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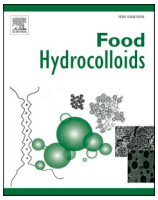

The effect of low vs. high temperature dry heating on solubility and digestibility of cow's milk protein

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ABSTRACT

Dry heating of cow's milk protein in the presence of the milk sugar lactose leads to a loss of solubility and digestibility. Most studies that investigated the loss of solubility in milk protein powders suggested that, besides structural changes, hydrophobic interaction, hydrogen bonds, disulphide bonds, and Maillard reaction-induced crosslinking are responsible for this. However, little is known about the direct contribution of these inter- and intramolecular interactions on loss of solubility and protein digestibility. Low temperature (60 °C) and high temperature (130 °C) dry heating of cow's milk protein in the presence of lactose was applied after which both the soluble and insoluble fractions were analysed with SDS-PAGE and LC-MS/MS. The Maillard reaction was monitored by quantification of N^{ε}-carboxymethyllysine, N^{ε}-carboxyethyllysine, and pentosidine with LC-MS/MS. Loss of solubility was analysed with solvent solubility tests. Protein hydrolysis after simulated infant *in vitro* digestion, and after hydrolysis with single enzymes, was monitored using SDS-PAGE and the o-phthaldialdehyde assay. The results indicated that caseins are the main proteins that become insoluble upon dry heating, independent of the heating temperature. The decreased solubility of low temperature dry heated cow's milk protein is induced by hydrogen bonds and hydrophobic interactions and did not impair protein hydrolysis. At the same time, covalent protein crosslinking is an important determinant in protein solubility and digestibility of high temperature dry heated cow's milk protein.

1. Introduction

Cow's milk protein (MP) consists of two main protein fractions: casein and whey protein. These two protein fractions show different susceptibility to heat induced denaturation and aggregation on their structure. Caseins have little secondary and tertiary structure and are organised in a micellar structure that makes them more resistant to heat induced aggregation than the globular whey proteins (De Wit, 1990; Walstra, 1990). Besides protein unfolding and aggregation as a result of hydrophobic interactions and aggregation via disulphide interactions, the Maillard reaction (MR) also occurs in both casein and whey proteins. Disulphide bond induced protein interaction is a key reaction in protein aggregation of MP in solution. The formation of the κ-casein/whey protein complexes on the surface of the casein micelle is the predominant disulphide bond-induced intermolecular reaction in MP. With respect to the whey proteins, both β-lactoglobulin and α-lactalbumin are involved in the aggregation and complexation on the casein micelle surface (Dalgleish, Senaratne, & Francois, 1997; Dalgleish, Van Mourik, & Corredig, 1997). However, the complexation of whey proteins with the casein micelle is mainly driven by the S–S/thiol disulphide interchange between the free cysteine residue of β-lactoglobulin and κ-casein (Corredig & Dalgleish, 1999). Next to κ-casein, also α_{s2}-casein contains free cysteine residues, which enables it to form disulphide bonds. Despite that α_{s2}-casein is mainly found in the interior of the casein structure, small amounts can migrate into the serum and form disulphide bonds with κ-casein and whey proteins (Donato & Guyomarc'h, 2009). Despite hydrophobic interactions and disulphide bond formation, the MR is one of the most abundant reactions occurring during thermal processing of MP. The MR is the reaction between α-amino groups of proteins, peptides, and amino acids and the reactive carbonyl

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group of a reducing sugar. In MP, the MR most often occurs between lysine and arginine residues of milk proteins and lactose, with the initial formation of the Amadori product that in the advanced stage of the MR reacts further to a pool of different advanced glycation end products (AGEs) (Van Boekel, 1998). Depending on the reaction conditions (reactants, pH, water activity (a_w), temperature, and time), different AGEs are formed, which can be both linear or crosslinked. Examples of linear AGEs are N^{ε}-carboxymethyllysine (CML) and N^{ε}-carboxyethyllysine (CEL), while pentosidine is a representative of a crosslinked AGE between lysine and arginine residues (Arena, Renzone, D'Ambrosio, Salzano, & Scaloni, 2017). Moreover, low a_w levels (<0.9) increase the reaction rate which leads to relatively more MR if heated under dry conditions compared to heating in a liquid system (Schong & Famelart, 2017). Recent studies showed that both the MR and MR-induced protein crosslinking can lead to loss of solubility of MP concentrates during storage (Fan et al., 2018; Le, Holland, Bhandari, Alewood, & Deeth, 2013). However, it was also indicated that protein interactions via hydrophobic forces, hydrogen bonds and disulphide bonds play a role in MP interactions, potentially resulting in a loss of solubility (Anema, Pinder, Hunter, & Hemar, 2006; Gazi & Huppertz, 2015; Yüksel & Erdem, 2005). Loss of solubility and structural changes of MP during thermal processing may also affect its digestibility and thereby the nutritional value of the proteins (Rudloff & Lönnerdal, 1992). While heat-induced protein unfolding may facilitate protein hydrolysis, it has been suggested that heat induced MP aggregation and high levels of glycation negatively affect digestibility (Pinto et al., 2014; Wada & Lönnerdal, 2014, 2015). Dry heating was applied in this study to create a model system that results in similar protein modifications as during the production of "baked milk", which refers to MP powder that was baked into a muffin or waffle and was previously shown to be involved in the accelerated resolution of cow's milk allergy symptoms in allergic children (Kim et al., 2011). Due to the low water activity, the high temperatures, and the high level of reducing sugars in these systems, it is hypothesised that protein aggregation and glycation via the MR plays an important role in modulating the immunoreactivity. These two factors have also previously been reported to affect both digestibility and immunoreactivity of MP (Deng et al., 2019; Nowak-Wegrzyn & Fiocchi, 2009; van Lieshout, Lambers, Bragt, & Hettinga, 2019). Therefore, MP was heated at low temperature (LT) in the presence of lactose to induce glycation with minimal aggregation as well as at high temperature (HT) to induce glycation and aggregation, to investigate the contribution of different molecular interaction on solubility and aggregation. This was done by using similar parameters as reported in previous studies that investigated the effects of dry heating on immunoreactivity and digestibility (Liu, Teodorowicz, Wichers, Van Boekel, & Hettinga, 2016; Zenker, Van Lieshout, Van Gool, Bragt, & Hettinga, 2020). Moreover, protein hydrolysis after infant *in vitro* digestion was monitored to assess the effect of these parameters on protein digestibility. The possible consequences on immunoreactivity were discussed against the background of existing literature.

2. Material and methods

2.1. Chemicals

NuPAGE® LDS sample buffer (4× concentrated), NuPAGE™ 12% Bis-Tris Protein Gel, 1.0 mm, NuPAGE™ 10x MOPS running buffer, NuPAGE® reducing agent, NuPAGE® 4–12% Bis-Tris gel, NuPAGE® 20× MES running buffer were obtained from Thermo Fisher Scientific (Massachusets, USA). CML, CEL, d4-CML, and d4-CEL were purchased from Iris Biotech (Marktredwitz, Germany). EMSURE® Anhydrous disodium hydrogen phosphate was purchased from Merck Millipore (Massachusets, USA). Glutaraldehyde and osmium tetroxide were purchased from Electron Microscopy Science (Hatfield, England). Sequencing grade trypsin used for protein identification was obtained from Roche (Mannheim, Germany). Corning® BioCoat™ poly-L-lysine coated plates were purchased from Corning (New York, USA). Coomassie brilliant blue R-250 was obtained from Biorad (California, USA). All other chemicals were obtained from Sigma aldrich (Missouri, USA).

2.2. Preparation of milk powder and heat treatment

Liquid concentrated raw cow's milk protein, composed of a mixture of micellar casein fraction (MCI88 liquid) and a whey protein fraction (acid WPC80 liquid), was received from FrieslandCampina (Wageningen, Netherlands). The protein composition and pH of the two main milk protein fractions was comparable to skim milk, while lactose had been reduced by membrane filtration processes at FrieslandCampina to 0.6%. Lactose monohydrate was added to the MP to achieve a lactose/protein ratio of 1.5/1.0 (w/w), as it is found in milk. The solution was stirred at room temperature until complete dissolution of the lactose and then lyophilised. The obtained powder was ground and sieved through a 425 μm round sieve (Retsch, Haan, Germany). The resulting powder was heated and glycated using two different procedures. For LT heat treatment, the powder was incubated for 3 weeks at 60 °C, over a saturated potassium fluoride solution to keep the a_w-level at 0.23. For HT heat treatment, the powder was incubated over a saturated potassium acetate solution at 10 °C until an a_w of 0.23 was reached. This powder was then heated for 10 min at 130 °C in a screw cap heating tube and immediately transferred to an ice bath to stop the heating. One part of the powder was non-treated (NT) to serve as an unheated control. All samples were kept at −20 °C until analyses were done. An overview of the sample preparation and analysis is shown in Fig. S1.

2.3. Dissolving of powdered MP and protein quantification

All powders were dissolved in simulated milk ultra-filtrate (SMUF). SMUF (pH 6.7) was prepared according to the protocol of Jenness & Koops (Jennes, 1983). Samples were dissolved at a protein/SMUF ratio of 1/12 (w/v) and left shaking for 1 h at room temperature. Subsequently, samples were centrifuged using an Eppendorf centrifuge 5430R (Eppendorf, Hamburg, Germany) for 15 min, 14 000×*g*, 20 °C and supernatants were filtered through a 0.45 μm CA syringe filter (Phenomenex, California, USA). The remaining pellet was washed 5 times with SMUF buffer. All compounds which were not dissolved after this procedure were considered as insoluble material. The insoluble material was lyophilised and grinded, while the soluble fraction was analysed for its protein content by DUMAS Flash EA 1112 Protein analyser (Thermo Fisher Scientific, Massachusetts, USA) in technical duplicates. The procedure was repeated twice on different days.

2.4. Acidic hydrolysis

To release (modified) amino acids from their protein structure, acid hydrolysis was performed according to (Kehm et al., 2019). Milk powder samples and the SMUF soluble protein fractions in an amount which equals 1 mg of protein were mixed with 100 μL ultrapure water and were directly incubated with 250 μL borate buffer (0.4 N, pH 10.2) and 250 μL sodium borohydride (1 M in 0.1 M sodium hydroxide) for 2 h at room temperature. The incubation was performed to reduce early glycation products such as fructosyllysine to avoid neo-formation of CML during heating in hydrochloric acid (HCl). Protein precipitation was achieved by adding 1 mL of trichloroacetic acid (20%, w/v) and centrifugation (4 °C, 10000 rpm, 10 min). The supernatant was discarded, and the precipitate was washed with trichloroacetic acid (5%, w/v) and centrifugation was repeated. After removal of the supernatant, 1 mL of HCl (6 M) and 10 μL internal standard mix containing 30 μM d4-CML and d4-CEL was added to the protein pellet after which the sample was heated at 110 °C for 23 h. The hydrolysed samples were evaporated to dryness with a vacuum concentrator (SpeedVac, Thermo Fisher Scientific, Karlsruhe, Germany). The residue was dissolved in 100 μL ultrapure water and after centrifugation (4 °C, 10000 rpm, 10 min) an aliquot was

used for MRM analysis of glycation end products by LC-MS/MS.

2.5. Analysis of advanced glycation end (AGE) products

AGE analysis was performed in acid hydrolysed samples. External calibration with the respective deuterated internal standards for CML and CEL was used for quantification, whereas d4-CML was used as an internal standard for pentosidine. UPLC analysis was performed with an Acquity Ultra Performance LC system coupled to a Waters Quattro Premier XE mass spectrometer (both Waters Corporation, Milford, MA, USA). For chromatographic separation, a Waters Cortecs C18 column (2.1 × 50 mm, 1.6 μm) at a column temperature of 30 °C was used. Solvent A was 10 mM nonafluoropentanoic acid in LC-MS grade water and solvent B was 10 mM nonafluoropentanoic acid in acetonitrile (ACN). The solvents were pumped at a flow rate of 0.4 mL/min in gradient mode (0 min, 1% B; 1 min, 1% B; 6 min, 50% B; 6.1 min, 1% B; 8 min, 1% B). The injection volume was 10 μL. The ESI source was operated in positive mode and nitrogen was utilized as the nebulizing gas with a gas flow of 650 L/h and a gas temperature of 450 °C. The capillary voltage was set to 2.6 kV and the source temperature was 150 °C. Analytes were measured in MRM mode with the following transitions and optimized collision energies (CE) and cone voltages (CV). CML: 204.9 → 84.2 (q, CV: 24, CE: 18 V), 204.9 → 130.2 (Q, CV: 24, CE: 12 V), d4-CML: 209.2 → 88.1 (q, CE: 20 V), 209.2 → 134.1 (Q, CE: 12 V); CEL: 219.1 → 84.1 (q, CV: 24, CE: 18 V), 219.1 → 130.1 (Q, CV: 24, CE: 12 V), d4-CEL: 223.2 → 88.1 (q, CE: 20 V), 223.2 → 134.1 (Q, CE: 12 V). pentosidine: 379.2 → 187.2 (q, CV: 42, CE: 36 V), 379.2 → 135.1 (Q, CV: 42, CE: 44 V). Transitions used for quantification are labeled with q and transitions used for the confirmation of the presence of the analyte are labeled with Q. Data were acquired and evaluated with the MassLynx Software (Waters, version 4.1).

2.6. Solvent dependent solubility test

The insoluble material from LT heated samples and HT heated samples was added to 4 different solvents, to determine the amount of material that could be solubilised under different conditions. Solvent 1 (S1) was composed of 10 mM phosphate buffer saline (PBS) at pH 7.4, solvent 2 (S2) was composed of 10 mM PBS and 1% sodium dodecyl sulphate (SDS), solvent 3 (S3) was composed of 10 mM PBS, 1% SDS, and 6 M urea, and solvent 4 (S4) was composed of 10 mM PBS, 1% SDS, 6 M urea, and 1% dithiothreitol (DTT). Ten milligrams of lyophilised insoluble material was accurately weighed in separate Eppendorf tubes using a micro balance XA105 (Mettler Toledo, Columbus, USA) and 300 μL of S1, S2, S3, and S4 were added, respectively. Samples were vortexed thoroughly and left at 70 °C for 10 min. To discriminate the effect of heating, which was used for all four solvents, 10 mM PBS (pH 7.4) without heating was used as a control. All solutions were centrifuged using an Eppendorf centrifuge 5430R (Eppendorf, Hamburg, Germany) and supernatants were collected. The procedure was repeated once and the supernatants were combined. Protein content of the supernatants was quantified using Pierce BCA protein assay kit (Thermo Fisher Scientific, Massachusetts, USA) after precipitation of the protein with 20% trichloroacetic acid and solubilisation of the pellet with 0.1 M sodium hydroxide. The procedure was repeated twice on the pellets obtained from the procedure described under 3.3.

2.7. SDS gel electrophoresis (SDS-PAGE) of undigested samples

To monitor the protein composition, formation of aggregates, and protein glycation, SDS-PAGE was conducted under both reducing and non-reducing conditions. Analyses were conducted on both the soluble and insoluble fraction of the unheated control, and heated samples. Samples were diluted with 5 μL NuPAGE® LDS sample buffer (4 × concentrated), and ultrapure water. After centrifugation (1 min, 500×*g*, 20 °C), the solutions were heated for 10 min at 70 °C and centrifuged again (1 min, 500×*g*, 20 °C). From each sample, 12.5 μg protein were loaded on a NuPAGE™ 12% Bis-Tris Protein Gel, 1.0 mm, and run at 120 V (constant) with NuPAGE™ 1x MOPS SDS running buffer. Gels were stained with Coomassie brilliant blue R-250 and unstained in 7.5% acetic acid, 10% ethanol. Images of each gel were obtained using a Universal Hood III (Biorad, California, USA) and Image Lab 4.1 software (Biorad, California, USA).

2.8. Protein identification

To identify specific bands on the SDS-PAGE of LT heated samples, in-gel based proteomics was applied. Briefly, gels were prepared as described previously and stained with Coomassie blue R-250. After destaining, gels were reduced in 50 mM ammonium bicarbonate solution and 100 mM DTT for 1 h at 60 °C. Subsequently, gels were alkylated in the dark for 30 min at 60 °C using 50 mM ammonium bicarbonate, 1 M Tris (pH 8), and acrylamide (9/1/0.014) (v/v/w). Bands were cut from the gel and digested with 5 ng/μL trypsin in 50 mM ammonium bicarbonate solution at room temperature for 14 h. All samples were adjusted to pH 2–4 with 10% trifluoroacetic acid and diluted with 150 μL 25 mM formic acid (HCOOH). Samples were desalted using in-house made C18 μcolumns and eluted with 50 μL ACN/25 mM HCOOH (1/1) (v/v). ACN was removed using an Eppendorf concentrator plus (Eppendorf, Hamburg, Germany) and sample volumes were adjusted to 50 μL final volume with 25 mM HCOOH.

Peptides were analysed on a Thermo nLC 1000 system (Thermo, Waltham, USA) coupled to a LTQ orbitrap XL (Thermo, Waltham, USA). Chromatographic separation was conducted over a 0.10 * 250 mm ReproSil-Pur 120 C18-AQ 1.9 μm beads analytical column with pre-concentration column prepared in house. Flow rate was set to 0.5 μL/min and ran with an ACN in water gradient spiked with 0.1% HCOOH. ACN increased from 9% to 34% ACN within 50 min. Full scan positive mode FTMS spectra were measured between m/z 380 and 1400 in the Orbitrap at high resolution (60000). CID fragmented (isolation width 2 m/z, 30% normalized collision energy, activation Q 0.25 and activation time 15 ms) MSMS scans of the four most abundant 2+ and 3+ charged peaks in the FTMS scan were recorded in data dependent mode in the linear trap (MSMS threshold = 5.000, 45 s exclusion duration for the selected m/z ± 25 ppm). LC-MS/MS runs were processed with MaxQuant 1.6.1.0 with the Andromeda search engine (Cox & Mann, 2008). using the "Specific Trypsin/P" digestion mode. Propionamide formation on cysteines was set as a fixed modification while variable modifications were set for acetylation of the N-terminus, deamidation of asparagine and glutamine, and oxidation of methionine.

To identify the proteins and peptides, a bovine database from Uniprot (downloaded in March 2016 from https://www.uniprot.org) was used together with a database for common contaminants. Trypsin/P was used as the specific digestion mode. The false discovery rate was set to 1% on both protein and peptide level. Reversed hits and proteins only identified by site were deleted from the protein group's results table. Non bovine derived contaminants, e.g. human keratins, were manually removed. Filtered MaxQuant data was analysed with Perseus 1.5.5.3 using the normalized LFQ intensities. Proteins with a minimum number of 2 distinct peptides and at least one unique peptide were displayed. Additionally, LFQ intensities of each protein were converted to a percentage of the total LFQ intensity of the sample, to identify the most abundant proteins. Only proteins with a relative LFQ intensity higher than 2.0% were shown.

2.9. Infant in vitro digestion

MP powders heated at LT and HT, as well as the NT MP powder, were applied to simulated infant *in vitro* digestion according to the method by Menard et al. (Ménard et al., 2018). The protein concentration was adjusted to 1.2% (w/v) with ultra-pure water. The meals were preheated for 10 min at 37 °C and applied to simulated gastric and intestinal

digestion. Briefly, in the gastric phase (GP), pH was adjusted to 5.3 while the pepsin activity was set to 268 U/mL digest. The meals were incubated for 60 min at 37 °C under continuous shaking (25 rpm) in an incubator (Venti-Line, VWR, Radnor, USA). In the intestinal phase (IP) the pH was adjusted to 6.6 and trypsin activity in the final digest was adjusted to 16 U/mL. The meals were incubated for 60 min under the same conditions as in the GP. Samples were taken after 60 min in the GP, 10 min in the IP, and 60 min in the IP. In addition to the simulation of infant *in vitro* gastrointestinal digestion, hydrolysis with different combinations of the single enzymes trypsin, chymotrypsin, and pepsin were conducted in the same manner as for the simulated infant *in vitro* digestion, with slight modifications. For tryptic digestion, pepsin stock solution was replaced by gastric fluid and trypsin TPCK treated (T1426) was added instead of pancreatin, keeping the same enzyme activity in the final digest. For chymotryptic digestion, digestion was conducted as for tryptic digestion but trypsin TPCK was replaced by chymotrypsin TLCK treated (C3142) to the same enzyme activity as was reached in the digest by the addition of pancreatin. Chymotrypsin activity in pancreatin was measured according to the protocol of Brodkorb et al. (Brodkorb et al., 2019). A combination of pepsin in the GP and trypsin in the IP was used with the same enzyme activities as described before. A combination of chymotrypsin and trypsin without pepsin was used with the same enzyme activities for each enzyme as used for the respective single enzyme digestion. Enzyme activity was stopped after the GP by increasing the pH to 6.6 and after the IP by the addition of 0.5 mM Pefabloc to the digest in the ratio Pefabloc/digest of 1/20 (v/v). Each digestion experiment was performed in duplicate. For determining the level of nitrogen transfer, an aliquot was taken directly after digestion, centrifuged and the supernatant was collected. For the o-phthaldialdehyde assay (OPA-assay), 0.5 mL digest was added to 0.4 mL cooled 10% trichloroacetic acid (w/v), vortexed and centrifuged. For the OPA-assay and nitrogen transfer, enzyme activity of the aliquots was not additionally stopped with Pefabloc to avoid interference of the enzyme blocker. An enzyme control was taken containing all substrates of the IP but sample was replaced by an equal amount of water.

2.10. Degree of hydrolysis

To monitor the degree of protein hydrolysis (DH) after digestion, the OPA-assay was conducted according to the method of Mulet-Cabero et al. (Mulet-Cabero, Rigby, Brodkorb, & Mackie, 2017). Briefly, supernatants of trichloroacetic acid precipitated undigested meals as well as meals after 60 min in the GP, 10 min in the IP, and 60 min in the IP were diluted in 10 mM PBS (pH 7.4) and mixed with OPA reagent in a ratio of 1/20 (v/v) to a final volume of 200 μL. The mixture was incubated in a transparent 96 well polystyrene plate (Greiner, Kremsmuenster, Austria) for 15 min in the dark. Absorbance was measured at 340 nm using Infinite® 200 PRO NanoQuant with i-control software (Tecan, Meannedorf, Switzerland). Degree of hydrolysis was calculated according to the following formula:

DH [%] = ([NH_2 (final) − NH_2 (initial)]/[NH_2 (acid) − NH_2 (initial)]) × 100.

Where NH_2 (final) is the concentration of free amino groups in the digested sample, NH_2 (initial) is the concentration of free amino groups in the undigested sample (at time 0 of digestion), and NH_2 (acid) is the total content of free amino groups in the sample after acid hydrolysis. Different concentrations of D-Leucine were used to obtain a calibration curve in the concentration range between 0.4 and 4 mM. Each sample of the in duplicate conducted digestion experiment was measured in technical triplicates with the OPA-assay.

2.11. SDS-PAGE of digested samples

Gel electrophoresis was conducted to monitor the remaining intact protein at each sampling point of digestion. Prior to analysis, meals were centrifuged to remove insoluble material. The supernatant was mixed with sample NuPAGE® LDS sample buffer (4 × concentrated) and NuPAGE® reducing agent. After heating for 10 min at 70 °C, 12.5 μg protein (based on nitrogen content measured with DUMAS) were loaded on a NuPAGE® 4–12% Bis-Tris gel. Gels were run at 140 V with NuPAGE® 2 × MES running buffer. Gels were stained and images were developed as described before.

2.12. Quantification of nitrogen transfer after in vitro digestion

Nitrogen transfer, as a measure for the total soluble protein, after 60 min in the GP, 10 min in the IP, and 60 min in the IP was quantified using DUMAS Flash EA 1112 Protein analyser (Thermo Fisher Scientific, Massachusetts, USA). Meals were centrifuged (10 000×*g*, 10 min, 4 °C) and 300 μL was dried at 60 °C before analysis. L-methionine was used as a standard and cellulose was used as a blank. The conversion factor was 6.38, as generally used for milk protein. Nitrogen transfer was calculated by the following formula and corrected for the enzyme control:

$$N_{transfer}\ [\%] = N_{measured}\ [mg/mL] / N_{theoretical}\ [mg/mL] * 100$$

Where $N_{measured}$ is the nitrogen concentration in the soluble fraction as obtained by DUMAS and $N_{theoretical}$ is the maximum possible concentration of nitrogen that can go into solution. Each sample of the in duplicate conducted digestions was measured in technical duplicates with DUMAS.

2.13. Scanning electron microscopy (SEM) images

SEM images of representative samples for each time point of digestion and each treatment were taken on the Magellan 400 XHR SEM (FEI, Lausanne, Switzerland). Samples were centrifuged (30 s, 13720×*g*, 20 °C) and diluted to a protein concentration between 0.1 and 0.25 mg/mL. Samples were fixed for 1 h on a poly lysine plate using 2.5% glutaraldehyde. A second fixation was done for 1 h using 1% osmium tetroxide followed by a stepwise dehydration with ethanol (10%, 30%, 50%, 70%, 80%, 90%, 100%). Finally, the samples were dried using critical point drying. Between steps samples were washed with 0.1 M phosphate/citrate buffer (pH 7.2).

2.14. Statistical analyses

Statistical analyses were conducted with IBM SPSS version 25 using one-way ANOVA, with the Tukey post hoc test. Differences were considered as statistically significant at $p < 0.05$.

3. Results

3.1. Recovery of SMUF soluble protein

Dry heating of the MP mixture used in this study caused the formation of SMUF insoluble material (Fig. 1a). The remaining soluble protein was quantified using DUMAS (Fig. 1b).

The soluble fraction of LT heated MP turned translucent, while the soluble fraction of HT sample turned dark brown. Protein solubility decreased by 67% in LT heated MP compared to NT MP reaching only 27.4 ± 0.9% soluble protein recovery. In HT heated MP, the protein solubility was 8.0 percent-point lower compared to LT heated MP, resulting in 19.4 ± 1.0% soluble protein recovery.

3.2. Quantification of AGEs

AGEs were quantified in both the MP powder and the SMUF soluble fraction. For CML and CEL absolute concentrations were obtained whereas for pentosidine relative concentrations based were obtained based on d4-CML as internal standard. The recovery of each AGE in the SMUF soluble fraction was calculated based on the total amount in the

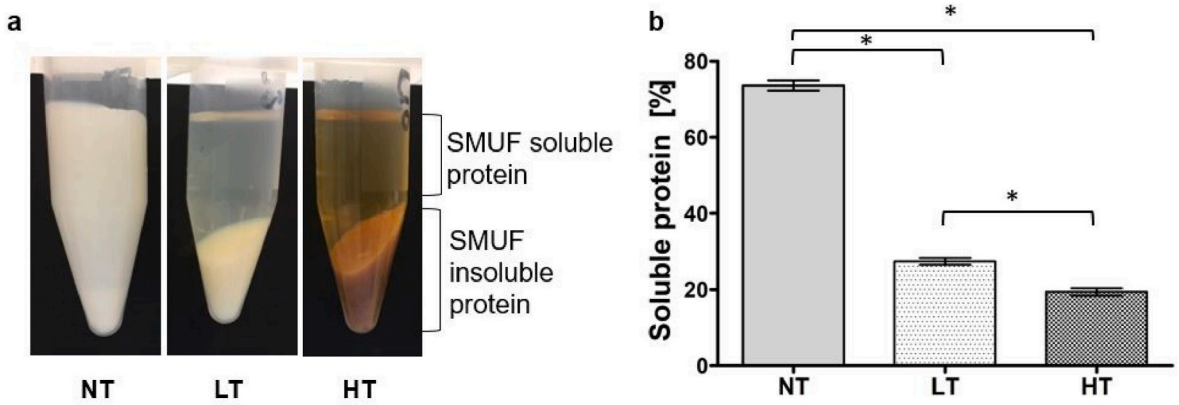

Fig. 1. (a) Images of cow's milk protein powders dissolved in simulated milk ultrafiltrate (SMUF), when non-treated (NT), heated at low temperature (LT), and heated at high temperature (HT). (b) Percentage of MP soluble in SMUF. Error bars represent standard deviation of technical duplicates. Statistical differences were analysed using one-way ANOVA and Tukey post hoc test, with *p < 0.05.

powder (Fig. 2).

The levels of CML and CEL increased with increasing heating temperature. Levels of pentosidine were ~28% higher in the HT heated MP powder compared to the other samples without significant difference between NT MP and LT heated MP. Levels of CML in the powder ranged between 1.13 ± 0.01 and 40.8 ± 0.96 nmol/mg protein, while CEL levels ranged from 0.20 ± 0.02 to 1.10 ± 0.01 nmol/mg protein. Pentosidine levels were observed between 16.6 ± 3.40 and 21.4 ± 0.01 pmol/mg protein in the powder and soluble fraction, respectively. For HT heated MP, but not LT heated MP, significantly lower levels of CEL and pentosidine were recovered in the soluble fraction. Interestingly, especially the recovery of pentosidine shows a drop in HT heated MP (51%) compared to NT MP and LT heated MP, where it showed a similar recovery of 66% and 67%, respectively. This was not observed for CML, which showed comparable levels between the powder and the soluble fraction in all samples.

3.3. Protein composition of SMUF soluble and insoluble fraction

The protein composition of soluble and insoluble material was analysed using SDS-PAGE (Fig. 3). To analyse the insoluble fraction, the pellet was dissolved in S3 (10 mM PBS + 1% SDS + 6 M urea). S3 was chosen to also identify the proteins that are part of the aggregates visible in the top of the gel (see also 4.4).

NT MP (lane 2) showed four dominant bands on the SDS-PAGE image under non-reducing conditions corresponding to five of the major milk proteins α-lactalbumin (10 kDa), β-lactoglobulin (15 kDa), α_{s1}-casein and β-casein (26 kDa), and α_{s2}-casein (29 kDa). A faint band at 22 kDa indicated the presence of κ-casein. The three bands on top correspond to other serum proteins, heavy IgG chain, bovine serum albumin (BSA), and lactoferrin, all of lower abundance. Once heating was applied, two intense bands at 20 kDa ad 30 kDa (indicated in lane 3 as a and b) and a faint band at 15 kDa (indicated as c) were visible in the soluble fraction of both LT heated MP and HT heated MP. Moreover, HT heated MP showed a slightly larger shift to a higher molecular weight than LT

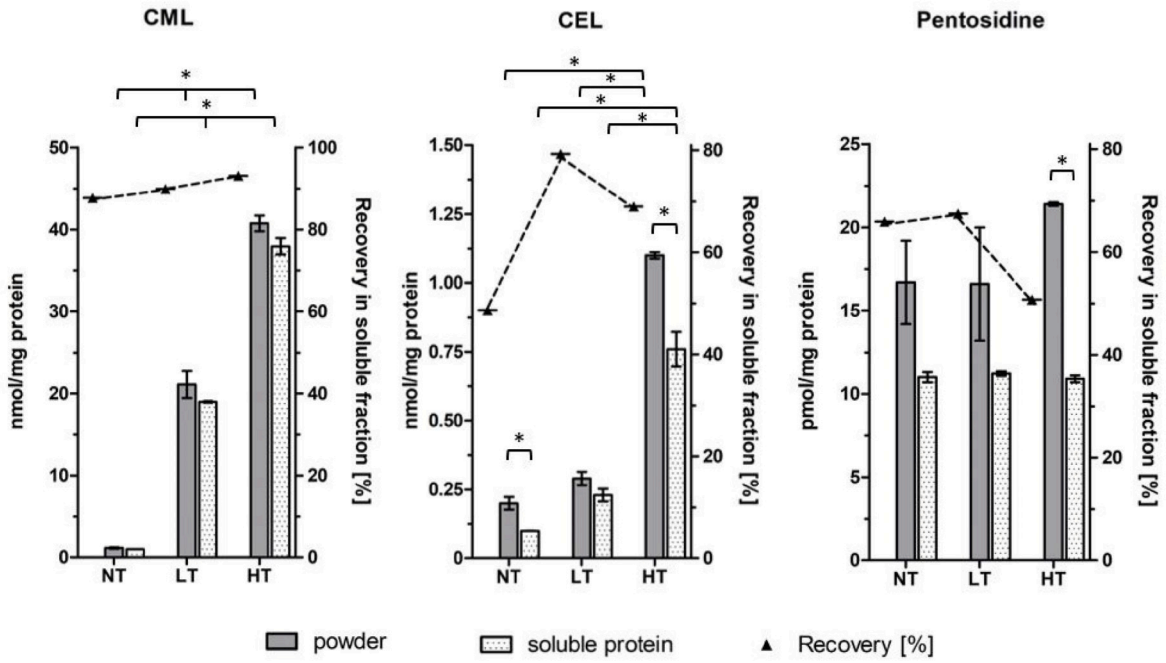

Fig. 2. N^{ε}-Carboxymethyllysine (CML), N^{ε}-carboxyethyllysine (CEL), and pentosidine quantities in cow's milk protein powder in non-treated (NT) sample, after glycation with lactose at low temperature (LT), high temperature (HT) with -▲- showing the recovery in SMUF soluble protein fraction. Error bars represent standard deviations of measurements of duplicate acidic hydrolysis. Statistical differences were analysed using one-way ANOVA and Tukey post hoc test, with *p < 0.05.

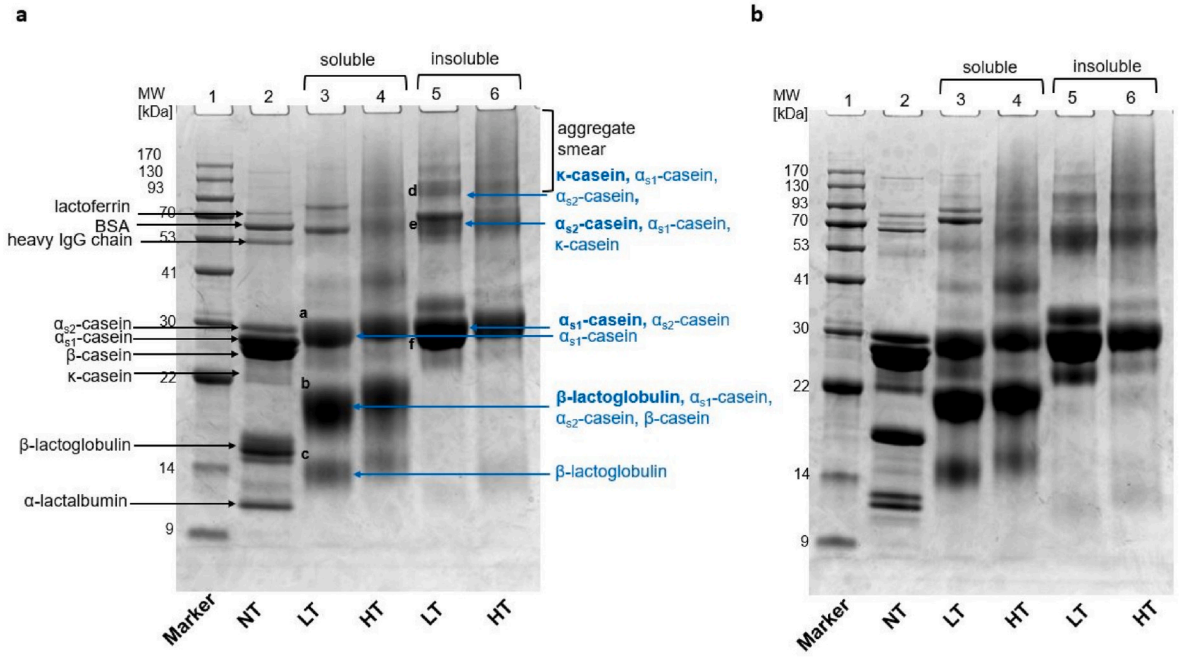

Fig. 3. (a) SDS-PAGE image of heated and glycated cow's milk protein (MP) under non-reducing conditions. (b) SDS-PAGE image of heated and glycated MP under reducing conditions. Lane 2–4: SMUF soluble fraction of MP not-treated (NT), glycated at low temperature (LT), and glycated at high temperature (HT). Lane 5 and 6: SMUF insoluble fraction of HT heated MP and LT heated MP, dissolved in solvent 3 (10 mM PBS + 1% SDS + 6 M Urea). Letters indicate the bands that have been analysed using LC-MS/MS. Identified proteins in blue with bold words indicating the main component of the band based on LFQ intensity. Identification of bands in NT MP was done in comparison with Uniprot data base (https://www.uniprot.org) as well as literature (Costa et al., 2014; Hinz, O'Connor, Huppertz, Ross, & Kelly, 2012) and represents the true mobility of the native proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

heated MP and a smear at higher molecular weight (>53 kDa). In the insoluble fraction (lane 5 and lane 6), one dominant band was observed at ~30 kDa (indicated as f). Additionally, both samples showed two more bands at 33 kDa and 60 kDa which were more intense in LT heated MP than in HT heated MP. Moreover, two additional bands at 34 kDa and 68 kDa (indicated as e) were observed as well as a smear at >53 kDa, indicating the presence of aggregates. Under reducing conditions the soluble fraction of all samples showed the same dominant bands as under non-reducing conditions. In the insoluble fraction of LT heated MP, the band at 33 kDa became more intense and an additional band at 25 kDa corresponding to κ-casein appeared, while the aggregate smear became less intense. This effect was also observed in the insoluble fraction of HT heated MP but to a much lesser extent.

Bands indicated with a letter in Fig. 3 have been analysed using LC-MS/MS to determine which proteins are present in which band. Broad bands were cut into smaller pieces to have multiple segments representing different molecular weights for better identification. The pieces were labeled according to their molecular weight. Results are shown in Fig. 4.

In the soluble fraction, β-lactoglobulin was the most abundant protein. Fig. 4a, band a, showed α_{s1}-casein as the main protein but also traces of β-lactoglobulin were found. Band b was divided in three parts. In the lower parts (18.5 kDa and 19 kDa) β-lactoglobulin was the major protein with traces of α_{s1}-casein in the 19 kDa part. In the top part (19.5 kDa) the highest abundant protein was the glycosylation-dependent cell adhesion molecule 1 (GLYCAM 1). Interestingly, the three major caseins and β-lactoglobulin were also found in the top part of band b with the following order of relative abundance: α_{s1}-casein > β-lactoglobulin > β-casein > α_{s2}-casein. Band c (14 kDa and 15 kDa) showed β-lactoglobulin as the most abundant protein as well as traces of α-lactalbumin at the lower part of the band. In the insoluble fraction, mainly caseins were found. In the aggregates on top of the gel (Fig. 4c, band d), κ-casein and α_{s2}-casein were the most abundant proteins but also α_{s1}-casein and traces of β-casein were found.

3.4. Dissolution of SMUF insoluble material in different solvents

To investigate the forces that lead to decreased solubility of heated and glycated MP, the solubility of the SMUF insoluble fraction in HT heated MP and LT heated MP in 4 different solvents was monitored. Solubility differences represent hydrophobic interactions (addition of SDS, S1 vs. S2), hydrogen bonds (extra addition of urea S2 vs. S3), and disulphide bonds (extra addition of DTT S3 vs. S4). The recovery of soluble protein in the solvents is shown in Fig. 5.

In LT heated MP, protein solubility continuously increased from S1 to S3, reaching 75% soluble protein, but did not further increase by the addition of DTT (S4). In HT heated MP, protein solubility increased 10 fold by the addition of SDS but showed only 1.5 fold additional protein solubility after the addition of urea. After the addition of DTT, the recovery of soluble protein further increased 1.6 fold to 53%. The protein composition of LT heated MP was similar in S1 to S3, as shown in Fig. 5b. Mainly caseins were observed in the insoluble fraction indicated by a dominant band at 30 kDa. Additionally, a smear from >53 kDa was observed, indicating the presence of soluble protein aggregates. A faint band at 35 kDa corresponding to α_{s2}-casein was observed in S2 as well as in S3 and became more intense in S4. In S4 also one additional band at 25 kDa corresponding to κ-casein appeared. Moreover, no bands corresponding to the molecular weight of whey proteins were detected in S1 to S4. For S1 to S3, the protein composition of the insoluble fraction of LT heated MP and HT heated MP were comparable. Interestingly, the smear at >53 kDa was not visible in S4 for LT heated MP. SDS-PAGE was also conducted under reducing conditions which allowed differentiating of the effect of S4 vs. S1–S3 (Fig. 5b, lane 11–15). Under reducing conditions, the smears of LT heated MP in S1 to S3 vanished from the SDS-PAGE image while the band at 25 kDa, corresponding to κ-casein, became more intense.

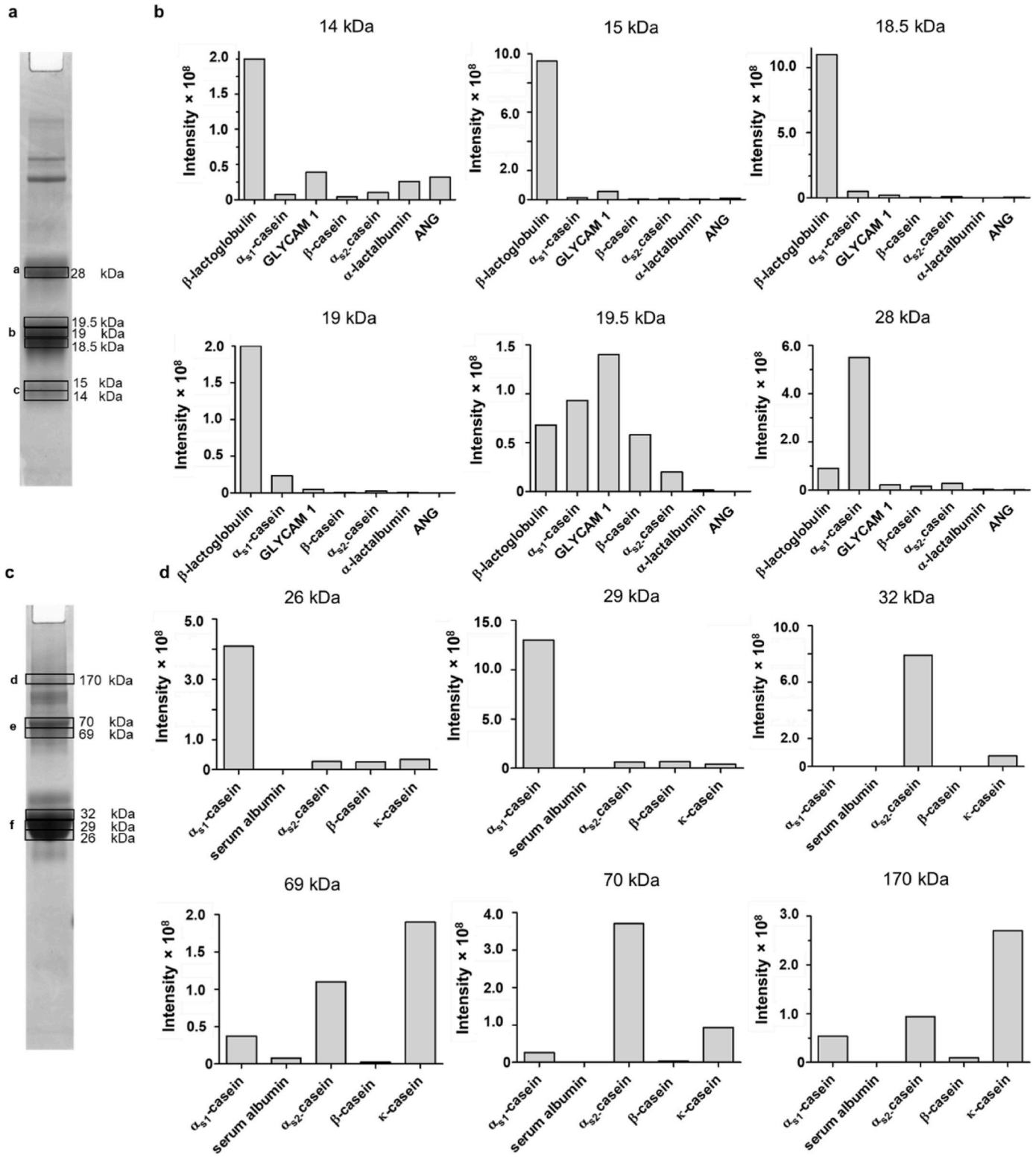

Fig. 4. (a) SDS-PAGE image of the SMUF soluble fraction of low temperature (LT) glycated cow's milk protein (MP) Letters indicate the bands that have been cut for protein identification. Squares indicate the cutting pattern. (b) LFQ intensities of the proteins identified in each band from the SMUF soluble fraction of LT MP. (c) SDS-PAGE image of SMUF insoluble fraction of LT MP dissolved in solvent 3. (d) LFQ intensities of the proteins identified in each band from the SMUF insoluble fraction of LT MP dissolved in solvent 3. All proteins that showed <2.0% LFQ intensity in all samples were not considered. GLYCAM 1: Glycosylation-dependent cell adhesion molecule 1, ANG: Angiogenin, ribonuclease.

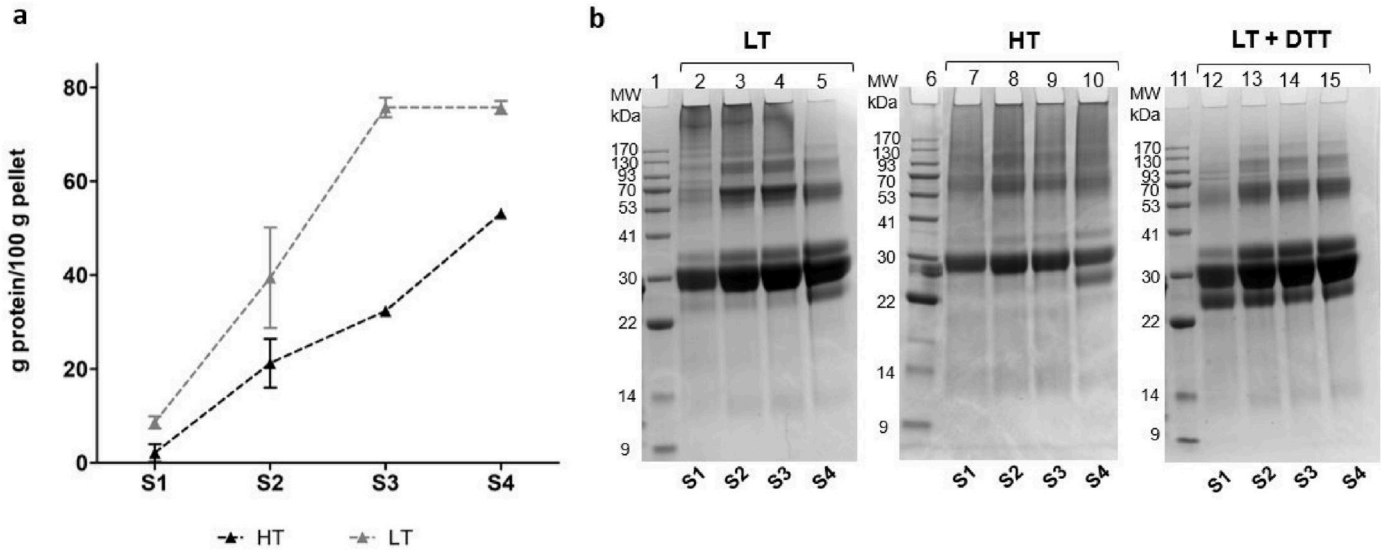

Fig. 5. (a) Soluble protein recovery from SMUF insoluble cow's milk protein formed after heating with lactose at low temperatures (LT) and at high temperature (HT) in 4 different solvents. S1: 10 mM PBS (pH 7.4), S2: S1 + 1% SDS (pH 7.4), S3: S2 + 6 M urea (pH 7.4), S4: S3 + 1% dithiothreitol (pH 7.4). Error bars represent standard deviation of technical duplicates. (b) SDS-PAGE image of soluble proteins after dissolving in S1 to S4, under non-reducing and reducing conditions (+DTT).

3.5. Nitrogen transfer after simulated infant in vitro digestion

Nitrogen transfer from the powder into the digestive fluid was determined after simulated infant *in vitro* digestion at different time points during digestion as a measure for the solubilisation of the heated and glycated MP (Fig. 6).

Gastric digestion led to an increase of total nitrogen in the soluble phase of the digested MP powders, where heated samples showed stronger increases than the unheated powder due to the initial lower solubility. Throughout the GP, HT heated MP showed lower solubility compared to the other samples. After 60 min in the IP, all samples reached between 91.9 ± 2.8% (HT) and 98.9 ± 1.1% (NT) nitrogen transfer, with no significant ($p < 0.05$) differences among samples.

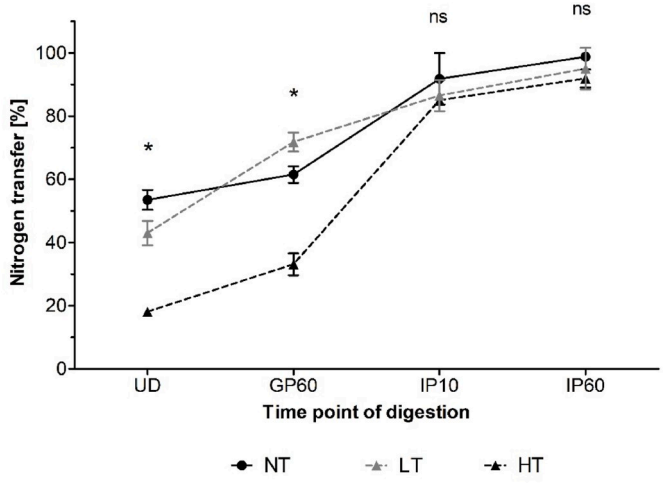

Fig. 6. Nitrogen transfer of cow's milk protein, non-treated (NT), glycated at low temperature (LT), and high temperature (HT) after simulated gastro intestinal infant *in vitro* digestion. UD: undigested (UD) meal, GP60: after hydrolysis in the gastric phase for 60 min, IP10 and IP60: after hydrolysis in the intestinal phase for 10 min (IP10) and 60 min (IP60), respectively. Error bars represent standard deviation of duplicate digestions. Statistical differences were tested using one way ANOVA and Tukey post hoc test. Differences were considered statistical significant at *$p < 0.05$ with ns: not significant.

3.6. Protein hydrolysis during simulated infant in vitro digestion

The extent of protein hydrolysis was monitored by measuring the degree of hydrolysis (DH) with the OPA-assay (Fig. 7).

The highest DH after 60 min in the GP was observed for NT MP with 2.9 ± 0.8%. While the DH of HT heated MP was significantly lower compared to NT MP, no differences were observed compared to LT-CMP. After 10 min in the IP, the highest DH was observed for LT heated MP followed by NT MP and HT heated MP but was only significantly different between LT and HT heated MP. At the end of intestinal digestion, DH reached 39.3 ± 2.6% for NT MP, 47.1 ± 4.2% for LT heating, and 27.1 ± 2.9% for HT heating, with significant differences

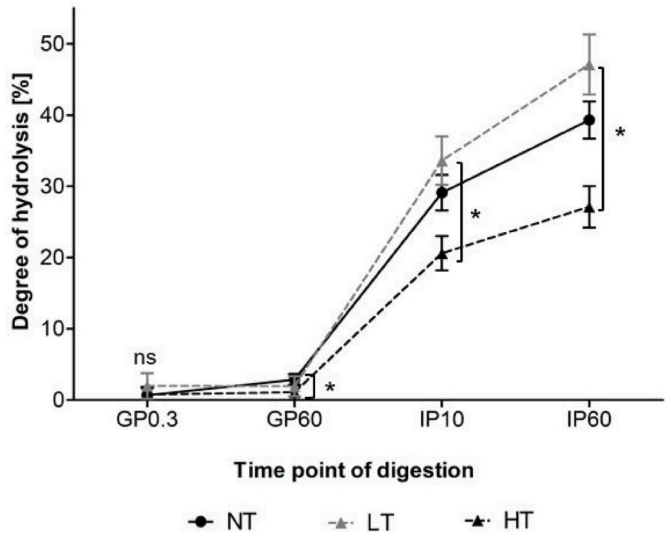

Fig. 7. Degree of hydrolysis of cow's milk protein, non-treated (NT), glycated at low temperature (LT), and high temperature (HT), after simulated gastro intestinal infant *in vitro* digestion. GP0.3: after hydrolysis in the gastric phase for 0.3 min, GP60: after hydrolysis in the gastric phase for 60 min, IP10 and IP60: after hydrolysis in the intestinal phase for 10 min (IP10) and 60 min (IP60), respectively. Error bars represent standard deviation between digestion duplicates. Statistical differences were tested using one way ANOVA and Tukey post hoc test. Differences were considered statistical significant at *$p < 0.05$ with ns: not significant.

between samples remaining the same as after 10 min in the IP.

Remaining intact proteins at different time points of gastro-intestinal infant *in vitro* digestion were visualized using SDS-PAGE (Fig. 8).

The casein bands disappeared in all samples after 60 min in the GP. Only one band from the caseins remained visible in NT MP at 28 kDa. However, this band was not observed in the glycated MP powders. The disappearance of the casein bands was accompanied by the appearance of large peptides at ~10 kDa indicating the formation of large peptides during the gastric hydrolysis of the caseins. In HT heated MP, a smear of aggregated protein over the whole lane appeared on the gel after 60 min in the GP. Whey proteins did not appear to be hydrolysed in the GP independent of the heat treatment. In NT MP, intact β-lactoglobulin remained until 10 min in the IP, while intact α-lactalbumin was still visible after 60 min in the IP. In HT heated MP, the bands of α-lactalbumin and β-lactoglobulin were not observed after intestinal digestion. At the same time, the bands between 10 and 15 kDa, indicating the presence of large casein-derived peptides, remained visible until the end of intestinal digestion. In LT heated MP, the band of β-lactoglobulin at 19 kDa and larger peptides at 15 kDa remained present until the end of intestinal digestion.

3.7. Protein hydrolysis after simulated in vitro digestion with chymotrypsin and trypsin

To disentangle the effect of heat induced protein modifications on the major enzymes in the applied *in vitro* digestion model, *in vitro* digestion with individual enzymes and with specific combinations of individual enzymes was conducted.

Remaining intact proteins after hydrolysis with single enzymes were monitored using SDS-PAGE (Fig. 9).

After chymotryptic hydrolysis of NT MP (Fig. 9a, lane 3–4), the band of β-lactoglobulin remained until 10 min in the IP, while α-lactalbumin and the bands corresponding to large peptides remained until the end of the IP. This was similar for all other used enzymes. However, when a combination of two enzymes was used, larger peptides were not observed. Additionally, the combination or pepsin and trypsin showed even higher density of the β-lactoglobulin band and it was still visible until the end of the IP. Once heating was applied to the MP (Fig. 9b and c), β-lactoglobulin remained until the end of the IP and larger peptides were observed regardless of the used enzyme combination. Additionally, a smear from 30 kDa to >140 kDa was observed for HT heated MP hydrolysed with trypsin as well as trypsin and pepsin, but not for other enzyme combinations.

Combinations of enzymes showed higher DH compared to single enzyme hydrolysis, but only in three cases they showed a potentially synergistic effect, defined as larger DH by the combination of enzymes compared to the sum of the DH values of the individual enzymes (supplementary material: Fig. S2). Especially in HT heated MP, the combination of pepsin with trypsin seemed to have a synergistic effect on the DH at both sampling points (supplementary material: Fig. S2b and Fig. 7 for DH after hydrolysis with pepsin). For the combination of chymotrypsin and trypsin, this promoting effect was only seen for LT heating after 10 min in the IP but not after 60 min in the IP.

3.8. Scanning electron microscopy (SEM) images

SEM images were taken from all samples before and after infant *in vitro* digestion from all sampling time points (Fig. 10).

Undigested NT MP showed several round particles (Fig. 10a), which appeared as white dots in the image. These particles were of various size in an estimated range between 50 nm and 200 nm (Fig. 10b) and showed a smooth surface indicating that these particles are casein micelles. In the background of the images, smaller particles with a less defined contrast were visible. After gastric digestion (Fig. 10c) no larger particles were visible, while in both IP10 (Fig. 10d) and IP60 (Fig. 10e) the majority of the particles was smaller than 50 nm with a tendency to become smaller in IP60. Nevertheless, some particles with a size around 100 nm were still detectable. In undigested LT heated MP (Fig. 10f), no particles large enough to be detected with SEM were visible. At the same time, GP60 samples of LT heated MP showed both, network-like structures (Fig. 10g) and spherical particles with a broad size range between 20 and 100 nm (Fig. 10h). The network-like structures were not detected at any time in the IP and only some spherical particles around 100 nm remained in the IP. In the undigested HT heated MP (Fig. 10m), larger particles were observed with a size around 300 nm. These particles showed a variable shape, either round or as unordered particle that seem to be composed of smaller protein aggregates. After gastric digestion of this sample (Fig. 10 n and o), network-like structures were observed that partly incorporated round particles with a size around 300 nm, as well as particles smaller than 50 nm (left top corner Fig. 10n). Additionally, larger unstructured particles around 5 μm that seemed to partly incorporate the round particles within other structures were found (Fig. 10o).

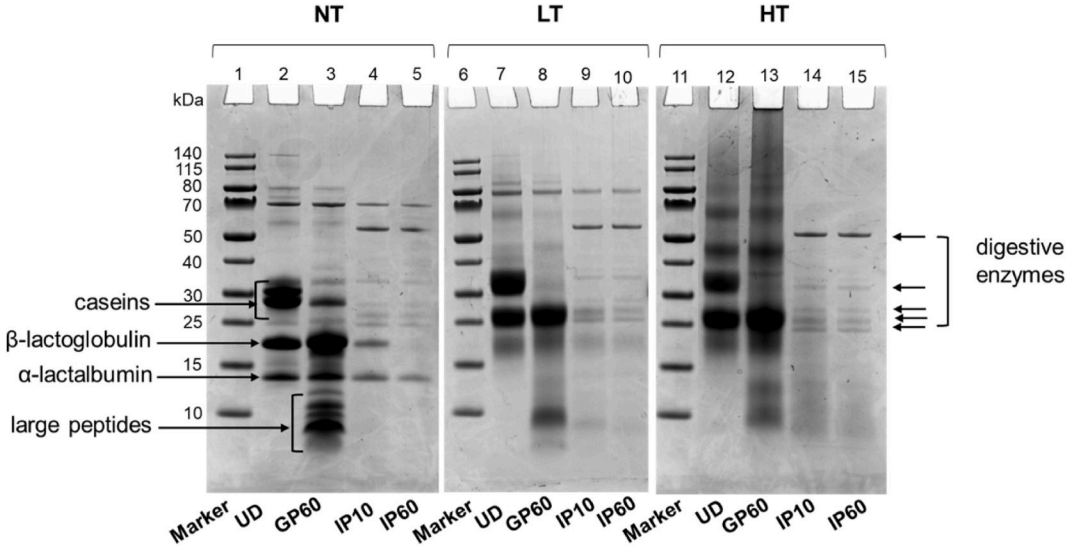

Fig. 8. SDS-PAGE image of cow's milk protein, non-treated (NT), glycated at low temperature (LT), and high temperature (HT)), after simulated gastro intestinal infant *in vitro* digestion. In comparison to the undigested (UD) meal, samples were taken after hydrolysis in the gastric phase for 60 min (GP60), after hydrolysis in the intestinal phase for 10 min (IP10) and 60 min (IP60), respectively.

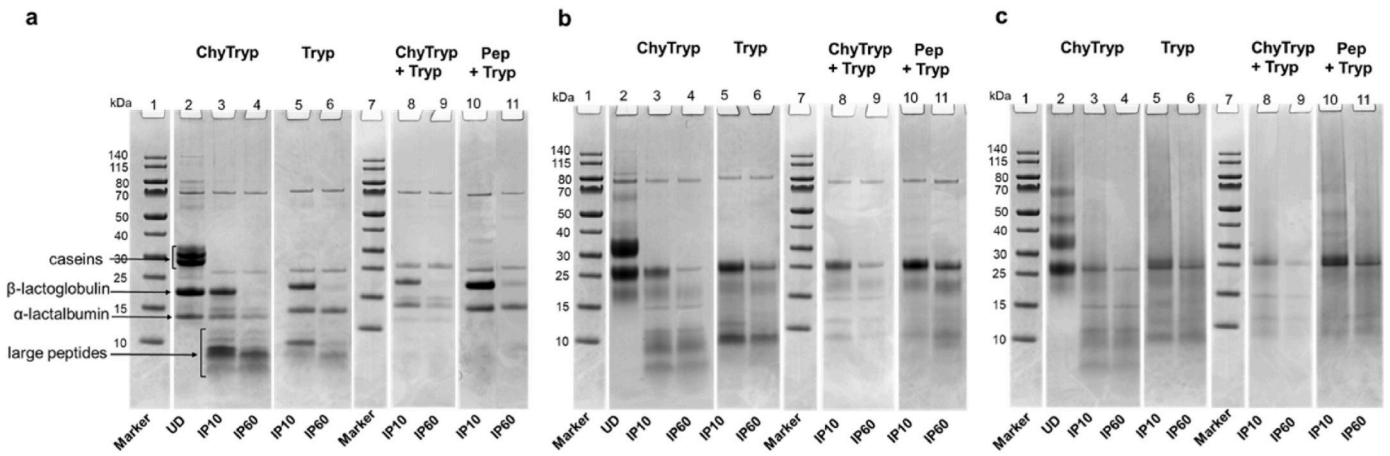

Fig. 9. (a) SDS-PAGE image after 0, 10 and 60 min in the intestinal phase (IP) of non-treated (NT) cow's milk protein (MP) with chymotrypsin (ChyTryp), trypsin (Tryp), respectively, as well as combinations of chymotrypsin and trypsin (ChyTryp + Tryp), and pepsin and trypsin (Pep + Tryp), respectively. (b) SDS-PAGE image of MP heated at low temperatures (LT) after simulated gastro intestinal digestion with various enzymes at different time points. (c) SDS-PAGE image of MP heated at high temperatures (HT) after simulated gastro intestinal digestion with various enzymes at different time points.

After 10 min in the IP, the network-like structures and particles above 500 nm disappeared. Many linear shaped particles of around 100 nm size were observed as well as some larger spherical particles around 250 nm size (Fig. 10p). After 60 min in the IP (Fig. 10q), the linear shaped particles seemed to be further broken down and only sometimes some larger particles were still observed. However, those were more frequently observed after 60 min in the IP in HT heated MP than in LT heated MP.

4. Discussion

Cow's milk contains two major protein fractions, casein and whey protein, that show different susceptibility to enzymatic hydrolysis as well as sensitivity to heat induced structural modifications. Both HT and LT heating led to decreased solubility of MP. In line with previous findings (Anema et al., 2006; Gazi et al., 2015), caseins were the main proteins that remained in the insoluble fraction under both heating conditions (Fig. 3). This is also in line with SEM images which showed no larger particles or casein micelles in the undigested LT heated MP (Fig. 10f). At the same time, undigested HT heated MP showed larger particles around 200 nm size (Fig. 10m), which could be either aggregated whey protein or whey protein coated casein micelles. Higher levels of glycation led to a significant decrease in protein solubility (Fig. 1), however also disulphide bond formation as well as non-covalent interactions such as hydrophobic interactions and hydrogen bonds contributed to the loss of solubility (Fig. 5). This is in line with previous findings suggesting that the MR, and especially MR-induced crosslinking, but also disulphide bond formation, hydrogen bonds and hydrophobic interactions can all contribute to loss of solubility in MP concentrates (Anema et al., 2006; Fan et al., 2018; Le, Bhandari, Holland, & Deeth, 2011; Le et al., 2013; Morgan, Appolonia Nouzille, Baechler, Vuataz, & Raemy, 2005). The contribution of these molecular interactions appeared to be heat treatment dependent (Fig. 5a). In LT heated MP, loss of solubility is mainly driven by hydrophobic interactions and hydrogen bonds, but not via MR or disulphide bond formation. However, disulphide bonds are the main interaction driving the formation of the high molecular weight aggregates in LT heated MP that are released by the use of urea and SDS, as is shown by the disappearance of these aggregates under reducing conditions and in S4 (Fig. 5). This is also supported by the relative abundance of α_{s2}-caseins and κ-caseins in the larger molecular weight aggregates of the insoluble fraction (Fig. 4d). These two caseins have a free cysteine residue which allows them to form disulphide bonds amongst each other and with whey proteins (Broyard & Gaucheron, 2015). The absence of β-lactoglobulin in the 170 kDa band of the insoluble fraction (Fig. 4d) does, not support the involvement of the κ-casein/β-lactoglobulin complex in dry heated samples. Overall, the low abundance of whey proteins in the insoluble fraction (Fig. 5b), indicates that they play a minor role in the loss of solubility. This is in agreement with previous findings showing that casein solubility in MP powders is more affected by heating and long term storage than whey protein solubility (Gazi et al., 2015). In HT heated MP, not only disulphide bonds but also the formation of other covalent bonds contribute to the decrease in solubility. The 47% of protein remaining insoluble after dissolving in S4 (Fig. 5a) and the low recovery of especially pentosidine of 51% in the soluble fraction (Fig. 2) indicates that MR-induced crosslinking contributed to the observed loss of solubility of especially caseins (Figs. 1b and 4). The large aggregates shown on the SDS-PAGE after tryptic hydrolysis of HT heated MP, but not after chymotryptic hydrolysis (Fig. 9c), also indicate the role of MR-induced crosslinking in aggregate formation, as trypsin specifically cleaves at the lysine and arginine residues which makes its hydrolysis prone to lysine blockage via the MR (Olsen, Ong, & Mann, 2004). While tryptic hydrolysis of LT heated MP is impaired (supplementary material: Fig. S2), it does not show hydrolysis-resistant aggregates on the SDS-PAGE (Fig. 9b), suggesting that MR-induced crosslinking is less involved in the formation of these aggregates. This is also supported by the results of the pentosidine analysis of the heat treated MP: LT heated MP contained less pentosidine than HT heated MP (Fig. 2), which may ultimately have influenced its degradability. Our findings showed that indeed several intramolecular interactions are involved in the loss of solubility upon dry heating, however their contribution strongly depends on the applied heating conditions. The combination of single enzyme digestions and solubility tests on heated and glycated MP allow two conclusions about the nature of the covalent interactions that result in insolubility and formation of large aggregates in MP heated in the presence of lactose (Fig. 3). First, covalent crosslinking, other than disulphide bond formation, seems to promote casein insolubility at HT dry heating but not at LT dry heating. Secondly, aggregates that are formed during heating are released from the insoluble material but the further hydrolysis/breakdown of these aggregates by trypsin is strongly impaired, especially when they are formed via covalent crosslinking, implying a role for the MR.

Aggregation and MR in this model system may not only have negative technological consequences, but also affect the overall digestibility of MP. After simulated infant *in vitro* digestion, the aggregates of HT heated MP were still visible at the end of the GP but not after 10 min in the IP (Fig. 8). This is in line with the results from the combined hydrolysis by chymotrypsin and trypsin, which showed that chymotrypsin

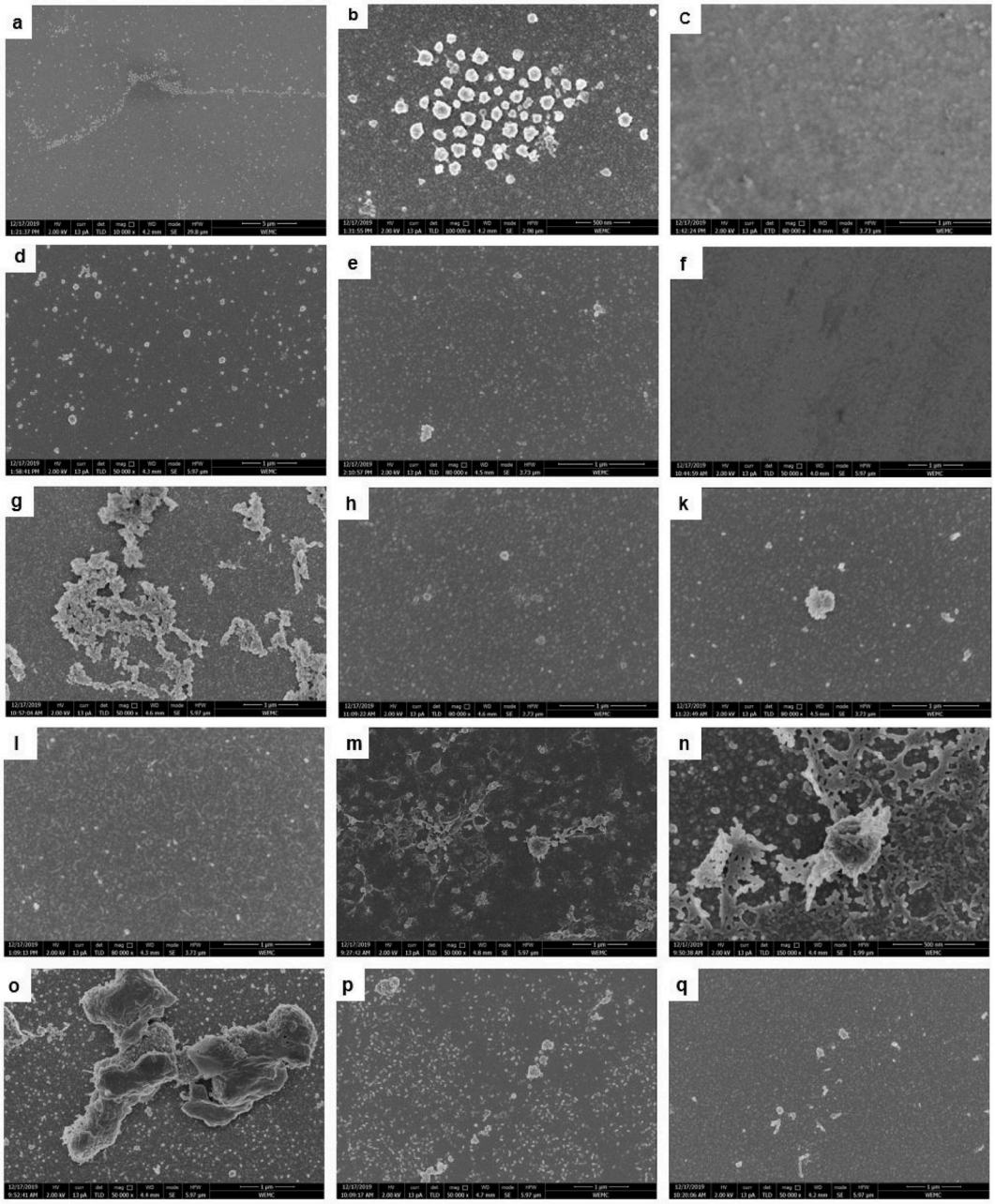

Fig. 10. Scanning electron microscopy images of non-treated (NT) cow's milk protein, heated at low temperature (LT), and heated at high temperature (HT) in the presence of lactose, after 60 min in the gastric phase (GP60), 10 min in the intestinal phase (IP10), and 60 min in the intestinal phase (IP60) of an infant *in vitro* digestion model. (a and b) NT MP undigested, (c) NT-GP60, (d) NT- IP10, (e) NT- IP60, (f) LT heated MP undigested, (g and h) LT- GP60, (k) LT-IP10, (l) LT- IP60, (m) HT heated MP undigested, (n and o) HT- GP60, (p) HT- IP10, (q) HT- IP60.

hydrolysed these aggregates and thereby partly compensated for the impaired trypsin hydrolysis (Fig. 9 and supplementary material: Fig. S2). In contrast to the single enzyme hydrolysis experiments, the degree of hydrolysis in infant *in vitro* digestion was impaired for HT heating, but not for LT heating (Fig. 7). This may be due to the use of pancreatin, which besides chymotrypsin and trypsin also contains other enzymes that can contribute to the protein hydrolysis. The experiments with specific enzymes suggest that adding another protease to trypsin can compensate for the impaired hydrolysis of trypsin in LT heated MP but not in HT heated MP (supplementary material: Fig. S2). Therefore, it is possible that all pancreatic enzymes together are also less affected by LT heating than HT heating of MP. The higher protein digestibility of LT heated MP compared to HT heated MP can be explained by the differences in the applied heating conditions and the resulting protein modifications. In general, thermal processing under either dry or wet conditions, of MP can both increase or decrease protein digestibility which is strongly dependent on the applied heating conditions that determine the structural protein modifications. Most *in vitro* studies reported an increase in protein digestibility after thermal processing of MP, possibly as a result of heat induced unfolding of the globular whey protein (van Lieshout, Lambers, Bragt, & Hettinga, 2019). However, when heating is conducted at low temperatures (<70 °C) and at low $_{aw}$-levels, that favour MR over protein unfolding, most studies report decreased *in vitro* protein digestibility (Culver & Swaisgood, 1989; Dupont, Boutrou, et al., 2010; Dupont, Mandalari, et al., 2010; Rudloff et al., 1992; Sanz, Corzo-Martínez, Rastall, Olano, & Moreno, 2007). In our study, we observed that LT dry heating, accompanied with an increase of MR, does not directly result in decreased protein digestibility.

However, HT dry heating decreased protein digestibility, probably due to the higher levels of advanced glycation and MR-induced crosslinking (Fig. 2). Additionally, SEM images showed relatively larger particles in undigested HT heated MP (Fig. 10m) than in LT heated MP (Fig. 10f) as well as a slower hydrolysis of these particles during the intestinal digestion of HT heated MP (Fig. 10p and q). This is in line with other findings showing that more severe heating in solution, resulting in relatively more aggregation and formation of e.g. lactosyllysine, leads to decreased protein digestibility *in vitro* (Wada et al., 2014, 2015). Interestingly, it seems that insolubility does not directly affect digestibility in the IP, as no significant differences were observed in nitrogen transfer (Fig. 6). The rapid disappearance of caseins on the SDS-PAGE, independent from the applied heating conditions and the network-like structures observed in the SEM images (Fig. 10), is in line with previous findings and a result of acidic depletion and rapid hydrolysis in the GP (Dupont, Mandalari, et al., 2010; Picariello et al., 2010). The observation of these network-like structures in the GP of the heated MP but not in NT MP is in line with previous findings that showed that unheated milk forms more firm clots than homogenised and heated milk (Ye, Cui, Dalgleish, & Singh, 2017). Moreover, the observation of larger spherical particles within these networks in the GP of HT heated MP (Fig. 10n) and the longer persistence of larger particles in the IP (Fig. 10p and q) indicate a higher resistance of caseins towards digestion in HT heated MP, which is also supported by the partly hydrolysed casein fragments on the SDS-PAGE (Fig. 8). This is in line with the findings by Dupont et al. (Dupont, Boutrou, et al., 2010), who reported that extensive heat treatment of milk powder results in a higher resistance of caseins to infant *in vitro* digestion. In contrast to this, whey proteins showed enhanced hydrolysis in both LT and HT heated MP compared to NT MP (Fig. 8). This is in agreement with previous findings, which reported that glycated α-lactalbumin (Joubran, Moscovici, & Lesmes, 2015; Joubran, Moscovici, Portmann, & Lesmes, 2017) and whey proteins in spray dried skim milk powders (Dupont, Boutrou, et al., 2010) showed enhanced protein hydrolysis in infant *in vitro* digestion. Heating and glycation of MP can both increase and decrease allergenicity (Nowak-Wegrzyn et al., 2009). Pinto et al. (Pinto et al., 2014) showed that the aggregates of glycated β-lactoglobulin survive until 60 min in the IP of adult gastrointestinal *in vitro* digestion. Moreover, another study reported that aggregation of β-lactoglobulin when glycated at HT and low a_w-levels resulted in the formation of binding ligands for the receptor of AGEs and immunoglobulin E (Zenker et al., 2019). This suggests that impaired MP digestibility, as well as the survival of aggregates in the gastrointestinal tract, can affect the allergenicity and immunogenicity of MP. Treating MP under such harsh conditions as in this study is not practiced in food industry. However, the ingestion of MP baked into a wheat matrix, such as a muffin or waffle, has been shown to positively correlate with the resolution of cow's milk allergy symptoms in allergic children (Kim et al., 2011). In these products, protein aggregation and MR are promoted due to the HT, low a_w-levels, and high sugar content. Our findings show that dry heating of MP in the presence of reducing sugars can affect protein digestibility and suggest that this could have an effect on the immunogenicity of the MP, although further studies would be needed to directly show the link between the digestibility and immunogenicity of dry heated MP.

5. Conclusion

Dry heating of a powdered mixture of casein and whey protein in the presence of lactose led to changes in the protein structure that affected both solubility and digestibility. Different inter-and intramolecular interactions lead to a decreased solubility of mainly caseins. Despite the high level of MR in both LT and HT heated MP, impaired protein digestibility after simulated infant *in vitro* digestion was only observed for HT heating, which is possibly related to the higher level of MR-induced covalent crosslinking. Moreover, large particles were present at the end of the GP and were slower hydrolysed in dry heated MP compared to NT MP. This will not only negatively affect the nutritional value of the MP but also determine in which form the protein will be presented to the mucosal immune system in the gastrointestinal tract, which may affect the immunological response towards ingested MP.

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Declaration of competing interest

None.

CRediT authorship contribution statement

Hannah E. Zenker: Conceptualization, Methodology, Investigation, Writing - original draft, Visualization, Validation, Formal analysis. **Jana Raupbach:** Formal analysis, Validation, Writing - review & editing. **Sjef Boeren:** Formal analysis, Validation, Data curation. **Harry J. Wichers:** Supervision, Writing - review & editing. **Kasper A. Hettinga:** Conceptualization, Project administration, Writing - review & editing, Funding acquisition.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2020.106098.

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