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Influence of storage and heating on protein glycation levels of processed lactose-free and regular bovine milk products

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ABSTRACT

Thermal treatment preserves the microbiological safety of milk, but also induces Maillard reactions modifying for example proteins. The purpose of this study was evaluating the influence of consumer behaviors (storage and heating) on protein glycation degrees in bovine milk products. Lactosylation and hexosylation sites were identified in ultra-high temperature (UHT), lactose-free pasteurized, and lactose-free UHT milk (ULF) and infant formula (IF) using tandem mass spectrometry (electron transfer dissociation). Overall, 303 lactosylated and 199 hexosylated peptides were identified corresponding to 170 lactosylation (31 proteins) and 117 hexosylation sites (25 proteins). In quantitative terms, storage increased lactosylation up to fourfold in UHT and IF and hexosylation up to elevenfold in ULF and threefold in IF. These levels increased additionally twofold when the stored samples were heated (40 °C). In conclusion, storage and heating appear to influence protein glycation levels in milk at similar or even higher degrees than industrial processing.

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1. Introduction

The high contents of proteins and reducing sugars (mainly lactose) in milk favor Maillard reactions during thermal processing (e.g., pasteurization and ultra-high temperature treatment) required to reduce the bacterial load of milk products and thereby extend its shelf life as well (Moatsou, 2013). The Maillard reaction is initiated by reducing sugars (e.g., aldoses) reacting with amino groups in proteins, i.e., mostly ε-amino groups of lysine residues, yielding relatively stable glycation products (e.g., Amadori products) (Hodge, 1955; Labuza, Monnier, Baynes, & O'Brien, 1998).

These products are present at low degrees in raw milk and colostrum, while their content increases during industrial processing (Milkovska-Stamenova & Hoffmann, 2016a, 2016b). They have been extensively studied in recent years with respect to their effect on the physicochemical properties of milk proteins, such as reduced digestibility and bioavailability, that may decrease the nutritional value of milk (Van Boekel, 1998). Furthermore, these reactions may alter protein functions, including reduced enzyme activity, receptor binding, and protein digestibility (Lorenzen, 1997). Glycated structures may also be recognized by the immune system and thereby trigger food allergies (Toda, Heilmann, Ilchmann, & Vieths, 2014; Verhoeckx et al., 2015). Moreover, Amadori products are precursors of advanced glycation end-products (AGEs) raising some concerns about potential health risks, as they may contribute to the *in vivo* AGEs pool possibly triggering inflammatory responses and oxidative stress with potentially damaging vascular and renal effects (Poulsen et al., 2013; Stirban & Tsch, 2015).

Recent studies relying on mass spectrometry have correlated ('early') glycation products derived from lactose (Meltretter, Wust, & Pischetsrieder, 2014; Milkovska-Stamenova & Hoffmann, 2016b; Wada & Lonnerdal, 2014) and hexoses (e.g., D-glucose and D-galactose) (Milkovska-Stamenova & Hoffmann, 2016a) to the harshness of the thermal treatment, considering the number of lactosylation and hexosylation sites as an indication of the glycation degree and the relative quantities of targeted glycation sites in different milk products. For example, lactose-free milk contains higher glycation levels than regular milk products, due to the high reactivity of D-glucose and D-galactose (obtained by enzymatic cleavage of lactose) compared to the disaccharide lactose (Milkovska-Stamenova & Hoffmann, 2016a). These products have been intensively studied in the context of industrial milk processing, whereas the influence of later, consumer-dependent handling steps, such as subsequent storage and meal preparation has not been investigated in detail. In 2000, Guyomarc'h et al. showed that

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the lactosylation degrees of β-lactoglobulin (β-LG) and β-casein (β-CN) increase in skim milk powder at temperatures above 4 °C (Guyomarc'h, Warin, Donald Muir, & Leaver, 2000). Similarly, the lactosylation degrees of whey proteins increased in regular UHT milk stored at 28 °C and 40 °C (Holland, Gupta, Deeth, & Alewood, 2011), compared to milk stored at 4 °C. LC-MS studies at the protein level indicated elevated lactosylation degrees for whey proteins β-lactoglobulin and α-lactalbumin (α-LA) and caseins in UHT stored for six months at room temperature (Rauh et al., 2015). Similarly, furosine contents were higher in lactose-free UHT after a storage period of nine months than in the fresh product (Jansson et al., 2014). Thus, higher temperatures and extended storage periods significantly increase protein glycation levels in milk products, indicating that proper guidance for consumers might reduce the burden of glycated proteins in milk products and ultimately in food. In this respect, comprehensive quantitative studies determining simultaneously lactosylation and hexosylation degrees at distinct sites in milk products, considering different storage and heating conditions, are desirable.

The objective of this study was to evaluate the impact of shelf-life storage and heating (40 °C) on protein lactosylation and hexosylation degrees in processed milk products. The glycation levels were determined in ultra-high temperature (UHT), lactose-free pasteurized (PLF), lactose-free UHT milk (ULF), and first-stage infant formula (IF) shortly after purchasing, UHT and ULF after storing for 3 months at room temperature, and IF for one year at 4 °C. Additionally, the influence of heating (10 min, 40 °C) was investigated for all samples.

2. Materials and methods

Materials were obtained from the following suppliers: AppliChem GmbH (Darmstadt Germany): iodoacetamide (IAA) and Tris (⩾99.9%); Biosolve GmbH (Valkenswaard, Netherlands): acetonitrile (ULC-MS grade, ⩾99.97%), formic acid (ULC-MS grade, ⩾99%), and methanol (ULC-MS grade, ⩾99.98%); Carl Roth GmbH (Karlsruhe, Germany): methanol (HPLC grade, ⩾99.9%), ethanol (HPLC grade, ⩾99.8%), urea (⩾99.5% p.a.), sodium dodecyl sulfate (SDS, ⩾99.5%), glycerol (⩾99.5%), and dithiothreitol (DTT) (⩾99%); Kmf Laborchemie Handels GmbH (Lohmar, Germany): ammonia solution (~25% p.a.); Promega GmbH (Mannheim, Germany): sequencing grade modified trypsin; Merck KGaA (Darmstadt, Germany): chloroform; Riedel-de Haën (Steinheim, Germany): bromophenol blue sodium salt; SERVA Electrophoresis GmbH (Heidelberg, Germany): bovine serum albumin (BSA), ammonium persulfate, acrylamide/bis solution (30%, w/v), tetramethylene diamine, Coomassie Brilliant Blue G 250, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); Sigma-Aldrich Chemie GmbH (Steinheim, Germany): *m*-aminophenylboronic acid-agarose, ammonium acetate (⩾98%), magnesium acetate tetrahydrate (⩾99%), thiourea (⩾99%), ammonium bicarbonate (⩾99.5%), acetic acid (LC-MS grade), and β-mercaptoethanol.

Water was purified in-house (resistance >18 mΩ/cm; total organic content <10 ppb) on a PureLab Ultra Analytic System (ELGA Lab Water, Celle, Germany).

2.1. Milk samples

PLF (one brand), ULF (one brand, two packs), UHT (one brand, two packs), and first-stage IF (one brand, one box containing two bags of 300 g) were bought in a local supermarket shortly after delivery. One replicate from each UHT milk sample was stored at room temperature (typically 22–25 °C) for three months and one sealed pack of IF was stored for one year in a cold room (4 °C), i.e., one week (ULF and UHT) and 1.5 months (IF) before the respective expiration dates. IF (first-stage, proteins 12.4 g/L, carbohydrates i.e., lactose 75 g/L, fat 36 g/L, various vitamins, and minerals) was prepared according to the manufacturer's instruction leaflet. Aliquots of each milk sample (40 mL) were incubated in 50-mL polypropylene tubes (40 °C, 10 min, water bath) simulating a bottle warmer. All milk samples were stored in aliquots at –80 °C and thawed only once prior to analysis.

2.2. Protein precipitation and tryptic digestion

Proteins were precipitated from three aliquots of each milk sample using Folch extraction (methanol/chloroform/water) (Milkovska-Stamenova & Hoffmann, 2016b) and quantified by a Bradford assay relative to bovine serum albumin. The protein contents were confirmed by a Coomassie stain of the major milk protein bands after SDS-PAGE (Milkovska-Stamenova & Hoffmann, 2016b). Sample aliquots (150 μg protein) were diluted with ammonium bicarbonate buffer (25 mmol/L) to obtain a final volume of 150 μL, reduced with DTT, alkylated with IAA, and digested with trypsin (3 μg) overnight (Milkovska-Stamenova & Hoffmann, 2016b). The digest was stopped with formic acid (0.5%, v/v) and the peptides were desalted by solid-phase extraction (SPE; Oasis HLB 1 cm^3, 30 mg, Waters GmbH, Eschborn, Germany) using aqueous acetonitrile (70%, v/v; 500 μL) containing formic acid (0.1%, v/v) for elution, and dried under vacuum.

2.3. Affinity chromatography

The tryptic digest (0.1 mg protein) was loaded on an *m*-aminophenylboronic acid-agarose column and washed with loading buffer (0.25 mol/L ammonium acetate, 0.05 mol/L magnesium acetate, pH 8.1, 4 °C, 3 × 4.5 mL). Glycated peptides were eluted with 0.1 mol/L (2 × 1 mL, 2 × 3 mL) and then with 0.2 mol/L aqueous acetic acid (2 mL) at 37 °C. The combined eluates were frozen (–80 °C), lyophilized (Edwards Freeze Dryer Modulyo, –60 °C, 0.2 mbar), reconstituted, desalted by SPE as described in the previous section, and dried under vacuum (Milkovska-Stamenova & Hoffmann, 2016b).

2.4. Protein analysis

The dried peptides were dissolved in aqueous acetonitrile (3%, v/v; 40 μL) containing formic acid (0.1%, v/v) before analysis and an aliquot (0.33 μL) corresponding to 0.83 μg proteins in the original milk sample was separated on a nano-Acquity UPLC (Waters GmbH, Eschborn, Germany) coupled on-line to an LTQ Orbitrap XL (ETD) mass spectrometer equipped with a nano-ESI source (Thermo Fisher Scientific, Bremen, Germany). Briefly, peptide samples (10 μL) were trapped (nanoAcquity Symmetry C_{18}-column, internal diameter 180 μm, length 20 mm, particle diameter 5 μm) at a flow rate of 10 μL/min (3% eluent **B**, 0.1% formic acid in acetonitrile) and separated on a BEH 130 column (C_{18}-phase, internal diameter 75 μm, length 100 mm, particle diameter 1.7 μm; 30 °C; flow rate of 0.4 μL/min). Peptides were eluted by a two-step gradient starting from 3% eluent **B** (0.1% formic acid in acetonitrile) to 30% eluent **B** within 18 min and then to 85% eluent **B** within 1 min. The transfer capillary temperature was set to 200 °C and an ion spray voltage of 1.5 kV was applied to a PicoTip™ on-line nano-ESI emitter (New Objective, Berlin, Germany) (Milkovska-Stamenova & Hoffmann, 2016b). Mass spectra were recorded from *m*/*z* 400–2000 in the orbitrap mass analyzer at a resolution of 60,000 at *m*/*z* 400. Tandem mass spectra were acquired in ETD mode (activation time 100 ms, isolation width 2 u, default charge state 2) using data-dependent acquisition (DDA) for the six most intense signals and a dynamic exclusion window of

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