**Effect of glycation on the gastrointestinal digestibility and
immunoreactivity of bovine β -lactoglobulin**

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ABSTRACT

Immunoreactivity of bovine β -lactoglobulin (β -Lg) hydrolysates obtained after a simulated gastrointestinal digestion and previously glycosylated via Maillard reaction with galactose, tagatose, and dextran of 10 or 20 kDa has been determined, with a view to study the effect of glycation and aggregation degree of β -Lg on its residual immunoreactivity. High levels of glycation impaired the β -Lg proteolysis and, consequently, increased the IgG- and IgE- reactivities of hydrolysates, regardless of the used carbohydrate. The protein aggregation during the advanced stages of Maillard reaction had a masking effect on β -Lg epitopes, counteracting the negative effect of the lower digestibility of glycosylated protein on its allergenicity. Finally, the use of polysaccharides as glycation agents did not contribute to enhancement of the masking effect of the attached carbohydrate on β -Lg epitopes. These findings stress the importance of evaluating the impact of glycation on protein gastrointestinal digestibility prior to investigation of the allergenicity of protein Maillard complexes.

1. Introduction

Beta-Lactoglobulin (β -Lg), the major whey protein, is a valuable ingredient in **food manufacturing** due to its high content **of** essential amino acids and versatility in terms of functional (emulsifying, foaming and gelling) properties (de Wit, 1998; Foegeding, Davis, Doucet, & McGuffey, 2002). In spite of this, its industrial application can be limited due to its high allergenicity, this protein being, together with caseins, one of the major allergens described in bovine milk (Wal, 2001). Thus, with the purpose of increasing its degree of applicability in the food industry, the reduction of its allergenicity has been **tested** over **recent** years by **use** of different methods such as chemical modification of the protein (Chobert, Briand, Grinberg, & Haertlé, 1995), proteolysis (Asselin, Hébert, & Amiot, 2006; Ehn, Allmere, Telemo, Bengtsson, & Ekstrand, 2005), and physical treatments **such** as heating (Guo, Fox, Flynn, & Kindstedt, 1995; Iametti et al., 2002) or high pressure (Chicón, Belloque, Alonso, & López-Fandiño, 2008; Peñas, Préstamo, Baeza, Martínez-Molero, & Gómez, 2006; Stapelfeldt, Petersen, Kristiansen, Qvist, & Skibsted, 1996).

On the other hand, considerable attention has been paid to the deliberately promoted Maillard reaction (MR) to improve protein functionality and, thus, obtain new modified proteins with high technological interest (Oliver, Melton & Stanley, 2006). Glycation of food proteins could also affect their allergenic activity at a structural level. Contradictory results have been reported so far about the effect of glycation on the IgE-binding ability of undigested food allergens, indicating that this effect might be allergen- and carbohydrate-dependent (Tahery-Kafrani et al., 2009). Whereas some authors have suggested that the interaction with sugars could mask the allergen epitopes,

reducing its allergenicity (Arita, Babiker, Azakami, & Kato, 2001; Bu, Lu, Zheng, & Luo, 2009; Gruber, Vieths, Wangorsch, Nerkamp, & Hofmann, 2004; Hattori et al., 2004; Kato, 2002; Nakamura et al., 2008; Tahery-Kafrani et al., 2009), other studies have pointed out that glycation of allergens increases their recognition by IgE antibodies (Beyer et al., 2001; Maleki, Chung, Champagne, & Raufman, 2000; Nakamura, Watanabe, Ojiva, Ahn, & Saeki, 2005; Pastorello, Pravettoni, Calamari, Banfi, & Robino, 2002; Simonato et al., 2001). One possible explanation for these dissimilar results might be that, given the complexity of the MR, the nature and extent of Maillard-induced changes in protein conformation also depend on the direct effects of temperature, water activity and duration of the thermal processing (Mills, Sancho, Moreno, & Kostyra, 2006).

Because most food allergens sensitize an individual via the gastrointestinal tract, digestibility and gut permeability are key factors to consider since they may affect the allergenic potential (Lehrer, Horner, & Reese 1996; Moreno, 2007). In fact, it is thought that one of the main factors contributing to β -Lg allergenicity is its exceptional resistance to digestive enzymes (Dalgalarondo, Dufour, Chobert, Bertrand-Harb, & Haertlé, 1995; Reddy, Kella, & Kinsella, 1988). As a result of the effect of glycation on the conformation and physico-chemical properties of proteins, it is expected that their susceptibility to proteolysis and subsequent intestinal absorption is altered. Nevertheless, none of the above reports studied the effect of glycation on the protein gastrointestinal digestibility and the subsequent IgG and IgE-binding abilities of the hydrolysates. According to several authors, glycated proteins, including human and bovine serum albumin (glycated with glucose) and β -Lg (glycated

with galactooligosaccharides), have been described to be more resistant to trypsin digestion, probably due to a lower reactivity of trypsin against glycated lysine and arginine residues (Lapolla et al., 2001, 2004; Moreno, Quintanilla-López, Lebrón-Aguilar, Olano, & Sanz, 2008; Sanz, Corzo-Martínez, Rastall, Olano, & Moreno, 2007). **In contrast**, glycation has also been reported to increase protein digestibility due to conformational changes produced during the advanced stages of the MR (Bouhallab, Morgan, Henry, Mollé, & Léonil, 1999; Yeboah et al., 2004). In this context, the use of pyridoxamine (PM), which is a potent inhibitor of the advanced stages of the MR (Booth, Khalifah, & Hudson, 1996; Corzo-Martinez, Moreno, Olano, & Villamiel, 2010; Voziyan, Metz, Baynes, & Hudson, 2002), could throw light on the effect of the MR evolution on digestibility and allergenic properties of proteins.

In the present study, bovine β -Lg was glycated with carbohydrates such as galactose (Gal), tagatose (Tag), and dextran of 10 (DX₁₀) or 20 kDa (DX₂₀) and then digested under simulated gastrointestinal conditions to evaluate the immunoreactivity of these glycoconjugates.

2. Materials and methods

2.1. Preparation and purification of β -Lg glycoconjugates

Carbohydrates (Gal, Tag, DX₁₀, DX₂₀) and β -Lg (mixture of A and B variants) (all from Sigma-Aldrich, St. Louis, MO, **USA**) were dissolved in 0.1 M sodium phosphate buffer pH 7.0 (Merck, Darmstadt, Germany) at the following weight ratios: β -Lg:Gal / Tag at 1:1, and β -Lg:DX₁₀ / DX₂₀ at 1:2. DX₁₀ and DX₂₀ were previously dialyzed versus deionised water using a Spectra/Por[®] 3 Biotech

dialysis membrane with a 3.5 kDa molecular weight cut-off (Spectrum Europe, Breda, The Netherlands) to remove low molecular weight oligosaccharides. After freeze-drying, the protein-carbohydrate solutions were incubated under vacuum in a desiccator equilibrated at a_w of 0.44 with a saturated K_2CO_3 solution (Merck) at 40 and 50 °C for 6 days for β -Lg:Gal / Tag conjugates (Corzo-Martinez, Moreno, Olano, & Villamiel, 2008), and at 60 °C for 36 and 60 h for β -Lg:DX₁₀ and β -Lg:DX₂₀ conjugates, respectively (Jiménez-Castaño, Villamiel, & López-Fandiño, 2007). With the purpose of studying the effect of pyridoxamine (PM) (Sigma-Aldrich) as inhibitor of the advanced stages of the MR, storage of β -Lg:Gal / Tag conjugates was also carried out in the presence of such inhibitor at a weight ratio of β -Lg:PM of 1:0.24 (Corzo-Martinez et al., 2010). In addition, the corresponding controls of β -Lg incubated without reducing sugars were prepared under the same incubation conditions described above. All incubations were performed in duplicate.

After incubation, the products were reconstituted in distilled water to a protein concentration of 1 mg mL⁻¹. To remove free carbohydrates and PM in excess, 2 mL-aliquots were ultrafiltered through hydrophilic 3 kDa (for conjugates of Gal and Tag) or 30 kDa (for conjugates of DX₁₀ and DX₂₀) cut-off membranes (Centricon® YM-3 and YM-30, Millipore Corp., Bedford, MA) by centrifugation at 1548 $\times g$ for 2 h. Finally, the retentates were reconstituted in distilled water to a protein concentration of 1 mg mL⁻¹ and kept at -20 °C for further analysis.

2.2. Characterization of β -Lg glycoconjugates

2.2.1. β -Lg:Gal / Tag conjugates

For determination of the extent of glycation, MALDI-TOF-MS analyses were performed on a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA, USA) 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 laser desorption of both control heated and glycated β -Lg were introduced into a time of flight analyzer (1.3 m flight path) with an acceleration voltage of 25 kV, 93% grid voltage, 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 10 - 35 ku. Apomyoglobin (Calibration Mixture 3 of Sequazyme Peptide Mass Standards Kit; Applied Biosystems) and carbonic anhydrase (Sigma-Aldrich) were used for external mass calibration. Sinapinic acid (> 99 %; Fluka, Buchs, Switzerland) at 10 mg mL⁻¹ in TFA 0.3 %: acetonitrile (70:30, v/v) was used as matrix. Undigested samples were mixed with the matrix at a ratio of 1:15 (v/v), and 1 μ L of this solution was spotted onto a flat stainless-steel sample plate and dried in air (Moreno et al., 2008).

For the study of aggregation degree, size exclusion chromatography (SEC) of β -Lg:Gal / Tag conjugates incubated with and without PM for 6 days at 50 °C was carried out under nondenaturing conditions (0.05 M sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl) using a Superdex 75 10/300 GL column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) on a FPLC system. Elution at room temperature of 1 mg mL⁻¹ sample (100 μ L) was achieved under isocratic mode at 0.8 mL min⁻¹ for 30 min and detection of eluting proteins was performed at 214 nm with an UV-Vis detector (Corzo-Martinez et al., 2010). The standard proteins used for calibration were 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. Analyses were performed in duplicate.

2.2.2. β -Lg:DX₁₀ / DX₂₀ conjugates

The extent of glycation of β -Lg:DX₁₀ / DX₂₀ complexes obtained under maximum glycation and low aggregation conditions was determined according to Jiménez-Castaño et al. (2007). After acid hydrolysis of these glycoconjugates, maximum formation of Amadori compound was estimated from the extent of Lys blockage determined by quantitative analysis of 2-furoylmethyl-Lys (furosine). Ion-pair RP-HPLC analysis of furosine was carried out using a C₈ (Alltech furosine-dedicated, Lokeren, Belgium) column (250 x 4.6 mm) and a variable wavelength detector set at 280 nm (LCD Analytical, SM 4000, Riviera Beach, FL, USA). Furosine concentration was transformed into mol of blocked Lys per mol of protein (Desrosiers, Savoie, Bergeron, & Parent, 1989). Free amino groups were measured using trinitrobenzenesulphonic acid (TNBS) (Sigma-Aldrich). The values of absorbance at 420 nm were transformed into μ moles of Leu / mL using a calibration curve within the range 0.25-2.1 μ M. The brown colour development was evaluated by the absorbance at 420 nm measured in a Beckman DU 70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). The samples were previously centrifuged at 6000 x g for 10 min to reduce the scattering effect due to protein aggregates.

For stoichiometry calculations, the amount of protein and dextran was determined in the ultrafiltration permeates and retentates by measuring the

absorbance at 280 nm and at 490 nm, after colour development with the phenol-sulphuric acid **method** (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), respectively. All analytical determinations were performed at least in duplicate.

2.3. In vitro gastrointestinal digestion

With the aim of studying the effect of glycation and aggregation of β -Lg on its digestibility, we simulated a **simplified *in vitro*** gastrointestinal digestion based on the method described by Moreno, Mellon, Wickham, Bottrill, and Mills (2005b). **This digestion model was based on *in vivo* data obtained by gastric and duodenal aspiration and from collection of effluent from ileostomy volunteers at the Institute of Food Research (Norwich, UK). Nevertheless, it should bear in mind that selection of the optimal digestive enzyme : substrate ratios reflecting those found physiologically is a difficult task, as humans show a wide variation in their gastric and pancreatic secretions and they also vary with type of food consumed or with gastrointestinal disorders (Moreno, 2007). Therefore, although the *in vitro* gastrointestinal digestion model here reported aims at being close to the physiological conditions found in adult humans, such a static and simplified model can not perfectly mimic what happens in the gut of humans.**

For the gastric digestion step, 4 mg of native, control heated and glycated β -Lg (in the absence and presence of PM), were dissolved in 1.33 mL of simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5 adjusted with 1 M HCl). Then, a 0.32 % (w/v) solution of porcine pepsin (EC 3.4.23.1, Sigma-Aldrich, 3300 activity units mg^{-1} of protein) in SGF was added to each sample at a simulated

physiological ratio of enzyme to substrate (1:20, w:w), and digestion was performed at 37 °C for 2 h. After this step, the pH was increased to 7.5 with 40 mM NH_4HCO_3 (Panreac, Barcelona, Spain) to inactivate pepsin. For the intestinal digestion, the pH was adjusted to 6.5 and a duodenal environment was simulated by adding: (i) a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate and glycodeoxycholic acid (both from Sigma-Aldrich), (ii) 1 M CaCl_2 (Panreac), and (iii) 0.25 M Bis-Tris buffer, pH 6.5 (Sigma-Aldrich). Solutions of porcine trypsin (0.05 %, w:v, EC 3.4.21.4; type IX-S; 14,300 activity units mg^{-1} of protein; Sigma-Aldrich) and bovine α -chymotrypsin (0.1 %, w/v, EC 3.4.21.1, type I-S; 62 activity units mg^{-1} of protein; Sigma-Aldrich) in water were then added at approximately physiological protein:trypsin:chymotrypsin ratios [1/(1/400)/(1/100), w/w/w]. Finally, samples were incubated at 37 °C, and aliquots were taken at 15, 30, 60, and 120 min for RP-HPLC-UV and SDS-PAGE analysis. Trypsin and chymotrypsin were inactivated either by heating at 80 °C for 5 min or by adding a solution of Bowman–Birk trypsin-chymotrypsin inhibitor from soybean (Sigma-Aldrich), at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix.

2.4. Analysis of digested samples

Analysis of digested samples was carried using a Beckman HPLC system equipped with a Phenomenex Jupiter Proteo column (250 mm × 4.6 mm, 4 μm particle size, 90 Å pore size). Samples were eluted using 0.1 % (v/v) trifluoroacetic acid in double-distilled water as solvent A and 0.1 % (v/v) trifluoroacetic acid in double-distilled water and acetonitrile of HPLC grade

(Scharlau Chemie, Barcelona, Spain) (1:9, v/v) as solvent B, following the method described by Moreno et al. (2004).

For SDS-PAGE analysis, 32.5 μL of the samples taken at different stages of the digestion were added to 12.5 μL of 4X NuPAGE® LDS Sample buffer (Invitrogen, CA, USA) and 5 μL of 0.5 M dithiothreitol (DTT, Sigma-Aldrich), and heated at 70 °C for 10 min. Samples (20 μL) were loaded onto a 12 % polyacrylamide NuPAGE® Novex Bis-Tris pre-cast gel and a continuous 2-(N-morpholino)ethanesulfonic acid (MES) SDS running buffer was used. Gels were run for 40 min at 120 mA / gel and 200 V and stained using the Colloidal Blue Staining Kit (Invitrogen).

2.5. ELISA experiments

Residual IgG binding of the samples was evaluated by an optimized indirect ELISA method, according to Chicón et al. (2008) using commercial antibodies as described below. High binding polystyrene microtiter plates (Corning, Cambridge, MA) were used as solid support. Single wells were coated with 50 μL of antigen (2.5 $\mu\text{g mL}^{-1}$ of native / control heated / glycated $\beta\text{-Lg}$ / $\beta\text{-Lg}$ hydrolysates) in 0.01 M phosphate buffer saline (PBS, Merck) solution, pH 7.4, and incubated overnight at 6 °C. Plates were washed with PBS containing 0.05 % Tween 20 (PBST). This washing system was used after each incubation step. Residual free binding sites were blocked with PBS containing 2.5 % of Tween 20 for 1 h at room temperature. Plates were then incubated with 50 μL per well of rabbit anti-bovine $\beta\text{-Lg}$ conjugated with horseradish peroxidase (HRP, Bethyl Laboratories, Montgomery, TX, USA) diluted 1:20,000 in PBS. Finally, a solution of freshly prepared o-phenylene-diamine dihydrochloride

(OPD, Dako, Glostrup, Denmark) containing H_2O_2 was added, following the instructions of the manufacturer. Plates were incubated for 30 min at room temperature in the dark, and the reaction was stopped by adding 50 μ L per well of 0.5 M H_2SO_4 . Optical densities (O.D) were read at 492 nm on an automated ELISA plate reader Multiskan Ascent (Labsystems, Helsinki, Finland).

The IgE binding of native β -Lg and hydrolysates of native / control heated / glycated β -Lg was evaluated by indirect ELISA, using the sera of three different patients. Individual serum samples from children with proven allergy to bovine milk proteins were collected with written consent at the Hospital Gregorio Marañón (Madrid, Spain) fulfilling ethical requirements. All patients showed specific IgE antibodies toward milk proteins, particularly β -Lg, determined by the FEIA-CAP System (Pharmacia Diagnostics, Uppsala, Sweden). The IgE binding assay was performed similarly to that of IgG but with slight modifications. Briefly, wells were coated with 50 μ L of antigen as described above. After the blocking step, plates were incubated overnight at 6 °C with 50 μ L per well of human serum diluted in PBST as follows: serum 1 at 1:1,000; serum 2 and 3 at 1:100. After blocking, 50 μ L per well of HRP-conjugated rabbit anti-human IgE (Dako) were added at a 1:1,000 dilution in PBST, and plates were incubated for 1 h at room temperature. Before the addition of enzyme substrate, a signal amplification system based on the subsequent addition of biotinyl-tyramide and streptavidin-HRP (ELAST ELISA amplification system, PerkinElmer Life Sciences, Waltham, MA, USA) was used following the instructions of the manufacturer. Finally, color development was with a solution of OPD as described above.

The ELISA determinations were carried out in triplicate and measurements averaged. Blanks (without antigen) containing PBS or the reaction mixture of *in vitro* gastrointestinal digestion, and a positive control containing intact β -Lg were included in each plate.

2.6. Statistical analysis

Statistical analysis was performed using SPSS for Windows version 17.0. Univariate analysis of variance (ANOVA) (significant minimum difference, SMD, test) was used for the statistical evaluation of IgG and IgE binding results of the glycated and unglycated β -Lg digested. Differences were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Determination of glycation extent and aggregation degree of undigested β -Lg conjugates

3.1.1. β -Lg:Gal / Tag conjugates

In agreement with Corzo-Martinez et al. (2008), MALDI-TOF-MS analyses revealed that the average number of carbohydrate molecules (Gal or Tag) covalently linked to the undigested β -Lg was higher for Gal compared to Tag throughout incubation at either 40 or 50 °C. The most remarkable difference was observed after 1 day of incubation at 40 °C, when average numbers of 14 Gal and 3 Tag molecules were linked to one β -Lg molecule. In

addition, up to 19 / 21 Gal and 15 / 20 Tag molecules linked to the protein chain were found after 2 / 6 days of storage at 50 °C, respectively.

Regarding the inhibitory effect of PM on protein aggregation determined by SEC under non-denaturing conditions, β -Lg:Gal / Tag conjugates incubated in the absence of PM eluted predominantly as a protein dimer upon storage at 40 °C (Fig. 1) and 50 °C for 1 day. Nevertheless, with increasing incubation time, particularly from the second day of storage at 50 °C, formation of important levels of β -Lg cross-linked products took place, indicating that glycation of β -Lg promoted its polymerization. However, as Corzo-Martinez et al. (2010) previously reported, the aggregation levels of β -Lg significantly decreased following incubation with Gal or Tag in the presence of PM (Fig. 1).

3.1.2. β -Lg:DX₁₀ / DX₂₀ conjugates

Optimal glycation conditions were chosen to provide a high degree of β -Lg glycation together with low brown colour development. In agreement with Jiménez-Castaño et al. (2007), the highest number of blocked lysine per mol of reactant protein (i.e., 2 for β -Lg:DX₁₀ and 1 for β -Lg:DX₂₀ conjugates), as estimated by the furosine levels, was obtained after incubation of mixtures of β -Lg and polysaccharide at 60 °C for 36 h (DX₁₀) and 60 h (DX₂₀). Data of free amino groups (14 for DX₁₀ and 15 for DX₂₀) and colour development (absorbance at 420 nm lower than 0.02) of glycoconjugates incubated under these conditions were consistent with a limited formation of advanced glycation end products (data not shown). Finally, the stoichiometry of the glycoconjugates was calculated from the reactant molar ratio, the results of purification (~ 50 % of recovered protein in ultrafiltration retentates) and the number of blocked

lysines / mol reactant protein, allowed to establish an average of 4 and 1.5 molecules of DX₁₀ and DX₂₀ covalently linked to β -Lg.

3.2. Effect of glycation on the IgG reactivity of undigested β -Lg:Gal / Tag conjugates

The antigenic response of glycated β -Lg at both 40 and 50 °C **estimated by ELISA** was similar or lower than that of control heated β -Lg, suggesting that no new epitopes were formed as a result of glycation (Fig. 2). While no important differences were observed between control heated and tagatosylated β -Lg, a significant reduction in the IgG response of β -Lg glycated with Gal was found from the first day of incubation either at 40°C or 50°C (Fig. 2). Moreover, this decrease was more evident as incubation proceeded. This fact was probably due to the higher number of adducts of Gal bound to β -Lg and, therefore, to the higher number of modified lysine residues which could be contained in the epitopes recognized by IgG antibodies. Taheri-Kafrani et al. (2009) recently described a notable carbohydrate-masking effect of epitopes on highly glycated β -Lg, whereas moderate glycation of β -Lg had only a small effect on antibody recognition. These authors attributed this behaviour to the fact that modification of lysyl residues present in the epitopes weakens or prevents antibody binding.

3.3. Effect of protein aggregation on the IgG reactivity of undigested β -Lg:Gal / Tag conjugates

Hardly any differences were found between the **IgG binding** of β -Lg:Tag complexes incubated in the presence and absence of PM, regardless of the storage conditions. However, a significant decrease (**$P<0.05$**) in the IgG

response of the undigested β -Lg:Gal conjugate obtained under the most favoured incubation conditions for the formation of protein aggregates (i.e., 50 °C, 6 days in the absence of PM) was observed (Fig. 2B). This may be related to important structural changes derived from extensive cross-linking reactions that occur during the advanced stages of the MR. Thus, the formation of protein aggregates might mask the antigenic regions of β -Lg, preventing their recognition by IgG antibodies. In good agreement with these results, Nakamura et al. (2006) found that conjugation of squid tropomyosin (TM) with ribose decreased its immunoreactivity, probably due to the structural modifications induced in TM during the advanced stages of the MR.

3.4. Effect of glycation on digestibility, IgG- and IgE-reactivities of β -Lg:Gal / Tag conjugates

After evaluating the effect of aggregation and glycation of undigested β -Lg with Gal or Tag at all incubation periods on its IgG antibody-binding properties, several samples were selected based on the results derived from section 3.1 for further analyses aimed at determining the digestibility and immunoreactivity of β -Lg:Gal / Tag / DX₁₀ / DX₂₀ Maillard complexes (Table 1).

The effect of glycation at early stages of the MR on IgG reactivity was tested on hydrolysates of β -Lg:Gal or Tag complexes obtained after incubations at 40 °C for 1 day. These Maillard complexes showed the highest difference in the average number of carbohydrate molecules linked to β -Lg and were non-aggregated. The chromatograms obtained by RP-HPLC-UV after pepsin digestion for 2 h showed a very limited hydrolysis extent of both control heated and glycated β -Lg, with the same pattern of minor peptides eluting within 20 and

40 min (Fig. 3). Similarly, Morgan, Léonil, Mollé, and Bouhallab (1999a) and Morgan et al. (1999b) did not find differences between native and glycated β -Lg regarding its susceptibility to pepsin. This exceptional resistance to degradation in the stomach, which makes possible to detect intact β -Lg in the upper portion of the small intestine (Kitabatake & Kinekawa, 1998; Mahe, Messing, Thuillier, & Tome, 1991), has been attributed to its structural stability at acid pH. Its peptic cleavage sites (hydrophobic or aromatic amino acid side chains) are buried inside its characteristic β -barrel structure, forming a strong hydrophobic core and preventing hydrolysis (Reddy et al., 1988; Dalgalarrrondo et al., 1995). Therefore, and in agreement with previous research (Corzo-Martínez et al., 2008), the results reported here indicate that the conformational state of β -Lg was not significantly affected by glycation at 40 °C for 1 day.

After 2 h of gastric digestion, the pH was raised to 6.5, irreversibly inactivating pepsin and mimicking the transfer of gastric contents into the duodenal compartment. Combined trypsin / chymotrypsin digestion of control heated and glycated β -Lg in presence of bile salts was followed for up to 120 min in the duodenal phase. However, a time of 15 min was chosen as the most relevant for this study, because this is approximately the transit time down the duodenum and into the jejunum / ileum to the site of the first Peyer's patch (Moreno, Mackie, & Mills, 2005a). According to SDS-PAGE analysis (Fig. 4A), control heated β -Lg that remained intact after pepsinolysis, was rapidly broken down during simulated duodenal digestion, and only traces of intact protein could be observed after 15 min of incubation with trypsin / chymotrypsin. Unlike unglycated β -Lg, a marked band corresponding to intact β -Lg glycated at 40 °C for 1 day could be clearly visualized at 15 min (Fig. 4A, lanes b and c), and

even after 1 h of trypsin / chymotrypsin digestion (results not shown). Furthermore, resistance to gastrointestinal digestion was particularly more evident in galactosylated β -Lg than in tagatosylated complexes, demonstrating that a higher degree of glycation could lead to a higher protective effect against proteolysis. Supporting this fact, a faint band corresponding to the dimeric intact β -Lg could only be visualized in the galactosylated glycoconjugate (Fig. 4A, lane b).

RP-HPLC analysis of hydrolysates (Fig. 4B) revealed the presence of a complex mixture of peptides eluting between 15 and 50 min. The intensity of chromatographic peaks was lower in glycated (particularly in β -Lg:Gal conjugates) compared to unglycated β -Lg. This lower protease activity could be explained as glycation protects β -Lg from trypsin / chymotrypsin proteolysis, probably due to a reduced reactivity of trypsin against glycated Lys and Arg residues (Henle & Klostermeyer, 1993; Morgan, Léonil, Mollé, & Bouhallab, 1997). These results are also in good agreement with those previously reported by Lapolla et al. (2001, 2004) in studies with human and bovine serum albumin, and by Sanz et al. (2007) and Moreno et al. (2008) in studies carried out with β -Lg-galactooligosaccharides conjugates.

Moreover, IgG- and IgE-binding properties of undigested native β -Lg and hydrolysates (derived from 2h of pepsin digestion followed by 15 min of trypsin / chymotrypsin digestion) of control heated / glycated β -Lg were also estimated by ELISA. Although the antigenic response of digested control heated β -Lg was significantly lower than that of undigested native β -Lg, the former still exhibited a clear response against IgG (Fig. 5A). Even if no remaining intact β -Lg was left after gastrointestinal digestion of control heated β -Lg (Fig. 4B), some of its

proteolysis products could retain antigenic epitopes. Likewise, the hydrolysates of β -Lg:Gal / Tag conjugates incubated at 40 °C for 1 day, which still contained intact β -Lg (specially those of β -Lg:Gal), exhibited a higher IgG reactivity compared to digested control heated β -Lg (Fig. 5A). These results were consistent with the greater IgE reactivity of digested galactosylated β -Lg compared to that of control heated β -Lg for the three human sera assayed (Table 2).

Jarvinen, Chatchatee, Bardina, Beyer, and Sampson (2001) identified seven epitopes of bovine β -Lg, covering residues 1-16, 31-48, 47-60, 67-78, 75-86, 127-144, 141-152, recognizable by IgE antibodies from children with persistent cow's milk allergy, whilst Ball et al. (1994) identified the region 97-108 as the main linear IgE epitope. In addition, Sélo et al. (1999) described the tryptic fragments 1-8, 25-40, 41-60, 102-124 and 149-162 as major IgE binding epitopes in a population of milk-allergic patients whose ages were not reported, whereas Adams et al. (1991) showed that the residue 124-134 of bovine β -Lg was responsible for 60 % of the IgE binding to the allergen in a single individual. In addition to the remaining intact protein detected following moderate and extensive glycation, some key peptides containing at least one entire IgE binding epitope, such as ¹L-K⁸, ²¹S-R⁴⁰, ⁴⁰R-Q⁵⁹, ⁴⁰R-K⁶⁰, ⁴¹V-K⁶⁰, ⁴³V-K⁶⁰, ⁶⁸Q-I⁷⁸, ⁷⁸I-L⁹³, ⁹⁰N-A¹¹¹, ¹²³V-F¹³⁶, ¹²⁸V-M¹⁴⁵ and ¹⁴⁹L-I¹⁶², have been shown to survive *in vitro* gastrointestinal digestion of either glycated or unglycated β -Lg (Corzo-Martínez, Lebrón-Aguilar, Villamiel, Quintanilla-López, & Moreno, 2009; Moreno et al., 2008). Hence, these fragments could also contribute to the IgE-reactivity of gastrointestinal digests.

3.5. Effect of protein aggregation on the digestibility, IgG- and IgE-reactivities of β -Lg:Gal / Tag conjugates

To investigate the effect of protein aggregation produced during the advanced stages of the MR, we analyzed the β -Lg:Gal / Tag conjugates incubated in the absence and presence of PM at 50 °C for 2 days (selected samples in Table 1), conditions that favoured the formation of soluble protein aggregates (Corzo-Martínez et al., 2010).

Similarly to the above non-aggregated glycoconjugates (section 3.4), control heated and glycated β -Lg at 50 °C for 2 days remained largely intact after pepsinolysis (data not shown). However, the yield of gastrointestinal digestion products of β -Lg:Gal / Tag conjugates was much higher when aggregation was partially inhibited in the presence of PM (Fig. 6). This suggests that protein aggregation induced by the MR may protect β -Lg during *in vitro* gastrointestinal digestion. These results were confirmed by SDS-PAGE (Fig. 7). In the absence of PM, two bands of intact protein, corresponding to β -Lg glycosylated with Gal and Tag in monomeric and dimeric forms, were clearly evident (Fig. 7, lanes b and d). In the presence of PM, these two bands were very diffuse and a number of bands corresponding to peptidic fragments were observed (Fig. 7, lanes c and e). The lower digestibility observed after glycation in the absence of PM could be attributed to masking of cleavage sites, as a consequence of cross-linking and aggregation of β -Lg induced by conjugation with Gal and Tag during the advanced stages of the MR. Similarly, Ara h 1 and Ara h 2, both major allergens from peanut, were also found to form intermolecular cross-links resulting in a certain protection against protease digestion due to the inaccessibility of potential cleavage sites (Shin et al., 1998;

Maleki et al., 2000). Consequently, once they had undergone the MR, Ara h 1 and Ara h 2 bound higher levels of IgE and were more resistant to heat and digestion by gastrointestinal enzymes.

Despite containing a higher amount of intact β -Lg, hydrolysates of β -Lg glycoconjugates incubated at 50 °C for 2 days either with (Fig. 5B) or without PM (Fig. 5C) showed: i) a lower IgG reactivity than those incubated at 40 °C for 1 day (Fig. 5A), and ii) a similar response against IgG antibodies to that of digested control heated β -Lg (Fig. 5B and 5C). These results strongly support that the cross-linking reactions induced by the advanced stages of the MR might partially mask the β -Lg epitopes, thus, counteracting the negative effect of the lower digestibility of glycated and aggregated β -Lg. In agreement with these findings, no significant differences ($P < 0.05$) were found in the serum IgE-binding of hydrolysates between control heated β -Lg and β -Lg glycated with Gal for 2 days at 50 °C in the absence of PM (Table 2).

3.6. Effect of glycation with high molecular weight carbohydrates on the digestibility and IgE-reactivity of β -Lg

As the shielding of epitopes by molecules having low IgG and IgE reactivities is thought to be important in reducing the immunoreactivity of the protein, the use of a modifier with a higher molecular weight could be more efficient (Hattori, Nagasawa, Ametani, Kaminogawa, & Takahashi, 1994; Hattori et al., 2000a, 2004; Sehon, 1982). Therefore, glycation of food allergens with polysaccharides could be an interesting alternative to reduce protein allergenicity. Previous studies have shown that either the covalent coupling, using water-soluble carbodiimide, of bovine β -Lg with chitosan (Hattori et al.,

2000a; Aoki, Iskandar, Yoshida, Takahashi, & Hattori, 2006) and carboxymethyl dextran (Hattori et al., 2000b; Kobayashi et al., 2001), or the modification via MR with alginic acid oligosaccharide and phosphoryl oligosaccharides (Hattori et al., 2004) were effective methods for reducing β -Lg immunogenicity. However, none of these studies investigated the impact of glycation with carbohydrates of high molecular weight on the β -Lg immunoreactivity after its simulated gastrointestinal digestion.

Thus, in the present study β -Lg was glycated with DX₁₀ and with DX₂₀ under conditions of maximum formation of Amadori compound between β -Lg and polysaccharide (selected samples in Table 1), and then digested under simulated gastrointestinal conditions. The glycation degree of β -Lg was slightly higher with DX₁₀ than with DX₂₀, as previously mentioned (section 3.1.2). Comparison of the HPLC patterns of hydrolysates of control heated β -Lg and β -Lg:DX₁₀ / DX₂₀ conjugates evident by their great resistance to pepsinolysis and showed no relevant differences between the unglycated and glycated protein (chromatograms not shown). This indicated that dry state glycation at 60 °C with the polysaccharides did not significantly affect the conformational state of β -Lg, in agreement with Jimenez-Castaño et al. (2007).

After *in vitro* gastrointestinal digestion, no substantial differences between the chromatographic patterns of control heated β -Lg and β -Lg glycated with DX₂₀ were found (Fig. 8C and 8D), probably because the protein was poorly glycated (1.5 molecules of Dx₂₀ per one molecule of β -Lg) due to the low reactivity of this polysaccharide. This may explain why no significant differences ($P < 0.05$) were found between the IgE reactivity of hydrolysates of control heated and of β -Lg glycated with DX₂₀ (Table 2). In contrast, the yield of

peptides derived from the gastrointestinal digestion of β -Lg glycosylated with DX₁₀ was lower than that observed in unglycosylated protein, and some traces of intact glycosylated β -Lg were still detected after 15 min of incubation with trypsin / chymotrypsin (Fig. 8A and 8B). Consequently, glycosylation with DX₁₀ significantly increased the response against IgE antibodies as compared to that of the unglycosylated β -Lg digested for the two human sera assayed (Table 2).

4. Conclusions

In general terms, moderate glycosylation with Gal and Tag during early stages of the MR barely altered the IgG reactivity of undigested β -Lg. A noticeable reduction in its IgG binding was mainly observed after incubation with Gal under conditions of a high degree of glycosylation and formation of protein aggregates, which could mask the antigenic regions of β -Lg. Moreover, the allergenic potential of the hydrolysates of β -Lg Maillard complexes after simulated gastrointestinal digestion has also been determined for the first time in this study. Whereas a high degree of glycosylation impaired the β -Lg proteolysis and, consequently, significantly increased the IgE-binding of the hydrolysates, the formation of protein aggregates during the advanced stages of the MR had a masking effect on the β -Lg epitopes, counteracting the negative effect of the lower digestibility of glycosylated protein on its allergenicity.

As with glycosylation with Gal, conjugation with dextran of 10 kDa decreased the proteolytic susceptibility of β -Lg and, consequently, significantly increased its immunoreactivity. In this case, the steric hindrance exerted by the molecules of dextran attached to β -Lg could also contribute to the lower reactivity of digestive enzymes toward this protein. The data indicate that studies addressing

the effect of glycation on protein allergenicity need to take into account the level of gastrointestinal digestibility of the protein Maillard complexes.

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Table 1. Samples selected for analyses aimed at determining the digestibility and immunoreactivity of β -Lg Maillard complexes.

GLYCOCONJUGATES	INCUBATION CONDITIONS	GLYCATION AND AGGREGATION DEGREE	
β -Lg:Gal	40 °C, 1 day	Highly glycated and non-aggregated	
	50 °C, 2 days	Highly glycated	Highly aggregated (absence of PM) Partial inhibition of aggregation (presence of PM)
β -Lg:Tag	40 °C, 1 day	Lowly glycated and non-aggregated	
	50 °C, 2 days	Highly glycated	Highly aggregated (absence of PM) Partial inhibition of aggregation (presence of PM)
β -Lg:DX ₁₀	60 °C, 36 hours	Maximum level of glycation and low aggregation	
β -Lg:DX ₂₀	60 °C, 60 hours	Maximum level of glycation and low aggregation	

Table 2. Effect of glycation with Gal, DX₁₀ and DX₂₀ on the ELISA response against IgE antibodies of β -Lg hydrolysates.

Sample		Incubation conditions	Human sera		
			Serum 1	Serum 2	Serum 3
undigested	Native β -Lg	---	1.50 $\pm$ 0.01 ^a	2.84 $\pm$ 0.06	3.30 $\pm$ 0.14
digested	Native β -Lg	---	0.85 $\pm$ 0.01	1.87 $\pm$ 0.02	1.90 $\pm$ 0.02
	Control heated β -Lg	40 °C, 1 day	0.83 $\pm$ 0.03	1.87 $\pm$ 0.07	1.88 $\pm$ 0.05
	β -Lg:Gal		0.95 $\pm$ 0.05*	2.17 $\pm$ 0.12*	2.11 $\pm$ 0.02*
	Control heated β -Lg	50 °C, 2 days	0.84 $\pm$ 0.04	1.89 $\pm$ 0.06	1.82 $\pm$ 0.02
	β -Lg:Gal		0.82 $\pm$ 0.04	1.94 $\pm$ 0.04	1.79 $\pm$ 0.06
	Control heated β -Lg	60 °C, 36 h	---	1.72 $\pm$ 0.09	1.74 $\pm$ 0.05
	β -Lg:DX ₁₀		---	1.91 $\pm$ 0.06*	1.92 $\pm$ 0.04*
	Control heated β -Lg	60 °C, 60 h	---	1.76 $\pm$ 0.03	1.77 $\pm$ 0.03
	β -Lg:DX ₂₀		---	1.75 $\pm$ 0.01	1.73 $\pm$ 0.03

An asterisk (*) indicates a significant difference between the sample and the corresponding control ($P < 0.05$).

^a Data represent the mean of triplicate determinations.

Figure captions

Figure 1. Size-exclusion chromatograms under non-denaturing conditions of β -lg glycosylated with Gal/Tag for 1 day at 40 °C, and for 2 days at 50 °C in the absence and presence of pyridoxamine (PM).

Figure 2. Effect of glycation on the IgG binding of β -Lg at 40 °C (A) and 50 °C (B). Control heated β -Lg with (—△—) and without (—▲—) PM; β -Lg:Gal with (—□—) and without (—■—) PM; β -Lg:Tag with (—◇—) and without (—◆—) PM. The graph shows means $\pm$ SD ($n = 4$).

Figure 3. RP-HPLC-UV chromatograms of control heated (A) and β -Lg glycosylated with Gal (B) and Tag (C), incubated for 1 day at 40 °C, after their digestion with pepsin for 0 and 2 hours.

Figure 4. SDS-PAGE analysis (A) and chromatographic profiles obtained by RP-HPLC-UV (B) of hydrolysates of control heated β -Lg (a) and β -Lg incubated with Gal (b) and Tag (c) at 40 °C for 1 day, after pepsin digestion for 2 h followed by trypsin/chymotrypsin digestion for 15 min. M: M_w markers.

Figure 5. IgG binding of undigested native β -Lg and of hydrolysates (pepsin digestion for 2 h followed by trypsin/chymotrypsin digestion for 15 min) of control heated and glycosylated β -Lg incubated at 40 °C for 1 day (A) and at 50 °C for 2 days in presence (B) and absence (C) of PM. The graph shows means $\pm$ SD ($n = 4$).

Figure 6. RP-HPLC-UV profiles of hydrolysates (pepsin digestion for 2 h followed by trypsin/chymotrypsin digestion for 15 min) of β -Lg glycosylated with Gal and Tag at 50 °C for 2 days in the absence and presence of pyridoxamine (PM).

Figure 7. SDS-PAGE analysis of control heated β -Lg without PM (a); β -Lg:Gal without (b) and with (c) PM; and β -Lg:Tag without (d) and with (e) PM incubated at 50 °C for 2 days, subjected to pepsin digestion for 2 hours followed by trypsin/chymotrypsin digestion for 15 min. M: M_w markers.

Figure 8. Chromatographic profiles obtained by RP-HPLC-UV of control heated and glycosylated β -Lg after gastrointestinal digestion (pepsin digestion for 2 hours followed by trypsin/chymotrypsin digestion for 15 min): (A) control heated and (B) β -Lg incubated with DX₁₀ at 60 °C for 36 hours; (C) control heated and (D) β -Lg incubated with DX₂₀ at 60 °C for 60 hours.

Figure 1. *Corzo-Martinez et al.*

Figure 2. *Corzo-Martinez et al.*

Figure 3. *Corzo-Martinez et al.*

Figure 4. *Corzo-Martinez et al.*

Figure 5. *Corzo-Martinez et al.*

Figure 6. *Corzo-Martinez et al.*

Figure 7. *Corzo-Martinez et al.*

Figure 8. *Corzo-Martinez et al.*