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Distribution of Protein Oxidation Products in the Proteome of Thermally Processed Milk

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ABSTRACT: During thermal milk processing, severe oxidation can occur, which alters the technological and physiological properties of the milk proteins. Due to differences in composition and physicochemical properties, it can be expected that the particular milk proteins are differently affected by oxidative damage. Therefore, the protein-specific distribution of oxidation products in the heated milk proteome was investigated. Raw and heated milk samples were separated by one-dimensional gel electrophoresis. Protein oxidation was visualized by Western blot after derivatization of protein carbonyls with 2,4-dinitrophenylhydrazine. Thus, α-lactalbumin displayed enhanced oxidation compared to β-lactoglobulin, despite its lower concentration in milk. Highly selective oxidation was detected for a previously unassigned minor milk protein. The protein was identified by its peptide mass fingerprint as a variant of α_{S1}-casein (α_{S1}-casein*). Similar oxidation patterns were observed in several commercial milk products.

KEYWORDS: *α_{S1}-casein, milk proteins, milk proteome, processed milk, protein carbonyls, protein oxidation*

■ INTRODUCTION

Milk is usually industrially processed prior to consumption including a more or less intensive heating step. As a result, severe protein damage can affect protein constitution as well as protein conformation. Constitutional changes are often linked with the formation of nonenzymatic posttranslational modifications (nePTMs), such as protein oxidation, glycation, and glycoxidation.[1] Nontargeted nePTM mapping of milk models and processed milk revealed that methionine oxidation to methionine sulfoxide is one of the most predominant amino acid modifications in processed milk.[2,3] In commercial milk products, such as caseinate preparations, up to 74% of methionine is converted to the sulfoxide.[4] In addition to methionine oxidation, oxidation of cysteine or tryptophan was detected as well as photooxidation of tyrosine.[2,5,6] Formation of protein carbonyls in milk can arise from the oxidation of lysine, arginine, proline, or threonine.[7] Furthermore, protein carbonylation can originate from the reaction of amino acid side chains with lipid oxidation products with dicarbonyl or α,β-unsaturated aldehyde structure.[8] nePTMs in milk, such as oxidation products, reduce the nutritional value of the milk proteins, for example, by degradation of essential amino acids. Moreover, important technological processes, such as protein coagulation and the cheese-ripening process can be impaired by covalent protein modifications.[9,10]

The physicochemical structures and composition of milk proteins are rather complex and diverse. Caseins are mainly present in supramolecular nanostructures, the so-called micelles, which reach particle sizes between 50 and 500 nm.[11] In contrast, whey proteins such as α-lactalbumin and β-lactoglobulin are dissolved as mono- or dimers in the milk serum. Furthermore, native whey proteins possess a defined tertiary structure, whereas a random coil structure is suggested for caseins in solution.[12] Therefore, it can be expected that different milk proteins are differently affected by oxidative damage. Mapping of the glycoxidation product N^{ε}-carboxymethyllysine (CML) in the heated milk proteome revealed, for example, that particularly high molecular aggregates, which are generated during heating, are modified by CML.[13] Likewise, it has been shown that photooxidation affects different milk proteins differently: whereas the flexible structure of β-casein favored the formation of dityrosine, the globular protein β-lactoglobulin mainly yielded 3,4-dihydroxyphenylalanine (DOPA).[6] Because the different milk proteins have very distinct nutritional properties and technological functions, it can be misleading to determine the overall protein oxidation by a sum parameter. Instead, it is important to identify which proteins in the milk proteome are predominately oxidized during thermal treatment and to differentiate the modification rate of the different protein fractions.

Therefore, the present study developed a method to analyze protein-specific oxidation in the proteome of heated milk and milk products. The mapping of oxidation products in the milk proteome complements previous studies that determined binding sites of oxidation products in the amino acid sequence of distinct milk proteins by mass spectrometry.[14,15]

■ MATERIALS AND METHODS

Chemicals. α-Cyano-4-hydroxycinnamic acid was obtained from Fluka (Taufkirchen, Germany) and Coomassie brilliant blue R-250 as well as Bio-Safe Coomassie from Bio-Rad (Munich, Germany). Molecular weight marker, Ponceau S, α-casein, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Taufkirchen, Germany). OxyBlot protein oxidation detection kit for the identification of protein carbonyls and ZipTip C_{18} were supplied by Millipore (Schwalbach, Germany). Endoproteinase Glu-C from *Staphylococcus aureus* V 8 and

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alkaline phosphatase (grade I) from calf intestine were obtained from Roche (Mannheim, Germany) and ECL detection reagent and Amersham Hyperfilm ECL from GE Healthcare (Munich, Germany).

Milk Samples. Bovine raw milk was purchased from a local dairy farm, and pasteurized, ultrahigh temperature (UHT), and condensed milks as well as liquid and powdered infant formulas were obtained from local retailers. Infant formulas IF_{L1} and IF_{L2} were growing-up milk products (12+ months), IF_{P1} was a follow-on product (10+ months), and IF_{P2} was a first product (0+ months). Infant formulas consisted mainly of milk and whey powder; IF_{P1} did not contain whey, but oligosaccharides as fibers. The products were sweetened with lactose (IF_{P2}), a mixture of lactose and maltodextrin (IF_{P1}, IF_{L1}), or maltodextrin alone (IF_{L2}). All infant formulas contained plant oils (IF_{P2} also contained a mixture of oils rich in long-chain poly unsaturated fatty acids) and were supplemented at least with minerals and vitamins. All information on the product composition was taken from the product labels. The powdered infant formulas were dissolved in bidistilled water following the manufacturer's instruction. All samples were centrifuged (Hettich centrifuge, Tuttlingen, Germany; 50 mL falcon tubes) at 1090*g* and 4 °C for 60 min so that the upper fat layer could be removed.

Preparation of Heated Raw Milk Samples. Aliquots of 150 μL of raw, defatted bovine milk were heated in closed vials at 72 °C for 60, 120, and 180 min and at 120 °C for 10, 30, and 60 min. The reaction was stopped by placing the vials in ice. Until analysis, the heated milk samples were kept at −18 °C.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Milk proteins were separated by discontinuous SDS-PAGE under reducing conditions as described before.[13] Briefly, milk samples were diluted 1:10; condensed milk and infant formulas were diluted to a protein concentration of 3.6 mg/mL. Ten microliters of each sample solution was separated by a separating gel (15% acrylamide, pH 8.8) and a stacking gel (6% acrylamide, pH 6.8) at 150 V for about 75 min.

Coomassie Staining. Protein bands were stained as described before using 0.1% Coomassie brilliant blue R-250 in 40% methanol containing 10% acetic acid.[13] The Coomassie stained gels have been used before to assess the distribution of CML in the heated milk protein, which has been analyzed in parallel to protein oxidation.[13]

Western Blot. The gels were electroblotted onto a nitrocellulose membrane, and protein transfer was checked by Ponceau staining as described before.[13] For the analysis of protein oxidation products, 5 μL of the defatted samples was diluted 20-fold with sample buffer and mixed with 5 μL of 10% SDS. Samples were derivatized for 15 min at room temperature by adding 10 μL of 2,4-dinitrophenylhydrazine (DNPH) solution from the OxyBlot kit. After neutralization with 7.5 μL of neutralization solution from the kit, proteins were separated by SDS-PAGE (10 μL loading capacity). Proteins were transferred to the membrane as described above. The membrane was blocked for 1 h with 1% bovine serum albumin (BSA) in phosphate-buffered saline−Tween 20 (PBST; 10 mM KH_2PO_4, 70 mM K_2HPO_4, 145 mM NaCl, 0.05% Tween 20, pH 7.4) and incubated overnight at 4 °C with the primary antibody from the kit (diluted 1:150 in PBST containing 1% BSA). The membrane was washed four times for 5 min with PBST and incubated with the secondary antibody from the kit (diluted 1:300 in PBST containing 1% BSA) for 1 h. The proteins were washed again four times for 5 min with PBST and then visualized using an ECL detection reagent and ECL Hyperfilm.

Data Analysis. The gel documentation system Versa Doc (Bio-Rad, Munich, Germany) was used.

Partial Enzymatic Protein Hydrolysis in Gel. For the identification of the selectively oxidized protein p22, peptide mass fingerprints were performed starting from underivatized raw milk and raw milk that had been heated for 30 min at 120 °C. Milk samples were diluted 10-fold with sample buffer, and 10 μL of this solution was loaded onto the gel. After protein separation by SDS-PAGE, the separating gel was washed three times for 5 min with bidistilled water. Proteins were stained for 1 h with Bio-Safe Coomassie under agitation, and the background was destained twice for 20 min and once for 40 min with bidistilled water under agitation.

Excision of interesting bands, in-gel digestion of the proteins, elution of the peptides, and ZipTip C_{18} purification was carried out as described previously with the exception that the gel was destained with 100 μL of 25 mM ammonium bicarbonate for 1 h including several changes of the destaining solution during this time.[16] The gel pieces were dried for 15 min, and Glu-C was used for in-gel digestion. For this purpose, 2 μL of a Glu-C solution (0.1 mg/mL) was added and incubated for 10 min at 4 °C. Gel pieces were covered with 20 μL of 25 mM ammonium bicarbonate buffer, pH 8.0, and incubated at room temperature for 16 h.

Matrix-Assisted Laser Desorption/Ionization (MALDI) Peptide Mass Fingerprint Analysis. An aliquot of 2 μL of peptide solution was mixed with an equivalent of 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 was spotted onto a stainless steel target and air-dried. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was performed on a Bruker Autoflex (Bruker Daltonik, Bremen, Germany) equipped with a nitrogen laser (λ = 337 nm) and operated in delayed extraction (80 ns) reflectron mode, with an accelerating voltage of 19 kV. External calibration was carried out with angiotensin I and II, substance P, bombesin, adrenocorticotropic hormone (ACTH) clip 1−17, and ACTH clip 18−39. For each spectrum, 300 laser shots were averaged from several positions on a spot.

Database Search. To assign the single peptides, the monoisotopic mass list from the database UniProtKB/Swiss-Prot and the software peptide mass, available at www.uniprot.org, were consulted (accession P02662, version 85). The following search parameters were applied: "with cysteines treated with nothing (in reduced form); with acrylamide adducts; with methionines oxidized; $[M + H]^+$; and monoisotopic". The selected enzyme was Glu-C (phosphate), and three missed cleavages were allowed.

Dephosphorylation of α-Casein. An aliquot of 1 μL of alkaline phosphatase was added to 60 μL of an α-casein (1 mg/mL) solution in buffer (pH 7.9, containing 584.4 mg NaCl, 788.0 mg TrisHCl, 203.3 mg $MgCl_2$ per 100 mL). After incubation for 60 min at 37 °C and at 600 rpm in a thermomixer (Eppendorf, Hamburg, Germany), the sample was transferred to an Amicon ultra-0.5 mL centrifugal filter and centrifuged at 10100*g* for 15 min. Five hundred microliters of water was added to the residue and centrifuged as described above, followed by addition of 500 μL of a 25 mM NH_4HCO_3 buffer, pH 8.0.

An aliquot of 3 μL of the supernatant was mixed with equal amounts of 2% TFA and DHAP matrix (7.6 mg of 2,5-dihydroxyacetophenone in 375 μL of ethanol mixed with 125 μL of a 80 mM diammonium hydrogen citrate solution). An aliquot of 1 μL was spotted onto a stainless steel target and air-dried. MALDI-TOF-MS analysis was performed in delayed extraction (140 ns) linear mode, with an accelerating voltage of 20 kV. External calibration was carried out with Bruker protein standard II. For each spectrum 300 laser shots were averaged from several positions on a spot.

Another 5 μL of the supernatant was separated by SDS-PAGE as described above.

RESULTS AND DISCUSSION

Mapping of Protein Oxidation Products in Heated Bovine Milk. To investigate the specific susceptibility of milk proteins to oxidation, raw milk was heated at 72 °C for 60−180 min and at 120 °C for 10−60 min. An unheated aliquot served as control.

After heating, the milk proteins were separated by one-dimensional (1D) SDS-PAGE and analyzed for protein-specific oxidation by combined chemical and immunochemical analysis. For this purpose, protein carbonyls, which are formed by oxidation, were derivatized with DNPH, and the resulting hydrazones were detected by an antibody specific for the DNP moieties (OxyBlot). As a control for unspecific binding, Western blot experiments were carried out without the derivatization step and did not result in any detectable signals (data not shown).

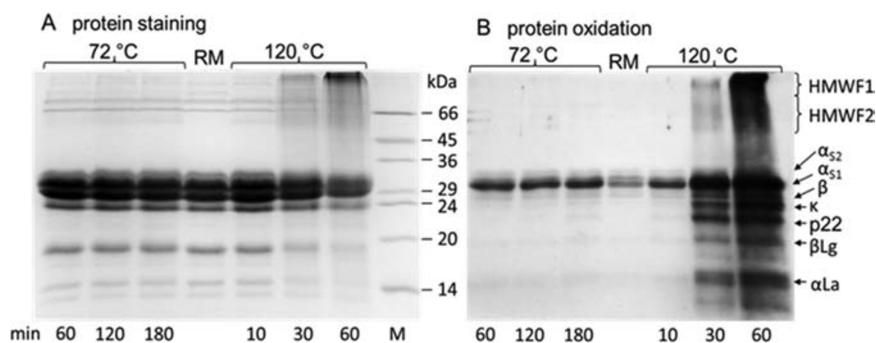

Figure 1. SDS-PAGE of raw milk heated at 72 and 120 °C for various time periods as indicated followed by Coomassie blue staining (A, see also ref 13) and Western blot for protein carbonyls (protein oxidation products) after derivatization with 2,4-dinitrophenylhydrazine (B). RM, raw milk; M, molecular weight marker; HMWF1, high molecular weight fraction 1; HMWF2, high molecular weight fraction 2; α_{S2}, α_{S2}-casein; α_{S1}, α_{S1}-casein; β, β-casein; κ, κ-casein; βLg, β-lactoglobulin; αLa, α-lactalbumin.

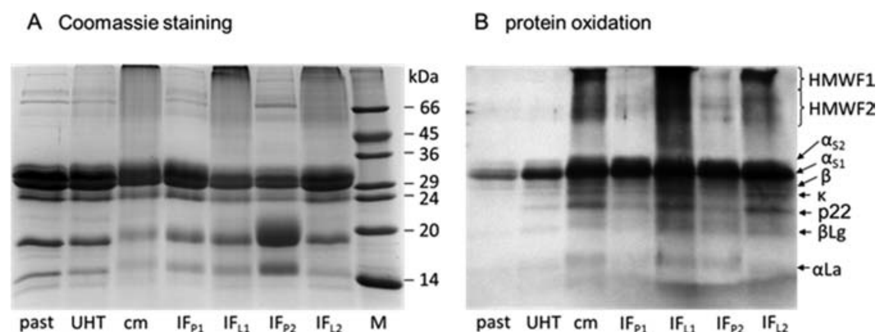

Figure 2. SDS-PAGE of commercial milk samples followed by Coomassie blue staining (A, see also ref 13) and Western blot for protein carbonyls (protein oxidation products) after derivatization with 2,4-dinitrophenylhydrazine (B). Lanes: past, pasteurized milk; UHT, UHT milk; cm, condensed milk; IF_{P1} and IF_{P2}, powdered infant formulas; IF_{L1} and IF_{L2}, liquid infant formulas; M, molecular weight marker; HMWF1, high molecular weight fraction 1; HMWF2, high molecular weight fraction 2; α_{S2}, α_{S2}-casein; α_{S1}, α_{S1}-casein; β, β-casein; κ, κ-casein; βLg, β-lactoglobulin; αLa, α-lactalbumin.

Whereas the protein oxidation in milk heated at 72 °C reached a maximal value already after 60 min, prolonged heating time at 120 °C increased the protein modification (Figure 1). After 60 min of heating, protein oxidation at 120 °C was more predominant than at 72 °C.

Protein oxidation products could already be detected in the casein fraction of raw milk (Figure 1). This background level may depict protein oxidation occurring during the storage or processing of raw milk, but also oxidation processes during sample workup and analysis cannot be excluded. Oxidation in raw milk, milk heated at 72 °C, and milk heated for 10 min at 120 °C was detectable only in the casein fraction. After further heating at 120 °C, however, protein oxidation was observed in all protein fractions. In these samples, α-lactalbumin appeared to be more intensively oxidized compared to β-lactoglobulin, particularly if it is taken into account that the α-lactalbumin concentration in milk is <50% of the β-lactoglobulin concentration. Additionally, a relatively strong band could be detected when the protein oxidation products were probed, which was hardly visible in the Coomassie stain. This protein (p22) appeared between the bands of κ-casein and β-lactoglobulin with an apparent molecular mass of 22 kDa and could not be assigned to any of the previously identified major milk proteins.

In contrast to protein carbonyl analysis after 1D gel electrophoresis, the derivatization of protein oxidation products with DNPH and subsequent Western blot analysis of 2D electrophoresis gels led to unselective protein staining independent from the heat treatment of the samples, indicating artifact formation. The artifact formation is probably related to the sample buffer used for isoelectric focusing, because 1D SDS-PAGE of samples dissolved in this buffer also led to inconsistent staining, which was not related to thermal treatment. Therefore, 2D gel electrophoresis could not be used to increase the resolution of protein separation.

Mapping of the Protein-Specific Oxidation in Commercial Milk Products. Next, protein-specific oxidation was analyzed in several commercial heat-treated milk products, including pasteurized, UHT, and condensed milks and powdered as well as liquid infant formulas. The results were very similar to those obtained for the heated milk samples (Figure 2). Protein oxidation was particularly intense in liquid infant formulas, followed by condensed milk and powdered infant formulas. These products gave signal intensities similar to those of raw milk heated for 30 or 60 min at 120 °C. In pasteurized milk, no additional modification compared to raw milk was recorded, similar to the milk samples heated for 60 min at 72 °C. Damage of UHT milk was consistent with raw milk heated at 120 °C for 10 min.

The distribution of protein oxidation products in the milk proteomes of the commercial products was also similar to that in heated milk samples, confirming a preferential oxidation of p22. Interestingly, in the whey-enriched formula (IF_{P2}), the higher concentration of whey proteins was not reflected by particularly intense bands of these proteins in the OxyBlot.

Identification of the Selectively Oxidized Protein by Peptide Mass Fingerprint Analysis. The preferentially oxidized unknown protein in heated milk and commercial milk

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products migrated in SDS-PAGE between κ-casein and β-lactoglobulin with an apparent molecular mass of 22 kDa (p22). To identify p22, unheated milk and raw milk heated at 120 °C for 30 min were separated by SDS-PAGE. Protein p22 was analyzed by MALDI-TOF-MS after in-gel digestion with Glu-C (Figure 3). A database search for the peptide mass fingerprint is summarized in Table 1. All major peptides could be assigned to α_{S1}-casein, leading to a sequence coverage of 56.3%. For comparison, the peptide mass fingerprint was also determined from the original α_{S1}-casein band, which yielded a similar mass spectrum, however, with sequence coverage of 63.3%. Thus, it can be concluded that p22 is a variant of α_{S1}-casein (see Discussion) and is, therefore, referred to as α_{S1}-casein*. Whether the native α_{S1}-casein is also more intensively oxidized than other milk proteins cannot be determined, because the resolution was not sufficient to determine the oxidation rates of distinct casein species.

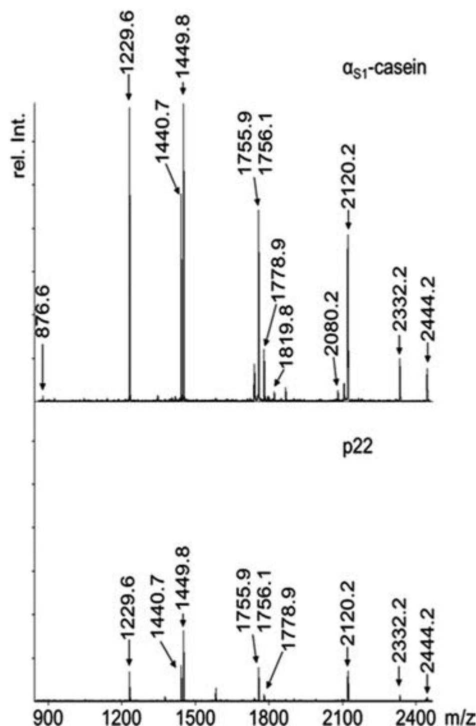

Figure 3. Peptide mass fingerprint of α_{S1}-casein (A) and p22 (B) obtained from milk (30 min/120 °C) by SDS-PAGE. Mass spectra were recorded by MALDI-TOF-MS after in-gel digestion with Glu-C.

Discussion. Apart from glycation products, protein oxidation products are very important protein modifications in heated milk,[3,4] which must be monitored to ensure the nutritional and technological quality of the milk proteins. The predominant protein oxidation products in milk have been identified as methionine sulfoxide, aminoadipic semialdehyde, cysteine sulfenic acid, and hydroxytryptophan.[2] Furthermore, arginine, proline, threonine, and tyrosine can be affected by oxidation reactions.[6,7] The analysis of protein-specific oxidation products is generally achieved by the analysis of protein carbonyls by OxyBlot. After derivatization of the carbonyl groups with DNPH and electrophoretic separation of the proteins, protein carbonyls can be immunoblotted using an antibody specific to the resulting hydrazones.[16] Carbonyl groups are introduced into proteins, for example, by the oxidation of lysine, arginine, proline, or threonine.[7] More recently, it was shown that also cysteine sulfenic acid, a key oxidation product of cysteine, is detected by this method.[17]

Mapping of protein oxidation products revealed that protein carbonyl formation was dependent on the protein structure. Thus, it was shown that α-lactalbumin was more prone to protein oxidation compared to β-lactoglobulin. Preferential oxidation can be caused, for example, by the protein conformation or by a higher content of amino acids, which can be oxidized to DNPH-reactive products.[18]

Protein-specific oxidation has been investigated before in milk products using OxyBlot analysis. Scaloni et al. investigated differently heated milk samples and obtained a similar staining pattern as in the present study.[19] Using a similar approach, Fenaille et al. investigated the protein oxidation in UHT milk and milk powder samples.[8] In contrast to the results of Scaloni et al. and the present study, the most intense staining was observed for the β-lactoglobulin band. Because the exact nature of the analyzed milk powder samples is not known, the reason for this discrepancy remains speculative.

Interestingly, preferential staining of α_{S1}-casein* was observed. The preferential oxidation of α_{S1}-casein* is more likely than a selective conversion of α_{S1}-casein into α_{S1}-casein* as a result of oxidation, because the band intensity increased with prolonged heating time in the OxyBlot, but not in the Coomassie stain. The nature of the α_{S1}-casein variant has not been fully established. The sequence coverage of α_{S1}-casein* was 56.3% compared to 63.3% for the native α_{S1}-casein. The missing peptide sequences may indicate a truncated form, which could arise by hydrolysis involving endogenous milk proteases. However, the sequence fragments (AA 142−148 and 193−199), which were not detected in the α_{S1}-casein*, are low-abundant peptides in native α_{S1}-casein. Thus, the absence of these peptides in the spectrum of α_{S1}-casein* may also be attributed to the generally lower peak intensity obtained for the variant due to its low concentration in milk. However, the presence of a truncated protein cannot be excluded. Another possible assignment of α_{S1}-casein* is based on its migration behavior in SDS-PAGE. α_{S1}-Casein shows a remarkably lower mobility in SDS-PAGE than expected on the basis of its molecular mass of 23.6 kDa. This anomalous behavior results from an unexpectedly large hydrodynamic size of the α_{S1}-casein/SDS complex, which could be caused, for example, by a high density of negative charge in the amino acid sequence 43−78.[20] Apart from negatively charged amino acid side chains, six phosphate groups are located in this area. The migration of α_{S1}-casein* resembles the expected behavior of the protein much more closely, suggesting that it represents a dephosphorylated form. However, the enzymatic dephosphorylation of α-casein, which was confirmed by MALDI-TOF-MS analysis, did not change the electrophoretic mobility of α_{S1}-casein (data not shown).

For the present study, milk samples were heated at 72 and 120 °C, temperatures that are applied in industrial processes for pasteurization and sterilization. Industrial heating is much more effective than the heating procedure applied in our laboratory. Previous studies have already searched for heating parameters that lead to heat damage similar to the damage observed in industrial products. Longer heating periods were chosen as compensation for the lower heating efficiency under laboratory conditions. It was detected that raw milk heated at 72 °C for 180 min under the applied conditions showed modification rates similar to those of UHT milk, and raw milk heated at 120 °C for 30 − 60 min showed modification rates similar to those of condensed milk.[13] Therefore, the formerly determined heating

Table 1. Peptides Obtained by MALDI-TOF-MS from α_{S1}-Casein and the Unknown Protein p22 after SDS-PAGE of Unheated Milk and Raw Milk Heated at 120 °C for 30 min and In-Gel Digestion of the Respective Bands with Glu-C

			measured mass (Da)			
			α_{S1}-casein		p22	
α_{S1}-casein position	modification[a]	theor. mass (Da)	unheated	heated	unheated	heated
193−199		876.5	876.5	876.6		
193−199	MSO: 196	892.5	892.5			
119−125		900.5		900.5		
142−148		902.4		902.5		
31−39		1049.6	1049.5	1049.6	1049.6	1049.6
149−157		1229.6	1229.5	1229.7	1229.6	1229.7
85−96		1440.7	1440.6	1440.7	1440.6	1440.7
19−30		1449.8	1449.7	1449.8	1449.7	1449.8
71−84		1648.9	1649.0			
1−14		1664.9	1664.8			
111−125[b]	MSO: 123	1755.9	1755.9	1756.1	1756.0	1756.1
97−110[b]		1756.1	1755.9	1756.1	1756.0	1756.1
126−141		1778.9	1778.8	1778.9	1778.7	1778.9
126−141	MSO: 135	1794.9	1794.8	1794.9		
111−125	PHOS: 115	1819.8	1819.7	1819.9		1819.8
97−110	lactose	2080.1		2080.2		
1−18		2120.2	2121.0	2120.2	2120.1	2120.2
78−96		2332.2	2332.1	2332.2	2332.0	2332.2
1−18	lactose	2444.2		2444.4		2444.2
α_{S1}-casein sequence coverage (%)			63.3	63.3	56.3	56.3

[a]The indicated modifications are as follows: MSO, methionine sulfoxide; PHOS, phosphate residue; lactose, lactose adduct. [b]Peptides cannot be distinguished with the available mass accuracy.

parameters were adopted for the present study. To confirm that the chosen conditions were relevant indeed, commercial milk products, namely, pasteurized milk, UHT milk, and condensed milk as well as powdered and liquid infant formulas, were analyzed in the same way as the laboratory-heated milk samples. The present investigation actually confirmed that protein oxidation obtained under the described laboratory heating conditions is in a similar range as the protein oxidation in industrially produced samples (Figures 1 and 2).

The highest staining of protein oxidation products was obtained in a powdered infant formula. In contrast, the highest overall content of CML had been detected in condensed milk, when the same set of samples was analyzed.[13] Thus, it can be concluded that not only the extent but also the nature of the protein modifications depends on the processing conditions. A similar trend was observed before, when the site-specific oxidation of whey proteins was analyzed in different milk products.[3] Oxidation may be promoted in infant formulas, for example, by pro-oxidative ingredients (such as iron) or by the production process (such as spray-drying).[21] Further studies, however, are warranted to understand the influence of different production parameters on various nePTMs in milk products.

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Notes

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ABBREVIATIONS USED

nePTM, nonenzymatic posttranslational modification; CML, N^{ε}-carboxymethyllysine; DOPA, 3,4-dihydroxphenylalanine; TFA, trifluoroacetic acid; UHT, ultrahigh temperature; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DNPH, 2,4-dinitrophenylhydrazine; BSA, bovine serum albumin; PBST, phosphate-buffered saline Tween 20 (10 mM KH_2PO_4, 70 mM K_2HPO_4, 145 mM NaCl, 0.05% Tween 20, pH 7.4); DTT, dithiothreitol; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ACTH, adrenocorticotropic hormone; 1D, one-dimensional.

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