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Identification and Site-Specific Relative Quantification of β-Lactoglobulin Modifications in Heated Milk and Dairy Products

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During milk processing, proteins can be severely modified by oxidation, condensation, and Maillard reaction, leading to changes in their nutritional and technological properties. In this study, major modifications of β-lactoglobulin, formed during the heating and processing of milk, were screened by mass spectrometry. For this purpose, β-lactoglobulin was isolated from the milk samples by gel electrophoresis and analyzed by matrix-assisted laser desorption/ionization mass spectrometry after in-gel digestion with endoproteinase AspN. In heated milk, lactulosyllysine was detected at lysine 47 and 138 or 141 as well as methionine sulfoxide at methionine 7, 24, and 145. All these modifications increased gradually when raw milk was heated for 20, 40, and 60 min at 120 °C. The major modifications were also relatively quantified in dairy products, such as raw, high-temperature, ultra-high-temperature, sterilized, and condensed milk as well as infant formulas. The highest contents of lactulosyllysine at Lys47 were detected in powdered infant formulas, whereas lactulosyllysine at Lys138/141 was predominant in condensed milk samples. Methionine sulfoxide at Met7 and Met24 showed a trend toward higher modification rates in more severely processed products.

KEYWORDS: β-Lactoglobulin; glycation; Maillard reaction; milk; oxidation; matrix-assisted laser desorption/ionization mass spectrometry

INTRODUCTION

Before consumption, milk is usually treated thermally in order to prolong shelf life or to obtain the required technological properties. Heat treatment, however, may also impair the sensory, nutritional, and technological quality of milk. Besides heat-labile vitamins and lactose, mainly the milk proteins are affected by processing. Protein modifications may then result in a decrease in their nutritional value by a major loss of essential amino acids and by a decrease in digestibility (*1*). Furthermore, the consumption of advanced protein modification products may have adverse or beneficial health effects (*2–4*). One of the first changes to milk proteins during processing regards the confirmation and aggregation, particularly of κ-casein (*5, 6*). Additionally, the amino acid side chains of milk proteins are modified during heating, predominantly the reaction of lactose with the ε-amino group of lysine to form the Amadori product lactulosyllysine (*7*). In milk-based infant formulas, for example, between 15% and 20% of lysine is blocked by the Amadori product (*8*). During severe heat treatment, lactulosyllysine can be degraded further and advanced Maillard products are formed. In commercial dairy products, for example, N^{ε}-carboxymethyllysine, oxalic acid monolysinylamide, pentosidine, and galactosyl-β-pyranone were detected (*9–12*). Furthermore, protein oxidation may occur, including formation of methionine sulfoxide or dityrosine (*13, 14*). And finally, the formation of several other protein modifications, such as lysinoalanine or histidinoalanine, in dairy products has been described (*15, 16*). In order to evaluate the nutritional, toxicological, and technological consequences of milk processing, it is crucial to determine systematically if important modifications of heated milk proteins have not been identified so far and which modifications are the most relevant ones. In a recent study, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis after partial enzymatic hydrolysis was applied to screen for whey modifications formed in a milk model (*17*). Thus, lactulosyllysine, N^{ε}-carboxymethyllysine, lysine aldehyde, methionine sulfoxide, cyclization of N-terminal glutamic acid, and oxidation of cysteine or tryptophan were identified as the major heat-induced alterations. The method allowed further determination of the preferred modification site in the protein sequence. In the present work, MALDI-TOF-MS analysis was coupled to electrophoretic

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protein separation and in-gel digestion in order to screen systematically for the major protein modifications in heated milk and dairy products.

MATERIALS AND METHODS

Bovine β-lactoglobulin was obtained from Sigma−Aldrich (Taufkirchen, Germany). Endoproteinase AspN (sequencing grade) was purchased from Roche (Mannheim, Germany). α-Cyano-4-hydroxycinnamic acid and dithiothreitol were obtained from Fluka (Taufkirchen, Germany). Ammonium dihydrogen phosphate was from Acros (Geel, Belgium). All chemicals were of the highest purity available. Bio-Safe Coomassie staining solution was obtained from Bio-Rad (Munich, Germany). C18 Zip Tips were from Millipore (Schwalbach, Germany).

Milk Samples. Raw milk was obtained from a local dairy farm. High-temperature milk (heated at minimum 85 °C until negative peroxidase test), ultra-high-temperature milk (heated at minimum 135 °C in a continuous flow-through process), sterilized milk (sterilized by in-bottle heating at 105 °C or higher), condensed milk (sterilized), and liquid and powered infant formulas were purchased from local supermarkets. The powdered sample was dissolved in water (2 mg in 12 mL). All samples were defatted by centrifugation in a Hettich centrifuge (Tuttlingen, Germany; 15 mL falcon tubes, 3850 rpm) at 1650*g* for 60 min at 4 °C and removal of the upper fat layer.

Heating of β-Lactoglobulin in a Milk Model. β-Lactoglobulin was incubated together with lactose in a milk-resembling system as reported previously (*17*). The commercially available β-lactoglobulin (standard) as well as the protein heated without lactose (heated control) served as controls.

Heating of Raw Milk. Aliquots of 200 μL of raw milk were heated at 120 °C for 10, 20, 30, 40, 50, and 60 min and cooled down on ice. The milk was defatted prior to heating, because it was not possible to remove fat sufficiently for SDS−PAGE from the small volume of the heated sample. The small sample volume was chosen to guarantee fast and homogeneous heating of the milk. Further experiments with commercially available milk products confirmed that an influence of the fat content on product formation is not evident.

SDS−PAGE of Dairy Products. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of the milk proteins of dairy products was performed according to Laemmli (*18*). Samples were diluted 10-fold with sample buffer (except the sterilized milk, which was diluted 1:5) and separated in a 6% stacking and a 15% separating gel (sample load 10 μL). The gel was stained by using a commercially available Coomassie staining solution following the manufacturer's instructions.

Partial Enzymatic Protein Hydrolysis in Solution. Partial enzymatic protein hydrolysis of the model protein in solution by using endoproteinase AspN and subsequent reduction with dithiothreitol were carried out according to Meltretter et al. (*17*).

Partial Enzymatic Protein Hydrolysis in Gel. The protein band of β-lactoglobulin was cut into small pieces (1 mm × 1 mm) and washed twice with 100 μL of water and once with 100 μL of acetonitrile, followed by equilibration with 100 μL of 25 mM ammonium bicarbonate buffer, pH 8.0. For destaining, 100 μL of acetonitrile and 100 μL of 25 mM ammonium bicarbonate buffer, pH 8.0, were added and the gel pieces were incubated for 30 min at room temperature during intensive shaking. After removal of the destaining solution, the gel pieces were incubated twice with acetonitrile and finally dried under the hood for 10 min. Three microliters of endoproteinase AspN solution (corresponding to 0.1 μg of enzyme) was added. Following incubation for 10 min at 4 °C, the gel pieces were covered with 25 mM ammonium bicarbonate buffer, pH 8.0, and incubated at 37 °C for 18 h.

Elution of Peptides from the Gel. After enzymatic hydrolysis, 100 μL of 60% acetonitrile was added to the gel. The mixture was vortexed and allowed to stand for 15 min. The supernatant was transferred to a clean tube. The gel was then incubated for 15 min with 100 μL of 80% acetonitrile/0.1% trifluoroacetic acid (TFA) and the supernatant was added to the first eluate. After incubation with 100 μL acetonitrile, the combined solutions were lyophilized and resolved in 10 μL of TFA (0.1%). In order to reduce the peptides, the solution was incubated for 30 min with 1 μL dithiothreitol (100 mM).

Cleanup of Peptide Solutions. Eluted peptides were cleaned up by C18 Zip Tips. The Zip Tips were wetted twice with 10 μL of acetonitrile and equilibrated with 0.1% TFA. Peptides were bound to the material by aspirating the sample 10 times. Following two washing steps with 100 μL TFA (0.1%) and with 100 μL of 5% methanol/0.1% TFA, peptides were eluted by aspirating 10 μL of 50% acetonitrile/0.1% TFA 10 times.

MALDI-TOF-MS Analysis. For measuring the peptides from in-solution hydrolysis, 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% TFA and a solution of 10 mM ammonium dihydrogen phosphate in 50% acetonitrile/0.1% TFA. An aliquot of 1 μL of the dilution was spotted twice onto a stainless steel target and air-dried. The purified peptides from in-gel hydrolysis were mixed 1:1 with the same matrix and spotted analogously.

The MALDI-TOF-MS analysis was carried out on a Bruker Autoflex (Bruker Daltonik, Bremen, Germany), equipped with a nitrogen laser ($\lambda = 337$ nm). Measurements were carried out by delayed extraction (140 ns). Laser-desorbed positive ions were analyzed after acceleration by 19 kV in the reflector mode. External calibration was performed with a mix of angiotensins I and II, substance P, bombesin, adrenocorticotropic hormone clips 1−7 and 18−39, and somatostatin 28. For each displayed mass spectrum, 300 individual spectra obtained from several positions on a spot were averaged.

For determination of the relative content of a single modification, the monoisotopic area of a specific modification was divided by the sum of the monoisotopic areas of the native and the modified peptide according to Kislinger et al. (*19*): rel content (lactulosyllysine) = $\text{area}_{\text{lactulosyllysine}}/(\text{area}_{\text{lactulosyllysine}} + \text{area}_{\text{methionine sulfoxide}}{}^{*} + \text{area}_{\text{native peptide}}) \times 100$; rel content (methionine sulfoxide) = $\text{area}_{\text{methionine sulfoxide}}/(\text{area}_{\text{methionine sulfoxide}} + \text{area}_{\text{lactulosyllysine}}{}^{*} + \text{area}_{\text{native peptide}}) \times 100$ (*, if present).

Statistical Data Evaluation. Statistical data evaluation was carried out with Microsoft Office Excel 2003. Levels of significance were calculated with a two-tailed, paired Student *t* test.

RESULTS

Comparison of the Analysis of Protein Modifications after In-Solution or In-Gel Digest. Whereas the identification of whey protein modifications in milk model solutions has been reported previously (*17*), the goal of this study was the systematical investigation of β-lactoglobulin modifications in dairy products. For this purpose, β-lactoglobulin was partially hydrolyzed by endoproteinase AspN, which specifically cleaves peptide bonds N-terminal to aspartic acid and, with lower affinity, to glutamic acid, and then the samples were subjected to MALDI-TOF-MS analysis. Whereas single model proteins can be analyzed directly after sample preparation, proteins of milk products must be isolated before enzymatic hydrolysis and MALDI-TOF-MS analysis. Otherwise, the mass spectra would be too complex to identify minor modifications. SDS−PAGE was used as a well-established standard method for protein separation, which allows digestion of the target protein directly in the gel. With this technique, β-lactoglobulin can be isolated well from other milk proteins, so that 2D gel electrophoresis was not necessary. After in-gel digest, interfering substances were removed from the peptides, which had been eluted from the gel. The resulting peptides were then submitted to mass spectrometric analysis. In order to investigate the influence of protein separation and in-gel hydrolysis on the detected protein modification, β-lactoglobulin was heated with lactose in a milk model. MALDI-TOF-MS spectra were recorded after direct hydrolysis in solution as described earlier (*17*) as well as after gel electrophoresis and in-gel digestion.

Table 1. Detection of β-Lactoglobulin Modifications by MALDI-TOF-MS[a]

amino acid	modification	mass shift (Da)	β-lactoglobulin hydrolysis in solution	β-lactoglobulin hydrolysis in gel	heated raw milk
Lys 47	Amadori product lactulosyllysine	+324	×	×	×
Lys 138 or 141	Amadori product lactulosyllysine	+324	×	×	×
Lys 47	N^{ε}-carboxymethyllysine	+58	×	×	
Lys 138 or 141	N^{ε}-carboxymethyllysine	+58	×	×	
Met 7	methionine sulfoxide	+16	×	×	×
Met 24	methionine sulfoxide	+16	×	×	×
Met 145	methionine sulfoxide	+16	×	×	×
Met 24/Trp 19	methionine/tryptophan oxidation	+32	×	×	
Lys 8	lysine aldehyde	−1	×	×	

[a] MALDI-TOF-MS was performed after partial enzymatic hydrolysis with endoproteinase AspN in solution (see also ref *17*) or after SDS−PAGE and in-gel hydrolysis. β-Lactoglobulin either was heated in the presence of lactose or was isolated from heated milk.

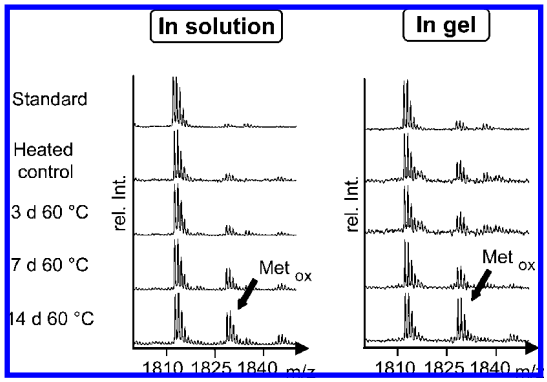

Figure 1. Comparison of MALDI-TOF-MS spectra of β-lactoglobulin, incubated with lactose, after partial enzymatic hydrolysis in solution or in gel. Incubated proteins were either directly digested in solution with endoproteinase AspN (see also ref *17*) or after separation by SDS−PAGE. After elution of the peptides from the gel and cleanup with C18 Zip Tips, peptides were analyzed via MALDI-TOF-MS. A representative mass range between *m*/*z* 1800 and 1860 is shown. The commercially available standard, as well as the protein heated without lactose, were used as controls. Met_{ox}, methionine sulfoxide.

All previously described oxidation and glycation products that could be detected in the model protein after in-solution digest (*17*) could still be detected after in-gel digest (**Table 1**), although a slightly decreased quality of the spectra was observed. **Figure 1** shows the mass spectra of β-lactoglobulin in the range *m*/*z* 1800−1860, resulting from partial enzymatic hydrolysis in solution and in gel. At the depicted peptide with a mass of 1812 Da, incubation with lactose yielded a new signal with a mass difference of 16 Da, corresponding to the oxidation of methionine at position 24 to methionine sulfoxide. Furthermore, formation of lactulosyllysine as well as N^{ε}-carboxymethyllysine at Lys47 and Lys138/141, methionine sulfoxide at Met7 and Met14, methionine/tryptophan oxidation at Met24/Trp19, and lysine aldehyde at Lys8 was detected. For details about the interpretation of the modifications, see Meltretter et al. (*17*). As a result of electrophoresis and enzymatic in-gel hydrolysis, the signal of the methionine sulfoxide increased (**Figure 1**). The oxidation due to the sample workup becomes obvious in the controls, in which methionine sulfoxide is hardly detected after in-solution digest, whereas it is clearly visible in the spectra generated after in-gel digest. Similar observations were made at the other peptides containing methionine residues (data not shown). Oxidation of methionine resulting from heat treatment in the presence of lactose, however, could easily be differentiated from the oxidation during sample workup.

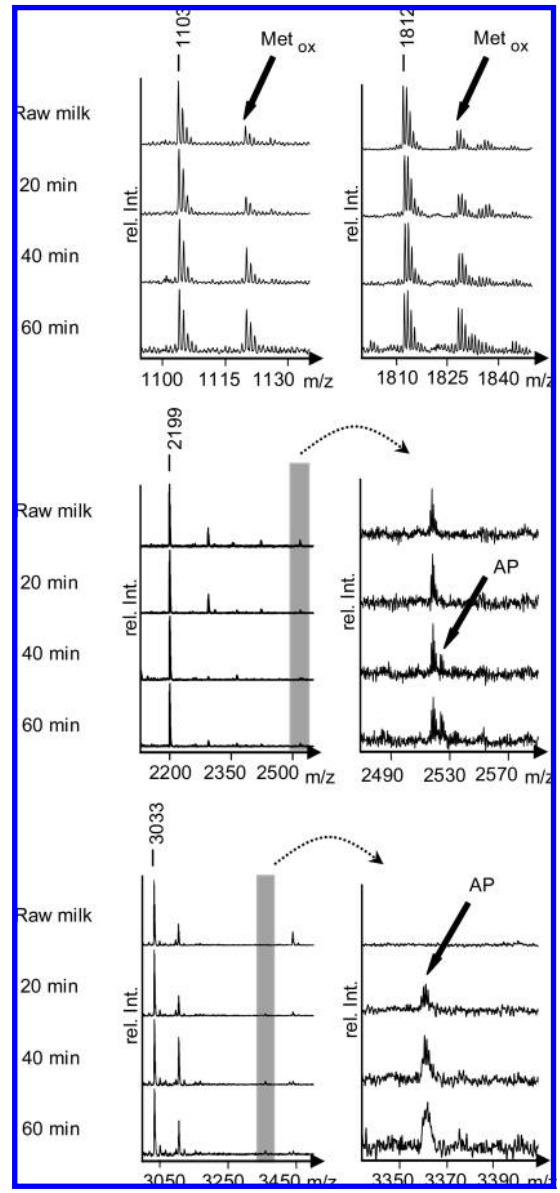

Figure 2. MALDI-TOF-MS spectra of β-lactoglobulin from heated raw milk after partial enzymatic hydrolysis. Raw milk was heated at a temperature of 120 °C for 20, 40, and 60 min. Milk proteins were separated by SDS−PAGE, and the band of β-lactoglobulin was digested with endoproteinase AspN. Eluted peptides from the gel were cleaned up with C18 Zip Tips before MALDI-TOF-MS analysis. Raw milk served as control. *m*/*z* values and detected modifications are indicated. Met_{ox}, methionine sulfoxide; AP, Amadori product.

Modifications of Heated Raw Milk. In the next step, raw milk was heated at 120 °C for 20, 40, and 60 min to monitor the formation of β-lactoglobulin modifications in milk dependent on the heating duration. **Figure 2** shows the spectra of β-lactoglobulin from the milk samples; detected modifications are summarized in **Table 1**.

The Amadori product with a mass shift of 324 Da was observed at lysine 47 and 138 or 141 as well as methionine sulfoxide with a mass shift of 16 Da at methionine 7, 24, and 145. In contrast to the model proteins, the degradation of lactulosyllysine to N^{ε}-carboxymethyllysine could not be detected, nor was a signal visible corresponding to lysine aldehyde,

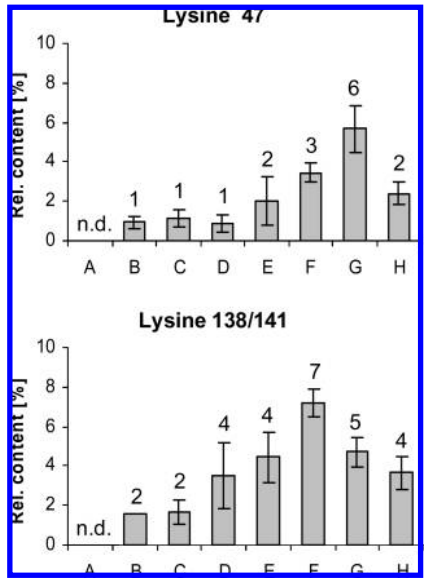

Figure 3. Relative quantification of lactulosyllysine in β-lactoglobulin from dairy products. For calculation of the relative content, the monoisotopic area of lactulosyllysine was divided by the sum of the monoisotopic areas of the native and modified peptides. The mean values ± standard deviations are shown. Samples were the following (with the number of replications given in parentheses): A, raw milk (10); B, high-temperature milk (2); C, ultra-high-temperature milk I (7); D, ultra-high-temperature milk II (3); E, sterilized milk (4); F, condensed milk (7); G, powdered infant formula (7); H, liquid infant formula (6). nd, not detectable.

methionine sulfone, or hydroxytryptophan. Obviously, the concentrations of these modifications were below the detection limit of the method.

Modifications of Commercially Available Dairy Products. To determine the major modifications of β-lactoglobulin formed during industrial heat treatment, several dairy products, including high-temperature, ultra-high-temperature, sterilized, and condensed milk and powdered as well as liquid infant formulas were analyzed in the same way as the heated raw milk. The Amadori product at lysine 47 and 138/141 and methionine sulfoxide at methionine 7, 24, and 145 were detected, confirming the results obtained for heated raw milk or β-lactoglobulin heated in the milk model. Obviously, neither the environment during the thermal treatment (milk model, real milk samples) nor the differences in the temperature control (varying heating processes in the milk model and the dairy samples) influenced the glycation or oxidation sites of β-lactoglobulin.

For gathering semiquantitative information about the modification rates, the relative content of each specific modification was calculated by dividing the area of the modified peptide by the sum of the areas of the native and the modified peptides (*19*). **Figure 3** depicts the relative content of Amadori products. At lysine 47, values between 0.9% ± 0.3% and 5.7% ± 1.2% were obtained, whereas at lysine 138/141, relative contents between 1.7% ± 0.6% and 7.2% ± 0.7% were observed. The highest contents of the Amadori product at lysine 47 were detected in powdered infant formulas, whereas the Amadori product at lysine 138/141 predominated in condensed milk.

Regarding methionine oxidation, a tendency toward increased mean values was observed at methionine residues 7 and 24 in products with more drastic heat treatment: Whereas in raw milk relative contents of 22% and 27%, respectively, were measured, the percentage of methionine sulfoxide reached values of 30% and 41% in liquid infant formula (**Figure 4**). The differences from raw milk, however, were not significant, which can probably be explained by additional protein oxidation during sample workup, leading to a higher variation of the results. Oxidation of Met145 did not show major differences between raw milk and the various dairy products.

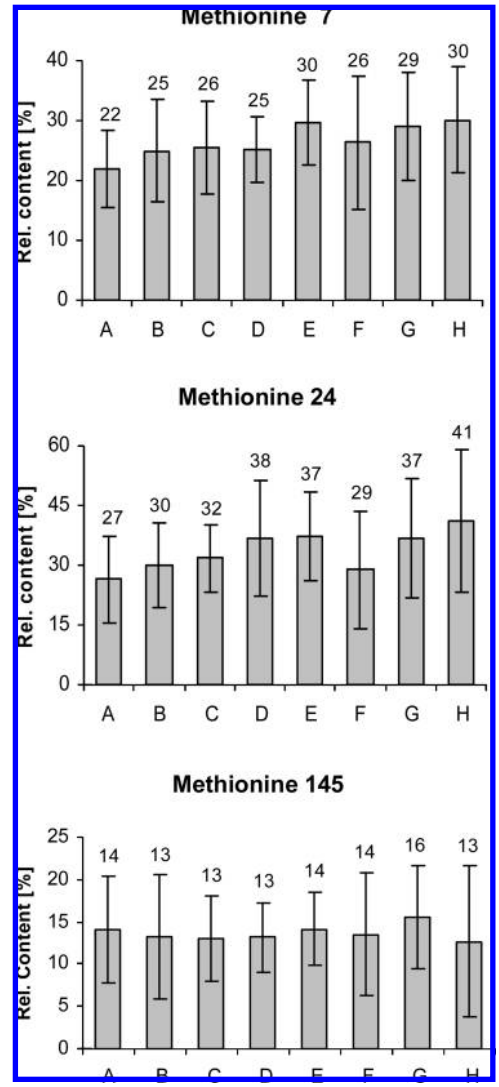

Figure 4. Relative quantification of methionine sulfoxide of β-lactoglobulin in dairy products. For calculation of the relative content, the monoisotopic area of methionine sulfoxide was divided by the sum of the monoisotopic areas of the native and modified peptides. The mean values of three independent measurements ± standard deviation are shown. Samples were the following: A, raw milk; B, high-temperature milk; C, ultra-high-temperature milk I; D, ultra-high-temperature milk II; E, sterilized milk; F, condensed milk; G, powdered infant formula; H, liquid infant formula.

DISCUSSION

Mass spectrometry is a very effective method to screen for protein glycation products. When soft ionization techniques are used, such as MALDI or electrospray ionization (ESI), milk proteins can be analyzed directly (*20*). Thus, in heated milk models or milk, formation of the Amadori product from whey proteins and caseins has been observed (*16, 17, 21–29*). When tryptic digestion was performed prior to mass analysis, binding sites of the Amadori products could be determined additionally in lactosylated whey proteins (*22, 30, 31*).

Trypsin cleaves proteins preferentially at lysine and arginine residues. Therefore, modification of lysine residues by the Maillard reaction usually leads to a loss of a cleavage site, so

that Amadori products of a peptide with higher molecular weight are observed (*32*). In contrast, the cleavage sites of AspN and GluC (aspartate and glutamate) are not affected by the Maillard reaction, so that the modified peptide appears as a satellite of the unmodified parent peptide. As a result, peak assignment is clearly facilitated, particularly when MALDI-TOF-MS is applied (*33*). Consequently, MALDI-TOF-MS analysis after GluC treatment allowed the identification of other protein modifications in addition to the Amadori product in glycated model proteins (*34, 35*). Thus, the formation of lactulosyllysine, N^{ϵ}-carboxymethyllysine, lysine aldehyde, and methionine sulfoxide, the cyclization of N-terminal glutamic acid, and the oxidation of cysteine or tryptophan were observed when whey proteins were heated in a milk model (*17*).

The purpose of this study was to establish a method that allows identification of major protein modifications which occur during heat treatment of milk. In order to verify whether the modifications that occurred in the milk model are also present in heat-treated milk samples, protein separation was necessary prior to mass analysis.

For this purpose, milk proteins were separated in previous studies by HPLC, allowing the identification of lactosylation sites of caseins or whey proteins from heated milk samples (*27–29*). Alternatively, 2D gel electrophoresis was applied in combination with mass spectrometry before and after trypsin digestion in order to characterize changes of proteins in milk powder (*36, 37*).

The main focus of the present study was on modifications of the whey protein β-lactoglobulin, which can be clearly separated from other milk proteins by 1D gel electrophoresis, so that the application of 2D gel electrophoresis was not necessary. The advantage of the electrophoretic separation is that protein digestion can be performed directly in the gel. However, electrophoresis may also lead to a decrease in mass spectra quality, due to artificial protein modifications, such as acrylamide and Coomassie adducts or methionine oxidation (*36*). Furthermore, signal intensity may be reduced due to higher salt concentrations or incomplete recovery of the peptides during sample preparation.

Therefore, β-lactoglobulin was first lactosylated and then digested once directly in solution and once in gel after SDS−PAGE. It was shown that the protein separation procedure reduced spectra quality, but all modifications, which were identified after digestion in solution, were also detectable after SDS−PAGE and in-gel digest. However, additional oxidation of methionine during the electrophoresis could not be avoided (*38*), but artificial formation of methione sulfoxide was low compared to the oxidation during the Maillard reaction. In some samples, acrylamide adducts were detected additionally. Overall, SDS−PAGE and in-gel digest was determined as an appropriate method for the screening of protein modifications in heated milk.

Therefore, raw milk was heated under sterilization conditions for several time periods and β-lactoglobulin was isolated by SDS−PAGE. MALDI-TOF-MS was recorded after in-gel digestion of the protein with AspN. As expected, lactulosyllysine was detected as the major protein modification in β-lactoglobulin from heated milk. Despite some background oxidation during workup, formation of methionine sulfoxide was clearly detectable as the second major protein modification occurring during heating of milk. These results indicate that the promotion of protein oxidation during the Maillard reaction, which has been observed previously in a milk model (*17*), is also of importance in real milk. Very recently, methionine sulfoxide levels up to 74% of total methionine were determined in milk products by HPLC (*13*).

Minor modifications, which were identified in the model solutions, were not detectable in the heated milk samples. Obviously, the sensitivity of the method was not sufficient to detect low-abundance adducts or the formation of these products is not favored in milk. Improvement of sensitivity may be achieved by the application of other mass spectrometric methods, such as ESI-MS.

Furthermore, it was shown that all modification sites in the amino acid sequence detected in the heated milk were also identified previously as main modification sites in the milk model (*17*). These results indicate that the heating conditions of the milk model were appropriate to predict changes in heated milk. Formation of the Amadori product at lysine 138/141 is in accordance with a study in which both lactosylation sites were detected in infant formulas by nano-ESI-MS/MS (*37*). On the other hand, formation of lactulosyllysine at lysine 47 has been reported for β-lactoglobulin, which had been reacted with lactose (*30, 31*). Modification sites for methionine sulfoxide have not been reported before.

The study was further expanded to commercially available dairy products, for which the results obtained for heated raw milk could be confirmed. Furthermore, the formation rate of the specified modifications in different dairy products was relatively quantified by a method of Kislinger et al. (*19*). With this approach, a comparison of the modification states of specific products at specific sites between the different dairy products is possible. However, it should be taken into account that the obtained values cannot be considered as absolute conversion rates, since the modifications might influence the ionization properties of the peptides and thus lead to a different detector response compared to native peptides.

For both lactulosyllysine residues, at lysine 47 and at lysine 138/141, significant differences between the product groups were observed dependent on the processing conditions. Interestingly, both modification sites showed differences in the distribution of lactulosyllysine among the product groups. Whereas the highest formation rate for lactulosyllysine at lysine 47 was detected in powdered infant formula, the highest formation rate for lactulosyllysine at lysine 138/141 was present in condensed milk. The processing conditions obviously determine not only the nature of the protein modifications but also the modification sites. This fact may explain why several studies, in which lactosylation sites in model solutions or heated milk were determined, do not show strict coherence.

For methionine sulfoxide at methionine 7 and 24, a trend toward higher modification rates in more severely processed products was visible. For position 7, for example, a 36% mean increase from raw milk to sterilized milk and liquid infant formula, and for position 24, a 52% mean increase from raw milk to liquid infant formula, were measured. Due to the relatively high standard deviation, however, the differences were not significant. In order to improve these measurements, minimization of background oxidation during sample preparation is required. In contrast, similar tendencies were not observed for methionine oxidation at position 145, which may indicate that oxidation during milk processing is also site-specific. However, further analyses with improved methodology are necessary to confirm these assumptions. The oxidation of methionine in dairy products may be caused by residual hydrogen peroxide, used for the sterilization of milk containers,

or by lipid oxidation (*39*). Furthermore, it was shown that the reaction with lactose also strongly promotes protein oxidation (*17*).

In conclusion, this study indicates that protein separation by gel electrophoresis combined with MALDI-TOF-MS peptide mapping allows monitoring of the modification states of defined sites and proteins in a complex food matrix like milk. As a consequence, very detailed and differentiated information on protein damage occurring during milk processing becomes available. Further studies are now required to increase the sensitivity of the method and to apply it to other milk proteins.

ABBREVIATIONS USED

MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; ESI, electrospray ionization.

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