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Glycation of heat-treated β-lactoglobulin: Effects on foaming properties

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ABSTRACT

In this paper, the effect of glucose-glycation of beta-lactoglobulin (monomer 88%, dimer 12%) on foaming properties has been studied in 20 mM phosphate buffer at pH 6.8. The results obtained by electrophoresis, circular dichroism and fluorescence quenching showed the presence of small amounts of aggregates of higher molecular weight, while only slight changes in the tertiary structure flexibility of the glycated protein were detected. The improvement on foaming properties (foamability and foam stability) after glycation was promoted by heat treatment in the dry state (96 h at 50 °C) rather than by sugar conjugation. On the other hand, treated beta-lactoglobulin was obtained by heating a protein solution (55 mg mL^{-1} in 20 mM phosphate buffer at pH 6.8) at 85 °C for 3 min. This heated protein sample (monomer 51%, dimer 27%, trimer 19%) formed foams with good stability. After glycation, spectroscopic measurements demonstrated that no significant changes were introduced in protein conformation, since the substrate used was composed by unfolded species. However, the additional presence of oligomers of protein found after sugar conjugation, decreased the volume foam stability, probably due to steric impediment that diminished the viscoelastic stiffness of the interfacial film. The variation of volume foam stability caused by the different treatments assayed in this work, resulted from the action of these treatments on disproportionation rather than on liquid drainage from the foam.

1. Introduction

The study of foam properties (foamability and foam stability) has a relevant application in food industry since many foods such as bread, meringue, ice cream or cake include foams as a vital component to improve their texture. Foamability can be understood as the capacity of achieving certain level of desired air phase volume and foam stability, as the foam endurance against destabilizing processes like mixing, cutting and heating or simply the aging process of the foam (Foegeding, Luck, & Davis, 2006).

Proteins are frequently used as foaming agents in foods since they contribute not only to the formation but also to the stability of foams. In a recent work (Moro, Báez, Busti, Ballerini, & Delorenzi, 2011), we have shown that the time of previous heating of beta-lactoglobulin (β-LG) solutions is a crucial variable for the features of this protein as a foaming agent, due to conformational changes and different proportion of species in solution that this treatment produces. In this cited work, 3 min was pointed as the critical time when 55 mg mL^{-1} β-LG solution was heated at 85 °C since the most significant conformational changes and aggregation processes occur at this time, producing non-native monomers and the greatest amount of dimers and trimers. Heat treatment affects foamability and even more foam stability. Both foam properties are closely linked to structural changes of the protein. In this case, the increase in surface hydrophobicity is considered as a decisive factor in the improved foamability. Also, the best foam stabilization was achieved at 3 min of heating treatment, coincidentally with the occurrence of those conformational changes.

On the other hand, numerous attempts were made to further improve the functional properties of whey proteins through chemical and/or enzymatic treatments (Haertlé & Chobert, 1999). However, most of these methods utilize toxic chemicals and are not permitted for potential industrial applications. Protein modification can also be obtained by glycation, via the Maillard reaction. Through this reaction, the conjugation of sugars to proteins does not require chemical catalysis. Thus, a well-controlled Maillard reaction can be a good method for protein processing in the food industry (Liu, Ru, & Ding, 2012). Several authors concluded that a moderate degree of β-LG glycation obtained with glucose appears to be the best condition for obtaining foams and oil–water emulsions with better stabilities (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001; Medrano, Abirached, Moyna, Panizzolo, & Añón, 2012; Medrano, Abirached, Panizzolo, Moyna, & Añón, 2009).

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The aim of this work was to investigate the effects that glycation of native and pre-heated β-LG with glucose produce on foaming properties. Since structural modifications can influence protein functional properties, structural studies on glycated proteins were carried out in order to obtain useful information about their structure–function relationship.

2. Materials and methods

2.1. Materials

β-LG AB and D-(+)-glucose were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). The protein was used without further purification. All other chemicals were of analytical grade.

2.2. Glycation experiments

A solution containing β-LG (1 mM) and glucose (100 mM) was prepared in 20 mM phosphate buffer at pH 6.8. After being freeze-dried, the protein–sugar powder was kept under 65% relative humidity (saturated KI solution) and 50 °C for a period of 96 h. After this treatment, the powder was dissolved and the free glucose was removed by extensive dialysis with phosphate buffer. The dialyzed solution was freeze-dried and the powder was stored at −20 °C until used. This sample was referred to as GLI. Control experiments were carried out with no added glucose (β-LGh sample).

Heat treated β-LG solution was prepared following the protocol employed by Moro et al. (2011). A 55 mg mL^{-1} β-LG solution was prepared in 20 mM phosphate buffer at pH 6.8. This solution was placed in a small glass tube and heated in a water bath at 85 °C for 3 min. The solution was cooled to room temperature and freeze-dried. This sample, referred to as TT, was then glycated as described above. The glycated sample and the control with no added glucose were referred to as TT-GLI and TTh, respectively.

For spectroscopic and foaming property measurements the different samples were dissolved to the desired concentration with 20 mM phosphate buffer at pH 6.8.

The protein content of samples was determined by the dye binding method (Bradford, 1976), using β-LG as standard.

2.3. Electrophoresis

SDS-PAGE of β-LG/β-LGh/GLI and TT/TTh/TT-GLI was performed as Laemmli (1970) described, using a stacking gel of 10% and a running gel of 15%. The amount of 10 μg of total protein was loaded in each lane. The gels were run under nonreducing conditions to avoid cleavage of intermolecular disulfide bonds formed during the heat treatment. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R250 and scanned using a Hewlett–Packard ScanJet 5p connected to a computer. The pixel densities of digitized protein bands were analyzed using software developed by our group (Palazolo, Rodriguez, Farruggia, Picó, & Delorenzi, 2000). These values were then used to calculate the relative amounts of monomers, dimers, trimers and oligomers found in each sample. The molecular weight of each protein band was matched to known standard proteins.

2.4. Determination of available amino groups

The quantity of available amino groups was determined by the colorimetric method based on the ortho-phthalaldehyde (OPA) reaction according to the technique described by García Alvarez-Coque, Medina Hernández, Villanueva Camañas, and Mongay Fernández (1989). The OPA reagent was prepared by dissolving 80 mg of OPA in 2 mL of ethanol which was mixed with 5 mL of 10% w/v sodium dodecyl sulfate, 0.2 mL of 2-mercaptoethanol and diluted to 100 mL with 0.1 M borate buffer (pH 9.9). The assay consisted of mixing 3 mL of OPA reagent with 50 μL of protein solution (10 mg mL^{-1}) and measuring the absorbance in a quartz cuvette at 340 nm after 30 min of incubation at room temperature. A calibration curve was obtained by using L-leucine as a standard. Measurements were performed in triplicate.

2.5. Circular dichroic spectral measurements

Circular dichroism (CD) of the solutions of different protein samples was measured using a Jasco J-810 automatic recording spectropolarimeter (Japan), with an integration time of 2 s and 2 nm bandwidth. Four scans at a rate of 50 nm min^{-1} were carried out and their results were averaged for the wavelength range employed. In far UV experiments (190–250 nm), 25 μM protein solutions (20 mM phosphate buffer at pH 6.8) were assayed in 0.1 cm path length cell. In near UV experiments (250–320 nm), 164 μM protein solutions (20 mM phosphate buffer at pH 6.8) were assayed in 1 cm path length cell. The CD data were reported as mean residue ellipticity ($[\theta]_{MRW}$) in units of deg cm^2 $dmol^{-1}$, using a mean residue weight of 113 g.

2.6. Measurement of β-LG surface hydrophobicity

Surface hydrophobicity of different protein samples was measured with the fluorescence quenching method of Moro, Gatti, and Delorenzi (2001). Three milliliters of a sample, 10 μM in protein (20 mM phosphate buffer at pH 6.8), was placed in the cell of a Jasco FP-770 spectrofluorometer, and the fluorescence intensity (F_0) was measured at 337 nm, using excitation at 295 nm. Aliquots of 7 M acrylamide, used as fluorescence quencher, were sequentially added in the cell content, and the new fluorescence intensities were measured (F). Acrylamide concentrations ranged from 0 to 0.2 M. At the concentration used, no corrections for acrylamide absorption were necessary. The F_0/F ratio was plotted versus the quencher concentration (Stern–Volmer plot). In the used range, this plot was linear and the Stern–Volmer equation can be expressed as

$$\frac{F_0}{F} = 1 + K_{app}[\text{acrylamide}]. \quad (1)$$

K_{app} is an apparent constant because β-LG has more than one tryptophanyl residue that can be quenched by acrylamide. The initial slope of Stern–Volmer plots, K_{app}, is an index of protein hydrophobicity.

2.7. Thermal unfolding of protein samples

Thermal unfolding of protein samples was followed as described by Busti, Gatti, and Delorenzi (2005). The UV-difference absorbance at 293 nm of each solution was measured using a Jasco V-550 double-beam spectrophotometer equipped with a thermostated cell (Peltier effect) controlled by a programmable unity. The sample cuvette was filled with protein solution whereas the reference cuvette was filled with buffer that was maintained at 20 °C. A 2.5 mL aliquot of 0.2% protein sample (20 mM phosphate buffer at pH 6.8) was placed into the sample cuvette which was sealed with a Teflon stopper to avoid evaporation during each experiment. UV-difference absorbance at 293 nm was recorded as a function of temperature over a range 20–98 °C. The heating rate in the experiments was 0.5 °C min^{-1}. The spectrophotometer compartment was continuously purged with nitrogen to avoid the condensation of water vapor on the sample cuvette.

2.8. Foaming properties

Foams were formed using a bubbling apparatus (Hagolle, Relkin, Popineau, & Bertrand, 2000; Loisel, Guéguen, & Popineau, 1993). Native and treated β-LG samples were dissolved to a final concentration of 0.1% (w/v) in 20 mM phosphate buffer pH 6.8. Determinations were made in a transparent acrylic tube (3.5 cm × 20.0 cm) equipped with a pair of electrodes located at the base of the column and with a porous disk

through which air, at a flow rate of 5 mL s^{-1}, was passed and forced through the liquid (V_{init} = 10 mL), creating foam. Bubbling stopped when the foam reached a fixed volume of 115 mL (V_f). During the test, the conductivity and the volume of foam were recorded by a computer and a digital camera Olympus DS-580 4.0 M pixel.

Conductivity measurements at different times (C_t) and with reference to the initial conductivity (C_{init}) were used to calculate the volume of liquid in the foam (V_{LF}) (Chevalier et al., 2001; Loisel et al., 1993):

$$V_{LF} = V_{init}\left[1 - \frac{C_t}{C_{init}}\right] \quad (2)$$

As it was previously reported (Croguennec, Renault, Bouhallab, & Pezennec, 2006; Fains, Bertrand, Baniel, & Popineau, 1997; Hagolle et al., 2000), foams are compared on the basis of (i) maximum foam density (FD), as a measure of foamability; (ii) half-life time of drainage ($T_{1/2}$) and (iii) volume variation with time, as measures of foam stability.

FD is defined as the ratio between the maximal liquid incorporated into the foam (V_{LFmax}) and the foam volume reached at the end of the sparging period (V_{Fmax}):

$$FD = \frac{V_{LF\,max}}{V_{F\,max}}. \quad (3)$$

It has been noted that $V_{Fmax} = V_f - (V_{init} - V_{LFmax})$.

$T_{1/2}$, the half-life time of drainage, is equal to:

$$T_{1/2} = t_{1/2} - t_0 \quad (4)$$

where $t_{1/2}$ is the time when half of the maximum volume of liquid in the foam came back to the solution ($V_{LFmax}/2$) and t_0 is the time at the end of bubbling.

On the other hand, in terms of volume variation with time, the longer it takes for the foam to collapse, the more stable the foam is (Wilde & Clark, 1996). This can be measured through the one quarter time of foam volume ($T_{1/4}$), which is defined as:

$$T_{1/4} = t_{1/4} - t_0 \quad (5)$$

where $t_{1/4}$ is the time required for V_{Fmax} to decay 25% and t_0 is the time at the end of bubbling.

Both times, $T_{1/2}$ and $T_{1/4}$, and FD were informed as relative ratios in reference to their values for the protein without any treatment: FD°, $T_{1/2}$° and $T_{1/4}$°.

All experiments were performed at 25 °C.

2.9. Statistical analysis

The statistical analysis was established by variance analysis and test of minimum significant difference, using statistical program StatgraphicPlus7.0 and the significance level was $p < 0.05$.

3. Results and discussion

3.1. Electrophoresis

The different protein samples (β-LG, β-LGh, GLI, TT, TTh and TT-GLI) were analyzed by SDS-PAGE (Fig. 1) and the different species of each sample (monomers, dimers, trimers and aggregates of higher molecular weight) were quantified by gel densitometry, Table 1. In this table, β-LGh can be seen to contain a slightly higher proportion of dimers and small amounts of trimers compared with untreated β-LG. The formation of these aggregates can be attributed to sulfhydryl/disulfide exchange reactions produced by heat treatment in the dry state. The glycation of β-LG (GLI) induced the presence of a small quantity of larger aggregates than trimers, indicating that this process also promoted the aggregation of the protein. This additional aggregation may also be due to the presence of covalent linkages different from disulfide bonds (Morgan, Léonil, Mollé, & Bouhallab, 1999). A similar tendency was observed when the electrophoretic behavior of TT, TTh and TT-GLI samples was analyzed. However, in this case, the formation of larger aggregates was higher than those obtained for β-LGh and GLI, a fact caused by a greater exposition of intramolecular sulfhydryl groups by protein unfolding.

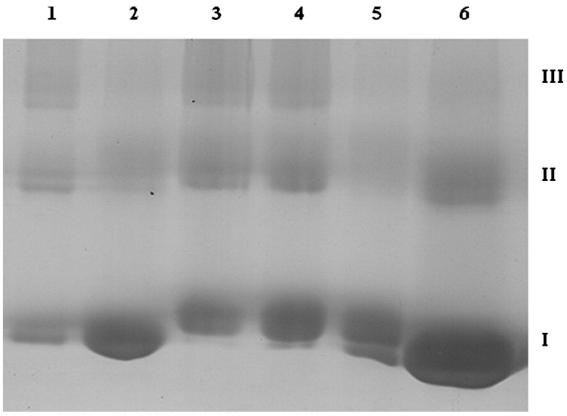

Fig. 1. SDS-PAGE electrophoresis under non-reducing conditions. TT-GLI (lane 1), β-LGh (lane 2), TTh (lane 3), TT (lane 4), GLI (lane 5), β-LG (lane 6). I: monomers; II: dimers and III: trimers. Oligomers of higher molecular weight did not enter the running gel.

On the other hand, the observed lower electrophoretic mobility of glycated proteins (GLI and TT-GLI) (electrophoretic patterns not shown) was explained by the higher molecular mass of the different species that contained glucose linked to the respective structure.

3.2. Determination of the extent of glycation

The extent of β-LG glycation was indirectly assessed using the colorimetric assayed based on the reaction between OPA and the free primary amine of the protein. The early stages of Maillard reaction consist of a condensation between the carbonyl group of a reducing carbohydrate with an amino group, mainly the ε-amino group of the lysine residues, to form a Schiff base. β-LG sequence contains 16 potential reactive amino-groups, including 1 α-amino and 15 ε-amino groups on lysil residues. In the present work, only 12.6 available amino groups per native β-LG monomer were found, while the number of available amino groups of GLI was 3.1, Table 2. Therefore, the average number of sugar residues bound per protein monomer was 9.5. This value was similar to that reported by some authors (Chevalier et al., 2001; Wooster & Augustin, 2007) but was fairly smaller than the value reported by Medrano et al. (2009). The latter determined the extent of glycation by MALDI-TOF mass spectrometry.

Table 1
Monomer, dimer, trimer and oligomer contents of the different β-LG samples, determined by SDS-PAGE electrophoresis under non-reducing conditions.

sample	monomer content (%)	dimer content (%)	trimer content (%)	oligomer content (%)
β-LG	88	12	–	–
β-LGh	79	18	2	1
GLI	76	19	3	2
TT	51	33	16	–
TTh	42	30	17	11
TT-GLI	38	27	19	16

Table 2
Number of carbohydrate molecules attached to β-LG after the glycation process.

sample	amino groups detected per β-LG molecule	average number of glucose molecules attached per β-LG molecule
β-LG	12.6 ± 0.4	0
GLI	3.1 ± 0.3	9.5
TT	10.6 ± 0.5	0
TT-GLI	1.9 ± 0.2	8.7

TT presented 10.6 available amino groups per β-LG monomer. This lower value compared with the one obtained for the native protein was possibly due to a masking of amino groups induced by protein denaturation and aggregation, impeding the access of OPA to reactive groups. The glycated species of the previously heated protein (TT-GLI) exposed only 1.9 amino groups. Therefore, the difference between the values obtained for these two latter species (TT and TT-GLI) may be attributed to the presence of 8.7 sugar residues bound per glycated protein monomer.

3.3. Circular dichroism

The far UV CD spectra largely reflect the secondary structure of proteins. Both β-sheet and α-helix secondary structures show CD peaks below 200 nm and troughs in the 200–235 nm region while "random" and turn structures exhibit deep troughs near 200 nm (Greenfield & Fasman, 1969; Johnson, 1990). The unfolding of native β-LG promoted by heat treatment produced a shift to shorter wavelengths in the spectra, a change which is related to the loss of β-sheet structure as well as α-helix with a corresponding increase in random coil (Kim, Cornec, & Narsimhan, 2005; Moro et al., 2011). Fig. 2 shows a selected portion of the far UV CD spectra of the different samples assayed in this work. Taking these results into account, CD signal at 205 nm was chosen to monitor changes in the backbone secondary structure of treated protein samples. As it can be seen in Fig. 3, the effects of glycation on the far UV CD spectra were practically negligible, implying that the reaction did not produce statistically significant changes in the secondary structure of the native β-LG and TT samples, respectively, in spite of the presence of larger unfolded aggregates. The analyses of near UV CD spectra were used to characterize the tertiary structure of proteins mainly because of the constrained asymmetries in the environment of the aromatic amino acids (Greenfield, 1996). The near UV CD spectra of the different samples assayed in this work are shown in Fig. 4. Two negative peaks of the ellipticity at ~285 and ~292 nm in the CD spectra of native β-LG are mainly due to Trp19 absorbance (Creamer, 1995; Gast, Siemer, Zirwer, & Damaschem, 2001). These two negative peaks diminished their intensities with the heat treatment of the native protein, reflecting structural changes within the calyx of the β-LG molecule, where the indole size chain of Trp19 is located (Manderson, Creamer, & Hardman, 1999). In this work, CD signal at 292 nm was followed to quantify changes in the tertiary protein structure promoted by glycation, Fig. 5. The difference between CD signal of β-LG and the signals corresponding to β-LGh and GLI samples was statistically significant. However, the slight difference indicated the presence of small conformational changes in protein structure. Fig. 5 also showed that the tertiary structure of native β-LG was more substantially disrupted by heating at 50 ºC in the dry-state (β-LGh) than by sugar addition (GLI). On the other hand, differences between TT, TTh and TT-GLI were not statistically significant, since the species present in TT sample have an unfolded structure (Moro et al., 2011).

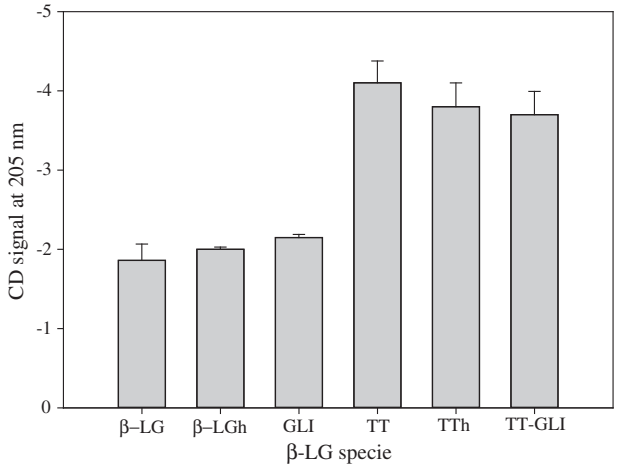

Fig. 3. CD signal at 205 nm of different protein samples. Error bars were calculated from the standard deviation of three replicates.

3.4. Fluorescence quenching

Fluorescence quenching of proteins by acrylamide is an accepted method to determine surface hydrophobicity (Hiller & Lorenzen, 2008; Moro et al., 2001). Protein unfolding causes a red shift on the

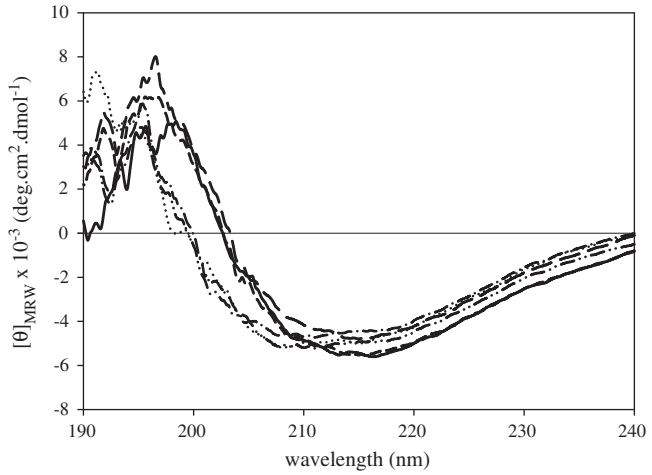

Fig. 2. Selected portion of far ultraviolet CD spectra of β-LG (———), β-LGh (– – – –), GLI (– – – – –), TT (.....), TTh (—·—·—), TT-GLI (—··—··).

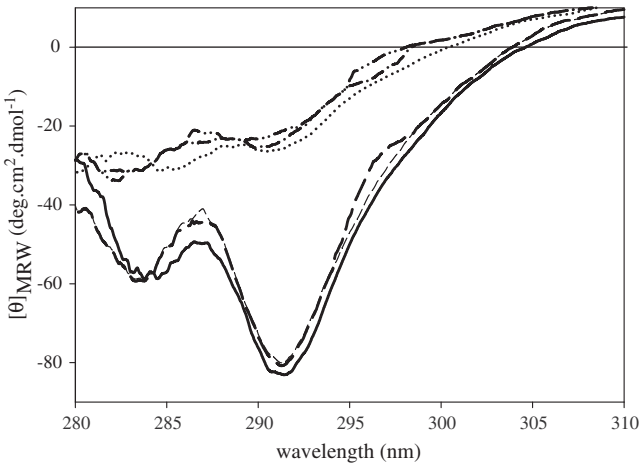

Fig. 4. Selected portion of near ultraviolet CD spectra of β-LG (———), β-LGh (– – – –), GLI (– – – – –), TT (.....), TTh (—·—·—), TT-GLI (—··—··).

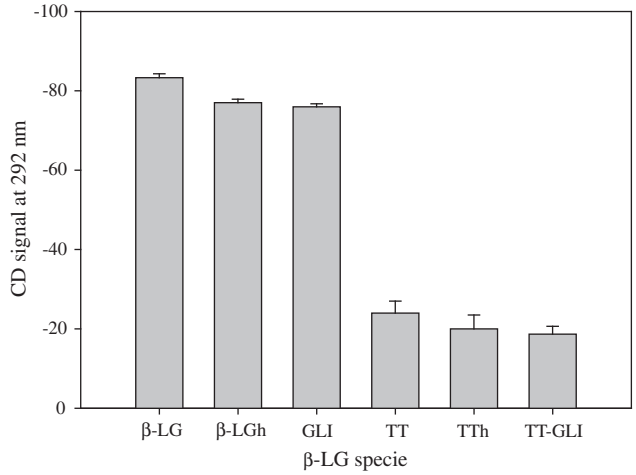

Fig. 5. CD signal at 292 nm of different protein samples. Error bars were calculated from the standard deviation of three replicates.

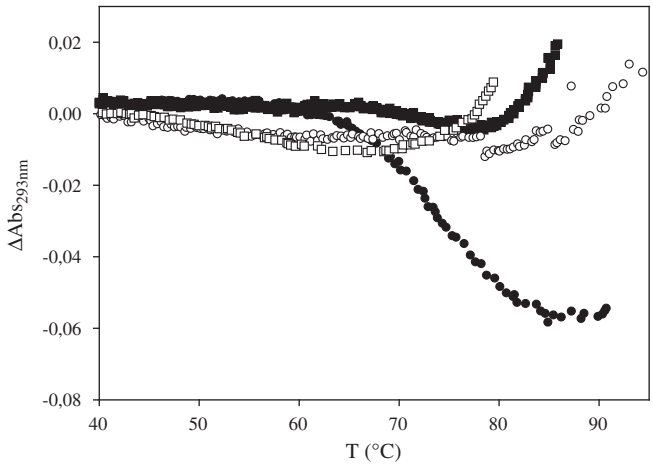

Fig. 7. Thermal unfolding of the different protein samples followed by UV-difference absorbance at 293 nm, ΔAbs_{293nm}. (●) β-LG, (■) GLI, (○) TT, (□) TT-GLI.

fluorescence emission spectrum due to the major exposition of Trp residues to the aqueous solvent, which promotes an increase in the fluorescence quenching of denatured proteins by acrylamide (Busti, Gatti, & Delorenzi, 2006; Moro et al., 2001; Palazolo et al., 2000). The more flexible the protein structure and/or the higher the unfolded species concentration, the more pronounced the slope in the Stern-Volmer plot and then, the greater the K_{app} is (Eq. (1)). Besides, during the unfolding process, the protein surface becomes more hydrophobic due to the appearance of non-polar amino acids, which were previously inside the protein structure.

The results of the present work were analyzed from Fig. 6. The surface hydrophobicity, measured trough the K_{app} values, significantly increased following the order: β-LG < β-LGh ~ GLI, while no significant differences were observed between TT, TT-h and TT-GLI. It can be seen that the slight modification of native β-LG conformation was more substantially disrupted by dry-state heating at 50 °C than by sugar conjugation (Fig. 6), which supports the results obtained by CD measurements.

The major exposition of Trp residues due to the partial unfolding of the protein after glycation, was also confirmed by the shift of maximal emission wavelength to higher values observed in the works of Corzo-Martínez, Moreno, Olano, and Villamiel (2008) and Medrano et al. (2009). In the latter studies, surface hydrophobicity was evaluated by using ANS (8-anilino-1-naphthalenesulfonic acid) as a fluorescent probe. Unlike the results observed in the present work, these authors reported that glycation produced a significant reduction of surface hydrophobicity. This reduction was attributed to protein aggregation as noted by Medrano et al. (2009). However, the decrease in surface hydrophobicity of the Maillard conjugates may be attributed not only to the formation of aggregates but also to the blocking of Lys residues by glycation, as ANS may also strongly bind cationic groups of proteins (Corzo-Martínez et al., 2008; Moro et al., 2001).

On the other hand, it was expected that the incorporation of sugar residues into the peptide chain could decrease the surface hydrophobicity. In that sense, Corzo-Martínez et al. (2008), performing RP-HPLC with a hydrophobic column C4, found that glycated β-LG had a lower retention time than the native protein. Nevertheless, it is important to note that C4 has a weak hydrophobic feature.

In conclusion, the results presented above were contradictory. Unfortunately, at present, there is no consensus on a standard absolute method for hydrophobicity measurements. Moreover, the distribution and periodicity of appearance of hydrophobic sites, or the location and distance between hydrophobic and hydrophilic sites may be important parameters which are not represented by the simple overall value of surface hydrophobicity.

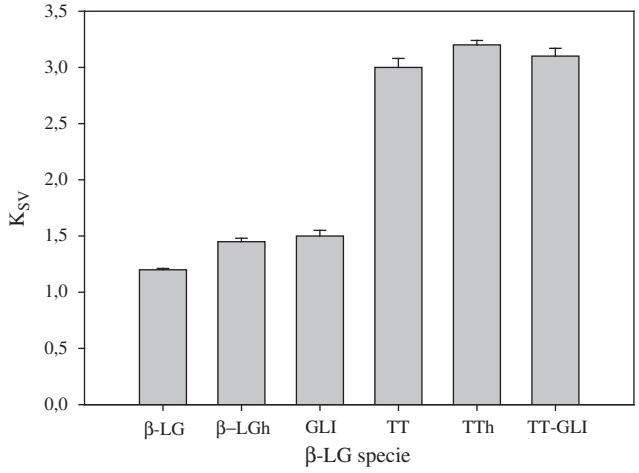

Fig. 6. K_{SV} of different protein samples. Error bars were calculated from the standard deviation of three replicates.

3.5. Thermal unfolding

Fig. 7 shows the changes in UV-difference absorbance at 293 nm for the different protein samples as a function of temperature. The unfolding of native β-LG monomer produced S-shaped denaturation profiles at temperatures between 60–90 °C, with an apparent temperature for 50% protein denaturation near 75 °C (Busti et al., 2005). ΔAbs_{293nm} measurements abruptly increased when the solution was heated above 90 °C, evidencing the formation of aggregates of different species of the protein (Busti et al., 2006). β-LG does not unfold to a random coil before aggregating in the final stage of the thermal denaturation (Cassal, Kohler, & Mantsch, 1988; Croguennec, Mollé, Mehra, & Bouhallab, 2004). Notwithstanding this, the buried sulfhydryl group of Cys121 is exposed with the partial unfolding of β-LG, initiating sulfhydryl/disulfide interchange reactions that lead to irreversible aggregation (Hoffmann & van Mil, 1997; Moro et al., 2011). However, there is evidence that in this aggregation reaction, non-covalent interactions must also be involved (Manderson, Hardman, &

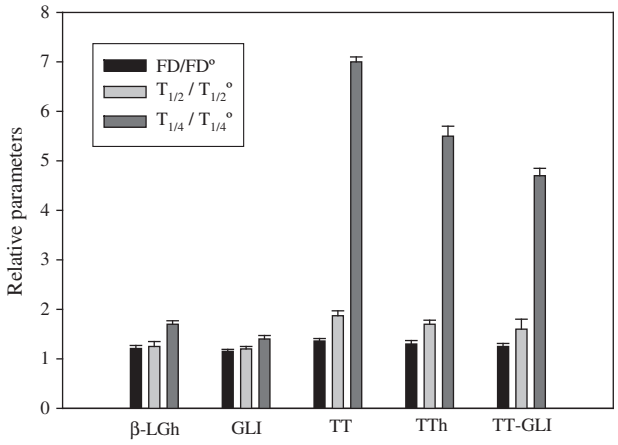

Fig. 8. Variation of relative foam density (FD/FD°) and relative decay times ($T_{1/2}/T_{1/2}^{\circ}$ and $T_{1/4}/T_{1/4}^{\circ}$) for different protein samples. FD°, $T_{1/2}^{\circ}$ and $T_{1/4}^{\circ}$ are the parameters for native β-LG. FD° = 0.0864 ± 0.0069, $T_{1/2}^{\circ}$ = 23.0 ± 1.8 s and $T_{1/4}^{\circ}$ = 11.0 ± 0.9 min. Error bars were calculated from the standard error of three replicates.

Creamer, 1998). The extent of their relative contribution to the overall aggregation process, however, is still unclear.

Spectroscopic measurements pointed to slight changes in the native conformation of β-LG produced by glycation. In spite of these results, GLI sample presented a very different thermal unfolding behavior when compared with native β-LG, Fig. 7. In this figure, it can be seen that GLI sample did not present a net S-shaped unfolding profile, showing a high increase in ΔAbs_{293nm} near 80 °C. This fact can be ascribed to protein aggregation, which involves sugar residues through non-covalent interactions before molecular unfolding took place. This aggregation masked the denaturation process that occurs prior to sulfhydryl/disulfide interchange reactions leading to irreversible aggregation.

3.6. Foaming properties

There are two distinct phases to protein foaming: (i) the effectiveness of gas encapsulation (foamability) and (ii) the lifetime of the foam (foam stability) (Foegeding et al., 2006; Wilde & Clark, 1996).

It is known that adsorption of a protein in a sufficient amount, within the time scale of foam production is a condition for efficient foam formation (foamability), strongly correlated with the rate at which surface tension can be lowered. Hence, one of the most important factors in foam formation is the protein adsorption rate, which depends on protein concentration, molecular weight of the protein, protein structure, and solution conditions such as pH (Martin, Grolle, Bos, Cohen Stuart, & van Vliet, 2002). Disordered, smaller and more flexible proteins are better surface agents than ordered, larger and rigid ones, leading to a greater affinity of the protein for the interface, which allows it to overcome the barrier against adsorption, which is developed at the interface while proteins are closely packed (Wilde & Clark, 1996). This fact promotes a rapid decrease in surface tension and then, an increase in protein foamability.

Fig. 8 shows the foamability of the different protein samples, measured as relative foam density (FD/FD°). It can be observed that native β-LG foamability was slightly improved by glycation (GLI). This improvement can be due to the partial increase in the tertiary structure flexibility of glycated protein, evidenced by electrophoretic, CD and fluorescence measurements. However, as β-LGh presented a similar value of relative foam density, it is possible that the moderate heat treatment applied during glycation in the dry state was actually responsible for this behavior, and not the sugar conjugation.

TT sample presents non-native β-LG monomers and other denatured species of higher molecular weights such as dimers and trimers (Moro et al., 2011). A previous heat treatment of β-LG solutions produces two important consequences simultaneously: (i) production of β-LG aggregates of higher molecular weights and (ii) thermal unfolding with exposure of hydrophobic patches on non-native β-LG. While the presence of aggregates produces a reduction in the diffusion coefficient, an unfavorable factor for foam formation, the exposure of hydrophobic patches is favorable for foamability. Taking into account the observed results, the increased surface hydrophobicity of TT sample became a more important factor, promoting a slight foam formation (Moro et al., 2011). Fig. 8 also shows that glycation did not modify TT foamability.

On the other hand, processes involved in foam destabilization are: liquid drainage, a close approach of adjacent bubble surfaces which leads to bubble coalescence and gas diffusion into the continuous phase (Ostwald ripening or disproportionation) resulting in bubble coarsening. All these mechanisms occur simultaneously after the air bubbling stops. While drainage and coalescence prevail at the beginning, when the bubbles are mainly spherical, disproportionation is more important at advanced stages, when the cells are polyedric. In the end, the foam collapses. It has been noted that the interfacial film of proteins formed during foam production assures the protection of foam against destabilization (Wilde & Clark, 1996).

Foaming stability for the different protein samples, estimated in this work either through $T_{1/2}/T_{1/2}^{\circ}$ or $T_{1/4}/T_{1/4}^{\circ}$ values, is also shown in Fig. 5. The observed increase of $T_{1/2}$ of treated samples is directly associated with an increase in protein solution viscosity due to the presence of aggregates of denatured β-LG with high hydrodynamic sizes, which slows the drainage rate (Foegeding et al., 2006; Moro et al., 2011). Electrophoretic measurements performed in this work (Table 1) confirmed the presence of relatively small quantities of aggregated proteins. Fig. 8 also evidenced that glycation did not produce significant modifications in the drainage rate of β-LGh, TTh and TT samples.

$T_{1/4}$ increased for all the treated samples even more than $T_{1/2}$ (Fig. 8). In this figure, it can be seen that the volume foam stability of β-LG was mainly improved by heat treatments rather than by sugar conjugation. The greatest changes were observed for TT and its treated samples. TT had an increased volume stability (~700%) value with respect to the native protein (Fig. 8), a fact which is consistent with the significant conformational changes that occur on β-LG molecules after protein solutions were heated (Kim et al., 2005; Moro et al., 2011) for 3 min at 85 °C.

While the drainage phenomenon finished, the foam volume was still considerable, since $T_{1/2} << T_{1/4}$ for all the samples assayed. The foam broke easily in the case of native β-LG, whereas better foam stability was observed for heated samples. These results agree with those reached by Martin et al. (2002) in that interfacial rheology plays an important role against disproportionation. Thus, aggregates formed by heating β-LG in the cited conditions led to more stabilized foams, slowing down disproportionation, because of the formation of stiffer films that resist compression and may reduce gas transport (Moro et al., 2011).

Croguennec et al. (2006) have suggested that the initial rheology (short-term) of the interfacial film is more important in the general mechanism of foam stabilization than the viscoelasticity that the interfacial film could reach on aging (long-term). Following the conclusions of these authors, it was assumed that the higher flexibility of treated β-LG species could facilitate their reorganization at the interface, leading to faster associations with neighboring molecules and thus to the rapid development (short-term) of a viscoelastic film. The main forces involved in the formation of this closely packed layer of adsorbed proteins could be hydrogen bonds, hydrophobic associations and electrostatic interactions rather than disulfide bonds formed by sulfhydryl-disulfide exchange reactions.

In the work of Moro et al. (2011) the presence of oligomers and polymers of higher molecular weight produced a steric impediment which

does not lead to the formation of a closely packed layer but to an open interfacial film, decreasing foam stability. The presence of these species, evidenced by electrophoretic measurements (Fig. 1 and Table 1), may be the cause of the observed decrease in foam volume stability of GLI, TTh and TT-GLI samples (Fig. 8). In summary, glycation in the assayed conditions showed a negative effect on the volume foam stability of pre-heated samples.

The results presented in this study were in agreement with the results achieved by Chevalier et al. (2001). However, these outcomes were opposite to the ones obtained by Medrano et al. (2009). These authors reported an increased foam stability of glucose-glycated samples even though similar reaction time and temperature (96 h at 50 °C) were used. The differences may be attributed to variations in the materials and methodology employed. Medrano et al. (2009) used a protein isolate that initially contained ~56% of aggregated species (dimer 50%, tetramer 3% and octamer 3%) as substrate of glycation. Besides, the authors reported that glucose binding produced an important amount of additional aggregates (~30 of oligomers).

On the other hand, while in the present work free sugars were removed by extensive dialysis against 20 mM phosphate buffer, pH 6.8, Medrano et al. (2009) used 20 mM ammonium carbonate for the same purpose. It is to be noted that the pH of 20 mM ammonium carbonate is near 9.5. Above pH 9.0, native β-LG undergoes irreversible base-induced unfolding (Taulier & Chalikian, 2001). It is possible that this base-induced change contributed to the presence of additional differences in the quality and quantity of the species formed in both works. In addition, Medrano et al. (2009) worked at pH 7.5 in the determination of foaming properties. At this pH, the Tanford transition occurs in the native β-LG molecule. As a consequence of this transition, the interior of the protein is opened and the carboxyl group of Glu89 becomes accessible to the solvent. This transition is accompanied by an increase in protein hydration and a loosening of the interior packing of the protein.

In summary, the contrary effects obtained by these authors when untreated β-LG was glycated may be ascribed to the initial presence of large amounts of aggregates and to the additional structural changes promoted by the pH used in some steps of their study. These protein species probably remain in solution during foam formation, increasing the viscosity of the medium. This increment results in foams that are more resistant to drainage than the non-glycated protein but less stable than the one only pre-heated TT sample.

4. Conclusions

Based on the results obtained in this work, it can be concluded that the effect of glucose-glycation on the foaming properties of β-LG strongly depends on the structural characteristics of the initial sample used. For protein samples containing a high proportion of monomers (~88%), the improvement on foaming properties (foamability and foam stability) was promoted by heat treatment in the dry state (96 h at 50 °C) rather than by sugar conjugation. Slight changes in the tertiary structure flexibility and the presence of small amounts of aggregates of higher molecular weight were detected after the glycation process.

When pre-heated β-LG (TT sample) was glycated with glucose, the presence of oligomers and polymers of the protein promotes a decrease in volume foam stability. This fact was ascribed to steric impediment that leads to the formation of an open interfacial film with diminished viscoelastic stiffness.

The variation of volume foam stability caused by the different treatments resulted from the action of these treatments on disproportionation rather than on liquid drainage from the foam.

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