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Effects of heat treatment and high pressure on the subsequent lactosylation of β-lactoglobulin

Maite Rada-Mendoza [1], Mar Villamiel *, Elena Molina, Agustin Olano

Instituto de Fermentaciones Industriales (CSIC), c/Juan de la Cierva 3, 28006 Madrid, Spain

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Abstract

The effects of a previous heat treatment (60 and 80 °C, 30 min) and high-pressure (400 MPa, 25 and 60 °C, 1 h) on the subsequent lactosylation of β-lactoglobulin (50 °C, 44% RH, 120 h) were investigated. A control of native β-lactoglobulin was also stored under the afore-mentioned conditions. The structural changes caused during these treatments were studied by the loss of amino groups, SE-HPLC and native-PAGE and the degree of lactosylation was evaluated by means of furosine determination. After thermal and high-pressure treatments, the greatest structural changes were observed in the case of samples of β-lactoglobulin treated at 80 °C, 30 min and 400 MPa, 60 °C, 1 h. During storage, the highest lactosylation degree was found in native β-lactoglobulin. In heat-treated samples, the increase of lactosylated lysines was lower than the decrease of free amino groups, probably due to the cross-linking reactions. A similar decrease of free amino groups of β-lactoglobulin was observed immediately after 400 MPa, 60 °C, 1 h and 80 °C, 30 min; however, the level of lactosylation during the storage period was lower in the former, indicating different types of conformational changes in the two treatments. These differences lead to a higher effectiveness of heat-treatment than high-pressure in denaturating β-lactoglobulin for subsequent lactosylation under the tested conditions (of temperature, time, high-pressure and storage).

Keywords: β-Lactoglobulin; Lactosylation; Maillard reaction; Denaturation; High-pressure; Heat-treatment; Structural changes

1. Introduction

Because of its nutritive, biological and functional properties, whey protein concentrates, derived from the dairy industry, constitute a valuable source of ingredients used in many processed foods, such as bakery, meat and dairy products (Akita & Nakai, 1990; Bouhallab, Morgan, Henry, Mollè, & Léonil, 1999; Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001b; Kinsella & Whitehead, 1989). β-Lactoglobulin is the most abundant protein found in the whey of milk and can be considered as responsible for the majority of its physicochemical characteristics (Zayas, 1997). During the manufacture of whey, heating results in complex conformational modifications of proteins, including, under severe conditions, aggregate formation and, consequently changes in the functionality of proteins (Hong & Creamer, 2002).

High-pressure treatments, recently used as an alternative to thermal treatments for food preservation, also give rise to structural changes in milk proteins, such as denaturation and formation of β-lactoglobulin aggregates (Funtenberger, Dumay, & Cheftel, 1997; Iametti et al., 1997). Some of these changes may improve the functionality of this protein (Belloque, López-Fandiño, & Smith, 2000).

Moreover, during recent years, a great deal of attention has been focussed on improving the functional properties of whey proteins by different methods, including the Maillard reaction. During this reaction, the conjugation of a

* Corresponding author. Tel.: +34 91 562 29 00x397; fax: +34 91 564 48 53.
E-mail address: mvillamiel@ifi.csic.es (M. Villamiel).
[1] AECI scholar on leave from University of Cauca, Popayán, Colombia.

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reducing carbohydrate to the ε-amino group of lysine occurs spontaneously under heating conditions without the utilization of toxic chemical products (Chevalier, Chobert, Dalgalarrondo, & Haertlé, 2001a). It is known that the Maillard reaction, carried out under dry state and well-controlled conditions (temperature, relative humidity, time), is an adequate method for improving functionality of proteins without important structural changes (Morgan, Léonil, Mollé, & Bouhallab, 1997). Thus, β-lactoglobulin has been conjugated by means of the Maillard reaction with mono- and disaccharides to improve functional properties, such as solubility and emulsifying capacity (Chevalier et al., 2001b; Fenaille, Morgan, Parisod, Tabet, & Guy, 2003; Matsuda, Kato, & Nakamura, 1991; Morgan, Léonil, Mollé, & Bouhallab, 1999).

Structural changes, such as those involved in a denaturation process, may influence the reactivity of the protein. Thus, it is important to know how different denaturation conditions may affect the susceptibility of the proteins to the Maillard reaction. In this work, a study was undertaken to investigate the effect of high-pressure and thermal treatments on the glycosylation of β-lactoglobulin with lactose.

2. Materials and methods

2.1. Sample preparation, thermal and high-pressure treatments and glycosylation experiments

β-Lactoglobulin (β-Lg) (a mixture of A and B variants) was supplied as a lyophilised sample by Sigma Chemical Co. (St. Louis, MO). Lactose monohydrate was obtained from Scharlau Chemie (Barcelona, Spain). All other reagents were of analytical grade. Solutions containing β-Lg (3 mg/ml) were prepared in phosphate buffer (0.1 M at pH 6.5).

Samples submitted to pressurization were placed in polyethylene bags (100 ml) avoiding headspace, vacuum-sealed in polyethylene bags, and pressurized using a 900 HP apparatus (Eurotherm Automation, Lyon, France). The pressure was raised to 400 MPa at a rate of 2.5 MPa s^{-1}, maintained for 1 h at 25 or 60 °C and released at the same rate. Samples (30 ml) submitted to thermal treatments were placed in sealed Pyrex glass tubes (25 × 100 mm) and heated for 30 min at 60 or 85 °C in a thermostatically-controlled temperature bath of water under continuous agitation. After treatments, samples were immediately cooled in an ice-water bath and lactose at 1:100 molar ratio (β-Lg:lactose) was then added. The resulting solutions were freeze–dried and kept at 50 °C in desiccators equilibrated under vacuum to 44% relative humidity (RH), using saturated solutions of K_2CO_3. Samples were taken for analysis after 8, 24, 48, 72, 96 and 120 h of storage. As control, lactose was added to solutions of native β-Lg (3 mg/ml) and treated in a similar manner. All glycosylation experiments were performed in duplicate. All the analyses performed in this work were done with lactosylated samples.

2.2. Determination of 2-furoyl-methyl-lysine (furosine)

The extent of β-Lg lactosylation was evaluated by the furosine assay (generated by acid hydrolysis of the Amadori compound, first stable compound of the Maillard reaction) (Finot & Mauron, 1972). Furosine determination was carried out after hydrolysis of the stored powders. 400 μl of 8 N HCl were added to 2 mg of protein in hydrolysis tubes and heated at 110 °C for 23 h under inert conditions, followed by the addition of 2 ml of 8 N HCl and filtering through Whatman No. 40 filter paper. Five hundred microlitres of the filtrate were applied to a previously activated Sep-Pack C_{18} cartridge (Millipore Corporate Headquarters, Billerica, Massachusets, USA). Furosine was eluted with 3 ml of 3 N HCl and 50 μl were injected. Analysis was by an ion-pair RP-HPLC method, using a C_8 (Alltech furosine-dedicated, Lokeren, Belgium) column (250 × 4.6 mm i.d.) and a variable wavelength detector set at 280 nm (LDC Analytical, SM 4000, Riviera Beach, FL, USA). Operating conditions were as follows: column temperature, 35 °C; flow rate, 1.2 ml/min; solvent A, 0.4% HPLC grade acetic (Sharlau Chemie, Barcelona, Spain) in double-distilled water; solvent B, 0.3% KCl (Merck, Darmstadt, Germany) in solvent A (Resmini, Pellegrino, & Batelli, 1990). Calibration was performed by using solutions of known concentrations (from 0 to 0.8 mg/l) of commercial pure furosine standard (Neosystem Laboratories, Strasbourg, France). Data were expressed as lactosylated Lys (Desrosiers, Savoie, Bergeron, & Parent, 1989).

2.3. Determination of free amino groups

The content of free amino groups was determined using trinitrobenzenesulfonic acid (Sigma Chemical Co., St. Louis, MO). The absorbance, measured at 420 nm in a Beckman, DU 70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA), was transformed into μmoles of leucine/ml, using a calibration curve within the range 0.25–2.1 μM.

2.4. Study of structural changes

SE-HPLC was carried out using a Beckman System Gold HPLC (Beckman Instruments Inc., Fullerton, CA, USA). A TSK G4000SW_{XL} column (7.8 i.d. × 300 mm, Tosoh, Tokio, Japan) was equilibrated with 0.020 M phosphate buffer containing 0.15 mM NaCl at pH 7. Samples (100 μl) were applied to the column and eluted at a flow rate of 0.4 ml/min. Absorbance was monitored at 280 nm.

Native polyacrylamide gel electrophoresis (Native-PAGE) was performed in a PhastSystem equipment (Pharmacia, Uppsala, Sweden) using homogeneous polyacrylamide gels (PhastGel Homogeneous 20, Pharmacia) and Phastgel Native Buffer Strips (Pharmacia). The PhastSystem Separation Technique No. 121 was followed for separation conditions and PhastSystem Developer Technique

No. 200 for staining. One microlitre of sample was applied at the anode.

3. Results and discussion

The structural changes due to the previous heat and/or high-pressure treatments applied to β-Lg were evaluated by SE-HPLC (Fig. 1). In native and heated (60 °C, 30 min) samples (Fig. 1(a) and (b), respectively), the major peak was found to correspond to the dimer of β-Lg (the predominant species at pH 7.0). In high-pressure (at room temperature)-treated β-Lg samples (Fig. 1(c)), aggregates of higher molecular weight started to be present in appreciable amounts. In β-Lg heated at 80 °C for 30 min and β-Lg pressurized at 400 MPa at 60 °C (Fig. 1(d) and (e), respectively), the most abundant forms were the aggregates of high molecular weight.

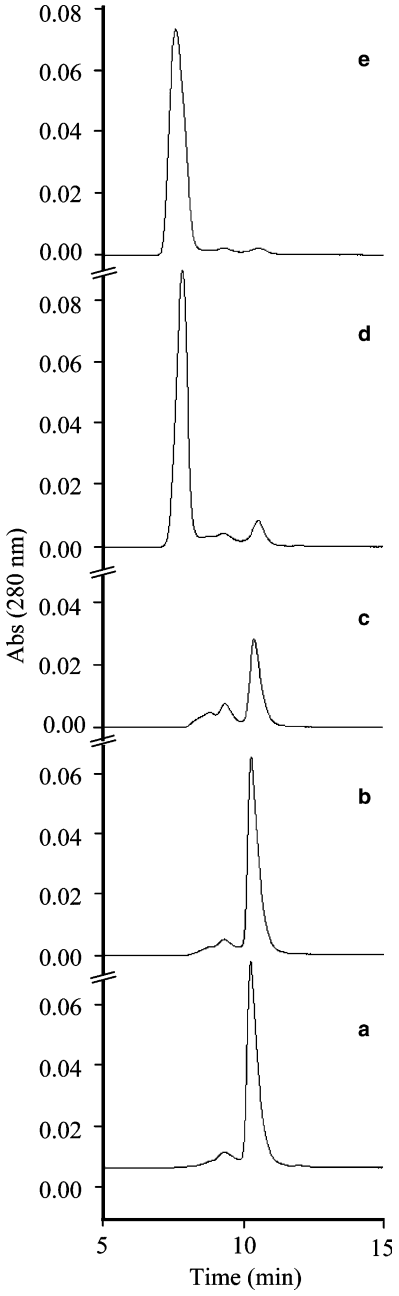

Fig. 1. SE-HPLC patterns of: (a) native β-Lg, (b) heat-treated β-Lg at 60 °C, 30 min, (c) pressurized β-Lg at 400 MPa, 25 °C, 60 min, (d) heat-treated β-Lg at 80 °C, 30 min, and (e) pressurized β-Lg at 400 MPa, 60 °C, 60 min.

Fig. 2 shows the native-PAGE plates of heat-treated and pressurized β-Lg at the initial stage and after storage during 96 h at 50 °C and 44% RH with lactose. The results are in agreement with those obtained by SE-HPLC. Moderate heat treatment did not result in appreciable changes since the patterns of native and heat-treated (at 60 °C) β-Lg are quite similar (lanes 2 and 3, respectively), and pressurization at room temperature led to the formation of aggregates with a slightly higher molecular weight than the former (lane 4). Aggregation was favoured upon application of higher temperatures, or pressurization combined with heat, since bands retained in the stacking gel (indicated by an arrow) (lanes 5 and 6, respectively), probably corresponding to aggregates of very high molecular weight, were observed. Storage at 50 °C and 44% RH and 96 h of native, heat-treated (at 60 °C), 30 min and pressurized at 25 °C, β-Lg samples in the presence of lactose (lanes 7, 8 and 9, respectively), produced similar patterns to their counterparts without lactose, although the aggregates showed higher molecular weights, probably due to the presence of attached lactose. Samples treated at 80 °C or at 400 MPa and 60 °C (lanes 10 and 11, respectively), and stored, showed bands retained in the stacking gel but with less intensity than the corresponding non-stored samples, that can be related to the formation of some insoluble high molecular weight aggregates. After further storage time, a similar trend was observed (data not shown).

Fig. 3 clearly shows that, during storage at 50 °C and 44% RH, native β-Lg is noticeably more reactive than pressure-treated or heat-treated β-Lg. Thirteen lysine residues of native β-Lg were lactosylated after 96 h of storage and this then started to decrease, indicating the progress of

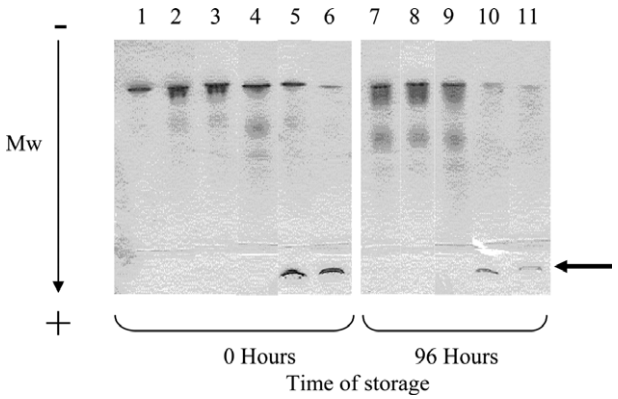

Fig. 2. Native polyacrylamide gel electrophoretic patterns of non-stored (lanes 1–6) and stored (at 50 °C and 44% RH) β-Lg in presence of lactose. Lanes: (1) standard β-Lg; (2 and 7) native β-Lg; (3 and 8) β-Lg treated at 60 °C, 30 min; (4 and 9) β-Lg pressurized at 400 MPa, 25 °C, 60 min; (5 and 10) β-Lg treated at 80 °C, 30 min; and (6 and 11) β-Lg pressurized at 400 MPa, 60 °C, 60 min.

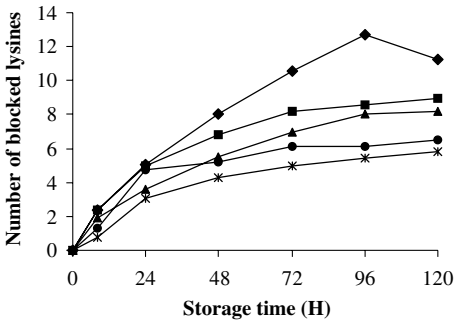

Fig. 3. Number of blocked lysines (estimated from the furosine concentration) during 120 h of dry-heating of β-Lg and lactose: (◆) native β-Lg, (■) heat-treated β-Lg at 60 °C, 30 min, (▲) heat-treated β-Lg at 80 °C, 30 min, (●) pressurized β-Lg at 400 MPa, 25 °C, 60 min and (∗) pressurized β-Lg at 400 MPa, 60 °C, 60 min.

the Maillard reaction into more advanced stages (Hurrell, Finot, & Ford, 1983). Lactosylation of native β-Lg in the dry state was previously studied (Morgan et al., 1999). The authors detected 7.7 lactose residues bound per β-Lg monomer after storage of β-Lg at 60 °C for 48 h at pH 7.2 and 65% RH; these different experimental conditions could be responsible for the differences found in the degree of lactosylation for β-Lg found in our results.

Heat-treated β-Lg reactivity towards lactose was lower than that observed for native β-Lg. The reactivity decreased with increasing thermal treatment intensity. Nine lysine residues of β-Lg heated at 60 °C were lactosylated after 120 h of storage, whereas ~8 lactosylated lysine residues of β-Lg heated at 80 °C were detected in the same period of time. The less reactive substrates towards lactose were high-pressurized β-Lg samples. The reactivity was decreased by combined use of high-pressure/temperature. A maximum of five lactosylated lysine residues were detected in high pressure-treated β-Lg (400 MPa at 60 °C, for 30 min) after the storage period.

Results obtained on the content of free amino groups determined by the trinitrobenzenesulfonic method are shown in Fig. 4. Initial free amino acid contents decreased as a consequence of heat and high-pressure treatments with especially marked losses in high-pressure/heat treated β-Lg. Considerable losses in free amino groups were also detected in high pressure-treated β-Lg at 25 °C and heat-treated β-Lg at 80 °C. These results generally show good agreement with the observed formation of aggregates in the SDS–PAGE study.

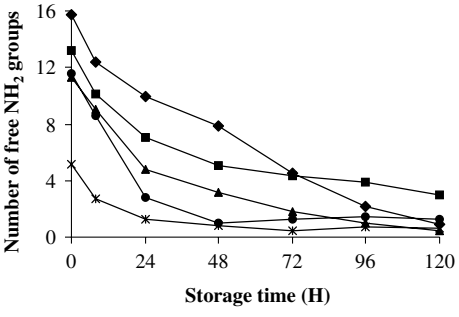

Fig. 4. Number of free amino groups during 120 h of dry-heating of β-Lg and lactose: (◆) native β-Lg, (■) heat-treated β-Lg at 60 °C, 30 min, (▲) heat-treated β-Lg at 80 °C, 30 min, (●) pressurized β-Lg at 400 MPa, 25 °C, 60 min and (∗) pressurized β-Lg at 400 MPa, 60 °C, 60 min.

Decrease of free amino groups of native β-Lg during the first 96 h of storage appeared to be well correlated with the extent of lactosylation shown in Fig. 3. However, further storage resulted in increased loss of free amino groups and there was no increase in lactosylated lysine. This may be attributed to the fact that, in the advanced stages of the Maillard reaction, furosine content decreases due to the decomposition of the Amadori compound (Hurrell et al., 1983).

In heat-treated β-Lg samples, the increase of lactosylated lysines was lower than the decrease of free amino groups, probably due to the formation of cross-linked amino acids (Friedman, 1999). Heat treatment of β-Lg for 30 min at 60 °C and 80 °C caused a decrease of 3 and 5 free amino groups, respectively, which explains, at least in part, the lower number of lactosylated lysines formed during storage of these samples with respect to that found in stored native β-Lg.

The decrease in free amino groups of β-Lg, due to high-pressure treatment at 25 °C, was similar to that due to heat-treatment at 80 °C. Although a lower degree of lactosylation was observed in high pressured β-Lg than in heat-treated β-Lg, the loss of free amino groups during storage proceeded faster in high pressure-treated β-Lg than in heat-treated β-Lg. This may suggest that conformational changes, caused by high-pressure treatment, facilitate the inter- and/or intramolecular cross-linking but may hinder the accessibility of lactose to the free amino groups of β-Lg resulting in a decrease of lactosylation degree.

Although more research is needed, the present results show the effect of a previous β-Lg denaturation on the subsequent lactosylation under dry-heating conditions. Since the structural changes in the β-Lg molecule affect its functionality, a study on the functional properties of products derived from lactosylation of β-Lg, which has been previously high pressure-or heat-treated, is currently underway.

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