JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

MALDI-TOF MS Characterization of Glycation Products of Whey Proteins in a Glucose/Galactose Model System and Lactose-free Milk

Saverio Carulli,* Cosima D. Calvano,* and Francesco Palmisano

Dipartimento di Chimica and Centro di Ricerca Interdipartimentale S.M.A.R.T. Università degli Studi di Bari "Aldo Moro", Via Orabona 4, 70126 Bari, Italy

Monika Pischetsrieder

Department of Chemistry and Pharmacy, Food Chemistry, University of Erlangen−Nuremberg, Schuhstrasse 19, 91052 Erlangen, Germany

ABSTRACT: The major modifications induced by thermal treatment of whey proteins α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) in a model system mimicking lactose-free milk (L$^-$ sugar mix) were investigated by matrix-assisted laser desorption ionization−time-of-flight mass spectrometry (MALDI-TOF MS). The analysis of the intact α-La revealed species with up to 7 and 14 adducts from lactose and sugar mix, respectively, whereas for β-Lg 3 and up to 5 sugar moieties were observed in the case of lactose and sugar mix experiments, respectively. A partial enzymatic hydrolysis with endoproteinase AspN prior to mass spectrometric analysis allowed the detection of further modifications and their localization in the amino acid sequence. Using α-cyano-4-chlorocinnamic acid as MALDI matrix, it could be shown that heating α-La and β-Lg with glucose or galactose led to the modification of lysine residues that are not glycated by lactose. The higher glycation degree of whey proteins in a lactose-free milk system relative to normal milk with lactose reflects the higher reactivity of monosaccharides compared to the parent disaccharide. Finally, the analysis of the whey extract of a commercial lactose-free milk sample revealed that the two whey proteins were present as three main forms (native, single, and double hexose adducts).

KEYWORDS: glycation, whey proteins, MALDI mass spectrometry, lactose-free milk, AspN

INTRODUCTION

Because of their biochemical, nutritional, and functional properties, the major whey proteins, α-lactalbumin (α-La) and β-lactoglobulins (β-Lg) A and B, belong to the most valuable food proteins.[1] Whey proteins are extensively used as additives (e.g., as foaming agents, emulsifiers, egg protein replacers, taste enhancers) in the food industry and, more recently, their introduction in clinical nutrition has also been proposed because of their beneficial effects on human health.[2,3] However, thermal treatments, such as pasteurization and ultrahigh-temperature (UHT) processes, required to reduce the bacterial load and increase the milk's shelf life, can alter some characteristics of the whey proteins.[4,5] Unfolding, glycation of lysine (and, to a lesser extent, of arginine and cysteine) via the Maillard reaction, oxidation and coaggregation with casein micelles are among the most important alterations occurring during milk heating.[6,7]

As a consequence, a reduced bioavailability of important amino acids such as lysine[8] and a general deterioration of whey proteins' functional properties,[9−11] with remarkable effects also on technological processes such as cheesemaking,[12] are observed.

Mass spectrometry (MS) techniques based on soft ionization processes, such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), have been extensively used[13−17] to study the effects of lactosylation on the major whey proteins. A +324 Da shift in the molecular mass of the intact protein gives an immediate indication of a single lactosylation event. Indeed, MS has provided evidence for single/multiple lactosylation of β-Lgs and α-La in either model solutions or commercial milk products.[18−22]

Some systematic investigations performed on whey protein lactosylation occurring in pasteurized or UHT milk[17−19] showed that even a mild heat treatment such as pasteurization can lead to an appreciable extent of lactosylation, involving one or two sites per protein molecule.

On the other hand, a much higher degree of lactosylation was reported for whey proteins subjected to severe thermal stress, involving a maximum of 5 and 10 lysine residues for α-La and β-Lgs, respectively, in infant formula powders[20] and 16 residues (i.e., 15 lysines plus a leucine at the amino terminus) for β-Lgs following solid state lactosylation.[23]

These findings indicate that the exposure of lysine residues to lactose originally embedded into the protein tertiary structure of β-Lg is significantly increased by protein unfolding resulting from thermal denaturation.

Very recently, Losito et al. studied the lactosylation of α-La and β-Lgs A and B occurring upon 1−5 h of heating at 70, 80, and 90 °C in the presence of a lactose excess, by means of HPLC-ESI-MS.[24] A significant amount of mono- and bilactosylated forms of the three proteins and their relative increase with heating temperature and time were observed. Lactosylation sites were

Received: October 23, 2010
Accepted: January 19, 2011
Revised: January 18, 2011
Published: February 14, 2011

identified by analyzing the tryptic digests of the heated protein solutions; moreover, a parallel, significant denaturation, involving partial tertiary structure unfolding, was also observed for β-Lgs. In particular, several new lactosylated lysine sites were identified for α-La, although their appearance was not accompanied by a significant modification of the protein tertiary structure. On the contrary, interesting differences were observed for the two variants of β-Lgs, suggesting a different denaturation pathway for these proteins.

Furthermore, a recent study[25] showed that, in addition to the well-known glycation reactions, oxidation of whey proteins plays a major role during the heating of model solutions containing lactose. Partial enzymatic hydrolysis, followed by MALDI-TOF MS, allowed the detection and localization of the most prominent modifications in the amino acid sequence, namely, lactulosyl- and *N*-carboxymethyl-lysine formation, lysine oxidation to aminoadipic semialdehyde, methionine oxidation to methionine sulfoxide, cyclization of N-terminal glutamic acid to a pyrrolidone, and oxidation of cysteine or tryptophan.

All of these studies focus on the investigation of modifications in milk or in model systems simulating lactose-containing milk. Lactose-free products are of emerging interest, as a response to the growing importance of lactose intolerance. Lactose intolerance is the inability to completely digest lactose;[26] approximately 75% of the world's population loses the ability to completely digest a physiological dose of lactose after infancy. Intestinal digestion of lactose involves its breakdown into glucose and galactose (rapidly absorbed into the portal circulation) by a membrane-bound lactase, located in the small intestine. In subjects suffering lactose maldigestion, a portion of the lactose load, not digested in the small intestine, passes into the large intestine, where it is fermented by the colonic microflora, producing short-chain fatty acids and gases, such as H_2, CO_2, or CH_4.[27−30]

To cope with this problem many dairy industries market lactose-free or so-called high-digestibility milk, in which lactose is almost quantitatively converted by an enzymatic process into glucose and galactose. Typically, the lactose content of untreated milk is around 4.9 g/100 mL; after enzymatic digestion, lactose content is reduced, by about 90%, to 0.5 g/100 mL with the concomitant formation of glucose and galactose (2.2 g/100 mL).[30] Hydrolysis with soluble or immobilized enzymes can be performed either before or after heating (pasteurization, UHT). The effects of intensity and sequence of heat and hydrolytic treatments could be assessed by monitoring the glucidic fraction (glucose, lactose, and galactose) and selected thermal treatment markers (furosine, lactulose, and fructose). For lactose-hydrolyzed milk, a higher reactivity toward Maillard reaction was found that could be ascribed to the higher reactivity of the reducing monosaccharides glucose and galactose compared to lactose.[31] Similar conclusions could also be drawn by analyzing the decrease of lysine availability that was greater for skim milk powder with hydrolyzed lactose compared with the normal skim milk powder.[32]

For instance, an increased reactivity of the monosaccharides has been already observed in a recent study on the glycation process of the bovine serum albumin (BSA) reacted with D-glucose, D-galactose, and D-lactose after dry-heating at 60 °C (for 30−240 min). The molecular mass increase and glycation sites of BSA were investigated by MS after digestion with trypsin and chymotrypsin. D-Galactose was more reactive than D-glucose or D-lactose, leading to the addition of 10, 3, and 1 sugar residues, respectively, after a reaction time of 120 min. Lys256 and Lys420 appeared to be the most available sites for conjugation.[33]

However, to the best of our knowledge, no detailed study on heat-induced whey protein modifications in lactose-hydrolyzed milk or in model systems has been reported. Fenaille et al.[34] have shown MALDI-TOF MS spectra of glycated α-La in different milk samples including one lactose-free powder infant formula.

This work aims at a systematic study of heat-induced modifications of whey proteins in a model system mimicking lactose-free milk. Solutions of α-La and β-Lg were thermally treated in the presence of lactose, glucose, galactose, or a sugar mix, resembling the carbohydrate fraction of lactose-free milk. The formation of protein modifications was monitored as a function of heating duration. Partial enzymatic digestion of the whey proteins followed by MALDI-TOF MS analysis allowed the identification of glycation and oxidation products and the modification sites in the amino acid sequence. Finally, a commercial lactose-free milk was analyzed and its glycation state assessed.

EXPERIMENTAL PROCEDURES

Materials. Bovine α-lactalbumin type I (α-La), bovine β-lactoglobulin (β-Lg), D-(+)-glucose (Glu), D-(+)-galactose (Gal), and D-(+)-lactose (Lac) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Sequencing grade endoproteinase AspN was obtained from Roche (Mannheim, Germany). ClinProt magnetic beads MB-IMAC-Cu and 2,5-dihydroxyacetophenone (DHAP) were purchased from Bruker (Bremen, Germany). Cyanoacetic acid and *p*-chlorobenzaldehyde used for α-cyano-4-chlorocinnamic acid (CClCA) synthesis were from *abcr* GmbH & Co. KG (Karlsruhe, Germany). α-Cyano-4-hydroxycinnamic acid (CHCA), diammonium citrate (DAC), and dithiothreitol (DTT) were obtained from Fluka (Taufkirchen, Germany). Ammonium dihydrogenphosphate was from Acros (Geel, Belgium).

Synthesis of CClCA. CClCA was synthesized according to a standard Knoevenagel condensation using cyanoacetic acid and *p*-chlorobenzaldehyde.[35] Ammonium acetate was used as a catalyst. Two grams of cyanoacetic acid (1 equiv), 0.9 equiv of the benzaldehyde, and 0.15 equiv of ammonium acetate were refluxed while stirring in sufficient amounts of toluene (ca. 50 mL) . After quantitative separation of the reaction water by a Dean−Stark apparatus (ca. 3 h), the reaction mixture was cooled to 50 °C and filtered. The crude product was washed with sufficient amounts of distilled water and purified by repeated recrystallization.

Heating of Whey Proteins in Different Milk Models. α-La (0.13 g/100 mL) and β-Lg (0.32 g/100 mL) were dissolved in together with Glu, Gal, and Lac in phosphate-buffered saline (10 mM sodium phosphate, 8 mM NaCl, pH 6.8). The sugars were either tested alone or mixed in a composition mimicking that of typical lactose-free milk. For single-sugar experiments, the model solutions had the following composition: lactose, 4.93 g/100 mL; glucose or galactose, 2.2 g/100 mL. For experiments involving the sugar mix, the following concentrations were used: lactose, 0.51 g/100 mL; glucose and galactose, 2.2 g/100 mL. In the following, the different samples used will be referred to as "single sugar (Glu, Lac, or Gal)", "sugar mix (Glu + Gal + Lac)", and "control (no sugar)".

The model solution (600 μL) was heated in a thermoshaker at 60 °C, and sample aliquots were taken after 0, 8, 46, 56, and 70 h and 3, 7, and 14 days, purified by OMIX C18 tips (Varian Inc., Palo Alto, CA), and lyophilized. Sugar-free protein solutions were treated in the same way and used as control. All experiments were performed in triplicate. Although during industrial manufacturing higher temperatures are normally reached in the lactose-free milk production, the thermal

treatment was carried out at 60 °C to prevent and/or reduce protein denaturation.

Partial enzymatic protein hydrolysis was performed according to the method of Meltretter et al.[25]

Sample Preparation for MALDI TOF Analysis. Before MALDI-TOF MS analysis, 1 μL of 100 mM DTT (reducing agent) was added to a 5 μL aliquot of the protein solution, followed by incubation for 30 min at room temperature. A 3 μL aliquot of the reduced sample was diluted 1:1 with 2% trifluoroacetic acid (TFA) and mixed with 3 μL of MALDI matrix solution prepared by mixing a DHAP saturated ethanolic solution with 10 μM diammonium hydrogen citrate (4 + 1 v/v). A 1 μL aliquot of the sample−matrix solution was spotted twice onto a stainless steel target and air-dried.

For peptide analysis, 1 μL of the protein digest was diluted with 14 μL of a matrix consisting of a 1:1 mixture of a CHCA saturated solution in 50% acetonitrile/0.1% TFA and a 10 mM ammonium dihydrogen phosphate solution in 50% acetonitrile/0.1% TFA. A 1 μL aliquot of the final solution was spotted twice onto a stainless steel target and air-dried.

A 10 mg/mL solution of CClCA, in 50% acetonitrile/0.1% TFA, was also used as an alternative matrix to CHCA in peptide analysis.[36] One microliter of the protein digest was mixed with an equal volume of CClCA solution; a 1 μL aliquot of the final solution was spotted onto a stainless steel target, air-dried, and washed on target with 2 μL of distilled water.

Extraction and Purification of Whey Proteins from Milk Samples. A sample of bovine, lactose-free UHT milk marketed in Italy was centrifuged at 3850 rpm for 60 min under controlled temperature (4 °C) conditions. Milk fat floated spontaneously and then solidified on the top layer so that it could be easily removed. Two milliliters of the defatted milk were treated with 250 μL of sodium acetate buffer solution (pH 4.2) to precipitate casein. After centrifugation at 3850 rpm for 5 min at 4 °C, the casein pellet was discarded, and the remaining solution (containing whey proteins) was filtered through PVDF filters (0.45 μm porosity), adjusted to pH 6.8, and then subjected to MB-IMAC purification protocol.

IMAC magnetic beads (5 μL) were pretreated with 50 μL of MB-IMAC binding buffer following the manufacturer's instructions. Then, 5 μL of the milk extract containing whey proteins was added, and the solution was carefully mixed. The tube was then placed in a magnetic beads separator, and the supernatant was carefully removed using a pipet. The magnetic beads were washed three times with 100 μL of washing buffer, and bound analytes were recovered using 10 μL of the elution buffer.

MALDI-TOF MS. MS experiments were performed using a Micromass M@LDI-LR time-of-flight mass spectrometer (Waters MS Technologies, Manchester, U.K.) or a Bruker Autoflex (Bruker Daltonik, Bremen, Germany), both equipped with a nitrogen UV laser (337 nm wavelength). The following voltages were applied (Micromass M@LDI): pulse, 2610 V (1550 V); source, 15000 V (15000 V); reflectron, 2000 V; MCP 1900 V (1900 V) for reflectron (linear) mode. A time lag focusing (TLF) delay of 500 ns was used. The laser firing rate was 5 Hz, and, unless otherwise specified, 80 laser shots, obtained by a random rastering pattern, were used for each well. The resulting spectra were averaged, background subtracted, and smoothed by a Savitzky−Golay algorithm in reflectron mode and a mean algorithm in linear mode. Mass calibration in reflectron mode was performed using a peptide mixture composed of the fragment 18−39 (2465.199 Da) of the adrenocorticotropic hormone (ACTH), renin (1758.933 Da), and angiotensin (1296.687 Da). Mass calibration in linear mode was performed using a protein mixture composed of insulin β-chain (3497.0 Da), insulin (5735.0 Da), and cytochrome *c* (12361.1 Da).

Using a Bruker Autoflex mass spectrometer, measurements of intact and digested proteins were carried out by delayed extraction (350 and 140 ns, respectively). Laser-desorbed positive ions were analyzed after acceleration by 20 kV in the linear mode for the intact proteins and by 19 kV in the reflector mode for the peptide digest. External calibration was performed using a mix of bovine α-La, chicken lysozyme, and bovine β-Lg variants A and B for the intact protein and a mix of angiotensins I and II, substance P, bombesin, ACTH clips 1−17 and 18−39, and somatostatin 28 for the digest. For each displayed mass spectrum, 150 laser shots from several positions on the spot were averaged.

Protein Identification. The mass spectra obtained from enzymatic digest of whey proteins were compared with the peptide sequences obtained in the nonredundant database Swiss-Prot/TrEMBL utilizing the Peptide Mass software, available at www.expasy.org (version 06/11/2009). The assignment was performed by setting the following parameters: monoisotopic peptide masses were indicated as $[M + H]^+$ with "cysteines treated with nothing". AspN and AspN + N-terminal Glu were selected as enzymes. One missed cleavage was considered. Peptides with a mass higher than 750 Da were displayed.

RESULTS AND DISCUSSION

Analysis of Intact Whey Proteins. To investigate the glycation reactions in a lactose-free milk model, solutions of whey proteins were heated with lactose, glucose, and galactose, separately or mixed, in concentrations typically occurring in milk samples (sugar mix). Figure 1 shows the MALDI-TOF MS spectra obtained for α-La heated at 60 °C for 0, 8, 46, 56, and 72 h in the presence of lactose (panel A), glucose (panel B), and a sugar mix (panel C), respectively. Spectra resulting from longer heating times, that is, 3, 7, and 14 days, are not displayed because only broad and unresolved peaks were obtained, probably due to severe modification, thermal degradation processes, or dehydration/oxidation processes of the condensed sugars.

The MALDI spectrum of native α-La showed (bottom figure in panel A) a main signal at m/z 14177 and two shoulders at higher masses shifted by 162 and 324 Da that can be likely assigned to the glucosyl and lactosyl adducts of the native standard protein. With increasing heating time, the formation of two series of signals was clearly observed (see uppermost spectrum in panel A). In particular, after 72 h of heating time, up to seven lactose adducts (mass shift +324) of the native and glycated protein (marked as solid and open squares, respectively) were formed. Similar experiments carried out by Meltretter et al.[25] gave slightly different results. However, in that case α-La type III was used, which was contaminated by its truncated form in which the C-terminal leucine was cleaved during the purification process. Moreover, after 3 days of incubation in the presence of lactose, only three adducts were clearly visible, instead of the seven adducts observed here. This discrepancy is most likely explained by the use of different heating conditions with a probably higher heating efficiency achieved in the present work.

Similarly, incubation experiments with glucose (panel B) up to 72 h led to the formation of up to 14 adducts (mass shift +162) of native and glycated α-La (marked as solid and open triangles, respectively). It is worth noting that the number of observed adducts (14) is higher than the total number (i.e., 12) of lysine residues, suggesting the possibility that α-La undergoes additional glycation probably at the N-terminus amino acid or at an arginine residue, as already observed by Fenaille et al.[37] For sugars mix experiments (panel C), the observed behavior was qualitatively similar even if one additional glyco adduct was formed compared with single sugar experiments up to 56 h. These results observed for α-La suggest that the modification

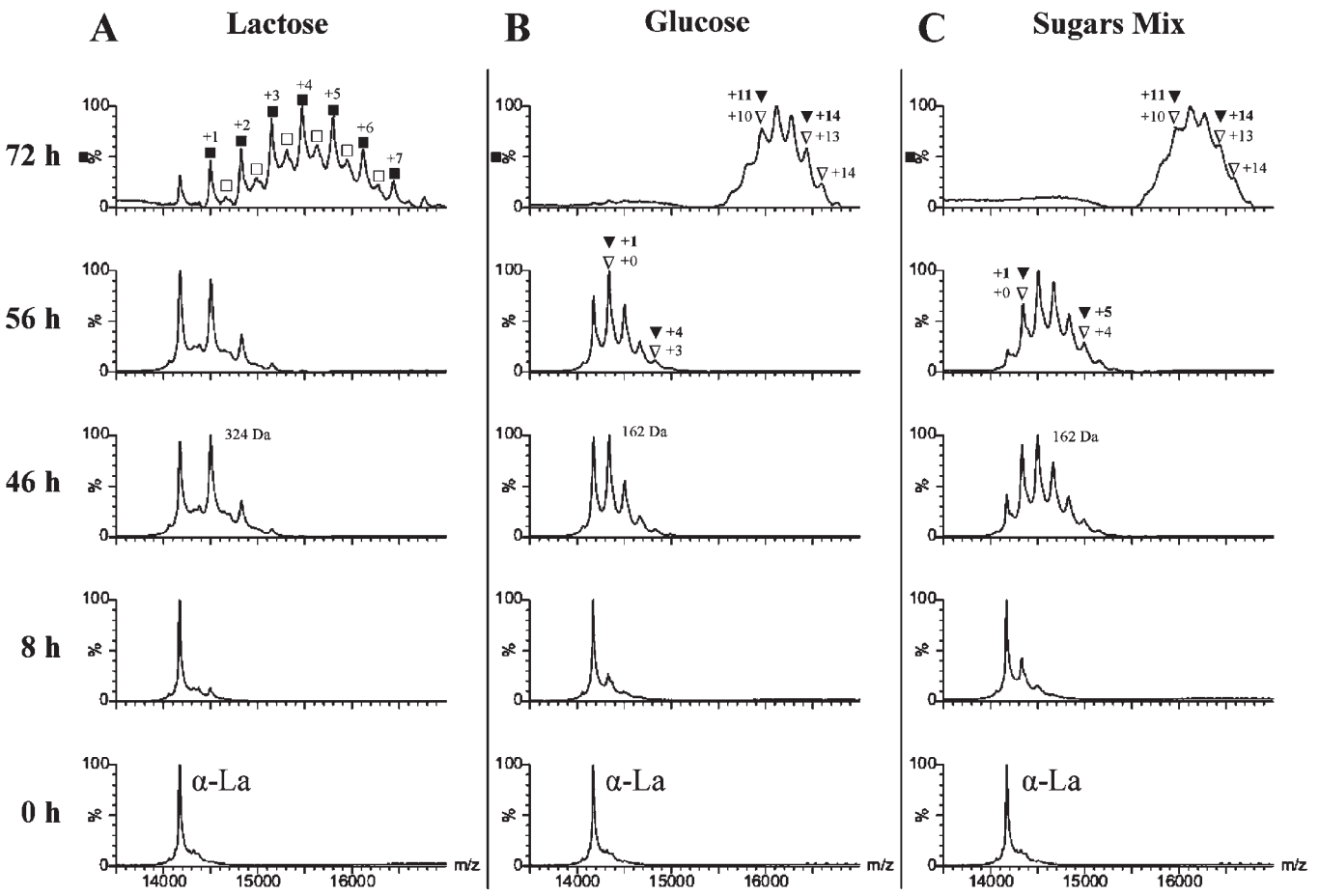

Figure 1. MALDI-TOF mass spectra of α-La heated at 60 °C for different times in the presence of (A) lactose, (B) glucose, and (C) sugars mix (D-glucose + D-galactose + D-lactose). Lactose adducts of the native and pre-existing glycated protein are marked as solid and open squares, respectively; hexose adducts of the native and glycated protein are marked as solid and open triangles, respectively.

pattern is different from that observed with the sugars mix or glucose (and galactose) alone and that glycation of α-La is more pronounced than lactosylation, in the same conditions of time and temperature.

Parallel experiments were carried out with β-Lg, the two main variants, A and B, of which were observed at m/z 18250 and 18336, respectively, with a mass difference of 86 Da. Figure 2 shows the results obtained after heating in the presence of lactose (panel A), glucose (panel B), and sugars mix (panel C).

After 8 h of incubation with lactose and glucose, one and two newly formed adducts could be resolved, respectively, from the native protein, whereas heating with the sugars mix led to the addition of four sugar moieties. In our case, after 56 h of incubation with glucose and sugars mix up to four and five adducts (mass shift 162 Da), respectively, were detected; on the other hand, during incubation with lactose up to three adducts (mass shift 324 Da) were identified. After 72 h of heating, three lactose adducts were still detectable over a much broader peak. For glucose and sugars mix experiments, only a broad peak was observed after heating for 72 h; a comparison with the signal obtained after heating for 56 h suggests that the adducts average number remained almost unchanged for glucose, whereas it increased (likely up to 6) for the sugars mix together with other severe modifications. In parallel, a decrease of the signal intensity was observed, probably due to the thermal degradation of protein, including denaturation and intermolecular coaggregation.

Typically, MALDI mass spectra of Amadori products between β-lactoglobulin and carbohydrates were characterized by a broad Gaussian peak shape because of the great heterogeneity of the glycated forms of β-lactoglobulin.[38−40]

Galactose experiments (data not shown) provided, for both whey proteins, results similar to those observed in the presence of glucose.

To evaluate the exact number of carbohydrates covalently linked to β-Lg, heating experiments were repeated at shorter times (5 h) but at higher temperatures (80 °C).

The protein was heated without sugar (Figure 3A) and in the presence of glucose (Figure 3B) and sugars mix (Figure 3C). After 5 h of heating in the absence of added sugar, the two main native variants A and B were still detected at m/z 18250 and 18336, respectively, but with lower absolute signal intensity. This result is not unexpected because it has been reported[24] that β-Lg undergoes a partial degradation during heating at relatively high temperatures, generating peptides with MW between 2000 and 10000 Da. MALDI-TOF MS spectra were acquired for heated samples; large peptides in the mass range m/z 2000−15000 were detected together with the less intense signal relevant to native protein (data not shown). In the presence of glucose (Figure 3B), heating for 5 h led to six additional adducts (mass shift +162 Da)

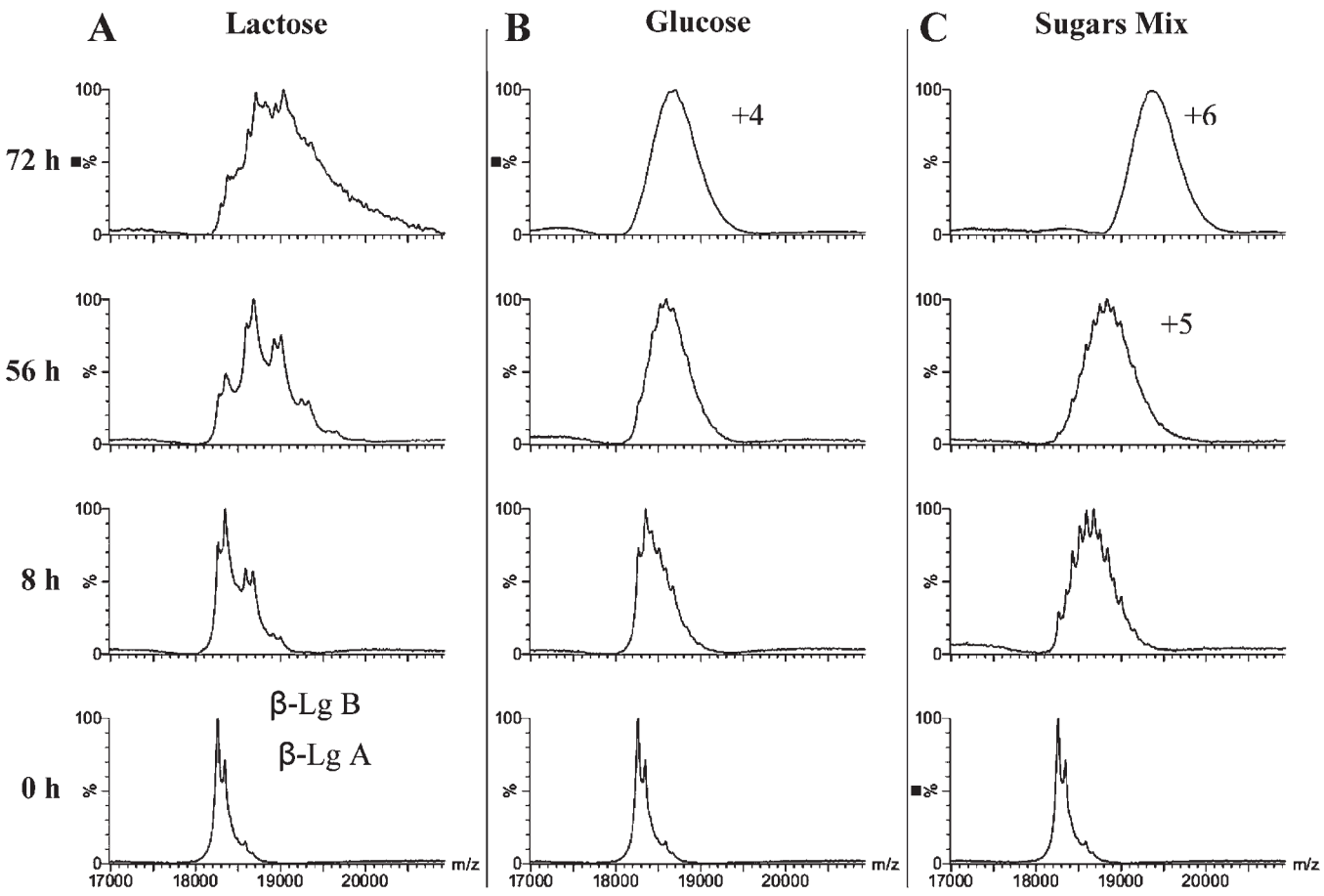

Figure 2. MALDI-TOF mass spectra of β-Lg heated at 60 °C for different times in the presence of (A) lactose, (B) glucose, and (C) sugars mix (D-glucose + D-galactose + D-lactose). Numbers of lactose and hexose adducts of both β-Lg variants are indicated.

clearly detectable on both variants and up to 11 adducts in the presence of the sugars mix (Figure 3C). Incubation for longer times led again to broad signals with average molecular weights around m/z 20500, indicating the addition of up to 15 glucose residues (data not shown).

The formation of a higher number of glycation adducts upon incubation at 80 °C compared to 60 °C can be explained, besides a higher reaction rate, by tertiary structure modification occurring on a major portion of β-Lg A and B populations at higher temperatures. The modified proteins likely expose new basic amino acids leading to a higher glycation degree.

In a next step, with the goal to get a more detailed insight into the structures and site of the modifications, the modified proteins were partially enzymatically digested prior to MS analysis.

MALDI-TOF MS Analysis of Whey Proteins after Partial Enzymatic Hydrolysis. The native and heated whey proteins were digested using endoproteinase AspN that specifically cleaves peptide bonds on the N-terminal aspartic acid, which is not affected by glycation.[20,41]

The use of CHCA as MALDI matrix led to unsatisfactory results in terms of the number of glycation adducts that could be detected (data not shown), likely because of changes in the ionization efficiency originating from a reduced basicity of the modified lysines.[42] A recently introduced new matrix, namely, CClCA, facilitates the ionization of less basic peptides compared to the classical CHCA.[36] Indeed, using CClCA, very informative MALDI spectra (see, e.g., Figure 4) were obtained. Tables 1 and 2 summarize the detected modifications of α-La and β-Lg and their localization in the amino acid sequence.

Similar to the α-La/lactose system, we could also show (see Table 1) a double oxidation of W118/C120, a probable glycation on N66/N71/N74, and K58/62 besides the modifications already reported by Meltretter et al. (ultimate assignments of these glycation sites would obviously require MS/MS experiments).[25]

The main modifications produced by the Maillard reaction involving glucose, galactose, and the "sugars mix" were oxidation, generation of N^{ε}-carboxymethyl-lysine (CML), lysine aldehyde formation, and glycation. Concerning glycation of α-La, the signals at m/z 1414.7 and 1784.9 were ascribed to glucose/galactose adducts on fragments 1−10 (m/z 1252.6) and 1−13 (m/z 1622.9 Da), respectively, of the native peptides. The glycation likely involved the lysine residue K5 as already reported for lactose experiments.[25] Moreover, for both native and modified peptides, byproducts with a mass difference of −18 Da were observed due to the heating-induced side reaction of the free amino group of the N-terminal glutamic acid with the side-chain carboxyl residue to form a pyrrolidone with loss of H_2O. In addition, a modification of the native peptide at m/z 2188.2 (fragment 97−115) with a mass shift of 162 Da was detected in the glucose, galactose, and sugars mix experiments, indicating a hexose adduct at Lys98 or Lys114.

The formation of a glyco adduct observed (see Table 1) for the fragment 64−77 (m/z 1531.6), where lysine is absent, deserves

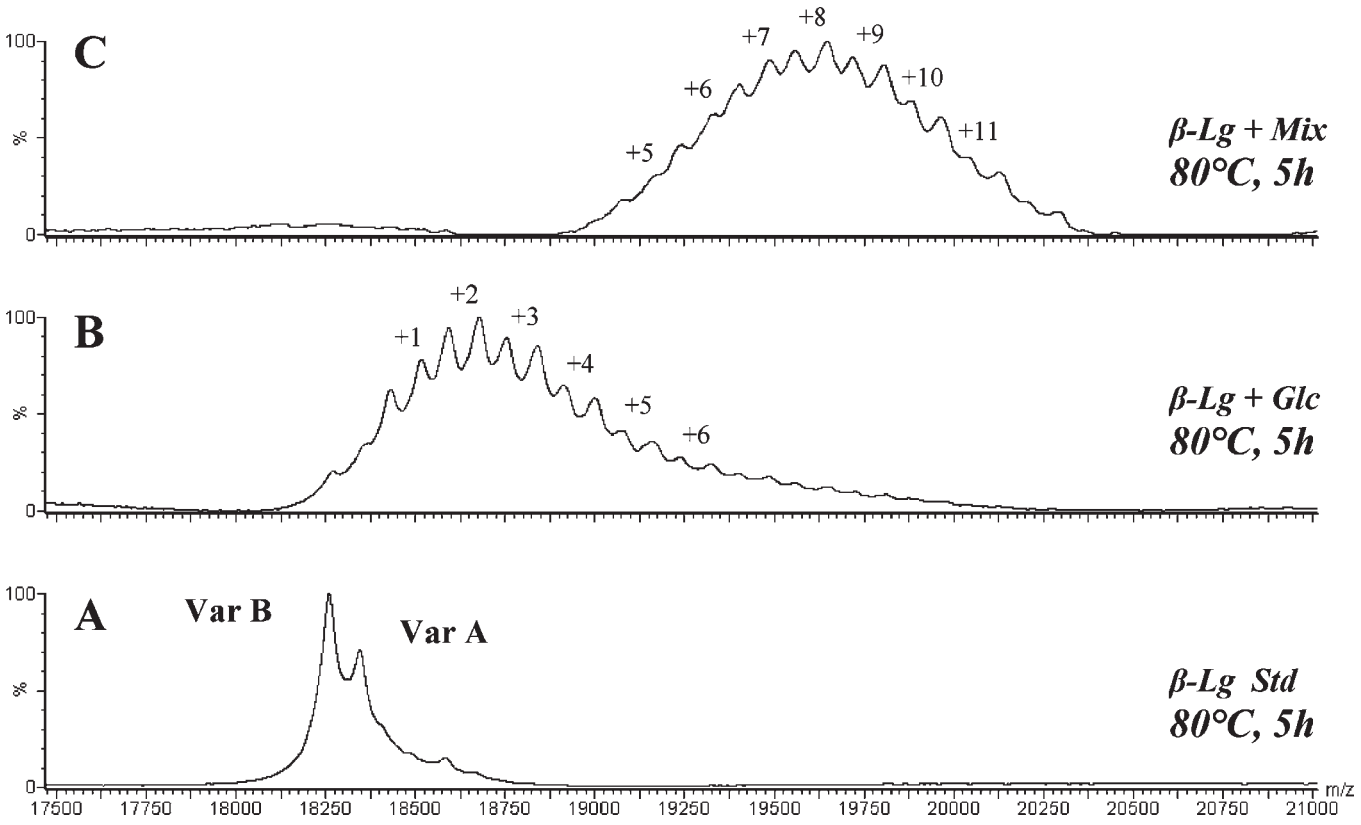

Figure 3. MALDI-TOF mass spectra of β-Lg heated at 80 °C for 5 h (A) in the absence of sugar, (B) in the presence of glucose, and (C) in the presence of the sugars mix (D-glucose + D-galactose + D-lactose). The numbers of hexose adducts are indicated.

special interest. This is in agreement with the analyses of the intact proteins, which revealed a number of hexose adducts higher than the lysine residues, suggesting that a different residue, for example, asparagine or glutamine, could be involved in the glycation reaction. Galactose behavior was similar to glucose.

For β-Lg, a similar reactivity was observed with some differences between lactose and glucose/galactose experiments (Table 2; Figure 4B). Sugar adducts of the native peptides at m/z 2199.1 (fragment 33−52) and 3032.7 (fragment 137−162) were observed for glucose, galactose, and the sugars mix experiments. These glycation sites have been previously reported when β-Lg was reacted with lactose.[24,25] Additionally, an adduct was detected at m/z 1265.7 (mass shift of +162 Da) involving Lys8 or Leu1 on the fragment 1−10 (m/z 1103.6). Indeed, a leucine residue (Leu1) is located on the N-terminus of the β-Lg sequence where the $-NH_2$ group is free and can react with sugar, giving the Amadori product. When a mild thermal treatment is adopted, the most probable glycation site is Leu1, but for longer time and higher temperature, further modification on Lys8 is also possible.[18,43,44] Furthermore, in agreement with Meltretter et al.,[25] we could detect one oxidation on M_7, a double oxidation on W_{19} and/or M_{24}, and the CML on K_{47} (see Table 2).

Other previous observations[16,18,43] indicated Lys91 and Lys100 as the most probable glycation sites of β-Lg; this discrepancy may be explained by the use of different heating conditions and/or the use of different enzymatic digestion conditions.

Study of Sugars Reactivity. To assess the different reactivity of each sugar toward a specific site in the protein sequence, some modified peptides were selected, and the MALDI signal intensities of modified and native peptides were recorded. The adduct percentage (%A) was calculated as

$$\%A = [M^*/(M^* + M)] \times 100 \quad (1)$$

where M and M^* are the intensities of the unmodified and modified peptides, respectively.

When both, native and glycated, forms occur (see above) together with the pyrrolidone byproduct (*pyrr*, mass shift −18 Da), %A was calculated according to

$$\%A = [(M^* + M^*_{pyrr}/((M^* + M^*_{pyrr}) + (M + M_{pyrr}))] \times 100 \quad (2)$$

Because other kinds of modifications such as oxidation, generation of CML, and lysine aldehyde formation are negligible compared to glycation, their contributions were omitted in the % A calculation. With this approach, relative quantification of each modification can be achieved, because the native peptide works as an internal standard correcting for variations in ionization efficiency of the single experiment. As a result, good reproducibility among the triplicate experiments was achieved. This method is particularly useful to record the time course of product formation.

Figure 5 shows the glycation degree (%A) versus time (days) for the native peptides at m/z 1252.6 (fragment 1−10 of α-La) and m/z 2199.1 (fragment 33−52 of β-Lg) heated at 60 °C in the presence of a single sugar. As can be seen, the %A value was higher for glucose and galactose than for lactose, suggesting that,

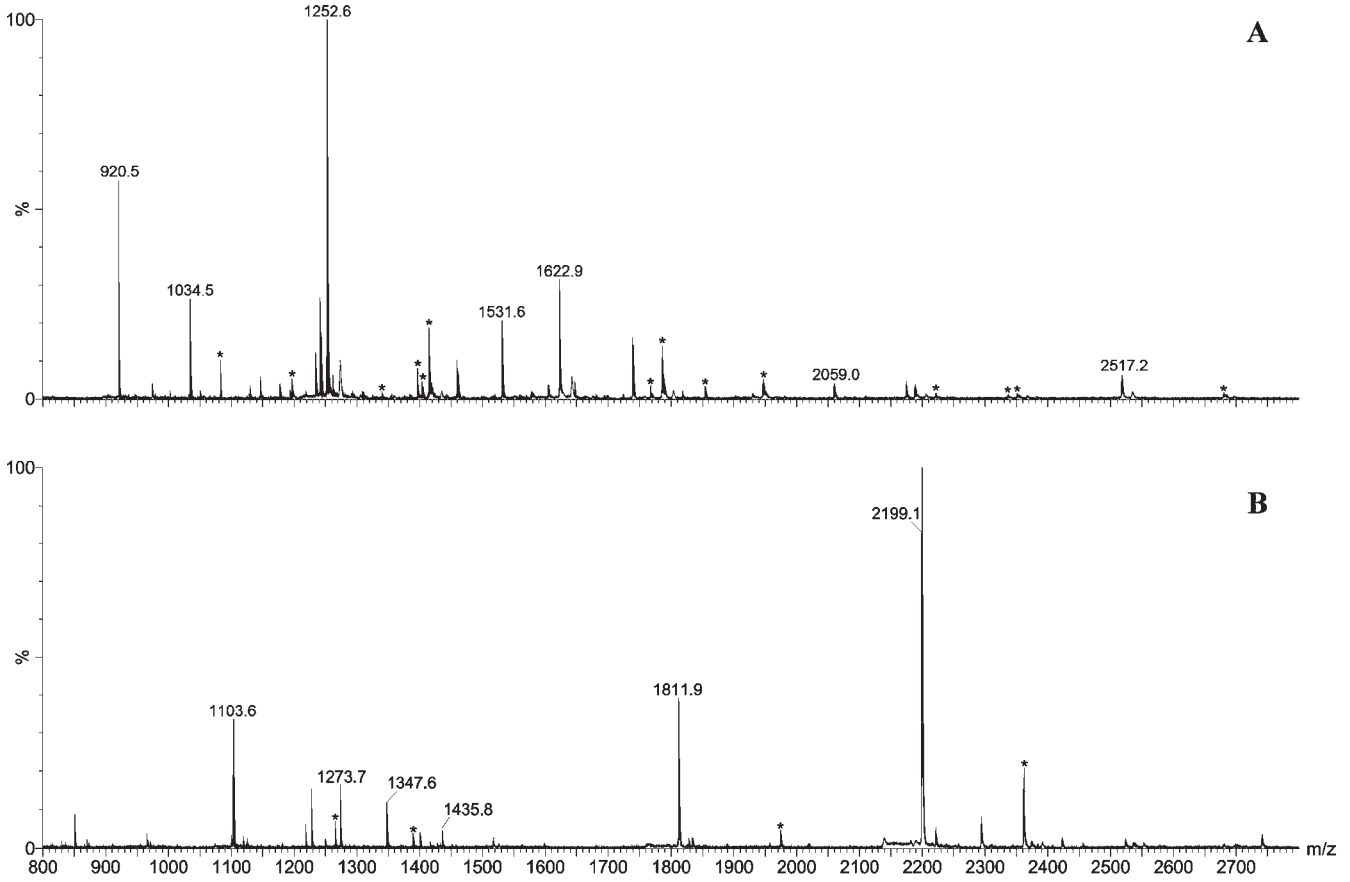

Figure 4. MALDI-TOF mass spectra of the AspN digest of α-La (A) and β-Lg (B) heated for 56 h at 60 °C in the presence of galactose. The glycated peptides are marked with an asterisk.

under the conditions used in this study, glucose and galactose were more reactive than lactose. In particular, at the longest observation time, glycation degrees of 25 and 35% were estimated for the peptide at m/z 1252.6 in the presence of lactose and glucose/galactose, respectively. Similar results, but with a lower glycation degree, were obtained for β-Lg (ca. 16% for glucose/galactose and ca. 8% for lactose).

As can be inferred from Figure 5, after a lag time (different for α-La and β-Lg), most of the glycation process takes place between the second and fourth days.

Analysis of a Commercial Lactose-free Milk Sample. Finally, a commercial UHT lactose-free milk sample purified by IMAC magnetic beads (see Experimental Procedures) was analyzed. The main goal of this experiment was to confirm the validity of our milk-simulating model solutions. The MALDI-TOF mass spectra of α-La and β-Lg obtained after IMAC cleanup are shown in Figure 6, panels A and B, respectively. For α-La, two hexose adducts (mass shift +162 Da) were observed. With regard to β-Lg, three hexose adducts could be detected for both protein variants.

It is worth noting that compared to our milk model sample the real system shows a lower glycation degree. As discussed before, this discrepancy is most likely explained by the use of different heating conditions in laboratory studies compared to industrial processing. In fact, UHT treatment is developed as a continuous flow process, whereas our investigations are carried out in a bulk static solution leading to a different heating transfer. Moreover, a probable competition for glycation by caseins or a protection by other milk components could contribute to decrease the glycation degree on whey proteins.

To the best of our knowledge, glycation of whey proteins in lactose-free milk has not been investigated. Only one study on lactose-free milk powder has reported the modification of α-La with six hexose moieties.[35] The lower glycation rate observed in the present study reflects very well the thermal treatment, which is less severe for UHT milk than for milk powders.

These results underscore once more that monitoring of Amadori products in milk samples by MALDI-TOF MS is a good indicator of protein damage during heating processes.

Conclusions. The main modifications occurring in whey proteins in a model system mimicking lactose-free milk were investigated by means of MALDI-TOF MS analysis of intact and partially digested proteins. Analysis of intact proteins reveals the formation of hexose/lactose adducts during heating, whereas the analysis of AspN digests, using α-cyano-4-chloro-cinnamic acid as MALDI matrix, allowed the identification of several (including new) glycation sites. In experiments mimicking lactose-free milk, additional modification sites were evidenced (compared with the lactose system) together with a higher glycation degree, indicating a higher reactivity of glucose and galactose compared to the parent disaccharide (lactose). Thus, under the same heating conditions, whey proteins in lactose-free milk should be more severely damaged than in regular milk.

A bovine lactose-free milk sample has been analyzed via MALDI-TOF MS after treatment with IMAC-Cu

Table 1. Main α-La Modifications Observed after Thermal Treatment (See Text for Details)

m/z native peptide	mass shift	attribution[a]	Lac $\Delta M = +324$	Glc $\Delta M = +162$	Gal $\Delta M = +162$	mix	position	peptide sequence	sites
920.5	+162	Glyco	ND[b]	●	●	ND	[7−13]	EVFRELK	K_{13}
1034.5	+16	Ox	●	●	●	●	[116−123]	DQWLCEKL	W_{118}, C_{120}
	+32	diOx	●	●	●	●	[116−123]	DQWLCEKL	W_{118}, C_{120}
	+324|+162	Glyco	●	●	●	●	[116−123]	DQWLCEKL	K_{122}
1177.6	+162	Glyco	ND	●	ND	ND	[87−96]	DDIMCVKKIL	K_{93}, K_{94}
1252.6	−1	Lys	●	●	●	●	[1−10]	EQLTKCEVFR	K_5
	−18	Pyr	●	●	●	●	[1−10]	EQLTKCEVFR	E_1
	+324|+162	Glyco	●	●	●	●	[1−10]	EQLTKCEVFR	K_5
	+306|+144	Pyr-Glyco	●	●	●	●	[1−10]	EQLTKCEVFR	K_5
1622.9	−1	Lys	●	●	●	●	[1−13]	EQLTKCEVFRELK	K_5, K_{13}
	−18	Pyr	●	●	●	●	[1−13]	EQLTKCEVFRELK	E_1
	+58	CML	●	●	●	●	[1−13]	EQLTKCEVFRELK	K_5, K_{13}
	+40	Pyr-CML	●	●	●	●	[1−13]	EQLTKCEVFRELK	K_5, K_{13}
	+324|+162	Glyco	●	●	●	●	[1−13]	EQLTKCEVFRELK	K_5, K_{13}
	+306|+144	Pyr-Glyco	●	●	●	●	[1−13]	EQLTKCEVFRELK	K_5, K_{13}
1531.6	+324|+162	Glyco	●	●	●	●	[64−77]	DQNPHSSNICNISC	N_{66}, N_{71}, N_{74}
1755.9	+162	Glyco	ND	●	ND	ND	[49−62]	EYGLFQINNKIWCK	K_{58}, K_{62}
1817.9	+324|+162	Glyco	●	●	●	ND	[97−112]	DKVGINYWLAHKALCS	K_{98}, K_{108}
2059.1	+324|+162	Glyco	●	●	●	●	[46−62]	DSTEYGLFQINNKIWCK	K_{58}, K_{62}
2174.0	+162	Glyco	ND	●	●	●	[46−63]	DSTEYGLFQINNKIWCKD	K_{58}, K_{62}
2188.2	+162	Glyco	ND	●	●	●	[97−115]	DKVGINYWLAHKALCSEKL	K_{98}, K_{108}, K_{114}
2517.2	+162	Glyco	ND	●	●	ND	[14−36]	DLKGYGGVSLPEWVCTTFHTSGY	K_{16}

[a] Modification type: Glyco, glycation adduct; Pyr-Glyco, glycation adduct with water loss; Ox/diOx, mono/di oxidation; Lys, lysine aldehyde; Pyr, pyrrolidone; CML, N^{ε}-carboxymethyl-lysine; Pyr-CML, N^{ε}-carboxymethyl-lysine with water loss. [b] ND, not detected.

Table 2. Main β-Lg Modifications in Lactose, Glucose, Galactose, and Sugars Mix Samples

m/z native peptide	mass shift	attribution[a]	Lac $\Delta M = +324$	Glu $\Delta M = +162$	Gal $\Delta M = +162$	mix	position	peptide sequence	sites
1103.6	−1	Lys	●	●	●	●	[1−10]	LIVTQTMKGL	K_8
	+16	Ox	●	●	●	●	[1−10]	LIVTQTMKGL	M_7
	+162	Glyco	ND[b]	●	●	●	[1−10]	LIVTQTMKGL	L_1, K_8
1811.9	+16	Ox	●	●	●	●	[11−27]	DIQKVAGTWYSLAMAAS	W_{19}, M_{24}
	+32	diOx	●	●	●	ND	[11−27]	DIQKVAGTWYSLAMAAS	W_{19}, M_{24}
2199.1	+58	CML	●	●	●	●	[33−52]	DAQSAPLRVYVEELKPTPEG	K_{47}
	+324|+162	Glyco	●	●	●	●	[33−52]	DAQSAPLRVYVEELKPTPEG	K_{47}
3032.7	+324|+162	Glyco	●	●	●	●	[137−162]	DKALKALPMHIRLSFNPTQLEEQCHI	K_{138}, K_{141}

[a] Modification type: Glyco, glycation adduct; Ox/diOx, mono/di oxidation; Lys, lysine aldehyde; CML, N^{ε}-carboxymethyl-lysine. [b] ND, not detected.

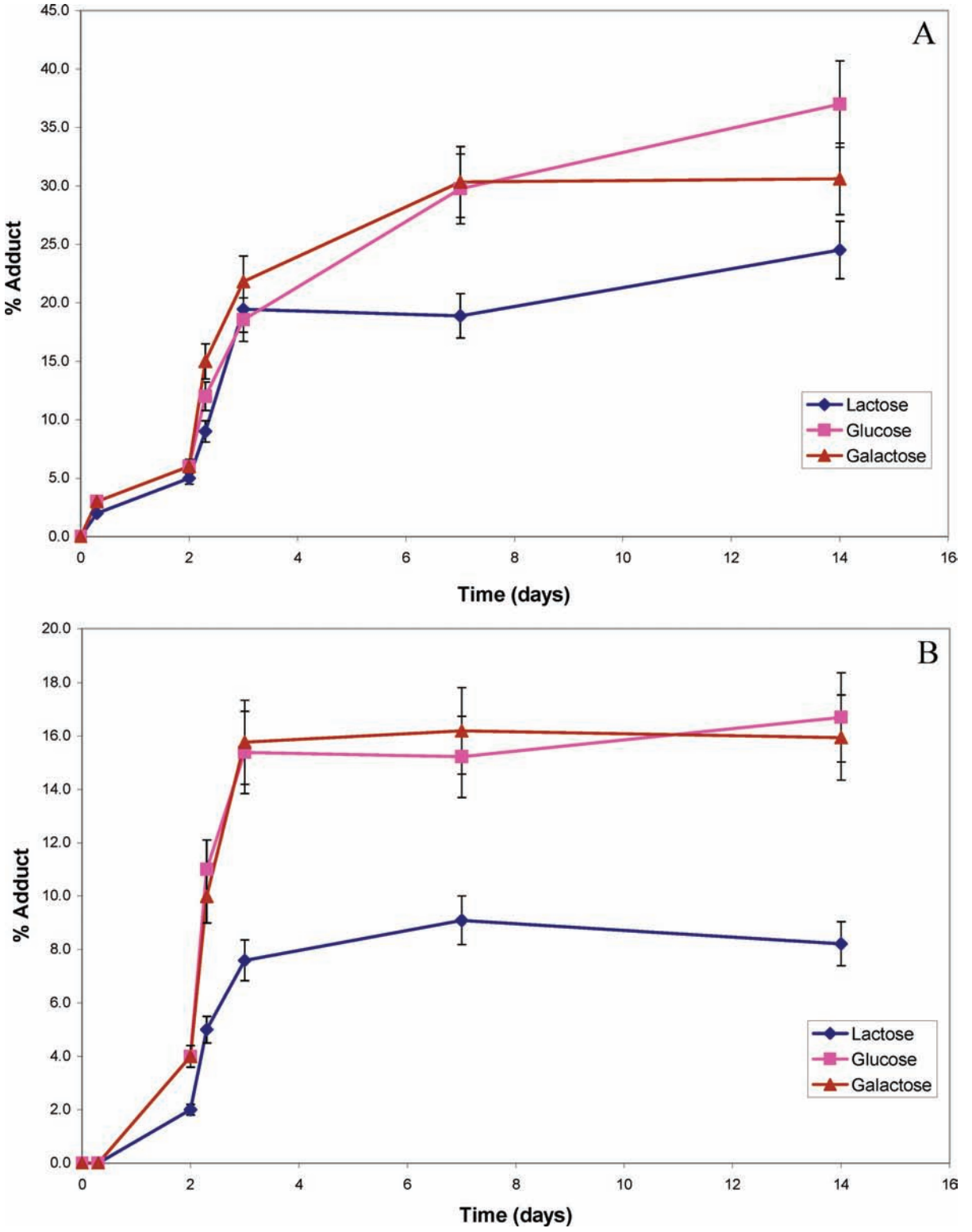

Figure 5. Glycation rate of selected α-La (A, native peptide at m/z 1252.6) and β-Lg peptides (B, native peptide at m/z 2199.1).

magnetic beads. Experimental evidence showed that up to two monosaccharide adducts can be detected in α-La, whereas up to three monosaccharide adducts were observed in both β-Lg variants.

This study demonstrates, once more, the usefulness of MALDI-TOF MS as a simple and fast technique for the high sample throughput analysis of heat-induced protein modifications in different milk products.

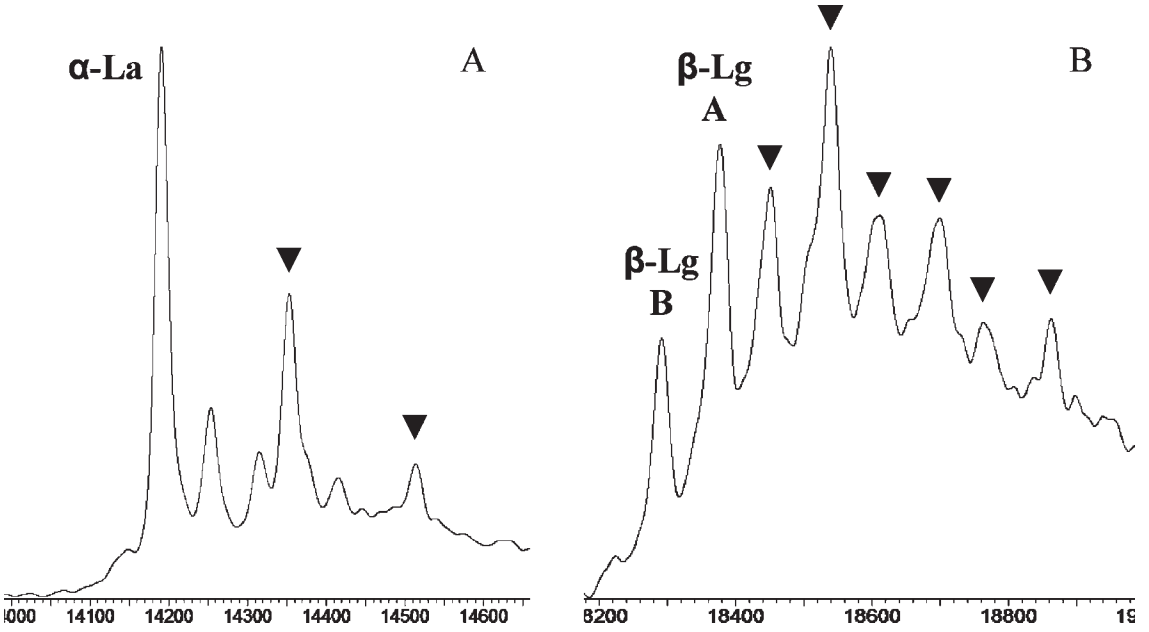

Figure 6. MALDI-TOF mass spectra of α-La (A) and β-Lg (B) extracted from a lactose-free milk sample and purified as described under Experimental Procedures. Hexose adducts on both whey proteins are marked with solid triangles.

AUTHOR INFORMATION

Corresponding Author

*Phone: +39-080-5443589. Fax: +39-080-5442026. E-mail: Saverio.Carulli@agenziadogane.it (S.C.); calvano@chimica.uniba.it (C.D.C.).

Funding Sources

Partial funding from Università degli Studi di Bari "Aldo Moro" is acknowledged.

ACKNOWLEDGMENT

We gratefully acknowledge Gregor Vollmer for his technical support, Dr. Jasmin Meltretter for the manuscript revision, and Dr. Antonio Monopoli for the matrix synthesis.

REFERENCES

(1) Kinsella, J. E.; Whitehead, D. M. Proteins in whey: chemical, physical and functional properties. *Adv. Food Nutr. Res.* **1989**, *33*, 343–438.

(2) Ewan, H.; Michael, B. Z. Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people (review). *J. Nutr. Biochem.* **2003**, *14*, 251–258.

(3) Chatterton, D. E. W.; Smithers, G.; Roupas, P.; Brodkorb, A. Bioactivity of β-lactoglobulin and α-lactalbumin − technological implications for processing. *Int. Dairy J.* **2006**, *16*, 1229–1240.

(4) Fox, P. F. Heat-induced changes in milk preceding coagulation. *J. Dairy Sci.* **1981**, *64*, 2127–2137.

(5) Singh, H.; Creamer, L. K. Heat stability of milk. In *Advanced Dairy Chemistry − 1. Proteins*; Elsevier Applied Science: London, U.K., 1992; pp 621−656.

(6) Mauron, J. The Maillard reaction in food; a critical review from the nutritional standpoint. *Prog. Food Nutr. Sci.* **1981**, *5* (1−6), 5–35.

(7) van Boekel, M. A. J. S. Effect of heating on Maillard reactions in milk. *Food Chem.* **1998**, *62*, 403–414.

(8) Mauron, J. Influence of processing on protein quality. *J. Nutr. Sci. Vitaminol. (Tokyo)* **1990**, *36* (Suppl. 1), S57–69.

(9) De Block, J.; Merchiers, M.; Mortier, L.; Braekman, A.; Ooghe, W.; Van Rentergem, R. Monitoring nutritional quality of milk powders: capillary electrophoresis of the whey protein fraction compared with other methods. *Int. Dairy J.* **2003**, *13*, 87–94.

(10) Finot, P. A. Metabolism and physiological effects of Maillard reaction products. In *The Maillard Reaction in Food Processing, Human Nutrition and Physiology*; Finot, P. A., Aeselbacher, H., Hurrell, R. F., Liardon, R., Eds.; Birkhaeuser: Boston, MA, 1990; pp 259−271.

(11) Horvat, S.; Jakas, A. Peptide and amino acid glycation: new insights into the Maillard reaction. *J. Pept. Sci.* **2004**, *10*, 119–137.

(12) Singh, H.; Waungana, A. Influence of heat treatment of milk on cheesemaking properties. *Int. Dairy J.* **2001**, *11*, 543–551.

(13) Marsilio, R.; Catinella, S.; Seraglia, R.; Traldi, P. Matrix-assisted laser-desorption ionization mass-spectrometry for the rapid evaluation of thermal-damage in milk. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 550–552.

(14) French, S. J.; Harper, W. J.; Kleinholz, N. M.; Jones, R. B.; Green-Church, K. B. Maillard reaction induced lactose attachment to bovine β-lactoglobulin: electrospray ionization and matrix-assisted laser desorption/ionization examination. *J. Agric. Food Chem.* **2002**, *50*, 820–823.

(15) Czerwenka, C.; Maier, I.; Pittner, F.; Lindner, W. Investigation of the lactosylation of whey proteins by liquid chromatography−mass spectrometry. *J. Agric. Food Chem.* **2006**, *54*, 8874–8882.

(16) Siciliano, R.; Rega, B.; Amoresano, A.; Pucci, P. Modern mass spectrometric methodologies in monitoring milk quality. *Anal. Chem.* **2000**, *72*, 408–415.

(17) Losito, I.; Carbonara, T.; Monaci, L.; Palmisano, F. Evaluation of the thermal history of bovine milk from the lactosylation of whey proteins: an investigation by liquid chromatography−electrospray ionization mass spectrometry. *Anal. Bioanal. Chem.* **2007**, *389*, 2065–2074.

(18) Leonil, J.; Molle, D.; Fauquant, J.; Maubois, J. L.; Pearce, R. J.; Bouhallab, S. Characterization by ionization mass spectrometry of lactosyl β-lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. *J. Dairy Sci.* **1997**, *80*, 2270–2281.

(19) Morgan, F.; Molle, D.; Henry, G.; Venien, A.; Leonil, J.; Peltre, G.; Levieux, D.; Maubois, J. L.; Bouhallab, S. Nonenzymatic lactosylation of bovine β-lactoglobulin under mild heat treatment leads to structural heterogeneity of the glycoforms. *Biochem. Biophys. Res. Commun.* **1997**, *236*, 413–417.

(20) Marvin, L. F.; Parisod, V.; Fay, L. B.; Guy, P. A. Characterization of lactosylated proteins of infant formula powders using two-dimensional gel electrophoresis and nanoelectrospray mass spectrometry. *Electrophoresis* **2002**, *23*, 2505–2512.

(21) Galvani, M.; Hamdan, M.; Righetti, P. G. Two-dimensional gel electrophoresis/matrix-assisted laser desorption/ionisation mass spectrometry of a milk powder. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1889–1897.

(22) Meltretter, J.; Birlouez-Aragon, I.; Becker, C. M.; Pischetsrieder, M. Assessment of heat treatment of dairy products by MALDI-TOF-MS. *Mol. Nutr. Food Res.* **2009**, *53*, 1487–1495.

(23) Fenaille, F.; Morgan, F.; Parisod, V.; Tabet, J. C.; Guy, P. A. Solid-state glycation of β-lactoglobulin by lactose and galactose: localization of the modified amino acids using mass spectrometric techniques. *J. Mass Spectrom.* **2004**, *39*, 16–28.

(24) Losito, I.; Stringano, E.; Carulli, S.; Palmisano, F. Correlation between lactosylation and denaturation of major whey proteins: an investigation by liquid chromatography−electrospray ionization mass spectrometry. *Anal. Bioanal. Chem.* **2010**, *396*, 2293–2306.

(25) Meltretter, J.; Seeber, S.; Humeny, A.; Becker, C. M.; Pischetsrieder, M. Site-specific formation of Maillard, oxidation, and condensation products from whey proteins during reaction with lactose. *J. Agric. Food Chem.* **2007**, *55*, 6096–6103.

(26) Harrington, L. K.; Mayberry, J. F. A re-appraisal of lactose intolerance. *Int. J. Clin. Practice* **2008**, *62*, 1541–1546.

(27) Levitt, M. D. Production and excretion of hydrogen gas in man. *N. Engl. J. Med.* **1969**, *281*, 122–7.

(28) Strocchi, A.; Corazza, G.; Ellis, C. J.; Gasbarrini, G.; Levitt, M. D. Detection of malabsorption of low-doses of carbohydrate − accuracy of various breath H_2 criteria. *Gastroenterology* **1993**, *105*, 1404–10.

(29) Bond, J. H.; Levitt, M. D. Use of breath hydrogen (H_2) to quantitate small bowel transit time following partial gastrectomy. *J. Lab. Clin. Med.* **1977**, *90*, 30–6.

(30) http://www.parmalat.it/prodotti/zymil/index.htm.

(31) Messia, M. C.; Candigliota, T.; Marconi, E. Assessment of quality and technological characterization of lactose-hydrolyzed milk. *Food Chem.* **2007**, *104*, 910–917.

(32) Rutherfurd, S. M.; Moughan, P. J. Effect of elevated temperature storage on the digestible reactive lysine content of unhydrolyzed- and hydrolyzed-lactose milk-based products. *J. Dairy Sci.* **2008**, *91*, 477–482.

(33) Ledesma-Osuna, A. I.; Ramos-Clamont, G.; Vázquez-Moreno, L. Characterization of bovine serum albumin glycated with glucose, galactose and lactose. *Acta Biochim. Pol.* **2008**, *55*, 491–497.

(34) Fenaille, F.; Parisod, V.; Visani, P.; Populaire, S.; Tabet, J. C.; Guy, P. A. Modifications of milk constitutents during processing: a preliminary benchmarking study. *Int. Dairy J.* **2006**, *16*, 728–739.

(35) Knoevenagel, E. *Ber. Dtsch. Chem. Ges.* **1898**, *31*, 2596–2619.

(36) Jaskolla, T. W.; Lehmann, W. D.; Karas, M. 4-Chloro-α-cyanocinnamic acid is an advanced, rationally designed MALDI matrix. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 12200–12205.

(37) Fenaille, F.; Campos-Gimenez, E.; Guy, P. A.; Schmitt, C.; Morgan, F. Monitoring of α-lactoglobulin dry-state glycation using various analytical techniques. *Anal. Biochem.* **2003**, *320*, 144–148.

(38) Corzo-Martınez, M.; Moreno, F. J.; Olano, A.; Villamiel, M. Structural characterization of bovine β-lactoglobulin-galactose/tagatose Maillard complexes by electrophoretic, chromatographic, and spectroscopic methods. *J. Agric. Food Chem.* **2008**, *56*, 4244–4252.

(39) Van Teeffelen, A. M. M.; Broersen, K.; De Jongh, H. H. J. Glucosylation of β-lactoglobulin lowers the heat capacity change of unfolding; a unique way to affect protein thermodynamics. *Protein Sci.* **2005**, *14*, 2187–2194.

(40) Sanz, M. L.; Corzo-Martınez, M.; Rastall, R. A.; Olano, A.; Moreno, F. J. Characterization and in vitro digestibility of bovine β-lactoglobulin glycated with galactooligosaccharides. *J. Agric. Food Chem.* **2007**, *55*, 7916–7925.

(41) Fenaille, F.; Morgan, F.; Parisod, V.; Tabet, J. C.; Guy, P. A. Solid-state glycation of β-lactoglobulin monitored by electrospray ionisation mass spectrometry and gel electrophoresis techniques. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1483–1492.

(42) Yeboah, F. K.; Yaylayan, V. A. Analysis of glycated proteins by mass spectrometric techniques: qualitative and quantitative aspects. *Nahrung* **2001**, *45*, 164–171.

(43) Morgan, F.; Bouhallab, S.; Mollè, D.; Henry, G.; Maubois, J. L.; Lèonil, L. Lactolation of β-lactoglobulin monitored by electrospray ionisation mass spectrometry. *Int. Dairy J.* **1998**, *8*, 95–98.

(44) Morgan, F.; Molle, D.; Henry, G.; Venien, A.; Leonil, J.; Peltre, G.; Levieux, D.; Maubois, J. L.; Bouhallab, S. Glycation of bovine β-lactoglobulin: effect on the protein structure. *Int. J. Food Sci. Technol.* **1999**, *34*, 429–435.