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Interfacial and foaming properties of bovine β-lactoglobulin:galactose Maillard conjugates

[1]Marta Corzo-Martínez, [2]Cecilio Carrera Sánchez, [1]F. Javier Moreno, [2]Juan M. Rodríguez Patino and [1]Mar Villamiel*

[1]Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM), Nicolás Cabrera 9, Campus de la Universidad Autónoma de Madrid, 28049, Madrid, Spain.

[2]Departamento de Ingeniería Química, Facultad de Química, Universidad de Sevilla, Prof. García González 1, 41012, Sevilla, Spain.

*Author to who correspondence should be addressed

Tel +34 910017951; Fax +34 910017905

E-mail: m.villamiel@csic.es

Current address: Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM), Nicolás Cabrera 9, 28049, Madrid, Spain.

Abstract

In this paper, the effect of the initial and advanced steps of glycosylation by Maillard reaction (MR) (glycation) of β-lactoglobulin (β-Lg) with galactose on the interfacial and foaming (foamability and foam stability) properties of this protein has been studied at both pH 7 and pH 5. Hardly any effect of glycation was observed at pH 7. However, a pH 5, due to its increased solubility, β-Lg glycated at 50°C during 48 h (advanced steps of MR) presented the best dynamic of adsorption which lead to an increase of the surface dilatational modulus of adsorbed film. This resulted in a better foaming capacity, as well as higher stability of foams of β-Lg glycoconjugates with respect to native and control heated protein. These results could extend the applicability of β-Lg as a foaming agent, particularly in acid foods.

1. Introduction

Foaming characteristics of food dispersions are important in determining quality attributes of many foods (milk, meat, mayonnaise, spreads, ice cream, frozen desserts, cakes, breads, whipped toppings, etc.). The structure of many of these products depends upon the formation and stability of foam which facilitates mixing, imparts structure and contributes to sensory qualities. These dispersions are thermodynamically unstable, and their relative stability depends on the properties of the surface-active components in the system (Carrera & Rodríguez Patino, 2005; Rodríguez Patino, Carrera & Rodríguez Niño, 2008).

In the food industry, foams are stabilized mainly by proteins (Rullier, Novales, & Axelos, 2008), milk proteins being one of the most utilized. In particular β-lactoglobulin (β-Lg), which represents 50% of the total mass of the whey proteins, is widely used due to its high capacity to be adsorbed at the air/water interface, to decrease surface tension and to build interfacial elastic networks after unfolding (Kinsella, 1984; Phillips, Whitehead, & Kinsella, 1994; Murray, 1998). This protein is known to form thick interfacial layers close to its isoelectric point (pI 5.2) (Kinsella, 1984; Phillips et al., 1994; Wilde & Clark, 1996) and, under heat treatment, a very strong aggregation at pH close to pI can be produced. Thus, the formation of covalently bound protein aggregates through disulphide bridges (Schmitt et al., 2005) might alter the foaming properties of protein. However, at neutral pH it has been shown that partial unfolding of β-Lg through heat treatment improves its foaming properties (Bals & Kulozik, 2003; Davis & Foegeding, 2004; Kim, Cornec, & Narsimham, 2005). In this context, the search for processes that can efficiently improve the functional properties of proteins and therefore increase their degree of applicability is of increasing interest.

Among the different physical, chemical, or enzymatic treatments, leading to the modification of protein functionality, a great deal of attention has been focussed on the covalent interaction protein/carbohydrate via the Maillard reaction (MR). During this reaction, the conjugation of a reducing carbohydrate to the ε-amino group of lysine occurs spontaneously under heating conditions without the utilization of toxic chemical products (Chevalier, Chobert, Dalgalarrondo, & Haertlè, 2001a). Moreover, it is well-known that the Maillard reaction, carried out under dry state and well controlled conditions (temperature, relative humidity and time), is an adequate method for improving functionality of proteins without important structural changes (Morgan, Leonil, Molle, & Bouhallab, 1997; Oliver, Melton, & Stanley, 2006a; Oliver, 2011). Several studies have shown that glycation under controlled conditions, in addition to improve the heat stability of food proteins, including whey proteins, favours the protein diffusion at the air/water interface and its adsorption to the same, especially due to an increase in exposed hydrophobicity and molecular unfolding, improving the protein ability to form and stabilize foams (Schmitt, Bovay, & Frossard, 2005; Medrano, Abirached, Panizzolo, Moyna, & Anon, 2009). In this sense, the study of glycosylation via the MR (glycation) of β-Lg as a tool to improve its foaming and stabilizing capacity, particularly at pH values close to its pI, could be of interest.

Several authors have described a direct relationship between the foam formation and stability and the interfacial properties of adsorbed protein films (Martin, Grolle, Bos, Cohen-Stuart, & van Vliet, 2002; Murray, 2002; Rouimi, Schorsch, Valentini, & Vaslin, 2005; Rodríguez Patino et al., 2008). Among them, the dynamic of adsorption and the rheological properties of interfacial films have been shown to influence foam properties, depending on the mechanisms causing foam destabilization (Baeza, Carrera, Rodríguez Patino, & Pilosof, 2005; Rodríguez Patino et al., 2008; Martínez, Carrera,

Rodríguez Patino, & Pilosof, 2009). To the best of our knowledge, studies in the literature about the impact of β-Lg glycation on the interfacial properties and, consequently, on the foaming properties of this protein are very scarce. Schmitt et al. (2005) in β-Lg:acacia gum conjugated by Maillard reaction at pH 4.2, 5.3 and 7.0 observed a higher capacity to form and stabilize foams of glycoconjugates than unglycated β-Lg, especially at pH 5.3. These authors needed 14 days at 60 ºC to obtain the maximum level (15%) of NH_2 loss. Because of the reaction with polysaccharides needs strong conditions and long incubation periods, which would be more expensive from the industrial standpoint, the use of monosaccharides such as galactose, might be of interest, since it allows obtaining modified proteins with a high yield under milder reaction conditions (Corzo-Martínez et al. 2008).

Thus, the aim of this work was i) to study the effect of glycation with galactose on the adsorption of β-Lg at the air/water interface and to characterize the rheological properties of the interfacial films; and ii) to evaluate foaming properties (foamability and foam stability) of β-Lg glycoconjugates in relation to their interfacial behaviour.

2. Materials and methods

2.1. Materials

Galactose (Gal) and bovine β-lactoglobulin (β-Lg) (mixture of A and B variants) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Preparation and purification of β-Lactoglobulin-galactose conjugates

Gal and β-Lg in a weight ratio of 1:1 were dissolved in 0.1 M sodium phosphate buffer, pH 7 (Merck, Darmstadt, Germany), and lyophilized. The β-Lg-Gal powders

were kept at 40 and 50 °C for 24 and 48 h, respectively (Corzo-Martínez, Moreno, Olano, & Villamiel, 2008), under a vacuum in a desiccator equilibrated at an a_w of 0.44, achieved with a saturated K_2CO_3 solution (Merck). In addition, control experiments were performed with β-Lg stored at 40 and 50 °C without galactose during the same periods (control heated β-Lg).

After incubation, the products were reconstituted in distilled water to a protein concentration of 1 mg/mL. To remove free carbohydrate, 2 mL portions were ultrafiltered through hydrophilic 3 kDa cut-off membranes (Centricon YM-3, Millipore Corp., Bedford, MA) by centrifugation at 1,548 x *g* for 2 h. After removal of free Gal, samples were lyophilized and stored at -20 ºC for further analysis.

Incubations were performed in duplicate, and all analytical determinations were performed at least in duplicate.

2.3. Solubility of β-lactoglobulin conjugates

For solubility evaluation, solutions of native, control and glycated β-Lg in distilled water (1 mg/mL) were adjusted to pH 5 and 7 using HCl or NaOH 1 N. After 30 min of stirring at room temperature, the samples were centrifuged for 15 min at 4 ºC and 15,000 x *g*. The protein content in the supernatants was determined by measuring the absorbance at 280 nm (A_{280}) in a Beckman DU 70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA) and the solubility was expressed as the percentage of the total protein content, considering as 100% the A_{280} of native β-Lg.

2.4. Interfacial properties measurement

Interfacial properties (dynamic of surface pressure and surface dilatational properties) of native, control heated and glycated β-Lg were determined at pH 7 and 5.

For this, samples were dissolved in Trizma-HCl buffer (0.05 M, pH 7.0) or acetic acid/acetate buffer (0.05 M, pH 5) (Sigma-Aldrich, St. Louis, MO), the final protein concentration being 5 mg/mL.

Time-dependent surface pressure and surface dilatational measurements of native, control heated and glycated β-Lg adsorbed films at the air/water interface were performed with an automatic pendant drop tensiometer (TRACKER, IT Concept, Longessaine, France) as previously described (Rodríguez Patino, Rodríguez Niño, & Carrera, 1999; Rodríguez Niño & Rodríguez Patino, 2002). The method involved a periodic automated-controlled, sinusoidal interfacial compression and expansion performed by decreasing and increasing the drop volume at a given desired amplitude ($\Delta A/A$) and angular frequency (ω), and the response of the surface pressure (π, $mN \cdot m^{-1}$) is monitored throughout the experiment, being:

$$\pi = \sigma^0 - \sigma \quad (1)$$

where σ^0 is the surface tension of aqueous solution, in the absence of protein (σ^0 = 72.5 $mN \cdot m^{-1}$), and σ ($mN \cdot m^{-1}$) is the surface tension in the presence of protein.

Since rate of increase of π is initially controlled by the protein diffusion from the bulk phase to the interface, in this work, dynamic of protein adsorption was evaluated considering the first stage of the protein diffusion, by determinating the apparent diffusion constant (K_{dif}). This was calculated as the slope of the line between the origin (point 0.0) and first point on the plot π vs. square root of time (θ).

Regarding surface rheological parameter, the surface dilatational modulus (E) derived from the change in interfacial tension (dilatational stress), σ (Eq. (2)), resulting from a small change in surface area (dilatational strain), A (Eq. (3)), may be described by Eq. (4) (Lucassen and van den Tempel, 1972):

$$\sigma = \sigma_0 \sin(\omega \cdot \theta + \phi) \quad (2)$$

$$A = A_0 \sin(\omega \cdot \theta) \quad (3)$$

$$E = \frac{d\sigma}{dA/A} = -\left(\frac{d\pi}{d \ln A}\right) = |E|e^{i\phi} = E_d + iE_v \quad (4)$$

where σ_0 and A_0 are the stress and strain amplitudes, respectively, θ is the time, ϕ is the phase angle between stress and strain, and |E|, the absolute modulus, a measure of the total unit material dilatational resistance to deformation (elastic + viscous), is the ratio (σ_0/A_0).

Surface dilatational modulus (E) is a complex quantity and it is composed of real and imaginary parts. The real part of the dilatational modulus (or storage component) is the dilatational elasticity, $E_d = |E| \cdot \cos\phi$. The imaginary part of the dilatational modulus (or loss component) is the surface dilatational viscosity, $E_v = |E| \cdot \sin\phi$. The phase angle (ϕ) between stress and strain is a measure of the relative film elasticity. For a perfectly elastic material stress and strain are in phase ($\phi = 0$) and the imaginary term is zero. In the case of a perfectly viscous material, $\phi = 90^o$ and the real part is zero.

Interfacial experiments were carried out at 20 ± 0.3 ºC. The temperature was maintained constant by circulating water from a thermostat. Sample solutions were placed in the syringe and subsequently in a compartment, and they were allowed to stand for 30 min to reach the desired constant temperature. Then a drop was delivered and allowed to stand for 10,800 s to achieve protein adsorption at the air–water interface. Surface rheological parameters (E, E_d, E_v and ϕ) were measured as a function of adsorption time (θ), at 10% of deformation amplitude ($\Delta A/A$) and at 0.1 Hz of angular frequency (ω). Sinusoidal oscillation for surface dilatational measurement was

made with five oscillation cycles followed by a time of 50 cycles without any oscillation up to the time required to complete adsorption. Measurements were made at least twice. The average standard accuracy of the surface pressure was roughly 0.1 mN/m. The reproducibility of the results was better than 0.5% and 5.0% for surface pressure and surface dilatational properties, respectively.

2.5. Foaming properties

The foaming properties of native, control heated and glycated β-Lg solutions were characterized through their foam formation and stability measured in a commercial instrument (Foamscan IT Concept, Longessaigne, France), based on the ideas by Popineau and co-authors (Guillerme, Loisel, Bertrand, & Popineau, 1993; Loisel, Guégan, & Popineau, 1993). With this instrument the foam formation, the foam stability and the drainage of liquid from the foam can be determined by conductimetric and optical measurements. The foam is generated by blowing gas (nitrogen) at a flow of 45 mL/min through a porous glass filter (pore diameter 0.2 mm) at the bottom of a glass tube where 20 mL of sample solution under investigation is placed. The foam volume is determined by use of a CCD camera. The drainage of water from the foam is followed via conductivity measurements at different heights of the foam column. A pair of electrodes at the bottom of the column was used for measuring the quantity of liquid that was not in the foam, while the volume of liquid in the foam was measured by conductimetry in three pairs of electrodes located along the glass column. In all experiments, the foam was allowed to reach a volume of 120 mL. The bubbling was then stopped and the evolution of the foam was analyzed. Foaming properties were measured at 20 ºC from protein aqueous solutions (5 mg/mL) at pH 5 and 7 and at an ionic strength of 0.05 M.

Four parameters were determined as a measure of foaming capacity. The overall foaming capacity (OFC, mL/s) was determined from the slope of foam volume curve till the end of the bubbling. The foam capacity (FC), a measure of gas retention in the foam, was determined by Eq. (5). The foam maximum density (MD), a measure of the liquid retention in the foam, was determined by Eq. (6). The relative foam conductivity (C_f, %) is a measure of the foam density and was determined by Eq. (7).

$$FC = \frac{V_{foam}(f)}{V_{gas}(f)} \quad (5)$$

$$MD = \frac{[V_{liq}(i) - V_{liq}(f)]}{V_{foam}(f)} \quad (6)$$

$$C_f = \left[\frac{C_{foam}(f)}{C_{liq}(f)}\right] \quad (7)$$

where $V_{foam}(f)$ is the final foam volume, $V_{gas}(f)$ is the final gas volume injected, $V_{liq}(i)$ and $V_{liq}(f)$ are the initial and final liquid volumes, and $C_{foam}(f)$ and $C_{liq}(f)$ are the final foam and liquid conductivity values, respectively.

The static foam stability was determined from the volume of liquid drained from the foam over time (Rodríguez Patino, Naranjo, & Linares, 1995; Rodríguez Patino, Rodríguez Niño, & Álvarez, 1997). For this, it was calculated the half-life time ($\theta_{1/2}$), referring to the time needed to drain the half the volume of liquid of foam.

2.6. Statistical analysis

Statistical analysis was performed using the Statgraphic CENTURION XV Program (Statistical Graphics Corporation, Rockville, MD, USA) for Windows. One-way analysis of variance (ANOVA) (least significant difference, LSD, test) was used

for the statistical evaluation of results derived from interfacial and foaming determinations of the glycated and unglycated β-Lg. Differences were considered significant when $P< 0.05$.

3. Results and discussion

On the basis of a previous paper of our research group (Corzo-Martínez et al., 2008), two types of glycoconjugates were prepared at different stages of the Maillard reaction, one of them, in early stages of the MR (β-Lg:Gal [24 h, 40 ºC]), consisted primarily of complexes with a high glycation degree and a low aggregation level, while the glycoconjugate obtained after incubation under more severe conditions (β-Lg:Gal [48 h, 50 ºC]), in the advanced stages of the MR, exhibited, in addition of a high glycation degree, an elevated content of protein aggregates.

In that paper, the progress of the Maillard reaction was evaluated by different methods. Thus, MALDI-TOF-MS analyses revealed that an average number of 14 and 22 molecules of Gal were covalently linked to β-Lg after incubation at 40 ºC for 24 h and at 50 ºC for 48 h, respectively. Isoelectric focusing (IEF) analysis also showed a high glycation degree of β-Lg, being observed a noticeable shift of the isoelectric point of β-Lg glycated especially at 50 ºC toward more acidic pH as a result of the loss of basicity and, consequently, the increase in negative charge of the β-Lg molecule due to the blocking of Lys and Arg residues with carbohydrates.

Concerning conformational characterization of glycoconjugates, Corzo-Martínez et al. (2008) also observed a slight shift of the tryptophan (Trp) emission maximum at 50 ºC, whilst no shift of the Trp emission maximum was detected after glycation of β-Lg at 40 ºC, suggesting that important structural changes in the three dimensional configuration of the protein occurred at 50 ºC. However, glycation at 40 ºC, although

partially affected the side chains of the protein in the tertiary structure, did not cause a great disruption of the native structure. According to this, a great decrease in surface hydrophobicity (S_0) of β-Lg:Gal [48 h, 50 ºC] was found, while glycation at 40 ºC only lead to a slight increase in β-Lg surface hydrophobicity, probably due to the exposition of hydrophobic patches on the protein surface, as a consequence of its partial denaturation. Likewise, results from size exclusion chromatography (SEC) showed that, unlike β-Lg:Gal conjugate at 40 ºC that eluted predominantly as a protein dimmer, SEC profile of conjugate at 50 ºC displayed trimeric and oligomeric forms, indicating that glycation under these reaction conditions of β-Lg promoted its polymerization.

3.1. Solubility

Since the solubility of a protein is a determining factor of its dynamic of adsorption at the interface and, consequently, of its foaming capacity, we determined solubility of all samples studied, previously to functionality studies.

Figure 1 depicts the solubility values obtained for native, control heated and glycated β-Lg at pH 5 and 7. Native β-Lg showed a maximum solubility at pH 7. At pH 5, close to its pI, it remained highly soluble, with a solubility of approximately ~ 86%, in agreement with other authors (Nacka et al. 1998; Chevalier et al. 2001b; Jimenez-Castaño et al. 2005, 2007). However, solubility at pH 5 of control β-Lg heated at 40 and 50 ºC significantly ($P<0.05$) decreased (a 30-35%).

With respect to the glycation effect, at pH 7, whereas conjugation with Gal at 40 ºC for 24 h did not modify the β-Lg solubility, glycation under more severe incubation conditions (48 h at 50 ºC) significantly ($P<0.05$) decreased solubility of such protein. This might be due to the formation of high molecular weight and insoluble aggregates during the advanced stages of the MR, according to results derived from SEC analyses

285 (Corzo-Martínez et al., 2008). At pH 5, nevertheless, β-Lg glycated at 40 and,
286 particularly, 50 ºC showed a significantly ($P<0.05$) higher solubility than that of native
287 and control heated β-Lg, which could be attributed to the shift of minimum solubility
288 (pI) of glycated protein to a lower pH, according to previous results derived from IEF
289 (Corzo-Martínez et al., 2008). Moreover, in the case of glycoconjugate obtained at 50
290 ºC, the fact that β-Lg aggregates formed during the advanced stages of the MR are more
291 soluble at pH 5 than at pH 7 (Figure 1) is particularly striking.

292 Some previous data in the literature have indicated that a higher formation of
293 insoluble moisture-induced whey protein aggregates were formed at pH 7 than at pH 5,
294 after storage for 14 days at 35 ºC. These authors indicated that these differences were
295 due to a different ratio between the thiolate anion and the thiol group (reactive form to
296 nonreactive form), which are responsible for the formation of intermolecular disulfide
297 bonds (Zhou et al., 2008).

298

299 ***3.2. Interfacial properties***

300 ***3.2.1. Dynamic of protein adsorption at air-water interface***

301 Dynamic of adsorption of native, control heated and glycated β-Lg was studied in
302 relation to its diffusion rate to the interface, represented by the apparent diffusion
303 constant (K_{dif}), and to its ability to increase the surface pressure (π) with the adsorption
304 time (θ) (Figure 2).

305 At pH 7 (Figure 2 (A)), surface activity of β-Lg glycated at 40 ºC was slightly
306 higher than that of native and control heated β-Lg and significantly higher than that of
307 β-Lg glycated at 50 ºC, probably due to better solubility of the conjugate in early stages
308 of the MR. Moreover, surface activity of β-Lg glycated at 50°C was very similar to that
309 of native and control heated β-Lg, no substantial differences being observed between

310 the values of surface pressure reached at long term adsorption (π at 10800 s, π_{10800}) and,
311 hence, between the amount of glycated and unglycated protein adsorbed to the air/water
312 interface.

313 However, when we studied the dynamic of adsorption during the first stage of
314 protein diffusion (Figure 2 (C)), we appreciated differences between the studied
315 systems. In particular, control β-Lg heated at 40 and 50 ºC and β-Lg glycated under
316 mild time and temperature conditions (24 h at 40 ºC) showed a K_{dif} value significantly
317 higher than that of native β-Lg. In agreement with the positive relation observed by
318 several authors between the diffusion rate of proteins and their surface hydrophobicity
319 (Wagner Sorgentini, & Añón, 2000; Moro, Gatti, & Delorenzi, 2001; Kim et al., 2005;
320 Pérez, Carrara, Carrera, & Rodríguez Patino, 2009), these results could be attributed to
321 the higher surface hydrophobicity (Corzo-Martínez et al., 2008) and, thus, higher
322 affinity for the air/water interface, of control heated and glycated β-Lg (24 h at 40 ºC) as
323 compared to native protein as a consequence of their partial heat denaturation. Likewise,
324 the lower surface hydrophobicity and solubility of β-Lg glycated with Gal at 50 ºC for
325 48 h, as a result of the formation of high molecular weight aggregates, could explain the
326 significantly ($P<0.05$) slower diffusion to the air/water interface of this conjugate, as
327 indicated by its lower K_{dif} value as compared to the rest of the assayed systems.

328 Regarding the results obtained at pH 5 (Figures 2 (B) and (D)), dynamic of
329 adsorption of native β-Lg, at both short and long times, was hardly altered by the pH
330 reduction, observing K_{dif} and π_{10800} values very similar to those obtained at pH 7. This
331 might be related to the high solubility showed by this protein in native form at pH 5.
332 Instead, control β-Lg heated at 40 and 50 ºC showed a lower K_{dif} than at pH 7 (Figure 2
333 (C) and (D)), probably due to its reduced solubility at pH 5 as a consequence of the

formation of protein aggregates that slow down the protein diffusion to the air/water interface.

Concerning glycation effect, dynamic of adsorption of β-Lg glycated at 40 ºC (Figure 2 (B)) was not altered as a result of the pH reduction, being its diffusion rate to the interface higher than that of control heated β-Lg (Figure 2 (D)). These results could be attributed to the high solubility at pH 5 of this conjugate as compared to that of control heated protein (Figure 1).

The most remarkable result was obtained with β-Lg glycated at 50 ºC (Figure 2 (D)), which showed a diffusion rate significantly ($P<0.05$) higher than that of control β-Lg heated at 50 ºC. In addition, a clear increase in its diffusion rate at pH 5 with respect to pH 7 was also observed, in agreement with the high solubility of this conjugate at pH 5 (Figure 1).

3.2.2 Surface dilatational properties

With the purpose of studying the rheological properties of adsorbed films of native, control heated and glycated β-Lg, their surface dilatational modulus (E) was plotted versus time (θ) (Figures 3 (A) and 4 (A)) and versus surface pressure (π) (Figures 3 (B) and 4 (B)), this second type of representation providing additional information on the extent of interactions between components of the adsorbed film.

In general, at pH 7, E-π plots (Figure 3 (B)) of all the systems studied were above the behaviour of an ideal fluid, not viscous (dashed line), suggesting the existence of relatively large interactions between components of the adsorbed film (Lucassen-Reynders, Lucassen, Garrett, & Hollway, 1975). According to several authors, this could be due to the partial denaturation of β-Lg, once adsorbed at the air/water

358 interface, allowing the intermolecular interaction via thiol-disulfide exchange, that
359 increase the rigidity and cohesion of the interfacial film.

360 Control β-Lg heated at both 40 and 50 ºC gave rise to the formation of a film with
361 higher E values than that of native β-Lg (Figure 3 (A)), probably due to its higher
362 efficiency of adsorption at the interface (higher K_{dif}) (Figure 2 (C)) (Bos & van Vliet,
363 2001; Rodríguez Patino et al., 2008).

364 Likewise, whereas glycation at 40 ºC hardly altered rheological characteristics of
365 adsorbed film of β-Lg (Figure 3 (A)), being only observed a slight decrease in the
366 dilatational modulus at long term adsorption (E at 10800 s, E_{10800}) with respect to native
367 β-Lg, protein glycated at 50 ºC led to the formation of a film with the lowest E values
368 for a given time as compared to films of native, control heated and glycated (24, 40 ºC)
369 protein. Wooster & Augustin (2007) obtained similar results in a study on the
370 rheological properties of the adsorbed films formed by WPI glycated with dextrans of
371 different molecular weights. In agreement with these authors and taking into account the
372 results of intrinsic fluorescence obtained in a previous work (Corzo-Martínez et al.,
373 2008), the decrease observed in the dilatational modulus (E) of the β-Lg:Gal [48 h, 50
374 ºC] adsorbed film might be due to structural changes undergone by protein during the
375 advanced stages of the MR, since alteration of the conformational state of protein is
376 responsible for the loss of its structural rigidity and, consequently, the loss of firmness
377 of the adsorbed film.

378 Moreover, as observed in Figure 3 (B), β-Lg:Gal [48 h, 50 ºC] conjugate showed
379 the lowest and closest values to the ideal behaviour E-π values, indicating the existence
380 of weak interactions between components of the adsorbed film.

381 On the other hand, the phase angle (ϕ) can be considered as a measure of the
382 relative elasticity of the adsorbed protein films. So the more pronounced the decline of

383 the phase angle values with the adsorption time (θ) or the surface pressure (π), the
384 greater the elasticity of the adsorbed protein film, and vice versa.

385 In general, for all the studied systems, including native, control heated and
386 glycated β-Lg, the phase angle (ϕ) decreased with increasing adsorption time (θ)
387 (Figure 3 (C)) and surface pressure (π) (Figure 3 (D)), indicating the formation of
388 elastic films. However, for a given time and pressure, the highest ϕ values were
389 observed with control heated β-Lg, indicating the formation of a film with a fluid
390 character. This result suggests that the higher E values observed with this system could
391 be due to its molecular packing as a result of the rapid protein adsorption at the
392 interface, and not due to the increase in the interaction degree between the adsorbed
393 molecules (Rodríguez Patino et al., 1999, 2003). Likewise, according to its low
394 dilatational modulus (E) (Figure 3 (A)), the film formed by β-Lg glycated at 50 ºC
395 showed a phase angle (ϕ) for a given time (θ) (Figure 3 (C)) and pressure (π) (Figure 3
396 (D)) higher than that of films of native and glycated (at 40 ºC) β-Lg, indicative of a
397 lower interaction degree between the film components and, hence, of a more fluid
398 character of this film (Horne & Rodríguez Patino, 2003; Rodríguez Patino et al., 2008).

399 At pH 5, the variation of the dilatational modulus (E) over time (θ) for native β-
400 Lg was little changed with respect to pH 7 (Figure 4 (A)). In other structural studies
401 carried out with β-Lg films, other authors have demonstrated that the pH effect on the
402 dilatational modulus and structure of β-Lg films is negligible as compared to that
403 observed for other proteins such as β-casein (Rodríguez Patino et al., 1999; Rodríguez
404 Patino, Carrera, Rodríguez Niño, & Cejudo, 2001; Rawel, Rohn, Kruse, & Kroll, 2002;
405 Zhang, Foegeding, & Hardin, 2004; Medrano et al., 2009). These authors related the
406 results obtained to the globular nature of β-Lg, since globular proteins generally retain
407 their native structure when they are initially adsorbed at the interface.

The film formed by control heated β-Lg showed, for a given time (θ) (Figure 4 (A)) and pressure (π) (Figure 4 (B)), E values lower than that of native β-Lg and those reached at pH 7, which could be related to its lower adsorption efficiency at pH 5.

Likewise, at pH 5, β-Lg glycated at 40 ºC led to the formation of a film with E values similar to those of native β-Lg film at short times of adsorption. Moreover, unlike at pH 7, surface dilatational modulus (E) of this film notably increased with the adsorption time, suggesting the formation of high intensity interactions between the film components.

At pH 5, the most remarkable differences with respect to pH 7 were observed with β-Lg:Gal [48 h, 50 ºC] conjugate, which gave rise to the film with the highest E values for a given time (θ) (Figure 4 (A)) and pressure (π) (Figure 4 (B)), suggesting, respectively, the formation of a highly elastic and cohesive film, with a great interaction degree between its components. These results are related to the improvement observed in the solubility and, subsequently, in the dynamic of adsorption of this conjugate at pH 5, so that this leads to an increase of the surface dilatational modulus of adsorbed film. In addition, β-Lg glycated at 50 ºC displayed the lowest ϕ values over the time (Figure 4 (C)) and pressure (Figure 4 (D)), which is indicative of the formation of a more elastic and resistant film than that of native, control heated and glycated (at 40 ºC) protein, in agreement with the high E values observed for this system.

3.3. Foaming properties

3.3.1 Foaming capacity

The values of the overall foaming capacity (OFC, mL/s), the foam capacity (FC), the foam maximum density (MD), and the relative foam conductivity (C_f, %) obtained with each of the systems assayed at pH 7 and 5 are shown in Figure 5.

At pH 7, native, control heated (40 and 50 ºC) and glycated (40 ºC) β-Lg showed the same foaming properties (no significant differences between values of OFC, FC and MD), only differing in the value of C_f. These results indicate that the increase produced in the protein diffusion rate (K_{dif}) as a result of the heat treatment or glycation at 40 ºC (Figure 2 (C)) has no significant effect on its foaming capacity, probably due to that the protein diffusion rate is already good enough for the system foams. This same behaviour can best be seen in Figure 6 (A), where a higher K_{dif} value did no result in significant increase ($P<0.05$) in the OFC value.

Glycation at 50 ºC, however, had a negative effect on β-Lg foaming capacity at pH 7, observing values for the formation parameters OFC and FC significantly ($P<0.05$) lower with β-Lg:Gal [48 h, 50 ºC] conjugate than with native, control heated (40 and 50 ºC) and glycated (40 ºC) protein. These results are related to the low K_{dif} and E values at short times previously observed for this conjugate (Figures 2 (C) and 3 (A)). This fact indicates that the low foaming capacity of β-Lg:Gal [48 h, 50 ºC] conjugate at pH 7 is likely due to that its rate of diffusion at the interface and dilatational characteristics of adsorbed film are not good enough to stabilize the bubbles during its formation.

At pH 5 (Figure 5), similar to adsorption efficiency (Figure 2 (D)), the foaming capacity of native β-Lg did not undergo substantial changes with respect to pH 7. Regarding the effect of the heat treatment in absence of Gal, foams formed with control protein heated at 40 and 50 ºC showed OFC and FC values significantly ($P<0.05$) lower

than that formed with native β-Lg, probably because its lower solubility and, consequently, worse adsorption efficiency at the air/water interface at this pH (Figure 5).

β-Lg glycated at 40 and 50 ºC displayed a foaming capacity significantly ($P<0.05$) higher than that of control heated protein and similar to that of native protein, observing no significant differences between OFC and FC values. These results are in good agreement with the dynamic of adsorption previously observed at pH 5 for these systems, which, regardless of being glycated or unglycated, showed a diffusion rate (K_{Dif}) and a surface activity (π-θ) very similar (Figures 2 (B) and (D)).

Moreover, by comparing the results obtained at pH 5 and at pH 7, we observed no important differences between the OFC and FC values of native and glycated (at 40 ºC) β-Lg, but a significant increase ($P<0.05$) in these parameters was found in the case of β-Lg glycated at 50 °C. This increase was probably due to the higher diffusion rate (K_{dif}) to the air/water interface displayed by this conjugate at pH 5 (Figure 2 (D)) with respect to that showed at pH 7. This behaviour can best be seen in Figure 6 (B), where it can be observed how systems with a higher K_{dif} also showed a higher OFC.

3.3.2 Foam stability

To evaluate the capacity to stabilize foams of β-Lg glycoconjugates, the half-life time ($\theta_{1/2}$, s) of foams formed with all the systems assayed was determined (Figure 7).

As observed in Figure 7 (A), at pH 7, stability of foam formed with native β-Lg was higher than that of foams with control heated and glycated protein, particularly at 50 ºC. This is consistent with the worse surface dilatational properties of adsorbed films formed by these systems (Figure 3).

At pH 5 (Figure 7 (B)), the half-life time of foam with native β-Lg (569 ± 26.87 s) did not substantially changed with respect to that obtained at pH 7 (575 ± 0.00 s), a fact that is related to the stability of surface dilatational modulus (E) of film of this protein against changes in pH. Likewise, the worse interfacial characteristics (dynamic of adsorption and surface dilatational properties) observed for the films formed by control heated β-Lg at pH 5 as compared to those of native and glycated protein resulted in a lower stability of foams containing control heated protein as foaming agent.

On the other hand, unlike at pH 7, glycoconjugates were found to be the best stabilizing agents at pH 5. Thus, the half-life time ($\theta_{1/2}$, s) of foam with β-Lg glycated, particularly at 50 ºC, was notably ($P<0.05$) higher than that of foams with native and control heated protein. This could be attributed to the increase observed in surface dilatational modulus (E) with increasing time (Figure 4 (A)) and pressure (Figure 4 (B)) for this system, suggesting the formation of an elastic film with a high degree of interaction between its components and, hence, with a high stability against mechanisms of foam destabilization such as drainage of fluid, diffusion or collapse.

4. Conclusions

Although at pH 7 glycation hardly changed the interfacial and foaming characteristics of β-Lg, at pH 5, both β-Lg:Gal glycoconjugates showed a better dynamic of adsorption to the air/water interface as compared to their corresponding controls of protein heated in absence of Gal. This resulted in a better foaming capacity of β-Lg glycoconjugates with respect to native and control heated protein. Likewise, the higher rigidity, cohesion (interaction degree in the interface) and elasticity of adsorbed films formed by β-Lg glycated at 40 and, particularly, 50 ºC led to a higher stability of

foams containing these complexes as stabilizing agents as compared to those foams with native and control heated β-Lg.

Therefore, from the findings described in this work we can infer that conjugation of β-Lg with galactose via the Maillard reaction could be a good alternative to consider when using this protein as a foaming agent. This reaction may extend the applicability range of β-Lg allowing its use as a foaming agent in acidic foods such as carbonated beverages, protein-fortified beverages (fruit juices, sports drinks and varieties of these beverages with long shelf-life), manufactured meats, reformed fish products, and a variety of formulated foods. In this way, a future work will be the study of the stability as foam agents of these potential ingredients during the processing and storage of acidic foods.

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686 **Figure captions**

687

688 **Figure 1.** Solubility at pH 5 and 7 of native, control heated and glycated β-Lg at
689 40 and 50 ºC during 24 and 48 h, respectively. Error bars indicate the standard deviation
690 of the mean. [a-e] Different case letters indicate statistically significant ($P<0.05$)
691 differences.

692

693 **Figure 2.** Surface pressure (π) as a function of time (θ) of adsorbed protein films
694 (**A** and **B**) and kinetic behaviour during the diffusion stage (**C** and **D**) of ✳ native β-Lg;
695 control heated β-Lg △ 24 h at 40 ºC and □ 48 h at 50 ºC; and glycated β-Lg ○ 24 h at
696 40 ºC and ◇ 48 h at 50 ºC at pH 7 (**A** and **C**) and pH 5 (**B** and **D**). Error bars indicate
697 the standard deviation of the mean. [a-c] Different case letters indicate statistically
698 significant ($P<0.05$) differences.

699

700 **Figure 3.** Surface dilatational modulus (E) and phase angle (ϕ) as a function of
701 time (θ) (**A** and **C**) and surface pressure (π) (**B** and **D**) of adsorbed films of ✳ native β-
702 Lg; control heated β-Lg △ 24 h at 40 ºC and □ 48 h at 50 ºC; and glycated β-Lg ○ 24
703 h at 40 ºC and ◇ 48 h at 50 ºC at pH 7.

704

705 **Figure 4.** Surface dilatational modulus (E) and phase angle (ϕ) as a function of
706 time (θ) (**A** and **C**) and surface pressure (π) (**B** and **D**) of adsorbed films of ✳ native β-
707 Lg; control heated β-Lg △ 24 h at 40 ºC and □ 48 h at 50 ºC; and glycated β-Lg ○ 24
708 h at 40 ºC and ◇ 48 h at 50 ºC at pH 5.

709

Figure 5. Values obtained for the parameters of overall foaming capacity (OFC, mL/s), foam capacity (FC), foam maximum density (MD), and relative foam conductivity (C_f, %) with native, control heated and glycated β-Lg at 40 and 50 ºC during 24 and 48 h, respectively, at pH 7 (solid bars) and pH 5 (hatched bars). Error bars indicate the standard deviation of the mean. [a-c] Different case letters indicate statistically significant ($P<0.05$) differences.

Figure 6. Relationship between the rate of diffusion (K_{dif}) at the air/water interface and the overall foaming capacity (OFC) of native, control heated and glycated β-Lg at 40 and 50 ºC during 24 and 48 h, respectively, at pH 7 (**A**) and pH 5 (**B**). ✳Native β-Lg; △ control heated β-Lg 24 h, 40 ºC; ○ β-Lg:Gal 24 h, 40 ºC; □ control heated β-Lg 48 h, 50 ºC; ◇ β-Lg:Gal 48 h, 50 ºC.

Figure 7. Stability (half-life time, $\theta_{1/2}$) at pH 7 (solid bars) (**A**) and pH 5 (hatched bars) (**B**) of foams formed with native, control heated and glycated β-Lg at 40 and 50 ºC during 24 and 48 h, respectively, as stabilizing agent. Error bars indicate the standard deviation of the mean. [a-e] Different case letters indicate statistically significant ($P<0.05$) differences.

Figure 1.

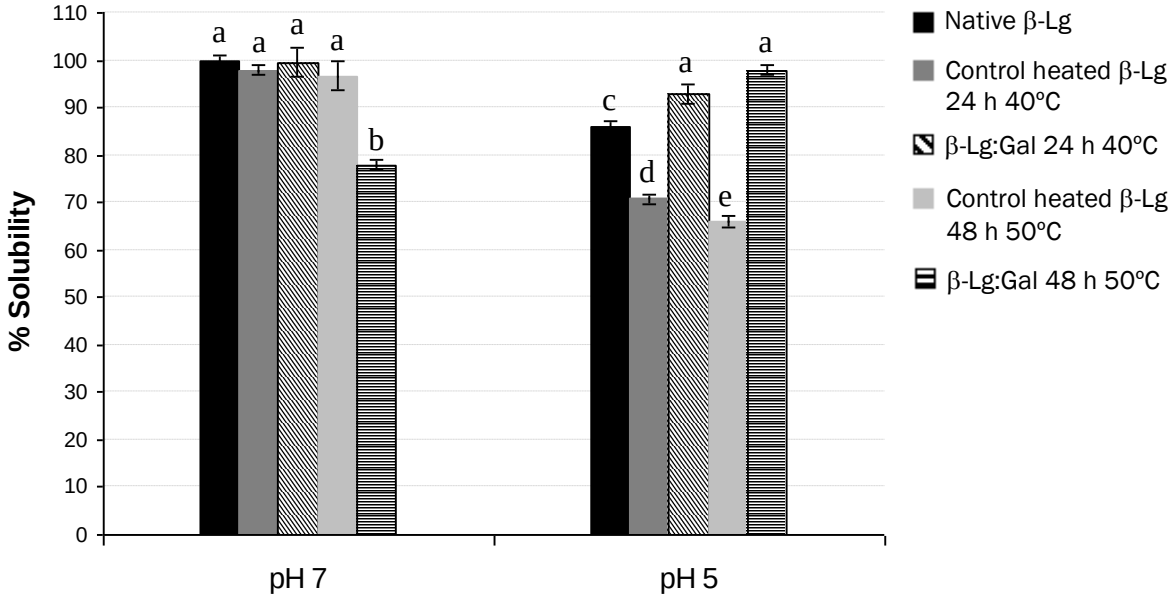

% Solubility
110
100
90
80
70
60
50
40
30
20
10
0
a
a
a
a
b
c
d
a
e
a
pH 7
pH 5
Native β-Lg
Control heated β-Lg 24 h 40°C
β-Lg:Gal 24 h 40°C
Control heated β-Lg 48 h 50°C
β-Lg:Gal 48 h 50°C

Figure 2.

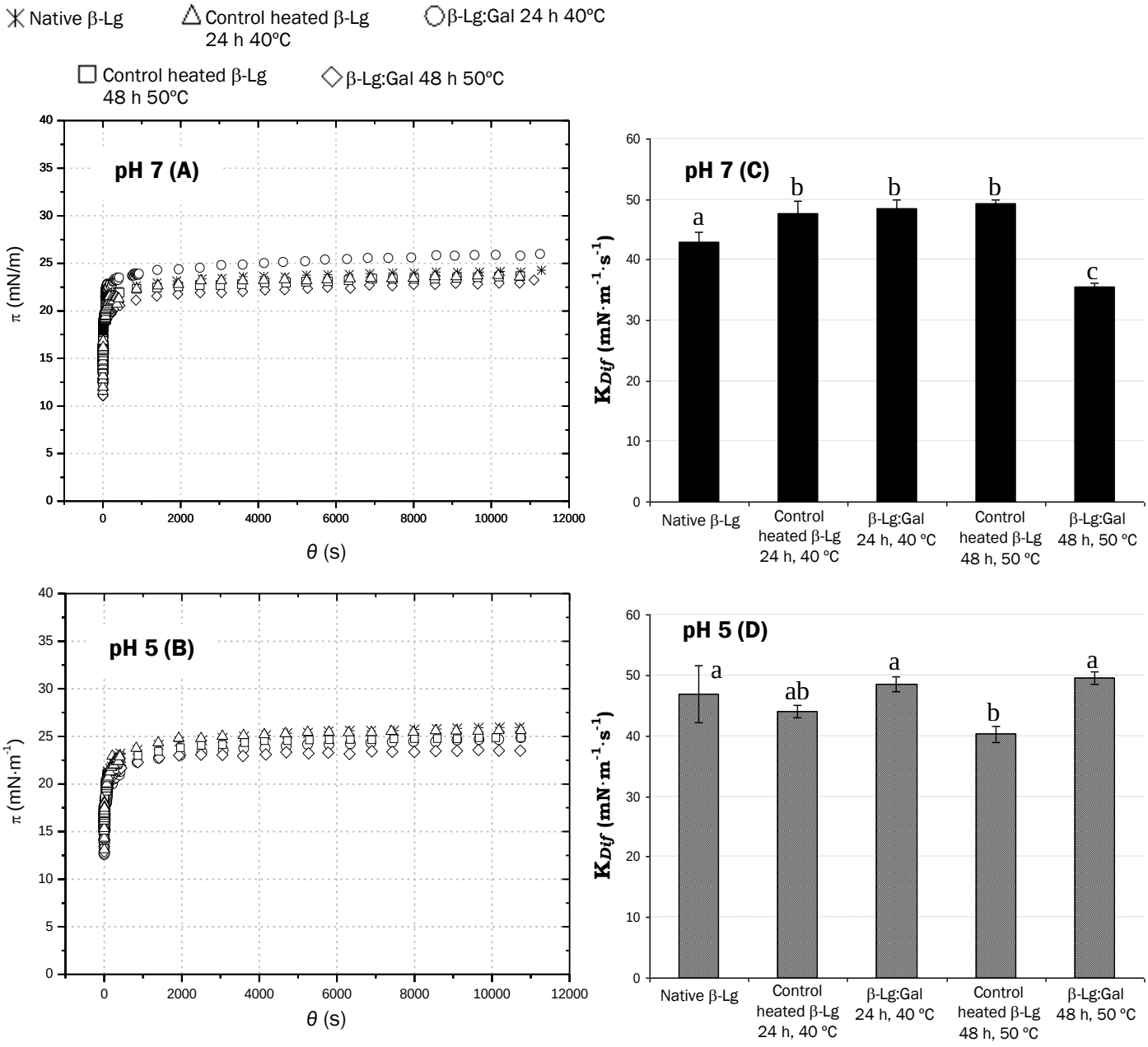

Native β-Lg
Control heated β-Lg 24 h 40°C
β-Lg:Gal 24 h 40°C
Control heated β-Lg 48 h 50°C
β-Lg:Gal 48 h 50°C
pH 7 (A)
π (mN/m)
θ (s)
pH 7 (C)
K_Dif (mN·m⁻¹·s⁻¹)
a
b
b
b
c
Native β-Lg
Control heated β-Lg 24 h, 40 °C
β-Lg:Gal 24 h, 40 °C
Control heated β-Lg 48 h, 50 °C
β-Lg:Gal 48 h, 50 °C
pH 5 (B)
π (mN·m⁻¹)
θ (s)
pH 5 (D)
K_Dif (mN·m⁻¹·s⁻¹)
a
ab
a
b
a

Figure 3.

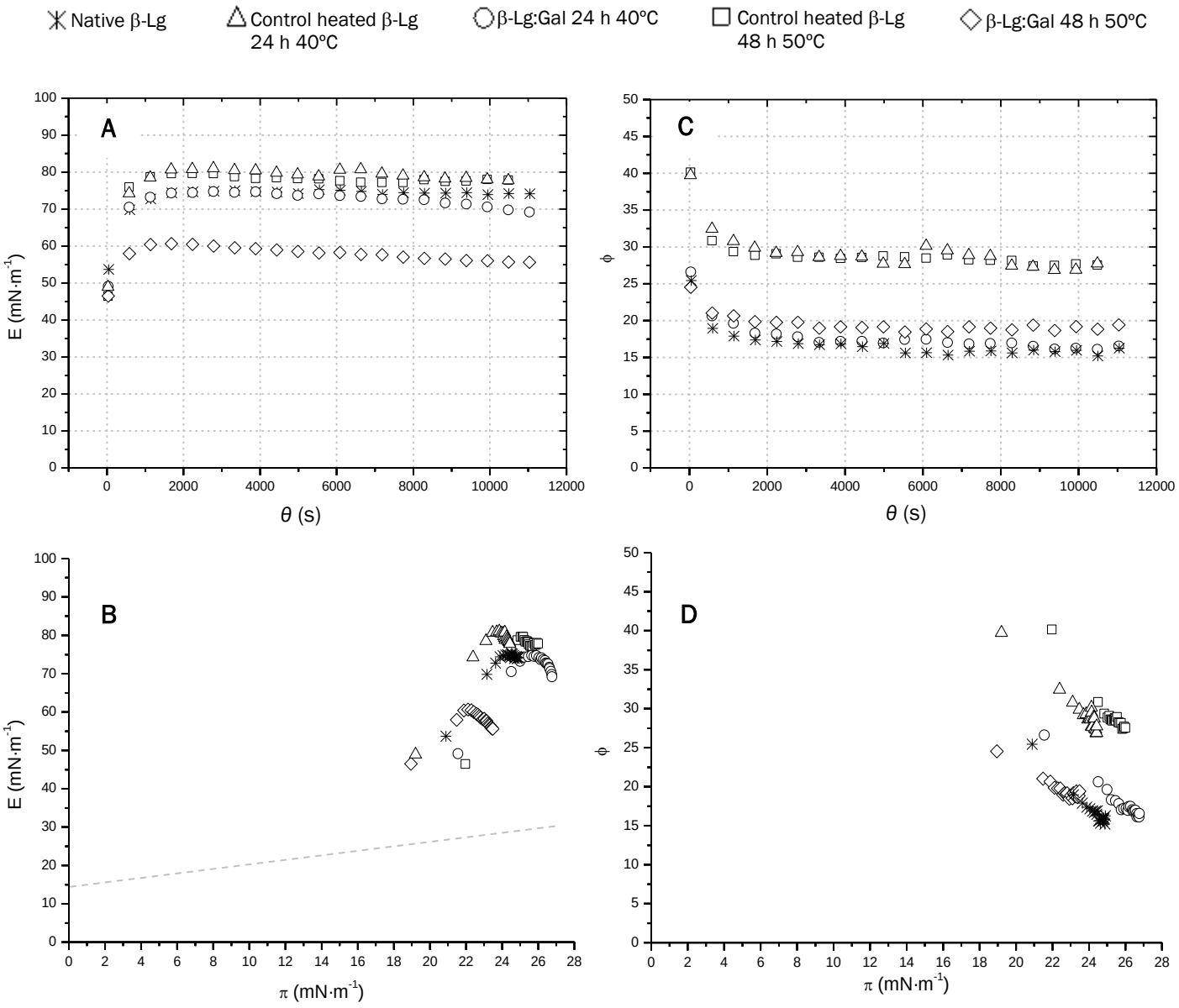

Native β-Lg
Control heated β-Lg 24 h 40°C
β-Lg:Gal 24 h 40°C
Control heated β-Lg 48 h 50°C
β-Lg:Gal 48 h 50°C
A
B
C
D
E (mN·m-1)
θ (s)
π (mN·m-1)
ϕ

Figure 4.

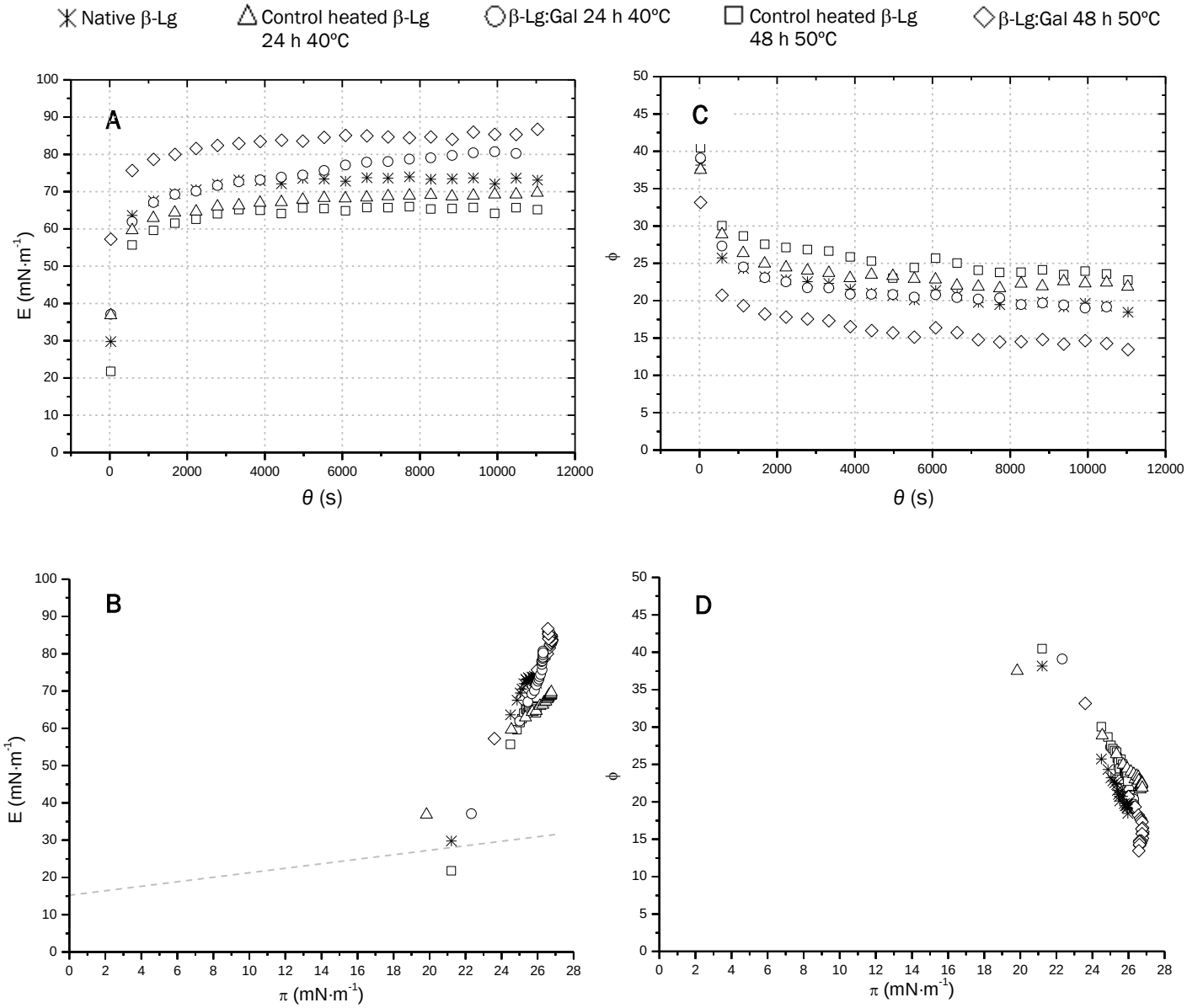

Native β-Lg
Control heated β-Lg 24 h 40ºC
β-Lg:Gal 24 h 40ºC
Control heated β-Lg 48 h 50ºC
β-Lg:Gal 48 h 50ºC
A
E (mN·m-1)
θ (s)
C
ϕ
θ (s)
B
E (mN·m-1)
π (mN·m-1)
D
ϕ
π (mN·m-1)

Figure 5.

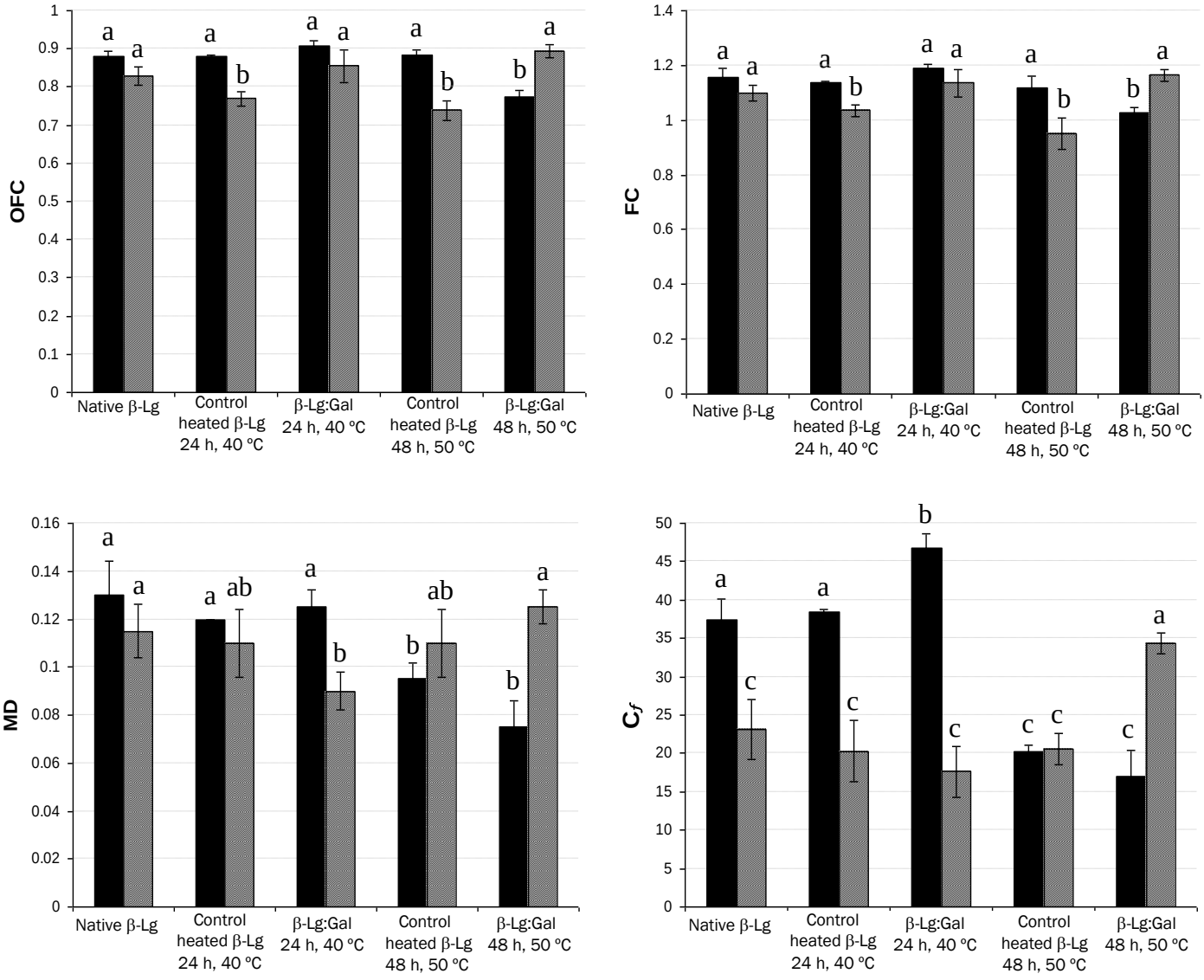

OFC
FC
MD
C_f
Native β-Lg
Control heated β-Lg 24 h, 40 °C
β-Lg:Gal 24 h, 40 °C
Control heated β-Lg 48 h, 50 °C
β-Lg:Gal 48 h, 50 °C

Figure 6.

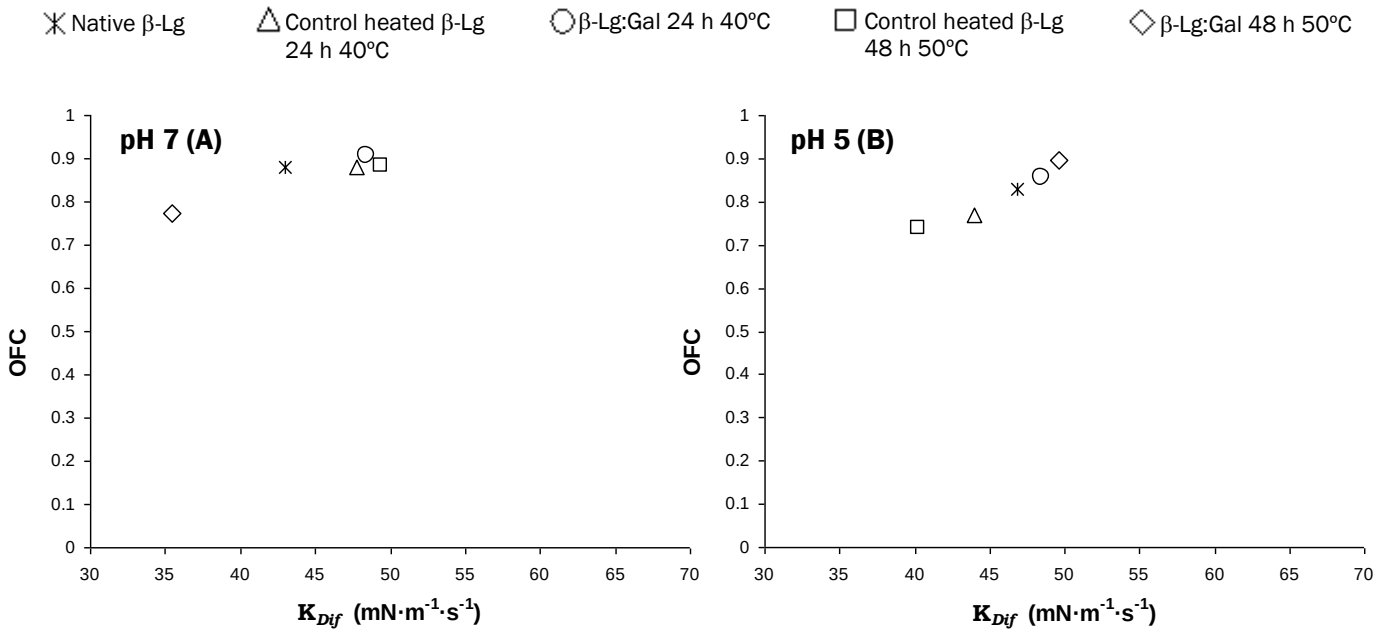
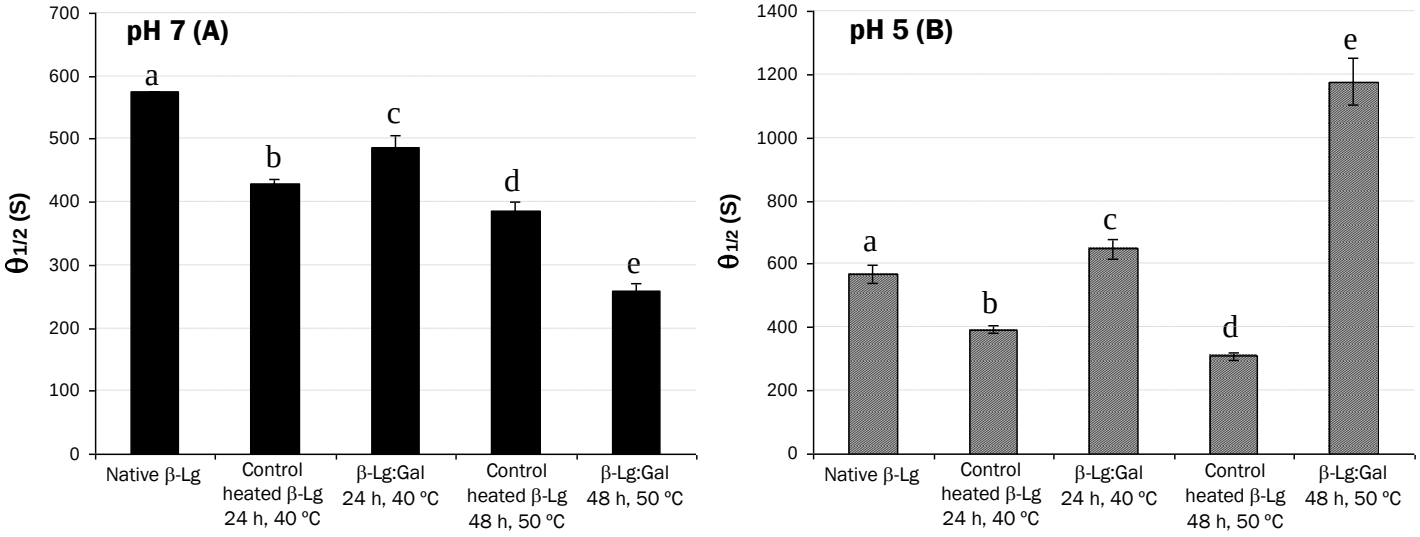

Native β-Lg
Control heated β-Lg 24 h 40ºC
β-Lg:Gal 24 h 40ºC
Control heated β-Lg 48 h 50ºC
β-Lg:Gal 48 h 50ºC
pH 7 (A)
pH 5 (B)
OFC
K_{Dif} (mN·m^{-1}·s^{-1})

Figure 7.


pH 7 (A)
θ1/2 (S)
700
600
500
400
300
200
100
0
a
b
c
d
e
Native β-Lg
Control heated β-Lg 24 h, 40 °C
β-Lg:Gal 24 h, 40 °C
Control heated β-Lg 48 h, 50 °C
β-Lg:Gal 48 h, 50 °C
pH 5 (B)
θ1/2 (S)
1400
1200
1000
800
600
400
200
0
a
b
c
d
e
Native β-Lg
Control heated β-Lg 24 h, 40 °C
β-Lg:Gal 24 h, 40 °C
Control heated β-Lg 48 h, 50 °C
β-Lg:Gal 48 h, 50 °C

*Graphical Abstract