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Site-Specific Formation of Maillard, Oxidation, and Condensation Products from Whey Proteins during Reaction with Lactose

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Heat treatment of dairy products leads to structural changes of proteins, which can severely decrease the nutritional value [Mauron, J. *J. Nutr. Sci. Vitaminol. (Tokyo)* **1990**, *36* (Suppl. 1), S57−69]. In this study, model solutions of the two main whey proteins, α-lactalbumin and β-lactoglobulin, respectively, were incubated with lactose, and modifications were monitored by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Lactulosyl residues were the most abundant modifications of α-lactalbumin and β-lactoglobulin. Up to four of these adducts were identified on the proteins. Enzymatical digest with endoproteinase AspN prior to mass spectrometric analysis allowed the detection of further modifications and their localization in the amino acid sequence. Most prominent modifications were lactulosyllysine, N^{ϵ}-carboxymethyllysine, oxidation of lysine to aminoadipic semialdehyde, oxidation of methionine to methionine sulfoxide, cyclization of N-terminal glutamic acid to a pyrrolidone, and oxidation of cysteine or tryptophan. The presence of methionine oxidation was deduced from a control protein that had been oxidized by hydrogen peroxide. These studies establish MALDI-TOF-MS as a reliable tool to monitor chemical modifications of nutritional proteins during food processing.

KEYWORDS: α-Lactalbumin; β-lactoglobulin; glycation; Maillard reaction; milk; oxidation; site specific; matrix-assisted laser desorption/ionization mass spectrometry

INTRODUCTION

Whey proteins represent a valuable protein source, providing a high content of essential amino acids in a ratio very similar to the required composition for human nutrition. Therefore, and also because of their useful technological properties, whey proteins find broad application in a variety of foodstuffs, such as infant formulas, dietetic foods, or beverages. During industrial processing, however, milk or dairy products are usually heated to achieve safe products with long shelf life, to prepare milk powders, or for enzymatic treatment. In the course of these thermal processes, a multitude of chemical reactions takes place. The latter can drastically decrease the favorable nutritional properties of whey and, as a consequence, their careful control represents an important challenge for food industries (*1*).

A fundamental reaction of milk proteins during thermal processing is the so-called nonenzymatic browning or Maillard reaction, during which the nitrogen of a protein-bound amino group, preferentially a lysine residue, reacts with the carbonyl function of the milk sugar lactose to form the Amadori product (**Scheme 1**), which subsequently undergoes series of further reactions leading to a great variety of chemical structures (*2*). As a result, lysine residues are blocked and no longer available for digestion, whereby the nutritional value can be severely decreased. In parallel, proteins are subjected to various oxidation reactions, leading to the degradation of essential amino acids such as lysine and tryptophan. Furthermore, cross-linking reactions in the advanced stage of the Maillard reaction additionally reduce protein digestibility by inhibiting the enzymatic proteolysis, implicating a general loss of bioavailability of amino acids (*1*).

Different attempts have been made to clarify the fate of milk proteins during thermal treatment. With model solutions or milk samples, the glycation of different milk proteins has been successfully analyzed by means of high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (*3*−*5*) or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (*6*, *7*). The combination with enzymatic digest prior to mass spectrometric analysis has allowed the identification of different modification

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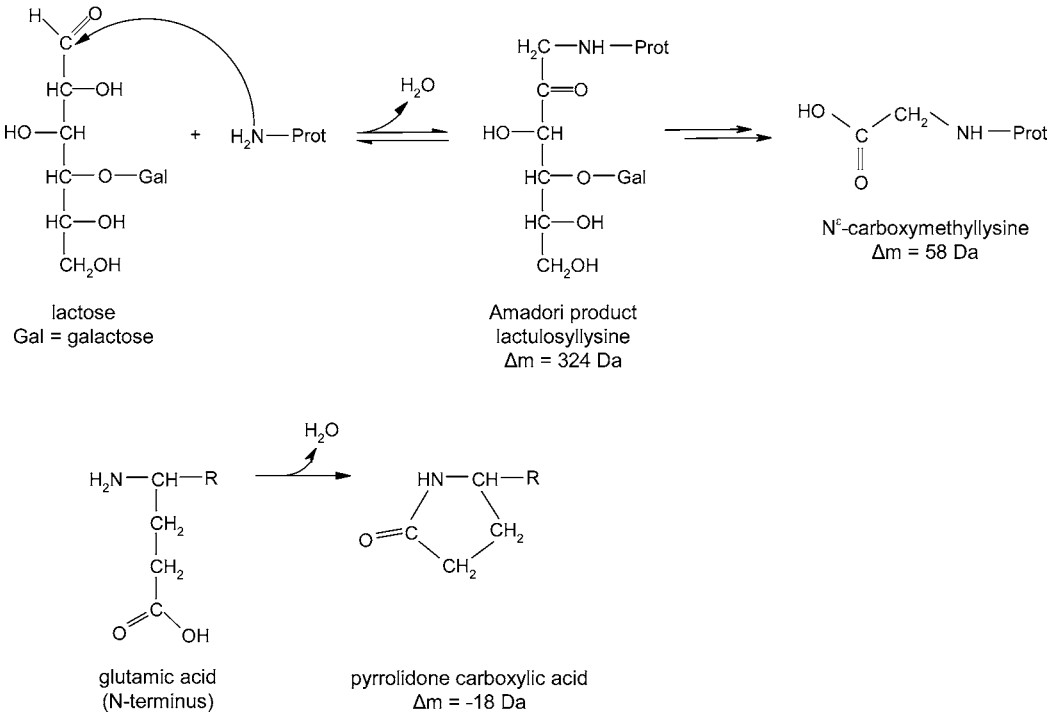
Scheme 1. Reactions Occurring during the Heating of Whey Proteins with Lactose



sites of the proteins (*8*−*12*). With this method, also site-specific relative quantification of the glycation products was achieved (*13*). The studies on milk proteins, however, concentrated on the detection of the Amadori product from the early Maillard reaction, whereas further glycation products or oxidized species have mainly been investigated by conventional techniques (*14*−*16*).

In this study, we used MALDI-TOF-MS to analyze modifications in model solutions of whey proteins simulating conditions in milk in the course of thermal treatment. This technique, coupled with an enzymatic digest before mass spectrometric measurement, allowed the detection of various glycation and oxidation products as well as the identification of modification sites in the protein structure.

MATERIALS AND METHODS

Materials. Bovine α-lactalbumin and bovine β-lactoglobulin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Endoproteinase AspN of sequencing grade and modified trypsin of sequencing grade were obtained from Roche (Mannheim, Germany). 2,5-Dihydroxyacetophenone (DHAP) was purchased from Bruker (Bremen, Germany). α-Cyanocinnamic acid, diammonium citrate, and dithiothreitol were obtained from Fluka (Taufkirchen, Germany). Ammonium dihydrogen phosphate was from Acros (Geel, Belgium). All chemicals were of the highest purity available.

Heating of Whey Proteins in a Milk Model. α-Lactalbumin (19.5 mg, 92 μM, equivalent to 1.1 mM lysine residues) and 740 mg of lactose (144 mM) were dissolved in 15 mL of milk-resembling phosphate-buffered saline (10 mM sodium phosphate with 8 mM NaCl at pH 6.8). Alternatively, 24 mg of β-lactoglobulin (175 μM, equivalent to 2.8 mM lysine residues) was dissolved with 370 mg of lactose (144 mM) in 7.5 mL of the milk resembling buffer. The solutions were heated in a shaking water bath at a temperature of 60 °C, and after 3, 7, and 14 days, samples were taken, cooled in ice, and dialyzed against doubly distilled water (molecular weight cutoff of 8000−10000 Da) followed by lyophilization. Furthermore, protein solutions without sugar were heated at 60 °C for 14 days in the same buffer (heated control). All experiments were performed in duplicate.

Oxidation of Whey Proteins. For the oxidation experiment, solutions of 19.5 mg of α-lactalbumin (92 μM, equivalent to 1.1 mM lysine residues) in 15 mL and 24 mg of β-lactoglobulin (175 μM, equivalent to 2.8 mM lysine residues) in 7.5 mL of the milk-resembling buffer were heated with 8.4 mM H_2O_2 in an oven at 120 °C for 10, 20, and 30 min. After the heating procedure, the solutions were treated as described before. As a control, protein solutions without H_2O_2 were heated at 120 °C for 30 min (heated control). All experiments were performed in duplicate.

Partial Enzymatic Protein Hydrolysis. Glycated or oxidized proteins (2 mg) were dissolved in 500 μL of 25 mM ammonium bicarbonate buffer (pH 8.0). An aliquot of 5 μL of the resulting solutions (corresponding to 20 μg of protein) and 5 μL (0.2 μg) of endoproteinase AspN (for α-lactalbumin) or 2.5 μL (0.1 μg) of AspN and 2.5 μL of water (for β-lactoglobulin) were incubated at 37 °C for 18 h. Trypsin digestion was carried out with 5 μL of the solution of oxidized α-lactalbumin (20 μg) and 2 μL of trypsin (0.2 μg), which were incubated at 37 °C for 15 h. Disulfide bonds in the proteins did not interfere with the enzymatic hydrolysis. The resulting peptides were reduced by adding 1 μL of 100 mM dithiothreitol and left for 30 min at room temperature.

MALDI-TOF-MS. For MALDI-TOF-MS analysis of the intact proteins, 5 μL of the protein solutions was reduced by the addition of 1 μL of 100 mM dithiothreitol. After 30 min at room temperature, 2 μL of the reduced sample was diluted with 2 μL of a DHAP matrix. For the DHAP matrix, a saturated solution of 2,5-dihydroxyacetophenone in 50% acetonitrile/0.1% trifluoroacetic acid was mixed 4:1 with 10 μM diammonium hydrogen citrate in 50% acetonitrile/0.1% trifluoroacetic acid. An aliquot of 1 μL of the dilution was spotted twice onto a stainless steel target and subsequently air-dried. For the measurement of the peptides, 1 μL of the reduced digest was diluted with 14 μL of a matrix consisting of a 1:1 mixture of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid and a solution of 10 mM ammonium dihydrogen phosphate in 50% acetonitrile/0.1% trifluoroacetic acid. An aliquot of 1 μL of the dilution was spotted twice onto a stainless steel target and air-dried. The MALDI-TOF-MS analysis was performed on a Bruker Autoflex (Bruker Daltonik, Bremen, Germany), equipped with a nitrogen laser (λ = 337 nm). Measurements of intact and digested proteins were carried out using 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-lactalbumin, chicken lysozyme,

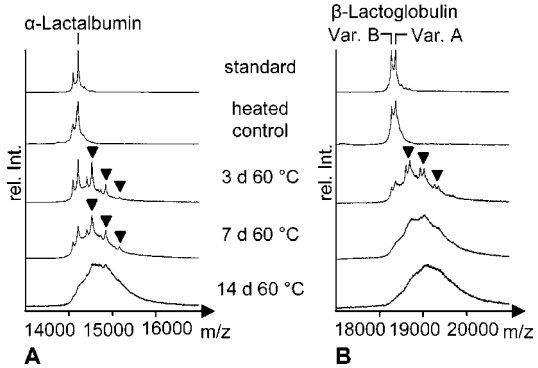


Figure 1. MALDI-TOF-MS spectra of glycated α-lactalbumin (**A**) and β-lactoglobulin (**B**). Model solutions of α-lactalbumin or β-lactoglobulin in a milk-resembling buffer at pH 6.8 were heated with lactose at 60 °C for 3, 7, and 14 days. The spectra of the commercial standard and of the proteins heated for 14 days without sugar used as control are shown. Amadori products (mass shift of 324 Da) are labeled with ▼.

and bovine β-lactoglobulin 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, at least 150 individual spectra obtained from several positions on a spot were averaged.

Database Search. The assignment of the observed signals to the corresponding amino acid sequences was carried out by means of the protein databases Swiss-Prot/TrEMBL and the software Peptide Mass, available at www.expasy.org (version 25/01/2007). Parameters for the search were the following: Monoisotopic peptide masses were indicated as $[M + H]^+$ with cysteines treated with nothing. As an enzyme, AspN and AspN + N-terminal Glu was chosen; for the analysis of oxidized α-lactalbumin, trypsin was selected. One missed cleavage was permitted. Peptides with a mass larger than 750 Da were displayed.

RESULTS

Analysis of Intact Whey Proteins from a Heated Milk Model. Model solutions of whey proteins and lactose with a composition similar to that of milk were heated to monitor protein modifications. Although higher temperatures are usually applied for industrial milk manufacturing, a temperature of 60 °C was chosen for these experiments to prevent thermal denaturation of the whey proteins. After 3, 7, and 14 days, samples were taken and analyzed after dialysis and lyophilization by MALDI-TOF-MS. The nontreated commercial standard (standard) as well as the standard heated without the addition of lactose (heated control) served as control.

Figure 1 depicts the spectra obtained from intact glycated α-lactalbumin (panel **A**) and β-lactoglobulin (panel **B**). The spectrum of the standard of α-lactalbumin showed two peaks. The predominant signal could be identified as α-lactalbumin. Because of the mass difference, the second signal was assumed to be the truncated form of α-lactalbumin, of which the C-terminal amino acid leucine is cleaved during the purification process. This hypothesis was supported by the appearance of a corresponding satellite peak of the C-terminal peptide after enzymatic hydrolysis (see below). The additional signal, however, did not interfere with the following analysis. After 3 days of incubation of α-lactalbumin with lactose, three additional signals were clearly visible, which were absent in the heated control. Each of the new signals was shifted by 324 Da to higher masses, corresponding to a multiple modification of the native protein with lactose. A further zoom of the spectra showed a fourth Amadori product (data not shown). Other signals seen in the spectra were due to analogous lactosylation of the truncated form of the protein. The incubation for 14 days led to a broad signal over the range of approximately *m*/*z* 14000−16000, indicating severe modifications forming a heterogeneous product spectrum that could no longer be resolved into individual signals. The analogous experiment with β-lactoglobulin, for which the two main variants A and B were discriminated with a mass difference of 86 kDa, showed similar results.

To get a more detailed insight into the structures and site of the modifications, a partial enzymatic hydrolysis of the modified proteins was performed prior to mass spectrometric analysis.

Analysis of α-Lactalbumin after Partial Enzymatic Hydrolysis. In the next step, the native and the incubated whey proteins were enzymatically digested using endoproteinase AspN. In 25 mM ammonium bicarbonate buffer (pH 8.0), AspN specifically cleaves peptide bonds N-terminally to aspartic acid and, with a much lower affinity, N-terminally to glutamic acid. In previous studies, the endoproteinase trypsin, which cleaves C-terminally to lysine and arginine, has been successfully used for the enzymatic hydrolysis of glycated whey proteins (*4*, *10*). However, in our approach, the enzyme AspN turned out to be more suitable because of the low rate of unspecific cleavages as well as the yield of clearly arranged signals of the peptide digest, which allowed an assignment of the resulting signals to the corresponding amino acid sequences by means of the software "peptide mass" (available on www.expasy.org). In more complex protein mixtures, such as milk, peptide fragmentation by collision-induced dissociation or electron-transfer dissociation would be required for definite peptide identification. Furthermore, hydrolysis by AspN was evidently not affected by glycation as it is known for trypsin (*4*, *17*). Sequence coverage after AspN digest was 70% of the glycated or oxidized α-lactalbumin and 94% (100%) of the glycated (oxidized) β-lactoglobulin.

The mass range from *m*/*z* 800 to 7000 was monitored, but only signals below *m*/*z* 4000 were detected. Heating of α-lactalbumin with lactose led to a modification of the peptide AA 1−13 (*m*/*z* 1623) with a mass difference of −18 Da. This modification was obviously not a Maillard product, because it was also formed when the protein was heated in the absence of lactose. The free amino group of a glutamic acid located at the N terminus of a protein, however, can easily react with the side-chain carboxyl residue to form a pyrrolidone (**Scheme 1**). This evidently takes place even at mild conditions and, indeed, the signal for the pyrrolidone form was also detected to a small extent in the unheated standard. Throughout the incubation, the signal of the pyrrolidone became almost as intense as the one of the native peptide (data not shown).

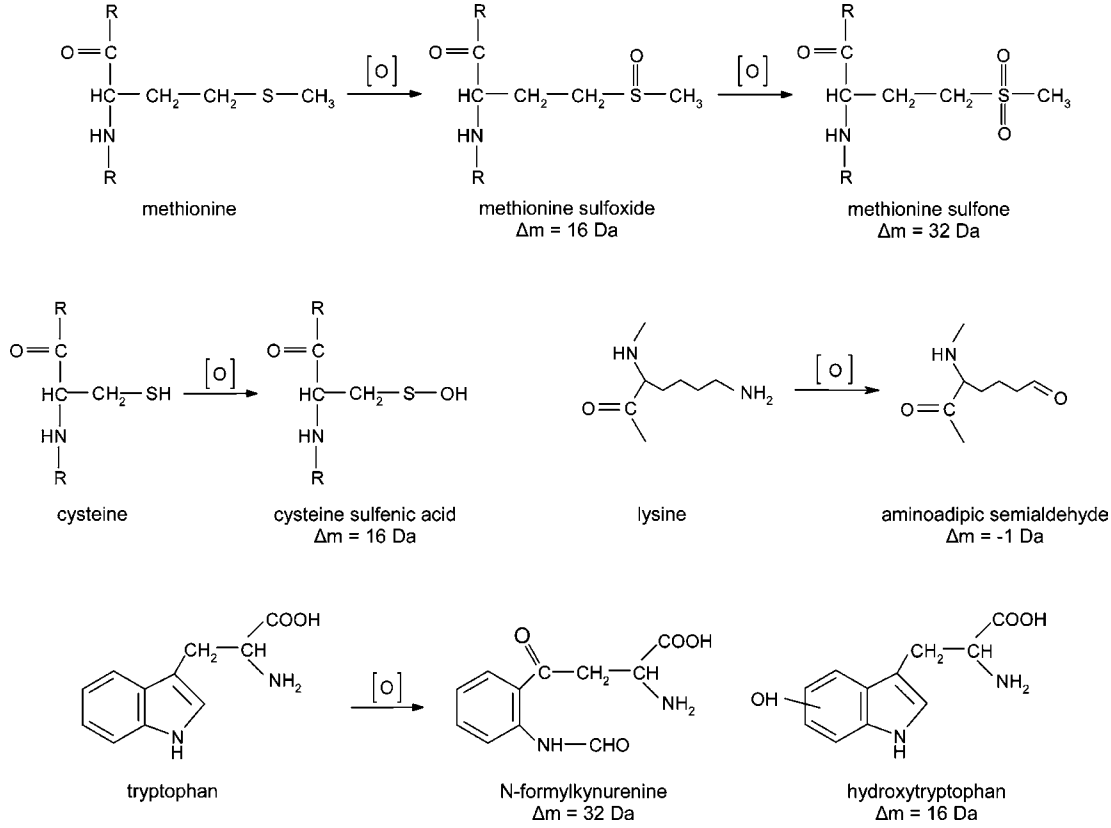
Two new signals with a mass difference of 342 Da from the native peptide AA 1−13 and its cyclic pyrrolidone form, respectively, suggest the formation of an Amadori product of lactose. After prolonged heat treatment, two other signals with a mass difference of 58 Da from the native peptide and the pyrrolidone, respectively, could be detected. It is likely that the Amadori product formed in the initial stage of the Maillard reaction underwent subsequent reactions which implicated the generation of N^{ϵ}-carboxymethyllysine (CML) entailing the observed mass increment (see **Scheme 1**). Exactly the same observations were made at peptide *m*/*z* 1253, a fragment of peptide *m*/*z* 1623 (see **Table 1**), where endoproteinase AspN additionally cleaved before glutamic acid at position 11 (data not shown).

Throughout the incubation with lactose, a new peak with *m*/*z* 1622 became evident, which was observed neither in the standard nor in the heated control to that extent. For a mass difference of 1 Da, the software suggests two possible modifica-

Table 1. Overview of Detected Modifications of α-Lactalbumin and β-Lactoglobulin after Incubation with Lactose and Suggested Interpretations

peptide	position	amino acid sequence	shift	interpretation
		α-Lactalbumin (Partially Hydrolyzed with AspN)		
1034	AA 116–123	DQWLCEKL	16	Cys/Trp oxidation
1253	AA 1–10	EQLTKCEVFR	–1	lysine aldehyde
			–18	pyrrolidone
			58	N^{ε}-carboxymethyllysine
			324	lactulosyllysine
1623	AA 1–13	EQLTKCEVFRELK	–1	lysine aldehyde
			–18	pyrrolidone
			58	N^{ε}-carboxymethyllysine
			324	lactulosyllysine
2188	AA 97–115	DKVGINYWLAHKALCSEKL	16	Cys/Trp oxidation
2517	AA 14–36	DLKGYGGVSLPEWVCTTFHTSGY	16	Cys/Trp oxidation
		β-Lactoglobulin (Partially Hydrolyzed with AspN)		
1104	AA 1–10	LIVTQTMKGL	–1	lysine aldehyde
			16	methionine sulfoxide
1812	AA 11–27	DIQKVAGTWYSLAMAAS	16	methionine sulfoxide
			32	Met/Trp oxidation
2199	AA 33–52	DAQSAPLRVYVEELKPTPEG	58	N^{ε}-carboxymethyllysine
			324	lactulosyllysine
3033	AA 137–162	DKALKALPMHIRLSFNPTQLEEQCHI	16	methionine sulfoxide
			58	N^{ε}-carboxymethyllysine
			324	lactulosyllysine

Scheme 2. Selective Overview of Some Protein Oxidation Products Relevant for This Study



tions. First, an amidation of carboxyl residues leads to a decrease in mass of 0.9840 Da. Second, an oxidation of a lysine residue leading to the formation of lysine aldehyde, also called aminoadipic semialdehyde (**Scheme 2**), results in a mass difference of 1.0316 Da. Because amidation appears to be highly unlikely under the conditions applied in the experiments, the modification was assigned to lysine aldehyde (see also results for β-lactoglobulin and discussion).

As a consequence of the incubation with lactose, new signals with a mass difference of 16 Da, respectively, slightly arose at peptides with *m*/*z* 1034, 2188, and 2517 (for assignment of the native signals to the amino acid sequence see **Table 1**). The mass shift of 16 Da led to the assumption that an oxidative reaction introduced oxygen into the protein structure. All peptides offer cysteine and tryptophan residues, which are known to be easily subjected to oxidation forming various products (**Scheme 2**) (*18*). Therefore, an oxidation of α-lactalbumin in the milk model is postulated to take place at these residues.

Analysis of β-Lactoglobulin after Partial Enzymatic Hydrolysis. Glycation of β-lactoglobulin with lactose and subsequent AspN digest was performed. The resulting spectra in the

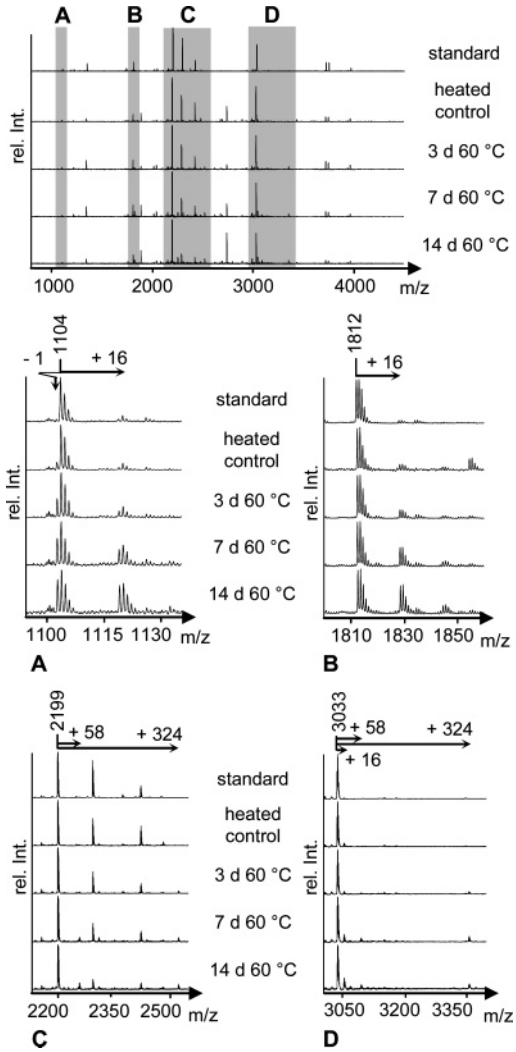


Figure 2. MALDI-TOF-MS spectra of the glycated β-lactoglobulin after digestion with endoproteinase AspN. A solution of β-lactoglobulin in a milk-resembling buffer at pH 6.8 was heated with lactose at 60 °C for 3, 7, and 14 days. After incubation, 20 μg of the dialyzed and lyophilized protein was digested with 0.1 μg of AspN at 37 °C for 18 h. Zooms of the interesting mass ranges are designated by letters **A**–**D**; *m*/*z* values are labeled.

mass range of 800−5000 Da are illustrated in **Figure 2**. Products that were assigned to lactulosyllysine, CML, and oxidation products (mass difference of 16 Da) are summarized in **Table 1**. Similar to the experiments with α-lactalbumin, a signal with a mass difference of −1 Da from the native peptide AA 1−10 at *m*/*z* 1104 was observed. The absence of a carboxylic acid as potential amidation site in this peptide confirms the assignment of the mass difference of −1 Da to the formation of lysine aldehyde. Signals at *m*/*z* 2293 and 2422 were attributed to fragments of peptide 3033, in which AspN additionally cleaved at glutamic acid. Their decrease throughout the incubation may be caused by changes in the protein structure leading to differences in the enzymatic hydrolysis.

Analysis of Oxidized Whey Proteins after Partial Enzymatic Hydrolysis. To confirm our hypothesis that protein oxidation occurs in the model solutions of α-lactalbumin and β-lactoglobulin with lactose, oxidized whey proteins were prepared by heating them at 120 °C in the presence of hydrogen peroxide. The purpose of this experiment was to confirm the presence of oxidation products in proteins incubated with lactose by detecting analogous mass differences in the oxidized proteins. The oxidized whey proteins were analyzed after enzymatic digestion by means of MALDI-TOF-MS.

The spectra of oxidized α-lactalbumin after digestion with endoproteinase AspN showed no significant changes (data not shown). However, the sequence coverage achieved with AspN was merely 70% and did not include the peptide with the only methionine residue in the sequence, which was expected to be the most susceptible toward oxidation. Hence, we used trypsin for enzymatic proteolysis, which allowed the detection of additional sequences. Incubation of α-lactalbumin with hydrogen peroxide led to the total conversion of the peptide AA 80−93 containing methionine (*m*/*z* 1643) into another peptide with a mass difference of 16 Da. This result is consistent with the formation of an oxidation product. Other changes due to the incubation with hydrogen peroxide were not observed.

Oxidation of β-lactoglobulin led to several structural modifications. As in the spectra of the glycated protein, the emergence of new peaks higher in mass by 16 Da was noted for the native peptides *m*/*z* 1104, 1812, and 3033, respectively. Furthermore, the incubation yielded the formation of two peaks higher in mass by 16 Da to the peptides *m*/*z* 3723 and 3751, which were apparently also oxidized (see **Table 2**).

DISCUSSION

It is well-established that industrial heat treatment of milk or milk products induces a variety of desired and undesired chemical reactions of the proteins. The latter include glycation and oxidation reactions leading to the loss of essential amino acids and to a decreased digestibility of the protein, both reducing the nutritional value of dairy products (*1*). This study focused on the structural analysis of modifications of whey proteins during thermal processing in model solutions. For this purpose, solutions of the two main whey proteins α-lactalbumin and β-lactoglobulin were heated with the milk sugar lactose in concentrations found generally in milk. The proteins were then analyzed by MALDI-TOF-MS.

After incubation of α-lactalbumin and β-lactoglobulin with lactose, several new signals appeared in the mass spectra of the intact proteins. A mass difference of multiples of 324 Da indicated the presence of several Amadori products, the first stable product in the Maillard reaction, which is formed through condensation of a lactose molecule with the amino group of a protein (*2*). Lactosylation has been identified as the most common modification of milk proteins (*17*) and has been detected in various dairy products (*6*, *19*−*21*). The resolution of the mass spectra may have been improved by changes in the experimental setup (*22*), but identification of distinct modifications other than the Amadori product could not be expected in the mass spectrum of the intact proteins. To overcome this problem, an enzymatic digest was performed before submitting the samples to the MALDI-TOF-MS analysis. Alternatively, other ion sources, such as electrospray ionization, could be applied to resolve minor modifications in the intact proteins. Detected modifications are summarized in **Table 1**.

In the digested α-lactalbumin only one lactose adduct with a mass increment of 324 Da was detected, which was located in AA 1−10 containing the N-terminal amino group and Lys5. Because the adduct was also detected in the pyrrolidone form of the protein, where the N terminus is blocked, Lys5 could be identified as a preferential glycation site of α-lactalbumin, which is in good agreement with the literature (*17*). An additional lactosylation of the N terminus, which cannot be excluded from the present experiments, has not been described so far. In contrast, site-specific lactosylation of lysine residues 98 and 114

Table 2. Overview of Detected Modifications of α-Lactalbumin and β-Lactoglobulin after Incubation with Hydrogen Peroxide and Suggested Interpretations

peptide	position	amino acid sequence	shift	interpretation
		α-Lactalbumin (Partially Hydrolyzed with Trypsin)		
1643	AA 80–93	FLDDDLTDDIMCVK	16	methionine sulfoxide
		β-Lactoglobulin (Partially Hydrolyzed with AspN)		
1104	AA 1–10	LIVTQTMKGL	16	methionine sulfoxide
1812	AA 11–27	DIQKVAGTWYSLAMAAS	16	methionine sulfoxide
3033	AA 137–162	DKALKALPMHIRLSFNPTQLEEQCHI	16	methionine sulfoxide
3723	AA 98–129	DYKKYLLFCMENSAEPEQSLACQCLVRTPEVD	16	methionine sulfoxide
3751	AA 98–129 (variant A)	DYKKYLLFCMENSAEPEQSLVCQCLVRTPEVD	16	methionine sulfoxide

or 122 has been reported previously (*11*). After digestion with endoproteinase AspN, the peptides containing Lys98 and Lys114 could not be detected by MALDI-TOF-MS, probably because of insufficient ionization properties. However, the peptide with Lys122 resulted in a very intensive signal, but a corresponding signal with a mass shift of 324 Da was not observed. Under the conditions applied in our experiments, therefore, lactosylation at this site was below the detection limit.

In β-lactoglobulin, lactosylation was detected at position 47, which is in good accordance with previous results from Leonil et al. (*10*). Furthermore, lactose adducts either on Lys138 or on Lys141 were observed, which cannot be differentiated under the present conditions. These residues have already been reported to react with lactose at low water activity (*23*). Lysine 100, which has been described as the second reactive lysine residue in β-lactoglobulin (*9*), was not covered in the spectra of the AspN digest. From the results of these studies, it can be concluded that the favored glycation site probably depends on the glycation conditions (solution or dry-state) and/or sample composition (model solutions or milk samples).

In contrast to the spectra of the protein samples hydrolyzed by AspN, where one or two Amadori products were detectable, MALDI-TOF-MS analysis of intact α-lactalbumin and β-lactoglobulin revealed the formation of four Amadori products in each protein. Thus, it can be concluded that the change of ionization properties due to glycation is of lower importance in the intact proteins as reported by Yeboah and Yaylayan (*24*) than in the peptides.

All peptides in which the formation of the Amadori product had been demonstrated showed new signals with a mass difference of 58 Da after prolonged heat treatment. These results indicate that the Amadori product underwent further degradation, which resulted in the generation of CML. CML has already been described as a good marker for the heat treatment of milk (*25*) and has been quantified in dairy products by immunochemical or chromatographic methods (*26*, *27*). Humeny et al. used a mass spectrometric technique for the identification of CML in glycated lysozyme after the digestion with endoproteinase GluC (*28*), but, to our knowledge, this is the first time that site-specific formation of CML was detected in milk proteins.

In addition to glycation reactions, several mass differences corresponding to oxidative modifications were observed during the incubation of whey proteins with lactose. **Scheme 2** gives a selective overview of some relevant structures resulting from oxidation.

Two new signals with mass shifts of −1.0 Da compared to the native peptide were detected in α-lactalbumin and β-lactoglobulin, respectively, and attributed to an oxidation of a lysine residue to aminoadipic semialdehyde. The formation of the aldehyde was unambiguously attributed to Lys5 in α-lactalbumin and Lys8 in β-lactoglobulin. Other lysine-containing peptides within the protein did not show analogous changes during incubation. Highly selective formation of aminoadipic semialdehyde has been demonstrated by Temple et al. for human serum albumin heated under metal-catalyzed conditions (*29*). Interestingly, incubation of α-lactalbumin and β-lactoglobulin with hydrogen peroxide at 120 °C for up to 30 min did not result in similar modifications, indicating that hydrogen peroxide, as a relatively mild oxidant, does not lead to lysine oxidation. In contrast, dicarbonyl compounds, which are formed from sugars during the Maillard reaction, promote the formation of aminoadipic semialdehyde by a Strecker-type degradation (*30*). Similar to previous studies with human serum albumin (*29*), an analogous oxidation of arginine to glutamic semialdehyde was not observed.

Furthermore, in the spectra of both digested glycated α-lactalbumin and β-lactoglobulin, several mass differences of multiples of 16 Da were measured, which indicate the introduction of oxygen. Hau et al. already showed mass shifts of 16 Da in electrospray ionization MS spectra of whey, which they associated with oxidation, but without enzymatic digest, they could not determine the modification site (*19*). In our study, a mass shift of 16 Da was detected for α-lactalbumin at peptides *m*/*z* 1034 (AA 116−123), *m*/*z* 2188 (AA 97−115), and *m*/*z* 2517 (AA 14−36) (see **Table 1**). The only methionine (Met90) in the protein was not covered by this method. To confirm that oxidation took place in our milk models, the incubation was repeated at 120 °C with hydrogen peroxide instead of lactose. However, after digestion with AspN, no difference between the native and the incubated proteins could be detected in the spectra of α-lactalbumin. Enzymatic hydrolysis of the oxidized protein was also repeated with trypsin, which yielded peptide *m*/*z* 1643 (AA 80−93), containing Met90, Cys91, and Phe80 (**Table 2**). An additional peak with a mass difference of 16 was detected for this signal. Methionine is the amino acid most readily oxidized by hydrogen peroxide (*18*), so we postulate that methionine is the only amino acid in α-lactalbumin which is oxidized under our incubation conditions by hydrogen peroxide to such an extent that the modified peptide could be detected.

Additionally to methionine, cysteine and tryptophan are most sensitive toward oxidative reactions (*18*), but post-translational modifications have been reported also for phenylalanine, tyrosine, and histidine, leading to an additional shift of 16 Da or multiples of it (*31*). In the glycated proteins, a mass shift of 16 Da in the peptides *m*/*z* 1034, 2188, and 2517 could result from oxidation of either cysteine or tryptophan, thus affecting Cys28/Trp26, Cys111/Trp104, and Cys120/Trp118 (**Table 1**). Cysteine sulfenic acid is stable in proteins under certain structural circumstances (*32*), but has not been found in milk proteins yet, whereas degradation products of tryptophan have been demonstrated in human milk (*33*). Likewise, oxidation products with a mass increase of 16 Da could be shown in the sequence of glycated β-lactoglobulin at *m*/*z* 1104 (AA 1−10), *m*/*z* 1812

(AA 11−27), and *m*/*z* 3033 (AA 137−162; **Table 1**). Incubation with hydrogen peroxide resulted in the same modifications. Additionally, a mass increase of 16 Da was detected in the peptides 3723 and 3751 (AA 98−129 of variants A and B; **Table 2**). Neither peptide was covered in the glycated β-lactoglobulin. Because all five oxidized peptides contain methionine, the formation of methionine sulfoxide was postulated to take place in the milk models at Met7, Met24, Met145, and possibly also Met107. However, an additional oxidation of other amino acids in these sequences, mainly Cys160, cannot be excluded. Furthermore, a mass increment of 32 Da could be detected at peptide *m*/*z* 1812 (AA 11−27), containing one methionine and one tryptophan. This can be attributed to either a double oxidation of methionine to methioninesulfone, an oxidation of tryptophan to *N*-formylkynurenine, or the formation of methionine sulfoxide and tryptophan oxidation (**Scheme 2**). Further studies are, therefore, required to identify unambiguously the nature of the oxidation products of α-lactalbumin and β-lactoglobulin in the milk models.

The oxidation products were not detected when the proteins were heated in the absence of lactose. The oxidative character of sugar−protein solutions is well-known. Glycation leads to the formation of the Amadori product, which promotes the generation of the superoxide radical ion (*34*). The latter is converted to hydrogen peroxide, which readily reacts with metal ions present in traces to form the hydroxyl radical. This species is highly reactive and attacks residues or the backbone of proteins, leading to grave damage (*35*).

In addition to the glycation and oxidation reactions that were mediated by lactose, modifications were observed that were induced only by the thermal treatment and were thus also present when the proteins were heated in the absence of lactose. In the spectra of α-lactalbumin, the appearance of a signal at 18 Da less than the N-terminal peptide (*m*/*z* 1623) was observed, which we attribute to the formation of a pyrrolidone through attack of the side-chain carboxylic group of glutamic acid by the N-terminal nitrogen (see **Scheme 1**). This reaction occurs nonenzymatically even at mild conditions (*36*), explaining the existence of the modified peptide even in the standard, which can be caused by the formation of the pyrrolidone in the commercial product either during the purification process or in the course of the digestion with AspN. During the heat treatment of the model solution, the corresponding peak increased severely.

Previous studies of milk proteins by MALDI-TOF-MS concentrated on the detection of the Amadori product formed during the first stage of the Maillard reaction. In this work, we attempted to enlarge the potential of this method by showing further modifications allowing a more detailed insight into the processes during thermal treatment of milk proteins. We showed that lactulosyllysine was the main glycation product, which was in part subsequently degraded to CML. This process was accompanied by a multitude of oxidation reactions. Furthermore, cyclization of the N-terminal glutamic acid to give the pyrrolidone was observed. Other signals in the spectra that appeared throughout the incubation experiments, but which could not be attributed to any known post-translational modifications, seem to be highly interesting targets for new studies. As a next step, the technique will be applied to milk samples to identify major modifications that occur during processing.

ABBREVIATIONS USED

MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; DHAP, 2,5-dihydroxyacetophenone; AA, amino acids; CML, N^{ϵ}-carboxymethyllysine.

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