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Abstract: The effect of limited hydrolysis followed by glycation with galactose, and vice versa, on interfacial and foaming (foamability and foam stability) properties of □-lactoglobulin (□-Lg) at pH 7 and pH 5 has been studied. Hardly any effect of the two treatments, hydrolysis and glycation (HG) or glycation and hydrolysis (GH), on □-Lg foaming capacity at both pH values was observed. Foam stability, however, was significantly enhanced after both HG and GH at pH 5. Particularly, system obtained after glycation at 50 °C followed by limited hydrolysis showed an exceptional stability, which might be related to the increase in elastic character and cohesion of the interfacial film, indicated by the increase of the surface dilatational modulus (E) and the decrease of the phase angle (□) over the time. These results indicated that glycation of □-Lg with galactose followed by limited hydrolysis might allow extending the use of this protein as foaming agent, although further research in complex food systems is needed to apply this method in the formulation of acidic foods and beverages.

Revised Cover Letter

November 7, 2016

P. A. Williams,
Materials and Analytical Science
Glyndwr University (UK)

Dear Prof. Williams,

Attached please find the revised manuscript entitled "EFFECT OF GLYCATION AND LIMITED HYDROLYSIS ON INTERFACIAL AND FOAMING PROPERTIES OF BOVINE β-LACTOGLOBULIN" by authors M. Corzo-Martínez, F. Javier Moreno, Mar Villamiel, Juan M. Rodríguez Patino and Cecilio Carrera Sánchez to be considered for publication in "Food Hydrocolloids".

We truly appreciate the efforts made by the reviewers to improve the content, clarity, and focus of the revised manuscript. All changes suggested by the reviewers were carefully considered and changes made accordingly in the revised version of the manuscript. Comments by the reviewers were constructive and relevant to improve the overall quality of the final manuscript.

Sincerely,

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*Detailed Response to Reviewers

FOODHYD-D-16-00799

Effect of glycation and limited hydrolysis on interfacial and foaming properties of bovine b-lactoglobulin

We would like to thank the Editor and the Reviewers for the constructive comments made on our manuscript that we found particularly relevant and will significantly improve the quality of the paper. Please find below the detail of the modifications made on the text and figures (modifications made on the text appear in yellow) and the replies to all questions raised by the Reviewers.

Reviewers' comments:

Reviewer #1

The paper is clearly presented and well differentiated from previous works and published works from other group. It clearly points out the hypothesis, the aim and the scientific approach.

There are little details to be taken care of prior to publication:

1. The terms glycosylation and glycation are both clear, but within one study, perhaps only one of these terms should consistently be used.

According to the referee's suggestion, the term "glycosylation" has been changed by "glycation" throughout the text.

2. The purities of the enzymes should be specified.

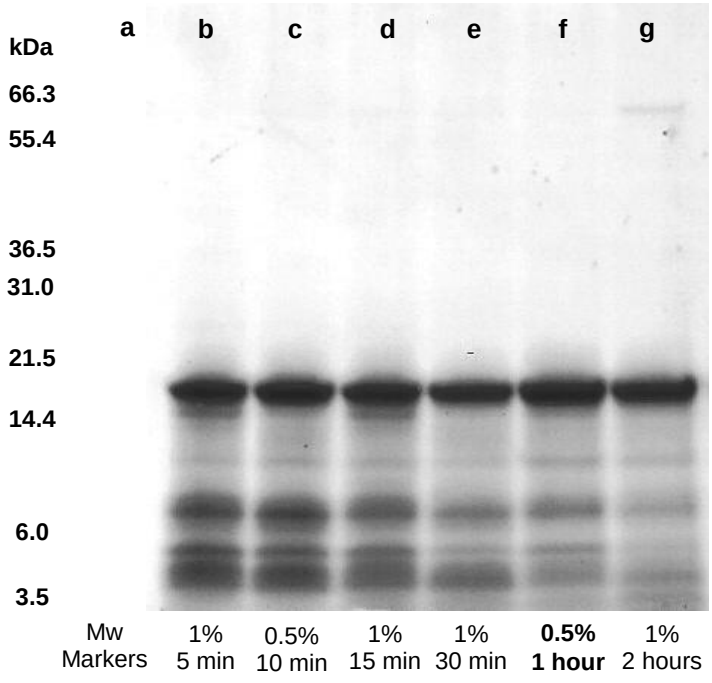
According to Referee's suggestion, purity of enzymes (as protein %) has been specify in the revised version of the manuscript (please, see lines 134-135).

3. Questions about ANS method:

3.1. The referee has the experience that the ANS method does not work for fully hydrolyzed proteins, i.e. for small peptides, which have become too small. The authors are invited to critically discuss the applicability of this method for peptides (perhaps as a function of DH).

Referee's expertise in the application of the ANS method for fully hydrolyzed proteins is, undoubtedly, greater than ours. We has only applied this method on non-hydrolyzed proteins, including β-Lg and sodium caseinate (Corzo-Martínez et al., 2008, 2010), and, in the present work, on systems derived from partial or limited hydrolysis, in which the DH was only about 4%, as indicated in the manuscript. Either with non-hydrolyzed proteins or with partially hydrolyzed systems, surface hydrophobicity values obtained using the ANS method were reproducible and feasible.

Although these data are not shown in the manuscript, hydrolyzed native b-Lg was analyzed by SDS-PAGE under reducing conditions (please, see Figure below). Separation is based primarily on size. However, a plot of log (molecular weight) vs. mobility of the reference proteins (lane a) can provide some information on the size of peptides formed after hydrolysis. At the selected hydrolysis conditions for native β-Lg (0.5% Trypsin, 60 min) (lane f), it was possible to see an intense band with an apparent Mw of ~18 kDa corresponding to intact β-Lg. At least 4 bands with lower intensity were also visible, which showed an apparent M_w ranging from 4.5 to 13 kDa, according to results obtained by Chobert et al. (1988) for the whey protein hydrolysate with a DH of 3.9%. These results confirm the degree hydrolysis determined as described in section 2.2.2 of the manuscript. Probably, the low DH of samples studied in this work is the reason why we do not have any problem with application of the ANS method for the determination of surface hydrophobicity.



References:

- Corzo-Martínez, M., Moreno, F. J., Olano, A., & Villamiel, M. (2008). Structural characterization of bovine b-lactoglobulin galactose/tagatose Maillard complexes by electrophoretic, chromatographic and spectroscopic methods. Journal of Agricultural and Food Chemistry, 56, 4244-4252.

- Corzo-Martínez, M., Moreno, F.J., Villamiel, M., & Harte, F.M. (2010). Characterization and improvement of rheological properties of sodium caseinate glycated with galactose, lactose and dextran. Food Hydrocolloids, 24, 88–97.

3.2. The evaluation of the ANS method was not made clear in the paper. Surface hydrophobicity is the slope of a calibration curve and normally has values of around 20,000 for native beta-lactoglobulin. The study showed values of around 40. Please describe in detail how the method was conducted.

In order to determine the most suitable concentration of samples to carry out the analysis, we firstly did a calibration curve by using known concentrations of native b-Lg (0.01-0.5 $mg \cdot mL^{-1}$). The relative fluorescence intensity (FI) value obtained for the solution with a protein concentration of 0.1 $mg \cdot mL^{-1}$ fell in the middle of the curve, so that this protein concentration was selected to prepare the rest of the samples for their further analysis. This, as described in section 2.4.2 of the manuscript, was as follows: 10 µL of ANS solution (8.0 mM in 0.1 M sodium phosphate buffer, pH 7.4) was added to 1 mL of sample (previously diluted with 0.1 M sodium phosphate buffer, pH 7.4, to a final protein concentration of 0.1 $mg \cdot mL^{-1}$). The resulting solution was mixed and equilibrated for 2 min and, finally, the fluorescence intensity measured at room temperature on a Shimadzu RF-1501 fluorescence spectrophotometer. The wavelengths of excitation (λexc) and emission (λem) were 390 and 470 nm, with slit widths of 10 nm.

3.3. In the paragraphs following line 361 and 366 it appears to be unclear whether the ANS method yields differences in surface hydrophobicity or not. While the first of these paragraphs sets no significant difference, the latter one does, even confirming expectations. Is the difference between these two chapters due to glycation/no or insufficient glycation? Why does the full glycation at 48h/50°C yield much lower S-values than 24H/40°C?

8-anilino-l-naphthalenesulfonate (ANS) binds to hydrophobic patches on protein surface, so that the value of fluorescence intensity obtained is directly related to the amount of hydrophobic patches at the protein surface (that is the protein surface hydrophobicity, S_0). The amount of hydrophobic patches at the protein surface and, hence, the protein surface hydrophobicity, depends on the structural features of protein, so it might be altered by any protein treatment modifying the protein structure. In the present work, the effect of different treatments, including

1º) limited hydrolysis + 2º) heating/glycation and 1º) heating/glycation + 2º) limited hydrolysis, has been studied.

As indicated in the paragraph 361-366, no differences were observed between S_0 values of control heated hydrolysate of b-Lg (1º) heating at 40 or 50 ºC + 2º) limited hydrolysis) and hydrolyzed control heated b-Lg (1º) limited hydrolysis + 2º) heating at 40 or 50 ºC). This indicates that both treatments altered in a similar way the protein structure and, hence, also the surface hydrophobicity. For this reason, we thought that not to show results from both systems could simplify the result explanation, since the high amount of different samples under study makes very hard to discuss the results in a concise and non-confusing way.

Not to see differences between S_0 value of control heated hydrolysate of b-Lg and hydrolyzed control heated b-Lg do not mean that these treatments do not alter surface hydrophobicity of the original protein, since, as observed in Table 2, S_0 of native or non-hydrolyzed b-Lg is significantly lower.

Similarly, changes observed in surface hydrophobicity values after hydrolysis and glycation (1º) limited hydrolysis + 2º) glycation at 40 or 50 ºC (GH)) or glycation and hydrolysis (1º) glycation at 40 or 50 ºC + 2º) limited hydrolysis (HG)) are also related to the effect of such treatments on protein structure. So that, the higher structural modification the higher alteration of protein surface hydrophobicity. Thus, as indicated in lines 347-349, protein hydrolysis has been shown to cause the unmasking of previously hidden hydrophobic groups and their exposition to the more polar surrounding environment, increasing the protein surface hydrophobicity. This agree with our results, since, as observed in Table 2, hydrolyzed systems showed significantly higher S_0 than non-hydrolyzed or native protein. Regarding the effect of heating and glycation, we observed that heating or glycation at 40 ºC did not significantly modified protein/peptide structure, so that S_0 values of b-Lg systems obtained after hydrolysis + heating at 40 ºC; hydrolysis + glycation at 40 ºC; and glycation at 40 ºC + hydrolysis, were similar to that of hydrolyzed native b-Lg.

At 50 ºC, however, important structural changes in the three-dimensional configuration of the peptides and protein take place. During advanced stages of the MR, as indicated in the manuscript (lines 374-378), cross-linking and aggregation reactions occur, leading to the masking of the protein hydrophobic patches and, hence, to the decrease of the protein surface hydrophobicity.

These results are in agreement with a previous study carried out with non-hydrolyzed b-Lg and glycoconjugates (Corzo-Martinez et al., 2008). In this work, we carried out an exhaustive characterization of the structural changes that occur during b-Lg glycation with Gal. We found that glycation at 40 °C (initial stages of the MR) partially affected the side chains of the protein in the tertiary structure without great disruption of the native structure, whereas at 50 °C (advanced stages of the MR) the conformational state of b-Lg was more affected.

References:

- Corzo-Martínez, M., Moreno, F. J., Olano, A., & Villamiel, M. (2008). Structural characterization of bovine b-lactoglobuline galactose/tagatose Maillard complexes by electrophoretic, chromatographic and spectroscopic methods. Journal of Agricultural and Food Chemistry, 56, 4244-4252.

3.4. What is the relation between glycation and ANS binding ability?

It has been reported that ANS may also strongly bind cationic groups of proteins (60) as lysine and/or arginine residues. During the Maillard reaction, the conjugation of the carbonyl group of a reducing sugar (galactose in this case) with an available amino group of a peptide or protein, mainly the ε-amino group of lysine residues and the δ-guanidino group of arginine residues, occurs spontaneously. Since Lys and Arg residues are potential binding sites of ANS, their blocking with galactose moieties during the MR might result in a lower fluorescence intensity (FI) value, as this is related to the amount of ANS bound to the protein. Thus, in the case of the glycated hydrolysates (GH) at 50 ºC and the hydrolyzed glycoconjugates (HG) at 50 ºC, with high glycation degree, the lower S_0 value observed as compared to the rest of systems, could be also attributed to the lower binding of ANS molecules due to the high blocking of Lys and Arg residues.

Reviewer #3

This paper investigated the effects of glycation and enzyme hydrolysis on beta-lactoglobulin foaming and interfacial properties. Results suggested that combining both techniques improves foam stability due to increasing interfacial elasticity. Enough data was provided to support conclusions; while a few questions can be further investigated. One is to compare viscosity of the pre-foam solution because both glycation and hydrolysis can influence MW and solution viscosity. Another question is to evaluate adding same amount of galactose to control sample as sugar can affect protein interfacial and foam properties. It is also interesting that even glycation increases the size of molecule and may hinder protein-protein interactions at interface; the hydrolyzed glycoconjugates still formed a more elastic interfacial film than hydrolyzed proteins. It might be worth to evaluate interfacial film thickness. Overall it is high quality paper and recommended to publish.

We would like to thank so much to the referee for his/her comment. We agree with him/her in that it would interesting to further study questions such as the effect of hydrolysis and glycation on Mw and solution viscosity, since they are factors that might also have any effect on the foaming and stabilizing capacity of the protein. In fact, as commented in answer to the question 3.1 of Referee 1, Mw of hydrolyzed native b-Lg was determined by SDS-PAGE to monitor the

hydrolysis extent and results agree with previous results in the Literature. Regarding the effect of galactose presence in the reaction medium, we also studied controls consisting in b-Lg plus galactose (in a weight ratio 1:1) without incubation; however, we did not observed notable effect of the sugar addition on foaming and interfacial properties of b-Lg. For that reason, we decided not to show those results, since we had enough samples and could result very confusing to the reader and difficult to write for ourselves.

Undoubtedly, we agree with the Referee that further studies could be carried out to gain more insight into the effect of hydrolysis and glycation on foaming and interfacial properties of b-Lg and we will have it into account for further works.

1. Highlights

1.1. Suggest to combined point 2 and 3. Point 2 description looks like pH 5 is reaction condition rather than measuring condition. It is clearer to combine point 2 and 3 and use pH 5 to describe foam/interface measurement condition.

According to the referee, we have combined points 2 and 3 and have use pH 5 to describe foam/interface measurement conditions (please, see revised version of highlights).

1.2. Be specific of 'improved surface dilatational properties". Is it refer to increasing dilatational elastic modulus?

When we say "improved surface dilatational properties" we refer to the increase observed in elastic character and cohesion of the interfacial film, indicated by the increase of the surface dilatational modulus (E) and the decrease of the phase angle (ϕ) over the time.

We consider it is not necessary to be so specific, mainly due to the limited length of highlights. However, in order to clarify, we have changed the highlight as follows: "Limited hydrolysis after glycation improved elastic character and cohesion of the protein interfacial film and, consequently, stabilizing capacity of β-Lg at pH 5."

2. Graphic abstract. The abbreviations of sample names are hard to understand for first time reader. Suggest to add full names or explanation.

According to Referee's suggestion, a legend with full names have been added to the graphic.

3. Line 141: What is the ratio of beta-lg and Galactose?

Galactose and β-lactoglobulin in a weight ratio of 1:1 were dissolved in 0.1 M sodium phosphate buffer, pH 7, and lyophilized. Then, the β-Lg-Gal powders were kept at 40 and 50 ºC for 24 and 48 h, respectively, under a vacuum in a desiccator equilibrated at an a_w of 0.44, achieved with a saturated K_2CO_3 solution.

A detailed protocol for glycoconjugate preparation is described in a previous work carried out in our laboratory (Corzo-Martínez et al., 2008), which reference is indicated in the text (please, see line 142). To facilitate reading, this information has been now included in the revised manuscript (please, see lines 140-151).

References:

- Corzo-Martínez, M., Moreno, F. J., Olano, A., & Villamiel, M. (2008). Structural characterization of bovine b-lactoglobuline galactose/tagatose Maillard complexes by electrophoretic, chromatographic and spectroscopic methods. Journal of Agricultural and Food Chemistry, 56, 4244-4252.

4. Line 145-146: It looks like the 'control heated beta-lg' is prepared using hydrolyzed beta-lg from later discussion. Please describe it in the method correctly to avoid confusion.

We agree with the Referee that the text is confusing. Therefore, “until their limited enzymatic hydrolysis” has been removed in the revised version of the manuscript.

5. Line 164-167: Is the Ht only refer to total hydrolysis of beta-lg and used for calculation of all samples? Please note in line 167.

Effectively, Ht only refers to the total hydrolysis of native b-Lg and the obtained value was used to calculate the degree of hydrolysis of the other samples subjected to limited proteolysis under the reaction conditions indicated in Table 1.

Following Referee’s suggestion, this has been specify in the new version of the manuscript (please, see lines 175-176).

6. Line 251-253: How is foam generated? Please provide more details of the equipment. Is the total gas volume controlled for all foam samples?

As we indicate in section 2.5 of the manuscript, foaming properties were evaluated by following the method previously described by Corzo-Martínez et al. (2012) in a commercial instrument (Foamscan IT Concept, Longessaigne, France), which is 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 total gas volume used until that moment being known for all foam samples. The bubbling was then stopped and the evolution of the foam was analyzed.

As indicated, this information is in the work Corzo-Martínez et al. (2012), however, we have included it in the new version of the manuscript (please, see lines 260-273).

References:

- Corzo-Martínez, M., Carrera, C., Moreno, F. J., Rodríguez Patino, J. M, & Villamiel, M. (2012). Interfacial and foaming properties of bovine β-lactoglobulin: Galactose Maillard conjugates. *Food Hydrocolloids*, 27, 438-447.

- Guillerme, C., Loisel, W., Bertrand, D., & Popineau, Y. (1993). Study of foam stability by video image analysis: relationship with the quantity of liquid in foams. Journal of Texture Studies, 24, 287-303.
- Loisel, W., Guéguen, J., & Popineau, Y. (1993). A new apparatus for analyzing foaming properties of proteins. In K. D. Schwenke, & R. Mothes (Eds.), Food proteins: Structure and functionality (pp. 320-323). Weinheim, Germany: VCH.

7. Line 396 and Table 2: How can the solubility% be above 100%?

Calibration was performed by using known concentrations (0.25-1.5 mg/L) of β-Lg in 0.1 M sodium phosphate buffer, pH 7. By interpolating in the calibrated curve the A_{280} of assayed native protein we obtained a protein concentration of 0.97 mg/mL, very close to the theorical protein content (1 mg/mL), indicating the nearly total solubility of protein. This absorbance value was considered as 100% of solubility and used to calculate the percentage of solubility of the rest of the samples studied. Thus, a solubility % above 100% means that solubility is higher than that of native β-Lg and, therefore, protein modification, limited hydrolysis and/or glycation, improve protein solubility.

Is there any reason that the solubility of glycated hydrolysates and hydrolyzed glycoconjugates of 50C/48hr treatment are higher at pH5 than at pH7; while the control heated hydrolysates are not different between at two pHs?

As indicated in line 406 of the manuscript, a previous study carried out with non-hydrolyzed b-Lg glycoconjugates (Corzo-Martínez et al., 2012) also show higher solubility at pH 5 than at pH 7 of glycoconjugates obtained at 50 ºC. We think that a plausible explanation for this behaviour is that the rate of formation of soluble/insoluble β-Lg aggregates is pH-dependent, among other factors. In consequence, our results point out that the β-Lg aggregates formed during the advanced stages of the Maillard reaction are more soluble at pH 5 than at pH 7 as it is shown in Table 2. As indicated in lines 407-411 of the manuscript, in good agreement with these results, some previous data in the literature have indicated that a higher formation of insoluble moisture-induced whey protein aggregates were formed at pH 7 than at pH 5, after storage for 14 days at 35 ºC. These authors indicated that these differences were due to a different ratio between the thiolate anion and the thiol group (reactive form to non-reactive form), which are responsible for the formation of intermolecular disulfide bonds (Zhou et al., 2008). Thus, below pH 7, the ratio thiolate anion/thiol group was low, which slowed the formation of intermolecular disulfide bonds. Certainly, further studies would be needed in order to better evaluate the nature of the Maillard aggregates formed during b-Lg glycation with galactose.

In an attempt to explain better this, the following sentence has been added in the new version of the manuscript: “So that, below pH 7, the ratio thiolate anion/thiol group was low, which slowed the formation of intermolecular disulfide bonds and, hence, the formation of water-insoluble aggregates” (please, see lines 579-581).

References:

- Corzo-Martínez, M., Carrera, C., Moreno, F. J., Rodríguez Patino, J. M, & Villamiel, M. (2012). Interfacial and foaming properties of bovine β-lactoglobulin: Galactose Maillard conjugates. *Food Hydrocolloids*, 27, 438-447.

- Zhou, P., Liu, X., & Labuza, T. P. (2008). Moisture-induced aggregation of whey proteins in a protein/buffer model system. Journal of Agricultural and Food Chemistry, 56(6), 2048-2054.

8. Line 457: Please describe how the diffusion constant is calculated.

Time-dependent surface pressure β-Lg adsorbed at the air/water interface were performed with an automatic pendant drop tensiometer (TRACKER, IT Concept, Longessaine, France) as previously described by Rodríguez Patino et al. (1999), Rodríguez Niño & Rodríguez Patino (2002) and Corzo-Martinez et al. (2012). 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 (ΔA/A) and angular frequency (ω), and the response of the surface pressure (π mN•m^{-1}) is monitored throughout the experiment, being:

$$\pi = \sigma^0 - \sigma$$

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

Since rate of increase of surface pressure (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 determining the apparent diffusion constant (K_{dif}). This was calculated as the slope of the line between the origin (point 0.0) and the first point on the plot π vs. square root of time (θ).

As indicated in the section 2.6 of the manuscript, it is possible to find this explanation in detail in a previous work carried out in our lab (Corzo-Martínez et al., 2012). However, we have included it in the new version of the manuscript (please, see lines 297-301).

References:

- Corzo-Martínez, M., Carrera, C., Moreno, F. J., Rodríguez Patino, J. M, & Villamiel, M. (2012). Interfacial and foaming properties of bovine β-lactoglobulin: Galactose Maillard conjugates. *Food Hydrocolloids*, 27, 438-447.

- Rodríguez Niño, M. R., & Rodríguez Patino, J. M. (2002). Effect of the aqueous phase composition on the adsorption of bovine serum albumin to the air–water interface. *Industrial and Engineering Chemistry Research,* 41, 1489–1495.

- Rodríguez Patino, J. M., Rodríguez Niño, M. R., & Carrera, C. (1999). Adsorption of whey protein-isolate at the oilewater interface as a function of processing conditions: a rheokinetic study. *Journal of Agricultural and Food Chemistry*, 47, 2241-2248.

9. Figure 5: Please indicate the dash line meaning in the figure captions.

According to the Referee, the meaning of dash line has been included in the figure caption of Figure 5 in the revised version of the manuscript.

Reviewer #4

This paper examines the effect of hydrolysis before or after glycation with galactose by Maillard reaction on interfacial and foaming properties of <beta>-lg. Its main conclusion

seems to be that glycation followed by limited hydrolysis increased the foaming stabilizing properties through the ability of the modified protein to increase the elasticity and cohesiveness of the interfacial films. In general terms, the experiments appear to have been carefully performed and they provide potentially interesting data. However, the manuscript needs to be thoroughly revised to clearly highlight the new or more relevant information. In particular, the experimental design, with many different samples under study, i.e. native and control heated <beta>-lg and their hydrolysates, hydrolysed glycoconjugates and glycated hydrolysates , is a bit complex and, as written, it is not always fully clear the type of modified protein the authors refer to. An example is shown in the abstract, highlights and conclusion sections:

1. Lines 27-29. As written, this sentence gives the impression that it was hydrolysis at pH 5 what enhanced foam stability, and that this was further improved by glycation at pH 5, but it probably means that hydrolysis and glycation improve foam stability at pH 5. The authors sometimes refer to "glycation combined with hydrolysis", but this wording could designate both glycation followed by hydrolysis and hydrolysis followed by glycation. It is advisable to name the glycated/hydrolyzed samples, clearly specifying the order of operation, using always the same terminology.

We completely agree with the Referee in that manuscript is a bit complex and, sometimes, confusing. However, as Referee said, the high amount of different samples under study makes very complicated to describe the results always clearly. We tried to do it, but, obviously, we have not fully got it. Thus, we have modified and unified the terminology with the aim to simplify it and try to help the understanding of the reader.

Regarding lines 27-29, we agree in that description of the results is not clear, so that, according to the Referee, the text has been rewritten as follows: “Foam stability, however, was significantly enhanced after limited hydrolysis followed by glycation and after glycation followed by limited hydrolysis at pH 5. Particularly, system obtained after glycation at 50 ºC followed by limited hydrolysis showed an exceptional stability,…” (please, see lines 28-30 of the revised version of the manuscript).

2. Lines 29-30. The sentence reads "Particularly, system obtained after glycation at 50 ºC and subsequent limited hydrolysis showed an exceptional stability" (the same is indicated in lines 576-577). However, the second and third highlights seem to indicate that the best results were obtained when <beta>-lg was first hydrolyzed and then glycated. The graphical abstract also appears to underscore this point, but it leads to confusion that the arrow points to the surface stability of samples labelled HG which, according to line 139 designates hydrolyzed glycoconjugates (first conjugated and then hydrolyzed).

As said in the previous comment, we agree with the Referee and the text as well as the highlights have been changed in the new version of the manuscript (please, see lines 28-30, 640 and 648 of the revised version of the manuscript as well as revised highlights).

3. In addition, it is strongly recommended that the authors re-write the results and discussion sections as separate sections. This might allow shortening the length of the paper, avoiding unnecessary repetitions. In addition, a separate discussion section might help to connect the different results (such as, for instance interfacial and foaming properties), but particularly, to focus on the interpretation and explanation of the more relevant ones.

Following Referee's suggestion, we have separated Results and Discussion sections in the revised version of the manuscript and have tried to explain and connect the results in a clearer way.

Specific points:

4. Line 289-299 and Table 1. What enzyme was used to hydrolyze heated <beta>-Lg, whose hydrolysates were also used as controls in the following sections? What was its degree of hydrolysis?

Conditions of hydrolysis of control heated b-Lg, including type of enzyme and ratio E/S, were the same that for native protein and the degree of hydrolysis observed for both controls was similar to that observed for native b-Lg. This is now indicated in the new version of the manuscript (please, see line 333) and Table 1. We thank the referee for advising that these data were missing. Sorry for the mistake.

5. Is it correct to assume that trypsin is less active than <alpha>-chymotrypsin or is it a question of susceptibility of the substrate? Is glycated <beta>-Lg a good substrate for trypsin?

Effectively, as Referee said, it is more a question of susceptibility of the substrate. Trypsin cleaves protein on the C-terminal side of lysine and arginine residues. These residues are blocked during glycation, especially under the more severe conditions (48 h at 50 ºC), which reduce reactivity of trypsin against glycated protein. Unlike trypsin, α-chymotrypsin hydrolyzes peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met, Leu) on the carboxyl end of the bond, and, hence, glycation does not block its cleavage sites. However, similarly to trypsin, α-chymotrypsin activity can be also affected by glycation due to the protein cross-linking and aggregation occurring during the advanced stages of the Maillard reaction, which may mask the cleavage sites.

This has been clarified in the revised version of the manuscript (please, see lines 326-332).

6. Is it correct to use different enzymes with different specificities for hydrolysis of the different substrates? In other words, would the authors have expected to get hydrolysates of native <beta>-Lg with equivalent surface hydrophobicity and solubility properties at the same degree of hydrolysis regardless of the enzyme?

Our objective was to hydrolyze native, control heated and glycated b-Lg until a DH of about 4%, which migh lead to the improvement of the foaming and stabilizing properties of b-Lg according to studies of Conde and Rodriguez Patino (2007) and Chobert et al. (1988). The use of porcine trypsin to hydrolysis of glycated b-Lg was not an option since, as commented above, galactose molecules are blocking its potential cleavage sites (lysine and arginine residues). Because of that, we considered that bovine α-chymotrypsin might be a good alternative to hydrolyze glycoconjugates.

Since cleavage sites of both enzymes are different, we agree with the Referee in that we cannot expect or assume similar surface hydrophobicity and solubility of hydrolysates just because they have similar DH. In fact, as our results depict, hydrolyzed native b-Lg, heated and hydrolyzed controls (HH controls) and GH systems showed different S_0 values and solubility. Taking into account that cleavage sites of α-chymotrypsin are aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met, Leu), this enzyme would release hydrophobic amino acids, leading to an increase of the surface hydrophobicity (S_0) of the glycated/hydrolyzed (GH) systems. However, this did not happen. GH [24 h, 40 ºC] systems showed no significant ($P > 0.05$) different S_0 values and solubility, respectively, respect to hydrolyzed native b-Lg and HH controls, whilst S_0 values at pH 7 and 5 and solubility at pH 7 of GH [48 h, 50 ºC] systems were significantly ($P < 0.05$) lower due to the cross-linking and aggregation reactions that occur during the advanced stages of the Maillard reaction. This suggest that the modification of the protein structure due to the Maillard reaction is the major responsible for the modification of physico-chemical properties of GH systems, rather than the DH, which is not high enough to greatly alter protein structure.

7. Have the authors taken into account that previous hydrolysis with trypsin not only provides higher amounts or free amino groups susceptible to glycation (line 325), but that it also releases peptides with Lys and Arg at the C-terminus?

Referee is right; hydrolysis realeases peptides with Lys and Arg residues at the C-terminus and, hence, with their NH2-terminal group blocked. However, many other available NH_2-terminal groups are created, which are susceptible to glycation, in addition of available NH_2 groups of the lateral chains of Lys, Arg, and, in the lesser extent, His and Trp residues. Moreover, the lower steric hindrance in the peptides might promote diglycation of some residues.

8. Lines 312-327. It is claimed that Eq. (2) allows an indirect estimation of the number of Gal molecules linked to <beta> -Lg protein/peptides but it is not fully clear on what basis this glycation degree was expressed.

As indicated in the text (lines 194-199), to calculate the constants 10^{19} and 2.99 10^{-19} we based on results from a previous work carried out in our lab (Corzo-Martinez, M. et al., 2010). In this work, the loss of total Gal during incubation with b-Lg was measured with the anthrone-sulfuric acid method and an estimated number of Gal adducts linked to b-Lg was determined by MALDI-TOF-MS, so that a relationship between both values, $\%Gal_{consumed}$ and Nº Gal adducts, could be established.

In Corzo-Martínez et al. (2010), MALDI-TOF-MS analysis allowed to estimate the number of molecules of galactose linked covalently to the b-Lg. The molecular masses of native b-Lg were 18 278 and 18 363 Da, in good agreement with the expected masses of the variants B and A, respectively (Léonil et al., 1997). Mass spectra of b-Lg incubated with galactose was characterized by a broad Gaussian peak shape without good resolution due to the great heterogeneity of the glycated forms of b-Lg (Broersen et al., 2004; Van Teeffelen et al., 2005; Sanz et al., 2007). Considering the maximum intensity of the Gaussian peaks, the average number of Gal molecules bound to b-Lg after glycation could be estimated. Thus, after 1 day of incubation at 40 °C, an average number of 12 galactose molecules were linked to the b-Lg. Additionally, up to 18 galactose molecules linked to the protein chain could be estimated after 2 days of storage at 50 °C.

In the present work, we say the glycation degree (number of Gal adducts bound to b-Lg) of hydrolyzed glycoconjugates [24 h, 40 ºC] and [48 h, 50 ºC] is 14 and 22, respectively; however, it is 12 and 18, respectively. Data have been corrected in the revised version of the manuscript (please, see line 351-352). Sorry for the mistake.

References:

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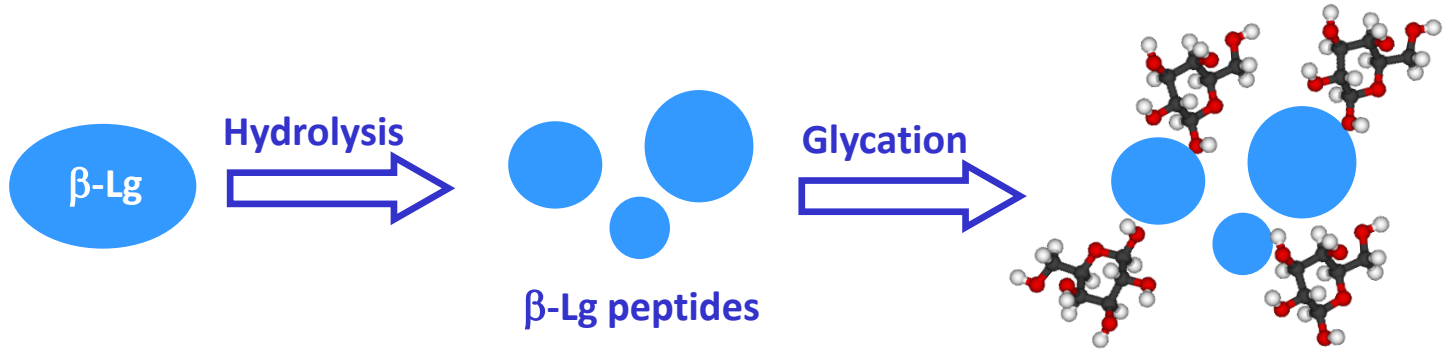
Highlights

- This work aimed to improve β-Lg interfacial and foaming properties at pH 5.
- β-Lg was subjected to glycation followed by hydrolysis (GH) and vice versa (HG).
- GH and HG hardly changed the foaming capacity with respect to that of β-Lg at pH 7.
- GH improved the viscoelastic character of β-Lg interfacial films at pH 5.
- Improvement of β-Lg interfacial properties after GH led to highly stable foams at pH 5.

Revised *Graphical Abstract



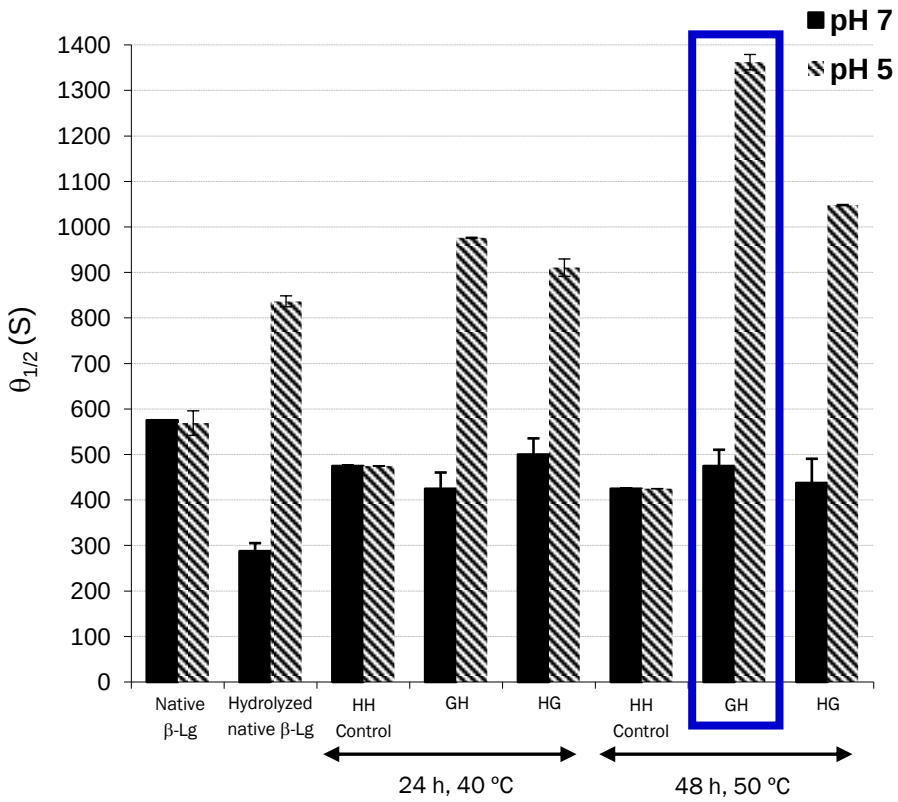
FOAM STABILIZATION

β-Lg = beta-lactoglobulin

HH = hydrolyzed/heated controls

HG = hydrolyzed/glycated systems

GH = glycated/hydrolyzed systems



Effect of glycation and limited hydrolysis on interfacial and foaming properties of bovine β-lactoglobulin

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ABSTRACT

The effect of limited hydrolysis followed by glycation with galactose, and vice versa, on interfacial and foaming (foamability and foam stability) properties of β-lactoglobulin (β-Lg) at pH 7 and pH 5 has been studied. Hardly any effect of the two treatments, hydrolysis and glycation (HG) or glycation and hydrolysis (GH), on β-Lg foaming capacity at both pH values was observed. Foam stability, however, was significantly enhanced after both HG and GH at pH 5. Particularly, system obtained after glycation at 50 ºC followed by limited hydrolysis showed an exceptional stability, which might be related to the increase in elastic character and cohesion of the interfacial film, indicated by the increase of the surface dilatational modulus (E) and the decrease of the phase angle (ϕ) over the time. These results indicated that glycation of β-Lg with galactose followed by limited hydrolysis might allow extending the use of this protein as foaming agent, although further research in complex food systems is needed to apply this method in the formulation of acidic foods and beverages.

Keywords: glycation, limited hydrolysis, β-lactoglobulin, interfacial properties, foaming properties

1. Introduction

In the last years, the interest toward foamed foods (e.g., meat, mayonnaise, spreads, ice cream, frozen desserts, whipped toppings, mousse, bakery products, etc.) has increased due to the soft and creamy mouth sensations provided by gas bubbles (Campbell, 2009; Foegeding, Çakır & Koç, 2010). Likewise, the demand by consumers for natural ingredients with desirable functional attributes is increasing. Thus, among surface-active agents used to impart structure in foamed products, milk proteins, namely whey proteins, are one of the most commonly utilized by the food industry due to their capacity to form stable foams. This is because they are natural, non-toxic, cheap, widely available, and have high nutritional value and good sensorial characteristics. In particular, β-lactoglobulin (β-Lg), which represents 50% of the total mass of the whey proteins, plays an important role as foaming agent in a wide variety of dairy and non-dairy foamed products due to its unique structural and physic-chemical properties, such as pronounced amphiphilic nature and relatively small molecule size. Thus, it has been reported that β-Lg quickly migrates from the bulk onto the interface, adsorbs and interfacial unfolds, so that the hydrophobic core tend to orientate towards the interface, and rearranges (inter-particle bonding between β-Lg molecules) within the interfacial layer, leading to the formation of foams with good overrun and stability (Lad, Birembaut, Matthew, & Green, 2006; Martínez, Carrera, Rodríguez Patino, & Pilosof, 2009). However, foaming properties of this protein can be altered by extrinsic physicochemical conditions. Particularly, it has been observed that at pH close to pI of β-Lg (pI 5.2) and under severe heat treatment a very strong aggregation through disulphide bridges and the subsequent formation of a thick and instable interfacial layer can be produced (Kinsella & Morr, 1984; Phillips, Whitehead, & Kinsella, 1994; Wilde & Clark, 1996; Murray, 1998). Heat and pH sensibility of β-Lg limits its use as foaming agent in formulated foods and beverages with acidic pH or subjected to thermal processing. In this context, the search for new processing techniques to enhance the functional properties of β-Lg and, therefore, increase their degree of applicability is of increasing interest.

Over the last years, different physical, enzymatic and chemical treatments, leading to the modification of protein functionality, have been studied. Among the latter, a great deal of attention has been focused on the covalent interaction protein/carbohydrate via the Maillard reaction (MR) or glycation. This reaction, carried out under dry state and well-controlled conditions (carbohydrate:protein ratio, temperature, relative humidity and time), has shown to be an adequate method for improving interfacial and foaming properties of proteins, including β-Lg (Matsuda, Kato, & Nakamura, 1991; Nakamura, Kato, & Kobayashi, 1991; Morgan, Leonil, Molle, & Bouhallab, 1999; Chevalier, Chobert, Dalgalarrondo, & Haertlé, 2001; Chevalier, Chobert, Dalgalarrondo, Choiset, & Haertlé, 2002; Moreno, Lopez-Fandiño, & Olano, 2002; Fenaille, Morgan, Parisod, Tabet, & Guy, 2003; Trofimova & Jongh, 2004; Rada-Mendoza, Villamiel, Molina, & Olano, 2006). Moreover, this reaction takes place without the use of toxic chemical products and may produce important changes in protein structure, like increasing the molecular unfolding, surface hydrophobicity and amphiphilic character (Medrano, Abirached, Panizzolo, Moyna, & Añón, 2009; Corzo-Martínez, Carrera, Moreno, Rodríguez Patino, & Villamiel, 2012). Previous studies developed in our research group (Corzo-Martínez et al., 2012) have demonstrated that limited glycation with galactose efficiently improves foaming and interfacial properties of β-Lg. However, a large extent of the MR promotes the formation of high molecular weight and insoluble protein aggregates that slow down the protein diffusion to the air-water interface and reduce the adsorption efficiency, impairing, hence, the protein ability to form and stabilize foams.

In addition to protein glycation, enzymatic hydrolysis has also shown to be a suitable mean to modify the foaming properties of proteins as a function of the molecular weight profile and composition of the different fractions which, in turn, depends on the degree of hydrolysis (Davis, Doucet, & Foegeding, 2005). Thus, according to Conde and Rodriguez Patino (2007), if the purpose is to improve foaming capacity and foam stability, a low degree of hydrolysis is favorable.

In several works with some isolated proteins, including whey protein isolate, β-Lg, α-lactalbumin, and β-caseins, enzymatic hydrolysis resulted in a reduction of molecular weight and, hence, in an increased solubility as well as in the exposure of hydrophobic groups previously buried inside the protein, which might increase the protein foaming capacity, due to the faster diffusion of molecules to the interface, as well as the protein stabilizing properties, due to the formation of rigid and stable interfacial films via hydrophobic induced aggregation of proteins at the interfacial layer (van der Ven, Gruppen, de Bont, & Voragen, 2002; Creusot, Gruppen, van Koningsveld, deKruif, & Voragen, 2006; Tamm, Sauer, Scampicchio, & Drusch, 2012). Based on this, we can hypothesize that limited hydrolysis might improve solubility of Maillard glycoconjugates, especially of those in the advanced stages of the MR, and, hence, further improve their foaming and interfacial properties. To the best of our knowledge, no works on the combined effect of both treatments have been carried out.

Thus, the aim of the present work has been to study the effect of i) the limited hydrolysis followed by glycation with galactose of bovine β-Lg (treatment called HG from now) and ii) the glycation with galactose followed by limited hydrolysis of bovine β-Lg (treatment called GH from now) on foaming properties, in an attempt to further improve the functionality of previously studied glycoconjugates and, hence, increase their applicability degree.

Likewise, the dynamics of protein adsorption at the air-water interface and the rheological properties of interfacial films have been shown to influence foam properties, depending on the prevalent instability foam mechanism (Baeza, Carrera, Rodríguez Patino, & Pilosof, 2005). Therefore, with the purpose of gaining more insight into the effect of glycation and proteolysis of β-Lg on protein functionality and further explaining results obtained from determination of foaming properties, interfacial properties were also studied in the present work.

2. Materials and methods

2.1. Materials

Galactose (Gal), bovine β-lactoglobulin (β-Lg) (mixture of A and B variants), porcine trypsin (EC 3.4.21.4; type IX-S; ≥ 90% protein) and bovine α-chymotrypsin (EC 3.4.21.1, type I-S; ≥ 85% protein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Glycation of β-Lg followed by limited hydrolysis: glycated/hydrolyzed (GH) b-Lg systems

2.2.1. β-Lg glycation and purification of glycoconjugates

Galactose and β-lactoglobulin in a weight ratio of 1:1 were dissolved in 0.1 M sodium phosphate buffer, pH 7, and lyophilized as previously described by Corzo-Martínez et al. (2008). Then, the β-Lg-Gal powders were kept at 40 and 50 ºC for 24 and 48 h, respectively, 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 reducing sugars during the same periods (control heated β-Lg). Incubations were performed in duplicate, and all analytical determinations were performed at least in duplicate. After incubation, the products were reconstituted in distilled water to a protein concentration of 1 mg/mL. To remove free carbohydrate, 2 mL portions were ultrafiltered through hydrophilic 3 kDa cutoff membranes (Centricon YM-3, Millipore Corp., Bedford, MA) by centrifugation at 1548g for 2 h. After removal of free Gal, samples were reconstituted in distilled water at a protein concentration of 1 mg·mL^{-1} and kept at 4 °C.

2.2.2. Limited hydrolysis of glycated β-Lg

To obtain the glycated/hydrolyzed β-Lg systems (GH systems), glycated β-Lg was dissolved in double-distilled water (Milli-Q water, Millipore, Bedfor, USA) to a final protein concentration of 3 mg·mL^{-1} and pH was adjusted to 8.0 with 1 M NaOH. Then, solutions bovine

α-chymotrypsin (62 activity units·mg^{-1} of protein) in double-distilled water adjusted at pH 8.0 were added to the reaction mixture in order to give a final enzyme/substrate (E/S) ratio of 0.5 and 1 % (w/w), as shown in **Table 1**. Samples were incubated at 37 ºC with stirring and aliquots were taken at different times for determination of the degree of hydrolysis (DH). In addition, native and control heated β-Lg were hydrolyzed in the presence of porcine trypsin (14,300 activity units·mg^{-1} of protein) at a final enzyme/substrate (E/S) ratio of 0.5 and 1 % (w/w) (**Table 1**) to obtain the hydrolyzed native β-Lg and the heated and hydrolyzed β-Lg controls (HH controls-1). DH was also determined at different times.

Trypsin and α-chymotrypsin were inactivated by heating at 80 ºC for 5 min after sample incubation.

The degree of hydrolysis (DH) was determined spectrophotometrically by the trinitrobenzenesulfonic acid (TNBS) method described by Adler-Nissen (1979) and adapted by van der Ven (2002) to 96-well microtitration plates (Microtest Plate 96-Well. Sarstedt, Inc. Newton, NC), which is based on the following equation:

$$DH = \frac{H_x}{H_t} \times 100 = \frac{(\text{nmol/mg protein})_x}{(\text{nmol/mg protein})_t} \times 100 \qquad (1)$$

where H_x are the nmols of free amino groups coming from limited hydrolysis of samples and H_t are the nmols of free amino groups after the total hydrolysis of the native β-Lg. H_t value was used to calculate the DH of all samples subjected to limited hydrolysis.

Total hydrolysis of β-Lg (4 mg) was carried out in Pyrex tubes by triplicate. Following the addition of 5 mL of 6 N HCl, tubes were mixed in a vortex and incubated at 100 °C for 24 h. Then, samples were concentrated under vacuum (Express SC250EXP SpeedVac™ Concentrator System, Thermo Scientific™) and, finally, concentrates were dissolved in 1% SDS at a protein concentration of 1 mg·mL^{-1}.

To determine the number of free amino groups after limited (H_x) and total (H_t) hydrolysis, hydrolysates were diluted in 1% SDS solution to a concentration of 0.5 mg·mL^{-1}. Then, sample

184 solution (15 μL) was mixed with 45 μL of 0.21 M sodium phosphate buffer (pH 8.2) and 45 μL
185 of 0.05% TNBS in a well. The well plate was incubated for 1 h in a 50 °C oven, covered with
186 aluminum foil to avoid light contact and evaporation. The reaction was stopped by addition of
187 90 μL of 0.1 M HCl, and absorption at 340 nm (A_{340}) was measured with a Beckman DU 70
188 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). To transform the A_{340} into nmols
189 of free primary amino groups, a calibration curve was done by using known concentrations (7-
190 50 nM) of a commercial pure standard of leucine (Sigma-Aldrich, St. Louis, MO, USA),
191 standards being treated in the same way as samples.
192
193 *2.3. Limited hydrolysis of β-Lg followed by glycation: hydrolyzed/glycated (HG) samples*
194 *2.3.1. Limited hydrolysis of native β-Lg*
195 Native β-Lg, in double-distilled water at 3 mg·mL^{-1} protein concentration, was subjected
196 to limited enzymatic hydrolysis with porcine trypsin as previously mention in section 2.2.2.
197
198 *2.3.2. Glycation of hydrolyzed native β-Lg*
199 Hydrolyzed native β-Lg was glycated with Gal under the same incubation conditions than
200 non-hydrolyzed protein (24 h at 40 ºC and 48 h at 50 ºC) (Chevalier et al., 2002) to obtain the
201 hydrolyzed/glycated β-Lg systems (HG systems). In addition, control assays with hydrolyzed
202 native β-Lg incubated without Gal under the same experimental conditions (hydrolyzed and
203 heated β-Lg controls, called HH controls-2) were carried out.
204
205 *2.3.3 Indirect determination of glycation extent of HG systems*
206 The disappearance of Gal from the reaction medium during incubation of hydrolyzed
207 native β-Lg (1 mg·mL^{-1}) was determined following the colorimetric method of anthrone-sulfuric
208 acid (with a maximum absorbance at 630 nm (A_{630})), previously described by Laurentin and
209 Edwards (2003) and used in a previous work of our group (Corzo-Martínez, Moreno, Olano, &
210 Villamiel, 2010a). To transform A_{630} values into mg·mL^{-1} Gal consumed, a calibration curve

was performed by using known concentrations of galactose (0.05-1.0 mg·mL^{-1}), which was represented by the equation y = 3.03x + 0.0705, with 0.999 as the coefficient of determination (R^2).

To transform mg·mL^{-1} Gal values into an estimated number of Gal molecules linked to peptides/proteins, the following equation was used:

$$N° \text{ Gal adducts} = (\%Gal_{consum} / 10^{19}) / 2.99 \cdot 10^{-19} \quad (2)$$

where, $\%Gal_{consum}$ is the percentage of consumed Gal during incubation with hydrolyzed native β-Lg; 10^{19} is a constant calculated from data obtained in a previous work carried out in our lab (Corzo-Martínez, M. et al., 2010a), in which the loss of total Gal during incubation with β-Lg was also measured with the anthrone-sulfuric acid method and the exact number of Gal adducts linked to β-Lg was determined by MALDI-TOF-MS; $2.99 \cdot 10^{-19}$ is a constant calculated by dividing the molar mass of Gal (180,156 mg·mol^{-1}) by the Avogadro constant (N_A, $6.022 \cdot 10^{23} \cdot mol^{-1}$).

2.4. Characterization of glycated/hydrolyzed (GH) and hydrolyzed/glycated (HG) systems

2.4.1. Browning determination

Absorbance at 420 nm of samples, including hydrolyzed native β-Lg, HH controls and GH and HG systems, at a protein concentration of 1 mg·mL^{-1} protein in doubly distilled water, was measured in a Beckman DU 70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA) at room temperature, as an index of the brown polymers formed in more advanced stages of the Maillard reaction (Ting & Rouseff, 1986).

2.4.2. Determination of the surface hydrophobicity

The surface hydrophobicity (S_0) of hydrolyzed native β-Lg, HH controls and GH and HG systems was investigated by binding of 8-anilino-l-naphthalenesulfonate (ANS). The relative fluorescence intensity (FI) of the ligand-protein conjugates was measured on a Shimadzu RF-

1501 fluorescence spectrophotometer at room temperature. The wavelengths of excitation (λ_{exc}) and emission (λ_{em}) were 390 and 470 nm, with slit widths of 10 nm. Samples were diluted with 0.1 M sodium phosphate buffer, pH 7.4, to a final protein concentration of 0.1 mg·mL^{-1}. Then 10 μL of ANS solution (8.0 mM in 0.1 M sodium phosphate buffer, pH 7.4) was added to 1 mL of the diluted sample, the resulting solution mixed and equilibrated for 2 min and, finally, the fluorescence intensity measured at room temperature. Solution of ANS in sodium phosphate buffer was prepared daily. All measurements were carried out at least in duplicate.

2.4.3. Solubility

For solubility evaluation, the pH of samples (hydrolyzed native β-Lg, HH controls and GH and HG systems), previously dissolved in distilled water to a final protein concentration of 1 mg·mL^{-1}, was adjusted to 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 *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 non-hydrolyzed native β-Lg (original protein).

2.5. Determination of foaming properties

Foaming properties of samples, including hydrolyzed native β-Lg, HH controls and GH and HG systems, previously 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) at a final protein concentration of 5 mg·mL^{-1}, were determined at pH 7 and 5 following the method previously described by Corzo-Martínez et al. (2012). Briefly, the foam formation, the foam stability and the drainage of liquid from the foam were determined by conductimetric and optical measurements 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). 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 total gas volume used until that moment being known for all foam samples. The bubbling was then stopped and the evolution of the foam was analyzed.

Two parameters were evaluated as a measure of the foaming capacity following the method described by Corzo-Martínez et al. (2012). The overall foaming capacity (OFC, mL/s) was determined from the slope of foam volume curve till the end of the bubbling; and the foam capacity (FC), a measure of gas retention in the foam, was determined by Eq. (3). Moreover, the static foam stability was determined from the volume of liquid drained from the foam over time by calculating the half-life time ($\theta_{1/2}$). This is the time needed to drain the half the initial volume of the foam, which is that reached when gas bubbling is stopped (Corzo-Martínez et al., 2012).

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

where $V_{foam}(f)$ is the final foam volume, $V_{gas}(f)$ is the final gas volume injected.

2.6. Measurement of interfacial properties

Interfacial properties of samples, including hydrolyzed native β-Lg, HH controls and GH and HG systems, were determined at pH 7 and 5. For that, 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 $mg \cdot mL^{-1}$.

The adsorption of samples at the air-water interface was evaluated by monitoring the dynamics of surface pressure (π) and surface viscoelastic parameters (surface dilatational modulus, E; surface dilatational elasticity, E_d; surface dilatational viscosity, E_v; and the phase angle, ϕ) as a function of adsorption time (θ), by using an automatic pendant drop tensiometer (TRACKER, IT Concept, Longessaine, France) as previously described in Literature (Rodríguez Patino, Rodríguez Niño, & Carrera , 1999; Rodríguez Niño & Rodríguez Patino, 2002; Corzo-Martínez et al., 2012). Since rate of increase of surface pressure (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 determining the apparent diffusion constant (K_{dif}). This was calculated as the slope of the line between the origin (point 0.0) and the first point on the plot π vs. square root of time (θ). The average standard accuracy of the surface pressure was roughly 0.1 $mN \cdot m^{-1}$. The reproducibility of the results was better than 0.5% and 5.0% for surface pressure and surface dilatational properties, respectively.

2.7. 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 results derived from interfacial and foaming determinations of all the samples studied. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Degree of hydrolysis of hydrolyzed native β-Lg, heated/hydrolyzed controls and glycated/hydrolyzed systems

As indicated in Introduction section, foam properties of hydrolysates may differ considerably from those of their parental proteins as a function of the DH. According to Conde

318 and Rodriguez Patino (2007), a low DH is favorable to improve foaming capacity and foam
319 stability. Likewise, Chobert et al. (1988) observed that solubility and emulsifying activity of
320 trypsin hydrolysates of whey proteins increased when DH increased until an optimum (3.9%)
321 and then decreased. Thus, assuming that the results obtained by Chobert et al. (1988) could be
322 extrapolated to foams, hydrolysis conditions (E/S ratio and incubation time) selected in the
323 present work were those leading to a hydrolyzed native β-Lg, HH controls-1 and GH systems
324 with a DH of ~ 4.0% (calculated with the Eq (1)), used later in interfacial and foaming
325 determinations. These conditions are highlighted in bold in **Table 1**.
326 Because of questions of susceptibility of the substrate, we used two types of proteolytic
327 enzymes, trypsin and α-chymotrypsin. Trypsin cleaves protein on the C-terminal side of Lys
328 and Arg residues. These residues are blocked during glycation, especially under the more severe
329 conditions (48 h at 50 ºC), which reduce reactivity of trypsin against glycated protein. Unlike
330 trypsin, α-chymotrypsin hydrolyzes peptide bonds with aromatic or large hydrophobic side
331 chains (Tyr, Trp, Phe, Met, Leu) on the carboxyl end of the bond, and, hence, galactose adducts
332 do not block its cleavage sites.
333 As observed in **Table 1**, under the same proteolysis conditions, native and control heated
334 β-Lg samples showed similar DH values, ranging from 2.40 to 6.11% and 2.33 to 5.99%,
335 respectively. Regarding glycoconjugates, those in advanced stages of the MR (β-Lg:Gal 48 h,
336 50 °C), showed the lowest DH values under the same proteolysis conditions. This may be
337 related to important structural changes derived from extensive cross-linking reactions that occur
338 during the advanced stages of this reaction (Corzo-Martínez et al., 2008, 2010b), which result in
339 the formation of protein aggregates that lead to a certain protection against enzyme activity due
340 to the inaccessibility of potential cleavage sites. Similar trend was found by Corzo-Martínez et
341 al. (2010b) and Maleki et al. (2000) who studied the digestibility of β-Lg glycoconjugates and
342 the major allergens from peanut (Ara h 1 and Ara h 2), respectively.

343 Hydrolyzed/glycated (HG) systems studied in the present work were obtained by
344 glycation of hydrolyzed native β-Lg with a DH of 4.87% (0.5% trypsin, 60 min), under the
345 same conditions as glycated/hydrolyzed (GH) systems, that is 40 °C for 24 h and 50 °C for 48 h.
346
347 *3.2. Determination of glycation extent of GH and HG systems*
348 Glycation degree (number of Gal adducts bound to peptide/protein) of
349 glycated/hydrolyzed (GH) systems is the same as that of non-hydrolyzed glycoconjugates
350 obtained under identical glycation conditions, which have been well-characterized in previous
351 works of our group by MALDI-TOF-MS (Corzo-Martínez et al., 2010a). That is 12 adducts of
352 Gal for GH [24 h, 40 ºC] system and 18 adducts of Gal for GH [48 h, 50 ºC] system.
353 Regarding hydrolyzed/glycated (HG) systems, to confirm that glycation efficiently took
354 place, the consumption of Gal present in the reaction medium during incubation of hydrolyzed
355 native β-Lg with Gal was determined. During incubation under controlled reaction conditions, a
356 loss of 54% (at 40 °C for 24 h) and 76% (at 50 °C for 48 h) of Gal present in the reaction
357 medium was detected, indicating that Gal covalently interacted with available free amino groups
358 of hydrolyzed native β-Lg. From these values, the number of Gal molecules covalently bound to
359 β-Lg protein/peptides was estimated with Eq. (2), previously described. Thus, an estimated
360 number of 18 and 25 Gal molecules were bound to the hydrolyzed native β-Lg after incubation
361 with Gal for 24 h at 40 °C and 48 h at 50 °C, respectively. At both incubation conditions,
362 glycation degree of HG systems was higher than that of GH systems, which is due to the higher
363 amount of free amino groups susceptible to glycation after hydrolysis in the former.
364 Moreover, absorbance at 420 nm (A_{420}) was measured as an index of the brown polymers
365 formed in more advanced stages of non-enzymatic browning (Ting & Rouseff, 1986; Corzo-
366 Martínez et al., 2008). A_{420} values at 50 °C of HG and GH systems (0.394 ± 0.006 and 0.233 ±
367 0.002, respectively) were significantly higher than at 40 °C (0.080 ± 0.002 for HG and 0.023 ±
368 0.001 for GH) because of the acceleration of reaction with the temperature (Ryu, Roh, Noh,
369 Kim, Oh, Lee, & Kim, 2003; Corzo-Martínez et al., 2008, 2010b). Likewise, according to the

370 glycation degree, A_{420} values of HG systems were significantly higher than those of GH systems
371 at all assayed reaction conditions, which indicates a higher advance of the MR with hydrolyzed
372 β-Lg than with non-hydrolyzed β-Lg.
373
374 *3.3. Solubility and surface hydrophobicity*
375 Surface hydrophobicity (S_0) values and solubility of heated and hydrolyzed controls (HH
376 controls-1) and hydrolyzed and heated controls (HH controls-2) were very similar, no
377 significant ($P > 0.05$) differences being observed. Thus, in order to simplify the tables and
378 facilitate the understanding of the results, only data obtained for HH controls-2 are shown.
379 **Table 2** shows ANS fluorescence (S_0) of non-hydrolyzed protein and hydrolyzed native
380 β-Lg, HH controls, and HG and GH systems. As expected, in general, all hydrolyzed systems
381 showed significantly ($P < 0.05$) higher S_0 than non-hydrolyzed ones, determined in a previous
382 work (Corzo-Martínez et al. 2008). This is probably due to the higher exposure of hydrophobic
383 groups on peptide surface after hydrolysis (Ipsen et al., 2001). Likewise, no significant ($P >$
384 0.05) differences were observed between ANS fluorescence values for hydrolyzed native β-Lg,
385 HH [24 h, 40 °C] control and HG and GH [24 h, 40 °C] systems, suggesting that heating and
386 glycation at 40 °C did not significantly modified protein/peptide structure. However, for GH
387 and HG [48 h, 50 °C] systems, S_0 values were significantly ($P < 0.05$) lower than the other
388 hydrolyzed systems, similar to that previously observed for non-hydrolyzed glycoconjugates
389 (Corzo-Martínez et al., 2008).
390 **Table 2** also depicts the solubility values obtained for all samples at pH 7 and pH 5. At
391 pH 7, no differences were observed among solubility of non-hydrolyzed and hydrolyzed native
392 β-Lg, probably due to unmodified protein is already completely soluble at pH 7. At this pH,
393 solubility of HH controls and HG and GH [24 h, 40 °C] systems did not show significant ($P >$
394 0.05) differences as compared to the native protein, indicating that heating and glycation under
395 mild conditions did not have an important effect on β-Lg solubility. Only for samples obtained
396 under severe incubation conditions, that is HG and GH [48 h, 50 °C] systems, a decrease of

397 solubility was observed probably due to their higher aggregation degree as compared to the
398 other systems, according to results from S_0 determination; however, in spite of this, such
399 samples remained highly soluble (> 89%).
400 At pH 5, hydrolyzed native β-Lg kept a maximum solubility (~100%) despite being near
401 its isoelectric point (pI 5.2). This solubility value was significantly higher than that of non-
402 hydrolyzed β-Lg, which also remained highly soluble (~86%), in agreement with other authors
403 who also reported the great structural stability to changes in environmental pH of native β-Lg
404 (Chevalier et al., 2001; Jimenez-Castaño, López-Fandiño, Olano, & Villamiel, 2005; Jimenez-
405 Castaño, Villamiel, & López-Fandiño, 2007; Corzo-Martínez et al., 2012). Moreover, unlike
406 non-hydrolyzed control heated and glycated β-Lg (Corzo-Martínez et al., 2012), HH controls as
407 well as HG and GH systems remained completely soluble even under severe incubation
408 conditions (48 h at 50 ºC).
409
410 *3.4. Foaming properties*
411 Similarly to surface hydrophobicity and solubility, foaming and interfacial properties of
412 heated and hydrolyzed controls (HH controls-1) and hydrolyzed and heated controls (HH
413 controls-2) were very similar, so that only results obtained for HH controls-2 are shown below.
414
415 *3.4.1. Foaming capacity*
416 **Figure 1** shows the values of the overall foaming capacity (OFC, mL/s) and the foam
417 capacity (FC) obtained with each of the systems assayed at pH 7 and 5.
418 For hydrolyzed native β-Lg HH controls and HG and GH [24 h, 40 °C] systems, at pH 7,
419 no substantial differences were observed among OFC and FC values of all of them (**Figures 1A**
420 **and 1B,** solid bars), according to no differences found among their surface hydrophobicity and
421 solubility values. Moreover, foam overrun of these systems was similar to that of non-
422 hydrolyzed native β-Lg, which could be attributed to that, regardless of the S_0, all of them
423 showed high solubility (**Table 2**). Particularly striking resulted that HG and GH [48 h, 50 °C]

systems, with the lowest S_0 values, showed OFC and FC values similar to those of the other systems.

At pH 5 (**Figures 1A and 1B**, hatched bars), the foaming capacity of non-hydrolyzed and hydrolyzed 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 HH [24 h, 40 °C] and [48 h, 50 °C] controls showed OFC and FC values similar to those of foams with non-hydrolyzed and hydrolyzed native β-Lg. Likewise, HG and GH [24 h, 40 °C] and [48 h, 50 °C] systems showed similar OFC and FC values than hydrolyzed native β-Lg and HH controls, and no significantly ($P > 0.05$) lower than at pH 7 (**Table 2**).

Likewise, no important differences in OFC and FC values were observed among hydrolyzed systems (hydrolyzed native β-Lg, HH controls and HG and GH systems) and non-hydrolyzed systems (Corzo-Martínez et al., 2012) at both pH 7 and 5.

3.4.2. Foaming stability

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

At pH 7 (**Figure 2**, solid bars), draining stability of foam formed with non-hydrolyzed native β-Lg was significantly higher than that of foams formed with hydrolyzed native β-Lg, HH controls and HG and GH systems, despite of foaming capacity of all of them was similar (**Figure 1**).

At pH 5 (**Figure 2**, hatched bars), similarly to the foaming capacity, stability of foams formed with non-hydrolyzed native β-Lg did not undergo substantial changes with respect to pH 7, in agreement with several authors (Kinsella & Morr, 1984; Schmitt, Bovay & Frossard, 2005) who reported that β-Lg forms thick interfacial layers close to its pI (5.2), leading to stable foams. Hydrolyzed native β-Lg gave rise to foams significantly more stable than at pH 7 and non-hydrolyzed protein at both pH values.

Moreover, foams of HH controls, which kept high solubility at pH 5, showed significantly lower stability than non-hydrolyzed and hydrolyzed native β-Lg, with a half-life time very similar to that observed at pH 7. Unlike HH controls, foams formed with HG and GH systems, especially [48 h, 50 ºC] systems, resulted to be the most stable foams at pH 5, showing $\theta_{1/2}$ values significantly higher than even foam formed by non-hydrolyzed native β-Lg at pH 7 and non-hydrolyzed glycoconjugates at pH 5, which already displayed improved stabilizing properties with respect native β-Lg (Corzo-Martínez et al., 2012).

3.5. Interfacial properties

3.5.1. Dynamic of protein adsorption at air-water interface

Dynamic of adsorption of non-hydrolyzed native protein, hydrolyzed native β-Lg, HH controls and HG and GH systems was studied in relation to their diffusion rate to the interface, represented by the apparent diffusion constant (K_{Dif}) (**Table 2**), and to its ability to increase the surface pressure (π) with the adsorption time (θ) (**Figure 3**).

At pH 7 (**Figure 3A**), surface activity of hydrolyzed native β-Lg, HH controls and HG and GH systems was very similar, no substantial differences being observed between the values of surface pressure reached at long term adsorption (π at 10,800 s, $\pi_{10,800}$) and, hence, between the amount of protein adsorbed at the air-water interface, according to results from OFC and FC determination.

However, when we studied the dynamic of adsorption during the first stage of protein diffusion (**Table 2**), in general, all the studied hydrolyzed systems showed significantly higher K_{Dif} values than non-hydrolyzed native β-Lg.

Regarding the results obtained at pH 5 (**Figure 3B**), dynamic of adsorption of hydrolyzed native β-Lg and HH controls at both short (**Table 2**) and long times (**Figure 3B**), was hardly altered by the pH reduction. K_{Dif} and $\pi_{10,800}$ values observed at pH 5 were very similar to those obtained at pH 7, which support OFC and FC results. For HG and GH [24 h, 40 °C] and [48 h,

477 50 °C] systems, a significant increase in K_{Dif} values with respect pH 7 was observed (**Table 2)**.
478 These results suggest that glycation, either after or before hydrolysis, increases the efficiency of
479 protein adsorption. However, as previously, this had no significant ($P > 0.05$) effect on foaming
480 capacity, as OFC and FC values of HG and GH systems were similar to those of non-
481 hydrolyzed and hydrolyzed native β-Lg and HH controls (**Figures 1A and 1B**).
482
483 *3.5.2. Surface dilatational properties*
484 Rheological properties of adsorbed films formed by hydrolyzed native β-Lg, HH controls
485 and HG and GH systems at pH 7 and 5 were evaluated by means of the evolution of the surface
486 dilatational modulus (E) with the adsorption time (θ) (**Figure 4**) and versus surface pressure (π)
487 (**Figure 5**). This second type of representation provides, at long adsorption time, additional
488 information of the degree of intermolecular interaction at the interfacial film after protein
489 adsorption (Martínez et al., 2009).
490 In general, at pH 7, E values showed by adsorbed films of hydrolyzed native β-Lg, HH
491 controls and HG and GH systems (**Figures 4A**) were lower than those of the film formed by
492 non-hydrolyzed native β-Lg (~70 mN/m) (Corzo-Martínez et al., 2012). These results suggest a
493 lower capacity of peptides to interact at the air-water interface and, hence, a lower capacity of
494 hydrolyzed β-Lg systems to form strong viscoelastic films as compared to non-hydrolyzed
495 native protein. Similar results had been obtained for others proteins (Pizones, Carrera, Pedroche,
496 Millan, & Rodríguez Patino, 2009). Thus, hydrolyzed native β-Lg, leading to the formation of a
497 film with the lowest E value at long term adsorption ($E_{10,800}$), gave rise to the foam with the
498 lowest stability (with the lowest $\theta_{1/2}$ value) (**Figure 2**). Adsorbed films formed with HH controls
499 and HG and GH systems showed similar E values at long term adsorption (**Figure 4A**),
500 according to results obtained for half-life values (**Figure 2**).
501 E-θ and E-π plots of hydrolyzed β-lg systems (hydrolyzed native β-Lg, HH controls and
502 HG and GH systems) showed an increase of E value with the adsorption time (θ) (**Figure 4A**)
503 and surface pressure (π) (**Figure 5A**), which suggested the existence of interactions between

film-forming components, especially non-hydrolyzed protein remaining after hydrolysis, which are created during protein adsorption, increasing the rigidity and cohesion of the interfacial film. As observed in **Figure 5A** and according to the theory of Lucassen-Reynders et al. (1975), all the hydrolyzed systems showed a behavior of a non-ideal fluid (slope of E-π plot > 1); however, in concordance with previous results, hydrolyzed native β-Lg showed the lowest E values with the surface pressure (π), close to the behavior of an ideal fluid (slope of E-π plot = 1; which is indicated in the graphic by a dashed line). This indicates the existence of weak interactions among components of the adsorbed film.

On the other hand, the phase angle (ϕ) at long term adsorption ($\phi_{10,800}$) can be considered as a measure of the relative elasticity of the adsorbed protein films. So that the higher drop of the phase angle values with the adsorption time (θ), the greater the elasticity of the adsorbed protein film, and vice versa. In general, for all the hydrolyzed β-Lg systems studied, the phase angle (ϕ) decreased with increasing adsorption time (θ) (**Figure 6A**), which is related to the formation of elastic films. Unexpectedly, the highest ϕ values at long adsorption time were observed with GH [24 h, 40 ºC] and [48 h, 50 ºC] systems and the HG [24 h, 40 ºC] system. This suggests that high E values of films formed by these systems could be due to their molecular packing as a result of the rapid protein adsorption at the interface, and not due to the increase in the interaction degree between the adsorbed molecules, which leads to the formation of films with fluid character (Rodríguez Patino et al., 1999; Rodríguez Patino, Molina-Ortiz, Carrera, Rodríguez Niño, & Añón, 2003) and, hence, not very resistant against destabilization, according to lower half-life values previously observed for these systems (**Figure 2**).

At pH 5, however, E-θ (**Figure 4B**) and E-π plots (**Figure 5B**) of GH and HG systems, especially [48 h, 50 ºC] systems, showed the highest E values of all studied samples, even at pH 7 (**Figures 4A and 4B**). These results suggest the formation of highly elastic and cohesive films, with a great interaction degree among their components. In agreement with these results, HG and GH systems, particularly the GH [48 h, 50 ºC] system, displayed the lowest ϕ values at long term adsorption (**Figure 6B**), which is indicative of the formation of a more elastic and

resistant film than that of native and control heated hydrolysates. These results are in concordance with foam stability results (**Figure 2**), as foams formed at pH 5 with HG and GH systems, especially with the GH [48 h, 50 ºC] system, showed the highest values of half-life, these being even higher that those showed by non-hydrolyzed glycoconjugates at pH 5 in a previous work (Corzo-Martínez et al., 2012).

4. Discussion

Hydrolyzed native β-Lg, HH controls and HG and GH [24 h, 40 °C] systems showed similar S_0 value, this being higher than that of non-hydrolyzed native β-Lg. According to this, protein hydrolysis has been also shown to cause the unmasking of previously hidden hydrophobic groups and their exposition to the more polar surrounding environment, increasing the protein surface hydrophobicity (S_0) (Creusot et al., 2006; Tamm et al., 2012). A significantly ($P < 0.05$) lower surface hydrophobicity was, however, observed for GH and HG [48 h, 50 °C] systems. These results are indicative of important structural changes in the three-dimensional configuration of the peptides and protein due to the cross-linking and aggregation reactions that occur during the advanced stages of the MR, resulting in protein hydrophobic patches buried inside of such aggregates. Moreover, such decrease in S_0 after glycation may be also attributed to the blocking of Lys and/or Arg residues by Gal in the Maillard conjugates, as ANS may also strongly bind cationic groups of proteins (Gasymov & Glasgow, 2007). This could also explain the lowest S_0 values of HG [48 h, 50 °C] systems, with higher glycation degree than GH [48 h, 50 °C] systems (**Table 2**).

According to surface hydrophobicity results, solubility at pH 7 of hydrolyzed native β-Lg, HH controls and HG and GH [24 h, 40 °C] systems was higher than that of non-hydrolyzed native β-Lg. It has been described that protein hydrolysis leads to an increased solubility of the peptide molecules due to the reduction of particle size, which gives rise to an increased number of charged groups (NH_4^+; COO^-) on the peptide surface and, consequently,

557 higher electrostatic forces that enhance protein-water interactions and, hence, solubility
558 (Damodaran, 1997a; Kilara & Panyam, 2003).
559 Solubility at pH 7 of HG and GH [48 h, 50 °C] systems was lower than that of the other
560 hydrolyzed systems, which could be also attributed to the cross-linking and aggregation
561 reactions that occur during the advanced stages of the MR; however, as commented above, such
562 samples kept still high solubility (> 89%), which could be related to their high glycation degree.
563 Thus, their high solubility might be due to the additional negative net surface charge and, hence,
564 increased electrostatic repulsion between molecules that keeps peptides/protein in dispersion.
565 Moreover, the higher surface hydrophilicity provided by covalently linked galactose increases
566 the electrostatic interactions between protein/peptide and water molecules and, hence, the
567 solubility (Turgeon, Gauthier, Mollé, & Léonil, 1992; caessens, Gruppen, Visser, Aken, &
568 Voragen, 1997).
569 At pH 5, solubility of non-hydrolyzed native β-Lg significantly ($P < 0.05$) dropped.
570 However, hydrolyzed native β-Lg and HH controls kept high solubility (similar to pH 7), and
571 solubility of HG and GH systems, especially [48 h, 50 ºC], increased after pH reduction (**Table**
572 **2**). It is noteworthy the fact that, in these later, β-Lg aggregates formed during the advanced
573 stages of the MR at 50 °C are more soluble at pH 5 than at pH 7. This was also observed with
574 non-hydrolyzed β-Lg glycoconjugates (Corzo-Martínez et al., 2012) and is in agreement with
575 results obtained by Zhou et al. (2008), who found a higher formation of insoluble moisture-
576 induced whey protein aggregates at pH 7 than at pH 5, after storage for 14 days at 35 °C. These
577 authors indicated that these differences were due to a pH-dependent ratio between the thiolate
578 anion and the thiol group (reactive form to non-reactive form), which are responsible for the
579 formation of intermolecular disulfide bond. So that, below pH 7, the ratio thiolate anion/thiol
580 group was low, which slowed the formation of intermolecular disulfide bonds and, hence, the
581 formation of water-insoluble aggregates.
582 Several authors have observed a positive relation between the diffusion rate of proteins
583 and their surface hydrophobicity (Nakai, 1983; Moro, Gatti,& Delorenzi, 2001; Kim, Cornec, &

584 Narsimham, 2005; Pérez, Carrara, Carrera, & Rodríguez Patino, 2009). Hydrophobic regions
585 have a high affinity by the air-water interface, so that their higher exposition on protein/peptide
586 surface promotes protein/peptide migration to and adsorption at the interface (Ipsen, Otte,
587 Sharma, Nielsen, Hansen, & Qvist, 2001), orienting the hydrophobic regions toward the air
588 phase and the hydrophilic ones toward aqueous phase and quickly reducing the interfacial
589 tension. Moreover, a direct relationship between solubility and the diffusion rate of proteins to
590 the air-water interface has been also observed (Damodaran, 1997b; Davis et al., 2005; Creusot,
591 Gruppen, van Koningsveld, deKruif, & Voragen, 2006), this being an important requirement of
592 good foaming agents during the migration phase.
593 Thus, the higher surface hydrophobicity and solubility at pH 7 of hydrolyzed native β-Lg,
594 HH controls and HG and GH [24 h, 40 °C] systems could explain the significantly ($P < 0.05$)
595 better efficiency of adsorption at the air-water interface (higher K_{Dif} values) found for these
596 systems as compared to non-hydrolyzed native β-Lg (**Table 2**). In the case of HG and GH [48 h,
597 50 °C] systems, their high adsorption efficiency might be also due to their high solubility (~
598 90%). However, the higher K_{Dif} value of hydrolyzed systems did not lead to a significant ($P >$
599 0.05) increase of OFC and FC values (foam overrun) at pH 7 with respect non-hydrolyzed
600 native β-Lg (**Figure 1**), likely due to this presents a diffusion rate already good enough for foam
601 formation.
602 At pH 5, hydrolyzed native β-Lg and HH controls showed similar adsorption efficiency at
603 short and long time (K_{Dif} and $\pi_{10,800}$ values, respectively) to that at pH 7, as they remained highly
604 soluble. The increase observed in solubility of GH and HG systems after pH reduction led to an
605 increase of their K_{Dif} value (**Table 2**). However, this had no significant ($P > 0.05$) effect on
606 foaming capacity, as OFC and FC values of HG and GH systems were similar to those of non-
607 hydrolyzed and hydrolyzed native β-Lg and HH controls (**Figures 1A and 1B**). Similarly to pH
608 7, this could be attributed to that the diffusion rate of these systems is also good enough to
609 stabilize the bubbles during their formation, leading to the formation of foams with good
610 overrun.

Regarding stabilizing properties, stability of foams ($\theta_{1/2}$ value) formed with hydrolyzed systems at pH 7 was significantly lower than that formed with non-hydrolyzed native β-Lg (**Figure 2**). This is related to the low E values observed for foams formed with hydrolyzed systems, indicating that dilatational characteristics of adsorbed film are not good enough to stabilize the bubbles. It has been reported that it might be due to the increased net surface charge of protein after hydrolysis (increased number of charged groups (NH_4^+; COO^-) on the peptide surface), which may increase intermolecular electrostatic repulsion at the interface giving rise to adsorbed films less rigid and stable against foam destabilization mechanisms (Turgeon et al., 1992; Ibanoglu & Ibanoglu, 1999). Among hydrolyzed systems, GH [24 h, 40 ºC] and [48 h, 50 ºC] systems and the HG [24 h, 40 ºC] system showed the lowest capacity to interact at the interfacial layer, as indicated by their high ϕ values at long adsorption time (**Figure 6A**). This might be due to their lower surface hydrophobicity, which is related to the intermolecular interaction of film-forming components during rearrangement phase, as well as to the glycation-induced electrostatic repulsion between molecules.

Interestingly, foams formed by GH and HG systems, especially [48 h, 50 ºC] systems, showed increased E values at pH 5, these being the highest E values of all of the studied hydrolyzed systems even at pH 7 (**Figures 4A and 4B**). This agrees with the improvement observed in the dynamic of adsorption (higher K_{Dif}) of these systems at pH 5 (Murray, 2002; Rodríguez Patino et al., 2003) (**Table 2**). Moreover, it might be possible that overcoming of repulsive electrostatic interactions between molecules produced at pH close to pI promotes intermolecular hydrophobic interactions and stronger ones, as hydrogen bonds and S-S bridges, at the interface, leading to the formation of a stronger viscoelastic film (Medrano et al., 2009; Kinsella & Morr, 1984; Wilde & Clark, 1996; Murray, 1998). This, as observed in **Figure 2**, gave rise to foams highly stable at pH 5, GH [48 h, 50 ºC] system being the one with the highest stabilizing properties against gravitational drainage.

5. Conclusions

No significant effect of glycation combined with hydrolysis on foaming capacity at both pH 7 and 5 was observed. However, a significant improvement of stabilizing properties of β-Lg at pH 5 took place after treatment, particularly after glycation at 50 ºC followed by limited hydrolysis (GH, 48 h at 50 ºC), as a result of the increase in elastic character and cohesion of the interfacial film. Thus, foams formed with the GH [48 h, 50 ºC] system showed an exceptional stability at pH 5 against gravitational drainage, higher than that of foams formed with non-hydrolyzed native β-Lg and even with non-hydrolyzed glycoconjugates, which, as observed in a previous work (Corzo-Martínez et al., 2012), already displayed better foam stability than native β-Lg.

Therefore, from the findings described in this work we can infer that conjugation of β-Lg with galactose via the Maillard reaction followed by limited hydrolysis is an efficient method to improve foam stabilizing properties of previously studied β-Lg glycoconjugates at pH 5 and, hence, increase their applicability degree as food ingredients with potential functionality. The use of these complexes is potentially of interest to the industry, as it might allow extending the use of β-Lg as foaming agent in the formulation of a wide range of acidic foods and beverages. In this way, a future work studying the stability as foam agents of these potential ingredients during the processing and storage of acidic foods is needed.

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Table 1. Degree of hydrolysis of native, control heated and glycated β-Lg under different hydrolysis conditions.

Sample (S)	Enzyme (E)	Ratio E/S	Incubation time (min)	Degree of Hydrolysis (%)
Native β-Lg	Trypsin	**0.5% *** 1%	10; **60** 5; 15; 30; 120	2.40; **4.87** 2.86; 3.29; 5.60; 6.11
Control heated β-Lg	Trypsin	**0.5% *** 1%	10; **60** 5; 15; 30; 120	2.33; **4.55** 2.54; 3.15; 5.43; 5.99
β-Lg:Gal 24 h, 40 ºC	α-chymotrypsin	1%	5; 15; 30; 120	2.86; 3.29; 5.60; 6.11
β-Lg:Gal 48 h, 50 ºC	α-chymotrypsin	**0.5%** 1%	30; **120** 15; 60	2.79; **4.73** 1.69; 5.56

* Conditions selected to study interfacial and foaming properties are indicated in bold.

Table 2. Surface hydrophobicity (S_0) and solubility and apparent diffusion constant (K_{Dif}) at pH 7 and 5 of β-lactoglobulin (β-Lg) systems, including hydrolyzed native β-Lg, hydrolyzed and heated controls (HH controls) and glycated/hydrolyzed (GH) and hydrolyzed/glycated (HG) systems.

	Samples	ANS fluorescence (S_0)	%Solubility		K_{Dif} (mN $m^{-1}s^{-1}$)	
			pH 7	pH 5	pH 7	pH 5
Native protein	Non-hydrolyzed β-Lg	40.8 ± 1.15 *a	100.00 ± 1.09 a	85.96 ± 1.20 b	42.95 ± 1.63 a	50.17 ± 4.69 d
	Hydrolyzed β-Lg	45.7 ± 0.68 bc	99.79 ± 0.92 a	99.67 ± 0.89 a	62.35 ± 2.08 b	65.93 ± 1.95 b
HH Controls	β-Lg 24 h, 40 ºC	46.40 ± 1.57 b	99.57 ± 1.13 a	99.82 ± 1.10 a	59.17 ± 1.52 bc	56.61 ± 2.55 bc
	β-Lg 48 h, 50 ºC	44.74 ± 0.47 c	100.89 ± 0.90 a	100.17 ± 1.18 a	57.40 ± 2.69 c	55.75 ± 1.87 c
HG systems	β-Lg:Gal 24 h, 40 ºC	46.69 ± 0.79 b	98.32 ± 1.73 a	102.99 ± 0.96 c	61.36 ± 1.02 b	70.34 ± 6.27 e
	β-Lg:Gal 48 h, 50 ºC	30.16 ± 0.24 d	89.45 ± 1.10 b	99.87 ± 0.97 a	63.67 ± 2.13 b	71.97 ± 5.86 e
GH systems	β-Lg:Gal 24 h, 40 ºC	47.68 ± 0.02 b	99.10 ± 1.01 a	102.1 ± 0.82 c	61.24 ± 1.19 b	67.07 ± 3.02 e
	β-Lg:Gal 48 h, 50 ºC	32.33 ± 0.79 d	90.07 ± 0.98 b	100.11 ± 1.07 a	62.95 ± 2.06 b	72.46 ± 2.49 e

*Data represent the mean of duplicate determinations ± standard deviation.

a-e Different case letters indicate statistically significant ($P < 0.05$) differences among values of S_0, solubility and K_{Dif} observed for β-Lg systems. For solubility and K_{Dif} values at pH 7 and 5 are being compared.

Figure captions

Figure 1. Values obtained for the parameters of overall foaming capacity (OFC, mL/s) **(A)** and foam capacity (FC) **(B)** with non-hydrolyzed native β-Lg, hydrolysed native β-Lg, hydrolyzed and heated controls (HH controls) and glycated/hydrolyzed (GH) and hydrolyzed/glycated (HG) systems at pH 7 (solid bars) and pH 5 (hatched bars). Data are average of two independent experiments ± standard deviation of the mean. $^{a-b}$ Different case letters indicate statistically significant ($P < 0.05$) differences.

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Figure 3. Surface pressure (π) as a function of time (θ) of adsorbed films of hydrolized β-Lg systems at pH 7 **(A)** and pH 5 **(B)**: ✳ native β-Lg; control β-Lg heated under △ mild and □ severe conditions; glycated/hydrolyzed (GH) systems obtained under ○ mild and ◇ severe conditions; hydrolyzed/glycated (HG) systems obtained under ● mild and ◆ severe conditions. Data are average of two independent experiments ± standard deviation of the mean.

Figure 4. Surface dilatational modulus (E) as a function of time (θ) at pH 7 **(A)** and pH 5 **(B)** of hydrolyzed β-Lg systems: ✳ native β-Lg; control β-Lg heated under △ mild and □ severe conditions; glycated/hydrolyzed (GH) systems obtained under ○ mild and ◇ severe conditions; hydrolyzed/glycated (HG) systems obtained under ● mild and ◆ severe conditions. Data are average of two independent experiments ± standard deviation of the mean.

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Figure 1.

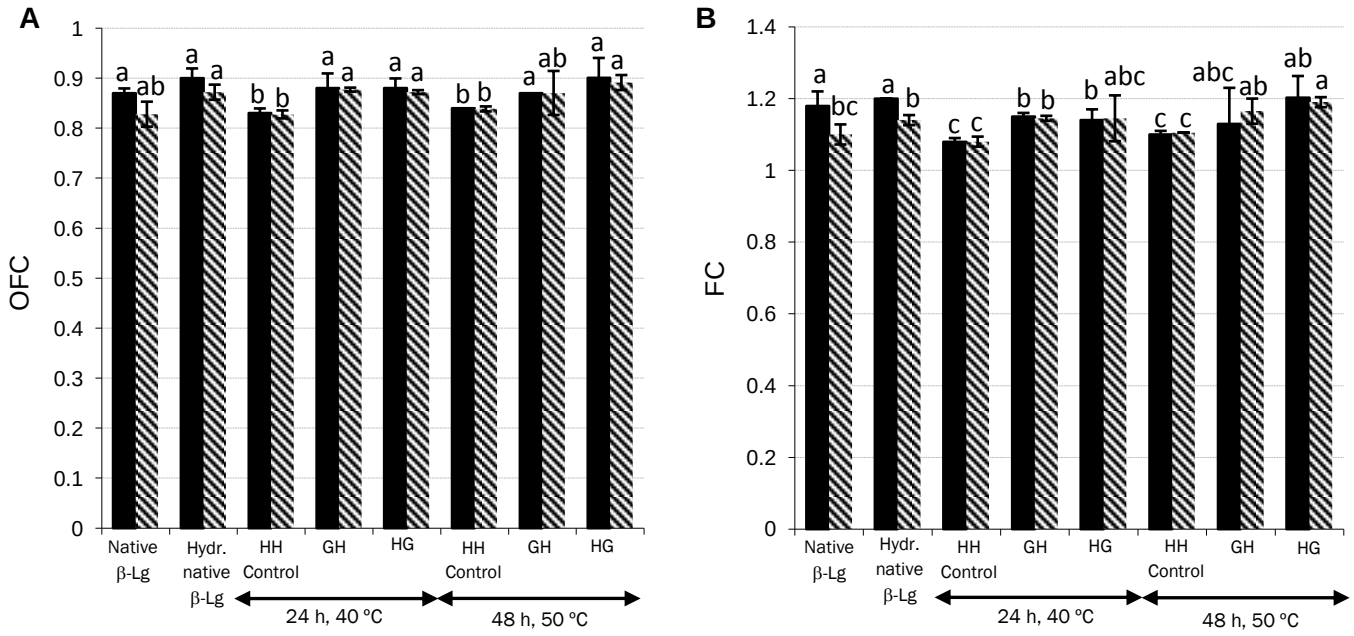
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Figure 2.

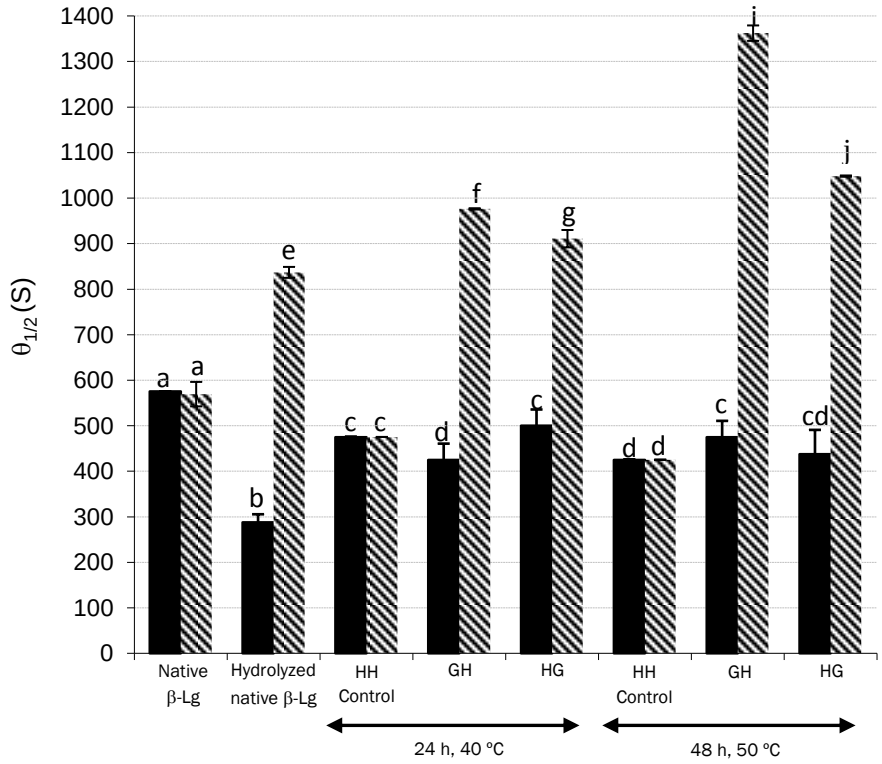
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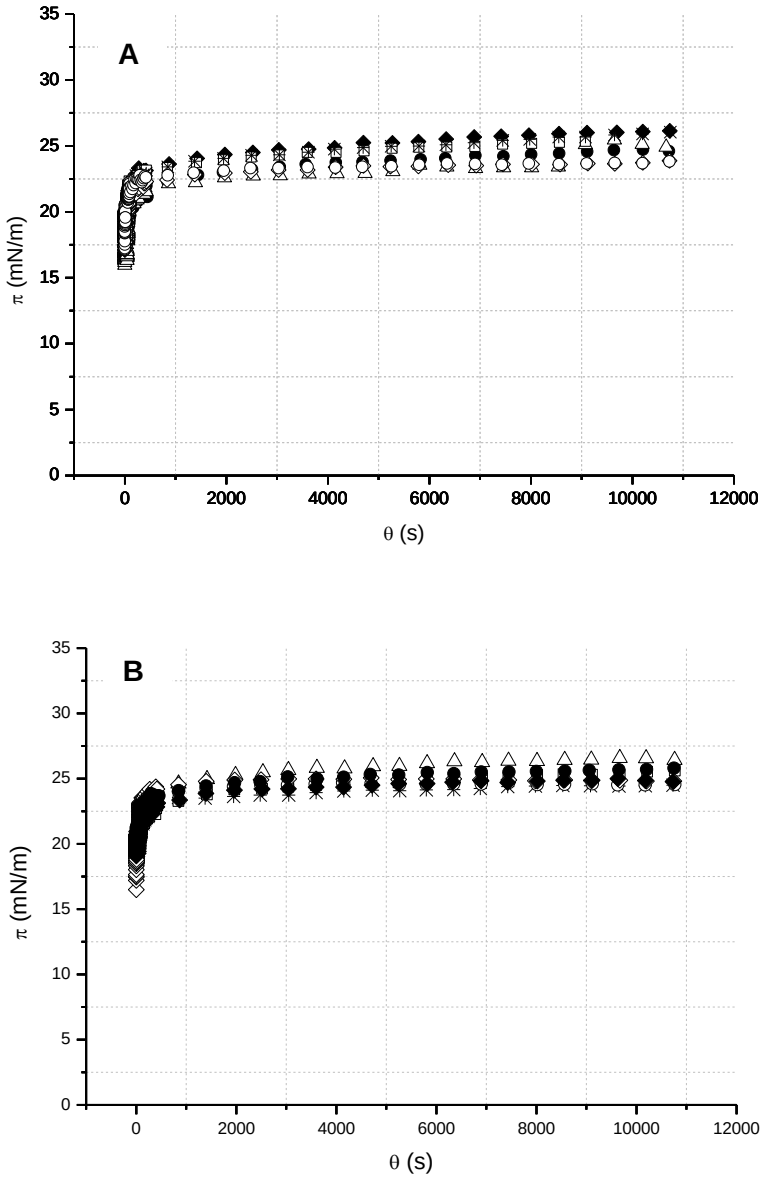
Figure 3.



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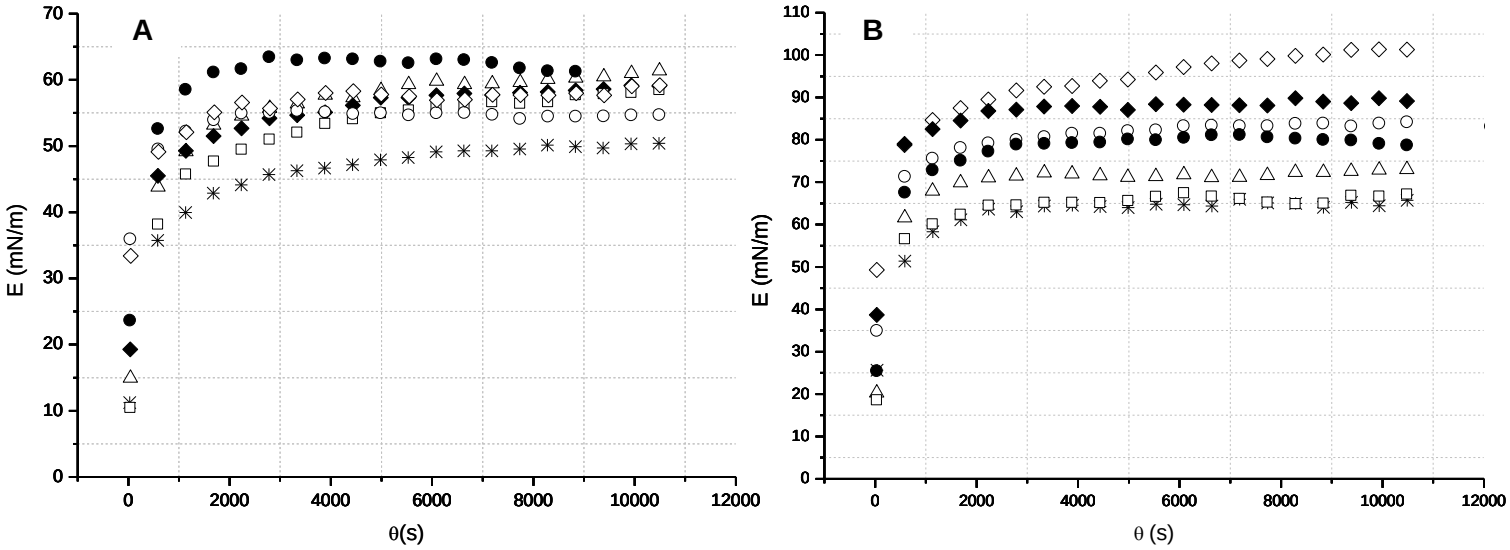

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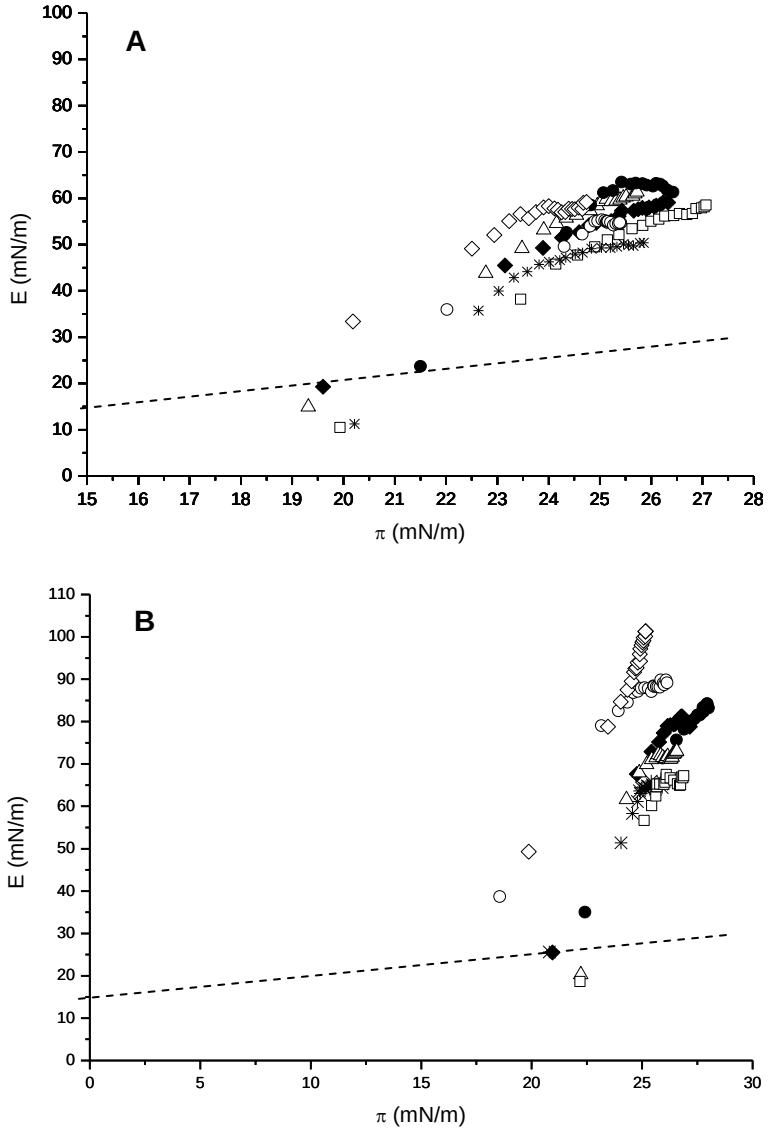

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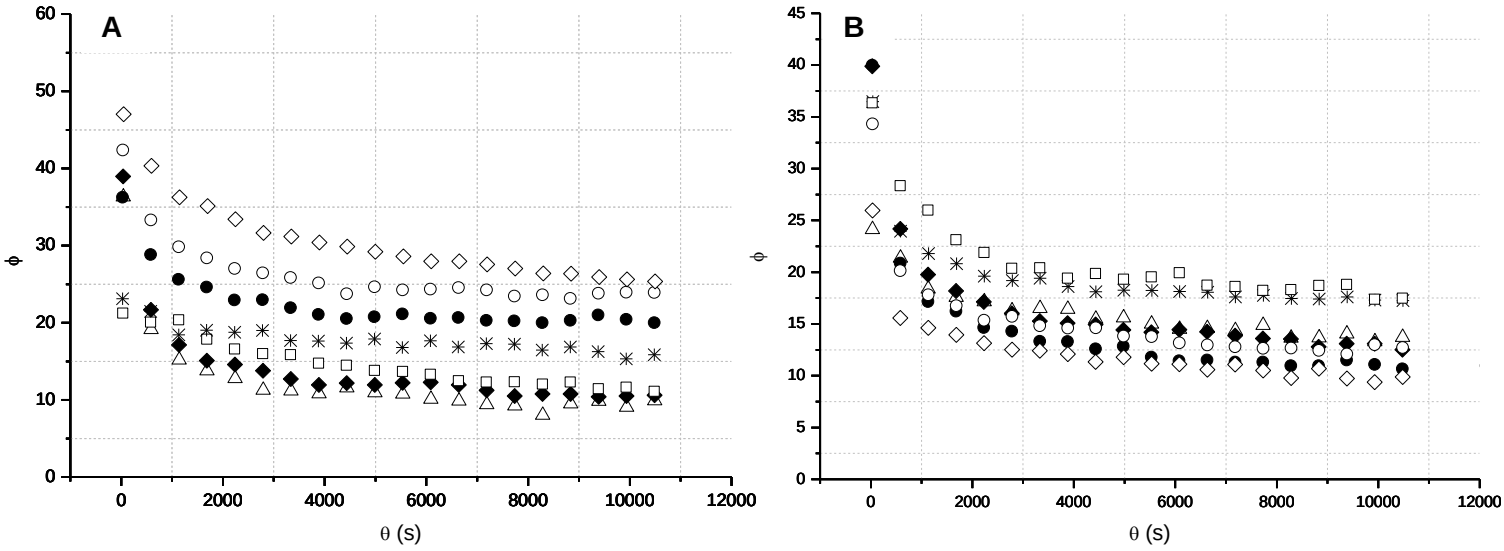
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