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# Structural Characterization of Bovine β-Lactoglobulin−Galactose/Tagatose Maillard Complexes by Electrophoretic, Chromatographic, and Spectroscopic Methods

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To investigate the influence of the type of carbonyl group of the sugar on the structural changes of proteins during glycation, an exhaustive structural characterization of glycated β-lactoglobulin with galactose (aldose) and tagatose (ketose) has been carried out. Conjugates were prepared via Maillard reaction at 40 and 50 °C, pH 7, and $a_w = 0.44$. The progress of the Maillard reaction was followed by indirect formation of Amadori and Heyns compounds, advanced glycation end products, and brown polymers. The structural characterization of glycoconjugates was conducted by using a number of analytical techniques such as RP-HPLC, isoelectric focusing, MALDI-ToF, SDS−PAGE, size exclusion chromatography, and spectrofluorimetry (tryptophan fluorescence). In addition, the surface hydrophobicity of the β-lactoglobulin glycoconjugates was also assessed. The results showed a higher reactivity of galactose than tagatose to form the glycoconjugates, probably due to the higher electrophilicity of the aldehyde group. At 40 °C, more aggregation was produced when β-lactoglobulin was conjugated with tagatose as compared to galactose. However, at 50 °C hardly any difference was observed in the aggregation produced by galactose and tagatose. These results afford more insight into the importance of the functional group of the carbohydrate moiety during the formation of protein−carbohydrate conjugates via Maillard reaction.

## INTRODUCTION

It is well established that an efficient and safe method to generate new modified proteins with great industrial and technological interest is the interaction of proteins with carbohydrates via Maillard reaction (*1*). The early stages of Maillard reaction consist of a condensation between the carbonyl group of a reducing carbohydrate and an available, unprotonated amino group, mainly the ϵ-amino group of the lysine residues, to form a Schiff base. Either Amadori or Heyns intermediate rearrangement products may be formed from the initial condensation reaction depending on whether the reducing sugar is an aldose or a ketose, respectively (*2*). The main degradation pathway of Amadori/Heyns rearrangement products is through dehydration of the sugar moiety to form deoxyosones (*3–5*). Further degradation reactions of the deoxyosones mark the onset of the advanced stages of the Maillard reaction, and it is characterized by oxidative fragmentation and dehydration of the sugar moieties and formation of fluorescent compounds, so-called advanced glycosylation end products (AGEs), and highly colored, water-insoluble, nitrogen-containing polymeric compounds called melanoidins.

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The kinetics of glycation is dependent, among other factors, on the type of sugar (*6*), the proportion of the reducing sugar existing in the acyclic or active form under the reaction conditions (*7, 8*), and the electrophilicity of the sugar carbonyl group (*9*). It is accepted that, in general, aldoses are intrinsically more reactive than ketoses and that ketoses could give rise to a greater protein aggregation through covalent cross-linking than aldoses. However, there have been conflicting reports on the influence of the sugar carbonyl group on glycation (*5, 10–15*). Such discrepancies have been attributed to differences in the conditions under which the Maillard reaction was conducted.

Under controlled conditions, whey proteins (β-lactoglobulin (β-Lg), α-lactalbumin, and bovine serum albumin) have been conjugated via Maillard reaction with carbohydrates of different sizes to improve their functionality (*6, 16–22*). The glycation with mono- and disaccharides is an industrially feasible method of preparing the glycoconjugates, since mild reaction conditions and short reaction times are required to obtain modified proteins with a reasonable output (*23, 24*). To the best of our knowledge, no comparative studies on the changes in the β-Lg structure have been carried out following its glycation with galactose (Gal) and tagatose (Tag), which are both derived from whey. Particularly, Tag has a great food interest due to its features as a low-calorie, low-glycemic, and noncariogenic sweetener,

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texturizer, stabilizer, and humectant, as well as its prebiotic character. Although small amounts of Tag are present in dairy products, it can be feasibly produced from lactose after hydrolysis and subsequent isomerization from galactose (*25–27*). Tag is a ketohexose, differing from its stereoisomer fructose only at C-4 (*28*). This small difference has important implications for the overall metabolism of Tag. Thus, the ingested Tag is hardly absorbed in the small gut, being mainly fermented in the cecum and proximal colon by colonic microbiota (*29, 30*). The fermentation of Tag may promote the growth of bifidobacteria and lactobacilli present in the colon, which are believed to have beneficial effects on the immune system, on absorption of minerals, and on several disorders such as constipation, diarrhea, and colorectal cancer (*31, 32*). Nowadays, studies on the interaction between Tag and proteins are very scarce and are focused on the kinetics of browning reactions (*33, 34*) as well as the effects of advanced Maillard reaction compounds (*35*). In the case of Gal, the corresponding Amadori compound (tagatosyllysine), formed during the early stages of the Maillard reaction, could have a higher prebiotic character than tagatose. Several studies have demonstrated the resistance to the digestive enzymes of compounds originated in the early stages of Maillard reaction with other carbohydrates and their ability to be fermented in the distal colon, the main site of onset of chronic gut disorders (*36*).

Since structural modifications of the native structure of protein induced by glycation can influence their functional properties, structural studies on glycated proteins can give useful information about their structure–function relationship. The aim of this study has been to investigate the influence of the sugar carbonyl group on the structural changes of $\beta$-Lg following glycation via Maillard reaction with Gal or Tag to dispose of new multifunctional ingredients obtained from byproducts of the dairy industry.

## MATERIALS AND METHODS

**Materials.** Galactose, tagatose, $\beta$-lactoglobulin from bovine milk (variants A and B), *all-trans*-retinol, and 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

**Preparation and Purification of $\beta$-Lg−Gal/Tag Conjugates.** Carbohydrates (Gal or Tag) and $\beta$-Lg (mixture of A and B variants) (Sigma-Aldrich) in a weight ratio of 1:1 were dissolved in 0.1 M sodium phosphate buffer, pH 7 (Merck, Darmstadt, Germany), and lyophilized. The protein−carbohydrate powders were kept at 40 and 50 °C for 6 days under a vacuum in a desiccator equilibrated at an $a_w$ of 0.44, achieved with a saturated $K_2CO_3$ solution (Merck). In addition, control experiments were performed with $\beta$-Lg stored at 40 and 50 °C without reducing sugars during the same periods (control heated $\beta$-Lg). Incubations were performed in duplicate, and all analytical determinations were performed at least in duplicate.

After incubation, the products were reconstituted in distilled water to a protein concentration of 1 mg/mL. To remove free carbohydrate, 2 mL portions were ultrafiltered through hydrophilic 3 kDa cutoff membranes (Centricon YM-3, Millipore Corp., Bedford, MA) by centrifugation at 1548*g* for 2 h. After removal of free Gal or Tag, samples were reconstituted in distilled water at a concentration of 2 mg/mL for further analysis.

**Assessment of Maillard Reaction Evolution.** The initial steps of Maillard reaction (formation of the Amadori (tagatosyllysine) and Heyns (galactosyllysine) compounds) were assessed by ion pair RP-HPLC after acid hydrolysis of the dry-heated products. Briefly, 400 $\mu$L of 8 N HCl was added to 2 mg of $\beta$-Lg in hydrolysis tubes and the mixture heated at 110 °C for 23 h under inert conditions (helium), followed by the addition of 2 mL of 8 N HCl and filtration through Whatman no. 40 filter paper. The filtered hydrolysate (500 $\mu$L) was applied to a previously activated Sep-Pak C18 cartridge (Millipore Corp., Billerica, MA). Compounds retained in the cartridge were eluted with 3 mL of 3 N HCl, and 50 $\mu$L was used for injection (*37*). Analysis was carried out via an ion pair RP-HPLC method using a $C_8$ (Alltech furosine-dedicated; Alltech, Nicolasville, KY) column (250 × 4.6 mm i.d.) and a variable-wavelength detector at 280 nm (LDC Analytical, SM 4000, Salem, NH). The operating conditions were as follows: column temperature, 35 °C; flow rate, 1.2 mL/min; solvent A, 0.4% HPLC grade acetic acid in doubly distilled water; solvent B, 0.3% KCl in solvent A (*38*). Calibration was performed by using known concentrations (0.52−5.2 mg/L) of a commercial pure standard of furosine (Neosystem Laboratories, Strasbourg, France). Data were expressed as milligrams per 100 mg of protein.

The fluorescence of the so-called AGEs was measured, as an indicator of the advanced stages of the Maillard reaction, in a Shimadzu RF-1501 fluorescence spectrophotometer (Kyoto, Japan) at an excitation wavelength of 340 nm and an emission wavelength of 415 nm, according to the method of Ponger et al. (1984) (*39*). Samples of native, control heated, and glycated $\beta$-Lg were dissolved in 0.1 M sodium phosphate buffer (pH 7.0) to give a final concentration of 1 mg/mL.

Absorbance at 420 nm of $\beta$-Lg−Gal/Tag conjugates, with a protein concentration of 1 mg/mL in doubly distilled water, was measured in a Beckman DU 70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA) at room temperature, as an index of the brown polymers formed in more advanced stages of nonenzymatic browning (*40*).

**Structural Characterization of Glycoconjugates.** RP-HPLC separations were carried out with a C4 Ace column (300 Å, 250 mm × 4.6 mm, 5 $\mu$m particle size) (Advanced Chromatography Technologies, Aberdeen, U.K.) at room temperature. The operating conditions were as follows: flow rate, 1 mL/min; solvent A, 0.1% (v/v) trifluoroacetic acid (TFA; Merck) in doubly distilled water; solvent B, 0.1% (v/v) TFA in doubly distilled water/HPLC grade acetonitrile (Scharlau Chemie, Barcelona, Spain) (1:9, v/v). The elution was performed with a linear gradient by increasing the concentration of solvent B from 40% to 65% in 25 min. The detection was performed at 214 nm using a Beckman 166UV detector (Beckman Instruments Inc.).

For isoelectric focusing (IEF) analysis, 25 $\mu$L of the stored samples was added to 25 $\mu$L of 2× Novex IEF pH 3–7 sample buffer (Invitrogen, Carlsbad, CA). The samples were loaded (20 $\mu$L) onto Novex pH range 3–7 gels (Invitrogen) and run according to the protocol from the manufacturer. The gels were stained using the colloidal blue staining kit (Invitrogen).

MALDI-ToF analyses were performed using a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed nitrogen laser ($\lambda$ = 337 nm, 3 ns pulse width, and 3 Hz frequency) and a delayed extraction ion source. Ions generated by the laser desorption were introduced into the flight tube (1.3 m flight path) with an acceleration voltage of 25 kV, a 93% grid voltage, a 0.05% ion guide wire voltage, and a delay time of 350 ns in the linear positive ion mode. Mass spectra were obtained over the *m/z* range 10000–35000. Myoglobin (horse heart) and carbonic anhydrase were used for external calibration, and sinapinic acid (10 mg/mL in 0.3% trifluoroacetic acid/acetonitrile, 70:30, v/v) was used as the matrix. The samples were mixed with the matrix at a ratio of approximately 1:15, and finally, 1 $\mu$L of this solution was spotted onto a flat stainless-steel sample plate and dried in air.

For SDS−PAGE analysis, 32.5 $\mu$L of the stored samples was added to 12.5 $\mu$L of 4× NuPAGE LDS sample buffer (Invitrogen) and 5 $\mu$L of 0.5 M dithiothreitol (DTT; Sigma-Aldrich) and the mixture heated at 70 °C for 10 min. The samples were loaded (20 $\mu$L) onto a 12% polyacrylamide NuPAGE Novex Bis-Tris precast gel, and a continuous MES−SDS running buffer was used. The gels were run for 41 min at 120 mA/gel and 200 V and stained using the colloidal blue staining kit (Invitrogen).

Size exclusion chromatography (SEC) was carried out under nondenaturing conditions (0.05 M sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl) using a 24 mL Superdex 75 column, HR 10/30 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), on an FPLC system. A 100 $\mu$L volume of a 1 mg/mL sample was applied to the column at room temperature. Elution was achieved in isocratic mode at 0.8 mL/min for 30 min, and detection of eluting proteins was performed at 214 nm. The standard proteins used for calibration were

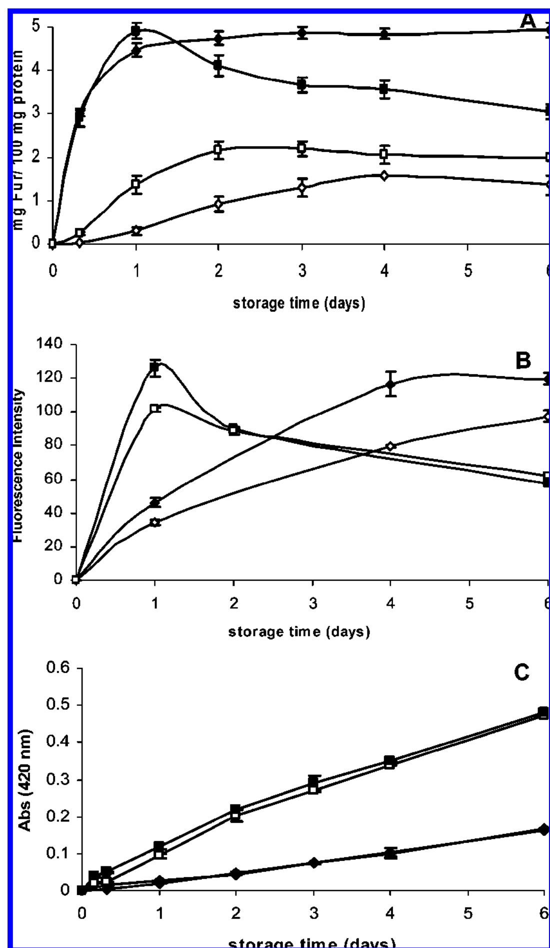


**Figure 1.** Evolution of the content of 2-(furoyl/furylmethyl)amino acid derived from the Amadori and Heyns products (**A**) and AGE fluorescence (**B**) and absorbance at 420 nm (**C**) during 6 days of storage of the $\beta$-Lg−Gal/Tag Maillard conjugates: (◆) $\beta$-Lg−Gal, 40 °C; (■) $\beta$-Lg−Gal, 50 °C; (◇) $\beta$-Lg−Tag, 40 °C; (□) $\beta$-Lg−Tag, 50 °C.

human serum albumin (67 kDa), ovalbumin (43 kDa), α-chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa) (GE Healthcare Bio-Sciences AB). The void volume was determined with blue dextran 2000.

Tryptophan fluorescence measurements were carried out in a Shimadzu RF-1501 fluorescence spectrophotometer (Kyoto, Japan) on 1 mg/mL native, control heated, and glycated $\beta$-Lg in 0.1 M sodium phosphate buffer (pH 7.4). Spectra of the samples were recorded from 300 to 400 nm upon sample excitation at 280 nm, with slit widths of 10 nm.

**Determination of the Surface Hydrophobicity of Glycoconjugates.** The surface hydrophobicity ($S_0$) of control heated and glycated $\beta$-Lg was investigated by binding of 8-anilino-l-naphthalenesulfonate (ANS) or retinol (RET). The relative fluorescence intensity (FI) of the ligand−protein conjugates was measured on a Shimadzu RF-1501 fluorescence spectrophotometer at room temperature. The wavelengths of excitation ($\lambda_{exc}$) and emission ($\lambda_{em}$) were 390 and 470 nm for ANS and 330 and 470 nm for RET, with slit widths of 10 nm.

Native, control heated, and glycated $\beta$-Lg samples were diluted with 0.1 M sodium phosphate buffer, pH 7.4, to a final concentration of 0.1 mg/mL. Then 10 $\mu$L of ANS solution (8.0 mM in 0.1 M sodium phosphate buffer, pH 7.4) was added to 1 mL of the diluted sample, the resulting solution mixed and equilibrated for 2 min and, finally, the fluorescence intensity measured at room temperature.

The RET binding activity of the same samples was measured by fluorescence titration according to the method of Hattori et al. (*41*). Retinol in ethanol was added to 2.0 mg of protein in PBS (in 0.1 M sodium phosphate buffer, pH 7.1, containing 0.04 M NaCl).

Solutions of ANS in sodium phosphate buffer and RET in absolute ethanol were prepared daily. In the case of RET binding, both ethanol

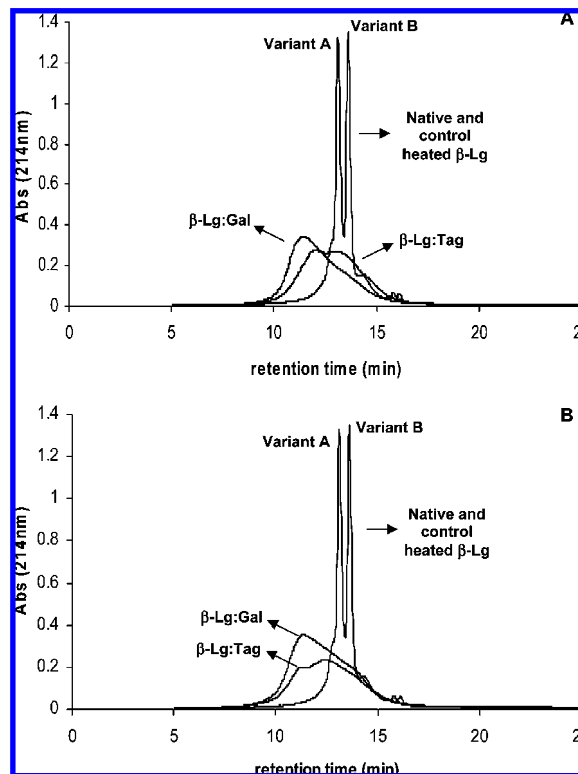


**Figure 2.** RP-HPLC patterns of control heated and glycated $\beta$-Lg incubated at 40 °C for 4 days with both sugars (**A**) and at 50 °C for 1 day with Gal and 2 days with Tag (**B**).

and the PBS used for protein dilution were flushed with helium to inhibit RET oxidation by air.

All measurements were performed at least in duplicate.

## RESULTS AND DISCUSSION

**Assessment of Maillard Reaction Evolution during the Formation of Glycoconjugates.** Amadori and Heyns products, as early indicators of Maillard reaction, were indirectly determined and quantified by ion pair RP-HPLC following acid hydrolysis. It is well-known that furosine (2-(furoylmethyl)lysine), formed upon acid hydrolysis of the protein-bound Amadori product of lysine, can be a useful indicator to measure the glycation extent of milk proteins (*19, 20, 24, 37, 42*). To the best of our knowledge, scarce information is available in relation to the compounds obtained from the acid hydrolysis of the Heyns product. In our case, an RP-HPLC peak with the same retention time as that for furosine was observed following hydrolysis of the $\beta$-Lg−Tag samples (data not shown). The absorbance at 280 nm exhibited by this compound is probably due to the presence of the furyl ring formed from the cyclization at carbons C-3, C-4, C-5, and C-6 of the Heyns product. Additionally, it is very likely that a small amount of furosine may also coelute with this compound as McPherson et al. (*43*) found that around 15% of the Amadori compound was formed from the incubation of fructose with BSA, since fructose and amino groups can undergo irreversible rearrangements to give not only two 2-amino-2-deoxyaldoses but also Amadori compounds (1-amino-1-deoxyfructose) (*44*).

**Figure 1A** shows the evolution of the furoyl and furyl compounds derived from the Amadori and Heyns products, respectively, upon storage at 40 and 50 °C for 6 days of the $\beta$-Lg−Gal/Tag conjugates. The results showed that galactose had a higher and faster initial rate of utilization of $\epsilon$-amino

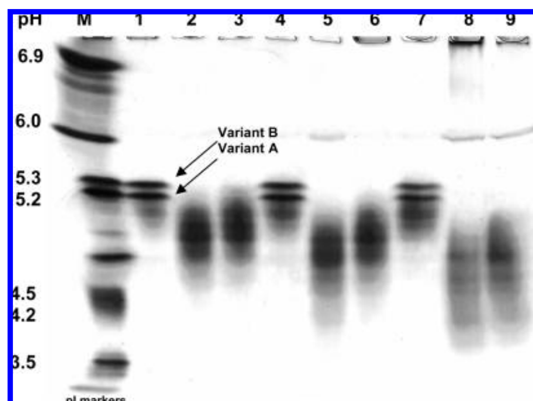


**Figure 3.** Isoelectric focusing performed in the pH range 3–7. Native $\beta$-Lg (lane 1), control $\beta$-Lg heated at 50 °C for 2 (lane 4) and 6 (lane 7) days, and $\beta$-Lg incubated at 50 °C with Gal or Tag for 1 day (lanes 2 and 3, respectively), 2 days (lanes 5 and 6, respectively), and 6 days (lanes 8 and 9, respectively).

groups of lysine residues than tagatose at both temperatures. The maximum levels of furosine in the glycated $\beta$-Lg−Gal system was achieved at 1 day of storage at both 40 and 50 °C, whereas the highest amounts of furyl derivative were found after the storage of $\beta$-Lg with tagatose for 4 days at 40 °C and 2 days at 50 °C. A noticeable decrease in furosine was also found from the first day of incubation of $\beta$-Lg with galactose at 50 °C, suggesting that the degradation of the Amadori compound prevailed over its formation.

The formation of fluorescent AGE compounds progressively increased during the entire storage period at 40 °C, and this was lower when Tag was used for glycation (**Figure 1B**). The maximum of fluorescence was achieved at 1 day of storage at 50 °C for both carbohydrates, although it was also higher for galactosyl derivatives. However, from the first day of incubation, the presence of fluorescence compounds sharply decreased, probably due to the reaction progress toward the formation of nonfluorescent aggregates. No differences were found between the $\beta$-Lg−Gal and $\beta$-Lg−Tag conjugates after 2 days of storage at 50 °C. Finally, the color development (**Figure 1C**) was also higher at 50 °C than at 40 °C, and hardly any difference was observed when $\beta$-Lg−Gal and $\beta$-Lg−Tag were compared.

**Structural Characterization of Glycoconjugates.** RP-HPLC−UV chromatograms of control heated and glycated $\beta$-Lg with Gal and Tag are shown in **Figure 2**. Control protein heated at 40 and 50 °C presented the same chromatographic profile as compared to native $\beta$-Lg, which displayed two well-defined peaks corresponding to the A and B genetic variants. However, the profiles corresponding to the glycated $\beta$-Lg showed a broad and less retained peak whose area increased with the incubation time, indicating that the changes in the RP-HPLC pattern of the incubated $\beta$-Lg−Gal/Tag mixtures were derived exclusively from the glycation of the protein. The shorter retention time of the peak corresponding to the glycated protein can be attributed to the incorporation of Gal/Tag residues into the peptide chain that leads to an increase in $\beta$-Lg hydrophilicity. A similar behavior was previously reported with the lactosylation of caseinomacropeptide (*37*) and $\beta$-Lg and α-lactalbumin (*22*). Moreover, the widening of the peak could be indicative of the great heterogeneity of $\beta$-Lg derivatives formed during the glycation process.

The blocking of Lys and Arg with carbohydrates induces a slight loss of basicity and, consequently, a moderate increase

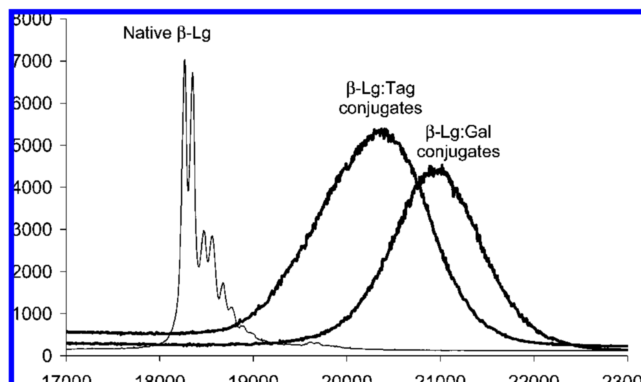


**Figure 4.** Superimposed MALDI-ToF-MS spectra of native $\beta$-Lg and $\beta$-Lg incubated with Gal or Tag at 40 °C for 6 days.

**Table 1.** Number of Gal and Tag Molecules Bound to Glycated $\beta$-LG Estimated by MALDI-ToF-MS

| | | $\beta$-Lg−Gal conjugates | | $\beta$-Lg−Tag conjugates | |
|---|---|---|---|---|---|
| temp (°C) | incubation time (days) | $m/z$[a] | av no. of Gal adducts | $m/z$[a] | av no. of Tag adducts |
| 40 | 1 | 20619.4 | 14 | 18845.3 | 3 |
| | 4 | 20971.5 | 16 | 20176.7 | 11 |
| | 6 | 21017.2 | 17 | 20368.3 | 13 |
| 50 | 1 | 21026.6 | 17 | 20448.2 | 13 |
| | 2 | 21350.7 | 19 | 20812.5 | 15 |
| | 6 | 21715.3 | 21 | nd | nd |

[a] Average mass values observed at the maximum intensity of the Gaussian peak.

in the negative charge of the $\beta$-Lg molecule, leading to a noticeable shift of the isoelectric point (pI) of the glycated protein toward more acidic pH (*24, 45, 46*). Thus, IEF can be a suitable method to monitor the glycation of proteins. Native $\beta$-Lg (**Figure 3**, lane 1) showed mainly two bands with pI values of 5.2 and 5.3 corresponding to the genetic variants A and B, respectively. A comparable pattern was observed after heating of $\beta$-Lg for 2 and 6 days in the absence of carbohydrates at 50 °C (**Figure 3**, lanes 4 and 7). The decrease of pI was more evident at 50 °C (**Figure 3**) than at 40 °C (profile not shown), confirming the greater degree of $\beta$-Lg glycation obtained at 50 °C. Several isoforms with pI values ranging from 5.2 to 4.0 could be detected (**Figure 3**, lanes 2, 3, 5, 6, 8, and 9); such diversity of bands with different charges is indicative of the formation of a wide range of glycated species of $\beta$-Lg. The pI shift was higher after incubation of $\beta$-Lg with Gal than with Tag, confirming that $\beta$-Lg was more extensively glycated with the former, in agreement with the results derived from the analysis of the acid hydrolysates of the Amadori and Heyns products (**Figure 1A**).

With the aim of knowing exactly the number of molecules of carbohydrate linked covalently to the $\beta$-Lg, a MALDI-ToF-MS analysis was carried out. The molecular masses of native $\beta$-Lg were 18 278 and 18 363 Da, in good agreement with the expected masses of the variants B and A, respectively (*47*) (**Figure 4**). Mass spectra of $\beta$-Lg incubated with both sugars were characterized by a broad Gaussian peak shape without good resolution due to the great heterogeneity of the glycated forms of $\beta$-Lg (*48–50*) (**Figure 4**). Considering the maximum intensity of the Gaussian peaks, the average number of Gal or Tag molecules bound to $\beta$-Lg after glycation could be estimated (**Table 1**). Thus, the most important difference between glycation with both sugars was observed after 1 day of incubation at 40 °C, where average

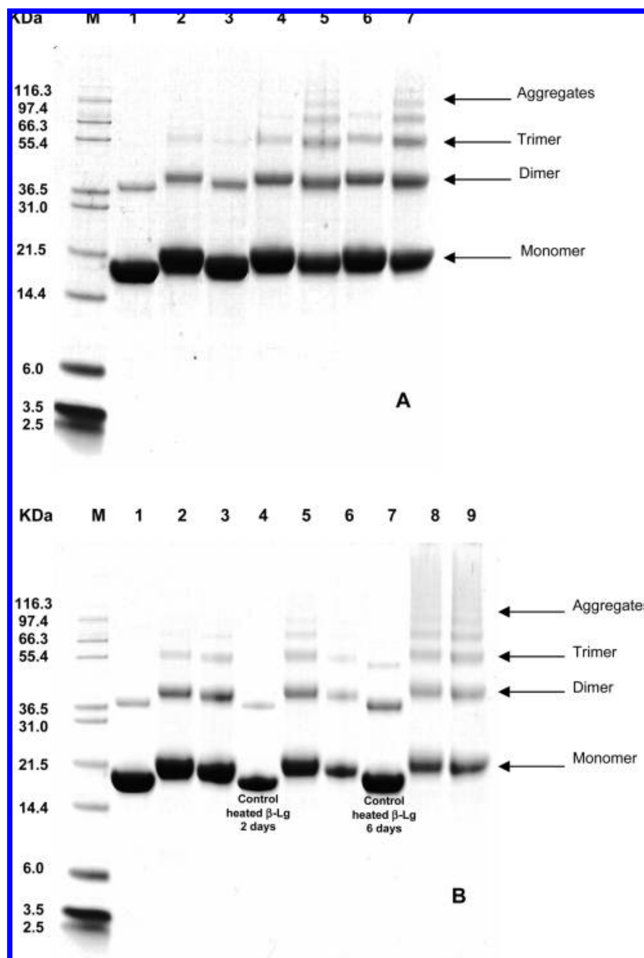


**Figure 5.** SDS−PAGE of native $\beta$-Lg (lane1) and $\beta$-Lg incubated with Gal or Tag for 1 day (lanes 2 and 3, respectively), 4 days (lanes 4 and 5, respectively), and 6 days (lanes 6 and 7, respectively) at 40 °C (**A**) and for 1 day (lanes 2 and 3, respectively), 2 days (lanes 5 and 6, respectively), and 6 days (lanes 8 and 9, respectively) at 50 °C (**B**). M = molecular mass markers containing $\beta$-galactosidase (116.3 kDa), phosphorylase B (97.4 kDa), BSA (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6.0 kDa), insulin B chain (3.5 kDa), and insulin A chain (2.5 kDa).

numbers of 14 galactose and 3 tagatose molecules were linked to the $\beta$-Lg. Additionally, up to 21 galactose molecules linked to the protein chain could be estimated after 6 days of storage at 50 °C, despite the fact that $\beta$-Lg has 19 potential sites of glycation (15 Lys residues, 3 Arg residues, and the $NH_2$-terminal Leu). Fenaille et al. (*51*), who determined 22 galactose adducts covalently linked to $\beta$-Lg following dry-way glycation at 60 °C, identified two diglycated lysine residues ($Lys_{47}$ and $Lys_{75}$), which could explain the high number of Gal moieties attached to the $\beta$-Lg.

The slower electrophoretic mobilities of the glycated $\beta$-Lg as compared to the control heated samples observed by SDS−PAGE under reducing conditions indicated the glycation of $\beta$-Lg at 40 °C (**Figure 5A**) and 50 °C (**Figure 5B**). Native $\beta$-Lg showed mainly the monomeric form ($M_r \approx$ 18 000), although the band corresponding to the dimeric form ($M_r \approx$ 36 000) was also detected in a much lower proportion (**Figure 5**, lane 1). Likewise, the detection of new bands of higher molecular weight after the incubation of $\beta$-Lg with both carbohydrates for longer times (**Figure 5A**, lanes 5–7, and **Figure 5B**, lanes 8–9) indicated that cross-linking reactions took place. Interestingly, a greater number of aggregation bands corresponding to the oligomeric forms were observed with tagatosylated $\beta$-Lg conjugates after incubation at 40 °C for 4 and 6 days as compared to galactosylated $\beta$-Lg (**Figure 5A**, lanes 4–7). These results suggested a higher trend of $\beta$-Lg for aggregation when it was incubated with the ketose (Tag) than with the aldose (Gal). Similar results were previously described for the $\beta$-Lg glycated with lactose and lactulose at 50 °C (*12*). Nevertheless, at 50 °C, a similar number of aggregation bands were observed with both carbohydrates (**Figure 5B**).

In an attempt to shed more light on the structural changes promoted by the reaction between Gal or Tag and $\beta$-Lg, the polymerization degree of $\beta$-Lg was investigated by means of SEC under nondenaturing conditions (**Table 2**). As expected, native $\beta$-Lg eluted mainly as a dimer with about 4% in a trimeric form at neutral pH. The control protein heated in the absence of sugars also contained some minor aggregated forms, whose formation can be attributed to noncovalent interactions or thiol/disulfide exchange reactions induced by increased exposure of previously buried hydrophobic groups and the thiol group (*52–54*). $\beta$-Lg−Gal/Tag conjugates eluted predominantly as a dimer upon storage at 40 °C for 1 day; however, with increasing incubation time, several peaks corresponding to the trimeric and oligomeric forms appeared, indicating that glycation of $\beta$-Lg promoted its polymerization (profiles not shown). At 40 °C, the ratio polymer (trimeric and oligomeric species):dimer was higher in $\beta$-Lg−Tag conjugates, as compared to the corresponding $\beta$-Lg−Gal conjugates. At 50 °C, protein aggregation was more noticeable than at 40 °C and a similar behavior with both monosaccharides was found (**Table 2**).

**Figure 6**shows the Trp fluorescence emission spectra of native, control heated, and glycated $\beta$-Lg with Gal or Tag at 40 °C (**A**) and 50 °C (**B**). When excited at 280 nm, native $\beta$-Lg exhibited a fluorescence emission maximum ($\lambda_{max}$) at 335 nm. At both temperatures, the intensity of Trp-related fluorescence (Trp-FI) increased in the control heated $\beta$-Lg, and this was accompanied by a light shift of the Trp emission maximum to 337 nm, which suggests a relatively increased exposure of tryptophan toward more hydrophilic surroundings. This means that $\beta$-Lg underwent conformational changes around the Trp residues ($^{19}$Trp and $^{61}$Trp) due to heat-induced denaturation (*19–21, 55*). In the case of $\beta$-Lg−Gal/Tag conjugates, the Trp-FI was lower than that of native protein at both temperatures, particularly at 50 °C, which can be attributed to the shielding effect of the carbohydrate bound to $\beta$-Lg (*19–21, 56*). Moreover, a slight shift of the Trp emission maximum to 338 nm (with Tag) and to 340 nm (with Gal) was observed at 50 °C, while no shift of the Trp emission maximum was detected after glycation of $\beta$-Lg at 40 °C. These results may suggest that the glycation at 40 °C with both reducing sugars partially affected the side chains of the protein in the tertiary structure without great disruption of the native structure, whereas at 50 °C the conformational state of $\beta$-Lg was more affected.

**Determination of the Surface Hydrophobicity of Glycoconjugates.** The three-dimensional structure of $\beta$-Lg is characterized by a hydrophobic region which confers to $\beta$-Lg the capacity to bind in vitro small hydrophobic ligands, such as ANS (*57*) and RET (*58*), among others. Therefore, the study of the capacity to bind both ligands (surface hydrophobicity, $S_0$) of $\beta$-Lg could be useful to determine the structural changes taking place in the protein after glycation.

With respect to the native $\beta$-Lg, the ANS fluorescence

**Table 2.** Dimer, Trimer, and Oligomer Contents of Native, Control Heated, and Glycated β-Lg with Galactose and Tagatose Incubated at 40 and 50 °C for 6 days, Estimated after Their Analysis by Size Exclusion Chromatography under Nondenaturing Conditions

| temp (°C) | sample | storage time (days) | dimer content (%) | trimer content (%) | oligomer content (%) | polymer:dimer ratio |
|---|---|---|---|---|---|---|
| 40 | native β-Lg | 0 | 96 ± 0.28 | 4 ± 0.28 | | |
| | heated β-Lg | 4 | 82.9 ± 0.33 | 17.1 ± 0.33 | | |
| | | 6 | 81.2 ± 1.66 | 18.8 ± 1.66 | | |
| | β-Lg−Gal | 1 | 84.0 ± 0.52 | 16.0 ± 0.52 | | |
| | | 4 | 59.1 ± 0.90 | 26.3 ± 0.33 | 14.6 ± 0.57 | 0.69 |
| | | 6 | 57.8 ± 2.84 | 24.3 ± 1.13 | 17.9 ± 1.70 | 0.73 |
| | β-Lg−Tag | 1 | 83.9 ± 0.28 | 16.1 ± 0.28 | | |
| | | 4 | 50.5 ± 0.85 | 23.9 ± 0.71 | 25.6 ± 1.56 | 0.98 |
| | | 6 | 50.2 ± 0.64 | 23.7 ± 0.07 | 26.1 ± 0.71 | 0.99 |
| 50 | native β-Lg | 0 | 96 ± 0.28 | 4 ± 0.28 | | |
| | heated β-Lg | 2 | 87.7 ± 0.78 | 12.3 ± 0.78 | | |
| | | 6 | 76.9 ± 0.49 | 23.1 ± 0.49 | | |
| | β-Lg−Gal | 1 | 58.1 ± 3.04 | 24.6 ± 1.48 | 17.3 ± 1.56 | 0.72 |
| | | 2 | 45.9 ± 1.56 | 23.0 ± 0.35 | 31.1 ± 1.20 | 1.18 |
| | | 6 | 28.9 ± 2.40 | 14.7 ± 1.20 | 56.4 ± 3.61 | 2.46 |
| | β-Lg−Tag | 1 | 60.3 ± 0.21 | 23.9 ± 0.35 | 15.8 ± 0.57 | 0.66 |
| | | 2 | 49 ± 2.26 | 23.3 ± 0.85 | 27.7 ± 1.41 | 1.04 |
| | | 6 | 31.4 ± 1.13 | 16.2 ± 1.48 | 52.4 ± 2.62 | 2.18 |

indicated an increase of $S_0$ during the heating of β-Lg without carbohydrates, particularly at 50 °C, probably due to the exposition of hydrophobic patches on the protein surface, as a consequence of its partial denaturation (*59*) (**Table 3**). The effect of protein glycation was mainly noted at 50 °C from the second day of incubation, particularly in the galactosyl conjugates, whose great decrease in $S_0$ suggested important structural changes in the three-dimensional configuration of the protein. The decrease in $S_0$ of these Maillard conjugates may be attributed not only to the formation of aggregates but also to

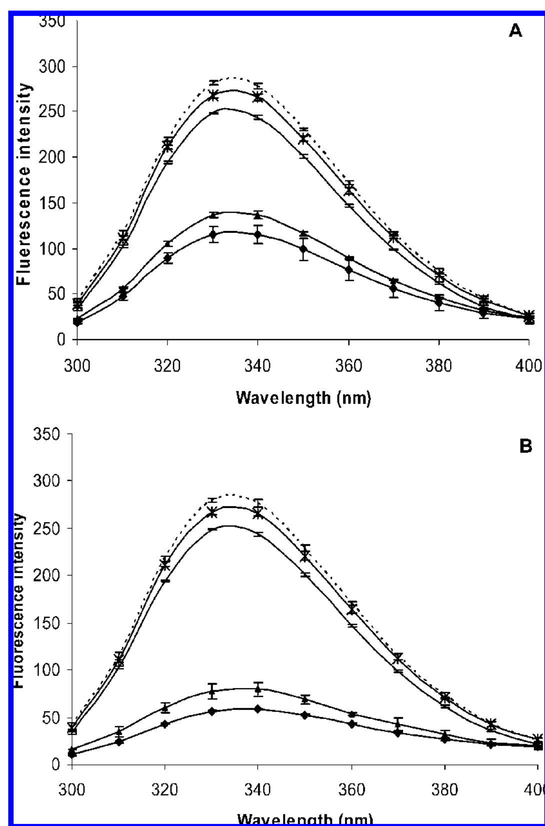


**Figure 6.** Intrinsic fluorescence emission spectra of native β-Lg, control heated β-Lg, and β-Lg incubated with Gal or Tag for 4 days at 40 °C (**A**) and for 2 days at 50 °C (**B**): (—) native β-Lg; (-∗-) control β-Lg heated for 4 days at 40 °C and for 2 days at 50 °C; (- - -) control β-Lg heated for 6 days at 40 and 50 °C; (◆) β-Lg−Gal; (▲) β-Lg−Tag.

**Table 3.** Surface Hydrophobicity ($S_0$) of Native, Control Heated, and Glycated β-Lg with Gal and Tag for 6 days at 40 and 50 °C

| temp (°C) | sample | storage time (days) | ANS fluorescence |
|---|---|---|---|
| 40 | native β-Lg | 0 | 40.8 ± 1.15 |
| | heated β-Lg | 4 | 46.7 ± 0.21 |
| | | 6 | 52.6 ± 3.93 |
| | β-Lg−Gal | 1 | 42.9 ± 0.16 |
| | | 4 | 40.0 ± 0.22 |
| | | 6 | 39.5 ± 0.13 |
| | β-Lg−Tag | 1 | 42.7 ± 1.81 |
| | | 4 | 33.7 ± 3.64 |
| | | 6 | 35.8 ± 3.20 |
| 50 | native β-Lg | 0 | 40.8 ± 1.15 |
| | heated β-Lg | 2 | 50.1 ± 3.79 |
| | | 6 | 60.5 ± 1.14 |
| | β-Lg−Gal | 1 | 38.8 ± 3.17 |
| | | 2 | 26.0 ± 0.99 |
| | | 6 | 10.8 ± 1.42 |
| | β-Lg−Tag | 1 | 43.0 ± 0.37 |
| | | 2 | 33.1 ± 0.16 |
| | | 6 | 17.1 ± 1.31 |

the blocking of Lys and/or Arg residues by Gal or Tag in the Maillard conjugates, as ANS may also strongly bind cationic groups of proteins (*60*).

**Figure 7**shows the results of the RET binding assays carried out at 50 °C. As the incubation time increased, the binding activity of control heated β-Lg decreased as compared to that of the native protein, indicating that the RET binding sites were not accessible probably due to protein aggregation induced by heat. At 40 °C (data not shown), no differences between the RET binding capacities of control heated β-Lg and the glycated samples were observed. Nevertheless, a stronger reduction of RET binding activity was observed in the glycated samples at 50 °C (**Figure 7**). In this case, such reduction was brought about by conformational changes in the RET binding site of β-Lg induced by conjugation with Gal or Tag.

**Conclusions.** The results exposed in this paper show the influence of the sugar carbonyl group (aldose or ketose) on the extent of glycation and the structural changes of glycated β-Lg at 40 and 50 °C. In the initial stages of the Maillard reaction, a higher reactivity of Gal than Tag was observed, probably because the aldehyde group of the acyclic form of Gal is more electrophilic than the keto group of the acyclic form of Tag. At 40 °C, it was clear that more aggregation

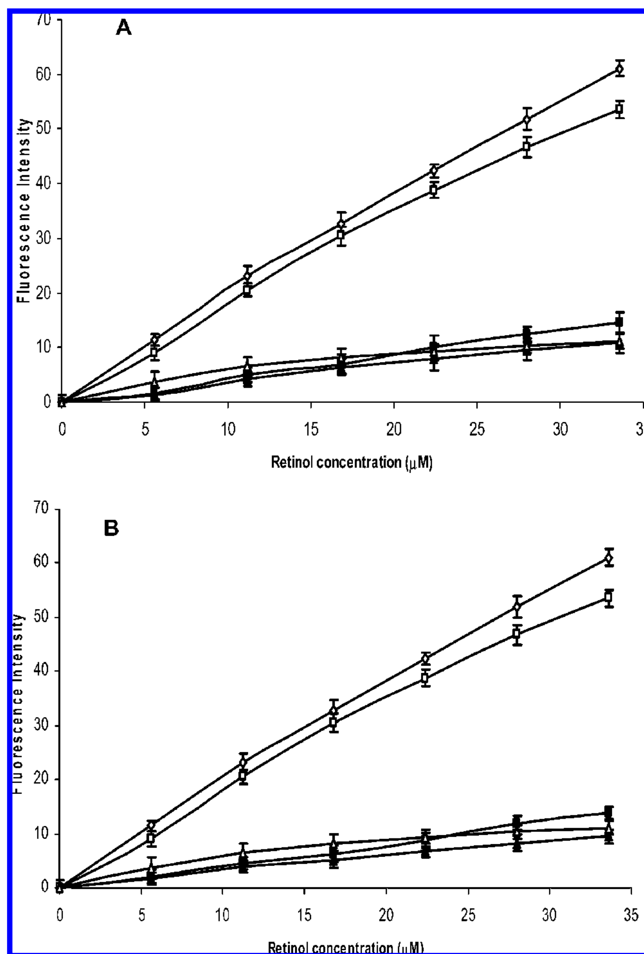


**Figure 7.** Retinol binding activity of the $\beta$-Lg−Gal (**A**) and $\beta$-Lg−Tag (**B**) conjugates at 50 °C: (◇) native $\beta$-Lg; (□) control $\beta$-Lg heated for 2 days; (△) control $\beta$-Lg heated for 6 days; (■) $\beta$-Lg−Gal/Tag incubated for 2 days; (▲) $\beta$-Lg−Gal/Tag incubated for 6 days.

was produced when $\beta$-Lg was conjugated with Tag as compared to Gal. This result is probably due to the fact that the Heyns product is more readily degraded to dicarbonyl compounds than the Amadori product (*61, 62*). These dicarbonyl intermediates can form different heterocyclic and small carbonyl compounds, which are susceptible to react with amino acids and/or proteins, leading to the formation of peptide-bound amino acid derivatives in the advanced and final stages of the Maillard reaction (*63–65*). However, at 50 °C, the results indicated a similar aggregation of glycated protein with Gal and Tag. This could be due to a higher susceptibility of the Amadori compound to the temperature as compared to that of the Heyns compound. The increase of temperature at 50 °C could produce the acceleration of enolization, so that, through this reaction, the keto group of the Amadori compound could migrate to C-3, C-4, or C-5 of the sugar moiety (*64*), eliminating, thus, the steric hindrance and promoting further condensation of the carbonyl group with another primary amino group. Yeboah et al. (*5*) demonstrated, in a study on the dry glycation of BSA with glucose and fructose at 50 °C, that the α-hydroxycarbonyl group of the Amadori product was more reactive than the aldehyde group of the Heyns product.

The findings obtained in this work have allowed an exhaustive characterization of the structural changes that occur during $\beta$-Lg glycation with Gal and Tag. Future work is needed to assess technological and biological activities in these new ingredients obtained from byproducts of the dairy industry.

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