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Glucose Slows Down the Heat-Induced Aggregation of β-Lactoglobulin at Neutral pH

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ABSTRACT: The behavior of β-lactoglobulin (β-Lg) during heat treatments depends on the environmental conditions. The influence of the presence or absence of a reducing sugar, namely, glucose, on the modification of the protein during heating has been studied using fluorescence, polyacrylamide gel electrophoresis (PAGE), size-exclusion chromatography (SEC), and transmission electron microscopy. Glycated products were formed during heating 24 h at 90 °C and pH 7. The fluorescence results revealed an accumulation of the advanced Maillard products and the formation of aggregates during heating. PAGE and SEC data suggested that the products in the control samples were essentially composed of covalently linked fibrillar aggregates and that their formation was faster than that for glycated samples. We showed that glucose affected the growing step of covalent aggregates but not the initial denaturation/aggregation step of native protein. Glucose-modified proteins formed a mixture of short fibrils and polydisperse aggregates. Our results revealed that β-Lg forms fibrils at neutral pH after heating and that glucose slows the formation of these fibrils.

KEYWORDS: *β-lactoglobulin, heat treatment, aggregation, fibrils, Maillard Reaction*

INTRODUCTION

Heat treatment is an essential operation in commercial processing of milk to provide acceptable safety, shelf life of dairy products, or to improve the functional properties of final products.[1] Heat treatments induce structural changes of the milk proteins leading to their denaturation and aggregation. This process is strongly related with the physicochemical characteristics of the proteins, environment, and processing conditions.[2,3] In the presence of reducing sugars, the most prominent change is the formation of glycoconjugated proteins via the Maillard reaction, a nonenzymatic condensation of reducing sugars with free amino groups of protein via an unstable Schiff's base. This product can oxidize and condense with lysine or arginine residues to form glycated products and unsaturated brown nitrogenous polymers and copolymers that have not been fully characterized yet.[4,5] As a consequence of the heat treatment of milk, several chemical pathways are interconnected, inducing significant protein modifications through the establishment of covalent protein−protein interactions, mainly due to a rearrangement of disulfide bridges linked to the protein unfolding, glycation of proteins with high polymers, as well as β-elimination, condensation reactions, and partial protein hydrolysis.[6] So far, all these pathways and modifications are incompletely understood.

To gain insight into the mechanisms of heat-induced protein aggregation, β-lactoglobulin (β-Lg) has been used as a model system, thanks to its availability in large amounts at high purity grades and its well known molecular structure. β-Lg is a single polypeptide chain of 162 amino acids with a molecular weight of 18.3 kDa. Its globular structure is composed of nine antiparallel β-sheets and one α-helix, and contains two intramolecular disulfide bonds and one free sulfhydryl group. β-Lg exists as a noncovalent dimer at neutral pH and dissociates into monomers at acidic pH.[7]

β-Lg forms several types of heat-induced aggregates whose morphology and size depend on the environmental conditions (concentration, pH, and ionic strength) and time−temperature couples.[8] At or near the isoelectric point, pI, spherical aggregates are formed, whereas far from pI, fibrils are observed: small curved strands at pH 7 and long rigid strands at pH 2.[8] In the presence of reducing sugars, the glycated β-Lg has been identified under different thermal conditions such as mild treatment (60 °C) by Morgan et al.[9] or at lower temperature and long time (40 °C for 24 h) by Corzo-Martinez et al.[10] Taken together, these results for β-Lg show the complexity of the mechanism of protein aggregation where multiple pathways can occur and small changes of environmental conditions can be critical in processing. The use of heat treatments is a current industrial practice in order to increase the functionality of dairy products. Considering the importance of the Maillard reaction, we have investigated how glucose affects the aggregation of β-Lg upon heating (90 °C for 24 h) at neutral pH to identify

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the different pathways and the complexes involved under these conditions.

MATERIALS AND METHODS

Materials. All of the chemicals and standards used were obtained from Sigma. Fresh bovine raw milk from homozygous cows was obtained from an experimental dairy farm (INRA, Rennes, France), and β-lactoglobulin was prepared using a modified method of Fauquant et al.,[11] as previously described by Leonil et al.,[6] in which membrane processes and low temperatures (below 56 °C) are involved to minimize chemical modifications, like the Maillard reaction. β-Lg A represented 98% of the protein in the freeze-dried powder based on reverse-phase high-performance liquid chromatography (RP-HPLC) analysis.

Glycation Experiments. Protein concentration was measured by the absorbance at 280 nm of samples centrifuged (30 min at 23,700*g*) and filtered through a 0.45 μM filter using a specific absorption coefficient of 16,730 L/mol/cm. Glycation was performed with glucose in aqueous system. Glucose−protein mixtures were prepared in sodium phosphate buffer 0.1 M, pH 7, containing 0.30 mM of β-Lg and 37.5 mM glucose, i.e., about 8 mols of glucose per mol of NH_2. Control samples were prepared as well without adding glucose. Model system solutions were heated at 90 °C for 24 h in Eppendorf tubes. After predetermined times between 0 and 24 h, the tubes were removed and cooled in an ice−water bath. Each sample was made in duplicate.

Characterization of Aggregates. *Protein Concentration.* The protein content of the solutions was determined by the Bradford method.[12] Experiments were carried out in 96-microwell plate format (Costar), with 200 μL of sample per plate on a Spectra Max M2 spectrophotometer (Molecular Devices, Sunnyvale, California). A pure β-Lg solution, whose concentration was determined by UV absorption at 280 nm, was used as a standard, at a range between 0 and 0.5 μM. All measurements were made in duplicate.

Advanced Glycation End Product (AGE) Fluorescence. The title process was according to the protocol described by Birlouez-Aragon et al.[13] except that experiments were carried out in 96-microwell black plate format. Fluorescence measurements were performed with 200 μL samples on a Spectra Max M2 fluorescence spectrophotometer (Molecular Devices, Sunnyvale, California). Excitation and emission wavelengths were 330 and 420 nm, respectively. When necessary, samples were diluted in pH 7 phosphate buffer.

Size Exclusion Chromatography (SEC). The separation was achieved by SEC using a BioSep-SEC-S4000 column, size 300 × 7.8 mm (Phenomenex, Torrance, USA), on a Waters system. Elution was achieved with 0.05 M sodium phosphate buffer, pH 7, at 0.5 mL/min for 30 min, and detection of eluting proteins was performed at 214 nm. The standard proteins used for calibration were apoferritin (481.2 kDa); BSA (66 kDa); and α-lactalbumin (14.2 kDa).

Gel Electrophoresis. The samples were analyzed by polyacrylamide gel electrophoresis (PAGE) using NuPage Novex (4−12%, Bis-Tris Mini Gels 1.5 mm, Invitrogen) under reducing and nonreducing conditions. Disulfide bonds were reduced by an overnight incubation in the NuPage lithium dodecyl sulfate (LDS) sample buffer containing 30 mM dithiothreitol (DTT) at room temperature. Samples containing approximately 12 μg of protein were analyzed. Gels were stained with Coomassie Brilliant Blue R250. A high molecular weight markers kit (3−200 kDa, Mark 12 Unstained Standard, Invitrogen) was used for calibration.

Transmission Electron Microscopy (TEM). Drops of 5 μM β-Lg suspensions were deposited onto glow-discharged carbon-coated microscopy grids. The liquid in excess was blotted with filter paper and a drop of distilled water was deposited on the preparation in order to rinse out the residual glucose and buffer salts. The water in excess was blotted, and prior to drying, the preparations were negatively stained with 2% (w/v) uranyl acetate. The samples were observed using a Philips CM200 microscope operating at 80 kV. Images were recorded on Kodak SO163 films.

Thioflavin T assay. Thioflavin T (ThT) can bind the amyloid fibrils by ionic and hydrophobic interactions changing the fluorescence excitation spectrum. An increase in ThT fluorescence intensity is related to a ThT bound along the length of the amyloid fibrils.[14] To perform the ThT fluorescence measurement, a ThT stock solution (0.63 mM) was prepared in phosphate buffer at pH 7 and stored at 4 °C.

To verify if fibrils were formed, experiments were carried out into a four-sided quartz cuvette: a working solution was prepared by diluting the ThT stock solution in phosphate buffer and adding it to the protein solutions so that the final dye and protein concentrations were 0.58 and 0.023 mM, respectively. Fluorescence measurements were performed on a Spectra Max M2 fluorescence spectrophotometer (Molecular Devices, Sunnyvale, California) at an excitation wavelength of 446 nm and an emission wavelength of 478 nm (475 nm cutoff). The net intensities were obtained after subtraction of the background signal. Results are expressed as the mean of five independent experiments.

RESULTS

The heat-induced aggregation of β-Lg was studied at pH 7 either in the absence or presence of glucose with a glucose/protein molar ratio of 125, which corresponds to an 8-fold excess of glucose over the amine groups of the protein.

Figure 1 shows the concomitant evolution of advanced glycation end products (AGE) and β-Lg denaturation-aggregation

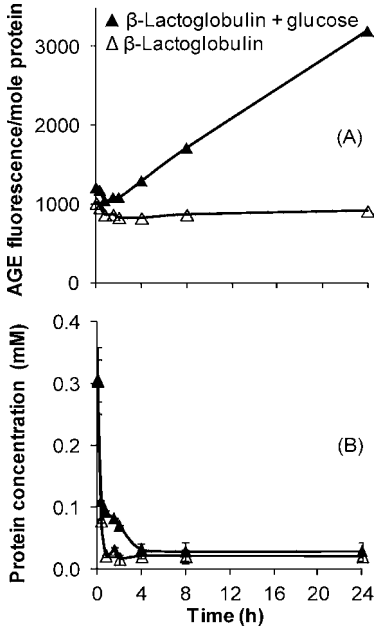

Figure 1. AGE fluorescence (A) and soluble protein at pH 4.6 (B) during heating of β-Lg solution at 90 °C, pH 7, with and without glucose. AGE were expressed per mole of protein.

after heating at 90 °C over 24 h with and without glucose. As expected, AGE formation increased with increasing heating time. After 4 h of heating, more than 80% of initial β-Lg precipitated at pH 4.6, a clear indication of denaturation and aggregation in the presence or absence of glucose. Before precipitation at pH 4.6, no precipitation was detected after 24 h of heating, an indication that the heat-induced aggregates were fully soluble at neutral pH in the presence as well as in the absence of glucose.

Native β-Lg exists as noncovalent dimers at pH 7 in equilibrium with native monomers.[15] The native β-Lg, as analyzed by SEC (Figure 2), is a mixture of monomers, prominent dimer forms, and minor oligomer forms. During heating

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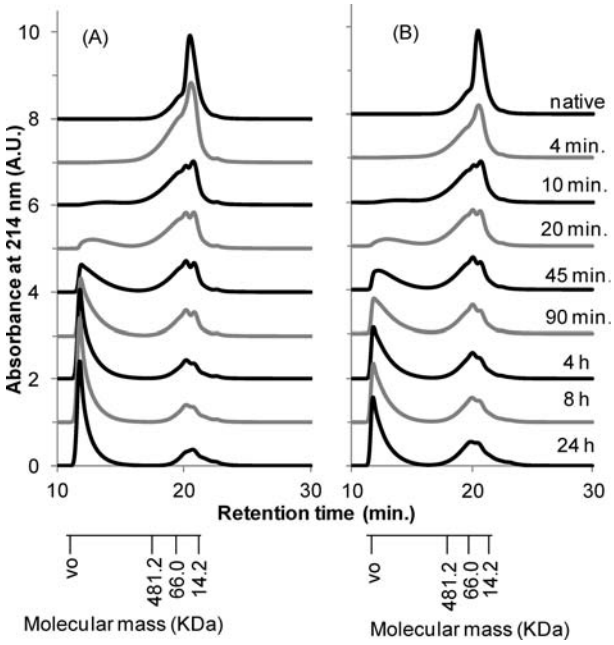

Figure 2. Evolution of SEC profiles of heated β-Lg samples (90 °C) without (A) and with (B) glucose. The holding time is indicated above each elution profiles. The secondary *x*-axis indicates the elution times of markers; vo = void volume.

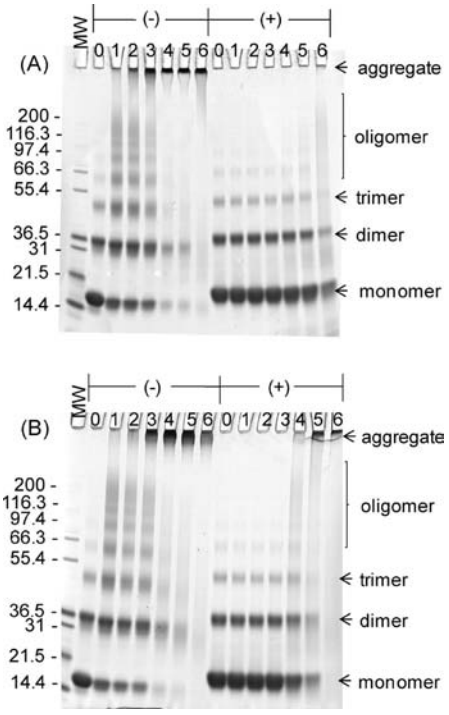

Figure 3. LDS−PAGE of heated β-Lg (90 °C, pH 7) without (A) or with (B) glucose before (−) and after (+) reduction with DTT. Native (0) and heated protein at different times: (1) 10 min, (2) 20 min, (3) 45 min, (4) 4 h, (5) 8 h, and (6) 24 h. MW = molecular weight marker.

and for both samples, a decrease of the peak height of native proteins (retention time = 21 min) and concomitant formation of high molecular weight products, eluted at the void volume (retention time = 12 min) were observed. Large aggregates eluted in the void volume started to be observed after 20 min heating, their amount getting significant after 45 min. The intensity of this peak was higher when β-Lg was heated without glucose. At the beginning of the heat treatment, the disappearance of species eluted at a retention time of 21 min was rather similar in the presence and absence of glucose. However, after 24 h of treatment, the high molecular weight species eluted at the void volume represented about 74% of the total product in the absence of glucose against 56% when glucose was present.

Nonreducing PAGE was performed to further characterize the β-Lg aggregates formed with and without glucose. Without glucose, a heating time-dependent decrease of the intensity of the initial bands (i.e., around 18 kDa for monomers, 36 kDa for dimers, and 54 kDa for trimers) was clearly observed, while oligomers and large aggregates progressively appeared at the top of the gel (Figure 3A). These aggregates did not enter the running gel and were the major species after 45 min of heating and above. As already shown by SEC analysis, the kinetic of disappearance of initial protein was not affected by the addition of glucose (Figure 3B). However, the addition of glucose affected subsequent aggregation steps, i.e., all the formed aggregates entered the electrophoresis gel and migrated as diffuse bands at the top of the gel.

To determine the nature of the aggregates formed during heating at pH 7, PAGE under reducing conditions was performed. The bands corresponding to oligomers (between 40 and 200 kDa) formed during heating disappeared in the gel for β-Lg heated both in the absence or presence of glucose. Comparatively, with no reduced samples, the intensity of monomer and dimer bands increased, indicating that most of these oligomeric species were made of monomers, held together by intermolecular disulfide bonds (S−S). These species seem to be the same for β-Lg heated in the absence and presence of glucose. The heat-induced large aggregates also disappeared after DTT treatment in the presence as well as in the absence of glucose. However, in the presence of glucose, some aggregated species still remained after reduction, indicating the occurrence of covalent bonds other than disulfide after long-time heating. Hence, the comparison of the electrophoretic profiles of β-Lg heated with and without glucose clearly shows the presence of new species for β-Lg heated with glucose.

We noted the presence of a dimeric form of β-Lg which progressively decreased during heating. This band was still present in unheated β-Lg under reduced conditions. This nonnative β-Lg dimer was not reduced by DTT whatever the experimental conditions tested (incubation time, DTT concentration) and therefore was not S−S-linked. This dimeric form could be attributed to the formation of covalent isopeptide bounds between protein molecules during purification or storage steps as already suggested by Mudgal et al.[16] These covalent dimers contributed to the aggregation process as their intensity decreased concomitantly to the increase of the intensity of large aggregates observed in the top of the gel.

β-Lg aggregates formed upon heating with and without glucose were observed by TEM. The morphological features of native β-Lg as well as samples heated in the absence and presence of glucose are clearly different (Figure 4). In the native sample, a mixture of aggregates of globular units and smaller individual objects were observed (Figure 4A). The presence of aggregated species in the native β-Lg TEM images is probably due to the effect of negative staining as no aggregates were detected by dynamic light scattering analysis of

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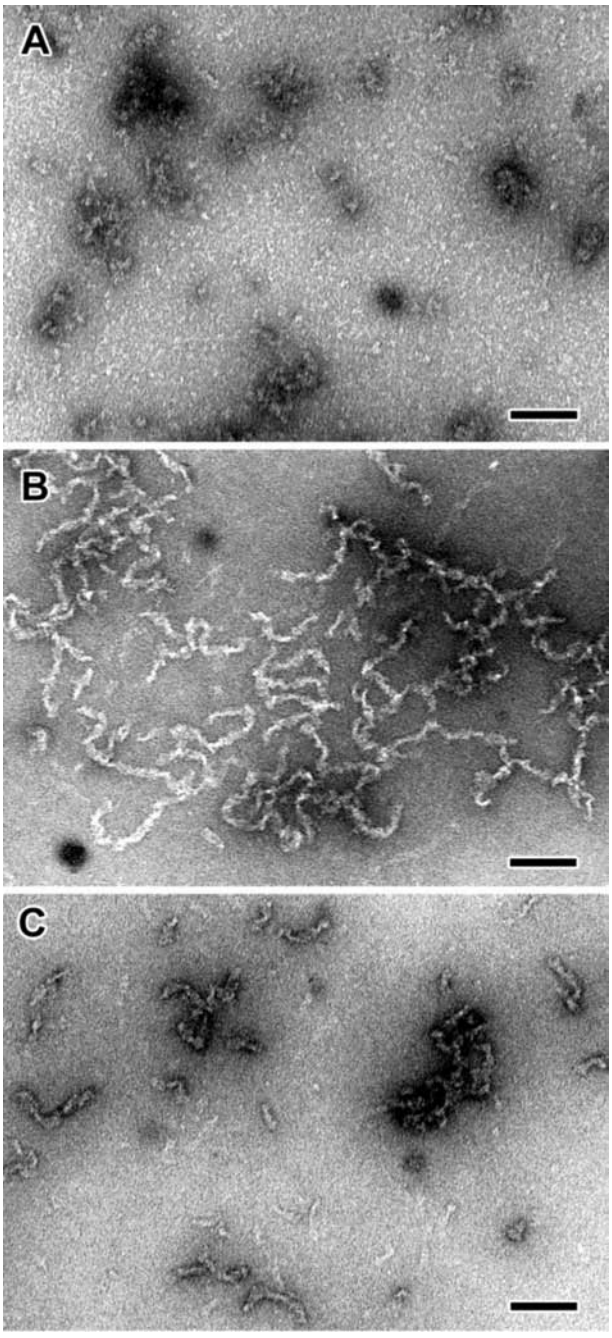

Figure 4. TEM images of negatively stained native β-Lg (A); heated for 4 h at 90 °C without (B) and with (C) glucose. Bars = 100 nm.

unheated β-Lg samples (data not shown). Images of the nonglycated sample treated at 90 °C during 45 min showed a mixture of aggregates and small fibril segments (not shown). The species seen in the native β-Lg sample almost disappeared after 4 h of heating, and in the sample heated without glucose (Figure 4B), a majority of twisted fibrils was observed. The TEM images of the β-Lg sample heated with glucose during 4 h showed a mixture of shorter fibrils (about 100 nm in length) and aggregates (Figure 4C). The width of the fibrils formed with or without glucose was constant and about 20 nm in both cases.

ThT fluorescence was used as a marker to identify the fibrillar nature of the aggregates and the kinetics of their formation. Figure 5 shows that the fluorescence intensity increased with time, with

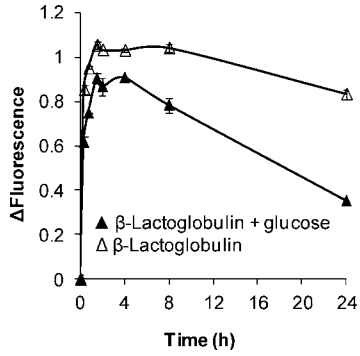

Figure 5. Thioflavin T (ThT) fluorescence of β-Lg heated at 90 °C and pH 7 both in the presence and absence of glucose. The emission intensity was measured at 478 nm upon excitation at 446 nm.

and without glucose, which is consistent with the TEM observations. The two curves have similar initial slopes, but the level at the plateau is higher in the absence than in the presence of glucose, indicating that glucose slowed down the initiation and/or the growth of β-Lg fibrils.

DISCUSSION

Nowadays, it is accepted, and more and more demonstrated, that the ability to form fibrillar structures is a common property of proteins. For a given protein, the formation of fibril specificity depends on the physicochemical conditions in relation to the proper protein properties. However, fibril formation has mostly been studied for pure proteins, and only few investigations focused on more complex systems containing, for example, additional molecules such as sugars and polysaccharides that can interact more or less specifically, more or less strongly with the proteins. To develop more applications, the challenge of ongoing studies is to better understand how protein fibrillation is affected by molecules or reaction that can be encountered in foods.

Many concepts and mechanisms of fibril formation from pure globular protein solutions were derived from studies on β-Lg, as the intimate mechanisms behind its denaturation–aggregation processes under a variety of experimental conditions are well described.[17,18] Electrostatic interactions have been proposed to be the main factor that controls the final morphology of heat-induced β-Lg aggregates. Variable particulate aggregates were formed under minimal electrostatic interaction, i.e., around the isoelectric point or at high ionic strength, while fibrillar structures developed under conditions favoring electrostatic interactions.[18] The morphology change as a function of pH was detailed by Jung et al.[19] using TEM. They showed that, after heating, the samples occurred as small curved strands at pH 7, large spherical particles at pH 5.8, and long semiflexible fibrils at pH 2. The structure and morphology of fibrils were shown to depend on other environmental parameters. However, the formation of pristine long β-Lg fibrils was promoted by a traditional thermal treatment at pH 2.[19]

Recent studies from different groups reported that the building blocks are small peptides derived from heat-induced hydrolysis rather than intact monomeric protein.[20−22] However, the exact nature of molecules leading to the pristine long fibrils after heating at pH 2 is still under discussion and probably depends on the exact heating conditions, as suggested by Jung and Mezzenga.[23] The fibrils were shorter and displayed a worm-like and granular appearance at pH 7 either in water[19]

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or in water−organic solvent mixtures.[24] Rather than fibrils, connected structural units containing visible β-Lg strands were observed when highly concentrated proteins (i.e., 8%) were heated at 85 °C at pH 7.[16] In our present work, we confirmed that β-Lg is able to fibrillate at pH 7 following heating. The twisted fibrils are long (up to 900 nm) with a width of about 20 nm. The shape of these fibrils is similar to that already reported at the same pH by Jung et al.,[19] but the overall dimensions are somewhat larger. The morphology that we observed may explain why some material was accumulated within the wells of the stacking gel during PAGE analysis (Figure 3). Interestingly, this means that the β-Lg fibrils were not completely dissociated by LDS detergent. These results are in agreement with those reported by Bolder et al.,[25] but they appear to contradict some previous studies that indicate protein fibrils (generally formed at pH 2) are easily dissociated and thus can enter PAGE.[16,21,26] The aggregates disappeared after DTT treatment indicating that covalent disulfide bonds are involved in the β-Lg fibrils formed at pH 7. The same conclusion was drawn by Jung and Mezzenga[23] to explain the isotropic−nematic transition found at pH 7 for β-Lg fibers formed at pH 2. They assumed that the higher isotropic−nematic phase transition may be related to the formation of covalent S−S bridges between neighboring fibers throughout activation of the free thiol groups favored at pH 7. However, covalent bridging of fibrils should probably be limited since randomly branched structures were not observed.

The addition of an excess of reducing sugar, namely, glucose, before heating induced the formation of AGE and influenced the β-Lg fibrillation process. This was revealed by SEC and PAGE analyses as well as by the shape of the aggregates observed by TEM. From a kinetics point of view, glucose did not seem to affect the initial step of protein denaturation−aggregation, corresponding to the initial disappearance of the native protein. In fact, the initial slopes of the disappearance of the native protein (SEC, PAGE) as well as that of the increase of ThT fluorescence are similar, regardless of the presence or absence of glucose. However, for longer heating time, glucose induced a significant decrease of ThT fluorescence. Similar decrease of ThT fluorescence was recently observed during heating of 10% β-Lg at pH 2 and high temperature (120 °C). Such decrease was attributed partly to local gelation or destruction of fibrils.[27] Given our LDS−PAGE results and the lower protein concentration used here, these assumptions do not seem to prevail in our case. We assume that nonfibrillar aggregation of worm-like fibrils could explain the observed decrease of ThT fluorescence. Glucose seems to decrease the growth rate of small aggregated species that accumulate, as shown by TEM images and PAGE. The progressive association of monomers into oligomers and then into small aggregates is clearly observed in the presence of glucose, as opposed to the rapid formation of very large aggregates in the control sample. The presence of glucose during heating clearly slows the formation of large molecular aggregates. A similar effect of β-Lg fibrillation by κ-carrageenan, an anionic polysaccharide, at pH 2 was recently reported.[28] Using TEM, AFM, and light scattering, the authors showed that κ-carrageenan complexes slowed the fibrillation kinetics probably through reduction of available protein monomers. Again, like in the control sample (see above), covalent bonds are involved in the aggregates. Interestingly, our results also show that part of the large aggregates is indeed resistant to LDS-reducing buffer (with DTT), even after a long incubation with a high excess of reducing agent. Consequently, disulfide and nondisulfide covalent bonds are involved in aggregated β-Lg in the presence of glucose. Several previous reports demonstrated that the formation of AGE throughout the Maillard reaction accelerated the fibrillation of amyloidogenic proteins such as β-amyloid peptide, human serum albumin, and τ-protein.[29−32] In contrast with these reports, we showed here that the presence of AGE slowed the formation of heat-induced β-Lg fibrils. Broersen et al.[33,34] reported that glycosylation improved thermostability of β-Lg at pH 7 but affects differently the aggregation behavior according to how the aggregation processes was induced: glucosylation was found to inhibit urea-induced aggregation, while on the contrary heat induced-aggregation was promoted.[33,34] However, the morphology of formed aggregates in both cases was not reported in this study. Our results are consistent with those of others showing a slow-down or even an inhibition of protein fibrillation after glycation and formation of AGE. Thus, according to Fernández-Busquets et al.,[35] only glucose favored the formation of globular oligomeric structures derived from aggregated species, while other tested carbohydrates promoted the formation of fibrillar structures. Lee et al.[36] showed that AGE inhibited the fibrillation of α-synoclein, an amyloidogenic protein. Interestingly, these authors showed that modified proteins predominantly exhibited nonfibrillar structures including spherical aggregates and atypical short fibrils. As was illustrated by the TEM image, heat treatment in the presence of glucose modifies the fibrillation process of β-Lg in a similar way, with a predominance of shorter fibrils (about 100 nm-long) mixed with spherical polydisperse aggregates.

The present results give new insight into the complex aggregation/fibrillation pathway of globular proteins at neutral pH and on how the process is affected by the Maillard reaction. From an academic point of view, our results could be explored for future understanding of controlled protein self-assembly processes, in particular the fibrillation process. Additional studies are in progress to identify the mechanism behind the observed changes and evaluate the nutritional and functional consequences. In fact, β-Lg and whole whey proteins are widely used as a functional ingredient. Our results showed that the presence of reducing sugar, as is the case in many whey protein powders, drive the aggregation kinetics and consequently the nature and morphology of heat induced protein aggregates. The use of these aggregates may be of interest to diversify textural properties, e.g., viscosity and shear thinning of formulated food products. These results are also relevant from a nutritional point of view because increasing demand exists for self-assembled structures that can be used for controlled delivery of bioactive substances.

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ABBREVIATIONS USED

β-Lg, β-lactoglobulin; PAGE, polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; RP-HPLC, reverse-phase high-performance liquid chromatography; AGE,

advanced glycation end products; LDS, lithium dodecyl sulfate; DTT, dithiothreitol; TEM, transmission electron microscopy; ThT, thioflavin T; pI, isoelectric point; SDS, sodium dodecyl sulfate; AFM, atomic force microscopy

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