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Effect of Heat Treatment on the Detection of Intact Bovine β-Lactoglobulins by LC Mass Spectrometry

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Lactoglobulin (LG) is the most abundant protein of the whey fraction of cow's milk, and due to its high nutritional value as well as its technological properties it is widely used as an ingredient in food preparation. As a consequence of heat treatment, milk proteins may undergo structural changes such as protein unfolding and aggregation, in addition to chemical modifications. This, in turn can change the allergenic potential of LG. In this study, the potential of mass spectrometry has been exploited to investigate LG protein modification and stability as a consequence of thermal treatments applied to both standard solutions and milk samples. An investigation into the charge-state distribution in ESI-MS source revealed that, in standard solutions, a higher degree of protonation accompanies increases in the severity of the heat treatment applied. In contrast, the analysis of milk samples revealed a higher stability of the charge-state distribution of LG. However, we observed modification of LG spectra after heating of standard solutions as well as milk samples caused by lactosylation. The degree of LG lactosylation has been investigated in raw milk samples by LC−MS and provides a potential marker to trace heat treatments.

KEYWORDS: Lactoglobulin; mass spectrometry; milk; heating; whey proteins; lactosylation

INTRODUCTION

Processing techniques usually applied by the food industry can severely impact the protein structure and the allergenic potential of allergenic foods (*1*). In the manufacturing of milk and milk products, most processes are based on heat treatments. Heating is known to induce many reactions within the food, which occur depending on the duration and severity of heat treatments (*2*, *3*). The first observation concerns structural changes in proteins that are thermally induced (*4*, *5*).

β-Lactoglobulin (LG) is the most abundant protein of the whey fraction, and its structure is characterized by an antiparallel β-barrel, a short β-strand, a short 3_{10}-helix, and a single major α-helix. It is present in cow's milk in two common variants A and B, which differ by two amino acids (*6*). It plays an important physiological role in the organism in its capability to bind a wide range of molecules such as retinol, β-carotene, and fatty acids. Despite this, it is also recognized as one of the most allergenic components of the whey fraction (*7*, *8*).

The heat-induced behavior of LG has been studied extensively as well as the mechanism of its aggregation (*9*−*11*). Thermal treatments are known to cause LG denaturation with loss of tertiary protein structure (*5*). Upon heating, the loss of tertiary protein structure can lead to a superficial exposure of the hidden allergenic sites, and therefore heating represents an essential factor capable of influencing the allergenic potential of whey proteins like LG (*12*). As a consequence, a change in the allergenic potential can be displayed through the apparition of neo antigens or the destruction of others (*13*). Discrepancies are reported in the literature whether the modified allergenic proteins would lead to an increase or decrease of this allergenic potential (*3*, *6*, *14*, *15*). The interaction of LG with other milk proteins might affect the overall heat stability of milk and that of individual allergenic proteins like LG. In fact, interactions between milk proteins are well known to occur (*9*, *17*).

To assess the impact of heating on the denaturation of milk proteins, Fourier transform infrared spectroscopy and differential scanning calorimetry have been employed and have proven to be capable of identifying changes in the secondary structure and the conformation of LG under various physicochemical conditions (*4*). In addition to this, efforts have been devoted to the development of immunological methods able to discriminate between the native and denatured form of whey proteins. By using immunoprobes, the effect of thermal treatment can be evaluated on the basis of changes in the immunoreactivity of thermally denatured proteins. An increased immunoreactivity of LG has been reported to be associated with thermal denaturation, which coincides with an alteration in secondary and tertiary protein structure (*12*).

Recently, a new surface plasmon resonance-based immunosensor has been developed, which allows a discrimination of different heat treatments based on a differential binding of α-lactalbumin (*18*).

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In addition to denaturation, thermal processing and storage also affect milk proteins via the so-called Maillard reaction, resulting in the attachment of reducing sugars to the amino group of lysine in cow milk proteins as a consequence of heating (*19*, *20*). Maillard reactions start with a nonenzymatic glycation of proteins, which has been reported to enhance the heat stability of proteins during the early stage of the reaction. Glycated proteins have a great importance also from an allergenic point of view because these modifications may account for a change in the allergenic potential (*21*, *22*). The identification of markers that provide insight into the heat treatment a milk sample has undergone is essential for process control, regulatory purposes, and especially for the assessment of the allergenic potential. A recent investigation aimed at the evaluation of potential tracers to discriminate among thermal treatments reported that the best discriminative tracers are those generally measuring the structural modifications of milk proteins rather than those employed to quantify Maillard reaction products (*23*, *24*).

In this study, the potential of mass spectrometry coupled to electrospray ionization for studying the heat stability of LG and their spectral modifications caused by thermal treatments has been investigated. Because charge-state distribution in ESI-MS is affected by the conformational state of a protein, this technique can provide information on protein folding (*25*, *26*). Additionally, the accurate mass information could provide insight into the association state of the protein (*27*). We employed an LC−MS method to analyze purified LG as well as whey samples. Parameters such as the protein ionization pattern and the extent of lactosylation have been taken into consideration and carefully evaluated to find a valuable marker discriminative for the extent of heat treatment.

MATERIALS AND METHODS

LG A and B (purity 92%) were purchased from Sigma Aldrich (St. Louis, MO). Stock standard solutions of LG (1 mg/mL) were prepared in water just prior to use. For heating experiments, a concentration of 50 μg/mL was prepared by dilution of the stock solution in a solution containing 5 mM ammonium acetate (adjusted with acetic acid to reach a final pH of 4.6).

Raw bovine milk was obtained from a local farm, while pasteurized and ultrahigh-temperature (UHT) bovine milk was obtained from local supermarkets. Lactoserum was obtained from the raw, pasteurized, and UHT milk samples after acidic precipitation by addition of 5% acetic acid to reach a pH of 4.6. All milk samples were prepared according to this method, which allows the separation of lactoserum from caseins after acidic precipitation and centrifugation. Aliquots of 1 mL of lactoserum were transferred to glass test tubes of 1.5 mL, closed with a screw cap to prevent evaporation, and all vials were placed in a heating block for the thermal treatments.

All analyses were performed in triplicate, and the mean of each set of measurements was taken into consideration.

Chromatographic separation was performed on a Discovery C5 column (Supelco, 100 × 2.1 mm, 3 μm packing). The mobile phase was composed of solvent A, a mixture of acetonitrile/water (90/10) + 0.1% formic acid; and solvent B, a mixture of acetonitrile/water (10/90) + 0.1% formic acid. The elution program was: from 75% to 64.5% of B in 9 min, from 64.5% to 63.5% in 3 min, from 63.5% to 61.5% in 5 min, and down to 60% in the following 2 min, then back to the initial conditions maintained for 15 min. The column temperature was 25 °C. The analyses were carried out by an online coupling between MS, UV, and RP-HPLC.

The chromatographic system consisted of a binary pump (Agilent HP1100) equipped with an autosampler (Agilent HP1100) and a diode array UV detector (Agilent HP 1100 PDA).

Mass measurements were carried out with a Triple Quadrupole mass spectrometer (Waters-Micromass, Ultima) equipped with an atmospheric pressure ionization source Z-spray. Solutions were sprayed through a capillary held at +3 kV and at a temperature of 100 °C, generating multiply charged ions. The cone voltage was set at 60 V with a sheath gas applied at a temperature of 200 °C. For direct injection experiments, the flow rate of the pump was set at 10 μL/min.

The system was operated in positive ion mode. For data acquisition, full scan mode was used, and the second quadrupole was scanned over the range 500−2000 *m*/*z* with a step size of 0.5 and a dwell time of 0.5 s. Protein spectra were obtained as an average of the spectra of multiple scans. Protein molecular mass was determined by using MaxEnt software (Waters-Micromass) from the measured *m*/*z* values of the protonated molecule. Quantification of LG was performed by using the peak area of the multicharged ions of the monomer in the range +12 to +21.

RESULTS AND DISCUSSION

Preliminary experiments were carried out to optimize all MS parameters such as cone and capillary voltages, source temperature, and the electric potential difference. Formic acid at a concentration of 0.1% was chosen as the most suitable acid for the mobile phase in terms of the best signal-to-noise ratio and the intensity of the multi-ion pattern generated.

Once the mass spectrometric parameters were set, standard solutions of LG were analyzed before and after heating using liquid chromatographic separation coupled to MS and UV detection, and the resulting chromatograms were compared. **Figure 1** shows two chromatograms of the same standard solution of LG A and B, injected before (**a**) and after heating (**b**) at 100 °C for 5 min. It is evident that a novel peak eluted between 16 and 17 min as a consequence of heating. Analysis of the spectra of the three chromatographic peaks appearing after heating provided three different ionization patterns as highlighted in **Figure 2**. The mass spectra corresponding to LG A and LG B are shown in **Figure 2a** and **b**, respectively. The different envelopes specific for both native proteins are characterized by a multiply charged pattern distributed in a Gaussian curve referring to either LG A or LG B in several charge states, and they proved to be conserved in nonheated as well as heated standard solutions. The most intense ions occurred in the charge states +15 and +16. **Figure 2c** represents the spectrum of the novel peak, eluting between 16 and 17 min, and shows a double envelope related to the presence of both LG B and LG A within this single chromatographic peak. Although the envelopes are attributed to LG A and LG B, a comparison with the spectra reported in **Figures 2** reveals several consistent changes in the spectra as a consequence of heating the LG solution at 100 °C for 5 min. As a result of this heat treatment, the dominating ions highlighted in the spectra of the novel peak have now shifted to +18 and +19 charge states, changing the appearance of the multicharge distribution pattern. Repetitive injections of standard solutions of LG in the concentration range of 5−50 μg/mL proved that the ion pattern did not fluctuate when the instrumental settings were kept constant. Therefore, the modifications observed in the distribution patterns are likely to be caused by protein unfolding as a consequence of the heat-induced denaturation of LG. This change in tertiary structure as indicated by the alterations in the ionization pattern probably accounts for an altered chromatographic behavior of the proteins as a consequence of a change in their spatial rearrangement.

Previously, it has been pointed out that ESI-MS allows a sensitive and selective monitoring of changes in protein tertiary structure, which is known to influence charge-state distribution patterns. Two hypotheses have been postulated to explain this effect (*28*, *29*). One of them supposes that the lower solvent accessibility of the basic and acidic residues in the folded structure makes them available to ionization compared to the

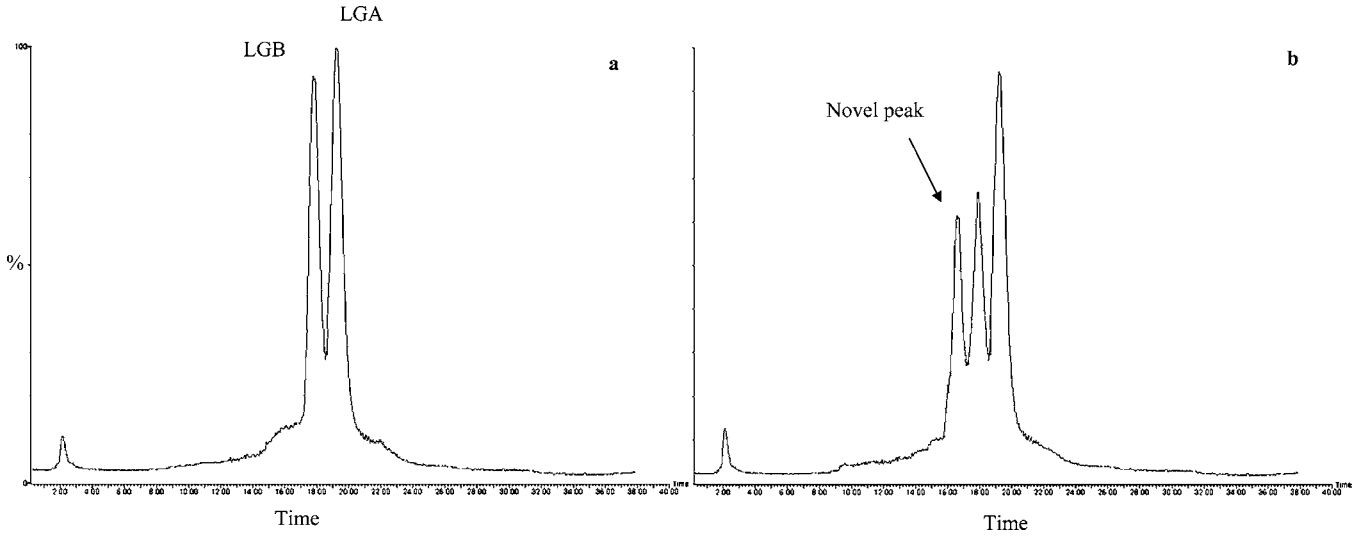

Figure 1. Total ion current chromatogram of LG A and B standard solution before (**a**) and after heating for 5 min at 100 °C (**b**). The novel peak is indicated in (b).

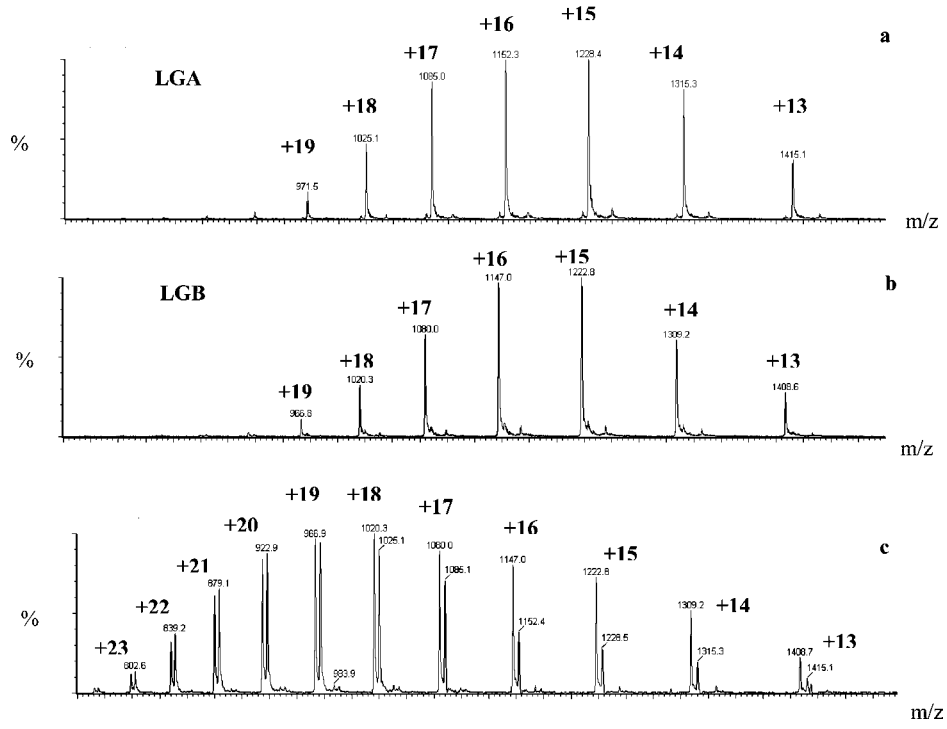

Figure 2. Multi-charged distribution pattern of LG A (**a**), B (**b**), and the novel peak (**c**) after heating at 100 °C for 5 min.

unfolded state. Alternatively, it has been suggested that unfolded conformations stabilize higher charge states by increasing the distance between charges of the same polarity on the polypeptidic chain (*29*, *30*). The different charge-state distribution patterns highlighted in **Figure 2** may account for a different protein conformation as a consequence of heat treatment. As shown in **Figure 2c**, the unfolded protein gives rise to a broad, bell-shaped distribution, whereas folded proteins are characterized by fewer more intense peaks (**Figure 2a**,**b**). This is in agreement with work described by Samalikova et al. (*31*). We therefore speculate that the different spatial rearrangement of LG upon heating leads to an exposure of the most polar residues on the surface masking the hydrophobic ones, and therefore chromatographic analysis results in a novel peak attributed to the denatured protein (**Figure 1b**).

It is well known that heating generally results in irreversible structural changes of LG that can be observed by heating it at 95 °C at different pH values (*32*, *33*). In addition to this, aggregation is likely to occur (*10*). When LG standard solutions were heated at 100 °C for longer periods of time, it was found that an increase in heating time correlated with an increase of the novel peak eluting between 16 and 17 min; consequently, the original peaks referring to the native LG decreased. This gradual heat-induced change is visualized in **Figure 3** that reports the peak area of the native protein (LG A + B) and that of the novel peak as a percentage of the total of those peaks after varying the duration of heating at 100 °C.

In addition to LG standard solutions, whey obtained from raw and UHT milk was analyzed under the same conditions to check whether the prolonged heating of the whey also led to the appearance of a novel peak as in the case of LG standard solutions. Our analyses showed that the appearance of this novel peak could not be observed after heating of whey. A detailed investigation into the relative abundance of the most representa-

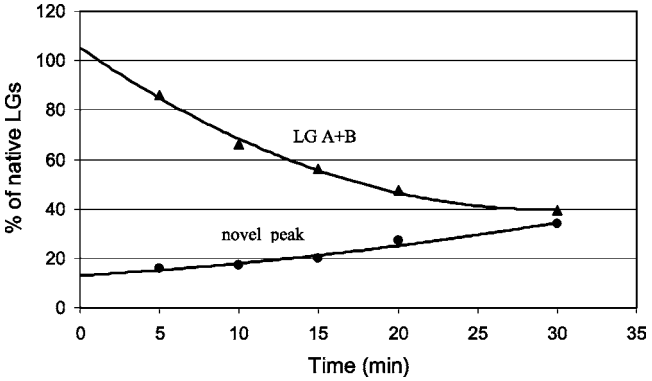

Figure 3. Effect of the heating period on the correlation between the sum of the peak area of LG A + B and that of the novel peak.

tive multicharged ions in standard solution as well as in whey was performed both before and after heating. The results of this analysis are represented in **Figures 4** and **5**. In the standard solution, the +20, +21, +22, and +23 ions appeared upon heating as analyzed by direct injection in the source (**Figure 4**), resulting in major changes in the relative abundance of all charge states.

In stark contrast to this, the ion distribution pattern of milk samples appears to be more conserved in all samples investigated: raw whey, heated whey at 100 °C for 5 min, and whey from UHT milk (see **Figure 5**). The only difference between raw and heated milk was the appearance of ions with +21 charges that could be detected at trace level both in UHT and after heating of samples derived from raw milk. That observation was in contrast to what was found for LG standard solutions: that the shape of the LG protein envelope did not vary in milk samples as a result of heat treatment points at a potential role of other milk constituents in stabilizing the native conformation of LG.

In the absence of clear qualitative changes in the chromatograms, or in the ion distribution pattern of milk samples, we investigated whether heat treatment resulted in quantitative effects. For this purpose, the most representative multicharged ions of LG (the range +13 +14 +15 + 16 +17 +18 +19) were monitored. Aliquots of standard solutions as well as whey obtained from raw milk were submitted to the same time−temperature treatment (5 min at x_1, x_2, x_3, ...°C) and analyzed by LC−MS. Peak areas of LG A and B were analyzed as a function of the temperature applied. **Figure 6a** and **b** shows the total current of the monitored ions and allows a comparison between the behavior of the protein in standard solution as well as in raw milk after submission to thermal treatments. Our results show that when a LG standard mixture undergoes heating, a relevant decrease in native protein is apparent (**Figure 6a**); the most crucial temperature for initiation of this effect lies in the range between 80 and 90 °C. In contrast to this, when raw milk is heated, native LG does not show any decrease (**Figure 6b**). These results were confirmed by analysis of the same samples by means of LC-UV. Heating the standard solution at 100 °C for 5 min produced a marked drop to around 40% of the original protein signal. In contrast to this, a loss of only 10% was observed after analysis of milk samples. These results again demonstrate a more thermo-resistant behavior of LG when it is in the presence of other milk components that likely stabilize the native structure of intact LG. It is well known that whey proteins are relatively heat labile and the susceptibility of LG to heat denaturation can be influenced by factors like the pH, Ca^{2+}, protein concentration, and the presence of sugars or

Table 1. Degree of Lactosylation upon Heating of Raw Milk Samples at 100 °C for Different Periods of Time (0, 5, 15, and 30 min)[a]

	LGA		LGB	
heating time (min)	mean (%)	s.d.	mean (%)	s.d.
0	2.4	±0.3	3.1	±0.2
5	7.2	±0.5	6.8	±0.4
15	17.4	±0.8	10.8	±0.5
30	18.0	±0.8	17.2	±0.8

[a] The values are expressed as the percentage of the sum of multicharged mono- and dilactosylated LG peaks divided by the sum of total LG peaks derived from the same spectrum at the retention time of LGA or LGB, respectively. s.d. = standard deviation (number of replicates = 3).

protein modifying agents (*34*, *35*). Previously, it has been reported that LG adduct formation with lactose or galactose can lead to an increased stability of the native protein toward denaturation (*20*). The extent of glycation can also be used to trace the severity of heating applied to milk samples as demonstrated by MALDI or ESI-MS analysis of tryptic digests of dairy products that had undergone heat treatments (*36*, *37*).

Several authors claimed that MS techniques are suitable for the quantitative measurements of glycoforms because extensive glycation seems neither to alter the protein ionization nor to influence the charge-state distribution (*20*, *38*, *39*). For instance, Nakanishi et al. have proposed such a way of quantification and demonstrated that the percentage of mono-glycated hemoglobin directly measured by ESI-MS was well correlated with the values obtained by another HPLC methodology (*38*). Moreover, Fenaille et al. showed strong correlations between the glycation extent of LG as analyzed by LC-ESI-MS and other markers for glycation (*40*). In addition, analysis of the charge states observed in ESI-MS has shown that solid-state glycation of LG tends to improve the stability properties of the native protein toward denaturation (*20*). On the basis of these reports, the LC−MS method developed here was employed to determine whether lactosylation of LG can be detected directly in the intact protein, and whether this LG modification can serve as a tracer for heat treatment. For this purpose, raw milk was submitted to prolonged heating at 100 °C for periods of 5, 15, and 30 min, and the overall content of unmodified and lactosylated LG was estimated by collecting the current of the predicted glycated ions. The degree of lactosylation was estimated by expressing the predicted glycated ions as a percentage of the total protein. **Table 1** reports the degree of lactosylation (the mean of three samples analyzed ± the standard deviation) with the increase of heating time. As shown, the lactosylation of LG A and B increases progressively with prolonged heating times. It should be pointed out that heating milk samples for prolonged periods at 100 °C results in precipitation and/or degradation of intact LG. This causes a drop of the signal, which was found to be halved after heating for a period of 15 min. The attachment of lactose to LG molecules is known to occur at the temperatures lower than 100 °C, and it has been demonstrated by Leonil et al. (*41*) that incubation of LG standard solutions with lactose leads to the formation of lactosylated LG that can reach levels of 40−45% for each LG variant after 3 h of incubation at 70 °C. The generation of such Maillard reaction products is time and temperature dependent, and a direct correlation exists between the severity of the heat treatment applied to milk and the abundance of whey protein adducts formed (*36*). Furthermore, the extent of LG lactosylation can dramatically increase under dry conditions as applied by the food industry (*37*).

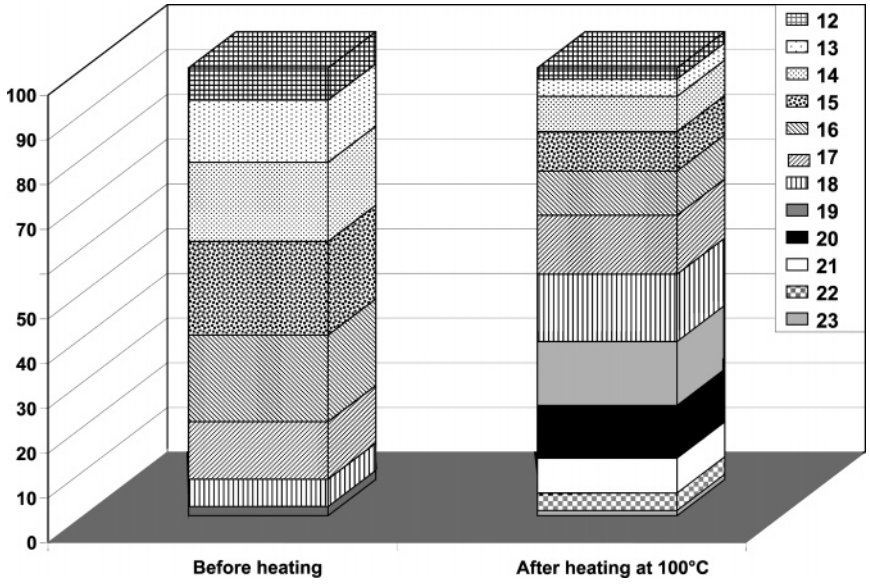

Figure 4. Relative abundance of multi-charged LG B ions detected in standard solutions before and after heating at 100 °C for 5 min.

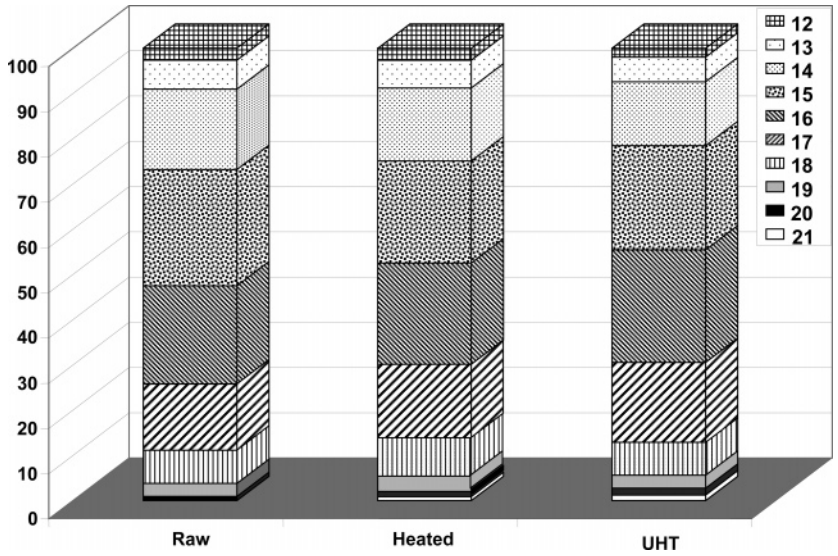

Figure 5. Relative abundance of multi-charged LG B ions detected in raw whey serum, whey serum heated for 5 min at 100 °C, and whey serum obtained from UHT milk.

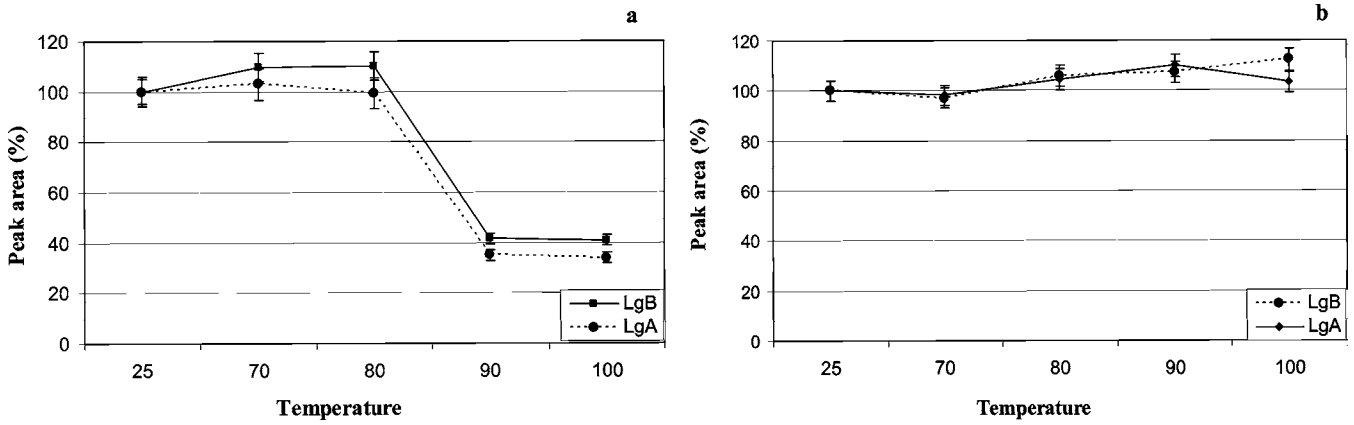

Figure 6. Peak area of LG A and B plotted against the heating temperature that was applied for a duration of 5 min, in standard solution (**a**) and whey from raw milk (**b**). The values are obtained by analysis of the masses of the most representative multicharged ions (in the range +12 to +20) and expressed as a percentage of the values of the unheated control samples.

UHT milk samples were also analyzed by LC−MS to determine the relative abundance of lactosylated/glycated forms of LG. Full scan acquisition performed on UHT samples upon LC separation typically produces a chromatogram with two peaks attributed to LG A and B. The glycated forms of LG A and B elute slightly earlier than their corresponding unmodified

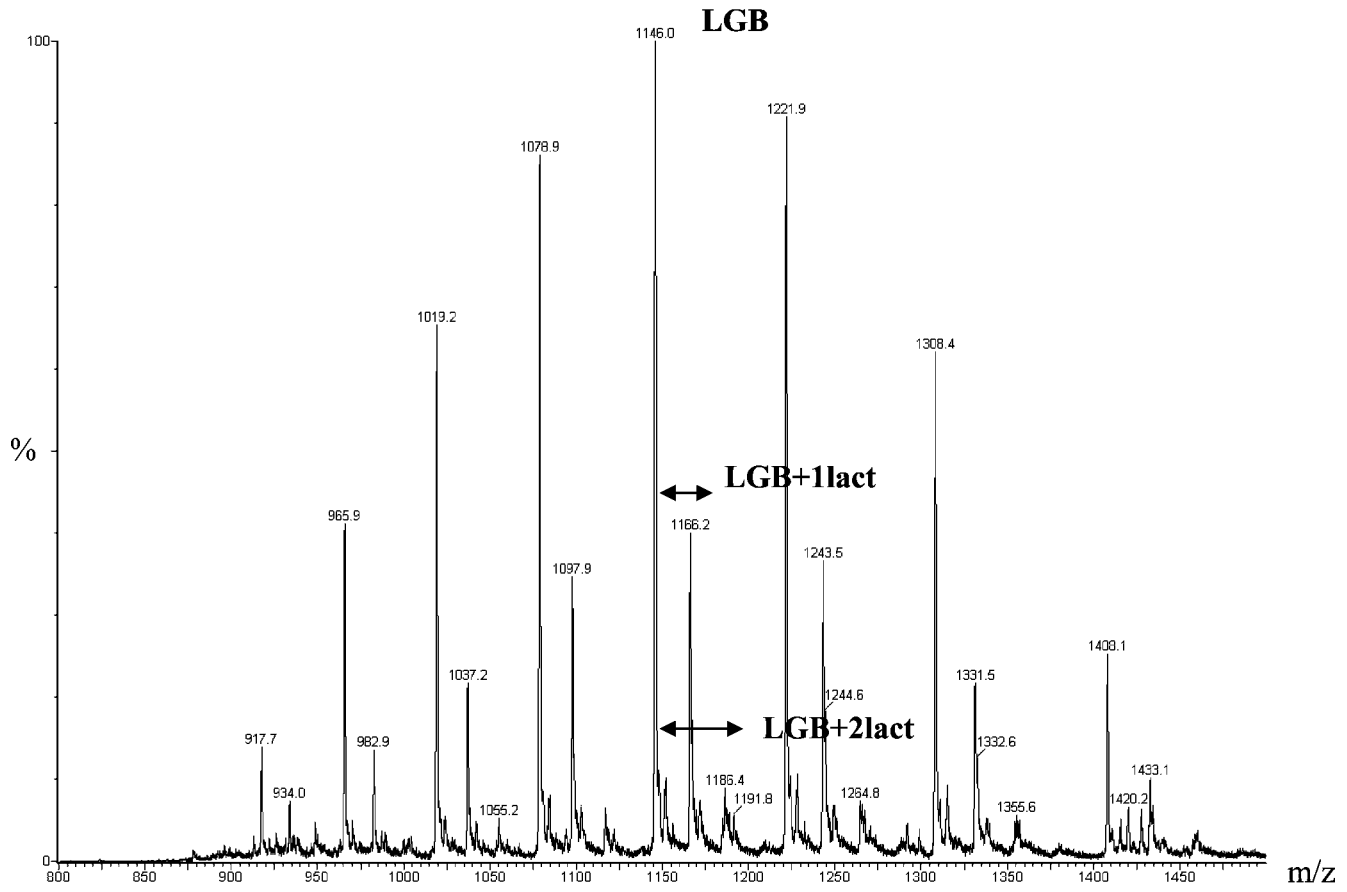

Figure 7. Mass spectrum of a UHT milk sample in which the presence of one of the multicharged LG B ions (+16) in its unglycated, monolactosylated, and bilactosylated forms is highlighted.

Table 2. Average Mass Spectra Charge State of LGA and LGB before and after Heating of UHT Milk at 100 °C for Different Periods of Time (0, 5, 15, and 30 min)[a]

time of heating (min)	LGA (mean ± s.d.)	LGB (mean ± s.d.)
0	16.00 ± 0.10	16.75 ± 0.10
5	16.04 ± 0.10	15.80 ± 0.10
15	15.71 ± 0.15	15.46 ± 0.15
30	14.69 ± 0.20	15.00 ± 0.15

[a] The intensity-weighted mean of all charge states in the range of +13 to +18 is given. The relative intensities of each charge-state peak (normalized to the total peak area for each LG variant) were multiplied by their respective charge-state value. The sum of the resulting values gives rise to the average mass spectra as reported here. s.d. = standard deviation (number of replicates = 3).

forms, and the retention time decreases with an increased degree of glycosylation. Yet, because these peaks to a large extent overlap with the native proteins, they cannot be adequately separated by conventional HPLC-UV (*42*). Mass spectrometry can overcome such difficulties, providing well-resolved peaks by selecting the masses of interest from the total ion chromatogram.

The spectra of both LG peaks showed the presence of unmodified LG, LG + 1, and LG + 2 lactose adducts as demonstrated by the mass difference accounting for the attachment of 1 or 2 molecules of lactose (324 and 648 Da, respectively) to the protein. **Figure 7** shows a typical mass spectrum obtained by LC−MS analysis of a UHT milk sample. The spectrum that was recalled under the LG B peak clearly shows three distinct envelopes of multicharged ions, representing the unmodified, the monolactosylated, and the dilactosylated forms of LG B. Similar results were obtained for LG A. To investigate the extent the impact of heating can have on the charge-state distribution of LG, UHT samples were submitted to prolonged heating at 100 °C for periods of 5, 15, or 30 min. The intensity-weighted mean of all observed charge states ranging from +13 to +18 was calculated, taking into account also the monolactosylated and dilactosylated forms of lactoglobulin. **Table 2** shows the average of the charge states and clearly indicates changes induced by prolonging the heating time. A slight shift of the charged-spectra distribution toward the lowest values was noticed for both variants A and B. This is in stark contrast with what found when heated standard solutions were analyzed as shown in **Figure 4**. The explanation for this difference in behavior is likely to be found in the capability of sugars to mask the protonable sites on the amino group of the LG moiety, resulting in a lower protonation extent. Similar results were found in case of raw milk submitted to such heating treatments.

Because the degree of lactosylation of intact LG as determined by LC−MS was found to be a potential tracer of the heat treatment a milk sample has undergone, we analyzed a number of pasteurized and UHT milks to determine their degree of LG lactosylation. Five different pasteurized milks were found to contain monolactosylated LG, accounting for 4−7% of their total LG content. For five different UHT milks, this value was found to be in the range of 33−44%. In addition to this, UHT milk was also found to contain dilactosylated LG in a range of 2−4% of its total LG, which could not be detected in pasteurized milk samples. These results indicate that analysis of intact LG

by means of LC−MS in principle allows an assessment of the thermal treatment the milk sample has undergone.

In conclusion, this study focuses on the detection of heat-induced changes of intact LG. Both purified LG in solution as well as whey serum obtained from bovine milk samples were subjected to heat treatments and analyzed by means of LC−MS. When solutions of pure LG were employed, two parameters were found to change upon thermal treatments. As a first observation, the chromatographic behavior of LG solutions was found to be altered with the appearance of a novel peak in the chromatogram of thermally treated samples. In addition to this, we observed an increase in protonation in such samples.

Subsequent analyses of milk revealed that those same two parameters were not affected in the same manner by heat treatments of milk. The observed relatively stable protonation pattern might turn out to be very useful for the development of methods designed to detect and quantify milk or whey traces in food samples, which does require the identification of (heat) stable markers.

A third parameter investigated, the extent of lactosylation of intact LG, could also be determined by LC−MS analysis. Our results clearly show that raw milk, pasteurized milk, and UHT milk samples can be differentiated on the basis of a heat-induced increase in lactosylation. Next, in addition to the qualitative advantages, MS seems to be also useful as a quantitative tool for monitoring protein glycation.

The analysis of milk samples by means of LC−MS allows an assessment of the severity of the heat treatment applied to milk, which is of relevance for process control and regulatory purposes. Mass spectrometric identification of LG might also form the basis for confirmational methods designed to detect allergenic milk or whey proteins in food products. Such methods are of paramount importance to safeguard the health of allergic consumers.

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