JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization and in Vitro Digestibility of Bovine β-Lactoglobulin Glycated with Galactooligosaccharides

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Galactooligosaccharides (GOS) are well-known prebiotic ingredients which can form the basis of new functional dairy products. In this work, the production and characterization of glycated *β*-lactoglobulin (*β*-LG) with prebiotic GOS through the Maillard reaction under controlled conditions (a_w = 0.44, 40 °C for 23 days) have been studied. The extent of glycation of *β*-LG was evaluated by formation of furosine which progressively increased with storage for up to 16 days, suggesting that the formation of Amadori compounds prevailed over their degradation. RP-HPLC–UV, SDS–PAGE, and IEF profiles of *β*-LG were modified as a consequence of its glycation. MALDI-ToF mass spectra of glycated *β*-LG showed an increase of up to ~21% in its average molecular mass after storage for 23 days. Moreover, a decrease in unconjugated GOS (one tri-, two tetra-, and one pentasaccharide) was observed by HPAEC-PAD upon glycation. These results were confirmed by ESI MS. The stability of the glycated *β*-LG to in vitro simulated gastrointestinal digestion was also described and compared with that of the unglycated protein. The yield of digestion products of glycated *β*-LG was lower than that observed for the unglycated protein. The conjugation of prebiotic carbohydrates to stable proteins and peptides could open new routes of research in the study of functional ingredients.

KEYWORDS: *β*-Lactoglobulin; galactooligosaccharides; Maillard reaction; gastrointestinal digestion; prebiotic

1. INTRODUCTION

The importance of the colonic microbiota in human health and nutrition is well known. The human large intestine plays an important role as a nutritional organ mainly due to the metabolic activities of the resident microbiota that represent a very complex profile of bacterial species. A large number of diseases are strongly related to the composition of colonic microbiota (*1*), and bifidobacteria and lactobacilli present in the colon 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 (*2, 3*). Since the introduction of prebiotics as "nondigestible oligosaccharides that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (*4*), an increased level of interest in the use of prebiotics in manipulating the composition and activity of the intestinal microbiota to improve the gastrointestinal health has been observed (*5–8*). Quantitative changes in bacterial groups, fermentation end products, and the site of fermentation, i.e., the proximal or distal colon, are important factors in assessing the prebiotic effect of nondigestible carbohydrates (*9, 10*). This is one reason why considerable efforts are being made to identify molecules that result in a more distal prebiotic type of fermentation, the principle site of onset of chronic gut disorders (*11*).

Many studies have demonstrated that glycation of proteins via Maillard reaction under controlled conditions can be successfully used to improve or produce new functional and biological properties, generating very useful glycoproteins for industrial applications (*12–14*). However, to our knowledge, no detailed biochemical studies of the glycation of proteins with prebiotic carbohydrates have been carried out to date.

Early stages of the Maillard reaction between proteins and carbohydrates give rise to protein glycation through the formation of the Amadori compound. Metabolic studies of protein-bound Amadori products performed with adult human volunteers showed very low levels of urinary (3%) and fecal (1%) excretions of casein-bound fructose-lysine (*15*). Likewise,

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10.1021/jf071111l CCC: $37.00 © 2007 American Chemical Society
Published on Web 08/21/2007

lactulose-lysine was also shown to be poorly digested and excreted (*16*). Although several reports indicate that nondigested Amadori compounds reach the hind gut where they are utilized by microorganisms (*17–19*), there is scant information about the ability of the colonic microbiota to act on Maillard reaction products, and it is principally limited to melanoidins derived from proteins glycated with glucose (*20, 21*).

Bovine β-lactoglobulin (β-LG), which is extensively used as a functional ingredient in food formulations because of its functional and nutritional properties, has been shown to be prone to reacting with reducing sugars through the Maillard reaction (*22–26*). Galactooligosaccharides (GOS) are prebiotic carbohydrates, also widely used in the food industry, which could participate in the glycation of proteins during food processing due to their reducing nature. This study reports the production and characterization of Maillard-type conjugates produced by the dry-heating of mixtures of bovine β-LG and GOS. The stability of the glycated β-LG to in vitro gastrointestinal digestion is also described and compared with that of the unglycated protein. The conjugation of prebiotic carbohydrates to proteins, through the Maillard reaction under controlled conditions, might lead to a new prebiotic product with properties different from those of the free carbohydrate/native protein. Additionally, they may have improved technological properties such as solubility, thermal stability, or emulsifying, foaming, textural, and gelation properties. The idea of combining these effects with prebiotic properties would add major functionality to the approach of modulating the gut microbiota.

2. MATERIALS AND METHODS

2.1. Preparation of Galactooligosaccharides (GOS). The original GOS source employed was a commercial product "Vivinal-GOS" kindly provided by Borculo Domo (Hanzeplein, The Netherlands), containing approximately 45% GOS and 30% digestible sugars: lactose (15%), glucose (14%), and galactose (1%) (*27*). Mono- and disaccharides were removed from the GOS sample using an activated charcoal treatment following the method of Morales et al. (*28*). In brief, 4 g of GOS was dissolved in 800 mL of a 15:85 (v:v) ethanol/water mixture and stirred with 24 g of activated charcoal Darco G60, 100 mesh (Sigma, St. Louis, MO), for 30 min to remove mono- and disaccharides. This mixture was filtered through Whatman No. 1 filter paper under vacuum, and the activated charcoal was further washed with 50 mL of a 15:85 (v:v) ethanol/water mixture. Oligosaccharides adsorbed onto activated charcoal were extracted when the mixture was stirred for 30 min in 800 mL of a 50:50 (v:v) ethanol/water mixture. Activated charcoal was eliminated by filtering as previously described, and the ethanol was evaporated under vacuum at 30 °C. The sample was dissolved in 5 mL of deionized water and filtered through 0.22 μm filters (Millipore Corp., Bedford, MA). Carbohydrates were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as indicated below. Glucose, galactose, and lactose (Sigma) were used as standards.

2.2. Glycation of β-Lactoglobulin (β-LG) with GOS. Aliquots of a solution consisting of 2.0 mg/mL β-LG (mixture of A and B variants) (Sigma) and 2.0 or 6.0 mg/mL GOS (GOS:β-LG weight ratio of 1 or 3, respectively) in 0.1 M sodium phosphate buffer (pH 7) (Merck, Darmstadt, Germany) were lyophilized. These were kept under vacuum in a desiccator at 40 °C and a water activity of 0.44, achieved with a saturated K_2CO_3 solution (Merck), for various periods (0, 5, 12, 16, 20, and 23 days). In addition, control experiments were performed with β-LG stored at 40 °C without GOS during the same periods (heated β-LG). Incubations were performed in duplicate, and all of the analytical determinations were performed in at least duplicate.

2.3. Determination of the Degree of β-LG Glycation. 2-Furoylmethyllysine (furosine) analysis was performed according to the method of Moreno et al. (*29*). Four hundred microliters of 8 N HCl was added to 2 mg of β-LG in hydrolysis tubes and heated at 110 °C for 23 h under inert conditions (helium), followed by the addition of 2 mL of 8 N HCl. After the solution had been filtered through Whatman No. 40 filter paper, 500 μL of the hydrolysate was applied to a previously activated Sep-Pak C18 cartridge (Millipore). Furosine was eluted with 3 mL of 3 N HCl, and 50 μL was used for injection. Analysis of 2-furoylmethyllysine was carried out via an ion-pair RP-HPLC method using a C8 (Alltech furosine-dedicated; Alltech, Nicolasville, KY) column [250 mm × 4.6 mm (inside diameter)] and a variable-wavelength detector at 280 nm (SM 4000, LDC Analytical, Salem, NH). Operating conditions were as follows: column temperature, 35 °C; flow rate, 1.2 mL/min; solvent A, 0.4% HPLC-grade acetic acid in double-distilled water; solvent B, 0.3% KCl in solvent A (*30*). Calibration was performed by using known concentrations of a commercial pure standard of furosine, from 0.52 to 5.2 mg/L (Neosystem Laboratories, Strasbourg, France).

The content of free primary amino groups was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS, Sigma) (*31*). The absorbance, measured at 420 nm in a Beckman DU 70 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA), was transformed into micromoles of primary amino groups of leucine per milliliter using a calibration curve within the range 0.03–0.50 μM. Absorbance at 420 nm of an aqueous solution of β-LG/GOS incubated mixtures was measured at room temperature, as an index of the brown polymers formed in more advanced stage intermediate products of nonenzymatic browning (*32*).

2.4. Analysis of the GOS Fraction. Oligosaccharides were analyzed by HPAEC-PAD using a Dionex DX-300 chromatograph containing a gradient pump and an eluent degas module. Separation of carbohydrates was carried out on a CarboPac PA 100 guard column (4 mm × 50 mm) and a CarboPac PA-100 anion-exchange column (4 mm × 250 mm), and 20 μL was injected. Carbohydrates were eluted by means of a gradient prepared with 100 mM NaOH (eluent A) and 1 M sodium acetate in 100 mM NaOH (eluent B). Eluent A was kept constant for 10 min at 100%, then changed to 90% at 50 min, and decreased to 60% at 55 min. From 60 to 63 min, 100% eluent B was kept constant. The flow rate was 1.0 mL/min, and carbohydrates were detected by pulsed amperometric detection (PAD) (Dionex Corp., Sunnyvale, CA) with a gold working electrode and a hydrogen reference electrode using triple-pulsed amperometry with the following potentials and durations: $E_1 = +0.15$ V ($t_1 = 400$ ms), $E_2 = +0.75$ V ($t_2 = 200$ ms), and $E_3 = -0.8$ V ($t_3 = 200$ ms). The sampling time (t_s) was 20 ms.

The unconjugated GOS fraction remaining after the storage of β-LG/GOS mixtures was separated from the protein fraction by ultrafiltration through a centrifugal filter (Centricon YM-3, 3000 nominal MW cutoff, Millipore Corp.) and, then, analyzed by HPAEC-PAD. Peaks which showed the greatest decrease following the incubation were manually collected after PAD from 20 injections of the original sample (Vivinal-GOS, 1 mg/mL), and salts were removed using a cation-exchange column (Dowex 50 4x, 20–50 mesh, Fluka). The resin was first activated using 1 M NaOH followed by 1 M HCl and washed with Milli-Q water to remove any excess acid. Carbohydrates were eluted from the column using 3 volumes of Milli-Q water, concentrated in a vacuum centrifuge (SpeedVac Concentrator A160, Savant Instruments, Farmingdale, NY), and analyzed by flow injection analysis mass spectrometry (FIA-MS) at a concentration of 3 mM in aqueous solution. The FIA-MS experiments were carried out on a Finnigan Surveyor (Thermo Electron, Alcobendas, Spain) pump coupled to a Finnigan LCQ Deca (Thermo Electron) ion trap mass spectrometer using an electrospray ionization (ESI) interface. Five microliters was injected into the LC system (without column) and carried through in a 0.2% formic acid/methanol (50:50, v:v) eluent at a flow rate of 100 μL/min. Mass spectra were acquired in positive mode, scanning from m/z 100 to 1200. The working conditions for the ESI source were as follows: spray voltage, 4.5 kV; heated capillary temperature and voltage, 275 °C and 20 V, respectively; sheath gas (N_2), 0.6 L/min; and auxiliary gas (N_2), 6 L/min.

2.5. Analysis of the β-LG Glycated Forms. After incubation, the powders were dissolved in distilled water and free (unconjugated) GOS were removed by ultrafiltration as explained above. After removal of GOS, protein samples were reconstituted in distilled water at 2 mg/mL and analyzed by SDS–PAGE, IEF, RP-HPLC–UV, and MALDI-ToF-MS.

For SDS–PAGE analysis, 22.5 μL of the stored samples was added to 10 μL of distilled water, 12.5 μL of 4× NuPAGE LDS sample buffer

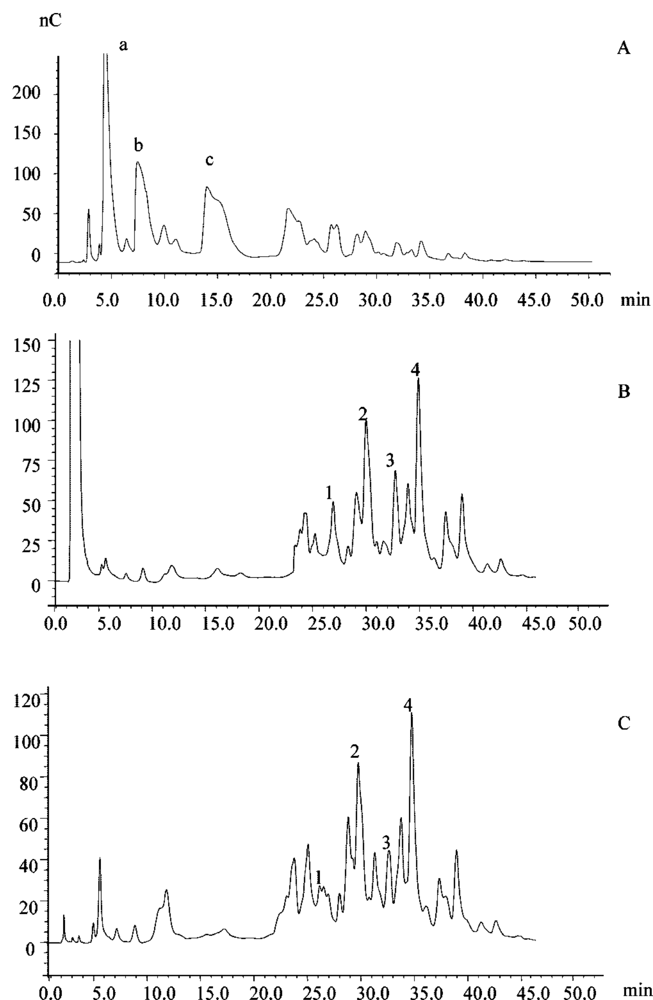

Figure 1. HPAEC-PAD profiles of (**A**) GOS, (**B**) GOS previously treated with activated charcoal, and (**C**) unconjugated GOS after glycation of β-LG (23 days, 40 °C): (a) glucose and galactose, (b) lactose, (c) unknown disaccharides, and (1–4) GOS which exhibited the most noticeable decrease after β-LG glycation.

(Invitrogen), and 5 μL of 0.5 M dithiothreitol (DTT, Sigma) and the mixture heated at 70 °C for 10 min. Samples were loaded (20 μL) on a 12% polyacrylamide–NuPAGE Novex Bis-Tris precast gel, and a continuous MES SDS running buffer was used. Gels were run for 40 min at 120 mA/gel and 200 V and stained using either the Colloidal Blue Staining Kit (Invitrogen) or the Periodic Acid Schiff (PAS) Staining protocol described by Zacharius et al. (*33*).

For isoelectric focusing (IEF) analysis, 20 μL of the stored samples was added to 20 μL of 2× Novex IEF pH 3–7 Sample Buffer (Invitrogen). Samples were loaded (20 μL) on Novex pH range 3–7 gels (Invitrogen) and run according to the protocol from the manufacturer. Gels were stained using the Colloidal Blue Staining Kit (Invitrogen).

RP-HPLC–UV followed the method described by Casal et al. (*34*). RP-HPLC separations were carried out with a C4 Jupiter column [300 Å, 250 mm × 4.6 mm (inside diameter), 5 μm particle size; Phenomenex, Cheshire, U.K.] at room temperature and with a flow rate of 1 mL/min, using 0.1% (v:v) trifluoroacetic acid (Merck) in double-distilled water as solvent A and 0.085% (v:v) trifluoroacetic acid in double-distilled water and acetonitrile (Scharlau Chemie, Barcelona, Spain) (1:9, v:v) as solvent B. The elution was performed as follows: from 0 to 60.0 min, a linear gradient by increasing the concentration of solvent B from 20 to 50%; from 60.0 to 65.0 min, 50% solvent B in an isocratic mode. Absorbance was recorded at 214 nm using a Beckman 166 UV detector (Beckman Instruments).

MALDI-ToF analyses were performed using a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped

Table 1. Formation of Furosine during Incubation of β-LG with GOS in Proportions of 1:1 and 1:3 (w:w) at 40 °C (a_w = 0.44) over 23 Days

storage time (days)	furosine content (mg/100 mg of β-LG) 1:1 (w:w) β-LG:GOS	1:3 (w:w) β-LG:GOS
0	not detected	not detected
5	1.83 (0.00)[a]	1.82 (0.03)[a]
12	2.21 (0.03)[a]	2.38 (0.04)[a]
16	2.43 (0.09)[a]	2.75 (0.02)[a]
20	2.33 (0.04)[a]	2.54 (0.03)[a]
23	2.39 (0.08)[a]	2.77 (0.09)[a]

[a] Standard deviation.

Table 2. Relative Abundance of GOS Peaks Mainly Involved in the Glycation of β-LG

storage time (days)	relative abundance (%) peak 1	peak 2	peak 3	peak 4
0	7.91	13.02	7.08	14.99
16	6.15	11.62	5.15	13.86
20	6.04	11.46	5.07	13.29
23	5.98	11.37	4.95	13.53

with a pulsed nitrogen laser (λ = 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 grid voltage of 93%, an ion guide wire voltage of 0.05%, and a delay time of 350 ns in the linear positive ion mode. Mass spectra were obtained over the m/z range of 10–35 ku. Myoglobin (horse heart) and carbonic anhydrase were used for external calibration, and sinapinic acid [10 mg/mL in 0.3% trifluoroacetic acid and acetonitrile (70:30, v:v)] was used as the matrix. Samples were mixed with the matrix at a ratio of approximately 1:15, and finally, 1 μL of this solution was spotted onto a flat stainless-steel sample plate and dried in air.

2.6. In Vitro Gastrointestinal Digestion. The native and glycated β-LG (after incubation with GOS at 40 °C for 16 days) were digested in vitro by following the simplified procedure described by Moreno et al. (*35*). Protein (3 mg) was dissolved in 1 mL of simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5) (*36*). The pH was adjusted to 2.5 with 1 M HCl if necessary. A solution of 0.32% (w:v) porcine pepsin (EC 3.4.23.1) in SGF (pH 2.5) (Sigma, activity of 3300 units/mg of protein) was added at an approximately physiological ratio of enzyme to substrate (1:20, w:w). The digestion was performed at 37 °C for 2 h. For the intestinal digestion step, the pH was increased to 7.5 with 40 mM NH_4CO_3 (Panreac) dropwise, and the following was added to adjust the pH to 6.5 and simulate a duodenal environment: (i) a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate (Sigma) and glycodeoxycholic acid (Sigma), (ii) 1 M $CaCl_2$ (Panreac), and (iii) 0.25 M Bis-Tris (pH 6.5) (Sigma). Finally, solutions of porcine trypsin (EC 3.4.21.4; 0.05%, w:v, Sigma, type IX-S, activity of 14 300 units/mg of protein) and bovine α-chymotrypsin (EC 3.4.21.1; 0.1%, w:v, Sigma, type I-S, activity of 62 units/mg of protein) in water were prepared and added at approximately physiological β-LG:trypsin: chymotrypsin ratios [1:(1/400):(1/100) (w:w:w)]. Simulated intestinal digestion of β-LG was carried out at 37 °C, and aliquots were taken at 15, 30, 60, and 120 min for RP-HPLC–UV and SDS–PAGE analysis. After protein hydrolysis, digestive enzymes were inactivated by adding a solution of Bowman-Birk trypsin–chymotrypsin inhibitor from soybean (Sigma) at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix. Digestions were performed without any derivatization of the sulfhydryl groups of cysteine residues in order to remain as close as possible to physiological conditions.

Digested samples were diluted five times with solvent A and applied (100 μL) to a Phenomenex Jupiter Proteo 90 Å pore size, 4 μm particle size [250 mm × 4.6 mm (inside diameter)] column attached to a Beckman HPLC system. Samples were eluted using 0.1% (v/v) trifluoroacetic acid in double-distilled water as solvent

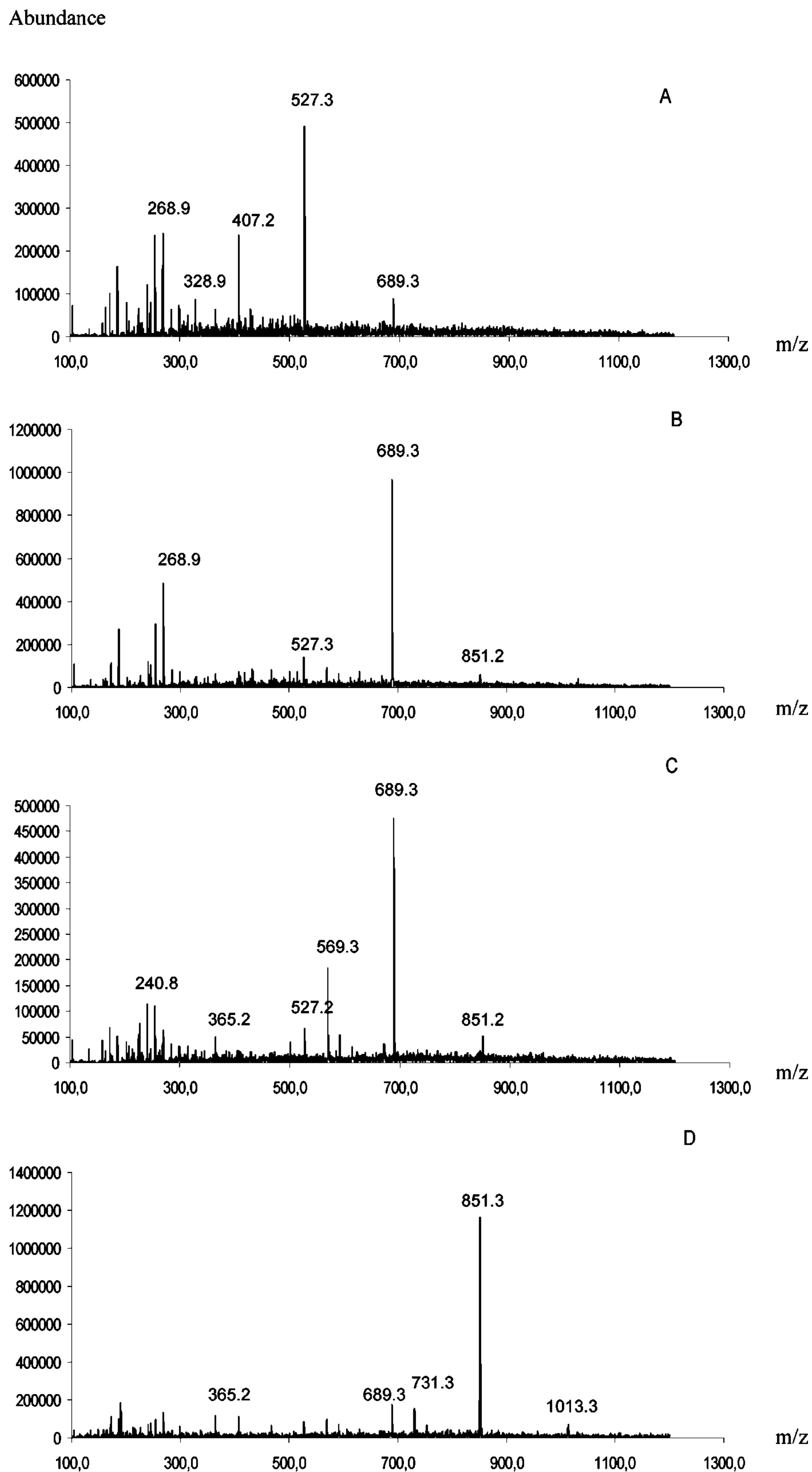

Figure 2. ESI mass spectra of oligosaccharides collected from HPAEC-PAD: (**A**) peak 1, trisaccharide; (**B**) peak 2, tetrasaccharide; (**C**) peak 3, tetrasaccharide; and (**D**) peak 4, pentasaccharide.

A and 0.085% (v/v) trifluoroacetic acid in double-distilled water and acetonitrile (1:9, v:v) as solvent B following the method described by Moreno et al. (*37*). Samples taken at different stages of the digestion were also analysed by SDS–PAGE as previously mentioned.

3. RESULTS AND DISCUSSION

3.1. Preparation of Galactooligosaccharides (GOS). The carbohydrate profile of Vivinal-GOS obtained by HPAEC-PAD is shown in **Figure 1A**. Glucose, galactose, and lactose were

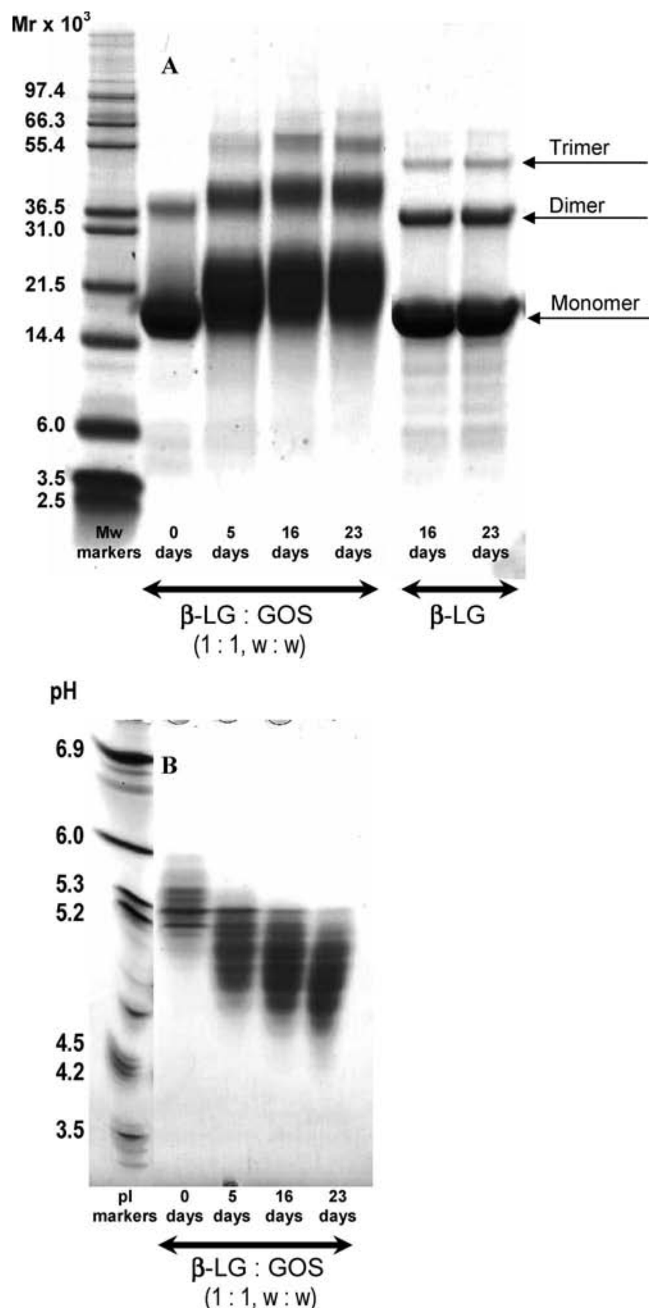

Figure 3. (**A**) SDS–PAGE analyses under reducing conditions of bovine β-LG incubated at 40 °C over 23 days in the presence or absence of GOS. (**B**) Isoelectric focusing of bovine β-LG incubated with GOS at 40 °C over 23 days.

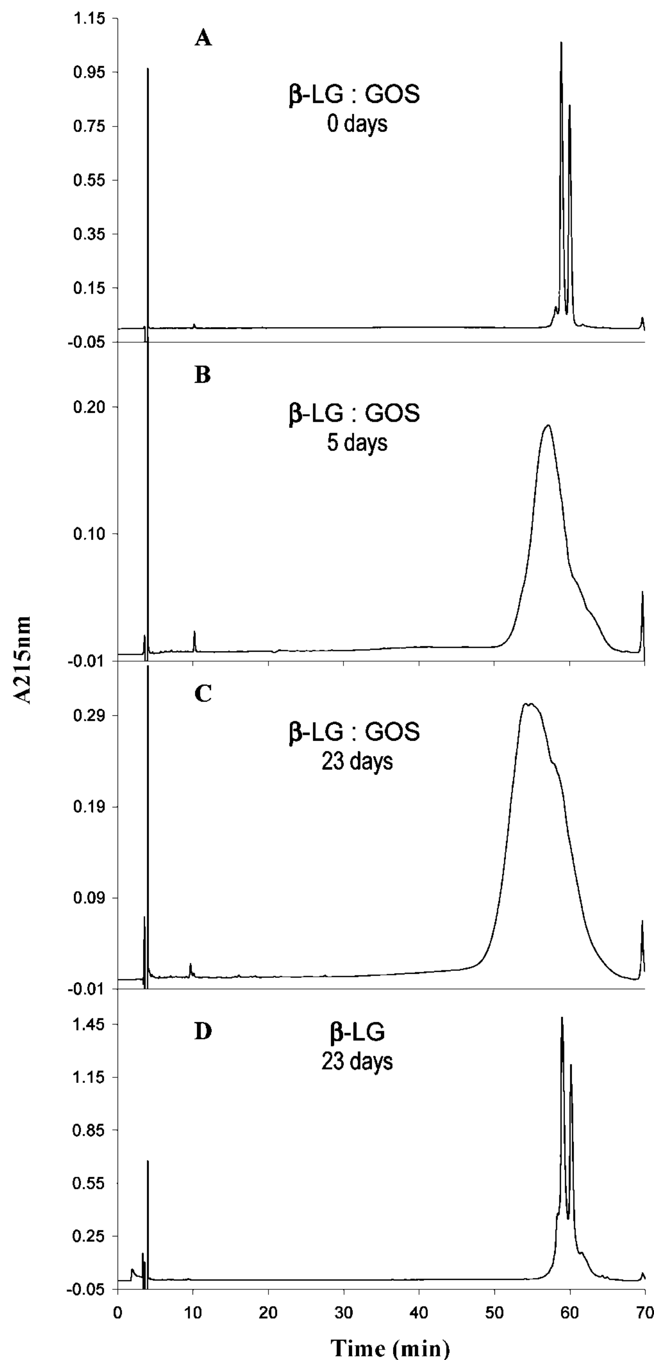

Figure 4. RP-HPLC patterns of nonreduced samples corresponding to bovine β-LG incubated with GOS at 40 °C for (**A**) 0, (**B**) 5, and (**C**) 23 days and without GOS at 40 °C for (**D**) 23 days.

identified via comparison of their retention times with those of commercial standards. Considering that these mono- and disaccharides are digested in the gastrointestinal tract (*38*), these carbohydrates were removed from the original sample using activated charcoal as described in Materials and Methods. **Figure 1B** shows the HPAEC-PAD profile of this sample. Those peaks corresponding to galactose and glucose were undetectable, whereas only traces of lactose remained in the sample. Moreover, the magnitude of a broad peak eluting after lactose also decreased after charcoal treatment which probably indicated they were two unknown disaccharides. This sample free from mono- and disaccharides was used for glycation assays.

3.2. Glycation of β-Lactoglobulin (β-LG). *3.2.1. Determination of the Extent of Glycation.* Furosine, formed upon acid hydrolysis of glycated lysine residues, was shown to be a useful indicator in assessing the extent of glycation of milk proteins (*26, 29, 39*). For both β-LG:GOS weight ratios that were tested, a progressive increase in the level of furosine was observed after incubation for 16 days, whereas furosine levels remained fairly constant between days 16 and 23 of incubation (**Table 1**). This suggested that after storage for up to 16 days the formation of the colorless Amadori compounds prevailed over their degradation. In good agreement, browning at 420 nm increased very moderately as storage proceeded, becoming more pronounced after the 16th day of storage (data not shown). Additionally, no substantial differences in furosine contents were found between β-LG:

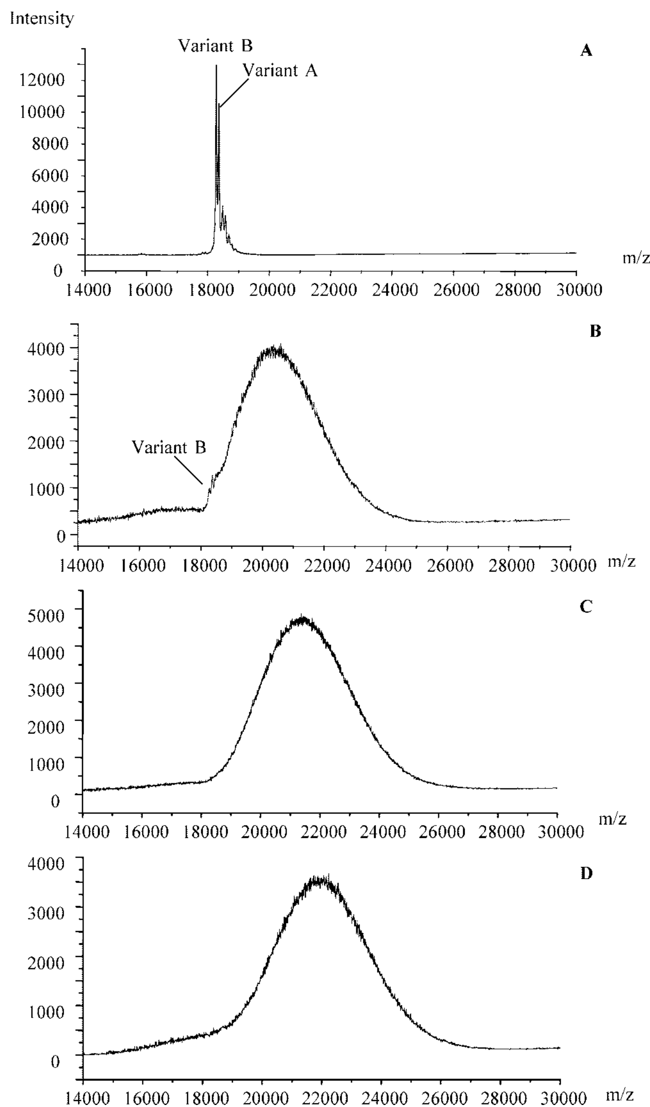

Figure 5. MALDI-ToF-MS spectra of bovine β-LG incubated with GOS at 40 °C for (**A**) 0, (**B**) 5, (**C**) 12, and (**D**) 23 days.

GOS weight ratios of 1:1 and 1:3. Thus, the 1:1 weight ratio was selected for further experiments.

Furthermore, the greatest decrease in the level of free primary amino acid groups took place during the first 12 days of storage, with only 20% of primary amino groups remaining unblocked. Additionally, no changes in the level of free amino groups were observed in the control β-LG stored at 40 °C without GOS over 23 days.

3.2.2. Identification of the Conjugated GOS Fraction. **Figure 1C** illustrates the HPAEC-PAD profile of unreacted GOS after incubation for 23 days with β-LG. The GOS fraction showed mainly four peaks to exhibit a gradual and marked decrease with storage, although minor changes in other carbohydrates were also observed. Peaks could not be accurately quantified due to the lack of standards; however, relative abundances to the total carbohydrates were determined (**Table 2**). Peak 3 showed the greatest decrease (30%), followed by peak 1 (24%), whereas peaks 2 and 4 presented diminutions of only 13 and 10%, respectively. These four oligosaccharides could have mostly participated in the glycation of β-LG. Analyses of these carbohydrates by ESI-MS revealed the existence of one trisaccharide (peak 1), two tetrasaccharides (peaks 2 and 3), and one pentasaccharide (peak 4), as shown in **Figure 2**.

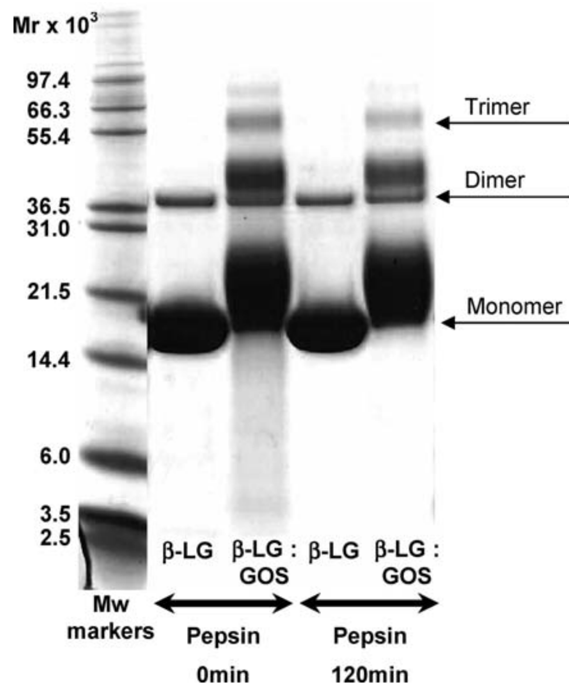

Figure 6. SDS–PAGE analysis under reducing conditions of bovine native and glycated β-LG subjected to pepsin digestion over 120 min.

3.2.3. Characterization of Glycated β-LG. As incubation with GOS progressed, β-LG bands exhibited a lower electrophoretic mobility on SDS–PAGE under reducing conditions, yielding an M_r value noticeably higher than that of β-LG incubated in the absence of GOS (**Figure 3A**). This suggested that the molecular weight of β-LG was increased due to the glycation with GOS. Unglycated β-LG (not incubated with GOS) exhibited mainly the monomeric form ($M_r \sim 18000$) following reduction with DTT, although the band corresponding to the dimeric form ($M_r \sim 36000$) was also detected in a much lower proportion. Upon storage, β-LG incubated in the presence and absence of GOS showed an additional minor band which might correspond to the trimeric form ($M_r \sim 54000$). Similar modifications of SDS–PAGE patterns of heated β-LG were described previously by other authors (*40, 41*) and could be explained by an incomplete reduction of the disulfide bonds (*23*) or by the prevalence of a covalent polymerization of the β-LG monomers different from the disulfide bridge formation (*40*). In addition to the monomer band, the dimer and trimer bands also exhibited a lower mobility, indicating glycation of β-LG (**Figure 3A**).

More evidence of glycation of β-LG was obtained by IEF (**Figure 3B**). Unglycated β-LG exhibited mainly two bands possessing isoelectric points of 5.2 and 5.3 corresponding to the genetic variants A and B, respectively. The blocking of Lys with carbohydrates would be expected to decrease the pK_a of the amino group, leading to a noticeable shift of the isoelectric point of glycated β-LG toward a more acidic pH (*22, 25, 42*). As the incubation time increased, the shift of isoelectric point was greater. In all cases, glycated β-LG showed the presence of a greater number of electrophoretic bands. Such charge

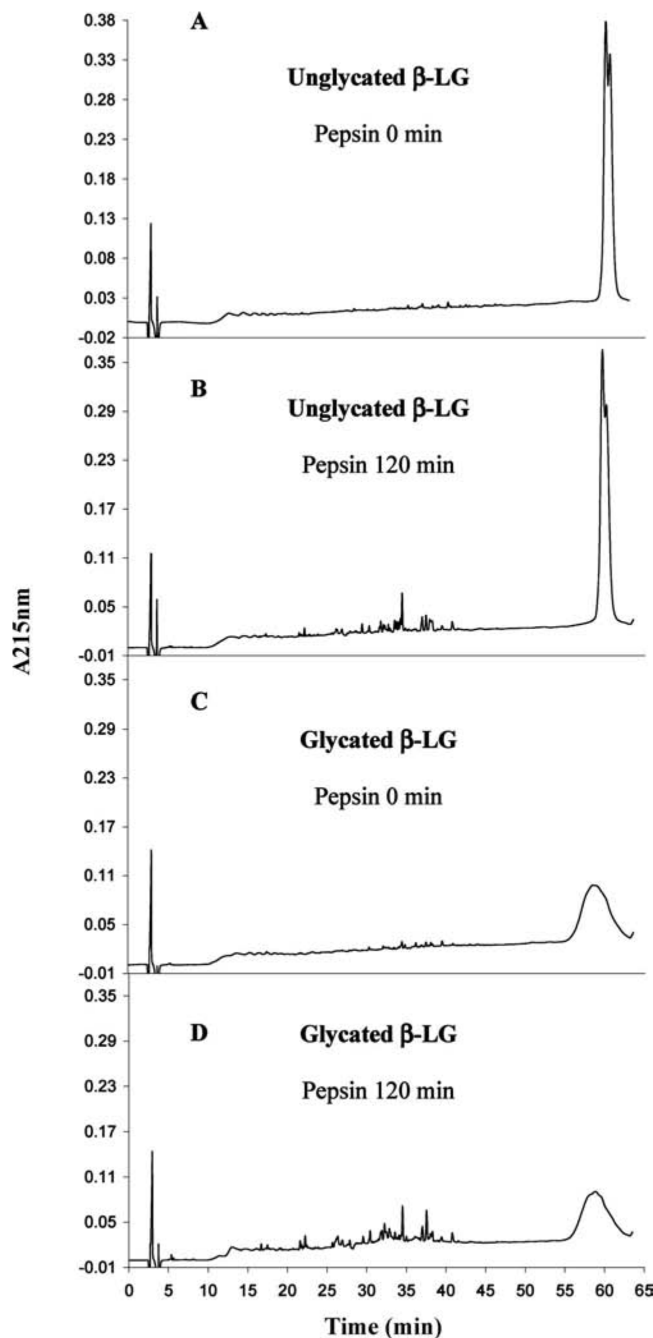

Figure 7. RP-HPLC patterns of nonreduced samples corresponding to bovine (**A** and **B**) native and (**C** and **D**) glycated pepsin-digested β-LG: digestion for (**A** and **C**) 0 and (**B** and **D**) 120 min.

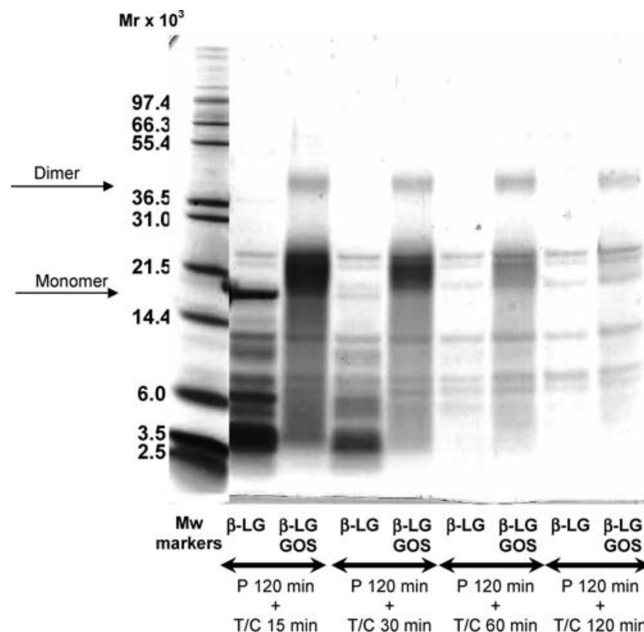

Figure 8. SDS–PAGE analysis under reducing conditions of bovine native and glycated β-LG subjected to pepsin digestion for 120 min followed by trypsin/chymotrypsin digestion over 120 min using the Colloidal Blue Staining Kit. P denotes pepsin digestion and T/C trypsin and chymotrypsin digestion.

heterogeneity is indicative of the formation of a wide range of glycated species of β-LG.

The chromatographic profiles of unglycated and glycated β-LG obtained by RP-HPLC are shown in **Figure 4**. Native β-LG (**Figure 4A**) presented two well-defined peaks corresponding to the A and B genetic variants. The chromatograms corresponding to the β-LG incubated with GOS indicated the appearance of a broad, less retained peak whose area increased with the incubation time (**Figure 4B**,**C**). The shorter retention time of this peak can be attributed to the incorporation of GOS residues into the peptide chain that leads to an increase in β-LG hydrophilicity. A similar behavior was previously reported with the lactosylation of caseinomacropeptide (*29*). Moreover, the width of the peak could be indicative of the great heterogeneity of glycated β-LG forms. β-LG incubated at 40 °C for 23 days without GOS and native β-LG exhibited similar profiles (**Figure 4A**,**D**), indicating that the changes in the RP-HPLC pattern of the incubated β-LG/GOS mixtures were derived from the glycation of the protein and not from some protein conformational changes. Morgan et al. (*23*) demonstrated that dry glycation of β-LG did not significantly alter its nativelike behavior.

MALDI-ToF-MS analyses were performed to confirm the presence of covalent β-LG–GOS conjugates (**Figure 5**). The molecular masses of native β-LG were 18278 and 18363 Da, in good agreement with the expected masses of variants B and A, respectively (*43*). The minor mass of 18688 Da corresponded to the lactosylated form of variant A, whereas other minor masses such as 18484, 18573, and 18778 arose from matrix adducts (sinapinic acid, **Figure 5A**). Mass spectra of β-LG incubated with GOS were characterized by a broad Gaussian peak shape without good resolution due to the great heterogeneity of the glycated forms of β-LG (**Figure 5B–D**). This heterogeneity is derived from the presence of two β-LG variants, each one with 19 sites (15 Lys, 3 Arg, and the NH_2-terminal Leu residues) susceptible to glycation, as well as the presence of GOS with different degrees of polymerization (from 3 to 9) (*27*). The Gaussian distribution of masses found by MALDI-ToF-MS has been previously reported by other authors following nonenzymatic glycation of β-LG (*44, 45*). The Gaussian peaks showed a maximum of intensity at ~20600 Da (storage for 5 days), ~21367 Da (storage for 12 days), and ~22238 Da (storage for 23 days), leading to an increase of ~12–21% in the molecular mass of the β-LG. Furthermore, while some traces of the molecular masses corresponding to the unmodified β-LG could be detected after incubation for 5 days (**Figure 5B**), from the 12th day of storage no occurrence of the mass values of unglycated β-LG was detected (**Figure 5C**,**D**).

3.3. Gastrointestinal Digestion of Native and Glycated β-LG. Native and glycated (incubated with GOS at 40 °C for 16 days) β-LG were found to be very resistant to pepsinolysis, with prominent bands evident on SDS–PAGE after digestion

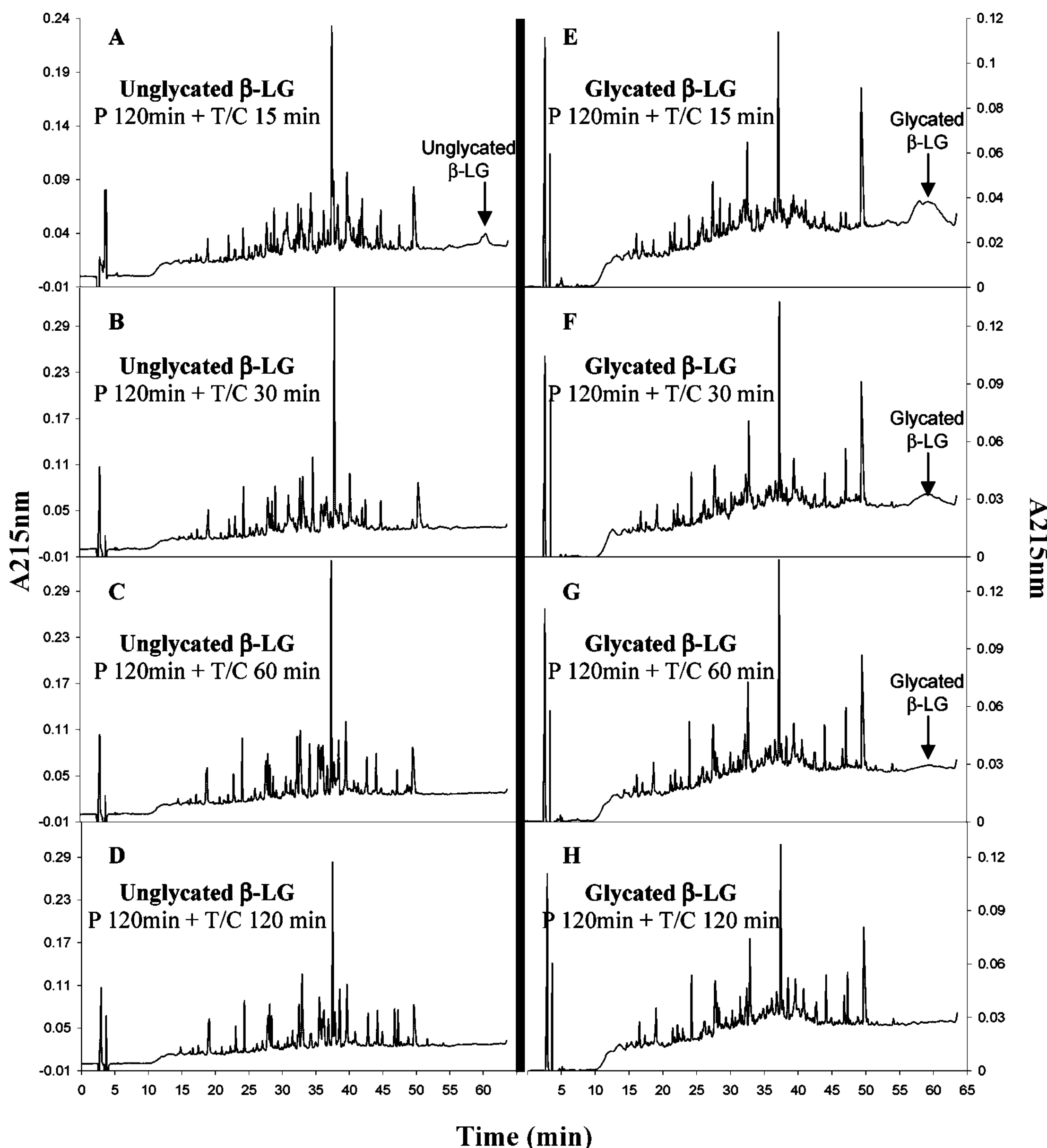

Figure 9. RP-HPLC patterns of nonreduced samples corresponding to bovine (**A**–**D**) native and (**E**–**H**) glycated β-LG subjected to pepsin digestion for 120 min followed by trypsin/chymotrypsin digestion for 120 min. P denotes pepsin digestion and T/C trypsin and chymotrypsin digestion.

for 2 h (**Figure 6**). Native bovine β-LG was shown to possess a remarkable stability in the gastric environment, resisting denaturation and hydrolysis by pepsin at low pH (*46, 47*). This means that β-LG would very likely remain intact after passing through the stomach to reach the upper portion of the small intestine (*48, 49*). The resistance of native β-LG to peptic digestibility has been explained by the fact that its peptic cleavage sites (hydrophobic or aromatic amino acid side chains) are buried well inside the characteristic lipocalin β-barrel, forming a strong hydrophobic core and preventing hydrolysis (*50, 51*).

RP-HPLC chromatograms showed a very limited peptic activity on both native and glycated β-LG, yielding the same pattern of minor peptides with retention times between 20 and 40 min (**Figure 7**). In good agreement with this, Morgan et al. (*23, 52*) did not find differences between native and dry glycated β-LG in terms of its susceptibility to pepsin, suggesting that the conformational state of β-LG was not significantly affected by the glycation process under dry-controlled conditions.

After gastric (pepsin) digestion for 2 h, the pH was increased and trypsin and chymotrypsin were added with bile salts to simulate a duodenal environment. According to SDS–PAGE analysis, the intact unglycated β-LG remaining after pepsinolysis was rapidly broken down during simulated duodenal digestion, and at 30 min, all trace of intact protein had disappeared (**Figure 8**). Nevertheless, a faint and diffuse band corresponding to the glycated β-LG could still be visualized at 60 min of trypsin/chymotrypsin digestion (**Figure 8**). HPLC analysis, in addition to confirming these observations, revealed the presence of a complex mixture of peptides eluting between 15 and 50 min in both glycated and unglycated β-LG (**Figure 9**). After simulated intestinal digestion for 2 h, all traces of intact unglycated or glycated β-LG had disappeared (**Figure 9D**,**H**). This fact is of particular importance because large amounts of intact proteins reaching the colon could be fermented by colonic microflora, leading to the production of a range of toxic metabolites such as certain amines, ammonia, and branched chain fatty acids (*21, 53*).

Due to the complexity of the peptide profile and to the incomplete chromatographic separation between nonglycated and glycated peptides, similar digestion patterns were apparently obtained for unglycated and glycated β-LG. A similar behavior was previously described for the tryptic hydrolysate of lactosylated and nonlactosylated β-LG (*43*). However, the PAS staining on SDS–PAGE gels revealed the glycation of the intact β-LG, as well as the presence of glycated peptides with lower molecular masses as digestion with trypsin and chymotrypsin progressed (data not shown). Furthermore, the yield of digestion products of glycated β-LG was lower than that observed in unglycated protein (**Figure 9**). It is very likely that the reactivity of trypsin against glycated Lys and Arg residues is largely reduced (*54, 55*). Lapolla et al. (*56, 57*) also showed a yield of digestion products of glycated human and bovine serum albumin lower than those observed in the unglycated proteins which was attributed to a lower proclivity of the glycated proteins to enzymatic digestion.

3.4. Conclusions. The results presented in this work show that β-LG can be efficiently glycated with prebiotic carbohydrates such as GOS with degrees of polymerization from 3 to 5 without substantial conformational changes. Moreover, β-LG–GOS conjugates could form stable glycated peptides surviving the in vitro gastrointestinal digestion. Considering that the Amadori compounds can reach the hind gut where they are degraded by the microflora, the conjugation between a prebiotic carbohydrate and the protein could potentially allow the carbohydrate to reach the distal parts of the colon where they can be fermented distally. These findings could open up new areas of research on prebiotics. Nevertheless, further studies will be carried out to address the potential prebiotic activity of these glycated peptides, as well as their bioavailability, absorption, and metabolism once they reach the intestinal epithelium.

ACKNOWLEDGMENT

We thank Dr. G. Klarenbeek and Dr. J. de Slegte from Borculo Domo for providing us with Vivinal-GOS. We also gratefully acknowledge Dr. Rosa Lebrón for her help in acquiring the ESI- and MALDI-MS spectral data.

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Received for review April 17, 2007. Revised manuscript received June 27, 2007. Accepted July 13, 2007. This work was supported by Projects 200670M027, ALIBIRD S-0505/AGR/000153 (financed by the Comunidad de Madrid), and AGL-2004-07227-C02-02 [supported by the Comisión Interministerial de Ciencia y Tecnología (CICYT)]. M.L.S. and F.J.M. are recipients of an I3P-CSIC and a Ramón & Cajal contract, respectively. M.C.-M. thanks the CSIC for an I3P Ph.D. grant.

JF071111L