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Comprehensive Analysis of Nonenzymatic Post-Translational β-Lactoglobulin Modifications in Processed Milk by Ultrahigh-Performance Liquid Chromatography−Tandem Mass Spectrometry

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Supporting Information

ABSTRACT: Nonenzymatic post-translational protein modifications (nePTMs) result in changes of the protein structure that may severely influence physiological and technological protein functions. In the present study, ultrahigh-performance liquid chromatography−electrospray ionization tandem mass spectrometry (UHPLC−ESI-MS/MS) was applied for the systematic identification and site-specific analysis of nePTMs of β-lactoglobulin in processed milk. For this purpose, β-lactoglobulin, which had been heated with lactose under conditions to force nePTM formation (7 d/60 °C), was screened for predicted modifications by using full scans and enhanced resolution scan experiments combined with enhanced product ion scans. Thus, the main glycation, glycoxidation, oxidation, and deamidation products of lysine, arginine, methionine, cysteine, tryptophan, and asparagine, as well as the N-terminus, were identified. Using these MS data, a very sensitive scheduled multiple reaction monitoring method suitable for the analysis of milk products was developed. Consequently, 14 different PTM structures on 25 binding sites of β-lactoglobulin were detected in different milk products.

KEYWORDS: *protein modification, β-lactoglobulin, glycation, oxidation, mass spectrometry, processed milk*

INTRODUCTION

The relevance of nonenzymatic post-translational protein modifications (nePTMs) in biochemical, clinical, and food technology processes is widely recognized. nePTMs change the structure of amino acid side chains and thus modulate biological and technological protein function.[1,2] Although the modification levels are often low, functional effects can be severe when functional domains are affected.[3] nePTMs are mainly caused by oxidation, glycation, or glycoxidation of amino acid side chains.[4−6] Depending on the reaction conditions, however, other mechanisms such as condensation, elimination, deamidation, or nitration also lead to post-translational modifications of amino acids.[7−9]

It is well-established that food processing can induce extensive nePTM formation. Milk and dairy products are particularly affected by protein modification because of their composition and the necessary thermal treatment to extend shelf life.[10] A comprehensive study of the structures, quantities, and binding sites of nePTMs in dairy products is not only important for the full evaluation of technological and nutritional but also of toxicological effects resulting from thermal treatment. However, this task is complicated by the low modification grade and the complexity of possible structures. Targeted analysis of several marker products, particularly of the glycation product lactulosyllysine, the glycoxidation product N^{ϵ}-carboxymethyllysine (CML), or oxidation products such as methionine sulfoxide, has been widely conducted for milk products.[11,12] Markers for the evaluation of heat treatment in different dairy samples include furosine (which reflects the formation of Amadori products) and lysinoalanine.[13,14] Furthermore, proteomic tools have been used to determine the protein targets in milk, which are mainly affected by the major glycation, glycoxidation, and oxidation products.[15,16] Site-specific analysis of the modification in the amino acid side chain of a distinct protein has been attempted. With the aim to search for novel modifications, untargeted matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was applied to a model protein, and major modifications of whey proteins could be detected.[8] However, the sensitivity of the method was not sufficient to analyze minor modifications in complex samples such as dairy products.[4] Another systematic untargeted approach for the identification of nePTMs was performed by multistep ultrahigh-performance liquid chromatography−tandem mass spectrometry (UHPLC−MS/MS) analysis of a model peptide covering the most reactive amino acids.[17] Since this study used a model peptide as glycation target and since the reaction conditions differed from those used for milk processing, it must still be confirmed if the products, which had been identified by this approach, are also present in heated milk.

The goal of the present study was, therefore, to develop a method for the systematic identification and site-specific analysis of multiple protein modifications in β-lactoglobulin from heated milk, which had been predicted by the untargeted analysis of models. For this purpose, a UHPLC−electrospray ionization (ESI)-MS/MS protocol was established by using a hybrid mass spectrometer that combined a linear ion trap for the detection of modifications and bindings sites in the model protein with a triple quadrupole for sensitive detection of these

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protein modifications in milk products. Thus, 14 different PTM structures on 25 binding sites of β-lactoglobulin were detected in different commercial milk products.

MATERIALS AND METHODS

Materials. Formic acid (eluent additive for LC−MS), acetonitrile (LC−MS Chromasolv), β-lactoglobulin variant A/B (from bovine milk, ≥85%), lactose, disodium phosphate (purum p.a.), and monosodium phosphate (purum p.a.) were purchased from Sigma (Taufkirchen, Germany). 1,4-Dithiothreitol (DTT) was obtained from Roth (Karlsruhe, Germany) and endoproteinase GluC from Roche (Mannheim, Germany). All water used was ultrapure water (18.2 MΩ cm resistivity).

Model Protein. To force heat- and lactose-induced modifications of β-lactoglobulin, a model protein was prepared as described previously.[8] Briefly, β-lactoglobulin (3.2 g/L) was heated with lactose (49 g/L) in phosphate-buffered saline (10 mM sodium phosphate, 8 mM sodium chloride, pH 6.8) for 7 d in a shaking water bath at 60 °C. Subsequently, the protein was dialyzed twice for 8 h against doubly distilled water (molecular weight cutoff 8000−10 000 Da, volume ratio 1:200) to remove salts and residual lactose, followed by lyophylization.

Milk Samples. Raw milk was provided by a local farmer. Commercial pasteurized, ultrahigh-temperature (UHT) treated, and sterilized milk samples were obtained from a German producer. The milk samples were defatted by centrifugation in a Hettich Universal 16/32 R centrifuge for 60 min at 1650*g* and 4 °C. The fat layer was carefully removed with a spatula. The defatted milk was stored at −80 °C prior to analysis.

Partial Enzymatic Hydrolysis. Aliquots of 25 μL of milk samples or of a solution of the model protein (400 μM) were transferred to Eppendorf tubes and partially hydrolyzed by addition of 75 μL of endoproteinase GluC (0.08 μg/μL) in 25 mM phosphate buffer pH 7.8. The tubes were kept in an Eppendorf thermo mixer for 16 h at 37 °C. Disulfide bonds in hydrolyzed proteins were reduced by adding 10 μL of 1 M DTT in order to avoid the presence of bridged peptides. After 30 min of incubation at room temperature, 890 μL of water was added and the samples were filtered through a PVDF filter (0.22 μm, Roth, Karlsruhe, Germany).

LC−MS/MS. LC−MS/MS experiments were carried out on a Dionex Ultimate 3000 UHPLC system, coupled to an AB Sciex QTrap 4000, equipped with an ESI source. Hydrolyzed proteins were separated on a C18 column (Waters Acquity UPLC BEH 300; 2.1 × 100 mm, 1.7 μm) at a flow rate of 0.3 mL/min, using the following gradient: 0 min 5% B, 5 min 5% B, 55 min 50% B, 55.5 min 95% B, 60 min 95% B, with 0.1% formic acid as eluent A and acetonitrile as eluent B. The column oven was set at a temperature of 30 °C. The injection volume was 10 μL. Before each injection, the system was equilibrated for 6 min using a mixture of 95% A and 5% B. The LC flow was directed into the mass spectrometer from minutes 1−55. All MS experiments were carried out in the positive mode. Source parameters were as follows: curtain gas, 30 psig; ion spray voltage, 5500 V; nebulizer gas, 60 psig; heating gas, 75 psig; heating gas temperature, 500 °C. Further QTrap specific parameters can be found in the Supporting Information. The QTrap 4000 allowed for a mass accuracy of 0.1 Da or better for singly charged ions in the range of 100−1500 Da, depending on the time gap between the last calibration of the instrument and the data analysis. Peak widths at half-height ranged from <0.25 Da in the enhanced resolution (ER) mode to <0.5 Da in the enhanced mass spectrum (EMS) mode.

For the development of the multiple reaction monitoring (MRM) method, the acquired enhanced product ion (EPI) spectra were searched for the presence of the expected b- and y-ions, calculated by the protein software tool BioAnalyst version 1.5 (AB Sciex). Identifier and verifier ions were selected for each modified peptide. In general, the most intense signal in the spectrum specific for the nePTM peptide was chosen as identifier (see Supporting Information, Table S1). Two further characteristic fragment ions were used as verifiers. Injecting β-lactoglobulin from the model protein, MS parameters were optimized for each single identifier and verifier. In detail, declustering potential and collision energy were systematically varied and the values leading to maximal signal-to-noise ratio were chosen as final parameter settings in order to maximize the sensitivity of the method.

Spectrum Interpretation. The spectra that were obtained in full scan mode or MS/MS fragmentation mode were interpreted using BioAnalyst version 1.5.

RESULTS AND DISCUSSION

The present study attempted a comprehensive analysis of PTMs in β-lactoglobulin from thermally processed milk. The MS/MS protocol applied for this purpose is outlined in Figure 1. Briefly, a model protein was used to determine the most intense mass signals of unmodified β-lactoglobulin peptides obtained by partial enzymatic hydrolysis and EMS, ER, and EPI scans. Combining the resulting *m*/*z* list of unmodified peptides with the information of possible nePTMs,

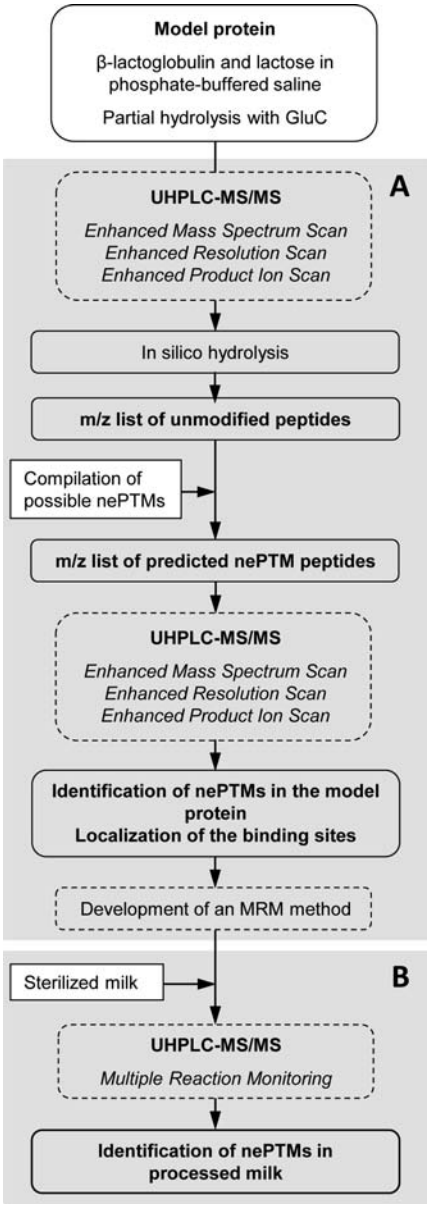

Figure 1. Workflow for the analysis of nePTMs in processed milk. Box A includes experiments with β-lactoglobulin from a model protein, while box B indicates the final steps performed with processed milk.

Table 1. Overview of Detected Modifications of β-Lactoglobulin in the Model Protein and in Milk Products and Assignment of Their Localization and Structures[a]

		mass (Da)							milk products			
amino acid	sequence	theor.	exp.	shift	localization	structure	t_R (min)	model	raw	past.	UHT	steril.
1−11	LIVTQTMKGLD	1217.67	1217.64	324.1	N-terminus	**1b**	21.7	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				324.2	K8	**1a**	22.3	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				57.9	N-terminus	**7a**	23.9	x (EMS)	nd	nd	t (MRM)	x (MRM)
				57.9	K8	**6a**	23.8	x (EMS)	nd	nd	t (MRM)	t (MRM)
				72.0	N-terminus	**7b**	31.0	x (EPI)	nd	nd	nd	nd
				16.0	M7	**2a**	17.7	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				32.0	M7	**2b**	18.9	x (EPI)	nd	nd	nd	nd
				−1.0	N-terminus/I2[b]	**8**	35.9	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
12−28	IQKVAGTWYSLAMAASD	1810.89	1810.84	324.1	K14	**1a**	28.4	x (EMS)	nd	nd	x (MRM)	x (MRM)
				58.1	K14	**6a**	29.8	x (EMS)	nd	nd	nd	t (MRM)
				16.1	M24	**2a**	26.5	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				32.1	M24	**2b**	27.2	x (EPI)	nd	nd	nd	nd
				16.1	W19	**10**	26.5	x (EMS)	nd	nd	nd	t (MRM)
				32.1	W19	**9a**	25.7	x (EPI)	nd	nd	nd	nd
				4.1	W19	**9b**	28.8	x (EPI)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
34−45	AQSAPLRVYVEE	1360.70	1360.68	72.1	R40	**5a**	21.7	x (EPI)	nd	nd	nd	nd
				54.1	R40	**5b**	21.4	x (EMS)	nd	nd	nd	x (MRM)
				144.1	R40	**5c**	20.4	x (EPI)	nd	nd	nd	nd
46−51	LKPTPE	683.39	683.48	324.0	K47	**1a**	3.9	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
46−55	LKPTPEGDLE	1226.60	1226.64	57.9	K47	**6a**	16.1	x (EPI)	nd	nd	t (MRM)	x (MRM)
56−62	ILLQKWE	928.54	928.52	324.2	K60	**1a**	22.5	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				58.1	K60	**6a**	24.4	x (EMS)	t (MRM)	t (MRM)	x (MRM)	x (MRM)
				72.1	K60	**6b**	23.7	x (EPI)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				32.0	W61	**9a**	21.7	x (EPI)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				4.0	W61	**9b**	21.9	x (EPI)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				20.0	W61	**9c**	20.1	x (EPI)	nd	nd	nd	nd
				16.1	W61	**10**	19.9	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
56−65	ILLQKWENGE	1228.65	1228.72	1.0	N63	**4**	22.1	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
66−74	CAQKKIIAE	1002.55	1002.60	324.2	K69/K70[b]	**1a**	7.0	x (EMS)	nd	nd	nd	nd
				57.9	K69	**6a**	10.4	x (EMS)	nd	nd	t (MRM)	x (MRM)
				57.9	K70	**6a**	10.7	x (EMS)	nd	nd	t (MRM)	x (MRM)
				72.0	K69/K70	**6b**	15.5	x (EPI)	t (MRM)	t (MRM)	t (MRM)	x (MRM)
				48.0	C66	**3b**	11.7	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
75−89	KTKIPAVFKIDALNE	1685.97	1686.00	324.1	K75/K77/K83[b]	**1a**	25.1	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				58.1	K75/K77[b]	**6a**	27.0	x (EMS)	nd	nd	t (MRM)	x (MRM)
				58.1	K83	**6a**	27.6	x (EMS)	nd	nd	t (MRM)	t (MRM)
90−96	NKVLVLD	799.48	799.48	324.0	K91	**1a**	18.0	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				58.1	K91	**6a**	20.2	x (EPI)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				72.0	K91	**6b**	19.9	x (EPI)	nd	t (MRM)	x (MRM)	x (MRM)
97−108	TDYKKYLLFCME	1552.73	1552.68	324.0	K100/K101[b]	**1a**	30.6	x (EMS)	nd	nd	nd	nd
				58.1	K100	**6a**	32.2	x (EMS)	nd	nd	nd	t (MRM)
				58.1	K101	**6a**	32.5	x (EMS)	nd	nd	nd	t (MRM)

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Table 1. continued

amino acid	sequence	mass (Da) theor.	mass (Da) exp.	mass (Da) shift	localization	structure	t_R (min)	model	milk products raw	milk products past.	milk products UHT	milk products steril.
				72.0	K100	**6b**	35.9	x (EPI)	nd	nd	nd	nd
				72.0	K101	**6b**	34.4	x (EPI)	nd	nd	nd	nd
				16.1	M107	**2a**	27.5	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				32.0	M107	**2b**	28.7	x (EPI)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
115–127	QSLACQCLVRTPE	1446.70	1446.68	32.1	C119	**3a**	23.1	x (EPI)	t (MRM)	t (MRM)	x (MRM)	x (MRM)
				32.1	C121	**3a**	22.7	x (EMS)	nd	nd	nd	nd
				48.1	C119	**3b**	22.8	x (EPI)	nd	nd	nd	nd
				48.1	C121	**3b**	22.9	x (EMS)	nd	nd	nd	nd
				72.1	R124	**5a**	23.7	x (EPI)	nd	nd	nd	t (MRM)
				54.1	R124	**5b**	23.8	x (EMS)	nd	nd	t (MRM)	x (MRM)
135–137	KFD	408.20	408.28	324.0	K135	**1a**	1.9	x (EPI)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
				58.0	K135	**6a**	2.5	x (EPI)	nd	nd	x (MRM)	x (MRM)
135–158	KFDKALKALPMHIRLSFNPTQLEE	2825.53	2825.60	16.0	M145	**2a**	27.3	x (EMS)	x (MRM)	x (MRM)	x (MRM)	x (MRM)
138–157	KALKALPMHIRLSFNPTQLE	2306.29	2306.32	58.0	K138/141[b]	**6a**	29.5	x (EMS)	nd	nd	nd	nd

[a]Sequence coverage was 100%. Only modified peptides are listed. Theor.: theoretical. Exp.: experimental. Past.: pasteurized. UHT: ultrahigh temperature. Steril.: sterilized. t_R: Retention time. Numbers of assigned structures refer to structures displayed in Figure 3. Detected modifications in the model protein or in commercially available milk are marked with an "x" or "t" (detected only in traces) with an indication of the MS mode for which the detection was achieved. nd: not detected. [b]Localization not possible.

an m/z list of predicted nePTM peptides was compiled. Predicted nePTM peptides were subsequently analyzed in the model protein in EMS, ER, and EPI mode. The identified PTM peptides were then included in a scheduled MRM (sMRM) method. Finally, the sMRM method was applied for site-specific analysis of low-abundant PTM peptides in processed milk.

Analysis of Predicted nePTMs in a Model Protein. A model protein was prepared to force modifications of β-lactoglobulin by heating it with lactose for 7 d at 60 °C in phosphate-buffered saline.[8] Since the MS results obtained from the model protein serve as a database for targeted nePTM analysis of milk products, relatively harsh reaction conditions were chosen to prepare the model protein. Thus, a maximum number of different nePTM structures could be considered for milk analysis. The model protein was partially hydrolyzed using the endoproteinase GluC, which specifically cleaves C-terminally to glutamic acid and, with lower affinity, to aspartic acid. Trypsin, the most commonly used endoproteinase in proteomics, was avoided. Trypsin specifically cleaves at lysine and arginine residues, which were expected to be modified during heating with lactose. Consequently, missed cleavages occur, which complicate data evaluation. The resulting peptide mixture was chromatographically separated and analyzed by UHPLC–MS/MS.

In a first step, unmodified peptide fragments of β-lactoglobulin were analyzed in the model mixture using the EMS scan mode. In this mode, the third quadrupole is set as a linear ion trap. Due to the ion trap functionality, the EMS scans are significantly more sensitive than a conventional scan obtained by a quadrupole. Detected signals were assigned to the corresponding amino acid sequences either by in silico digestion of β-lactoglobulin and comparison of the theoretical with the experimental masses and charge states or by additional fragmentation of the peptides in the EPI scan mode. The sequence coverage of β-lactoglobulin was 100%.

For the identification of nePTM peptides of β-lactoglobulin from the model protein, an m/z list of predicted modifications was established by considering the most prevalent charge states. For this purpose, possible nePTMs were compiled from untargeted studies and the literature. Some of these modifications had already been detected in the model protein used in this study by MALDI-TOF-MS,[8] and others were systematically screened for since they had been detected in similar model proteins or model peptides after heating with sugars or sugar degradation products,[17−19] after forced oxidation [20] or in heated or stored milk.[21−23] Signals of predicted nePTM peptides were searched in the spectra by extraction from the EMS spectrum. If a peak was detected in the extracted ion chromatogram, the monoisotopic mass and the charge state in the corresponding spectrum were checked to confirm the postulated identity of the signal. For further confirmation of the charge state and a more precise mass of the detected peaks, ER scans were acquired. Here, the target ions were selected in quadrupole 1, trapped in quadrupole 3, and then scanned out at a slow scan rate (250 Da/s), allowing for a higher mass accuracy and resolution (<0.25 Da peak width at half-peak height) and thus for a more reliable signal assignment.

EMS scans in combination with ER scans provide a very rapid overview of peptides present in a sample and their major modifications. Using this scan mode, 33 modifications could be detected in one single run by their characteristic mass shift (see Table 1). However, if several reactive amino acids are present in a peptide, the unambiguous localization of the modification is not possible and MS/MS scans are required. Furthermore,

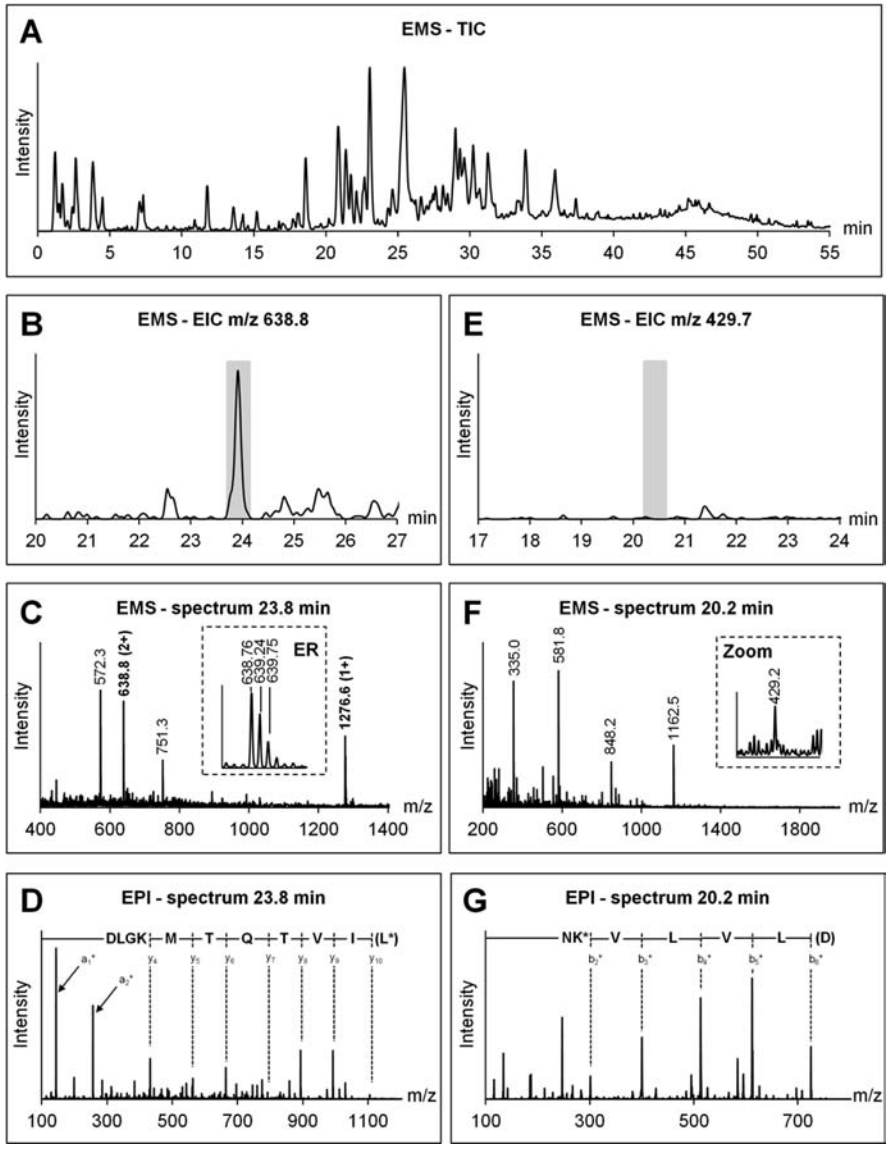

Figure 2. (A) Total ion chromatogram of an enhanced mass spectrum scan of β-lactoglobulin heated with lactose. (B) Zoomed extracted ion chromatogram of m/z 638.8 and (C) the spectrum at 23.8 min, corresponding to N^{ε}-carboxymethyllysine (CML)-modified peptide AA 1−11 (LIVTQTMKGLD). The inset displays the corresponding spectrum acquired in enhanced resolution mode. The enhanced product ion scan (D) identified the N-terminal leucine, not lysine K8, being modified. (E) Zoomed extracted ion chromatogram of m/z 429.7 Da and (F) the spectrum at 20.2 min, where the CML-modified peptide AA 90−96 (NKVLVLD) with m/z 429.7 ± 0.1 Da cannot be detected. In contrast, the more sensitive enhanced product ion scan (G) allows the detection and identification of the CML-modified peptide by its characteristic fragment pattern. Modified amino acids or fragments are marked with an asterisk.

the sensitivity of EMS scans is limited, since all ions present in a sample are detected, and consequently, minor modifications may remain hidden in the noise.

For a more sensitive detection and identification of the binding site of the detected PTMs, EPI scans of all detected modifications were performed. In this mode, the mass of doubly or triply charged predicted modified peptides were set as precursor masses. Singly charged ions are typically not accessible to MS/MS fragmentation and were, therefore, not considered as precursor ions. EPI scans are more sensitive than EMS scans since the number of ions entering the ion trap is reduced due to ion-selection in Q1. Consequently, these target masses can be much more enriched using the dynamic fill time option of the instrument. Furthermore, the EPI mode allows for the localization of the modification in the amino acid sequence by comparing the b- and y-ion series of the native and the modified peptide, which reveals the amino acid bearing the modification. The allocation of the peptide mass shift to a specific amino acid supports the structure interpretation of the observed mass difference.

Generated EPI spectra were searched for the characteristic b- and y-ion series that were expected for modified peptides and could be calculated by protein software tools (see Materials and Methods). By this approach, the localization of modifications previously identified in the EMS scan was achieved. Additionally, 23 further modifications could be assigned, which were not covered by the EMS scan (see Table 1).

Figure 2 exemplarily compares the potential of EMS and EPI scans. In the EMS scan, the singly charged ion with m/z 1276.6 Da and the doubly charged ion with m/z 638.8 Da at 23.8 min suggest the modification of peptide AA 1−11 (LIVTQTMKGLD, native mass 1217.67 Da) with CML, resulting in a mass shift of

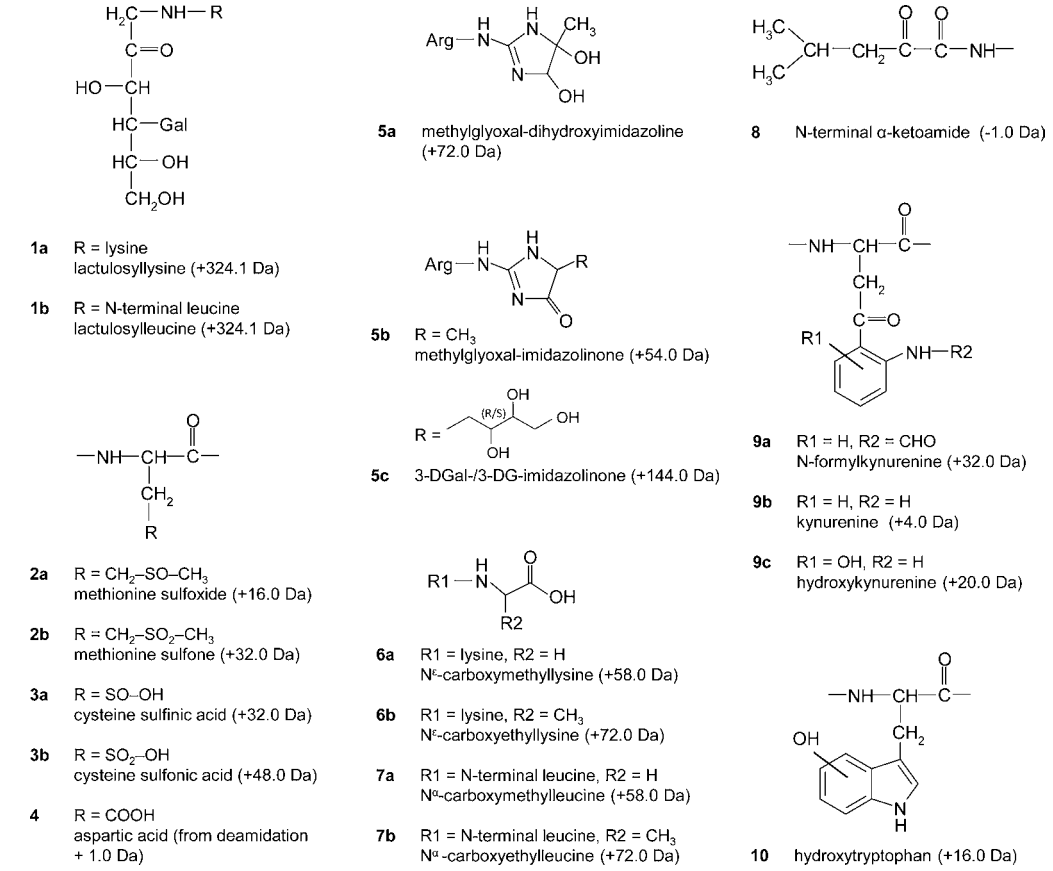

Figure 3. Structures of postulated modifications detected in β-lactoglobulin heated with lactose in phosphate-buffered saline for 7 d at 60 °C and/or in milk products. Resulting mass shifts are indicated.

58 Da (see Figure 2A–C and Table 1). The EPI scan (Figure 2D) reveals the information that the N-terminal amino acid is carboxymethylated, since y-ions 4–10 are detected with their native mass, whereas a-ions 1–2 are shifted by +58 Da.

Additionally, EPI scans provide higher sensitivity, leading to the detection of modified peptides that are not visible in the EMS scan. Thus, for example, the modification of Lys91 at peptide 90–96 with CML could only be detected by an EPI scan, but not in the EMS mode: While no signal from this peptide with m/z 429.7 ± 0.1 Da could be detected in the EMS scan (Figure 2E,F), the peptide gave a signal in the EPI scan at 20.2 min. The EPI scan further showed the characteristic pattern of b- and y-ions of the CML-modified peptide and allowed for the identification of the binding site (Figure 2G).

Table 1 gives an overview of all modifications that were detected in β-lactoglobulin from the model protein. The structures are displayed in Figure 3. Lysine, arginine, methionine, tryptophan, cysteine, and asparagine, as well as the N-terminal leucine, were shown to be the most reactive amino acids. The observed mass shifts and the localization of the modifications indicate the presence of lactulosyllysine and N-terminal lactulosylleucine (theoretical mass shifts Δm = +324.1 Da), CML (Δm = +58.0 Da), carboxyethyllysine (CEL, Δm = +72.0 Da), methionine sulfoxide (Δm = +16.0 Da), methionine sulfone (Δm = +32.0 Da), hydroxytryptophan (Δm = +16.0 Da), *N*-formylkynurenine (Δm = +32.0 Da), kynurenine (Δm = +4.0 Da), hydroxykynurenine (Δm = +20.0 Da), methylglyoxal-dihydroxyimidazoline (Δm = +72.0 Da), methylglyoxal-imidazolinone (Δm = +54.0 Da), 3-deoxygalactosone-/3-deoxyglucosone-imidazolinone (Δm = +144.0 Da), cysteine sulfinic acid (Δm = +32.0 Da), and cysteine sulfonic acid (Δm = +48.0 Da). In addition, deamidation of asparagine to aspartic acid (Δm = +1.0 Da) was assigned, as well as the N-terminal oxidative deamination generating an α-ketoamide structure (Δm = −1.0 Da) (see Table 1).

Development of the sMRM Method. In general, the detection of protein modifications in food requires a very sensitive method, since some of the modifications are expected to be present only in low concentration. Additionally, the complexity of the matrix further impairs the mass spectrometric detection. Therefore, we developed an MS method in MRM mode. For a further increase of sensitivity, the sMRM mode was applied, so that target transitions were only measured during short, user-specified retention time windows. To check whether the β-lactoglobulin modifications that had been detected in the model protein were also present in processed milk, commercial pasteurized, UHT, and sterilized milk samples were analyzed using the developed sMRM method (Table 1).

Modification Products of Lysine. In the EMS scans, mass shifts of 324 Da at peptides containing lysine residues indicated the presence of lactulosyllysine (Figure 3, **1a**). Lactulosyllysine (also called Amadori product) is formed during the Maillard reaction by condensation of the milk sugar lactose with the amino group of lysine with subsequent keto–enol and imine–enamine tautomerism.[24] Lactulosyllysine has been shown to be the predominant modification formed during thermal treatment of milk proteins.[4] At six lysine residues (K8, K14, K47, K60, K91, and K135), lactulosyllysine could be clearly detected. Furthermore, the three peptides containing K69/70, K75/77/83, and K100/101 showed the typical mass shift of

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lactosylation. The EPI spectra, however, did not allow the unambiguous localization, and thus, it remained open which lysine residues bore the modification. Lactulosyllysine could not be detected at lysine K138/141, but the signal of the native peptide was also of low intensity.

Furthermore, signals with mass shifts of 58 Da indicated that CML (**6a**) was formed at lysine K8, K14, K47, K60, K69, K70, K75/77, K83, K91, K100, K101, K135, and K138/141. CML is an established advanced glycation end product of proteins and can be formed in protein−sugar solutions either by oxidative cleavage of the Amadori product[25] or by direct modification of the lysine residues by glyoxal generated during sugar degradation.[26] It has been shown before that CML is formed at lysine residues which were also lactosylated.[8] Therefore, it can be assumed that the Amadori product is also formed in the model protein at K138/141 but was below the detection limit of the method, although the corresponding EPI spectra did not confirm that conclusion (see above). Possibly, the intensity of the K138/141-lactosylated peptide of the model protein could be low because of its extensive further degradation under the applied heating conditions.

Mass shifts of 72 Da were detected at lysine K60, K69/70, K91, K100, and K101, indicating the presence of CEL (**6b**). This modification has been detected for the first time in model compounds by Buettner et al.[27] and is formed from lysine and methylglyoxal, the latter being generated by degradation of sugars. A previous study from Ahmed et al. already demonstrated that CEL was present in milk and that its total concentration increased during heating.[21]

Glyoxal- or methylglyoxal-derived hemiaminals, which are formed in early stages of the Maillard reaction and which have been reported in previous studies with model incubations,[17] could not be detected in our model protein. Most likely, the long reaction time applied for the preparation of the model protein favored the degradation of possible intermediately formed hemiaminals.

Modification Products of the N-Terminal Amino Group. Since the N-terminus of a protein contains a free amino group, it can also react with lactose, similar to lysine.[28,29] Indeed, a mass shift of 324 Da revealed a lactosylated N-terminal leucine (Figure 3, **1b**), which was obviously further oxidized to N^{α}-carboxymethylleucine (Figure 3, **7a**). Furthermore, the N-terminal amino group was also modified to form N^{α}-carboxyethylleucine (see **7b** in Figure 3 and Table 1).

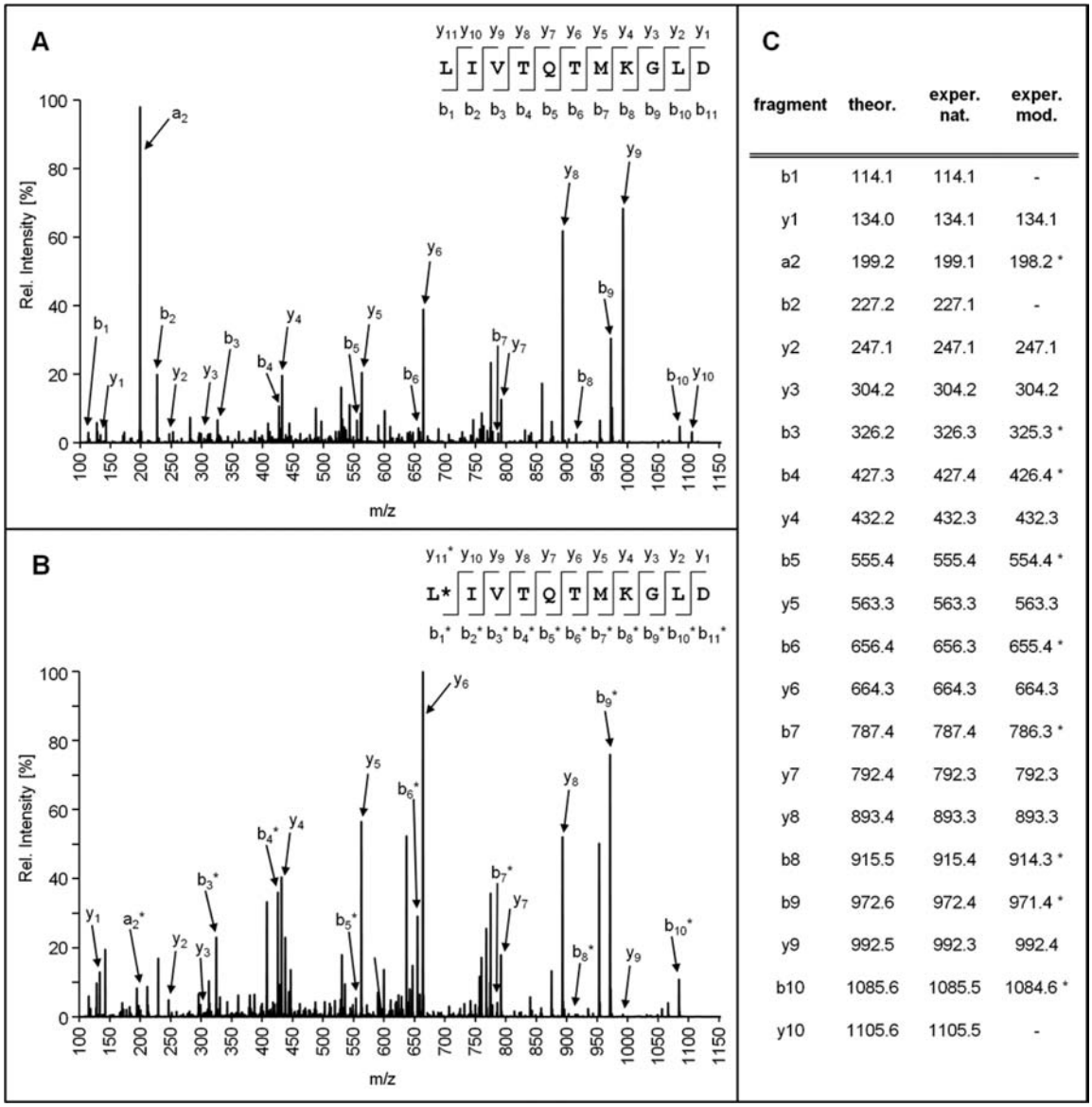
Moreover, a mass shift of −1 Da was measured at the N-terminal peptide. A previous study analyzing the model protein by MALDI-TOF-MS postulated that lysine aldehyde was formed at K8 lactose-dependently during heating.[8] The analysis by LC−MS/MS, however, revealed now that the modification was not located at lysine K8, but at amino acids 1−2 (see Figure 4). When the N-terminal peptide (AA 1−11) was fragmented, y-ions 1−9 were measured with their native mass, while the detected a-ions 1−2 and b-ions 3−10 were all detected with a mass reduced by 1 Da. Since leucine and isoleucine both represent very unreactive amino acids, it can be assumed that the N-terminus with its very reactive amino group was oxidatively deaminated to yield α-ketoamide (see Figure 3, **8**). Oxidation may be either mediated by reactive oxygen species which are set free during glycation reactions[30] or, alternatively, the N-terminal amino group may also have reacted in a Strecker-type reaction with a dicarbonyl compound generated from lactose, according to the reaction pathway proposed by Akagawa et al.[31]

Modification Products of Arginine. Heat treatment of milk leads to the degradation of lactose, forming α-dicarbonyl compounds such as glyoxal, methylglyoxal, and 3-deoxygalactosone,[32] which can further react with arginine to dihydroxyimidazolines and dehydrate to imidazolinones.[33,34] Methylglyoxal-derived imidazolinone has already been detected in milk.[21] Mass shifts of +72 and +54 Da observed in the model protein at R40 and R124 indicate the presence of the methylglyoxal-derived dihydroxyimidazoline (Figure 3, **5a**) and imidazolinone (Figure 3, **5b**), respectively. It should be noted that a mass shift of +72 Da may also be a consequence of modification to carboxyethylarginine. However, the fragmentation of carboxyl residues (−44 Da), which was expected for carboxyethylarginine, was not observed in the EPI spectra. Signals corresponding to water elimination, however, further supported the hypothesis that dihydroxyimidazoline was formed rather than carboxyethylarginine. Furthermore, a mass shift of 144 Da at R40 suggested an imidazolinone derived from a hexose. Although hexoses were not present in the initial preparation of the model protein, galactose is released from lactose and/or lactulosyllysine during the heating of milk.[35] Therefore, it can be assumed that the observed mass shift indicated the presence of 3-deoxygalactosone-imidazolinone (Figure 3, **5c**). Although glucose levels in milk are very low,[35] it cannot be fully excluded that the signal also represented glucose-derived 3-deoxyglucosone-imidazolinone (Figure 3, **5c**). Previous studies with model proteins, which were incubated with sugar or sugar degradation products, reported the formation of tetrahydropyrimidine (+144 Da) and/or its dehydration product dihydropyrimidine (+126 Da).[17,19,33] However, we could not detect the characteristic neutral loss of carbon dioxide of tetrahydropyrimidine in the product ion spectra,[36] and therefore, it seems more likely that the observed mass shift of +144 Da was due to formation of the 3-deoxyglucosone/3-deoxygalactosone-imidazolinone rather than tetrahydropyrimidine. This hypothesis is in agreement with the findings by Brock et al., who showed that the imidazolinone was the major product from incubation of proteins with methylglyoxal, while tetrahydropyrimidine was only built when a relatively high concentration of methylglyoxal was used.[33]

Simple products from lactosylation of arginine-containing peptides, as reported for models with milk proteins,[37] were not detected (data not shown). Either their concentration was below the detection limit or the detected advanced glycation products of arginine were not formed via the lactosylation product but via the formation of α-dicarbonyl compounds by sugar degradation and their subsequent attack of arginine.

No difference in the modification state between genetic variant A and B of β-lactoglobulin was detectable (data not shown), and thus, the replacement of alanine at position 118 by valine does not seem to have an important influence on reactions of R124 during heating.

Modification Products of Methionine. The formation of methionine sulfoxide (Figure 3, **2a**) was shown for M7, M24, M107, and M145 based on the findings that mass shifts of 16 Da could be unambiguously identified at these amino acids (see Table 1). Methionine is known to be easily oxidized by two- or one-electron oxidants,[38] a process that already occurs during sample preparation. However, a previous systematic analysis of the model protein by MALDI-TOF-MS has already shown the heat- and lactose-dependence of methionine oxidation,[8] and therefore, we assume that the presence of methionine sulfoxide could be at least partially ascribed to the



C

fragment	theor.	exper. nat.	exper. mod.
b1	114.1	114.1	-
y1	134.0	134.1	134.1
a2	199.2	199.1	198.2 *
b2	227.2	227.1	-
y2	247.1	247.1	247.1
y3	304.2	304.2	304.2
b3	326.2	326.3	325.3 *
b4	427.3	427.4	426.4 *
y4	432.2	432.3	432.3
b5	555.4	555.4	554.4 *
y5	563.3	563.3	563.3
b6	656.4	656.3	655.4 *
y6	664.3	664.3	664.3
b7	787.4	787.4	786.3 *
y7	792.4	792.3	792.3
y8	893.4	893.3	893.3
b8	915.5	915.4	914.3 *
b9	972.6	972.4	971.4 *
y9	992.5	992.3	992.4
b10	1085.6	1085.5	1084.6 *
y10	1105.6	1105.5	-

Figure 4. Enhanced product ion spectrum of the precursor mass with (A) m/z 609.3 at 22.8 min (native peptide AA 1−11) and (B) m/z 609.8 at 35.9 min (modified peptide with the N-terminus oxidized to an α-ketoamide, $\Delta m = -1$. Da). For simplification, signals corresponding to water loss are not annotated. Theoretical and experimental masses of characteristic a-, b-, and y-ions (from native or modified peptides) are displayed (C). Modified amino acids or fragments are marked with an asterisk.

heating process. A mass shift of 32 Da at M7, M24, and M107 indicated that methionine sulfoxide was further oxidized to the sulfone (Figure 3, **2b**).

Modification Products of Tryptophan. Tryptophan can be enzymatically and/or chemically oxidized via *N*-formylkynurenine (Figure 3, **9a**) and kynurenine (Figure 3, **9b**) to form hydroxykynurenine (Figure 3, **9c**).[39,40] Indeed, we could detect the corresponding mass shifts of 32, 4, and 20 Da, respectively, at W19 and W61 (see Table 1). Furthermore, hydroxylated tryptophan has been reported.[39,40] In accordance with these findings, a mass shift of 16 Da at W19 and W61 could be explained by hydroxylation of the aromatic ring of tryptophan, yielding hydroxytryptophan (Figure 3, **10**). Although it was already shown that the tryptophan content decreased when milk was heated[41] or when solutions of caseins were exposed to radicals[42] or photooxidation,[20] little is known about the structures of the generated compounds.

Modification Products of Cysteine. β-Lactoglobulin carries five cysteine residues, four of them forming disulfide bridges (C66/160, C106/119). Cysteine occurs in different oxidation states in proteins, mainly in its reduced form as thiol or as part of disulfide bonds. Furthermore, cysteine can be oxidized to cysteine sulfenic, sulfinic, and sulfonic acid. Thermally induced disulfide bridges were not covered by this study, since disulfide bridges were reduced with DTT during sample preparation. Mass shifts of 16 Da corresponding to sulfenic acid could not be detected at any of the five cysteine residues. If cysteine sulfenic acid residues were present in the model protein, they were probably also reduced by DTT prior to LC−MS/MS analysis. However, mass shifts of 32 Da could be detected at C119 and C121, indicating the presence of cysteine sulfinic acid (Figure 3, **3a**). Furthermore, cysteine sulfonic acid (Figure 3, **3b**) was detected at C66, C119, and C121. At C106 and C160, no cysteine oxidation products were observed. It can be expected that the oxidation of cysteine residues, which are normally involved in disulfide bridges, influences the tertiary structure of β-lactoglobulin. This might also change the microenvironment

and the accessibility of other reactive amino acids and thus modulate the formation rate of other protein modifications. Replacement of alanine 118 by valine in β-lactoglobulin variant A did not have a detectable influence on cysteine oxidation (data not shown).

It is known that, upon heating of β-lactoglobulin, cysteine can eliminate hydrogen sulfide, yielding dehydroalanine.[43] However, this modification was not detected in the present study.

Modification Products of Tyrosine. Tyrosine is susceptible to oxidation because the aromatic ring can be attacked by reactive oxygen species, forming dityrosine.[20] Since we detected signals corresponding to peptides AA 12−28, AA 34−45, and AA 97−108 (containing Y20, Y42, Y99, and Y102) with mass shifts of 16 Da, hydroxylation of tyrosine was postulated. Although oxidation of other easily oxidizable amino acids such as W19, M24, or M107 could be excluded by the EPI spectrum, the spectrum quality was, however, not sufficient to directly prove the oxidation of tyrosine residues (data not shown).

Modification Products of Asparagine. An observed mass shift of +1 Da at N63 was assigned to deamidation (see Table 1). The side chain of asparagine is easily attacked by the nitrogen from a vicinal peptide bond, intermediately forming the succinimide (Δm = −17.03 Da), which further reacts to aspartic acid (Figure 3, **4**; Δm = +0.98 Da) or isoaspartic acid (Δm = +0.98 Da) by hydrolysis of the lactame.[44] Since the native peptide and the deamidated peptide coeluted in the chromatograms, the signal of the latter overlapped with the ^{13}C isotope of the native peptide, as a consequence of the mass shift of +1 Da. Therefore, the isotopic pattern of the signals indicated the occurrence of deamidation. Deamidation was almost complete in variant B, in which N63 is vicinal to G64 (signal m/z 1230.64, Figure 5). In variant A, in which G64 is replaced

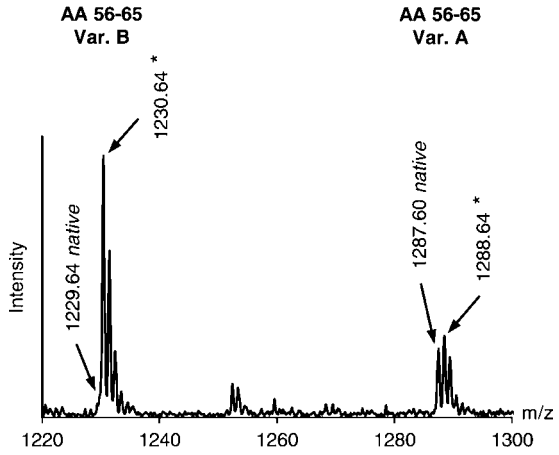


Figure 5. Enhanced mass spectrum scan of peptide AA 56−65, variants A and B. The signals corresponding to the native peptides are indicated. Signals that result from the overlapping of the ^{13}C-isotope of the native peptide and the ^{12}C-isotope of the deamidated form are marked with an asterisk. In variant B, only the deamidated molecule was clearly detected (m/z 1230.64), indicating almost complete deamidation, while in variant A, the native peptide at m/z 1287.60 was still detectable.

by aspartic acid, the nondeamidated peptide (signal m/z 1287.60, Figure 5) could still be detected. Still, the isotope pattern clearly indicates that deamidation also took place (signal m/z 1288.64, Figure 5). Thus, it can be concluded that the presence of glycine in the vicinal position promotes deamidation of asparagine N63. Other asparagine residues in the sequence of β-lactoglobulin A or B were not detectably affected by deamidation. These findings are in good accordance with literature reporting rapid deamidation of asparagine followed by small and flexible amino acids such as glycine, while the steric hindrance of more bulky residues such as aspartic acid prevents asparagine deamidation.[45] Deamidation is further promoted by slightly alkaline pH and elevated temperature during sample preparation.[45] Therefore, it cannot be fully differentiated whether deamidation occurred during heating of the model protein with lactose, respectively the industrial heating step, or during partial enzymatic hydrolysis prior to LC−MS analysis. Deamidation during thermal treatment of the milk sample is supported by a study showing that thermal treatment of milk proteins leads to deamidation.[46]

Detection of β-Lactoglobulin Modifications in Different Commercial Heat-Treated Milk Products. In a next step, commercially available milk products were analyzed for the presence of β-lactoglobulin modifications. Although milk products are much more complex in their composition than the model protein, the selective nature of the UHPLC−MS/MS(MRM) approach choosing characteristic b- and y-ions as identifiers and verifiers (see above) allows for sensitive analysis of β-lactoglobulin modifications without interference of other milk proteins, such as different whey proteins or caseins. Forty-one out of the 56 modifications, which have been observed in the model protein, were also identified in commercial sterilized milk (Table 1). Thirty-five of the modifications were further detected in UHT milk and 25 in pasteurized milk. Thus, it can be concluded that the applied model protein was valid to predict protein modifications in different commercial milk products and that the developed LC−MS/MS method is suitable for complex matrices such as milk. In general, modifications were less pronounced in commercial milk products than in β-lactoglobulin from the model protein and, in some cases, their concentration was below the detection limit of the applied method. Lower signal intensities in commercial milk compared to the model protein are most likely caused by a lower modification rate. Additionally, an excess of casein-derived peptides over β-lactoglobulin-derived peptides present in milk, but not in the model, may lead to signal suppression.

Physiological Relevance of β-Lactoglobulin Modifications. Whey proteins are of high nutritional value due to their high content of essential amino acids. However, the detected modification of essential amino acids, such as lysine, tryptophan, and methionine, and semiessential amino acids, such as arginine, may lead to a significant decrease of the nutritional value of milk products. For example, lactosylation reduces the bioavailability of lysine.[47] In severely heated milk products such as condensed milk, up to 36.2% of the lysine residues has been reported to be blocked by the Maillard reaction.[48] Furthermore, glycation of milk proteins leads to a reduced digestibility by gastrointestinal enzymes, which further reduces the amount of essential amino acids available for absorption.[49] It can be expected that steric hindrance by other modifications that were detected in the present study have a similar impact on protein digestibility.

It is also known that adducts that are located in a functional domain of β-lactoglobulin may have particular influence on protein function. For example, K8, K75, K77, K83, and K135 have been identified as critical amino acids of IgE-binding epitopes of β-lactoglobulin.[50]

Quantitative studies will be necessary to determine to what extent the detected PTM are naturally present in milk or generated during the processing step. Furthermore, the targeted nature of the study did not allow the detection of nePTMs that had not been described before and had not been formed in the model protein. Therefore, highly sensitive untargeted approaches will be required in the future to detect modifications that have escaped identification so far.

The present method can easily be adopted to other proteins or to other food or biological matrices. Comprehensive analysis of process-induced protein modifications is an important prerequisite of physiological and toxicological evaluation of processed food. Identification of the binding site of the modifications allows the identification of adducts in functional protein domains, which may have a particular impact on physiological and technological protein properties.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental conditions for MS analysis and Table S1: Overview of detected modifications of β-lactoglobulin and their identifier and verifier signals used for the sMRM method. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

nePTM, nonenzymatic post-translational protein modification; CML, N^{ϵ}-carboxymethyllysine; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; UHPLC–MS/MS, ultrahigh-performance liquid chromatography tandem mass spectrometry; ESI, electrospray ionization; DTT, 1,4-dithiothreitol; UHT, ultrahigh temperature; ER, enhanced resolution; EMS, enhanced mass spectrum; EPI, enhanced product ion; (s)MRM, (scheduled) multiple reaction monitoring; CEL, carboxyethyllysine.

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