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Identification of sixteen peptides reflecting heat and/or storage induced processes by profiling of commercial milk samples

Jennifer Ebner, Florian Baum, Monika Pischetsrieder *

Food Chemistry Unit, Department of Chemistry and Pharmacy, Emil Fischer Center, Friedrich-Alexander Universität Erlangen-Nürnberg (FAU), Schuhstr. 19, 91052 Erlangen, Germany

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ABSTRACT

Peptide profiles of different drinking milk samples were examined to study how the peptide fingerprint of milk reflects processing conditions. The combination of a simple and fast method for peptide extraction using stage tips and MALDI–TOF–MS enabled the fast and easy generation and relative quantification of peptide fingerprints for high-temperature short-time (HTST), extended shelf life (ESL) and ultra-high temperature (UHT) milk of the same dairies. The relative quantity of 16 peptides changed as a function of increasing heat load. Additional heating experiments showed that among those, the intensity of peptide β-casein 196–209 (*m/z* 1460.9 Da) was most heavily influenced by heat treatment indicating a putative marker peptide for milk processing conditions. Storage experiments with HTST- and UHT milk revealed that the differences between different types of milk samples were not only caused by the heating process. Relevant was also the proteolytic activity of enzymes during storage, which were differently influenced by the heat treatment. These results indicate that the peptide profile may be suitable to monitor processing as well as storage conditions of milk.

Significance: In the present study, peptide profiling of different types of milk was carried out by MALDI–TOF–MS after stage-tip extraction and relative quantification using an internal reference peptide. Although MALDI–TOF–MS covers only part of the peptidome, the method is easy and quick and is, therefore, suited for routine analysis to address several aspects of food authenticity. Using this method, 16 native peptides were detected in milk that could be modulated by different industrial processes. Subsequent heating and storage experiments with pasteurized and UHT milk confirmed that these peptides are indeed related to the production or storage conditions of the respective products. Furthermore, the heating experiments revealed one peptide, namely the β-casein-derived sequence β-casein 196–209, which underwent particularly sensitive modulation by heat treatment. The present results indicate that the modulated peptides, and especially β-casein 196–209, may be suitable markers to monitor processing parameters for industrial milk production. Furthermore, the model experiments suggest mechanisms leading to the formation or degradation of peptides, which help to evaluate putative marker peptides.

1. Introduction

In general, raw milk has to be heat-treated before consumption to guarantee microbiological safety and stability. Different technological processes are available for this purpose, ranging from very mild thermization over different pasteurization technics, like high-temperature short-time (HTST) pasteurization, to the more severe ultra-high temperature (UHT) treatment and in-container sterilization [1]. More recently, extended shelf life (ESL) milk has been introduced into the market in addition to the well-established pasteurized und UHT varieties. ESL milk combines low heat damage and the fresh taste of pasteurized milk with prolonged shelf life. Since the legal definition of this milk type is missing, however, various techniques are used for production [2]. HTST pasteurization combined with a microfiltration step through ceramic filters with 1.4 μm pore width is widely used [3]. Other methods rely on milk treatment at higher temperatures, either by indirect heating or direct heat infusion or injection processes [2].

Apart from sensory and technological changes, heating can have negative impact on the nutritive value of milk, for example by vitamin degradation and reduced bioavailability of certain essential amino acids [4,5]. Furthermore, amine groups of proteins may react with lactose or its degradation products to form Amadori and other Maillard reaction products, leading, for example, to the loss of the essential amino acid lysine [6]. Additionally, toxic effects of some dietary Maillard reaction products have been discussed [7,8]. Therefore, it is necessary to keep the heat impact on milk as low as possible. Consequently, analytical methods are required which reliably monitor the industrial heating

Abbreviations: HTST, high-temperature short-time; UHT, ultra-high temperature; ESL, extended shelf life; ACE, angiotensin-converting enzyme.

* Corresponding author at: Henriette Schmidt-Burkhardt Chair of Food Chemistry, Department of Chemistry and Pharmacy, Emil Fischer Center, Friedrich-Alexander Universität Erlangen-Nürnberg (FAU), Schuhstr. 19, 91052 Erlangen, Germany.
E-mail address: monika.pischetsrieder@fau.de (M. Pischetsrieder).

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process and the quality of raw materials. Furthermore, food safety authorities need stable marker compounds to detect incorrect labeling and adulteration.

In general, two possible types of heat markers reflect the heat impact on milk. Firstly, natural milk components may be degraded or, secondly, novel substances can be formed as a consequence of the heat treatment. Thus, several markers have been suggested to differentiate between different kinds of commercial milk samples, such as the formation of 5-hydroxymethylfurfural or lactulose, various non-enzymatic whey protein modifications, as well as the loss of acid-soluble β-lactoglobulin or the inactivation of indigenous enzymes like lactoperoxidase [9–13].

More recently, it has been suggested that the composition of the peptide fraction may be a sensitive and selective marker reflecting heat treatment of milk. Based on an early observation that trichloroacetic acid-soluble (glyco)-peptides are released during the heating of milk [14], Morales and Jiménez-Pérez could detect two peptides in this fraction that showed a time-dependent increase during heating [15]. Later, a pilot study on the native peptide fraction of raw milk combining IMAC and MALDI–TOF–MS analysis detected five peptides, which were highly influenced by heating in a time- and temperature-dependent manner [16]. Very recently, MALDI–TOF–MS peptide profiling combined with a principle component analysis revealed 28 peptides that were characteristic either for raw, pasteurized, UHT, or powdered milk [17]. The use of indigenous peptides as heat markers possibly has advantages compared to other indicators because the peptide profile is very complex. In several studies, >250 components have been identified [18,19]. Since the indigenous peptide pattern is not only dependent on the heat treatment, but also on other parameters, such as species [17], the health state of the cow [20], product storage [21], or fermentation processes [22], it can be assumed that quantitative and qualitative profiling of indigenous milk peptides can be used as a fingerprint, reflecting different parameters of the milk sample simultaneously.

The goal of the present study was, therefore, to monitor how the profile of indigenous milk peptides is modulated by different heating regimens and storage conditions. For this purpose, peptide profiles of HTST-, ESL- and UHT-milk samples from the same dairies were recorded by MALDI–TOF–MS and peptides reacting to the heat treatment were identified. For a better understanding of the mechanistic background of peptide liberation or degradation in milk, additional heating and storage experiments were conducted.

2. Materials and methods

2.1. Sample purification

All milk samples were purchased in different supermarkets. The declaration on the milk packages provided the following information on the applied heat treatment: All HTST milk samples were heated at 72–75 °C for 15–30 s; the ESL milk of manufacturer 1 was microfiltered and subsequently heated at 72–75 °C for 15–30 s, whereas the second ESL milk was gently short-time heated using steam. The UHT milk of manufacturer 2 was produced using a mild heating method which accounts for 50% of the conventional UHT heat load, while further information on the heating regime applied for the other two UHT milk brands was not available. For milk defatting, samples were centrifuged twice at 1100*g* and 4 °C for 60 min and the upper fat layer was removed. Traces of residual fat remaining in the industrially homogenized milk samples after this step were removed during the directly following extraction step and did not interfere with further analysis. Some samples were mixed with angiotensin I solution (Fluka, Taufkirchen, Germany) prior to extraction for relative quantification experiments (see Section 2.4).

Peptide extraction was carried out by stage-tip microextraction according to Rappsilber et al. [23], modified by Baum et al. for the application in milk peptide analysis [24] with some further minor changes. The stage tips contained three layers of 3M empore disk-C18 material (3M, Neuss, Germany). For their assembly, small pieces of extraction material were stamped out of the empore disk by a biopsy punch (Kai Industries, Seki, Japan) and inserted into a conventional 2–200 μL pipette tip. In the following steps, different solutions were added to the stage tips followed by centrifugation at room temperature. The centrifugation parameters indicated for each step ensured liquids to pass, while the extraction material remained wet. For equilibration of the stage tips, first 100 μL of ACN (LC–MS grade, VWR International, Darmstadt, Germany) was pipetted into the reservoir, followed by 100 μL of 0.1% TFA (HPLC grade, Sigma-Aldrich, Taufkirchen, Germany) in purified water (MilliporeSynergy 185, Millipore, Billerica, USA), each followed by a centrifugation step at 1840*g* for 1 min. Then, 40 μL of the defatted milk sample was added with subsequent centrifugation at 3610*g* for 5 min. The tips were washed twice with 25 μL of 0.1% TFA and subsequently centrifuged each time at 3610*g* for 3 min. Finally, peptides were eluted with 10 μL of a mixture of ACN and 0.1% TFA (60:40) and following centrifugation at 3610*g* for 3 min. The eluate was directly used for MALDI–TOF–MS analysis.

2.2. Sample preparation for MALDI analysis

α-Cyano-4-hydroxycinnamic acid (HCCA, Sigma-Aldrich, Taufkirchen, Germany) was used as MALDI matrix. For this purpose, a solution of HCCA in 60% ACN/0.1% TFA (5.0 mg/mL) was mixed with an equal quantity of eluate from the stage-tip extraction. An aliquot of 0.7 μL was spotted onto a ground steel target (Bruker Daltonik, Bremen, Germany) and dried at room temperature.

2.3. MALDI–TOF–MS

MALDI–TOF analysis was performed with an Autoflex mass spectrometer from Bruker Daltonik (Bremen, Germany), equipped with nitrogen laser ($\lambda = 337$ nm). The measurement was conducted in reflectron mode and by delayed extraction (140 ns) of the positive ions. Mass range was set to 600–5000 Da. A total of 300 spectra per spot, obtained from different raster positions, were accumulated automatically, starting in the spot center and spirally moving outwards. For removal of matrix from the surface, 12 initial laser shots were fired with higher energy to every newly addressed raster position. Then 15 single spectra were recorded at each position after setting the laser energy to a lower level, which had been predetermined by manual test measurements to ensure good resolution and high signal intensity. The external calibration was performed using the monoisotopic masses of the calibration standard peptide mix containing bradykinin 1–7, angiotensin I, angiotensin II, substance P, bombesin, renin substrate, adrenocorticotropic hormone clip 1–17 and 18–39, and somatostatin-28 (Bruker Daltonik, Bremen, Germany). Spectra processing, baseline subtraction and smoothing were performed using *FlexAnalysis* software from Bruker Daltonik. Peaks with a signal-to-noise ratio ≥ 8 were marked using *mMass* software [25]. To improve mass accuracy, internal calibration was carried out by *mMass* using the monoisotopic masses of ten indigenous milk peptides, which had previously been identified as major components of the MALDI–TOF peptide profile of milk [18].

2.4. Relative quantification of selected peptides

HTST-, ESL- and UHT-milk from different manufacturers was investigated. Prior to stage-tip extraction, 150 μL of 4 μM aqueous solution of angiotensin I was added to 600 μL of each milk sample. Stage-tip extraction and MALDI–TOF–MS measurement were conducted as mentioned above (see Sections 2.1 and 2.3) six times per sample. Initially, the angiotensin I signal was used as reference and its intensity was set as 100%. For the final relative quantification, however, an internal milk peptide, α_{S1}-casein 80–102, was selected, which had previously been identified in raw milk [18] and whose intensity was not influenced by the type of heat treatment in the present study.

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