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Analysis of the Peptide Profile of Milk and Its Changes during Thermal Treatment and Storage

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In this study a new method was developed for analysis of the low molecular weight protein fraction of milk, allowing a simple and fast overview of the peptide profile of various milk samples. For this purpose, immobilized metal affinity chromatography (IMAC) was coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). By this technique, two major peptides in milk could be identified as fragments of α-s1-casein. During heat treatment of raw milk, five new peptides were generated, the origin of which could be assigned to the casein fraction. Storage experiments with extended shelf life milk at 4 °C did not show any changes in the peptide profile, whereas in ultra high temperature milk stored at room temperature, one peptide increased significantly, which was identified as the N-terminus of α-s1-casein. The peptide was assumed to be formed in an enzymatic reaction, which was confirmed in a storage experiment with sterilized milk. Analyses of different commercially available milk samples confirmed the results obtained with the heated and stored milk. Furthermore, differences in the peptide profiles of the samples, probably due to different cow breeds or lactation stages, were observed. These results establish IMAC prior to MALDI-TOF-MS as a valid tool for the rapid analysis of the peptide profile of milk.

KEYWORDS: Milk; peptides; proteolysis; heat treatment; storage; immobilized metal affinity chromatography; matrix-assisted laser desorption/ionization mass spectrometry

INTRODUCTION

Recent studies showed that the milk proteome is much more complex than anticipated. Although the main proteins α-s1-casein, α-s2-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin are well-studied, many questions about the details of the protein composition still remain. The presence of genetic variants, posttranslational modifications, and proteolytic reactions in milk lead to a striking complexity, which is not fully understood (*1*).

In particular, the peptide fraction that results from hydrolytic activity of several milk proteinases is a wide field that has not been clarified yet. However, this protein class is of high importance with respect to nutritional, technological, and sensory properties of milk. Encrypted bioactive peptides in milk proteins with, for example, opioid, immunostimulating, or antithrombotic characteristics have been studied extensively (*2*) and are discussed for use as nutraceuticals (*3*). On the other hand, a high content of hydrolytic fragments of milk proteins is not wanted in the milk industry as it has a negative impact on the gelation behavior, for example, of stored ultra high temperature (UHT) milk or on the clotting properties in cheese production (*4, 5*). Furthermore, proteolysis is linked with astringent or bitter off-flavors in milk and cheese (*6, 7*) and should, therefore, be minimized.

Hence, proteolysis has been the focus of many studies in which the non-protein nitrogen in the acid-soluble fraction served as an indicator for hydrolytic protein breakdown (*8*). In parallel, casein hydrolysis can be followed by gel electrophoretic methods (*9*). However, those techniques are not very sensitive and do not allow a satisfying separation of the peptides. Another approach to analyze the peptides in milk was high-performance liquid chromatography (*10*), which revealed the formation of two new compounds during thermal treatment. The structure of these compounds, however, was not determined. In this context, mass spectrometric techniques offer a clear advantage, as they allow additional structural characterization of the peptide

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fraction in milk. In the past, mass spectrometry has been successfully applied to analyze modifications of milk proteins that occur during thermal treatment (*11, 12*). For milk peptides, however, this method has been used only for the investigation of milk in the involution stage (*13*) or for cheese (*14–16*), whereas further studies with consumer milk do not exist.

The goal of our study was to develop a simple and fast method for obtaining an overview of the peptide profile of milk. For this purpose, we used immobilized metal affinity chromatography (IMAC) for a cleanup of the milk samples before they were submitted to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). With this method, it was possible to get an overview of the peptide profile of raw milk. Furthermore, changes were analyzed that occur during heat treatment of milk as well as during storage. Additionally, MALDI-TOF/TOF-MS measurements were performed to identify some of the detected peptides. The analysis of commercial milk samples confirmed the validity of the model experiments.

MATERIALS AND METHODS

Materials and Milk Samples. The magnetic beads IMAC-Cu kit was purchased from Bruker Daltonics (Bremen, Germany). α-Cyano-4-hydroxycinnamic acid (HCCA, purriss p.a.) was obtained from Fluka (Taufkirchen, Germany). Poly(vinylidene difluoride) (PVDF) membrane filters (0.45 μm) were from Roth (Karlsruhe, Germany). Raw milk samples from different cows, collected at different points during 1 year, were obtained from a local dairy farm. Extended shelf life (ESL) milk and UHT milk for storage experiments were provided by a local producer. Sterilized milk and commercial milk products (pasteurized milk, high-temperature milk, and UHT milk) were purchased from several supermarkets. In the following, the term "UHT milk" will be used for milk that was heated at minimum 135 °C for 2−20 s in a continuous flow-through process, while sterilized milk was sterilized by in-bottle heat treatment at a temperature of 105 °C or higher for 20−40 min.

Defatting and Isolation of Casein and Whey Fractions. The milk was defatted by centrifugation at 3850 rpm and 4 °C for 60 min. The upper fat layer was removed. Sodium acetate buffer (0.5 M, pH 4.0, 250 μL) was added to 2 mL of raw milk, resulting in a pH of 4.6. After 5 min at room temperature, the milk was centrifuged for 5 min at 3850 rpm and 4 °C, and the supernatant was filtered through a PVDF membrane filter (0.45 μm). After the pH was adjusted to 6.8 with potassium hydroxide, the filtrate was used directly for the incubation experiment of the whey fraction. Remaining whey proteins in the centrifugation pellet were removed by washing the pellet twice with 2 mL of 0.1 M sodium acetate buffer, pH 4.6, and centrifuging for 5 min at 3850 rpm and 4 °C. The pellet was suspended in a buffer resembling the salt composition of milk (10 mM phosphate buffer with 8 mM sodium chloride, pH 6.8). This suspension was used for the heating of the casein fraction.

Heating of Raw Milk, Casein, and Whey Fraction. Heat treatments were all done in triplicate. Aliquots of 200 μL of raw milk were heated in 2 mL plastic tubes at temperatures of 72, 85, and 120 °C, respectively. After 10, 20, and 30 min, the tubes were taken and immediately cooled down in ice. In an analogous experiment, the whey and casein fractions were heated at a temperature of 120 °C. The described heating experiments were repeated with raw milk from different cows collected at different points during 1 year as well as with commercially available pasteurized milk.

Storage Experiments. The storage experiments were performed in triplicate. Packages of ESL and UHT milk were provided directly after production by a local manufacturer. The former was stored at a temperature of 4 °C for up to 21 days. The storage of the latter was carried out at room temperature for up to 12 weeks. For the storage experiment with sterilized milk, the milk was stored at room temperature for up to 12 weeks. For sample drawing, packages were opened and the milk was defatted as described above and stored at −20 °C prior to analysis.

Purification of Milk by Immobilized Metal Affinity Chromatography. The milk samples were purified by use of IMAC-Cu magnetic beads. The purification was performed according to the manufacturer's instructions. Briefly, 5 μL of the magnetic beads were rinsed three times with 50 μL of binding solution. Then, 20 μL of binding solution and 5 μL of milk sample were added, and the mixture was mixed carefully. After 10 min of incubation, the magnetic beads were washed three times with washing solution. Finally, the purified peptides and proteins were retrieved by incubation with the elution solution for 10 min.

MALDI-TOF Mass Spectrometry. For MALDI-TOF-MS analysis, 1 μL of the elution solution was mixed with 5 μL of a saturated solution of HCCA in 50% acetonitrile/0.1% trifluoroacetic acid (TFA). An aliquot of 1 μL was spotted twice on a stainless steel target and air-dried. MALDI-TOF-MS analysis was performed on a Bruker Autoflex (Bruker Daltonik, Bremen, Germany), equipped with a nitrogen laser ($\lambda = 337$ nm). Measurement of the low molecular weight proteins was carried out by delayed extraction (140 ns). Laser-desorbed positive ions were analyzed after acceleration by 20 kV in the linear mode. External calibration was performed with a mix of angiotensin I and II, substance P, bombesin, adrenocorticotropic hormone clip 1−17 and 18−39, somatostatin, and insulin. For each displayed mass spectrum, at least 150 individual spectra obtained from several positions on a spot were averaged. Mass accuracy was 150 ppm.

MALDI-TOF/TOF Analysis and Data Processing. For selected peptides, MALDI-TOF/TOF-MS analyses were performed at the Institute of Molecular Systems Biology (Swiss Federal Institute of Technology Zurich) in order to determine the amino acid sequence. An aliquot of 1 μL of each peptide solution was spotted on a stainless steel MALDI plate. After drying, 1 μL of the matrix solution (2.5 mg of HCCA in 1 mL of 50% acetonitrile/0.1% TFA) was put on each peptide-containing spot on the MALDI target. Mass spectra were acquired on a Proteomics Analyzer 4800 (MALDI-TOF/TOF) mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a 200 Hz solid-state laser ($\lambda = 337$ nm). Totals of 1500 and 2500 shots were accumulated for each MS- and MS/MS-spectrum, respectively. Peptide identification was carried out by searching against the SWISS-PROT-database (release 11.2; taxonomy = other mammalia) by use of an in house version of MASCOT (*17*). The following search parameters were applied: precursor mass tolerance =100 ppm; fragment mass tolerance =0.25 Da. No enzyme specificity and modification were allowed. Only peptides identified with a confidence of at least 95% were considered to be correct calls. In addition, all MS/MS-spectra that could be assigned to a peptide sequence were manually checked and the results were verified.

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 AND DISCUSSION

Analysis of Peptide Profile of Raw Milk. This study aimed at the development of a simple and fast method for characterization of the peptide profile of milk. For this purpose, milk peptides and proteins were purified from defatted raw milk by means of metal affinity chromatography magnetic beads and subsequently directly analyzed by MALDI-TOF-MS. In order to obtain optimal coverage of the peptide fraction below 6000 Da, α-cyano-4-hydroxycinnamic acid was used as matrix.

Figure 1 depicts a typical spectrum of raw milk after purification by metal affinity chromatography. It shows several distinct signals, the predominant ones between approximately *m*/*z* 1900 and 4500 (see also **Table 1**). After treatment with IMAC-Cu beads, the signal-to-noise-ratio was considerably improved. On the other hand, however, a reduction or increase in the intensity of some signals was observed, obviously due to lower or higher affinity of the corresponding peptides for copper (data not shown).

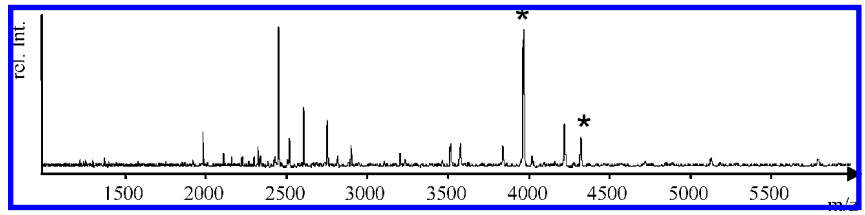

Figure 1. MALDI-TOF-MS spectrum of raw milk after purification with IMAC-Cu magnetic beads. Peptides that were structurally identified by means of MALDI-TOF/TOF-MS as discussed in the text are marked with asterisks.

Table 1. Most Intensive Peptides That Were Detected in the Various Milk Samples after Purification by Metal Affinity Chromatography[a]

peptide (Da)	raw milk	heated milk	raw whey	heated whey	raw casein	heated casein	stored ESL milk	stored UHT milk	stored sterilized milk
1974.4		xx				xx		x	x
1993.2	x	x			x	x	x	x	x
2218.7		xx				xx		x	x
2235.8	x	x	x	x			x	x	x
2462.0	x	x	x	x			x	x	x
2618.2	x	x	x	x			x	x	x
2765.4	x	x	x	x	x	x	x	x	x
2912.4	x	x	x	x	x	x	x	x	x
3730.1		xx				xx		x	x
3856.2	x	x			x	x	x	x	x
3984.2	x	x			x	x	x	x	x
4241.3	x	x			x	x	x	x	x
4297.8		xx						x	x
4340.4	x	x			x	x	x	xxx	x
4436.8		xx				xx		x	x

[a] Listed peptide masses were measured in the linear mode. Mass accuracy was 150 ppm. Newly formed peptides are indicated with xx, peptides with higher intensity compared to raw milk with xxx.

MALDI-TOF-MS has been suggested as a useful tool for monitoring the protein profile of milk (*18, 19*) or dairy products (*20–23*). However, those studies focused on analysis of the main proteins of milk, whereas the low molecular weight protein fraction was not further discussed. Furthermore, MALDI-TOF-MS was applied for the detection of peptides in cheese after extraction with ethanol (*14*) or in milk during involution (*13*), but for consumer milk, a similar study has not been reported yet.

Immobilized metal affinity chromatography is already an established technique for the cleanup of biological samples such as plasma (*24*) and has been shown to reproducibly enrich a high amount of peptides and proteins from complex sample matrices (*25*). To our knowledge, however, its application for milk has not been reported yet. This cleanup technique allowed a significant improvement of the signal-to-noise ratio, since additional milk ingredients that interfere with the mass spectrometric analysis (e.g., lactose) were obviously successfully removed. Other established techniques for the recovery of peptides in milk, such as precipitation with TCA or ultracentrifugation, mainly aim at the separation of the peptides from the milk proteins, whereas low molecular weight substances are not removed. Further advantages of the IMAC beads are very easy handling as well as high sample throughput and short analysis time. Proteins and peptides are bound to copper immobilized at the IMAC beads via carboxylic and phosphate groups, as well as via amino acid side chains with an electron donor group, such as histidine, cysteine, or tryptophan. The presence of many binding partners for the IMAC beads in the protein structure thus allows an unspecific enrichment of the peptide fraction. However, it should be borne in mind that a certain part of the information is lost during the purification step, since peptides that do not offer accessible electron donor groups are not recovered by the IMAC beads. The number of lost peptides, however, is surprisingly small and thus acceptable, in view of the parallel improvement of spectral quality, which allows a much more sensitive analysis of minor components. Therefore, the method described herein is a valid, fast, and simple tool for obtaining a peptide fingerprint of milk.

The prominent signal at *m/z* 3984 was further analyzed by MALDI-TOF/TOF-MS. The appearance of y- and b-fragments in the second mass spectrum allows the identification of the amino acid sequence. Thus, it was possible to assign the amino acid sequence RPKHPIKHQGLPQEVLNENLLRFFVAPF-PEVFGK to the prominent signal at *m/z* 3984 (see **Figure 2**), a fragment of α-s1-casein (amino acid position 1−34). Milk contains a number of proteolytically active enzymes like plasmin, which lead to protein hydrolysis even in the mammary gland (*26*). The dominating proteolytic products in milk are the γ-caseins and the proteose peptones, which are generated through plasmin activity from β-casein (*27, 28*). Furthermore, the susceptibility of α-s1-casein to plasmin hydrolysis was reported (*29*) and amino acid positions 34/35 were identified as one of several preferred cleavage sites of plasmin in a model solution (*29, 30*). Therefore, it is very likely that the detected peptide resulted from enzymatic cleavage at amino acids 34/35 of α-s1-casein by plasmin in the mammary gland before the milking process.

The sequence corresponding to the signal at *m/z* 4340 was identified as RPKHPIKHQGLPQEVLNENLLRFFVAPF-PEVFGKEKV, also a fragment of α-s1-casein (amino acids 1−37). As plasmin cleaves highly specifically at lysine and arginine residues (*31*), the observed cleavage between amino acids (aa) 37/38 (valine/asparagine) cannot be explained by plasmin proteolysis. Kelly et al. (*32*) detected an α-s1-casein fragment aa38 − *x* in raw milk, supporting the presence of the cleavage site aa 37/38. Therefore, the peptide is likely generated through the action of another heat-stable proteinase, for example, a lysosomal proteinase in milk with cleavage specificity for this site. It has been suggested that active proteinases other than plasmin are present in milk, as the pattern of plasmin proteolysis of caseins was different from that of stored milk (*27*). Further

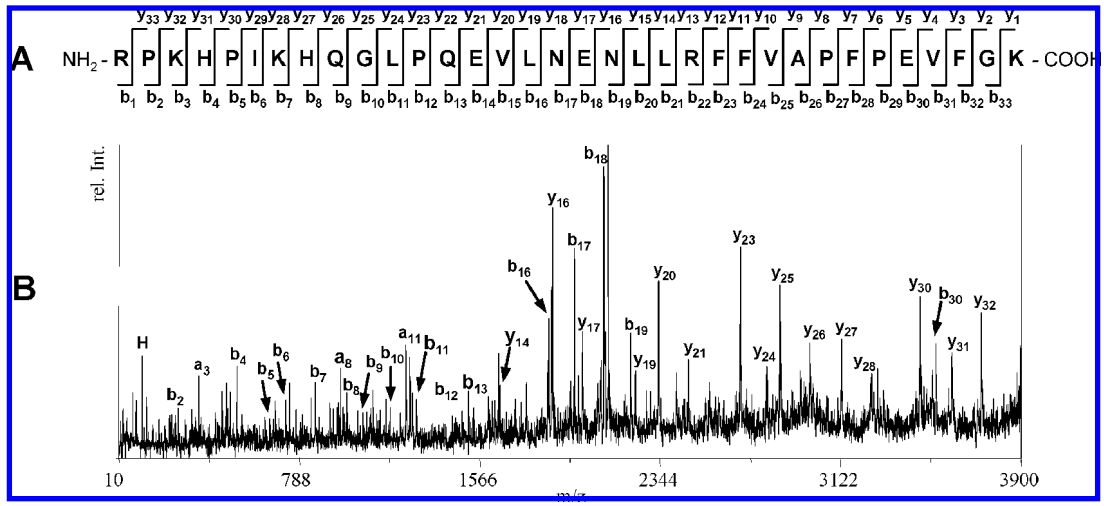

Figure 2. MALDI-TOF/TOF analysis of the MS peak detected at *m*/*z* 3981.19 (in the reflector mode). (A) Sequence of the identified α-S1 casein fragment and its fragmentation pattern observed under collision-induced dissociation. (B) Fragmentation of the peptide backbone during tandem mass analysis, resulting in the characteristic b- and y-ions. Other dominant fragment ions like the immonium ion of histidine (H) and some a-ions that represent typical byproducts of the high-collision energy fragmentation are also displayed.

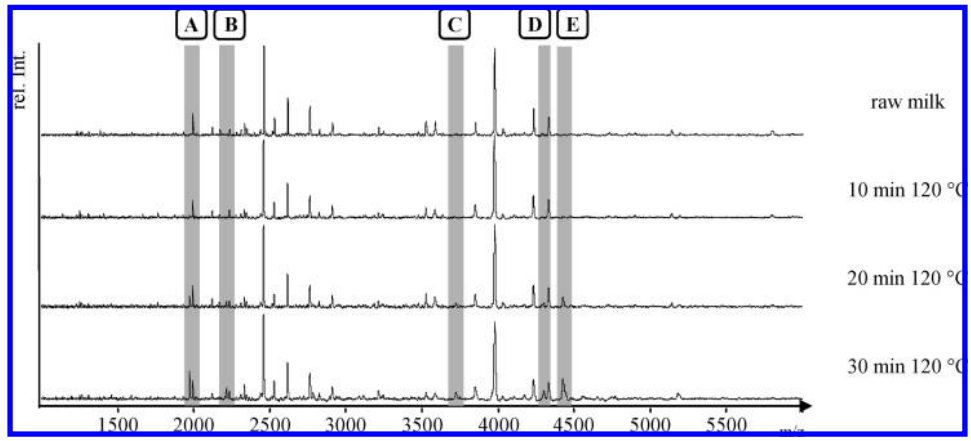

Figure 3. MALDI-TOF-MS spectra of heated raw milk. Raw milk was defatted and heated at a temperature of 120 °C for 10, 20, and 30 min. After heat treatment, the milk was purified by means of metal affinity chromatography magnetic beads and directly analyzed via MALDI-TOF-MS. Changes are highlighted by gray boxes.

detected peptides in raw milk are highly interesting targets for sequence analysis by mass spectrometry.

The peptide fraction of milk is very important for the nutritional, technological, and sensory properties of milk and milk products (*2, 4–6*). Therefore, a systematic analysis of the composition of this fraction is necessary to fully evaluate physiological and technological consequences of the milk peptides. This could be achieved by MALDI-TOF/TOF analysis after treatment of the samples with IMAC-Cu beads as demonstrated for the peptides at *m*/*z* 3984 and 4340.

Heating Experiment with Raw Milk. Milk is usually heated in order to obtain a safe product with long shelf life or to prepare milk powders or hypoallergenic products. In order to investigate changes in the peptide pattern of milk during thermal treatment, raw milk was heated at three typical temperatures applied in the dairy industry (72, 85, and120 °C). The peptide profile was monitored after metal affinity chromatography via MALDI-TOF-MS. The spectra obtained after heating at 120 °C are shown in **Figure 3**. Throughout the heating, five new signals were detected at *m*/*z* 1974.4, 2218.7, 3730.1, 4297.8, and 4436.8, respectively, that had not been present in raw milk. The same observations were made in the spectra of the milk heated at 72 and 85 °C, but to a lower extent (data not shown). Hence, the formation of the new peptides seems to be clearly dependent on the heating time as well as on the applied temperature.

For acquiring semiquantitative information about the newly formed peaks at 120 °C, relative quantification of the peak intensities was performed (see **Figure 4**). The signal at *m*/*z* 3984 was chosen as reference and its intensity was defined as 100%. The concentration of this peptide was considered as constant since its ratio to all other signals in the spectra, except for the five peptides that were generated during the heating process, did not change significantly throughout the thermal treatment. The signal intensities of the peptides depend on parameters such as amino acid sequence or ionization properties, so that the obtained values do not represent absolute concentrations of the peptides in the heated milk. Even so, they can be considered as semiquantitative estimation, reflecting the formation of the peptides during heat treatment. The intensities of the signals at *m*/*z* 1974.4, 2218.7, 3730.1, 4297.8, and 4436.8 increased significantly within 30 min and yielded relative intensities of 38%, 17%, 10%, 12%, and 27%, respectively, as a consequence of the increasing heat load. Absolute quantification of the peptides was beyond the objectives of this study.

To investigate the origin of the peptides formed during the heating experiment, the raw milk was divided into casein and

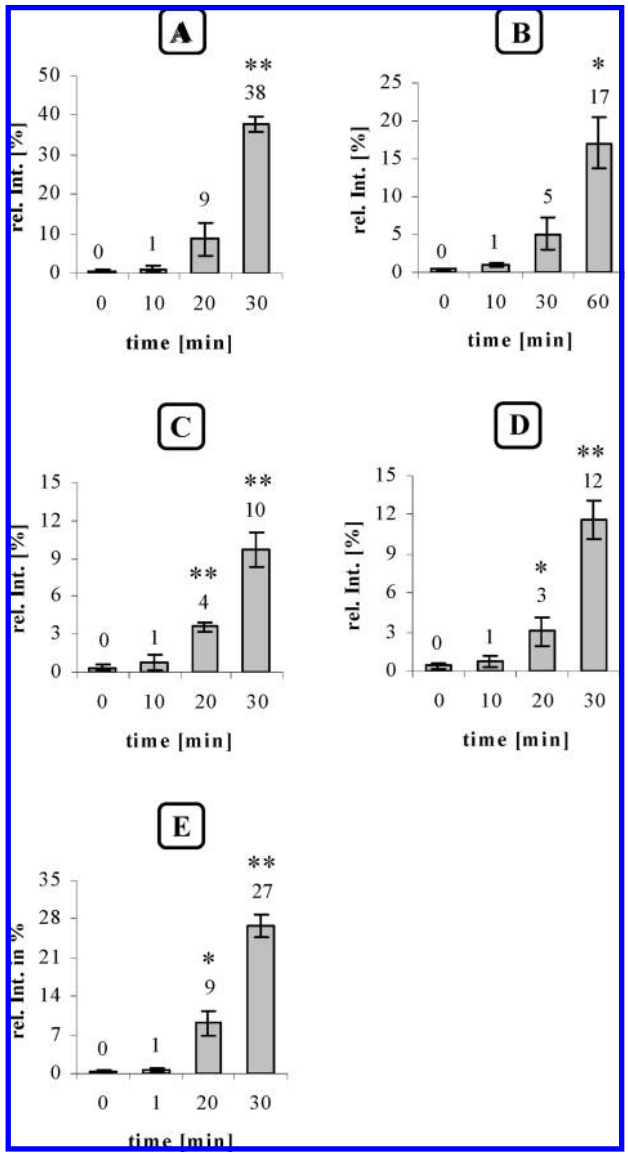

Figure 4. Relative quantification of selected peptides in milk heated at 120 °C. The signal at *m/z* 3984 was chosen as reference and its intensity was set as 100%. The mean values of an independent triplicate ± standard deviation are presented. (A) Peptide 1974.4; (B) peptide 2218.7; (C) peptide 3730.1; (D) peptide 4297.8; (E) peptide 4436.8. *$p < 0.05$; **$p < 0.01$

whey protein fractions by adjusting the pH to 4.6, the isoelectric point of the caseins. The precipitated caseins were separated from the soluble whey proteins and resuspended in a milk-resembling buffer, whereas the pH of the whey protein solution was readjusted to 6.8. The casein suspension as well as the whey protein solution was subsequently heated at 120 °C, analogously to the experiment with raw milk. The spectra of the whey proteins did not show changes after the heating process (see **Table 1**). However, when the heated caseins were analyzed, the same peptides were formed as in the heated milk (see **Table 1**). Therefore, it was concluded that the newly formed peptides result from a fragmentation of the caseins. All heating experiments were repeated with several raw milk samples that had been collected from different cows within 1 year, as well as with pasteurized milk from the supermarket, and yielded similar results.

Studies with isolated α-, *β*-, and *κ*-caseins showed an increase of non-protein nitrogen when subjected to temperatures of 100 °C or higher (*8*). Similar results were obtained by Hindle and Wheelock (*33*), who confirmed the release of peptides by the action of heat on bovine milk. A more detailed study was presented by Morales and Jiménez-Pérez (*10*) by means of high-performance liquid chromatography; they reported two newly formed peptides after heating of milk . As they noticed the same peptides in a milk-resembling system that did not contain whey proteins but only caseins and lactose, they attributed the source of the peptides to the casein fraction. However, the structure of the peptides was not available.

In general, protein degradation can occur via two principal pathways: either by hydrolysis, enzymatic or acid-catalyzed, or through radical fragmentation of the protein backbone. Enzymatically induced formation of the peptides can be excluded because of the high temperature treatment (120 °C). Furthermore, enzymatic hydrolysis would lead to a different peptide profile, as shown below. A measurable acid-catalyzed proteolysis also seems less likely under the given conditions. Whereas no selectivity would be expected in an acid-catalyzed proteolysis, an evidently selective formation of particular peptides was observed in the heating experiment. On the other hand, it is well established that radicals that are generated during the Maillard reaction can promote a selective attack on the protein backbone (*34*). Hence, we assume that the heat treatment of the milk led to the Maillard reaction, which induced radical formation and subsequent selective attack of the protein backbone, yielding the five peptides observed. In a previous study, it has already been demonstrated that the Maillard reaction highly promotes the oxidation of side chains of whey proteins (*12*). On the other hand, we cannot exclude that the Maillard reaction modifies some peptides, which had not been recovered from raw milk, in such a way that they bind to the IMAC beads.

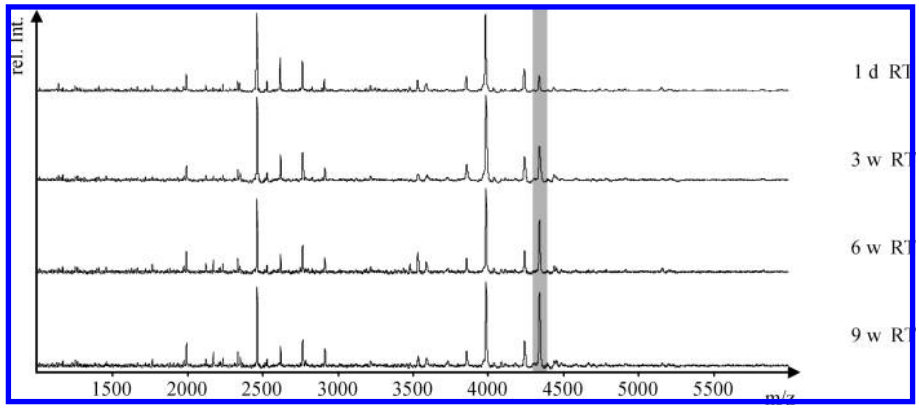

Figure 5. MALDI-TOF-MS spectra of stored UHT milk. The UHT milk was kept at room temperature for 3, 6, and 9 weeks, and mass spectra were recorded after purification by means of metal affinity chromatography magnetic beads. Changes are highlighted by gray boxes.

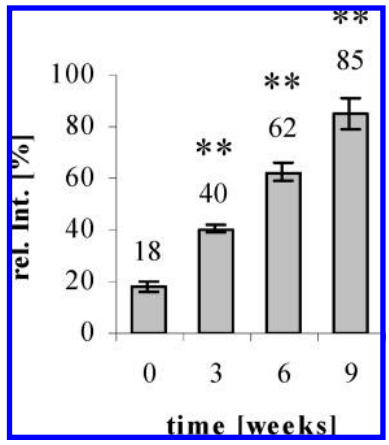

Figure 6. Relative quantification of the peptide detected at *m/z* 4340.4 during storage of UHT milk. The signal at *m/z* 984 was chosen as reference and its intensity was set as 100%. The mean values of an independent triplicate ± standard deviation are presented. $^{**}p < 0.01$.

Storage Experiments. In a next step, changes in the low molecular weight fraction of milk during storage were analyzed by applying the developed method. For this purpose, two kinds of processed milk were provided from a local producer: on the one hand, ESL (high-temperature) milk, which can be stored in the refrigerator up to 3 weeks, and on the other hand UHT milk, with a shelf life of at least three months at room temperature. The samples were obtained on the day of production and stored till the end of the expiration date. Samples were drawn at regular intervals by opening new packages each time: the milk was defatted and stored at −20 °C till the analysis. Storage of the ESL milk at 4 °C did not lead to changes in the peptide spectra (data not shown). In contrast, storage of the UHT milk at room temperature resulted in a significant increase ($p < 0.01$) of the signal at *m/z* 4340 (**Figure 5**; for relative quantification see **Figure 6**.) Other changes in the signal intensities were not significant. By use of MALDI-TOF/TOF-MS, the corresponding sequence was identified as a fragment of α-s1-casein (amino acids 1−37; see also above).

It can be assumed that proteolytic activity, which could lead to changes in the peptide profile of ESL milk, is suppressed by the low temperatures applied in the present storage experiment. This hypothesis is supported by a similar study with raw milk, which reported lower importance of plasmin hydrolysis in milk at refrigerator temperatures (*35*). Residual proteinase activity at low temperatures obviously did not influence the group of peptides investigated in our study. In contrast, in stored UHT milk, a notable enzymatic proteolysis was monitored although the proteinases are partially denatured due to the heat treatment (*36*). Plasmin has been reported to be surprisingly heat-stable in milk (*37*). However, the observed cleavage between aa 37/38 (valine/asparagine) cannot be explained by plasmin proteolysis, as plasmin shows high specifity for lysine and arginine residues (*31*). Therefore, the increasing peptide is assumed to be generated through the action of another heat-stable proteinase in milk with cleavage specifity for this site. Additionally, sterilized milk was stored at room temperature for up to 12 weeks. The goal was to verify that the peptide formed during the storage of UHT milk was of enzymatic origin. It is expected that the enzymes are completely inactivated in the sterilized milk due to the severe heat treatment. Indeed, no significant increase of the intensity of the peptide at *m/z* 4340 in the sterilized samples was observed within 12 weeks (see **Table 1**). This is in good agreement with the hypothesis that the increase was caused by proteinase activity.

Analysis of Commercial Milk Samples. Several commercially available milk samples, including pasteurized as well as ESL and UHT milk, were analyzed by MALDI-TOF-MS after purification with IMAC-Cu magnetic beads.

In **Figure 7**, four spectra are given, representing pasteurized, ESL, and two UHT milk samples. None of the formed peptides that had been observed in the heated raw milk could be detected in any of the pasteurized samples that were analyzed ($n = 12$). Obviously, the heat charge during the pasteurization process is not sufficient for significant changes in the low molecular weight protein profile covered by our method. In contrast, one of the six analyzed ESL milk samples showed all the heat-induced peptides, according to the higher temperatures applied in the course of the industrial processing. The UHT milk samples confirmed the formation of five peptides as a result of heat treatment: In all samples analyzed ($n = 16$), the peptides could be detected to varying extents. Differences in the relative intensity among the samples are thought to be due to different heating technologies (direct/indirect heating, variable heating duration and temperatures). Furthermore, clear differences were visible between the samples with regard to the intensity of peptides that had not shown any changes during heating of the raw milk (see peptides in **Figure 7** that are marked with arrows). It can be assumed that these differences are caused by different cow breeds or lactation stages; these factors are known to influence the peptide composition of milk (*4*).

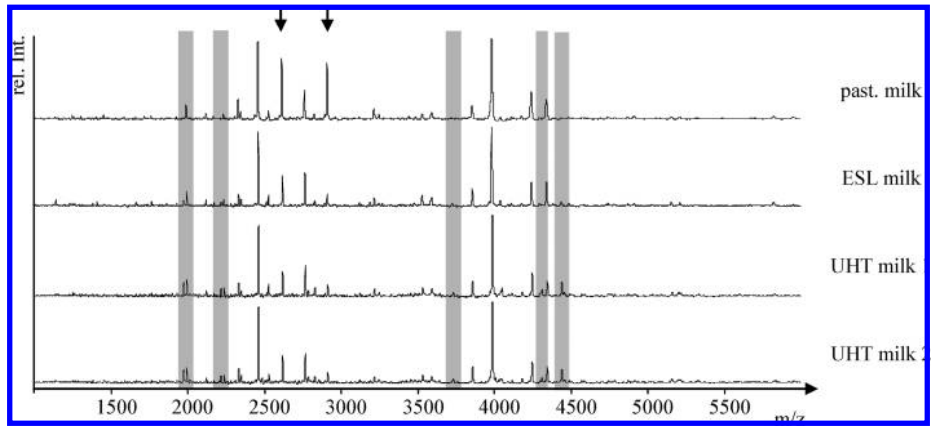

Figure 7. MALDI-TOF-MS spectra of commercially available milk products. Defatted milk was purified by metal affinity chromatography magnetic beads and directly analyzed via MALDI-TOF-MS. Signals that were also observed in the heated raw milk samples are highlighted with a gray box. Arrows indicate differences between individual milk samples as discussed in the text.

In this study, a method was developed to analyze the low molecular weight protein fraction of milk via MALDI-TOF-MS after treatment with IMAC-Cu magnetic beads. Application of this technique is simple and fast and allows a high sample throughput. Further investigations are now required to elucidate the structures of this protein fraction, which is mostly unknown. It was shown that the concentrations of the newly formed peptides depend on heating duration and temperature. In-depth analyses will be needed to show if these peptides may be applied as new indicators of the heat damage of milk products. For this purpose, however, a more detailed study on the formation of the peptides and their concentration in a broad range of milk samples is necessary.

ABBREVIATIONS USED

UHT, ultra high temperature; IMAC, immobilized metal affinity chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ESL, extended shelf life; HCCA, α-cyano-4-hydroxycinnamic acid; TFA, trifluoroacetic acid; PVDF, poly(vinylidene difluoride).

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