (POX) has allowed food scientists to expand their research horizons into innovative fields.

As targets of reactive oxygen species (ROS), proteins are oxidatively modified through multiple mechanisms and pathways.[1] Consequently, the oxidative damage is manifested as a variety of physicochemical modifications including protein unfolding and denaturation, peptide scission, formation of cross-links, and loss of functionality.[2] Specific amino acid side chains are particularly susceptible to oxidation, leading to various chemical modifications such as loss of sulfhydryl and amino groups and the formation of carbonyl compounds and other oxidation derivatives.[1] Protein carbonylation has been highlighted as the most remarkable modification in oxidized proteins and the major source of direct oxidative attack to proteins.[1,3] In food and biological systems, the α-aminoadipic and γ-glutamic semialdehydes (AAS and GGS, respectively) are regarded as the most abundant protein carbonyls.[4] In accordance with the existing literature, these compounds are formed in food proteins upon the oxidative deamination of lysine, arginine, and proline residues in the presence of radical species and transition metals such as iron and copper.[5] These compounds have been found to be reliable markers of protein oxidation in food systems.[4,6,7] However, protein carbonyls are holders of highly reactive moieties and may be involved in advanced reactions.[3] According to studies in biological systems,[8] AAS, in particular, undergoes a further oxidative degradation in the presence of peroxides to yield the α-aminoadipic acid (AAA). This compound, however, has never been described in food systems. The AAS may also react with neighboring amino groups from other amino acid side chains (e.g., lysine) to form an azomethine structure, also known as Schiff bases.[3] As a result, protein carbonyls may also contribute to the formation of intra- and intermolecular cross-links in proteins. So far, it is unknown whether this carbonylation pathway, including the formation of advanced lysine oxidation products such as AAA and Schiff bases, takes place in food proteins or not. In the same line, the factors and mechanisms involved in these reactions and their impact on protein functionality or food quality remain indefinite.

In medical research, oxidative damage to proteins is usually a reflection of pathological conditions and various age-related disorders such as Alzheimer's disease.[9] In food systems, however, the impact of oxidized proteins on food quality and safety is still poorly understood. The overall lack of knowledge of the basic chemistry behind protein oxidation has hindered a full comprehension of the scientific and technological meaning of such phenomena in food systems. However, promising progress has recently been made in muscle foods through which POX has been established as a topic of increasing interest.[1] POX is recognized as a leading cause of meat quality deterioration as it leads to undesirable modifications of meat color, texture, and nutritional value.[7,10,11] In particular, the potential impact of POX on the functionality of myofibrillar proteins (MP) was suggested by early studies carried out by Decker et al.,[12] among others. The functional properties of MP in meat systems, including their water-holding capacity

Received: May 15, 2012
Revised: July 24, 2012
Accepted: July 25, 2012
Published: July 27, 2012

(WHC), largely determine the quality of fresh meat and the success of numerous technological processes applied during the manufacture of meat products. Significant correlations between general protein oxidation measurements (i.e., total protein carbonyls) and the alleged POX-driven changes, are usually regarded as proof of the impact of POX on particular meat quality traits. However, the real causality with specific chemical changes induced by oxidative reactions is typically missing.

The present study was conceived to investigate (i) the formation of lysine-derived oxidation products, namely, AAS, AAA, and Schiff bases, during in vitro oxidation of MP by iron (Fe^{3+}), copper (Cu^{2+}), or myoglobin (Mb) and (ii) the potential impact of such chemical modifications on the functionality of MP.

MATERIALS AND METHODS

Chemicals and Meat Supply. All chemicals and reagents used for the present work were purchased from Panreac (Panreac Química, S.A., Barcelona, Spain), Merck (Darmstadt, Germany), or Sigma Chemicals (Sigma-Aldrich, Steinheim, Germany). Water used was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA, USA). Porcine meat (muscle longissimus dorsi) was obtained from a local slaughterhouse.

Extraction of MP. MP were extracted from porcine longissimus dorsi muscle according to the procedure used by Estevez et al.,[13] with minor modifications. Minced muscle was homogenized for 30 s with 4 volumes (v/w) of a cold isolation buffer (10 mM potassium phosphate, 0.1 N NaCl, 2 mM $MgCl_2$, and 1 mM EGTA at pH 7). Samples were centrifuged (2000 rpm for 15 min at 4 °C), and the supernatant was discarded; the pellet was washed twice with 4 volumes (v/w) of the same buffer. Then, the myofibrillar pellet was washed three times with 4 volumes of 0.1 N NaCl. Before the third centrifugation, the myofibrillar suspension was filtered through gauze, and the pH was adjusted to 6.0 with 0.1 N HCl. MP suspensions (5 mg/mL) were prepared in 15 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6) buffer containing 0.6 N NaCl.

Oxidation Systems. MP suspensions (5 mg/mL) were oxidized in vitro. Three different oxidation systems were considered depending on the addition of an oxidation promoter (25 μM), namely, iron ($FeCl_3$), copper ($CuSO_4$), or equine skeletal muscle myoglobin in combination with hydrogen peroxide (2.5 mM). Three experimental units (replicates) were prepared for each of the oxidation systems. All concentrations are expressed as final concentrations in the experimental units. Suspensions were oxidized in the dark at 37 °C for 12 days with constant stirring. According to preliminary studies, the chosen conditions of time and temperature guarantee the oxidation of myofibrillar proteins and the loss of MP functionality. Sampling was carried out at days 0, 3, 6, 9, and 12 for analyses.

Protein Oxidation. *HPLC-FLD Analysis of AAS.* Samples were derivatized with 50 mM aminobenzoic acid (ABA) and subsequently hydrolyzed with 6 N HCl according to the procedure described by Utrera et al.,[4] with minor modifications. An aliquot of MP suspensions (200 μL) was dispensed in 2 mL screw-capped eppendorf tubes. Proteins were precipitated with 2 mL of cold 10% TCA and subsequent centrifugation at 2000 rpm for 30 min. The resulting pellets were treated again with 2 mL of cold 5% TCA and proteins precipitated after centrifugation at 5000 rpm for 5 min. Pellets were then treated with 0.5 mL of 250 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) containing 1% sodium dodecyl sulfate (SDS) and 1 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 mL of 50 mM ABA in 250 mM MES buffer (pH 6.0), and 0.25 mL of 100 mM $NaBH_3CN$ in 250 mM MES buffer (pH 6.0). The derivatization was completed by allowing the mixture to react for 90 min while tubes were immersed in a water bath at 37 °C and stirred regularly. All solutions employed for the derivatization procedure were freshly made on sampling days. The derivatization reaction was stopped by adding 0.5 mL of cold 50% TCA followed by a centrifugation at 5000 rpm for 5 min. Pellets were then washed twice with 1 mL of 10% TCA and 1 mL of ethanol/diethyl ether (1:1, v/v). Centrifugations at 5000 rpm for 5 min were performed after each washing step. Protein hydrolysis was performed at 110 °C for 18 h in the presence of 6 M HCl. Hydrolysates were finally dried in vacuo at 40 °C using a Savant speed-vac concentrator. Hydrolysates were finally reconstituted with 200 μL of Milli-Q water and filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 μm pore size, Pall Corp., USA) for HPLC analysis.

Samples were injected in a HPLC equipped with a Cosmosil 5C18-AR-II RP-HPLC column (5 μm, 150 × 4.6 mm) and a guard column (10 × 4.6 mm) filled with the same material. The Shimadzu "Prominence" HPLC apparatus (Shimadzu Corp., Kyoto, Japan) was also equipped with a quaternary solvent delivery system (LC-20AD), DGU-20AS online degasser, SIL-20A autosampler, RF-10A XL fluorescence detector, and CBM-20A system controller. Sodium acetate buffer (50 mM, pH 5.4) (eluent A) and acetonitrile (ACN) (eluent B) were used as eluents. A low-pressure gradient program was used, varying B concentration from 0% (min 0) to 8% (min 20). The volume injection was 1 μL, the flow rate was kept at 1 mL/min, and the temperature of the column was maintained constant at 30 °C. Excitation and emission wavelengths were set at 283 and 350 nm, respectively. Identification of the derivatized semialdehyde in the FLD chromatograms was carried out by comparing their retention times (Rt) with that from a standard AAS (9), injected and analyzed under the above-mentioned conditions. The peak corresponding to AAS−ABA were manually integrated from FLD chromatograms, and the resulting areas were plotted against an ABA standard curve (ranging from 0.1 to 0.5 mM). Regression coefficients >0.99 were obtained. The estimation of the quantities of AAS−ABA through an ABA standard curve was accomplished by assuming that the fluorescence emitted by 1 mol of ABA is equivalent to that emitted by 1 mol of derivatized protein carbonyl. Results are expressed as nanomoles AAS per milligram protein.

HPLC-FLD Analysis of AAA. An aliquot of MP suspensions (600 μL) was dispensed in 2 mL screw-capped eppendorf tubes. Proteins were precipitated with 1.6 mL of cold 10% TCA and subsequent centrifugation at 10000 rpm for 5 min. Protein hydrolysis was carried out at 110 °C for 18 h in the presence of 6 M HCl. Hydrolysates were finally dried in a vacuum centrifuge using a Savant speed-vac concentrator and finally reconstituted with 150 μL of 200 mM tetraborate buffer (pH 8.5). The derivatization procedure involved the addition of 50 μL of 0.2 mM 9-fluorenylmethyl chloroformate (FMoc) dissolved in acetonitrile. The tube was agitated and the derivatization allowed to proceed for 90 s; 500 mL of heptane was added and again vortex-mixed for 1 min to stop the reaction. The upper phase was discarded by aspiration followed by second and third extractions according to the same process. The lower phase was filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 μm pore size, Pall Corp., USA) for HPLC analysis. An aliquot (1 μL) from the reconstituted protein hydrolysates was injected and analyzed in the above-mentioned HPLC equipment using a Zorbax Eclipse AAA column (3.5 μm, 4.6 × 150 mm) and a guard column (10 × 4.6 mm) filled with the same material. Eluent A was 20 mM ammonium acetate (pH 6.5) and 15% methanol, and eluent B was 90% acetonitrile. The flow rate was constant at 1.0 mL/min, and the column was maintained at 35 °C. The gradient profile was as follows: 0−1.5 min, 12% B; 1.5−2.0 min, 12−18% B; 2.0−9.0 min, 18% B; 9.0−9.5 min, 18−25% B; 9.5−12.5 min, 25% B; 12.5−13.0 min, 25−30% B; 13.0−16.0 min, 30% B; 16.0−17.0 min, 30−40% B; 17.0−20.0 min, 40% B; 20.0−22.0 min, 40−50% B; 22.0−23.0 min, 50% B; 23.0−24.0 min, 50−99% B. Excitation and emission wavelengths were set at 263 and 313 nm, respectively. Identification of the derivatized aminoadipic acid in the FLD chromatograms was carried out by comparing their retention times (Rt) with that from a standard AAA injected and analyzed in the above-mentioned conditions. The peak corresponding to AAA-FMoc was manually integrated from FLD chromatograms and the resulting areas plotted against an AAA−FMoc standard curve with known amounts (ranging from 5 to 0.4 pM). Regression coefficients >0.98 were obtained.

Table 1. Evolution of AAS (Nanomoles per Milligram Protein) during in Vitro Oxidation of Myofibrillar Proteins (37 °C/12 Days) in the Presence of Iron, Myoglobin, and Copper (Mean ± Standard Deviation)[a]

	day 0	day 3	day 6	day 9	day 12	p value[b]
iron	0.03 d,z ± 0.01	0.34 b,y ± 0.05	0.58 a,y ± 0.07	0.17 c,y ± 0.03	0.25 bc,y ± 0.03	***
myoglobin	0.12 c,y ± 0.01	0.31 a,y ± 0.03	0.19 b,z ± 0.02	0.21 b,y ± 0.03	0.12 c,y ± 0.01	***
copper	0.38 c,x ± 0.02	1.55 a,x ± 0.15	1.49 a,x ± 0.25	0.93 b,x ± 0.05	1.04 b,x ± 0.03	***
p value[c]	***	***	***	***	***	

[a]Different letters (a–c) within the same row denote significant differences. Different letters (x–z) within the same column denote significant differences. [b]Significance level for the effect "sampling time": ***, $p < 0.001$. [c]Significance level for the effect "catalyst": ***, $p < 0.001$.

Schiff Base Structures. The emission of fluorescence by protein oxidation products (Schiff base structures) was assessed by using fluorescence spectroscopy. [13] A 1 mL aliquot of the MP suspensions was redissolved in 3 mL of the 15 mM PIPES buffer and then dispensed in a 4 mL quartz spectrofluorometer cell. Emission spectra of tryptophan were recorded from 400 to 500 nm with the excitation wavelength set at 350 nm (LS 55 Perkin-Elmer luminescence spectrometer). Excitation and emission slit widths were set at 10 nm, and data were collected at 500 nm per minute in both measurements. The content of Schiff base structures was expressed as fluorescence intensity units emitted at 460 nm.

Lipid Oxidation. Lipid oxidation was assessed by measuring the levels of thiobarbituric acid-reactive substances (TBARS) according to the method described by Chelh et al.[14] One milliliter of MP suspension was incubated with 1% (w/v) 2-thiobarbituric acid in 50 mM NaOH (0.50 mL) and 2.8% (w/v) trichloroacetic acid (0.50 mL) in a boiling water bath for 10 min. After cooling at room temperature for 20 min, the pink chromogen was extracted with *n*-butanol (2 mL) and its absorbance measured at 535 nm against a blank of *n*-butanol. TBARS concentrations were calculated using 1,1,3,3-tetraethoxypropane as standard. Results were expressed as milligrams malondialdehyde (MDA) per kilogram sample.

Protein Functionality. *Protein Solubility.* Changes in protein solubility were measured according to the method of Liu et al.[15] MP suspensions were incubated at 10 °C for 1.5 h, and then suspensions were centrifuged (2 °C) at 4000 rpm for 15 min. Protein solubility was defined as the protein concentration in the supernatant divided by the protein concentration of the original myofibril suspension. The protein concentration was determined according to the biuret method.[16]

Aggregation and Gelling. MP suspensions (1 mg/mL protein in 0.6 M NaCl, 50 mM PIPES buffer, pH 6.0) were assessed for aggregation and for the ability to form gels during heating from 30 to 80 °C in a water bath. Solution turbidity was monitored during 16 min by measurement of the absorbance at 600 nm according to the method of Xia et al.[17] Aggregation was measured as the turbidity of the sample before heating. Gelling was expressed as the percentage of the turbidity gained by the sample after heating calculated as % gelling = $[(Abs_{16} - Abs_0)/Abs_0] \times 100$, where Abs_0 is the initial turbidity of the suspension and Abs_{16} represents the turbidity of the sample after heating.

Water-Holding Capacity (WHC). An aliquot of MP suspensions was heated at 70 °C for 30 min in a water bath. After heating, suspensions were cooled in an ice slurry for 1 h. The WHC was determined by a centrifugal method. Briefly, samples (5 mL) were centrifuged at 2000 rpm for 15 min at 4 °C. WHC (%) was expressed as gel weight after centrifugation divided by gel weight before centrifugation multiplied by 100.

Foaming Capacity. Foaming capacity of the protein was determined according to the method described by Mohan et al.[18] Known volumes of MP suspension were dispensed in tubes and stirred in a vortex mixer at 18000 rpm for 1 min. Foaming capacity (% FC) was expressed as the percent volume increase after mixing and calculated as follows: % FC = (foam volume (mL) × 100)/initial MP suspension volume. The foam stability was calculated as the percentage of foam remaining after 30 min at room temperature as % foam stability = (volume of foam (mL) retained after 30 min × 100)/volume of foam after stirring.

Statistical Analysis. Analyses of variance (ANOVA) and Tukey tests were carried out to study the effect of the three oxidation promoters on lipid and protein oxidation and protein functionality. Differences were considered to be significant at $p \leq 0.05$. Relationships among oxidation parameters and protein functionalities were calculated using Pearson's correlation coefficients. A principal component analysis (PCA) was also performed using SPSS for Windows (v. 15.0).

RESULTS AND DISCUSSION

Effect of Iron, Myoglobin, and Copper on the Carbonylation Pathway. *Formation of AAS.* AAS and GGS are the most abundant protein carbonyls, which are usually employed as indicators of protein oxidation in biological systems.[3] Carbonylation of MP can be induced in vitro by several ROS-generating systems including metal-catalyzed oxidation (MCO) or myoglobin-mediated oxidation systems.[3,19] According to the results of the present study, the presence of the three pro-oxidants analyzed, Fe^{3+}, Cu^{2+}, and Mb, induced the formation of AAS throughout the oxidation assay (Table 1). The three pro-oxidants displayed different abilities to promote the carbonylation of MP (Table 1). Cu^{2+} displayed the most intense pro-oxidant action toward the formation of AAS from MP followed by Fe^{3+} and Mb. The formation of AAS involves an eventual oxidative deamination of lysine residues, which could be induced by the presence of metal ions.[20] The metals tested as pro-oxidants in the present study would be implicated in the formation of ROS through the reaction with H_2O_2 (Fenton reaction). Furthermore, both metals, Cu^{2+} and Fe^{3+}, could be involved in the formation of ROS in the presence of molecular oxygen by a chemistry similar to that which occurs in the Fenton reaction.[21] The resulting ROS, such as the hydroxyl radical, would initiate the oxidative degradation of lysine by abstracting a susceptible hydrogen atom and inducing the formation of a protein carbon-centered radical.[3] The hydroxyl radicals as well as the protein radicals perpetuate the oxidative reactions by abstracting a new hydrogen atom from a neighboring amino acid side chain. As a result, the combination of transition metals with H_2O_2 results in a highly effective pro-oxidant system for generating AAS from MP. In agreement with the present results, copper ions have been previously described as more efficient than their iron counterparts in promoting the formation of AAS during the in vitro oxidation of MP.[19] The enhanced pro-oxidant activity of copper could be ascribed to its ability to bind to specific binding sites in proteins, also observed during the oxidation of collagen[22] and low-density lipoproteins.[23] According to Lauritzsen and Martinsen[24] positively charged copper ions are able to bind to negatively charged proteins or other tissue components, where they can exert a caged pro-oxidant action. In addition, autoxidation of the metal may reduce oxygen to a superoxide radical anion, which can dismutate to hydrogen peroxide or oxidize to singlet oxygen.[24]

Table 2. Evolution of AAA (Picomoles per Milligram Protein) during in Vitro Oxidation of Myofibrillar Proteins (37 °C/12 Days) in the Presence of Iron, Myoglobin, and Copper (Mean ± Standard Deviation)[a]

	day 0	day 3	day 6	day 9	day 12	p value[b]
iron	0.03 c ± 0.0[c]	39.3 b,y ± 8.7	71.6 a,x ± 10.1	65.6 a,y ± 19.5	60.4 a,y ± 11.4	***
myoglobin	0.0 d ± 0.0	71.9 c,x ± 10.5	78.9 c,x ± 11.3	95.0 b,x ± 6.7	131.3 a,x ± 25.5	***
copper	0.0 c ± 0.0	22.1 b,z ± 4.4	44.9 a,y ± 8.2	45.6 a,y ± 5.8	53.2 a,y ± 10.7	***
p value[d]	ns	*	*	*	*	

[a]Different letters (a–c) within the same row denote significant differences. Different letters (x–z) within the same column denote significant differences. [b]Significance level for the effect "sampling time": ***, $p < 0.001$. [c]The amount of AAA at day 0 was below the quantification limit. [d]Significance level for the effect "catalyst": *, $p < 0.05$; ns, nonsignificant.

Table 3. Evolution of Schiff Bases (Fluorescence Intensity) during in Vitro Oxidation of Myofibrillar Proteins (37 °C/12 Days) in the Presence of Iron, Myoglobin, and Copper (Mean ± Standard Deviation)[a]

	day 0	day 3	day 6	day 9	day 12	p value[b]
iron	78 c ± 17	124 c,y ± 23	124 c,y ± 10	284 b,z ± 66	455 a,y ± 91	***
myoglobin	60 e ± 17	324 d,x ± 39	1016 c,x ± 115	1251 b,x ± 55	1757 a,x ± 163	***
copper	61 e ± 12	93 d,y ± 11	126 c,y ± 19	484 b,y ± 63	1926 a,x ± 132	***
p value[c]	ns	**	***	***	***	

[a]Different letters (a–c) within the same row denote significant differences. Different letters (x–-z) within the same column denote significant differences. [b]Significance level for the effect "sampling time": ***, $p < 0.001$. [c]Significance level for the effect "catalyst": **, $p < 0.01$; ***. $p < 0.001$; ns, nonsignificant.

On the other hand, the initiation of protein oxidation by Mb/H_2O_2 systems is known to involve the formation of hypervalent species such as ferrylmyoglobin, an intermediate with radical character.[25,26] This intermediate is thought to result from the one-electron reduction of H_2O_2 by heme with concomitant formation of a hydroxyl radical that rapidly reacts with an amino acid residue and forms a protein radical.[25] An alternative mechanism involves the heterolytic cleavage of H_2O_2, giving a porphyrin radical cation, which also may induce the formation of a protein radical.[25] A number of formation sites have been reported for these species (e.g., tyrosine, tryptophan, and cysteine residues).[26] For this reason, Mb/H_2O_2 systems have been highlighted to be more efficient at promoting the loss of tryptophan, tyrosine, and sulfhydryl groups than at promoting the carbonylation of muscle proteins.[1] However, Park et al.[27] reported that metmyoglobin (MetMb) promoted the formation of carbonyls from MP to a greater extent than an iron-catalyzed oxidation system (Fe^{3+}/ascorbic acid/H_2O_2).

Formation of AAA. The evolution of AAS during the in vitro oxidation of MP highlights the reactivity of the carbonyl moiety. The highest concentration of AAS was found at day 3 for Mb and Cu^{2+} systems and at day 6 for Fe^{3+} systems and was followed by a significant decrease in the subsequent sampling points. This finding is consistent with that previously reported by Estévez et al.[5] and Estévez and Heinonen[19] during the in vitro oxidation of MP in the presence of copper acetate, iron(III) chloride, or Mb in combination with H_2O_2. Similar results have also been observed in complex muscle foods such as ground meat subjected to frozen storage[28] and porcine patties subjected to cooking and chilled storage.[6] The loss of AAS at prolonged oxidation rates may be caused by the implication of the semialdehyde in further reactions as recently reviewed by Estévez.[3] To this regard, the formation of AAS from lysine may be the first step of a more complex oxidation pathway, which is, so far, largely unknown in biological systems. Recent scientific evidence supports the occurrence of a further oxidation step by which the aldehyde moiety of the semialdehyde is oxidized into a carboxylic acid. This further oxidation step would take place under intense oxidative conditions (i.e., induced by hydroxyl radical generating systems) and leads to the formation of AAA from AAS.[3] The formation of AAA has been described in cell cultures and model systems[8] and also recently in a muscle food matrix.[6] This latter study reveals that meat processing (mincing, cooking, ...) creates the suitable oxidation conditions for the formation of AAA from the corresponding semialdehyde.

Certainly, in the present study, the formation of AAA was confirmed to occur during the in vitro oxidation of MP by Fe^{3+}, Cu^{2+}, and Mb (Table 2). Measurable quantities of AAA were obtained at day 3, and successive increments of this compound were observed during the subsequent sampling times. It is worth noting that the formation of AAA from AAS requires the presence of peroxides,[3] and the H_2O_2 may play this role in the conditions of the present experiment. Compared to the transition metals, Mb displayed the most intense pro-oxidant action toward the formation of AAA (131.3 ± 25.5 pmol/mg protein). In fact, around 50% of the AAS formed in Mb/H_2O_2 systems was subsequently transformed into the corresponding AAA. Direct comparison of quantities between oxidation products should be made with caution as recoveries for AAA were found to be lower than that of AAS due to the nature of the analytical procedure (acid hydrolysis prior to derivatization). A potentially higher amount of H_2O_2 molecules may be available in Mb/H_2O_2 systems to induce the oxidation of the aldehyde moiety into the carboxylic group. In fact, in the presence of Mb, the H_2O_2 may not be consumed as intensively as in MCO systems. In such systems, H_2O_2 is permanently decomposed to regenerate the redox cycle. This hypothesis would reasonably explain the higher efficiency of Mb/H_2O_2 systems at promoting the formation of AAA compared to the MCO. Meanwhile, no significant differences were observed between Fe^{3+} and Cu^{2+} systems for the amount of AAA (60.4 ± 11.4 and 53.2 ± 10.7 pmol/mg protein, respectively) (Table 2). Although AAS has been identified as a suitable protein oxidation marker in a variety of meat products,[7,29] AAA, a stable end oxidation product, might be a more reliable marker for protein oxidation than its precursor, as proposed by Sell et

Table 4. Evolution of TBARS (Milligrams MDA per Kilogram Sample) during in Vitro Oxidation of Myofibrillar Proteins (37 °C/12 Days) in the Presence of Iron, Myoglobin, and Copper (Mean ± Standard Deviation)[a]

	day 0	day 3	day 6	day 9	day 12	p value[b]
iron	0.25 b,y ± 0.05	0.57 a,x ± 0.04	0.49 a,x ± 0.02	0.56 a,x ± 0.05	0.56 a,x ± 0.05	***
myoglobin	0.12 c,y ± 0.04	0.40 a,y ± 0.04	0.31 b,y ± 0.03	0.30 b,z ± 0.03	0.28 b,z ± 0.01	***
copper	0.73 a,x ± 0.06	0.65 ab,x ± 0.11	0.53 bc,x ± 0.05	0.43 c,y ± 0.04	0.48 c,y ± 0.01	***
p value[c]	***	***	***	***	***	

[a]Different letters (a–c) within the same row denote significant differences. Different letters (x–z) within the same column denote significant differences. [b]Significance level for the effect "sampling time": ***, $p < 0.001$. [c]Significance level for the effect "catalyst": ***, $p < 0.001$.

al.[8] Because AAS is liable to undergo further oxidation reactions, its usage as an indicator of protein oxidation may lead to an underestimation of the actual extent of the oxidation damage. The reliability of AAA as a protein oxidation marker may also be dependent on the proportion of AAS transformed into the carboxylic acid, which is, in turn, affected by the oxidative conditions. It is also worth noting that AAA may also be involved in further reactions (i.e., esterification) depending on the reaction environment. Therefore, the claim of the suitability of AAA as a reliable indicator of protein oxidation in food systems should also be made with prudence.

Formation of Schiff Bases. In addition, the carbonyl moiety of an AAS residue may react with an ε-amino group from a neighboring protein-bound amino acid or with another protein-bound AAS residue to form a covalent bond via Schiff base or aldol condensation structure, respectively.[3,6] The potential formation of these condensation products was also assessed by recording fluorescence at 400–500 nm when excited at 350 nm (Table 3). As expected, a significant increase in the level of the aforementioned condensation structures was detected in the systems under study (Table 3). Mb/H_2O_2 systems were found to be the most efficient promoters of Schiff base formation, whereas the lowest potential was displayed by the Fe^{3+}/H_2O_2 system (Table 3). The accumulation of Schiff bases as induced by the Mb/H_2O_2 system at day 12 was similar than that induced by the Cu^{2+}/H_2O_2 system (1757 ± 163 vs 1926 ± 132 fluorescence intensity units). However, the formation of Schiff bases in the former system occurred gradually during the in vitro experiment, whereas the formation of such structures in the latter mostly occurred at the end of the oxidation assay (Table 3). It is worth noting that the formation of Schiff bases involves the formation of intra- or intermolecular cross-links, depending on the location of the reacting residues. In such a manner, the formation of these condensation structures not only depends on carbonyl production and the availability of amino groups in protein side chains but also requires a favorable stereochemical distance between reacting groups and may take place in the absence of oxygen.[3] The increases of Schiff base structures in the MP suspensions under study are consistent with the previously reported decrease of AAS ($r = -0.61$). The formation of Schiff bases could be at least partially attributed to the reaction of AAS between them or with amino groups of MP. Similar findings were reported by Utrera et al.[6] in a previous study devoted to the assessment of the formation of lysine oxidation products in processed porcine patties.

The enhanced formation of advanced protein oxidation products (AAA and Schiff base structures) in Mb/H_2O_2 systems could be attributed to the rapid electron transfer between the radicals generated by the reaction of Mb and H_2O_2.[30] Mb and H_2O_2 undergo a reaction that does not require catalysis by trace transition metals.[31] It is generally accepted that this reaction initially yields a transient compound in which the ferryl is coupled to a porphyrin.[30,31] Therefore, it is likely that the protein radical is generated by subsequent rapid electron transfer from the protein to the porphyrin radical.[30] The resulting protein radical appears to transfer efficiently from one residue to another over considerable distances.[30] Consequently, a mechanism for the dispersal of the free radical center is therefore not a prerequisite for radical formation at a distal residue in MetMb, unlike MCO systems. This efficient oxidation mechanism may explain why Mb has been recurrently described as an effective promoter of the formation of cross-links as an expression of protein oxidation. Lund et al.[1] described the role of Mb oxidation systems in the formation of disulfide bonds and dityrosines in muscle foods. According to the present results, Mb may also be actively implicated in the formation of cross-links via Schiff base formation.

Formation of TBARS. Lipid oxidation, measured as TBARS, was also assessed (Table 4) to evaluate the potential influence of this phenomenon on the carbonylation pathway. Whereas the evolution of the TBARS numbers during the oxidation assay depended on the pro-oxidant applied, the overall trend was similar to that of the AAS. In this manner, the highest concentration of TBARS was found within the first 3 days of the oxidation assay in all systems and showed a significant decrease in the case of suspensions oxidized by Mb and Cu^{2+}, but remained constant in suspensions oxidized by Fe^{3+} (Table 4). The decrease of the concentration of TBARS at the last stages of the oxidation assay may be attributed to the likely implication of these carbonyls in further reactions such as the formation of adducts with protein side chains as reported by Chelh et al.[14] These authors demonstrated that the interaction between proteins and lipid-derived aldehydes mainly led to the production of fluorescent pigments in cooked meat via Schiff base formation. This fact could explain that suspensions oxidized by Cu^{2+} had higher amount of TBARS, AAS, and Schiff base structures. Interestingly, there is a timely coincidence between the loss of TBARS over time and the sudden increase of Schiff bases by the end of the oxidation assay in suspensions oxidized by Cu^{2+}. In agreement with the present results, copper has been reported to be more effective than iron in stimulating the decomposition of peroxides and the formation of fluorescent lipid complexes.[32]

Effect of in Vitro Oxidation on the Functionality of MP. Protein solubility, aggregation, WHC, and foaming and gelling capacities were measured in the protein suspensions during the oxidation assay and used as indicators of the functionality of MP. In general, the functional properties of MP in the suspensions were altered in accordance with the oxidation extent of the samples.

The solubility of proteins oxidized with Cu^{2+} decreased progressively during the course of oxidation (Figure 1). In the case of proteins suspensions oxidized with Fe^{3+}, the solubility

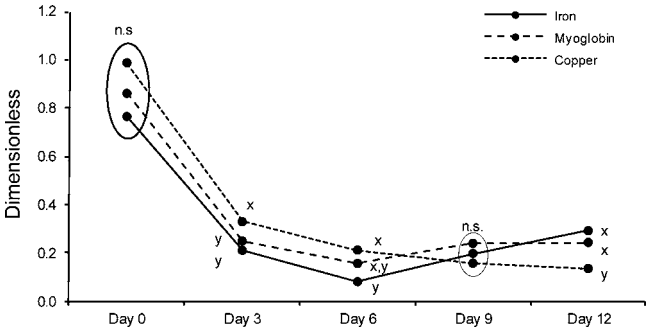

Figure 1. Evolution of the solubility of myofibrillar proteins during their in vitro oxidation (37 °C/12 days) in the presence of iron, myoglobin, or copper.

gradually diminished until day 6 of the oxidation period (Figure 1). The apparent improvement observed in protein solubility oxidized by Fe^{3+} at day 6 could be ascribed to regaining protein−water interaction. As observed by Liu et al.,[15] who studied the influence of different NaCl concentrations on protein solubility, the loss of protein charges as a result of carbonyl formation could be compensated by Cl^- ions available in the oxidation environment. On the other hand, a significant and irreversible loss on the solubility of MP oxidized by Mb was observed at day 3 (Figure 1). Similar results were found in iron- and copper-oxidized turkey MP,[12] in iron-oxidized porcine MP,[15] and in chicken breast proteins in which carbonylation was induced by cooking treatments.[33]

Turbidity measurement (absorbance at 600 nm) was used to monitor protein aggregation. Protein molecules may aggregate as a consequence of oxidation, leading to steady increases in optical density. In fact, MP suspensions exhibited increasing turbidity as the oxidation assay progressed (Figure 2). The

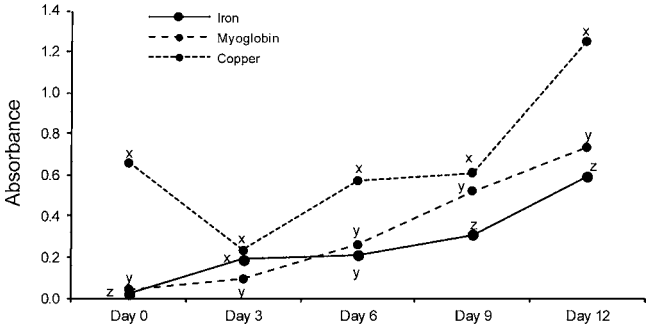

Figure 2. Evolution of the aggregation of myofibrillar proteins during their in vitro oxidation (37 °C/12 days) in the presence of iron, myoglobin, or copper.

aggregation rate was significantly different among oxidation systems. The formation of protein aggregates was more intense in suspensions oxidized by Cu^{2+} followed by those oxidized by Mb and Fe^{3+}. Increased aggregation displayed by MP was comparable with that suffered by soy proteins subjected to oxidation by hydroperoxides.[34] Similar results were also observed in surimi protein suspensions with increased amounts of protein oxidation carbonyls.[35] Many of the functional properties of MP are dependent on the association among individual proteins and, hence, oxidative modifications leading to polymerization and massive aggregation may cause significant deleterious effects in muscle foods. [2]

Consistent with the increase in protein oxidation products, the WHC of protein suspension decreased during the course of oxidation in the presence of Fe^{3+}, Mb, and Cu^{2+} (Figure 3).

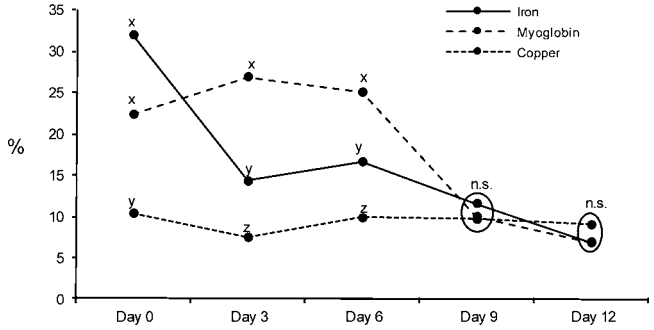

Figure 3. Evolution of the WHC of myofibrillar proteins during their in vitro oxidation (37 °C/12 days) in the presence of iron, myoglobin, or copper.

The faster and more intense loss of WHC was induced by Cu^{2+} (Figure 3). The loss of the WHC was previously reported in turkey MP oxidized in vitro by Fe^{3+} and Cu^{2+}.[12] Recently, Estévez et al.[28] reported that the loss of WHC during frozen storage of porcine muscles occurred concomitantly with intense protein oxidation phenomena.

Little is known about the potential effect of oxidation on the foaming ability of myofibrillar proteins. In agreement with other functional properties previously described, Cu^{2+} ions induced a faster and more intense loss of the foaming capacity of MP than Fe^{3+} and Mb. Already at day 0, the foaming capacity of MP suspensions was reduced 4-fold more intensely in the presence of Cu^{2+} than in the presence of Fe^{3+} and Mb. The foaming capacity was completely lost at day 6 in suspensions oxidized by Cu^{2+} (Figure 4A). On the other hand, both Fe^{3+} and Mb induced the reduction on the foaming capacity of MP to a lower extent during the same oxidation period (around half of the initial values) (Figure 4A). As well as the foaming capacity, the foaming stability of MP was affected as the oxidation progressed (Figure 4B). According to our results, the foaming stability of proteins was more susceptible to oxidation than the foaming capacity. In this manner, foaming stability was completely lost at days 3, 9, and 12 in suspensions oxidized by Cu^{2+}, Fe^{3+} and Mb, respectively (Figure 4B). In contrast to the present findings, Liu et al.[15] reported an increase of the foaming ability and a decrease of the foaming stability of oxidized egg white protein induced by irradiation.

The gelling ability of MP was one of the most impaired functional properties by oxidation. The gelling capacity of proteins oxidized by Cu^{2+} and Mb was lost at day 3, whereas it was lost at day 6 in proteins oxidized by Fe^{3+} (Figure 5). These observations contrast with the enhanced gelling capacity observed in MP oxidized in vitro by iron and MetMb for 24 h at 4 °C.[36] In this regard, Xiong[2] hypothesized that slow and mild-to-moderate oxidation of MP could lead to an improvement of the stability and rheological properties of gels through the formation of cross-links via carbonyl−amine condensations.[36] In contrast, extensive protein oxidation, such as that induced in the present experiment, would result in decreased functionality due to random and excessive aggregation phenomena.

Relationship between Protein Oxidation and MP Functionality. The potential implication of protein oxidation

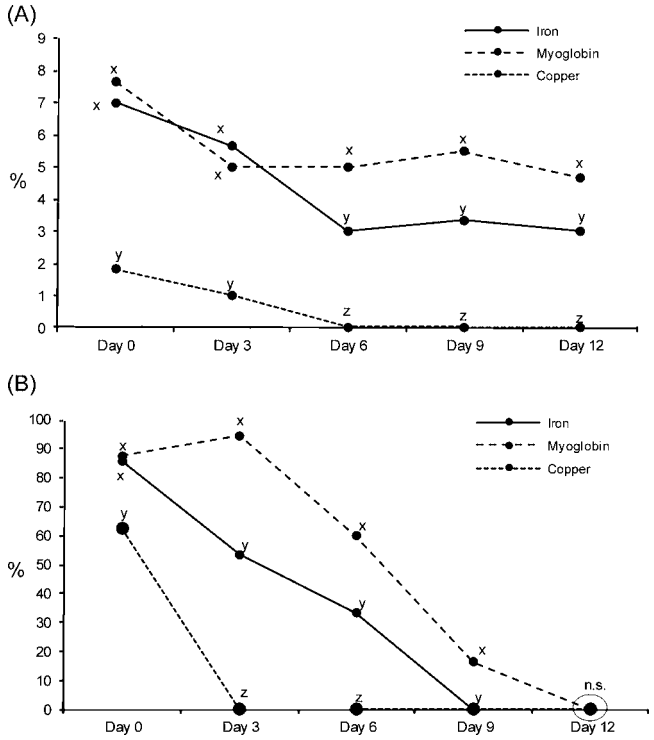

Figure 4. Evolution of the foaming capacity of myofibrillar proteins (A) and the stability of the foam (B) during their in vitro oxidation (37 °C/12 days) in the presence of iron, myoglobin, or copper.

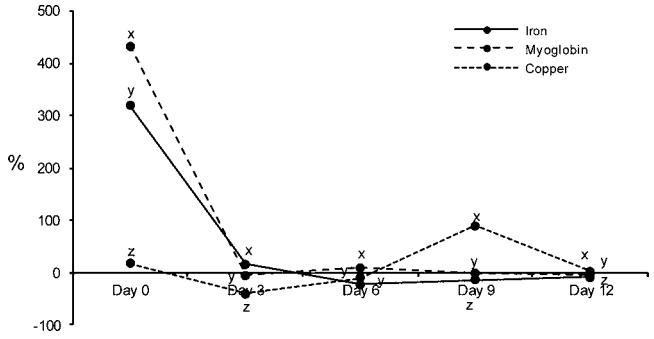

Figure 5. Evolution of the gelling capacity of myofibrillar proteins during their in vitro oxidation (37 °C/12 days) in the presence of iron, myoglobin, or copper.

on particular food quality traits has been a recurring challenge for food chemists as the discovery of such an implication would highlight the scientific and technological significance of protein oxidation. Already in early studies, the onset of protein oxidation in muscle-based systems was initially linked to loss of protein functionality, including alterations in solubility, viscosity, gelation, emulsification, and water-holding capacities.[2,12] The link between protein oxidation and the alleged impaired functionality was usually established on the basis of significant correlations between protein carbonyl measurements (DNPH method) and the assessed functionality.[3] In most of those previous studies, however, the potential causality was rarely supported by scientific arguments explaining the precise implication of protein carbonyls in the loss of protein functionality. The simplicity of the method traditionally applied for measuring protein carbonylation (DNPH method) probably hindered the possibility of accomplishing an in-depth discussion of the results. In agreement with those previous studies, significant correlations were found between the protein oxidation measurements and the functional properties assessed in the present study (Table 5). These data suggest that the impaired functionality of MP could be a consequence of the severe chemical changes resulting from the in vitro oxidation. In the present study, the precise description of the carbonylation pathway, which includes detection of specific protein carbonyls and the formation of advanced oxidation products, may enable a more precise understanding of the potential impact of protein oxidation on protein functionality. It is worth mentioning that other expressions of the oxidative damage to proteins not considered in the present study (e.g., formation of disulfide bonds or cleavage of peptide bonds) could also have contributed to impair the functionality of myofibrillar proteins.

Table 5. Pearson Correlations (r) between Protein Oxidation Measurements and Protein Functionality Indices[a]

	TBARS	AAS	AAA	SB
solubility	−0.11	−0.29*	−0.68***	−0.40**
WHC	−0.52***	−0.45**	−0.30*	−0.30*
foaming capacity	−0.54***	−0.80***	−0.01	−0.10
foam stability	−0.37*	−0.51***	−0.43**	−0.39**
aggregation	0.26	0.33*	0.30*	0.69***
gelation capacity	−0.64***	−0.35*	−0.55***	−0.28

[a]Significance level: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, nonsignificant.

Significant correlations were found between the formation of AAS and the loss of WHC and the foaming capacity and stability of proteins (Table 5). As explained before, the formation of AAS involves the loss of amino groups from amino acid side chains. As polar residues, the side chains of basic amino acids such as lysine would be orientated outward with respect to the bulk water and, hence, more exposed and accessible to potent oxidation promoters such as metal ions and myoglobin.[3,37] An intense loss of protonable amino groups as a result of severe carbonylation would reasonably lead to an alteration of the distribution of the electrical charges and the overall electrical arrangement of MP as previously observed in bovine serum albumin and other animal proteins.[20,38] The modification of the electronic arrangement of MP may cause the alteration of the molecules interactions and, hence, the modification of some functional properties of protein. The WHC of proteins may be altered by the loss of interactions between polar groups from myofibrillar proteins and water molecules, which is essential for their binding abilities.[37] In accordance, Estévez et al.[28] reported significant correlations between protein carbonylation and WHC of proteins from porcine muscles subjected to frozen storage. On the other hand, the foaming capacity is modified by the loss of protein−air interactions. Foaming refers to the proteins' capacity to form a flexible cohesive film to entrap air in the formed air/liquid interface during air-bubbling.[39] The configuration of the aforementioned film is enabled by the formation of protein−air interactions, which depend on the electronic arrangement of proteins.[39] Therefore, a great foam capacity and stability is expected when a large number of protein and air molecules interact. Therefore, it is plausible to hypothesize that at high oxidation rates, random protein−air interactions may occur that would lead, in turn, to a decreased foam stability. The loss of functional properties that correlated with the formation of AAS also correlated significantly with TBARS measurements. The

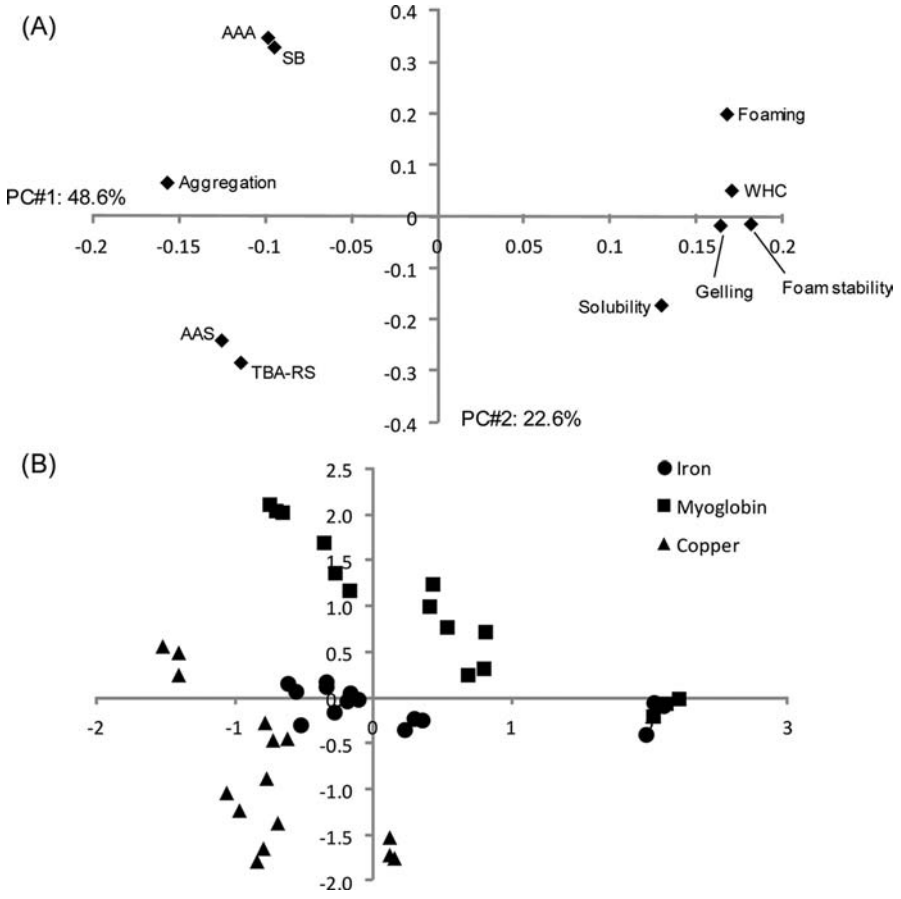

Figure 6. In vitro oxidation of myofibrillar proteins: projection of the oxidation and functionality indices (A) and the samples from the oxidation systems, iron, myoglobin, or copper (B), onto the space defined by the principal components (PC#1/PC#2).

TBA test involves the reaction between TBA and MDA produced during lipid oxidation. It is known that AAS and MDA are secondary protein and lipid oxidation products, respectively, so it is plausible that MDA is formed concurrently with the physicochemical changes of proteins that promoted the loss of WHC and foaming capacity.

On the other hand, significant correlations were found between the formation of AAA and the loss of solubility, gelation, and foaming stability (Table 5). The formation of AAA involves an initial deamination of protein-bound lysine residues and a subsequent oxidation to form carboxylic moieties. Unlike the original protonable amino groups in lysine residues, the newly formed carboxylic moieties can hold a negative charge.[8] Thus, the formation of AAA may also contribute to impair the electronic arrangement of the proteins. It is plausible that this severe chemical modification may have several consequences, namely, (i) a decrease of protein solubility possibly derived from the loss of protein−water interactions;[15] (ii) protein−protein interaction that might be favored by the aforementioned decrease in protein solubility and exposure of hydrophobic amino acid side-chain groups that would increase aggregation of proteins;[17] and (iii) a disruption in the protein−air interactions at the air/water interface, reducing the foaming stability.[39] It is remarkable that relevant technological properties of MP such as gelation are particularly affected by this carboxylation process.

Likewise, Schiff base formation was highly correlated to the loss of protein solubility, aggregation, and foaming stability (Table 5). These correlations could be ascribed to protein modifications caused by the intra- and/or intermolecular cross-linking of proteins. One of the most important consequences of cross-linking is that protein conformation would be altered.[3,26] These changes on the tertiary and quaternary protein structures would affect proteins hydration[26] and thereby the disruption of water−protein interactions and the subsequent loss of solubility. On the other hand, cross-linking via carbonyl−amine condensations may promote the formation of protein−protein interactions, enhancing the formation of protein aggregates.[2,26,36] In addition, the formation of new cross-links might enhance the rigidity of proteins at the air/water interface and decrease the foaming stability of proteins.[39]

A PCA was performed using data obtained from the oxidation and functionality measurements in MP suspensions to determine the relationship between such variables. Figure 6A shows the similarity map of the measured parameters defined by the two first principal components (PC#1 and PC#2, respectively) that accounted for the 71.2% of the total variability. The opposite location of the oxidation and the functionality parameters along the PC#1 axis support the hypothesis previously formulated regarding the impact of the oxidation-induced chemical changes on the loss of MP functionality. The aggregation, as an expression of the polymerization of individual MP as a result of the loss of effective water−protein interactions, is located on the negative axis of PC#1, together with the oxidation parameters. Interestingly, both lipid and protein carbonyls are located close to each other on the left lower quadrant and in clear

divergence from the secondary protein oxidation products, AAA and Schiff bases, located in the left upper quadrant.

The projection of the samples onto the space of PC (Figure 6B) shows a clear discrimination between oxidation promoters, Fe^{3+}, Cu^{2+}, and myoglobin. This result confirms that the pro-oxidant action of transition metals and the myoglobin is governed by different mechanisms and leads to different oxidation expressions within the carbonylation pathway. Cu^{2+} ions, which were found to induce the most intense alteration of MP functionality, are located in the plane area of TBARS and AAS. Suspensions intensively oxidized in the presence of Mb are related to high values of AAA and Schiff bases. Iron samples are in an intermediate position close to the origin.

In conclusion, intense lipid and protein carbonylations, principally induced by Cu^{2+}/H_2O_2 systems, lead to a fast and severe loss of MP functionality, including impaired water-holding, foaming, and gelling capacities. However, considering that meat systems are particularly rich in iron and myoglobin, these pro-oxidants are more likely the main promoters of protein oxidation muscle foods. In particular, the implication of Mb in the oxidation events enhances the production of AAA and Schiff bases, compromising to a larger extent the solubility of MP and worsening the aggregation and the gelling capacity. The understanding of the factors and consequences of the carbonylation pathway on MP would enable the development of antioxidant strategies to control the negative impact of protein oxidation on muscle food quality. Taking into consideration the limitations of the present study to reproduce the oxidative conditions during meat storage/processing, future studies should confirm whether these chemical changes have such an impact in real meat systems.

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Funding

M.E. receives support from the Spanish RYC-MICINN program (RYC-2009-03901), from the Spanish Ministry of Economics and Competitiveness through Project AGL2010-15134, and from the Executive Research Agency from the European Community through a Marie Curie Reintegration Fellowship (PERG05-GA-2009-248959; Pox-MEAT). M.U. is supported by the University of Extremadura through a predoctoral grant (Human Resources Recruitment Program "C Action").

Notes

The authors declare no competing financial interest.

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