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Carbonylation of Myofibrillar Proteins through the Maillard Pathway: Effect of Reducing Sugars and Reaction Temperature

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ABSTRACT: Carbonylation is recognized as one of the most remarkable chemical modifications in oxidized proteins and is generally ascribed to the direct attack of free radicals to basic amino acid residues. The purpose of this work was to investigate the formation of specific carbonyls, α-aminoadipic and γ-glutamic semialdehydes (AAS and GGS, respectively), in myofibrillar proteins (MP) through a Maillard-type pathway in the presence of reducing sugars. The present study confirmed the concurrent formation of protein carbonyls and advanced glycation end-products (AGEs) during incubation (80 °C/48 h) of MP (4 mg/mL) in the presence of reducing sugars (0.5 M). Copper irons (10 μM) were found to promote the formation of protein carbonyls, and a specific inhibitor of the Maillard reaction (0.02 M pyridoxamine) blocked the carbonylation process which emphasize the occurrence of a Maillard-type pathway. The Maillard-mediated carbonylation occurred in a range of reducing sugars (0.02−0.5 M) and reaction temperatures (4−110 °C) compatible with food systems. Upcoming studies on this topic may contribute further to shed light on the complex interactions between protein oxidation and the Maillard reaction and the impact of the protein damage on food quality and human health.

KEYWORDS: *α-aminoadipic semialdehyde, γ-glutamic semialdehyde, AGEs, protein oxidation, Maillard reaction*

INTRODUCTION

The complex protein oxidation pathways and mechanisms described in medical research[1] are still largely ignored by food scientists. This lack of knowledge of basic food chemistry affects not only specific protein oxidation routes and mechanisms but also the potential connection of protein oxidation pathways and products with lipid oxidation and the Maillard reaction. Nowadays, protein oxidation is recognized to have an impact on protein conformation, functionality, and digestibility and on the overall quality of muscle foods.[2] In food systems, protein oxidation has been assessed through several of its multiple chemical manifestations including loss of sulfhydryl groups,[3] loss of tryptophan fluorescence,[4] gain of carbonyl derivatives,[6] and formation of intra- and intermolecular cross-links.[6] Among the aforementioned changes, the formation of carbonyl compounds has been highlighted as one of the most salient modifications in oxidized proteins.[7,8] The identification of the routes and mechanisms involved in the formation of specific protein carbonyls is essential to establish the potential implication of such compounds on particular food quality traits.

The oxidation of the side chains of certain amino acids (lysine, threonine, arginine, and proline) by metal-catalyzed oxidation (MCO) systems has been highlighted as the main route for protein carbonylation and the most potent and major source of direct oxidative attack to proteins.[9] According to this mechanism, hydroxyl radicals formed through the Fenton reaction induce the oxidative deamination of the side chain of basic amino acids leading to the formation of the carbonyl derivative (Figure 1). As a consequence of MCO, threonine is converted into α-amino-3-keto butyric acid, lysine into α-amino adipic semialdehyde (AAS), and arginine and proline into γ-glutamic semialdehyde (GGS). The two latter (AAS and GGS) have been identified as the main carbonyl in meat proteins with both semialdehydes accounting up to 70% of the total carbonyl compounds.[10] Recently, these two compounds (AAS and GGS) have been detected in various muscle foods by high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS)[5,11,12] or by fluorescent HPLC as p-aminobenzoic acid (ABA)-derivatives.[13,14] The formation of carbonyls by MCO systems is, to our knowledge, the only mechanism that has been proved to yield carbonyls in food proteins.[15]

In medical research, Akagawa et al.[16] revealed the existence of a novel mechanism for the formation of specific protein carbonyls *in vivo*. As reported by these authors, the concentration of AAS and GGS in blood samples from diabetic rats was higher than in samples from normal controls. These researchers hypothesized that circulating reducing sugars may have contributed to form AAS and GGS in plasma proteins from diabetic rats through a Maillard-type mechanism. According to this mechanism, α-dicarbonyl compounds derived from the Maillard reaction may react with the ϵ-amino group from protein-bound lysine residues inducing the oxidative deamination of the amino acid and, hence, the formation of the corresponding semialdehyde (AAS).[17] To our knowledge, this alternative route of protein carbonylation has never been described before in the food science field, and it is currently unknown whether or not food proteins undergo carbonylation in the presence of reducing sugars. On the other hand, it is well-known that the carbonylamine condensation between carbonyls from monoand disaccharides and amino groups from proteins takes place in food systems as a starting point for the Maillard reaction. This reaction is responsible for the formation of a

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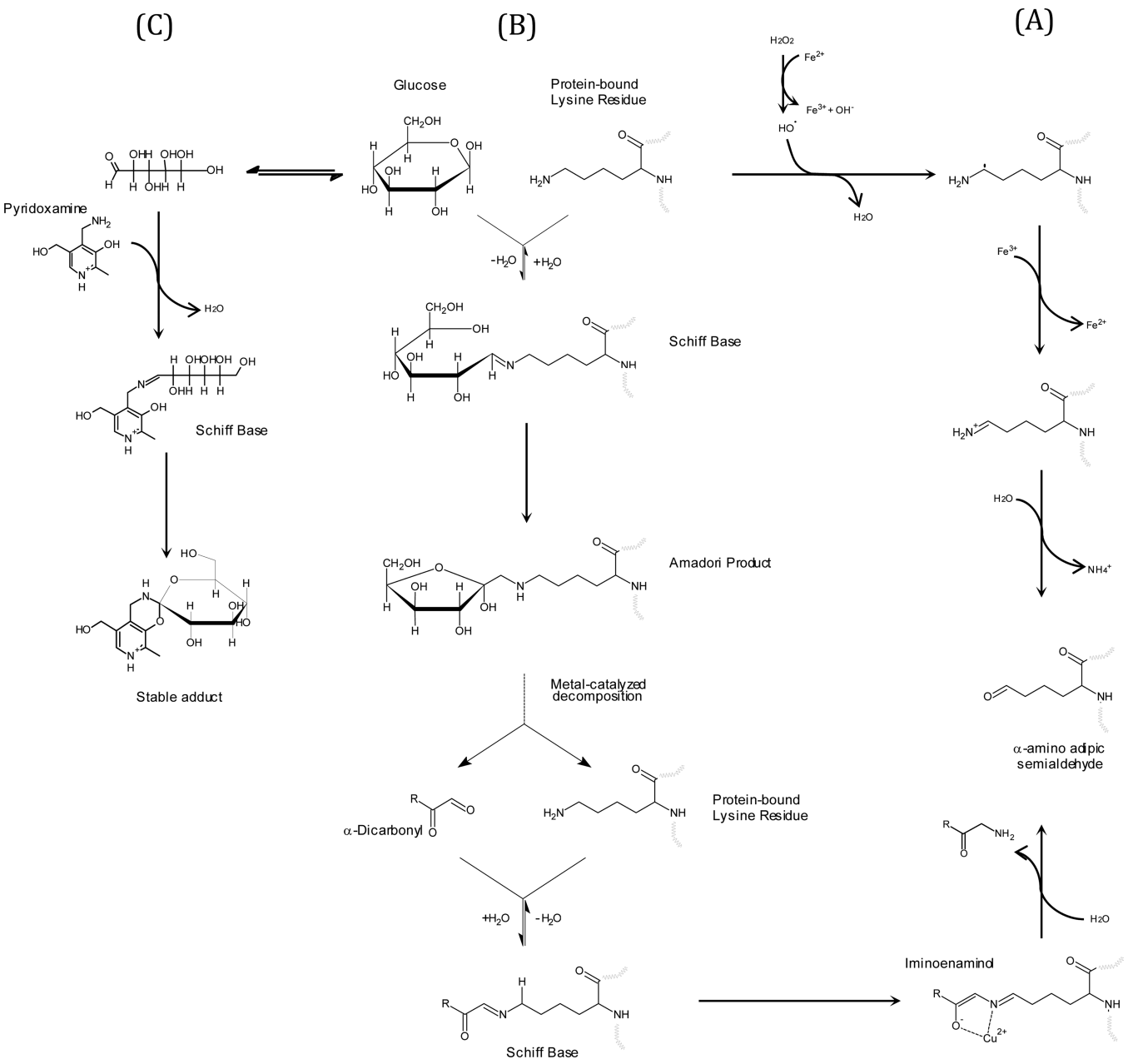

Figure 1. (A) Metal-catalyzed oxidation of lysine residues to yield AAS as reported by Estévez et al.[17] (B) Plausible formation pathway of AAS in the presence of glucose and copper ions in accordance with the mechanism proposed by Akagawa et al.[18] (C) Glucose sequestering by pyridoxamine in accordance with the reaction proposed by Adrover et al.[27]

large variety of compounds including Strecker aldehydes and advanced glycation end-products (AGEs).[18] The impact of the Maillard products in muscle foods is indisputable and involves influence on multiple nutritional and sensory aspects.[18] Reducing sugars are naturally present in postrigor muscle[19] and commonly used as ingredients in the manufacture of some meat products.[20] While the Maillard reaction is known to take place during handling, processing, and storage of meat and meat products,[21,22] the occurrence of protein carbonylation through the aforementioned Maillard-pathway is unknown.

Hence, the objectives of the present study are the following: (i) to prove the formation of specific protein carbonyls (AAS and GGS) in myofibrillar proteins through the Maillard pathway, (ii) to compare the effectiveness of diverse reducing sugars to promote this Maillard-mediated carbonylation, and (iii) to investigate the occurrence of this reaction in a range of sugar concentrations and temperatures of interest in food systems.

MATERIALS AND METHODS

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

Synthesis of AAS and GGS Standard Compounds. *N*-Acetyl-L-AAS and *N*-acetyl-L-GGS were synthesized from *Nα*-acetyl-L-lysine and *Nα*-acetyl-L-ornithine using lysyl oxidase activity from egg shell membrane following the procedure described by Akagawa et al.[23]

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Briefly, 10 mM Nα-acetyl-L-lysine and Nα-acetyl-L-ornithine were independently incubated with constant stirring with 5 g egg shell membrane in 50 mL of 20 mM sodium phosphate buffer, pH 9.0 at 37 °C for 24 h. The egg shell membrane was then removed by centrifugation and the pH of the solution adjusted to 6.0 using 1 M HCl. The resulting aldehydes were reductively aminated with 3 mmol ABA in the presence of 4.5 mmol sodium cyanoborohydride ($NaBH_3CN$) at 37 °C for 2 h with stirring. Then, ABA derivatives were hydrolyzed by 50 mL of 12 M HCl at 110 °C for 10 h. The hydrolysates were evaporated at 40 °C *in vacuo* to dryness. The resulting AAS-ABA and GGS-ABA were purified by using silica gel column chromatography and ethyl acetate/acetic acid/water (20:2:1, v/v/v) as elution solvent. The purity of the resulting solution and authenticity of the standard compounds obtained following the aforementioned procedures have been checked by using MS and ^1H NMR.[15,23]

Extraction of Myofibrillar Proteins. A 10 g portion of porcine meat was weighed and chopped finely with a knife. There were 4 volumes of 10 mM potassium phosphate buffer, pH 7, added. The mixture was homogenized in the ultraturrax for 30 s and then centrifuged at 670*g* for 15 min. Then, the supernatant was carefully removed, and 4 volumes of 10 mM potassium phosphate buffer, pH 7, were added again. The vials were shaken vigorously and centrifuged under the aforementioned conditions and the supernatant removed. There were 4 volumes of 0.1 M NaCl added, and the samples were stirred and centrifuged as in the previous steps. This procedure was repeated three times. Before the last centrifugation the solution was filtered by passing through a gauze. The pH of this solution was adjusted to 6 with 0.1 N HCl, and the vials were centrifugated. Finally, the supernatant was carefully withdrawn, and 200 mL of 100 mM sodium phosphate buffer, pH 7, with 0.6 M NaCl was added to obtain a concentration of myofibrillar protein (MP) of 4 mg/mL.

Experimental Setting. In order to fulfill the three objectives of the present study, three consecutive experiments were planned. The molarities of all reactants are referring to the final concentration in the reaction mixture. For the first experiment, 4 different types of reaction units containing MP (4 mg/mL) were prepared as follows: Reaction 1 included MP and 0.5 M glucose; reaction 2 included MP and 10 μM $CuSO_4$; reaction 3 included MP, 0.5 M glucose, and 10 μM $CuSO_4$; and reaction 4 included MP, 0.5 M glucose, 10 μM $CuSO_4$, and 0.02 M pyridoxamine. All the reaction mixtures were prepared in triplicate and incubated at 80 °C for 48 h. Samples were taken at fixed times (0, 1, 3, 8, 24, and 48 h) and subsequently analyzed for protein carbonyls.

For the second experiment, 6 different reaction units were prepared by adding 10 μM $CuSO_4$ and 0.5 M of diverse reducing sugars, namely, glucose, fructose, ribose, galactose, maltose, and lactose to MP suspensions (4 mg/mL). These reaction units were prepared in triplicate and incubated at 80 °C for 48 h. Samples were taken at fixed times (0, 1, 6, 12, 24, and 48 h) and subsequently analyzed for protein carbonyls and AGEs.

For the third experiment, 12 reaction units were prepared by adding ribose at 3 different concentrations (0.02 M, low concentration, LC; 0.1 M, medium concentration, MC; and 0.5 M, high concentration, HC) and 10 μM $CuSO_4$ to a MP suspension (4 mg/mL) and by incubating the resulting mixtures at 4 different temperatures (5, 37, 80, and 110 °C). All reaction units were prepared in triplicate, and a single sampling was performed after 48 h of incubation for the analysis of protein carbonyls and AGEs.

Analysis of AAS and GGS by HPLC. A 400 μL sample of protein suspension was dispensed in eppendorf tubes and treated with a cold 10% TCA solution. Each eppendorf was vortexed and then subjected to centrifugation at 2000*g* for 30 min at 4 °C. The supernatant was removed, and the pellet was treated with a cold 5% TCA solution. A new centrifugation was performed at 5000*g* for 5 min at 4 °C. The supernatant was removed, and the pellets were incubated with the following freshly prepared solutions: 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 eppendorfs were vortexed and then incubated in an oven at 37 °C for 90 min. The samples were stirred every 15 min. After derivatization, samples were treated with a cold 50% TCA solution and centrifuged at 5000*g* for 10 min. The pellet was then washed twice with 10% TCA and diethyl ether−ethanol (1:1). Finally, the pellet was treated with 6 N HCl and kept in an oven at 110 °C for 18 h until completion of hydrolysis. The hydrolysates were dried *in vacuo* in a centrifugal evaporator. The generated residue was reconstituted with 200 μL of Milli-Q water and then filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 μm pore size, Pall Corporation) for HPLC analysis.

A Shimadzu "Prominence" HPLC apparatus (Shimadzu Corporation, Kyoto, Japan), equipped with a quaternary solvent delivery system (LC-20AD), a DGU-20AS online degasser, a SIL-20A autosampler, a RF-10A XL fluorescence detector, and a CBM-20A system controller, was used. An aliquot (1 μL) from the reconstituted protein hydrosylates was injected and analyzed in the above-mentioned HPLC equipment. AAS-ABA and GGS-ABA were eluted in a Cosmosil 5C18-AR-II RP-HPLC column (5 μm, 150 mm × 4.6 mm) equipped with a guard column (10 mm × 4.6 mm) packed with the same material. The flow rate was kept at 1 mL/min, and the temperature of the column was maintained constant at 30 °C. The eluate was monitored with excitation and emission wavelengths set at 283 and 350 nm, respectively. Standards (0.1 μL) were run and analyzed under the same conditions. Identification of both derivatized semialdehydes in the FLD chromatograms was carried out by comparing their retention times with those from the standard compounds. The peaks corresponding to AAS-ABA and GGS-ABA were manually integrated from FLD chromatograms and the resulting areas plotted against an ABA standard curve with known concentrations that ranged from 0.1 to 0.5 mM. Results are expressed as nmol of carbonyl compound per mg of protein.

Analysis of AGEs by Fluorescence Spectroscopy. AGEs were analyzed in reaction mixtures from experiments 2 and 3 using a LS-55 Perkin-Elmer fluorescence spectrometer (Perkin-Elmer, Beaconsfield, U.K.). Prior to the analysis, reaction mixtures from experiment 2 were diluted (1:50) with 8 M urea in 100 mM sodium phosphate buffer, pH 7. Reaction mixtures from experiment 3 were diluted using the same buffer. The extent of dilution depended on the temperature at which the samples were incubated (1:600 for samples incubated at 110 °C; 1:100 for samples incubated at 80 °C; 1:12.5 for samples incubated at 37 °C; 1:1.5 for samples incubated at 4 °C). AGEs were excited at 350 nm, and the emitted fluorescence was recorded at 450 nm. The excitation and emission slit widths were set at 10 nm, and data was collected at 500 nm per minute. The height of the peaks corresponding to AGEs spectra was recorded. After taking into consideration the applied dilutions, the results were expressed as fluorescence units.

Statistical Analysis. Data from the analysis of protein carbonyls and AGEs ($n = 3$) were collected and subjected to statistical analyses. In order to assess the effect of the different types of reducing sugars (experiment 2), the effect of the concentrations of ribose and the effect of the reaction temperatures (experiment 3), an analysis of variance (ANOVA) was applied to data (SPSS v. 15.5). A Tukey test was applied when ANOVA found significant differences between treatments. The statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Carbonylation of MP through the Maillard Reaction. Figure 2A,B shows the concentration of AAS and GGS in MP suspensions (4 mg/mL) incubated with diverse reactants (0.5 M glucose, 10 μM $CuSO_4$, and 0.02 M pyridoxamine) According to these results, carbonylation of MP occurred in the presence of 0.5 M glucose as significant increases of AAS and GGS were found after 48 h of incubation with MP at 80 °C (0.80 and 3.04 nmol/mg protein, respectively). The formation of AAS and GGS in the presence of glucose was greatly enhanced by the addition of 10 μM $CuSO_4$. In this reaction

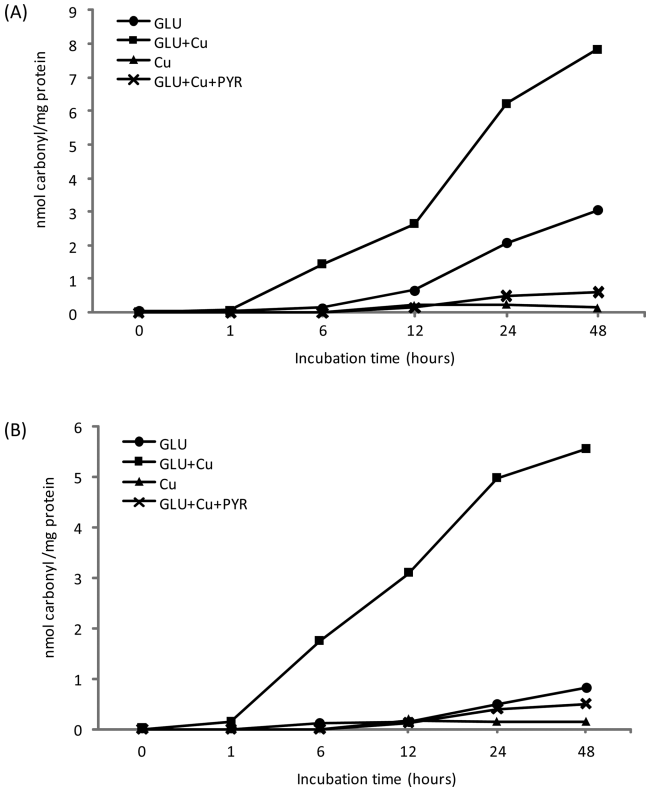

Figure 2. Evolution of protein carbonyls, GGS (A) and AAS (B), during incubation of myofibrillar proteins (4 mg/mL; 80 °C; 48 h) with 0.5 M glucose.

mixture, the concentrations of AAS and GGS after 48 h incubation were 5.55 and 7.83 nmol/mg protein, respectively. The addition of the copper solution, alone, had no impact on the formation of protein carbonyls. These results confirm the findings by Akagawa et al.[17] in 10 mg/mL bovine serum albumin (BSA) suspensions incubated with 0.05 M glucose and 5 μM CuSO4 at 37 °C for 3 weeks. The amount of carbonyls reported by these authors at the end of the assay was 0.13 nmol/mg protein. Despite the differences between the present study and Akagawa's work in terms of incubation conditions, concentration of glucose, and the nature of the protein, consistent results were obtained in both experiments, confirming the formation of AAS and GGS through a Maillard-type pathway. Figure 1 shows the plausible reaction pathway between glucose and amino groups from MP for the formation of AAS and GGS in accordance with the proposal by Akagawa et al.[17] In an early stage, the carbonylamine condensation leads to the formation of a Schiff base (aldosamine) which is converted to a ketosamine through the Amadori rearrangement. The decomposition of glucose is consummated by the degradation of Amadori products to reactive carbonyls such as keto-aldehydes, dicarbonyls, reductones, etc. In a subsequent stage, the reactive carbonyls may condense with the ϵ-amino group from the side chains of basic amino acids to form a Schiff base adduct (iminoketone). The ϵ-proton of the basic amino acid would then be abstracted by basic media, and the enolization might give an iminoenaminol. Finally, spontaneous hydrolysis of the iminoenaminol would lead to the release of an enaminol and the formation of the carbonyl moiety in the side chain of the susceptible amino acid. It is known that transition metals catalyze the autoxidation of glucose and the formation of α-dicarbonyls from the Maillard reaction.[18] As shown in Figure 1, metal ions also serve as electron-pair acceptors favoring the formation and stabilization of the iminoenaminol prior to its hydrolysis and the formation of the semialdehyde.[17] In the present study, the remarkable promotion of carbonylation accomplished by copper ions in the presence of glucose may plausibly respond to these mechanisms. The occurrence of the above-described Maillard pathway is supported by the fact that the addition of pyridoxamine, a specific inhibitor of the Maillard reaction, significantly diminished the formation of AAS and GGS (Figure 2). Pyridoxamine sequesters reducing sugars by reacting with their carbonyl moieties to form stable adducts and, hence, hindering the involvement of such moieties in the Maillard reaction [24] (Figure 1). Therefore, the present results highlight that the carbonyl moiety from glucose plays a major role in the formation of AAS and GGS in MP incubated with this reducing sugar. Furthermore, this study confirms that specific protein carbonyls can be formed in a food protein as a result of the Maillard reaction. The plausible involvement of Maillard-derived α-dicarbonyls in the oxidative deamination of ϵ-amino groups from basic amino acids was already substantiated by Akagawa et al.[17] According to these authors, the incubation of specific Maillard-derived dicarbonyls such as glyoxal and methylglyoxal (1 mM) with BSA (10 mg/mL) yielded AAS.

It is worth noting that this Maillard-type mechanism is analogous to a well-known reaction in food systems: the Strecker degradation of amino acids. α-Dicarbonyls from the Maillard reaction[25] and from lipid oxidation[26] induce the oxidative deamination and decarboxylation of free amino acids to yield Strecker aldehydes, with these compounds having a carbon atom less than the degraded amino acid. Whereas the Strecker degradation involves the α-amino group of certain free amino acids, the Maillard-mediated mechanism described in this study involves the ϵ-amino group located in the side-chain of basic amino acids. Unlike the formation of Strecker aldehydes, the formation of AAS and GGS through the Maillard pathway takes place in protein-bound amino acids and, hence, does not require a previous proteolysis. Interestingly, a recent study provided evidence of the reactivity of AAS and GGS and their implication in the Strecker-type degradation of free leucine and isoleucine.[8] This previous study and the present one highlight the complex, and, until recently, unknown interactions between protein oxidation and the Maillard reaction. The protein oxidation markers, AAS and GGS, could also be regarded as Maillard products and, furthermore, be promoters, as reactive carbonyls, of the Strecker degradation of free amino acids.

Reactivity of Reducing Sugars. The second experiment aimed to compare diverse reducing sugars, namely, glucose, fructose, ribose, maltose, lactose, and galactose, for their ability to induce the carbonylation of MP through the Maillard-type pathway. In addition, the formation of AGEs during the incubation of MP with the reducing sugars was also assessed. Figure 3A,B shows the concentration of GGS and AAS, respectively, in MP suspensions incubated for 48 h at 80 °C with the previously described carbohydrates (0.5 M) and 5 μM $CuSO_4$. All the tested carbohydrates promoted the formation of AAS and GGS in MP. However, not all sugars were equally reactive toward the carbonyl formation, with the ribose being the most reactive, followed by fructose, glucose, and galactose, and finally by the disaccharides maltose and lactose. The

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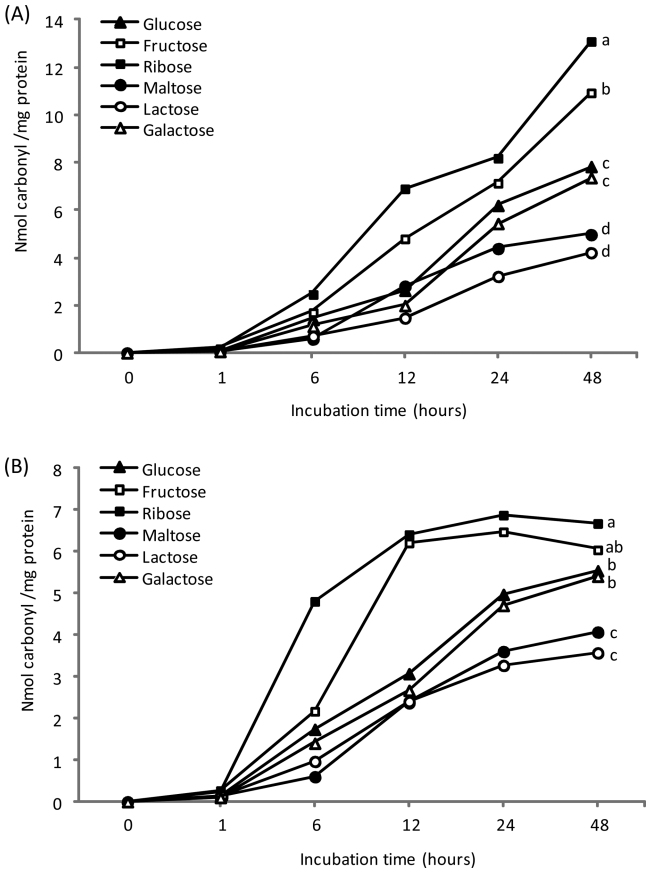

Figure 3. Evolution of protein carbonyls, GGS (A) and AAS (B), during incubation of myofibrillar proteins (4 mg/mL; 80 °C; 48 h) with various reducing sugars (0.5 M).

differences were more noticeable as the incubation time increased. At the end of the assay, the amounts of GGS and AAS in suspensions incubated with ribose (13.1 and 6.7 nmol/mg protein, respectively) were from 2- to 3-fold higher than in suspensions incubated with the least reactive carbohydrates, lactose (4.2 and 3.6 nmol/mg protein, respectively) and maltose (5.0 and 4.1 nmol/mg protein, respectively). Assuming that carbohydrates induced the carbonylation of MP through the aforementioned pathway, their ability to promote the oxidative deamination of basic amino acids may be related to their susceptibility to undergo decomposition in the Maillard reaction and, hence, yield reactive α-dicarbonyls. The rate of the Maillard reaction is usually dependent on the size (number of carbon atoms) and chemical nature of the reducing carbonyl moiety of the carbohydrate.[18] The reaction is generally faster and more intense when the degraded sugar is small. Therefore, it was expected that ribose, a pentose, yielded dicarbonyl compounds to a larger extent than hexoses and disaccharides which was eventually reflected in a more effectively formation of protein semialdehydes. Furthermore, within the same group (hexoses), sugars have different reactivity, with aldoses such as glucose being more reactive than ketoses such as fructose.[18] This fact contradicts the results obtained in this experiment, as MP incubated with fructose had larger amounts of protein carbonyls than MP incubated with the hexoses (glucose and galactose). Likewise, there is a large difference in the reactivity of the different amino acids. Lysine is more reactive than arginine, due to its basic character, particulary in the early stages of the Maillard reaction.[18] In the medical field, Akagawa et al.[17] showed similar results after incubating serum proteins such as BSA (10 mg/mL) with 0.05 mM of various sugars (glucose, galactose, fructose, and ribose) and 5 μM $CuSO_4$ for 3 weeks at 37 °C. These authors reported that ribose was the most reactive sugar, followed by galactose, fructose, and glucose. The present study shows that other carbohydrates with interest for the food industry, including disaccharides such as lactose and maltose, are also able to induce carbonylation of MP.

It is worth noting that the formation of both semialdehydes followed different trends during the course of the reaction. The concentration of GGS increased gradually during the entire assay while the concentration of AAS tended to reach a plateau by the end of the experiment. In addition, the concentration of GGS was considerably higher than that of AAS, regardless of the sampling point or reaction mixture. Other authors such as Requena et al.[27] and Armenteros et al.[28] have reported similar results when the formation of both carbonyls was assessed in diverse biological and food systems. The apparent deceleration of the AAS formation in advanced stages of the assay could respond to the implication of this semialdehyde in further reactions. Requena et al.[27] reported that protein carbonyls may interact with nonmodified amino acid residues and form cross-links via Schiff base formation. This potential implication has also been suggested in food systems.[8] On the other hand, AAS may also have undergone further oxidative degradation to yield its end-product, the α-aminoadipic acid (AAA).[29] Recently, Utrera et al.[13,14] described the formation of this novel protein oxidation marker in MP subjected to an intense metal-catalyzed oxidation[13] and in meat products subjected to severe processing and storage conditions.[14] Whereas the fate of AAS in the present experiment remains unknown, our results suggest that GGS may be a more reliable indicator of the oxidative damage caused by reducing sugars to meat proteins.

The study of food protein glycation through the formation of particular protein carbonyls may be regarded as an innovative approach. In fact, the occurrence of the Maillard reaction in food systems is commonly assessed by the analysis of fluorescent AGEs.[30] In the present study, the incubation of MP with reducing sugars led to the formation of AGEs in all reaction mixtures (Figure 4). In agreement with the analysis of protein carbonyls, the formation of AGEs was dependent on the reducing sugar. In this case, the tested carbohydrates behaved similarly with ribose being the most reactive followed by hexoses (fructose, galactose, and glucose) and the

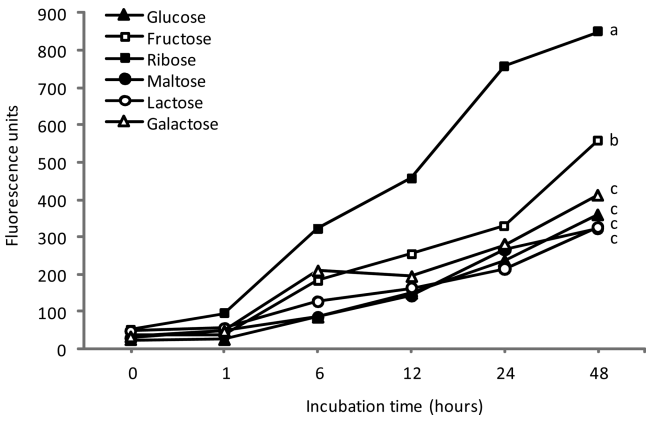

Figure 4. Evolution of AGEs during incubation of myofibrillar proteins (4 mg/mL; 80 °C; 48 h) with various reducing sugars (0.5 M).

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disaccharides (lactose and maltose). Similar results were found by other authors during glycation assays with food proteins such as β-lactoglobulin.[31,32] AGEs are generated in the late stages of the Maillard reaction in foods and biological systems. Several mechanisms have been postulated for the formation of AGEs from glycated proteins, and they all feature protein-bound carbonyl intermediates.[33] Using 2,4-dinitrophenylhydrazine (DNPH) these intermediates have been detected on BSA, lysozyme, and β-lactoglobulin after *in vitro* glycation by glucose or fructose.[34] In the present study, specific carbonyls (AAS and GGS) were also formed in parallel with AGE-fluorophores. In fact, according to several medical scientists, AAS may be candidate to the formation of AGEs and other cross-linked glycation products under pathological conditions.[35,36] However, in the conditions of the present study, protein carbonyls were found to be more precise indicators of the ability of reducing sugars to undergo the Maillard reaction. The analysis of protein carbonyls enabled the detection of significant differences in reactivity between the pentoses, hexoses and the disaccharides while the amount of AGEs induced by the aldoses and the disaccharides was similar at the end of the assay (Figures 3 and 4). Therefore, AAS and GGS may be employed, under particular circumstances, as early and reliable indicators of the modification induced by reducing sugars to food proteins.

Effect of Sugar Concentration and Temperature. In order to study the potential occurrence of these glycation reactions in real muscle-based foods, the most reactive sugar (ribose) was incubated with MP for 48 h in a range of concentrations (0.5 M, HC; 0.1 M, MC; 0.02 M, LC) and temperatures (4, 37, 80, and 110 °C) compatible with a food system. Results show that the carbonylation of MP took place at all ribose concentrations and at all temperatures. As expected, higher amounts of GGS and AAS (Tables 1 and 2, respectively) were found in reaction mixtures with higher sugar concentrations and incubated at higher temperatures. It is worth noting that the amount of AAS in reactions mixtures incubated at 4 and 37 °C is, at all ribose concentrations, higher than the amount of GGS. At higher temperatures (80 and 110 °C), the opposite situation is observed as the amounts of GGS are, as reported in the first experiment, larger than those of AAS. These results emphasize the plausible conversion of the more reactive semialdehyde (AAS) in further species as promoted by high temperatures. Therefore, AAS may be a more suitable marker of Maillard-mediated carbonylation of MP at low temperatures while GGS would be more reliable of such damage at higher temperatures.

Table 1. Effect of Sugar Concentration and Reaction Temperature on the Formation of AAS (nmol/mg protein) During Incubation (48 h) of Myofibrillar Proteins (4 mg/mL) with Ribose[a]

	LC	MC	HC
4 °C	0.30b,z ± 0.04	0.39b,z ± 0.05	0.79a,z ± 0.07
37 °C	0.73c,z ± 0.06	1.93b,z ± 0.27	2.75a,z ± 0.39
80 °C	2.39c,y ± 0.33	4.05b,y ± 0.68	6.71a,y ± 0.67
110 °C	6.13b,x ± 1.04	11.67a,x ± 1.48	13.58a,x ± 1.95

[a]LC: Low concentration of ribose (0.02 M). MC: Medium concentration of ribose (0.1 M). HC: High concentration of ribose (0.5 M). (a–c) Different roman letters within a row denote significant differences ($p < 0.05$) between concentration levels. (x–z) Different roman letters within a column denote significant differences ($p < 0.05$) between reaction temperatures

Table 2. Effect of Sugar Concentration and Reaction Temperature on the Formation of GGS (nmol/mg Protein) During Incubation (48 h) of Myofibrillar Proteins (4 mg/mL) with Ribose[a]

	LC	MC	HC
4 °C	0.11b,z ± 0.01	0.16b,z ± 0.03	0.60a,z ± 0.05
37 °C	0.29c,z ± 0.03	1.31b,z ± 0.07	1.83a,z ± 0.26
80 °C	5.20c,y ± 1.06	9.56b,y ± 1.90	13.08a,y ± 1.71
110 °C	9.68b,x ± 1.31	13.51b,x ± 1.74	20.84a,x ± 2.60

[a]LC: Low concentration of ribose (0.02 M). MC: Medium concentration of ribose (0.1 M). HC: High concentration of ribose (0.5 M). (a–c) Different roman letters within a row denote significant differences ($p < 0.05$) between concentration levels. (x–z) Different roman letters within a column denote significant differences ($p < 0.05$) between reaction temperatures.

As for carbonylation, the formation of AGEs was similarly affected by both variables (Table 3). As mentioned previously, the consistency between results from protein carbonyls and AGEs reflects the timely coincidence of both chemical changes that may be expressions of a common chemical pathway. Interestingly, the results showed that the carbonylation of MP even takes place at the lowest concentration of ribose (0.02 M), which coincides with the total amount of reducing sugars in post-mortem muscle.[19] The higher concentrations of reducing sugar employed in the present experiment are also commonly found in a varied range of processed muscle-based products such as fermented sausages and cooked meats.[20] The carbonylation reaction would also take place at refrigeration temperatures as low as 4 °C in the tested range of ribose concentration. So far, the occurrence of protein carbonyls in muscle and muscle foods has been ascribed to the MCO of the side chains of basic amino acids.[9] In particular, the pathways and mechanisms by which MCO systems (≈hydroxyl radical-generating systems) and H_2O_2-activated myoglobin promote the formation of AAS and GGS in muscle proteins have been described in detail.[15,37,38] Consistently, numerous previous studies have interpreted the carbonylation process in post-mortem muscle,[39] chilled meat,[8] and processed meat products[5,11] as an expression of the direct attack of free radicals to meat proteins. The present study suggests that the formation of AAS and GGS during storage and processing of muscle-based foods may also be a consequence of the

Table 3. Effect of Sugar Concentration and Reaction Temperature on the Formation of AGEs (Fluorescence Units) During Incubation (48 h) of Myofibrillar Proteins (4 mg/mL) with Ribose[a]

	LC	MC	HC
4 °C	10c,z ± 1	17b,z ± 1	31a,z ± 1
37 °C	22b,z ± 2	38b,z ± 8	78a,z ± 7
80 °C	384b,y ± 14	462b,y ± 16	840a,y ± 24
110 °C	279c,x ± 8	504b,x ± 36	1522a,x ± 83

[a]LC: Low concentration of ribose (0.02 M). MC: Medium concentration of ribose (0.1 M). HC: High concentration of ribose (0.5 M). (a–c) Different roman letters within a row denote significant differences ($p < 0.05$) between concentration levels. (x–z) Different roman letters within a column denote significant differences ($p < 0.05$) between reaction temperatures.

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implication of reducing sugars through a Maillard-type pathway. The formation of protein carbonyls has relevant consequences in foods, including the irreversible modification of essential amino acids, the loss of protein functionality,[38] and the impairment of the digestibility and nutritional value of dietary proteins.[8]

In conclusion, reducing sugars are involved in the formation of specific carbonyls in MP through a Maillard-type pathway that would likely involve the oxidative deamination of the side chains of basic amino acids in the presence of reactive α-dicarbonyls. This extent must be ascertained since, to our knowledge, the outcome of the interaction of reactive α-dicarbonyls (i.e., glyoxal or methylglyoxal) with ϵ-amino groups in food protein-bound amino acids is yet unknown. Furthermore, the relative contribution of this Maillard pathway to the overall carbonylation phenomena occurred in food systems is yet to be determined. The concentration of reducing sugars and transition metals, the temperature applied during processing/storage, as well as other factors such as pH, water activity, and the presence of other redox-active compounds may have a plausible influence on this Maillard pathway. The identification of the impact of protein carbonyls and their end-products on food quality and human health requires clarification of the chemistry behind these novel pathways. Only by accomplishing further studies on this topic will the complex interactions between protein oxidation and the Maillard reaction be fully unveiled.

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Notes

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