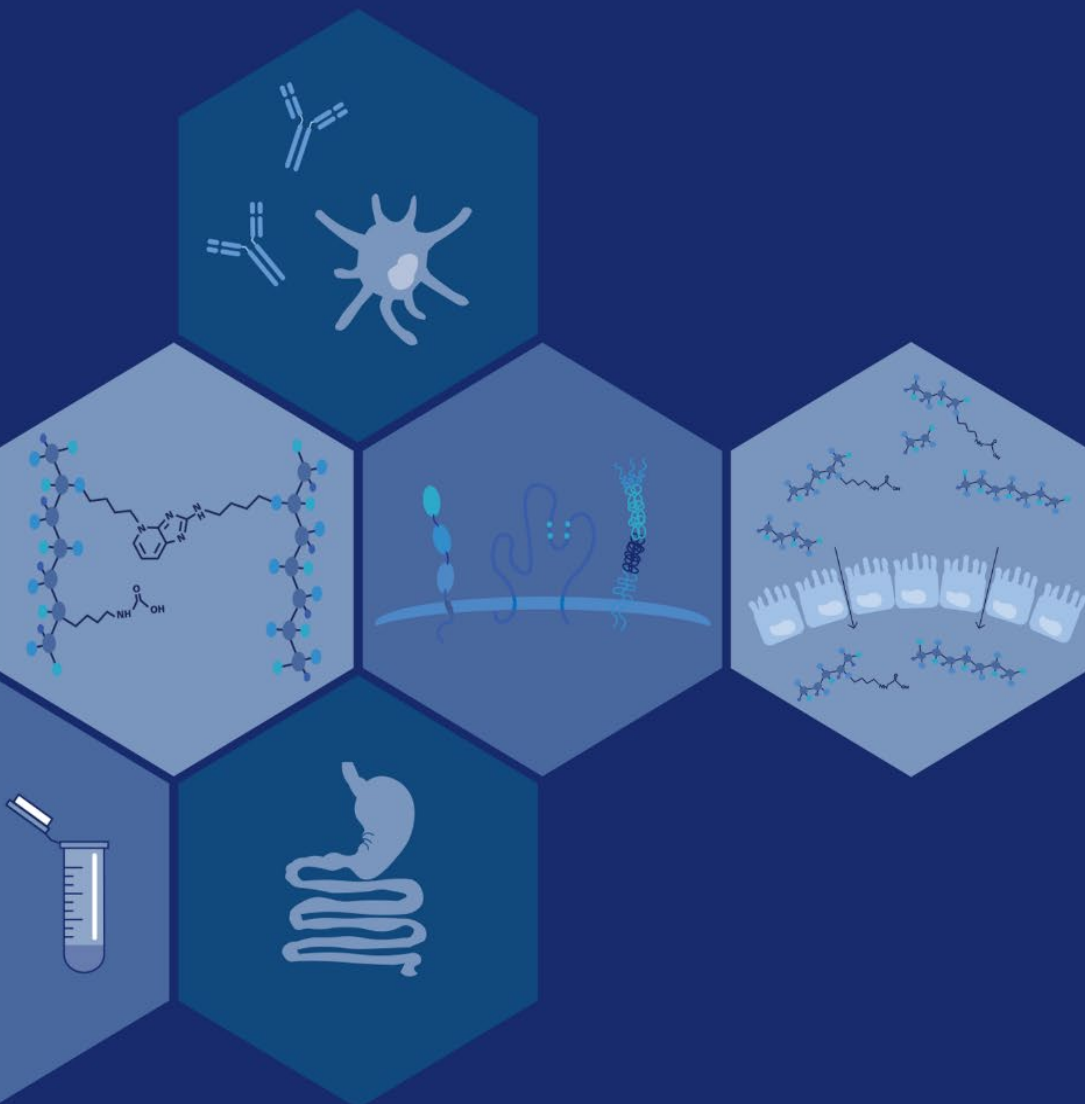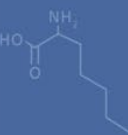# Towards understanding the role of heat-induced structural changes on immunoreactivity and digestibility of cow's milk protein

Hannah E. Zenker

Propositions:

1. The sum of structural modifications, occurring upon heating, affects the immunoreactivity of cow's milk protein and not a single modification by itself. (This thesis)
2. Immunoreactivity and digestibility are linked by the digestion resistance of immunoreactive structures (This thesis).
3. Being a multidisciplinary researcher is another form of being an expert.
4. The biggest challenge of doing a PhD is telling your friends about your work.
5. Increasing availability of information alone does not make us more educated.
6. Commercialism of news is the biggest threat to fact-based reports.
7. Justice in a society is better served by the consensus to act as a community than by its laws.

Propositions belonging to the thesis, entitled

Towards understanding the role of heat-induced structural changes on immunoreactivity and digestibility of cow's milk protein

Hannah E. Zenker

Wageningen, 23rd of October 2020

Towards understanding the role of heat-induced structural changes on immunoreactivity and digestibility of cow's milk protein

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Towards understanding the role of heat-induced structural changes on immunoreactivity and digestibility of cow's milk protein

Hannah E. Zenker

Thesis

Submitted in the fulfilment of the requirements for the degree of doctor

at Wageningen University

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in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

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List of contents

Chapter 1

General Introduction

1.1 Project overview

This thesis investigated the physical-chemical changes that occur during heating of cow's milk protein and the potential effects on digestibility and immunogenicity. It was part of a larger NWO-TTW project (Immunogenic Properties of Heated and Glycated Cow's Milk Protein – Effect on Initiation and Resolution of Cow's Milk Allergy) and was carried out in collaboration with the Allergology & Clinical Immunology group at Erasmus Medical Centre in Rotterdam and the Cell Biology and Immunology group at Wageningen University. This larger project consisted of 4 work packages:

- Work package 1 is described in this thesis and focused on the physicochemical modifications of heated and glycated cow's milk protein.

- Work package 2 investigated the immunogenicity and allergenicity of heated and glycated (MP) *in vitro*.

- Work package 3 developed a product of heated and glycated MP that can be added to the daily diet of cow's milk (CM) allergic children.

- Work package 4 studied the induction of tolerance by the in work package 3 developed product in a clinical trial.

Combining data from different disciplines (physicochemical analysis and molecular data) leads to new insights in the possible causes for heating induced changes in cow's milk immunoreactivity and the role of the Maillard reaction (MR).

1.2 CM protein and application in infant nutrition

CM protein is a commonly used basis of infant formula that is used as a substitute for human milk when breastfeeding is not possible or commonly introduced between the age of 6-12 months [1]. CM can roughly be divided in two main protein fractions: 80% caseins and 20% whey proteins (WPs). WPs are globular proteins and in contrast to caseins soluble at pH 4.6. The major WP in CM is β-lactoglobulin (BLG) (~50% (w/w) of all WPs), followed by α-lactalbumin (ALA) ~20% (w/w), immunoglobulins ~10% (w/w), and bovine serum albumin (BSA) ~6% (w/w) [2]. The globular structure of both BLG and ALA is stabilised via disulphide bonds. While ALA exists as monomer, BLG exists in a monomer-dimer equilibrium in solution and is predominantly dimeric under physiological conditions. Moreover, it contains the free cystein-121 that is buried in the interior of the protein, however upon denaturation becomes exposed and plays an important role in the aggregation of BLG [3]. The casein fraction covers four proteins, α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein in the ratio of 4/1/3.5/1.5 [4]. Caseins are unstructured proteins with little secondary and tertiary structure. All caseins contain clustered phosphorylated serine residues, except κ-casein that only contains one phosphoserine in its sequence [5]. Most of the κ-casein is located on the surface of the casein micelle. The C-terminal κ-casein macropeptide, which has a variable degree of glycosylation, extends on the

outside of the casein micelle to form a hairy layer that, via steric repulsion, inhibits self-association of casein micelles. The size of the casein micelles depends on the milk composition as well as the glycosylation degree of the κ-casein and usually ranges between 100 nm and 300 nm [4]. Both $α_{s2}$-casein and κ-casein contain free cysteine residues that enable them to form intermolecular disulphide bonds. These disulphide bonds occur naturally but can also be redistributed during thermal processing of MP, for instance the heat induced interaction of BLG with κ-casein via S-S/thiol disulphide interchange [6,7]. Three caseins, $α_{s1}$-casein, β-casein, κ-casein, and ALA in CM are structurally similar to their counterparts in human milk, while BLG and $α_{s2}$-casein are not part of the natural components of human milk [8,9]. Moreover, protein composition of human milk and the contribution of the individual proteins differ compared to CM. For example, while $α_{s1}$-casein is highly abundant in CM it is only a minor component in human milk. The ratio of casein to WPs in human milk is estimated to 40:60 compared to 80:20 in CM, however also shows large variation during lactation [9]. In early infant nutrition the ratio of casein to WP in CM based infant formula is adjusted closer to the composition of human milk, while at a later stage a higher ratio of casein to whey is chosen. Next to compositional adjustment of MP based infant formulas, heat treatment is applied to ensure product safety. This is of great importance in infant nutrition as their immune system still develops. However, the thermal processes also change protein structure which affects its digestibility and immunoreactivity.

1.3 Heat induced modifications of MP

Thermal processing of CM is often applied in industry to obtain powdered products and ensure product safety. Upon heating, protein structure may change and chemical modifications occur. The type and relative abundance of these modifications, as well as the extent to which these modifications occur, depends on the applied conditions during heating, such as pH, salt concentrations, a_w-level, temperature, and time as well as on the native structure of the protein. The threshold temperature for irreversible structural changes (aggregation) in isolated BLG and α-lactalbumin, in solution is between 65-70 °C [10,11], however heat induced changes to BLG are strongly affected by pH and ionic strength [12]. Caseins, in contrast, are more heat-stable due to their minimal secondary and tertiary structure. Their arrangement in the casein micelle stabilises them against precipitation and makes the casein molecules more resistant towards changes of environmental conditions. In the presence of both micellar casein and WPs, a complex between WPs and the casein micelle is formed resulting in the coverage of the casein micelle surface with aggregated WP upon heating in solution. This complex formation is triggered via disulphide bond formation and predominantly occurs between κ-casein and β-lactoglobulin. Although ALA is also involved in the formation of this complex on the casein micelle surface, it does not interact with the casein micelle in the absence of BLG [13].

While heat induced modifications in solution have been object of many studies in the past, only a few studies investigated dry heating of WPs [14-21]. Dry heating of WPs, induces relatively

more glycation and less denaturation than wet heating. For WPs it is suggested that dry heating temperatures <55 °C do not result in significant changes of the protein structure while temperatures >55 °C may lead to structural modifications [22]. Significantly increased aggregation was reported at temperatures above 80 °C, however this is also strongly depending on the presence of reducing sugars [23]. For example, Deng *et al.* [21] showed that dry-heated BLG at 130 ºC in the presence of lactose is composed of ~60% oligomers and polymers, while heating in the absence of a reducing sugar lead to only ~10% polymerisation. Moreover, they observed the formation of insoluble material for high temperature dry-heated BLG in the presence of reducing sugars which was also supported by previous findings [20]. For casein micelles, only few studies can be found that report dry heating conditions for milk protein concentrates (micellar casein together with WPs) at temperatures below 50 °C [24-35] and micellar casein in the presence or absence of lactose at 60 °C [36,37]. Dry heating of milk protein concentrates at elevated temperatures results in loss of solubility of caseins while WPs are less affected. Next to hydrophobic interactions, hydrogen bonds, disulphide bonds [34], and high calcium ion activity [31], many studies attributed the loss of solubility to glycation via the MR [27,30].

During thermal processing of food products, side chain modifications on the amino acids will occur, of which MR, oxidation, isopeptide bond formation, and crosslinking following a β-elimination of cysteines or serine residues are most commonly described [38]. For milk and dairy products, the MR is considered as the predominant reaction. The MR is the reaction between the reactive carbonyl group of a reducing sugar with the primary or secondary amino group of proteins, peptides and free amino acids to form the Amadori product. Due to the high levels of the reducing milk sugar lactose, and the high availability of lysine, milk is particularly prone to this reaction. The MR is often divided into three stages: the early MR, the advanced MR, and the late MR [39]. For each stage, representative marker molecules are defined (Figure 1.1). Especially the products of the advanced stage, the so called advanced glycation end products (AGEs) that can be categorised in linear and crosslinking AGEs. An example for a crosslinking AGE is pentosidine, while examples for linear AGEs are N^{ε}-carboxymethyllysine (CML) and pyrraline that have also previously been studied in relation to their immunomodulatory properties and metabolic transit [41-44]. However, due to the temperature dependence of the MR, it is often not possible to separate the effects of heat and glycation on modifications of the protein structure. Moreover, the occurrence of the MR has been described to have a promoting effect on disulphide bond formation and protein aggregation [45,46] and has been shown to significantly affect the morphology of the formed aggregates [47]. Next to the MR, dephosphorylation reactions of caseins can also occur resulting in the formation of serine and threonine adducts. However, significant changes are only observed after prolonged heat at temperatures >100 °C [48] and therefore uncommon in most dairy products.

Early MR

$$Prot-(CH_2)_4-\underset{H}{N}-C-\underset{H_2}{C}-\overset{O}{\underset{OH}{C}}-\underset{Glu}{CH}-CH-\overset{OH}{CH}$$

Amadori product with lactose

Advanced MR

$$Prot-(CH_2)_4-\underset{H}{N}-\underset{H_2}{C}-COOH$$

N$_\varepsilon$-carboxymethyl lysine

$$Prot-(CH_2)_4-N$$
Pyrralin

Pentosidine

$$Prot-(CH_2)_4-\underset{H}{N}-\overset{CH_3}{\underset{H}{C}}-COOH$$

N$_\varepsilon$-carboxyethyl lysine

Late MR

Mellanoidines
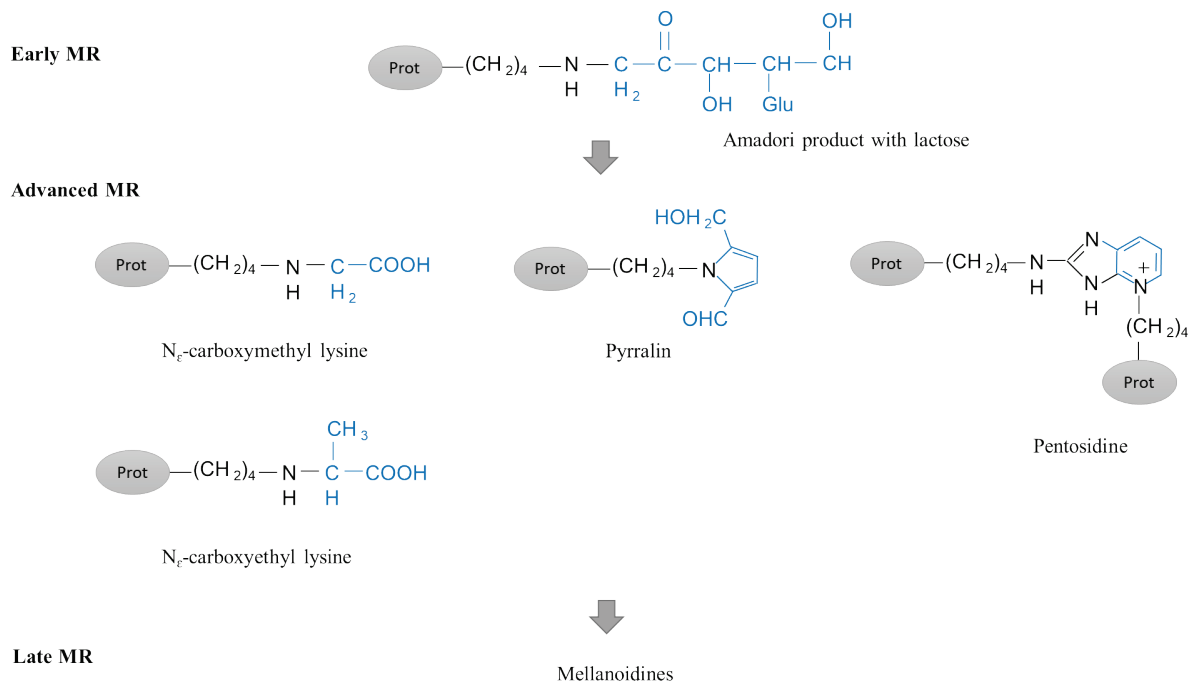
Figure 1.1: Selected protein bound (Prot) Maillard reaction (MR) products representative for each state of the MR. Adapted from Arena *et al.* [40].

These structural modifications of milk proteins during heat treatment either in solution or under dry conditions can affect the immunoreactivity of the protein and may alter the immunological response of CM allergic children [49].

1.4   CM allergy

CM allergy (CMA) is an adverse reaction of the immune system towards one or more CM derived proteins. CMA occurs as immunoglobulin E (IgE) mediated, non-IgE mediated, or mixed forms of CMA. Symptoms of CMA usually affect one or more organs, especially the skin, the respiratory tract, and the gastrointestinal tract. In IgE mediated CMA, symptoms usually occur within 30 min of ingestion, while in non-IgE mediated CMA symptoms are delayed beyond 2 h of ingestion. While IgE mediated CMA can be indicated by the presence of specific IgE (sIgE) in the serum together with a positive skin prick test, the diagnosis of non-IgE mediated CMA can only be confirmed via oral challenge [50]. Clinical manifestation of CMA symptoms usually occurs, within 2- 3 days after the first introduction of MP based infant formula. Prognosis for natural resolution of CM allergic symptoms is estimated to be at the age of 1 year for non-IgE mediated CMA, while it is likely to be of longer persistence in IgE mediated CMA [50]. For IgE mediated CMA, the natural resolution rate of symptoms has been estimated to be 70% by the age of 3 years [51,52].

1.5   Management of CMA

Despite the natural resolution of CMA symptoms in the majority of CM allergic children, a careful management of CMA is required for the period in which symptoms frequently occur.

The most common approach is the strict avoidance of MP and the replacement of MP based infant formula with other products, such as extensively hydrolysed MP based formulas and amino acid formulas as well as plant based infant formulas such as soy or rice-based products. On the other hand, the introduction of dry-heated MP, often referred to as "baked milk", to the diet of allergic children was positively correlated with the resolution of CMA symptoms in 70% of the tested children. These studies often use milk powder which was baked into a food matrix (waffle or muffin) [53-55], however, also other matrices (cheese on pizza) [54,55], fermented products [56], and liquid baked milk with and without wheat matrix (30 min at 180 °C) have been used [57]. These studies showed that the heat treatment of MP under dry or semi-dry conditions can, in a certain subgroup of MP allergic children, result in the development of a tolerogenic immune response to MP. The choice of the most suitable diet depends on, amongst others, the severity of the symptoms of the child; it is for instance recommended to only use amino acid formulas for patients with a high risk for anaphylaxis. Moreover, dietary requirements of the infant, for instance the availability of essential amino acids as well as potential cross-reactivity to soy-based formulas, need to be carefully evaluated [50].

1.6   Cow's milk allergens

BLG was often described as the major MP allergen due to the high frequency CM allergic children to β-lactoglobulin, as well as its high quantity in CM but its absence in human milk. Nevertheless, Wal *et al.* [58] pointed out that the majority of MP allergic children also shows reactivity to caseins. Moreover, in a later study it was shown that sensitisation to α-caseins and κ-casein is equally frequent in 20 MP allergic children as to BLG [59]. Next to this, Docena *et al.* [60] reported a higher prevalence of sensitisation towards caseins than WPs by monitoring IgE and immunoglobulin G (IgG) in 80 CM allergic children. Table 1.1 shows an overview over common CM protein allergens.

Table 1.1: Properties of cow's milk allergens, adapted from Hochwallner *et al.* [61]. Relative amount of the milk proteins was calculated from another source [2] and for caseins was estimated based on 80% of total cow's milk proteins and their relative ratio [4]. Post translational modifications (PTMs) and disulphide bonds were based upon the Uniprot database (https://www.uniprot.org/ January 2020)

Milk protein fraction	Allergen name	Protein name	Amount of total protein [%]	Size [kDa]	Prevalence [% of patients]	PTMs
Whey proteins (20%, ~ 5 g/L)	Bos d 4	α-Lactalbumin	~3%	16.247	0-67	
	Bos d 5	β-Lactoglobulin	~7%	19.883	13-62	
	Bos d 6	Bovine serum albumin	~0.9%		0-76	Phosphoserine 29, 82, 89, 296, 442, 512 Phosphothreonine 107, 443, 445, 569 Glycosylation 151
	Bos d 7	Ig	~ 1.4%		12-36	
Caseins (80%, ~30 g/L)	Bos d 9	α_{s1}-casein	~ 32%	24.529	65-100	Phosphoserine 56, 61, 63, 79, 81, 82, 83, 90, 130
	Bos d 10	α_{s2}-casein	~ 8%	26.019	N/A	Phosphoserine 23, 24, 25, 28, 46, 71, 72, 73, 76, 144, 146, 150, 158
	Bos d 11	β-casein	~ 28%	25.107	35-44	30, 32, 33, 34, 50
	Bos d 12	κ-casein	~ 12%		35-41	Phosphoserine 148, 170, 187 Phosphothreonine 166 Glycosylation 152, 153, 154, 157, 163, 170, 186

To summarise, literature repeatedly reported that not only BLG but also other MP and especially caseins are important allergens in CM. This highlights the importance to not only study the immunogenicity and allergenicity of BLG but to also include caseins and other WPs in the studies of CMA induction and prevention.

1.7   Mechanism of food allergy

Food allergy is an adverse reaction of the immune system towards a food derived antigen. The overall self-reported prevalence of food allergy in Europe is estimated to be 5.9% and is higher in children than in adults [62]. Food antigens can lead to sensitisation via the cutaneous route, the gastrointestinal tract, oral cavity, and the respiratory tract [63]. After oral intake the first contact with antigen presenting cells already starts in membrane of the mouth which can cause mild oral allergic symptoms. Subsequently, the antigens are hydrolysed by digestive enzymes in the gastrointestinal tract. Once they reach the small intestine, they can cross the intestinal barrier where they are exposed to the gastrointestinal immune system. The molecular mechanism from the first contact with the antigen to the allergic reaction can roughly be divided in two phases, the sensitisation phase and the effector phase (Figure 1.2) [64]. In the sensitisation phase, the antigen is recognised and taken up by antigen presenting cells (APCs), such as dendritic cells (DCs), macrophages, and activated B-cells [65]. Amongst these APCs, DCs display a unique role due to their ability to migrate to the mesenteric lymph nodes to present the antigen to naive T-cells [66]. To make the antigen accessible for naive T-cells, DCs

present it on their surface via the major histocompatibility complex class II molecule (MHC-II). Naive T-cells recognise the antigen via the T-cell receptor and present it to naive B-cells. These subsequently undergo a class switch from immunoglobulin M (IgM) to IgE and differentiate to effector B-cells. Finally, IgE released by effector B-cells binds to the FcεRl receptor on the surface of mast cells and basophils [64,67].
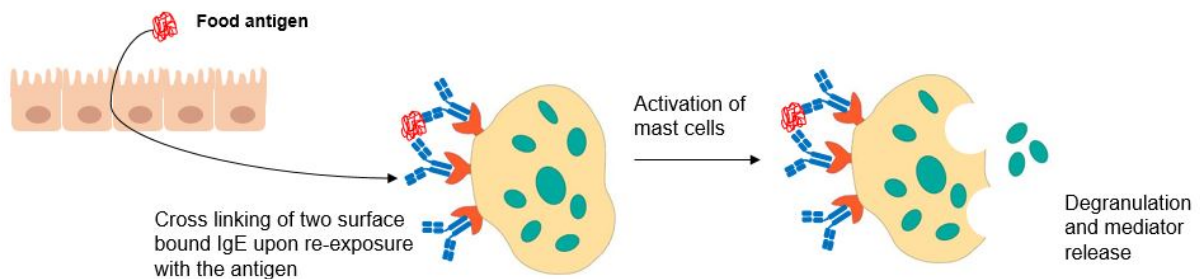
Figure 1.2: Simplified molecular mechanism showing the sensitisation phase (a) and the effector phase (b) of food allergy, modified from Ontiveros *et al.* [64] and Larché *et al.* [67]. DC: dendritic cell, MHC-II: major histocompatibility complex class II molecule, TCR: T-cell receptor, T_H: T helper cell, IgE: Immunoglobulin E, IgM: Immunoglobulin M.

Upon re-exposure (Figure 1.2b), two mast cell surface-bound IgE molecules crosslink due to the binding to the antigen. This results in the activation of the mast or basophilic cell and their subsequent degranulation, which ultimately leads to the release of pro-inflammatory mediators such as histamine [68].

1.8   Induction either pro-inflammatory or tolerogenic responses

The interaction of activated DCs with naive T-cells and the resulting T-cell proliferation and differentiation determines whether a pro-inflammatory or tolerogenic immune response is induced. The T-cell recognises the antigen that is presented on the MHC-II complex of the DC via the T-cell receptor, however also other costimulatory and adhesion molecules, as well as mediator release are important determinants in DC-T-cell interaction. The dynamics and duration of this interaction as well as the activation state of the DC has been described as crucial

factor for the subsequent T-cell maturation [69]. During this step different subsets of T-helper cells (Th)-1, Th2, Th17 as well as regulatory T-cells (T_{reg}) are produced. The balance between these subsets determines the elicitation of a pro-inflammatory or tolerogenic immune response [70]. While differentiation and proliferation towards Th2 and Th17 subsets results in a pro-inflammatory response, differentiation towards Th1 and T_{reg} subsets redirects to a tolerogenic innate immune response [71]. The receptor mediated recognition and uptake of antigens by APCs is a crucial step in T-cell immunogenicity. The potential role of glycated food proteins in T-cell immunogenicity has previously been demonstrated in a study by Heilmann *et al.* [42] where uptake of pyrraline-modified ovalbumin by murine DCs was mediated via scavenger receptor class A and resulted in an enhanced Th2 polarisation. However, little is known about the modulatory role of other heat induced modifications as well as other receptors in T-cell activation.

1.9 Antigen sampling in the small intestine

The intestinal epithelium is the physicochemical barrier between the lumen of the small intestine and the underlying *lamina propria*, hence the mucosal immune system. Most of the intestinal epithelial cells are adsorptive enterocytes that are organised in crypts and microvilli that facilitate the sampling of nutrients from the lumen due to its largely extended surface. Next to the epithelial cells, microfold cells (M-cells), which are located in the follicle associated epithelium of Peyer's Patches are also present in the intestinal barrier. Their morphology differs in three main characteristics from intestinal enterocytes: a rather unorganised brush border with short microvilli, a lower enzymatic activity, and an M-cell underlying pocket enriched with B- and T-cells, macrophages and DCs. They are thus considered a key player in antigen sampling from the intestinal lumen [72]. This morphological variability of the intestinal epithelium already indicates that proteins and their digestive breakdown products, can pass the intestinal epithelial tissue via different pathways (Figure 1.3) which can also affect the form in which they are presented to the immunes system. The transport pathway of ingested proteins can roughly be divided into paracellular (between the tight junctions) and transcellular routes. Moreover, specialised DCs and macrophages can actively sample antigens from the lumen by reaching in between the tight junctions of the epithelial cells, while follicle associated epithelium transport routes occur mainly via M-cells [74,75]. The transport pathway, determines whether the antigen reaches the *lamina propria* intact or hydrolysed to smaller fragments and can also predispose the immunological response.
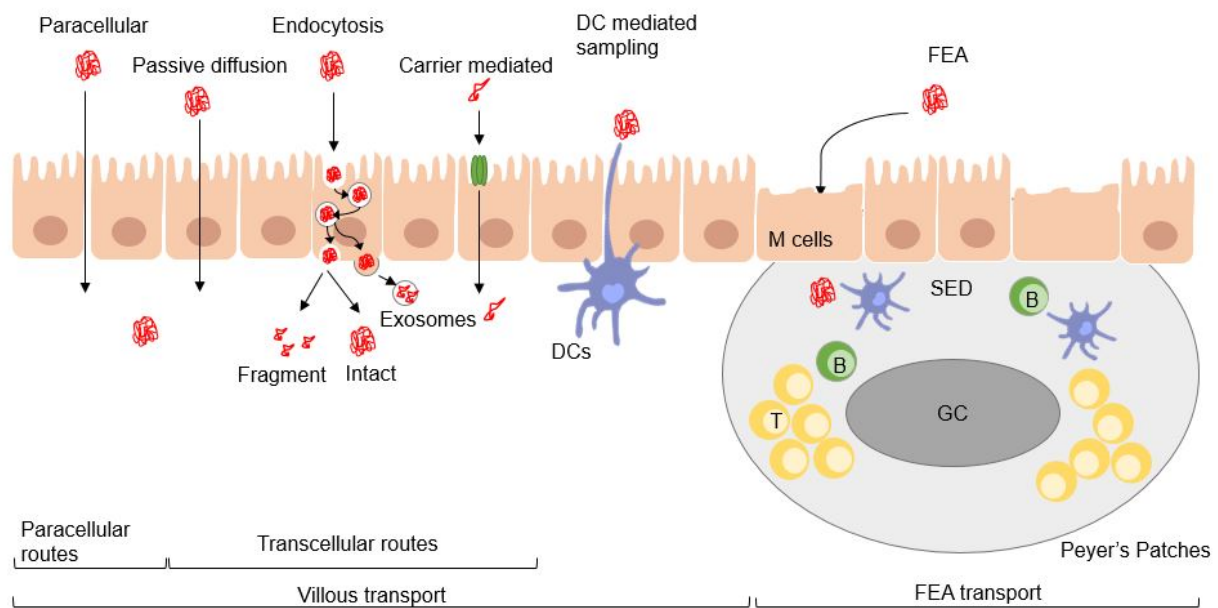
Figure 1.3: Epithelial transport routes of food proteins and antigens in the small intestine adapted from Corr *et al.* [72] and Reitsma *et al.*[73]. DC: dendritic cell, FEA: follicle-associated epithelium, microfold cells (M)-cells, B: B-cells, T: T-cells, SED: subepithelial dome, GC: germinal centre.

1.10 Effect of thermal processing on cow's MP immunogenicity and allergenicity

Immunogenicity describes the potential of a material to initiate a cellular immune response [76]. Heating of food protein results in several structural changes that can affect immunogenicity. The presence of AGEs on glycated food proteins can affect the immunogenicity via binding to the receptors for AGEs [41,42]. Moreover, their presence in heated food proteins has been linked to the induction of inflammatory responses in APCs [77-79]. Next to the presence of AGEs, heating-induced aggregation has been shown to modulate immunogenicity. For example, pasteurisation (30 min at 63 °C) of BLG has been shown to skew the uptake towards Peyer's Patches, indicating a higher immunogenicity [80]. Moreover, binding to receptors of AGEs can also be initiated by the formation of aggregates [20] and can occur independent from the presence of a reducing sugars, as shown on BLG heated in solution [81]. Moreover, aggregation as well as increasing hydrophobicity and the formation of amyloid-like structures during heating in solution has been shown to enhance the receptor-mediated uptake of BLG by THP-1 macrophages [21]. Next to immunogenicity, thermal processing of food allergens can also modify their allergenicity [49]. Industrial heat treatments of liquid dairy products have been shown to both decreased and increased IgE and IgG binding to WPs. At the same time, casein allergenicity is mostly unaffected by these heat treatments due to their relative heat stability [76]. As discussed before, the structural modifications strongly depend on the applied

heating conditions and thus also determine whether epitopes are exposed, hidden in the interior, or if neo-epitopes are formed. Bu *et al.* [82] showed that heating of WPs resulted in an initial increase of IgE binding and subsequent decrease if heated >90 ºC which was related to the unfolding and subsequent aggregation. Next to this, the MR showed a masking effect on IgE binding at a high degree of glycation [83].

The accelerated resolution of CM allergy symptoms in allergic children after ingestion of "baked milk" has been shown repeatedly in the past [53-55]. The applied heating conditions (low a_w-level, high temperatures, and presence of reducing sugars) during the preparation process, indicate a potential role of the MR as well as aggregation in these products. However, the changes that occur to the protein during these processes and the underlying immunological mechanisms are not well established. Especially regarding the role of AGEs, disentangling the effect of structural modifications and the MR on immunogenicity is often difficult as the conditions used to glycate the protein as well as MR itself promote protein aggregation [45] and thus requires further investigation.

1.11 The MR and immunity - the role of cell surface receptors of APCs

Several AGE binding cell surface receptors have been described in previous studies, such as the receptor for AGEs (RAGE), CD36, galectin-3 (Gal-3) as part of the AGE-R-complex, and scavenger receptor class A type I and type II [84-88]. Next to endogenously formed AGEs, also food derived AGEs have been described as ligands for AGE receptors, indicating the role of exogenous AGEs in inflammatory responses [41,42,81]. RAGE is involved in endocytosis of antigens as well as in cellular signalling pathways that determine the maturation and differentiation of macrophages and DCs and therefore plays a key role in the innate immune system [89]. The CD36 pattern recognition receptor belongs to the scavenger receptor class B family, and is predominantly found on macrophages, endothelial cells, and smooth muscle cells. In contrast to RAGE it has a key role in endocytosis and cell adhesion, rather than cellular signalling [90]. Gal-3 is highly expressed on cells of the innate immune system, such as neutrophils, macrophages, and DCs [91]. Its role in the differentiation and proliferation mechanisms of antigen presenting cells has been demonstrated by a number of studies and indicates the importance of Gal-3/ligand interactions in the immunological response to an antigen [92-95]. The binding of food derived AGEs to these receptors is hence a crucial step in the initiation of an immune response.

1.12 Cow's MP digestion

Most studies showed the binding of AGE receptor to glycated food proteins prior to digestion. However, Liu *et al.* [20] also observed binding of glycated WPs after simulated infant *in vitro* digestion. Upon digestion, MP is hydrolysed into peptides and amino acids by the digestive enzymes. The digestive system of an infant still develops and enzyme activity as well as bile acid concentrations are lower, while the pH of the stomach and the intestine is higher compared

to adult digestion [96]. While the WPs are relative resistant to gastric digestion, caseins rapidly disappear from the soluble fraction of the digestive juice. This is partly related the clotting of caseins upon acidic precipitation in the stomach (pH ~5.5 in infants) but also to the rapid hydrolysis of caseins as pepsin cleavage sites in caseins are easy accessible for the enzyme due to their open structure. In the intestine, the pH slightly increases (~6.5 in infants) and both caseins and WPs are hydrolysed by the two main proteolytic enzymes trypsin and chymotrypsin. Moreover, other carboxypeptidases and brush border enzymes located on the luminal side of the intestinal epithelial cells further hydrolyse the proteins and digestion-derived peptides [97]. Thermal processing of MP can have both a negative or positive effect on its digestibility depending on the heating conditions and the heat-induced modifications on the protein structure. Denaturation may facilitate digestibility as unfolding makes the protein's cleavage sites more accessible to the enzymes, while aggregation rather shows a negative effect on protein digestion by reducing its accessibility. Moreover, glycation impairs digestibility possibly by blocking cleavage sites of trypsin which specifically cleaves after lysine residues [98]. Next to the overall digestibility, also the peptides that are released upon digestion differ depending on the heat treatment of the digested product. Both, decreasing and increasing numbers of peptides have been described [99,100], as well as an increased average peptide length at 20.5% blocked lysine in infant formula [101]. The presence of relatively larger fragments may increase the risk of CM allergy symptoms. This has been demonstrated with a screening ELISA measuring residual immunoreactivity with specific monoclonal antibodies [100,102] but has also been shown using IgE and IgG ELISA on *in vitro* digests of CM with various thermal treatments [103]. However, there is no direct link between the released peptide patterns upon digestion and an increasing or decreasing immunoreactivity, as this strongly depends on the bioavailability of the peptides and the immunological mechanism under investigation.

1.13   Research objectives and outline of the thesis

The consumption of "baked" milk products has raised increasing attention as a possible alternative to strict avoidance diets for CM allergic children. However, the underlying (molecular) mechanisms that result in the enhanced resolution of allergic symptoms upon ingestion of baked milk compared to raw milk are yet unclear. As reviewed in the previous paragraphs heat treatment causes several structural modifications of milk proteins which may alter immunoreactivity of the protein. To describe the molecular consequences of the particular heat treatment, the detailed structural analysis of the protein needs to be combined with functional read-outs assessing immunoreactivity of modified protein. Such a multidisciplinary approach is so far rare and therefore more studies involving physicochemical analysis together with molecular immunological assays are needed to understand the baked milk-induced tolerance. The hypothesis of this thesis is that heat-induced molecular alterations in the proteins that are contained in CM, notably MR products (MRPs), are responsible for the accelerated resolution of CMA in CMA-children that are exposed to baked milk. This thesis aims to acquire

knowledge about the occurring structural changes, and how they may affect the immune response. This is crucial for predicting the outcome of food challenges with baked milk, and to better understand the potential mechanism of oral tolerance induction. The objective of this work was thus to gain more insights in the structural modifications of dry-heated MP, to understand how these may affect digestibility and immunoreactivity, and to determine the role of the MR within the observations made.

In chapter 2, the composition of the buffer soluble and insoluble fraction of MP, heated under controlled humidity conditions in the presence of lactose, is studied. Furthermore, it aims to gain better understanding which molecular interactions are involved in the formation of insoluble protein upon dry heating at low and high temperatures and the resulting effects on infant *in vitro* digestion.

In chapter 3, the role of aggregation and glycation on antigenicity and immunogenicity of BLG is investigated. As protein aggregation and the extent of glycation is determined by the applied heating conditions, different heat treatments at low and high temperatures, as well as in solution and under dry conditions, are applied. The treatment-dependent effects on allergenicity and immunogenicity are examined by means of IgE binding and sRAGE binding capacity.

In chapter 4, the chemical modification of BLG with CML is conducted to better understand the role of this specific AGE in immunogenicity, independent from changes in the secondary and tertiary protein structure. Therefore, the modified BLG is exposed to the receptors for AGEs, CD36, Gal-3, and sRAGE using ELISA and possible structural changes are closely monitored and related to the receptor binding.

In chapter 5, the receptor binding and internalisation by APCs of wet heated and glycated BLG to CD36, Gal-3, scavenger receptor class A type I, and sRAGE are investigated. Additionally, the solutions of heated BLG are fractionated into two size ranges (above and below 100 kDa) to evaluate the relevance of large aggregates in binding and internalisation. After initial screening with receptor binding ELISAs, binding to the cell surface bound receptors is tested in THP-1 cells. Internalisation experiments are conducted on THP-1 cells and DCs which are isolated and differentiated from human peripheral blood mononuclear cell. To indicate the physiological relevance of the aggregates *in vivo*, experiments are also conducted on *in vitro* digests of heated and glycated BLG.

In chapter 6, the peptides released upon simulated infant *in vitro* digestion of MP heated under dry conditions at low and high temperature are monitored. sIgE binding epitopes are identified based on literature, while T-cell epitopes are identified using a prediction software. Moreover, the transport across a Caco-2 cell monolayer is investigated. Both non-modified and glycated peptides are monitored before and after Caco-2 cell transport. This study gives and overview over potential immunoreactive peptides and glycated peptides generate upon simulated infant *in vitro* digestion of differentially heated MP and their transport across the Caco-2 cell monolayer.

Finally, in the General Discussion presented in chapter 7, the results are summarised as well as interpreted in coherence and against the background of existing literature. An interpretation for the relevance of MR based on the presented findings is given. The main conclusion and further perspectives are given in this chapter.

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Chapter 2

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.

2.1 Introduction

Cow's milk protein (MP) consists of two main protein fractions: casein and whey protein (WP). 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 WPs [1,2]. 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 WPs. Disulphide bond induced protein interaction is a key reaction in protein aggregation of MP in solution. The formation of the κ-casein/WP complexes on the surface of the casein micelle is the predominant disulphide bond-induced intermolecular reaction in MP. With respect to the WPs, both β-lactoglobulin (BLG) and α-lactalbumin (ALA) are involved in the aggregation and complexation on the casein micelle surface [3,4]. However, the complexation of WPs with the casein micelle is mainly driven by the S-S/thiol disulphide interchange between the free cysteine residue of BLG and κ-casein [5]. 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 WPs [6]. 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 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) [7]. 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 [8]. 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 [9]. Recent studies showed that both the MR and MR-induced protein crosslinking can lead to loss of solubility of MP concentrates during storage [10,11]. 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 [12-14]. Loss of solubility and structural changes of MP during thermal processing may also affect its digestibility and thereby the nutritional value of the proteins [15]. 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 [16-18]. 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 [19]. Due to the low a_w, 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 [20-22]. 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 [23,24]. 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.

2.2   Material and Methods

2.2.1   *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 (Waltham, Massachusetts, USA). CML, CEL, d4-CML, and d4-CEL were purchased from Iris Biotech (Marktredwitz, Germany). EMSURE® Anhydrous di-sodium hydrogen phosphate was purchased from Merck Millipore (Burlington, Massachusetts, 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 (Hercules, California, USA). All other chemicals were obtained from Sigma Aldrich (St. Louise, Missouri, USA).

2.2.2   *Preparation of milk powder and heat treatment*

Liquid concentrated raw MP, composed of a mixture of micellar casein fraction (MCI88 liquid) and a WP 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. To obtain LT heated MP

(LT-MP), 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. To produce HT heat MP (HT-MP), the powder was incubated over a saturated potassium acetate solution at 10 °C until an a_w-level 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-MP) 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 the supplementary material (Figure 2-S1).

2.2.3 *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 [25]. 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 cellulose acetate syringe filter (Phenomenex, Torrance, 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, Waltham, Massachusetts, USA) in technical duplicates. The procedure was repeated twice on different days.

2.2.4 *Acidic hydrolysis*

To release (modified) amino acids from their protein structure, acid hydrolysis was performed according to Kehm *et al.* [26]. 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. Protein precipitation was achieved by adding 1 mL of trichloroacetic acid (20%, w/v) and centrifugation (4 °C, 10000 ×g, 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 hydrochloric acid (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, Waltham, Massachusetts, USA). The residue was dissolved in 100 µL ultrapure water and after centrifugation (4 °C, 10000 ×g, 10 min) an aliquot was used for MRM analysis of glycation end products by LC-MS/MS.

2.2.5   *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, Massachusetts, 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 energy (CE) as well as cone voltage (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 labelled with q and transitions used for the confirmation of the presence of the analyte are labelled with Q. Data were acquired and evaluated with the MassLynx Software (Waters, version 4.1).

2.2.6   *Solvent dependent solubility test*

The insoluble material from LT-MP and HT-MP 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, while solvent 4 (S4) was composed of 10 mM PBS, 1% SDS, and 6 M urea, and 1% dithiothreitol (DTT). Ten milligrams of lyophilised insoluble material were accurately weighed in separate Eppendorf tubes using a micro balance XA105 (Mettler Toledo, Columbus, Ohio, 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 bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, 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 2.2.3.

2.2.7   *Sodium dodecyl sulphate 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, Hercules, California, USA) and Image Lab 4.1 software (Biorad, Hercules, California, USA).

2.2.8   *Protein identification*

To identify specific bands on the SDS-PAGE of LT-MP, 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. Samples were desalted using in-house made stage tip C18 µcolumns and eluted with 50 µL ACN/25 mM formic acid (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 formic acid.

Peptides were analysed on a Thermo nLC 1000 system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled to a LTQ orbitrap XL (Thermo Fisher Scientific, Waltham, Massachusetts, 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% formic acid. ACN increased from 9% to 34% ACN within 50 min. Full scan positive mode spectra were measured between m/z 380 and 1400 in the Orbitrap at high resolution (60000). Collision-induced dissociation fragmented (isolation width 2 m/z, 30% normalised 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 full scan positive mode spectra 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 [27]. 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 were analysed with Perseus 1.5.5.3 using the normalised label free quantification (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.

2.2.9   *Infant in vitro digestion*

MP powders heated at LT and HT, as well as an unheated control (NT-MP), were applied to simulated infant *in vitro* digestion according to the method by Ménard *et al.* [28]. 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, Pennsylvania, 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.* [29]. 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.

2.2.10 *Determination of 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.* [30]. 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). DH was calculated according to the following formula:

$$DH\ [\%] = ([NH_2\ (final) - NH_2\ (initial)] / [NH_2\ (acid) - NH_2\ (initial)]) \times 100$$

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.

2.2.11 *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.

2.2.12 *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.

2.2.13 *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 seconds, 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).

2.2.14 *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 $p < 0.05$.

2.3   Results

2.3.1   *Recovery of SMUF soluble protein*

Dry heating of the MP mixture used in this study caused the formation of SMUF insoluble material (Figure 2.1a). The remaining soluble protein was quantified using DUMAS (Figure 2.1b).
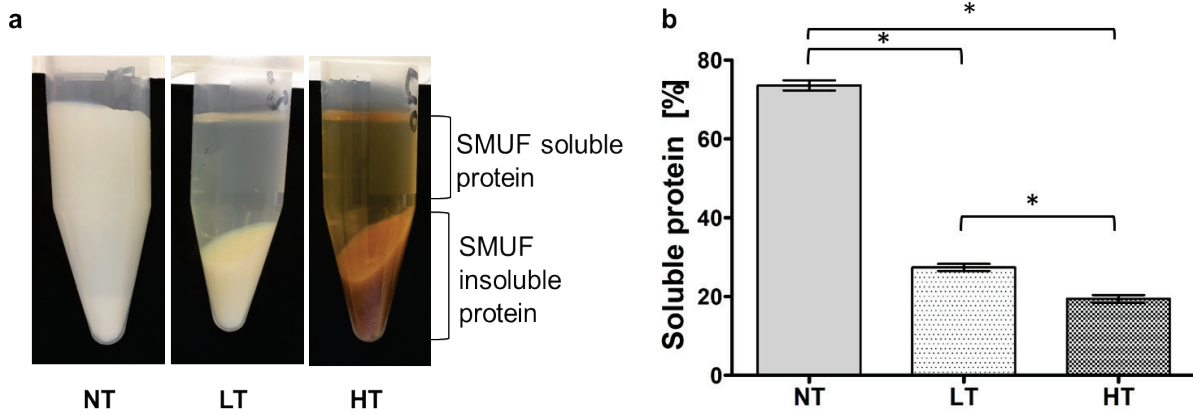
Figure 2.1: (a) Images of cow's milk protein powders dissolved in simulated milk ultrafiltrate (SMUF), when non-treated (NT), heated at low temperature (LT), and heated at high temperature (HT). (b) Percentage of MP soluble in SMUF. Error bars represent standard deviation of technical duplicates. Statistical differences were analysed using one-way ANOVA and Tukey post hoc test, with $*p < 0.05$.

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

2.3.2 *Quantification of AGEs*

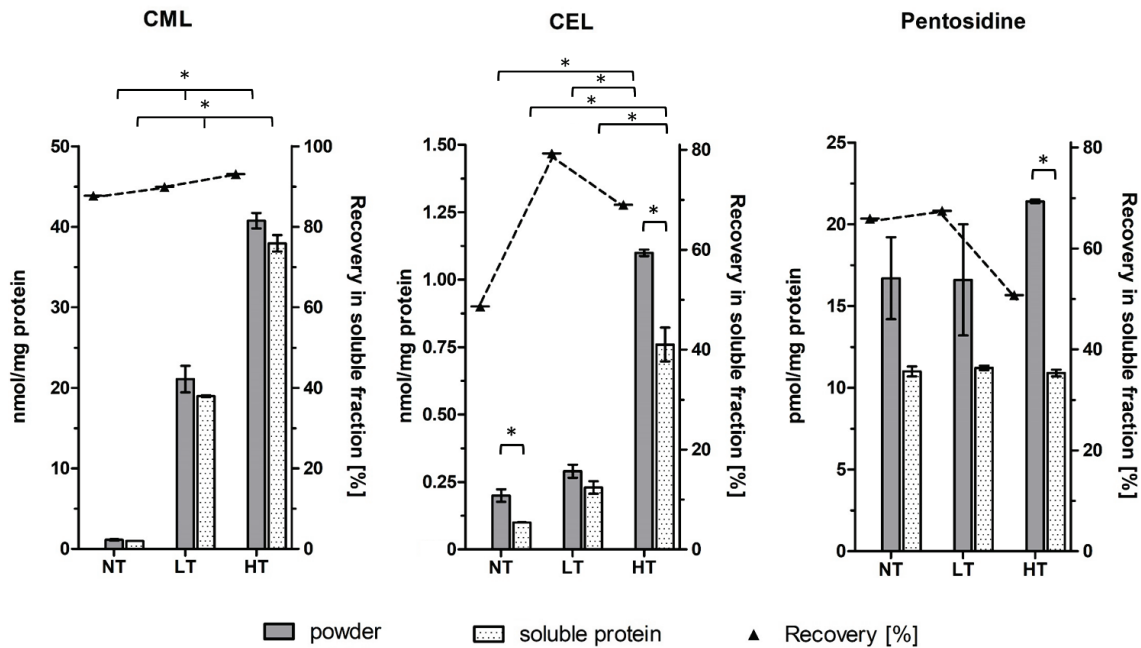AGEs were quantified in both the MP powder and the SMUF soluble fraction. For CML and CEL absolute concentrations were obtained whereas for pentosidine relative concentrations based were obtained based on d4-CML as internal standard. The recovery of each AGE in the SMUF soluble fraction was calculated based on the total amount in the powder (Figure 2.2).

Figure 2.2: N$^{\varepsilon}$-Carboxymethyllysine (CML), N$^{\varepsilon}$-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 $*p < 0.05$.

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-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-MP, but not LT-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-MP (51%) compared to NT-MP and LT-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.

2.3.3 *Protein composition of SMUF soluble and insoluble fraction*

The protein composition of soluble and insoluble material was analysed using SDS-PAGE (Figure 2.3). 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 paragraph 2.3.4).
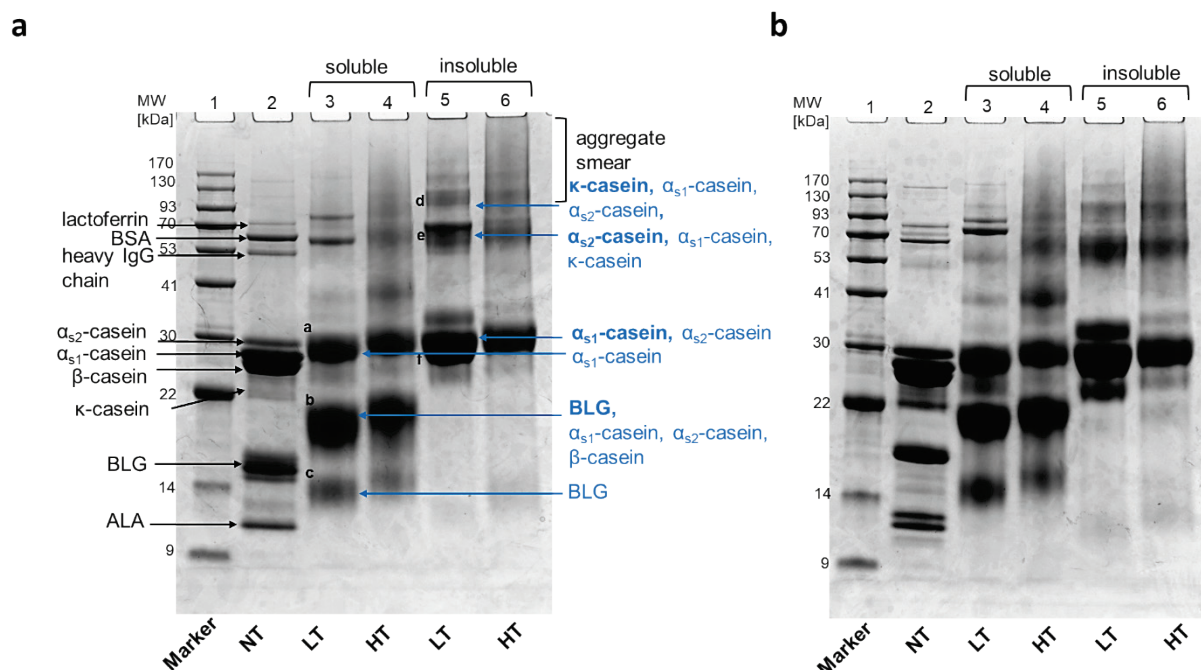
Figure 2.3: (a) SDS-PAGE image of heated and glycated cow's milk protein (MP) under non-reducing conditions. Bands are indicated as α-lactalbumin (ALA), β-lactoglobulin (BLG), bovine serum albumin (BSA), and immunoglobulin G (IgG). (b) SDS-PAGE image of heated and glycated MP under reducing conditions. 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 the LFQ intensity. Identification of bands in NT-MP was done in comparison with Uniprot database (https://www.uniprot.org) as well as literature [31,32] and represents the true mobility of the native proteins.

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 ALA (10 kDa), BLG (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 and 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-MP and HT-MP. Moreover, HT-MP showed a slightly larger shift to a higher molecular weight (MW) than LT-MP and a smear at higher MW (>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-MP than in HT-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-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-MP but to a much lesser extent.

Bands indicated with a letter in Figure 2.3 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 MW for better identification. The pieces were labelled according to their MW. Results are shown in Figure 2.4.
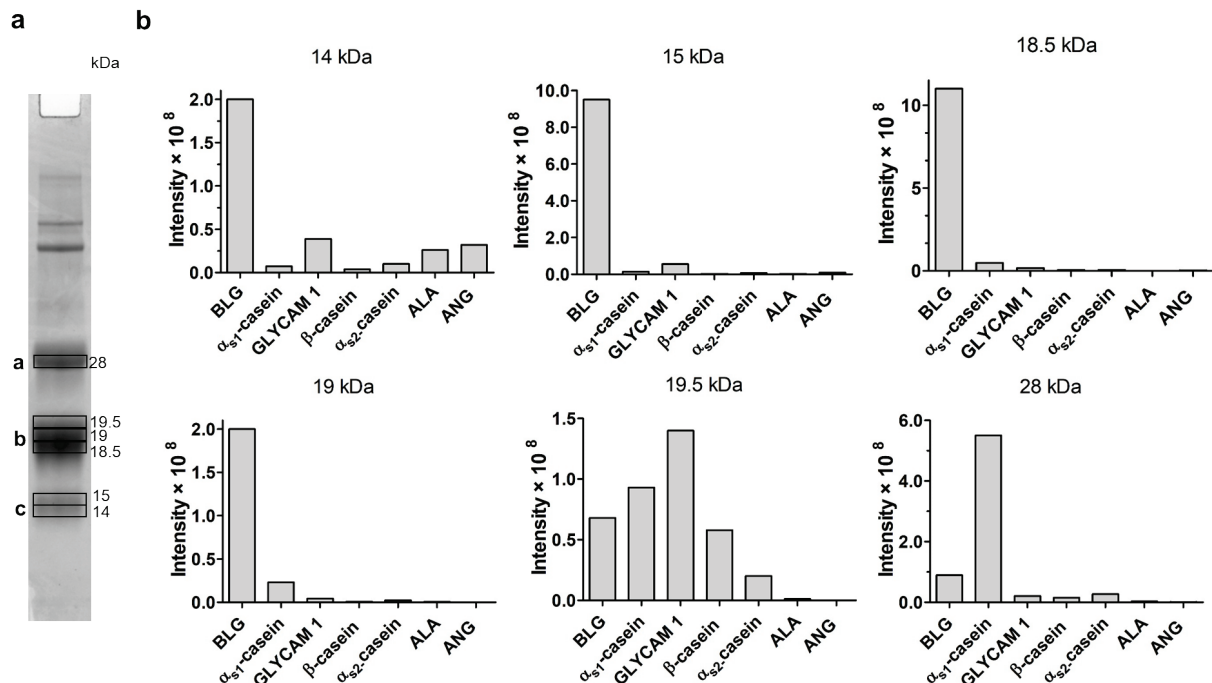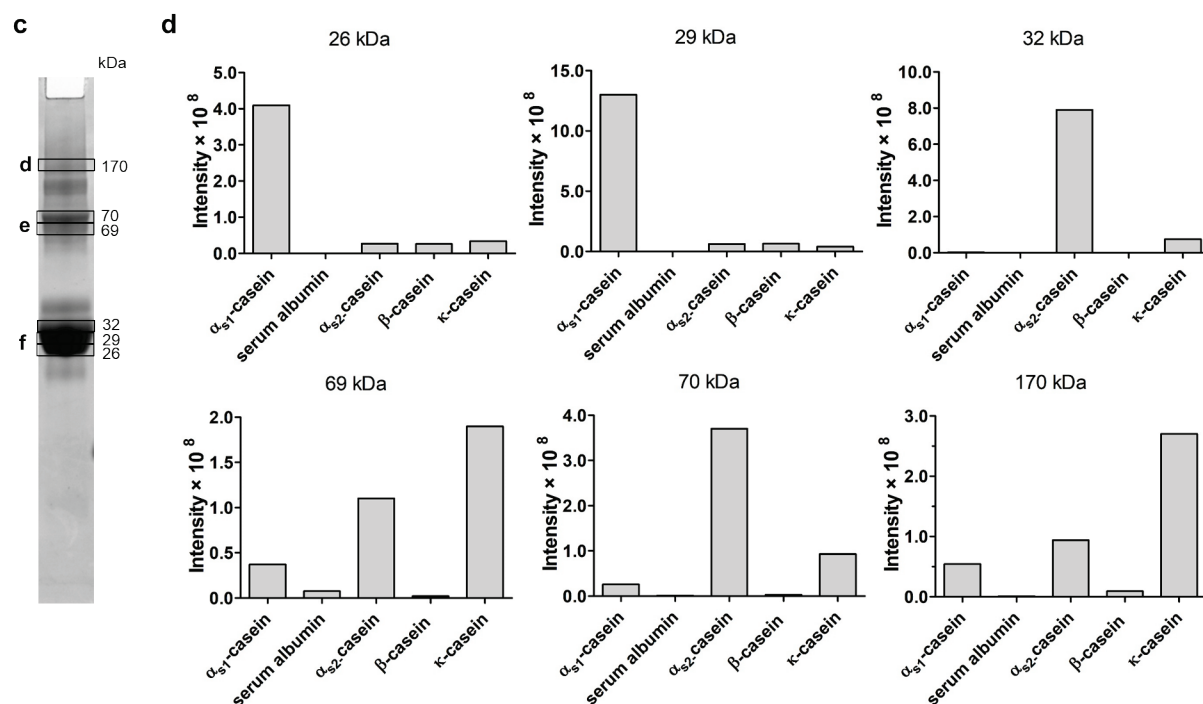
**Figure 2.4:** (a) SDS-PAGE image of the SMUF soluble fraction of low temperature (LT) glycated cow's milk protein (MP) Letters indicate the bands that have been cut for protein identification. Squares indicate the cutting pattern. (b) LFQ intensities of the proteins identified in each band from the 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 displayed. Proteins are indicated as α-lactalbumin (ALA), β-lactoglobulin (BLG), 1 Glycosylation-dependent cell adhesion molecule 1 (GLYCAM), Angiogenin ribonuclease (ANG).

In the soluble fraction, BLG was the most abundant protein. Figure 2.4a, band a, showed α_{s1}-casein as the main protein but also traces of BLG were found. Band b was divided in three parts. In the lower parts (18.5 kDa and 19 kDa) BLG 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. Interestingly, the three major caseins and BLG were also found in the top part of band b with the following order of relative abundance: α_{s1}-casein > BLG > β-casein > α_{s2}-casein. Band c (14 kDa and 15 kDa) showed BLG as the most abundant protein as well as traces of ALA at the lower part of the band. In the insoluble fraction, mainly caseins were found. In the aggregates on top of the gel (Figure 2.4c, 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.

2.3.4 *Dissolution of SMUF insoluble material in different solvents*

To investigate the molecular interactions that lead to decreased solubility of heated and glycated MP, the solubility of the SMUF insoluble fraction in HT-MP and LT-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 Figure 2.5.

a
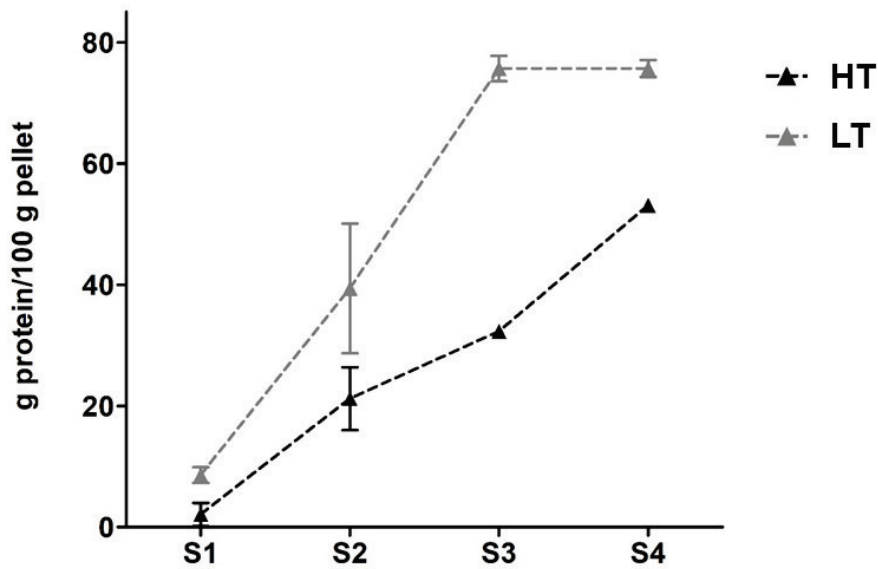
b
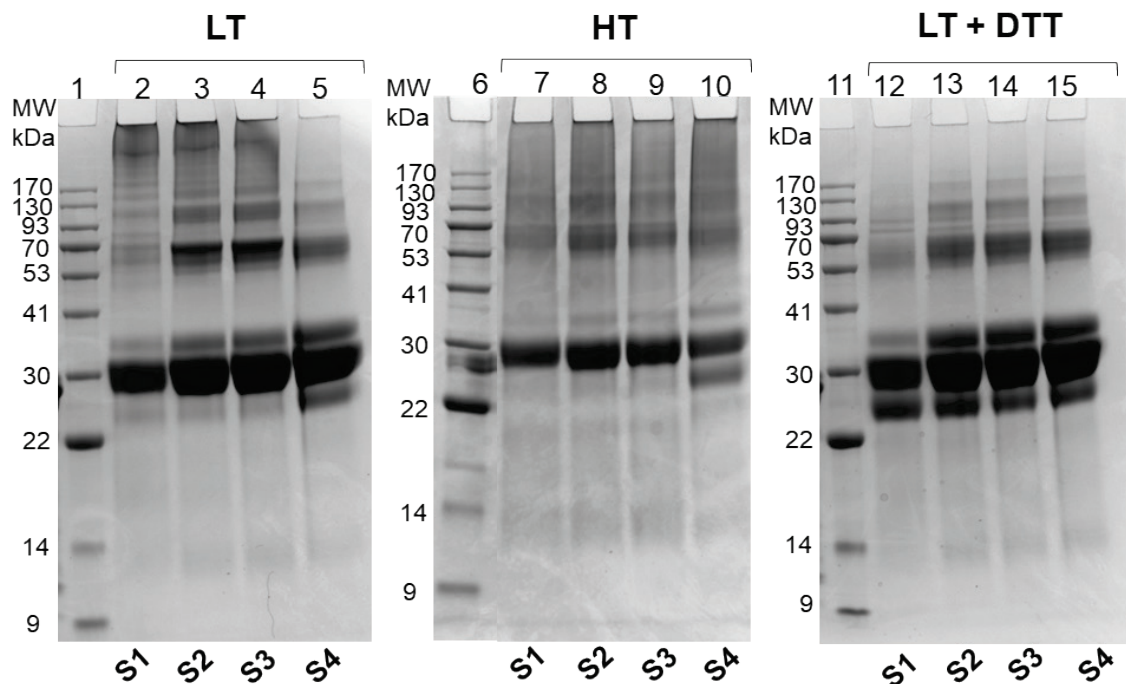
Figure 2.5: (a) Soluble protein recovery from SMUF insoluble cow's milk protein formed after heating with lactose at low temperatures (LT) and at high temperature (HT) in 4 different solvents. S1: 10 mM PBS (pH 7.4), S2: S1 + 1% SDS (pH 7.4), S3: S2 + 6 M urea (pH 7.4), S4: S3 + 1% dithiothreitol (pH 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).

In LT-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-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-MP was similar in S1 to S3, as shown in Figure 2.5b. 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 MW of WPs were detected in S1 to S4. For S1 to S3, the protein composition of the insoluble fraction of LT-MP and HT-MP were comparable. Interestingly, the smear at >53 kDa was not visible in S4 for LT-MP. SDS-PAGE was also conducted under reducing conditions which allowed differentiating of the effect of S4 vs. S1-S3 (Figure 2.5b, lanes 11-15). Under reducing conditions, the smears of LT-MP in S1 to S3 vanished from the SDS-PAGE image while the band at 25 kDa, corresponding to κ-casein, became more intense.

2.3.5 *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 (Figure 2.6).
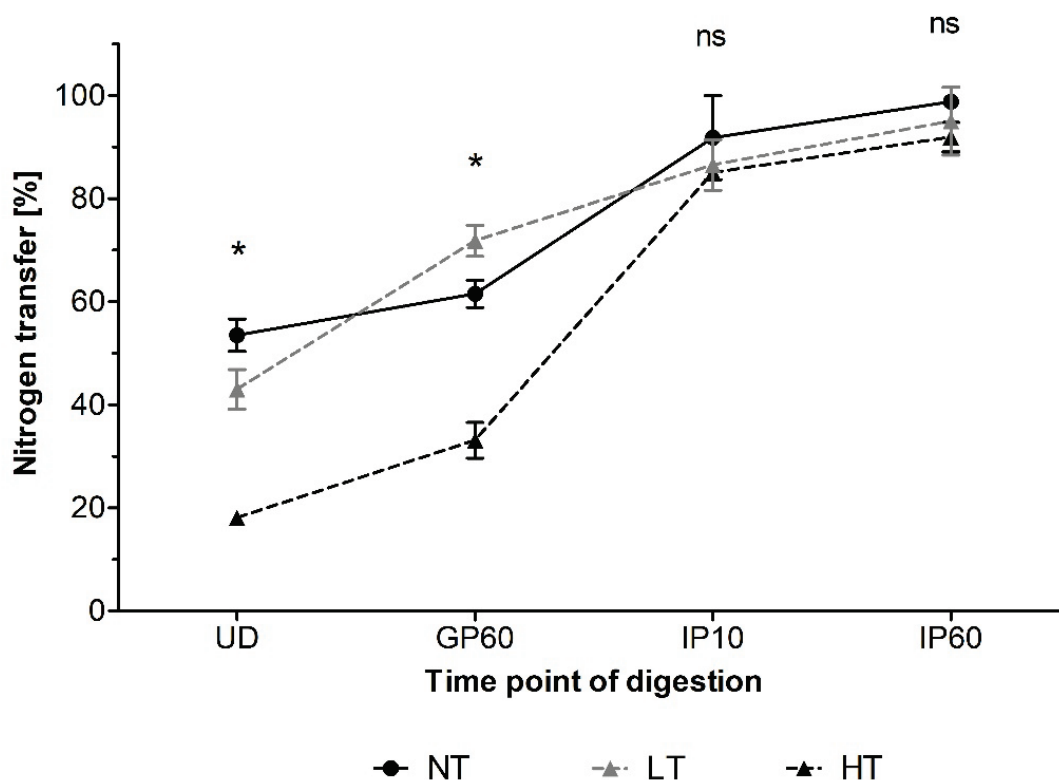
Figure 2.6: Nitrogen transfer of cow's milk protein, non-treated (NT), glycated at low temperature (LT), and high temperature (HT) after simulated gastro intestinal infant *in vitro* digestion. UD: undigested (UD) meal, GP60: after hydrolysis in the gastric phase for 60 min, IP10 and IP60: after hydrolysis 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 statistically significant at *$p < 0.05$ with ns: not significant.

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-MP showed lower solubility compared to the other samples. After 60 min in the IP, all samples reached between 91.9 ± 2.8% (HT-MP) and 98.9 ± 1.1% (NT-MP) nitrogen transfer, with no significant (p < 0.05) differences among samples.

2.3.6   *Protein hydrolysis during simulated infant in vitro digestion*

The extent of protein hydrolysis was monitored by measuring the DH with the OPA-assay (Figure 2.7).
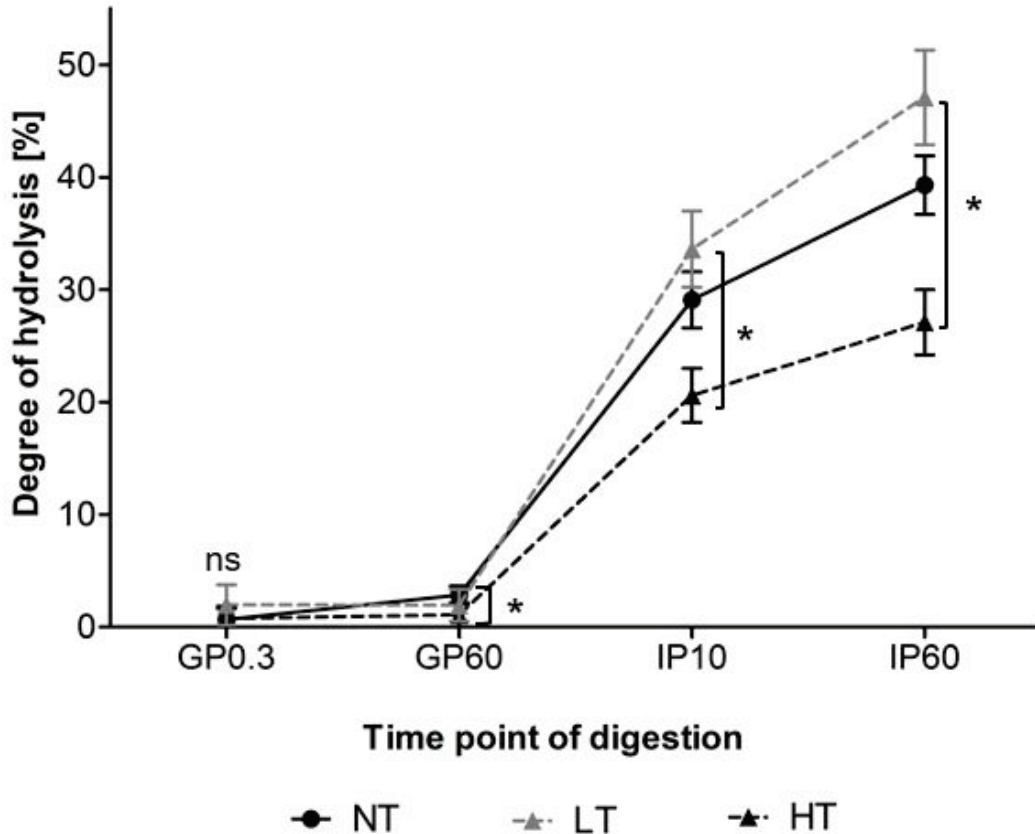
Figure 2.7: Degree of hydrolysis of cow's milk protein, non-treated (NT), glycated at low temperature (LT), and high temperature (HT), after simulated gastro intestinal infant *in vitro* digestion. GP0.3: after hydrolysis in the gastric phase for 0.3 min, GP60: after hydrolysis in the gastric phase for 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.

The highest DH after 60 min in the GP was observed for NT-MP with 2.9 ± 0.8%. While the DH of HT-MP was significantly lower compared to NT-MP, no differences were observed compared to LT-MP. After 10 min in the IP, the highest DH was observed for LT-MP followed by NT-MP and HT-MP but was only significantly different between LT-MP and HT-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 between samples remaining the same as after 10 min in the IP.

Remaining intact proteins at different time points of gastrointestinal infant *in vitro* digestion were visualized using SDS-PAGE (Figure 2.8).
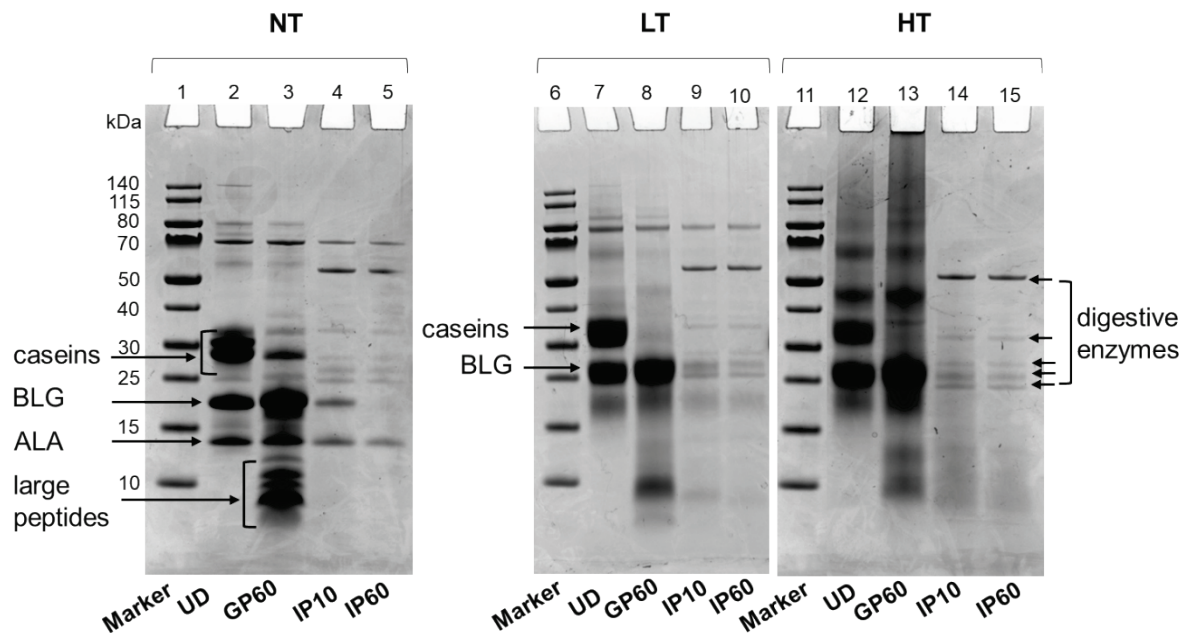
Figure 2.8: SDS-PAGE image of cow's milk protein, non-treated (NT), glycated at low temperature (LT), and high temperature (HT)), after simulated gastro intestinal infant *in vitro* digestion. In comparison to the undigested (UD) meal, samples were taken after hydrolysis in the gastric phase for 60 min (GP60), after hydrolysis in the intestinal phase for 10 min (IP10) and 60 min (IP60), respectively. Bands are indicated as β-lactoglobulin (BLG) and α-lactalbumin (ALA).

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-MP, a smear of aggregated protein over the whole lane appeared on the gel after 60 min in the GP. WPs did not appear to be hydrolysed in the GP independent of the heat treatment. In NT-MP, intact BLG remained until 10 min in the IP, while intact ALA was still visible after 60 min in the IP. In HT-MP, the bands of ALA and BLG 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-MP, the band of BLG at 19 kDa and larger peptides at 15 kDa remained present until the end of intestinal digestion.

2.3.7 *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 (Figure 2.9).
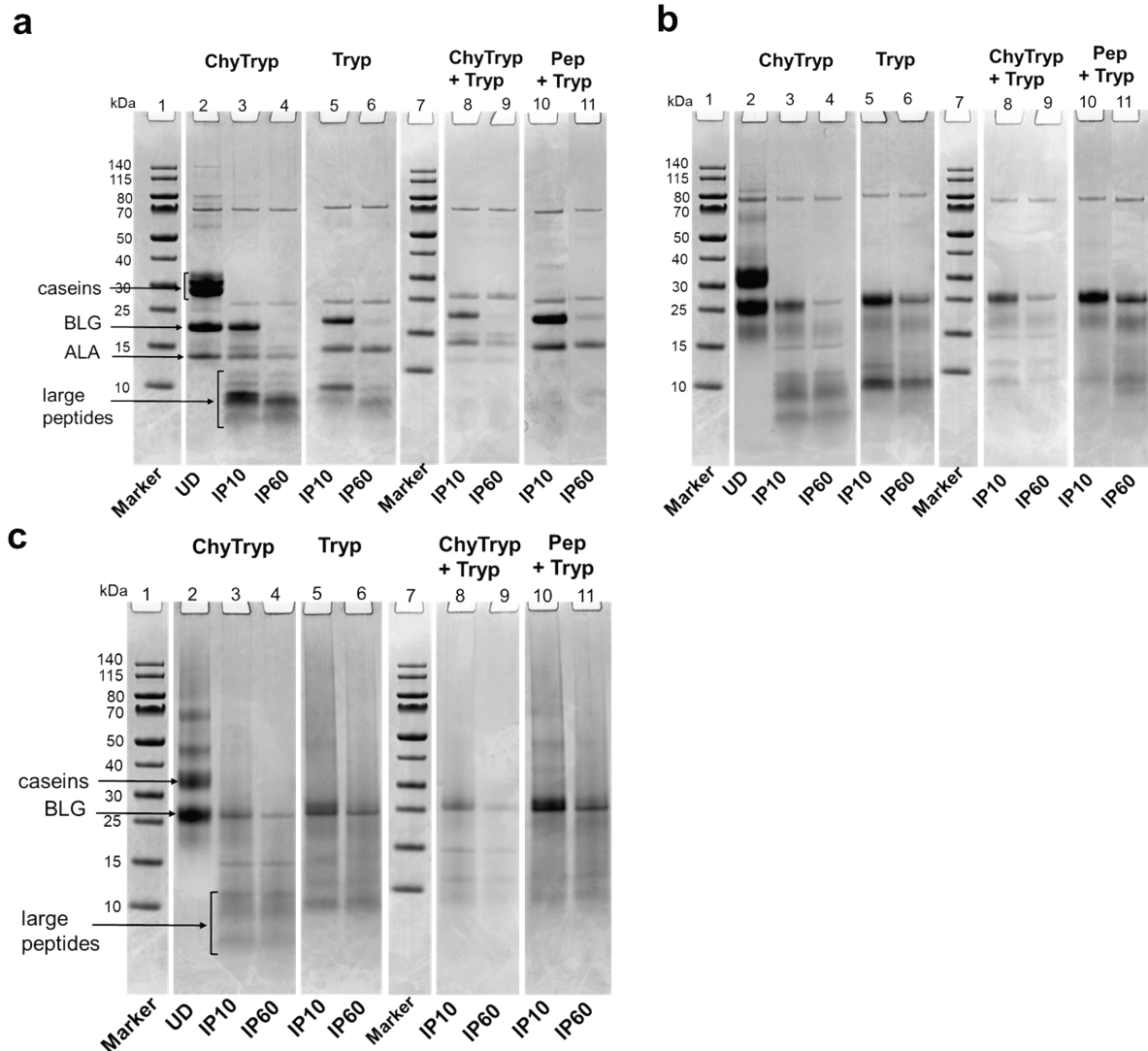
Figure 2.9: (a) SDS-PAGE image after 0, 10 and 60 min in the intestinal phase (IP) of non-treated (NT) cow's milk protein (MP) with chymotrypsin (ChyTryp), trypsin (Tryp), respectively, as well as combinations of chymotrypsin and trypsin (ChyTryp+Tryp), and pepsin and trypsin (Pep+Tryp), respectively. (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. Bands are indicated as β-lactoglobulin (BLG) and α-lactalbumin (ALA).

After chymotryptic hydrolysis of NT-MP (Figure 2.9a, lane 3-4), the band of BLG remained until 10 min in the IP, while ALA 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 BLG band and it was still visible until the end of the IP. Once heating was applied to the MP (Figure 2.9b and c), BLG 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-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: Figure 2-S2). Especially in HT-MP, the combination of pepsin with trypsin seemed to have a synergistic effect on the DH at both sampling points (supplementary material: Figure 2-S2b and Figure 2.7 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.

2.3.8   *SEM images*

SEM images were taken from all samples before and after infant *in vitro* digestion from all sampling time points (Figure 2.10). Undigested NT-MP showed several round particles (Figure 2.10a), which appeared as white dots in the image. These particles were of various size in an estimated range between 50 nm and 200 nm (Figure 2.10b) 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 (Figure 2.10c) no larger particles were visible while in both IP10 (Figure 2.10d) and IP60 (Figure 2.10e) 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-MP (Figure 2.10f), no particles large enough to be detected with SEM were visible. At the same time, GP60 samples of LT-MP showed both, network-like structures (Figure 2.10g) and spherical particles with a broad size range between 20 to 100 nm (Figure 2.10h). 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-MP (Figure 2.10m), 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 (Figure 2.10n 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 Figure 2.10n). Additionally, larger unstructured particles around 5 μm that seemed to partly incorporate the round particles within other structures were found (Figure 2.10o). 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 (Figure 2.10p). After 60 min in the IP (Figure 2.10q), 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-MP than in LT-MP.
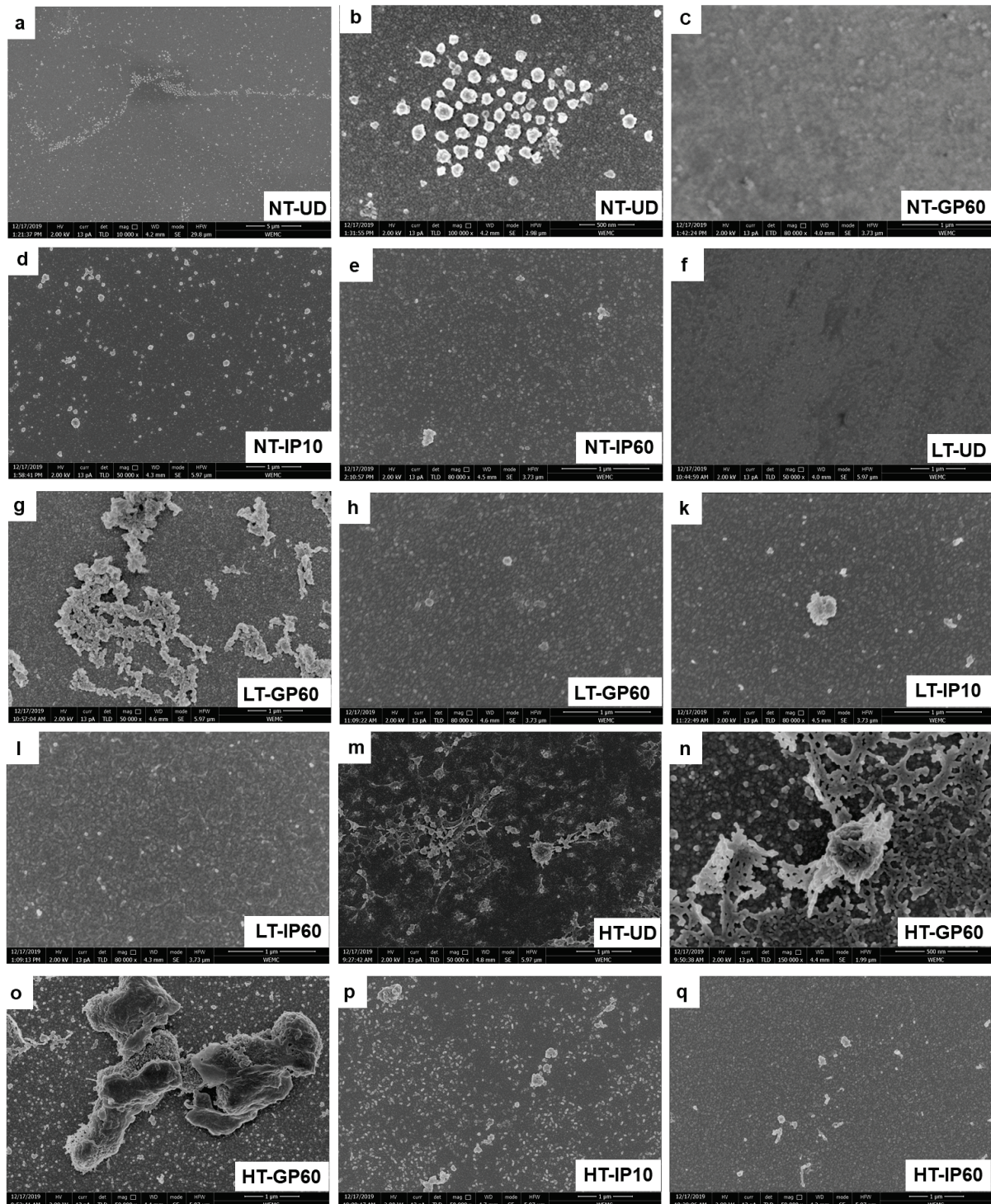
Figure 2.10: Scanning electron microscopy images of non-treated (NT) cow's milk protein (MP), dry heated at low temperature (LT), and dry heated at high temperature (HT) in the presence of lactose, after 60 min in the gastric phase (GP), 10 min in the intestinal phase (IP), and 60 min in the IP of an infant *in vitro* digestion model. (a and b) NT-MP undigested (NT-UD), (c) NT-MP after 60 min in the GP (NT-GP60), (d) NT-MP after 10 min in the IP (NT-IP10), (e) NT-MP after 60 min in the IP (NT-IP60), (f) LT heated MP undigested (LT-UD), (g and h) LT heated MP after 60 min in the GP (LT-GP60), (k) LT heated MP after 10 min in the IP (LT-IP10), (l) LT heated MP after 60 min in the IP (LT-IP60), (m) HT heated MP undigested (HT-UD), (n and o) HT heated MP after 60 min in the GP (HT-GP60), (p) HT heated MP after 10 min in the IP (HT-IP10), (q) HT heated MP after 60 min in the IP (HT-IP60).

2.4 Discussion

Cow's milk contains two major protein fractions, casein and WP, 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 [13,14], caseins were the main proteins that remained in the insoluble fraction under both heating conditions (Figure 2.3). This is also in line with SEM images which showed no larger particles or casein micelles in the undigested LT-MP (Figure 2.10f). At the same time, undigested HT-MP showed larger particles around 200 nm size (Figure 2.10m), which could be either aggregated WP or WP-coated casein micelles. Higher levels of glycation led to a significant decrease in protein solubility (Figure 2.1), however also disulphide bond formation as well as non-covalent interactions such as hydrophobic interactions and hydrogen bonds contributed to the loss of solubility (Figure 2.5). 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 [10,11,13,33,34]. The contribution of these molecular interactions appeared to be heat treatment dependent (Figure 2.5a). In LT-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 MW aggregates in LT-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 (Figure 2.5). This is also supported by the relative abundance of α_{s2}-caseins and κ-caseins in the larger MW aggregates of the insoluble fraction (Figure 2.4d). These two caseins have a free cysteine residue which allows them to form disulphide bonds amongst each other and with WPs [35]. The absence of BLG in the 170 kDa band of the insoluble fraction (Figure 2.4d) does, not support the involvement of the κ-casein/BLG complex in dry-heated samples. Overall, the low abundance of WPs in the insoluble fraction (Figure 2.5b), 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 WP solubility [14]. In HT-MP, not only disulphide bonds but also the formation of other covalent bonds contributed to the decrease in solubility. The 47% of protein remaining insoluble after dissolving in S4 (Figure 2.5a) and the low recovery of especially pentosidine of 51% in the soluble fraction (Figure 2.2) indicates that MR-induced crosslinking contributed to the observed loss of solubility of especially caseins (Figure 2.1b and Figure 2.4). The large aggregates shown on the SDS-PAGE after tryptic hydrolysis of HT-MP, but not after chymotryptic hydrolysis, (Figure 2.9c) 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 [36]. While tryptic hydrolysis of LT-MP is impaired (supplementary material: Figure 2-S2), it does not show hydrolysis-resistant aggregates on the SDS-PAGE (Figure 2.9b), 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-MP contained less pentosidine than HT-MP (Figure 2.2), which may ultimately have influenced its degradability. Our findings showed that indeed several intramolecular interactions were 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 (Figure 2.3). 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-MP were still visible at the end of the GP but not after 10 min in the IP (Figure 2.8). This is in line with the results from the combined hydrolysis by chymotrypsin and trypsin, which showed that chymotrypsin hydrolysed these aggregates and thereby partly compensated for the impaired trypsin hydrolysis (Figure 2.9 and supplementary material: Figure 2-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 (Figure 2.7). 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-MP but not in HT-MP (supplementary material: Figure 2-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-MP compared to HT-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 WP [20]. However, when heating is conducted at low temperatures (<70 °C) and at low a_w-levels, that favour MR over protein unfolding, most studies report decreased *in vitro* protein digestibility [15,37-40]. 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 (Figure 2.2). Additionally, SEM images showed relatively larger particles in undigested HT-MP (Figure 2.10m) than in LT-MP (Figure 2.10f) as well as a slower hydrolysis of these particles during the intestinal

digestion of HT-MP (Figure 2.10p 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* [16,17]. Interestingly, it seems that insolubility does not directly affect digestibility in the IP, as no significant differences were observed in nitrogen transfer (Figure 2.6). 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 (Figure 2.10), is in line with previous findings and a result of acidic depletion and rapid hydrolysis in the GP [39,41]. 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 [42]. Moreover, the observation of larger spherical particles within these networks in the GP of HT-MP (Figure 2.10n) and the longer persistence of larger particles in the IP (Figure 2.10p and q) indicate a higher resistance of caseins towards digestion in HT-MP, which is also supported by the partly hydrolysed casein fragments on the SDS-PAGE (Figure 2.8). This is in line with the findings by Dupont *et al.* [38], 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, WPs showed enhanced hydrolysis in both LT-MP and HT-MP compared to NT-MP (Figure 2.8). This is in agreement with previous findings, which reported that glycated ALA [43,44] and WPs in spray dried skim milk powders [38] showed enhanced protein hydrolysis in infant *in vitro* digestion. Heating and glycation of MP can both increase and decrease allergenicity [22]. Pinto *et al.* [18] showed that the aggregates of glycated BLG survive until 60 min in the IP of adult gastrointestinal *in vitro* digestion. Moreover, another study reported that aggregation of BLG when glycated at HT and low a_w-levels resulted in the formation of binding ligands for the receptor of AGEs and immunoglobulin E [45]. 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 [19]. 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.

2.5   Conclusion

Dry heating of a powdered mixture of casein and WP 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-MP and HT-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.

Supplementary material
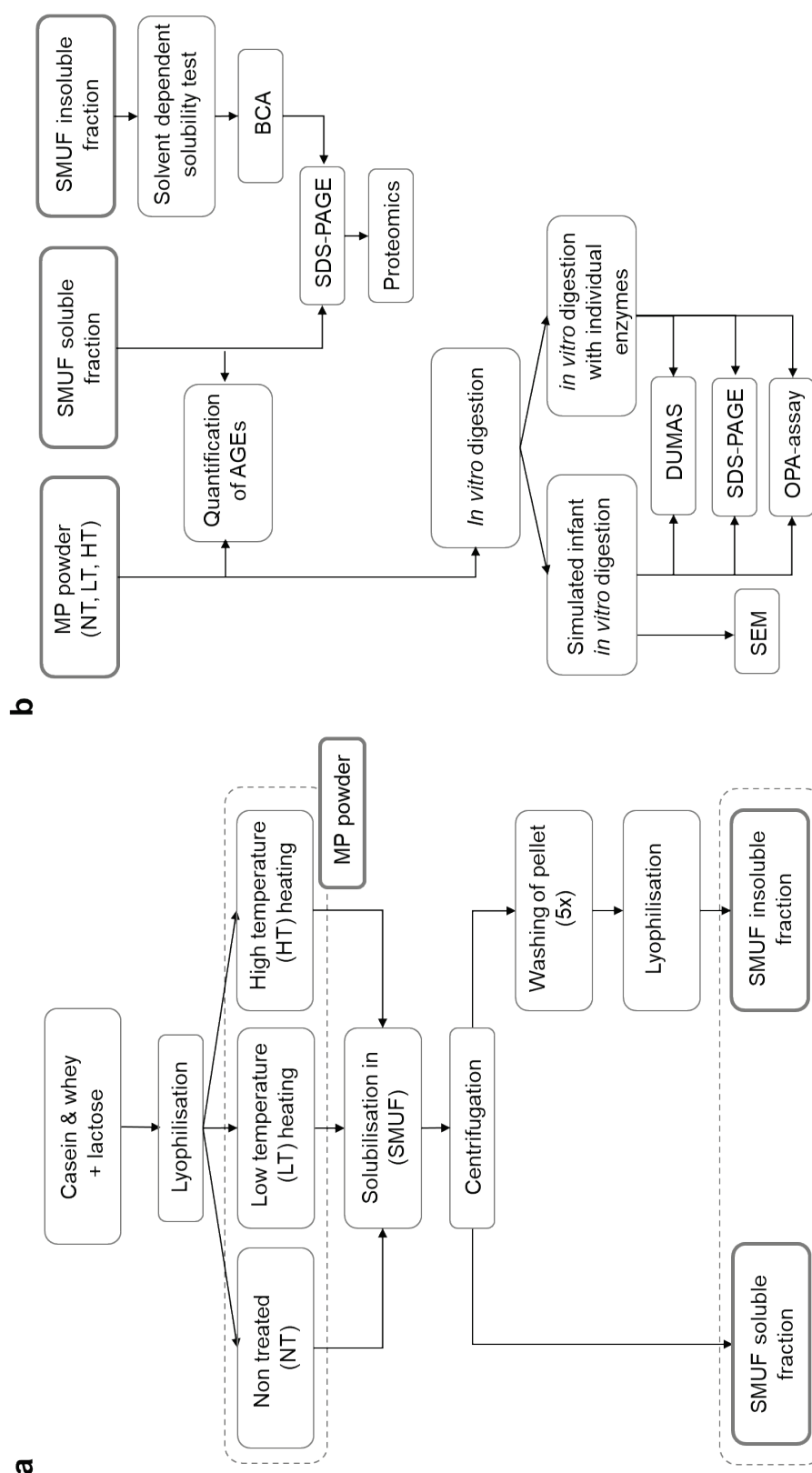
Figure 2-S1: (a) flow chart for the preparation of heated and glycated milk protein (MP) powder and the simulated milk ultrafiltrate (SMUF) soluble and SMUF insoluble fraction. (b) flow chart for sample analysis and *in vitro* digestion experiments.

a

b
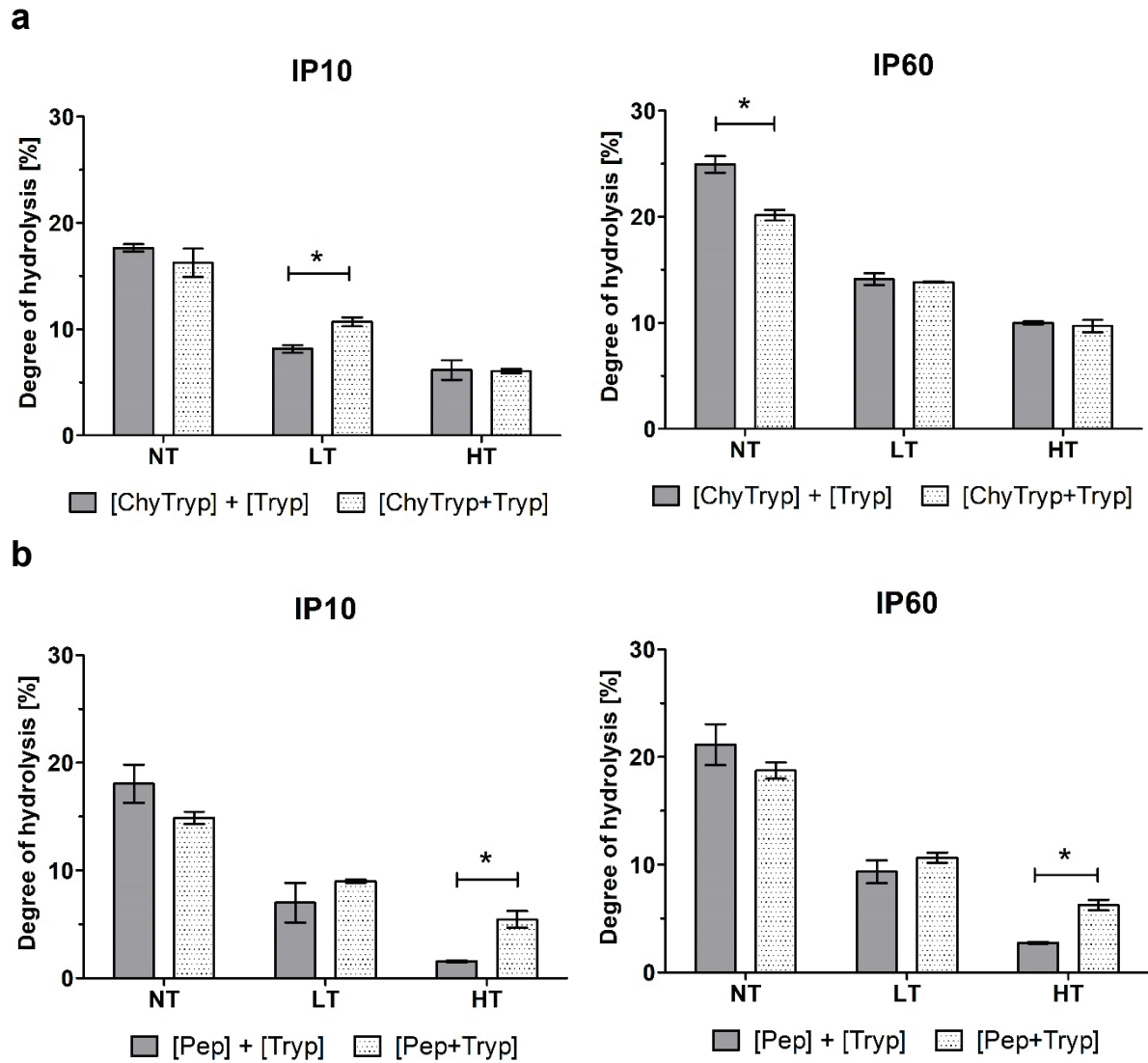
Figure 2-S2: (a) Comparison of sum of DH from chymotryptic and tryptic hydrolysis ([ChyTryp] + [Tryp]) to combined hydrolysis of both enzymes ([ChyTryp + Tryp]) after 10 min and 60 min in the intestinal phase (IP). (b) Comparison of sum of DH from pepsin and trypsin hydrolysis ([Pep] + [Tryp]) to combined hydrolysis of both enzymes ([Pep+ Tryp]) after 10 min and 60 min in the IP. Error bars represent standard deviation of technical duplicates. Statistical differences were analysed using one-way ANOVA with *p < 0.05.

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Chapter 3

Differential effects of dry vs. wet heating on the formation of sRAGE binding ligands and IgE epitope recognition

This chapter has been published as:

Abstract:

The effect of glycation and aggregation of thermally processed β-lactoglobulin (BLG) on binding to sRAGE and immunoglobulin E (IgE) from cow milk allergic patients were investigated. BLG was heated under dry conditions (water activity <0.7) and wet conditions (in phosphate buffer at pH 7.4) at low temperature (<73 °C) and high temperatures (>90 °C) in the presence or absence of the milk sugar lactose. N^{ε}-Carboxymethyllysine (CML) western blot and glycation staining were used to directly identify glycation structures on the protein fractions on SDS-PAGE. Western blot was used to specify sRAGE and sIgE binding fractions. sRAGE binding was highest under wet-heated BLG independent of the presence of the milk sugar lactose. Under wet heating, high-molecular-weight aggregates were most potent and did not require the presence of CML to generate sRAGE binding ligands. In the dry system, sRAGE binding was observed only in the presence of lactose. IgE binding affinity showed large individual differences and revealed four binding profiles. Dependent on the individual, IgE binding decreased or increased by wet heating independent of the presence of lactose. Dry heating required the presence of lactose to show increased binding to aggregates in most individuals. This study highlights an important role of heating condition-dependent protein aggregation and glycation in changing the immunogenicity and antigenicity of cow's milk BLG.

3.1 Introduction

The manufacturing of many dairy products implies heating at moderate (<73 °C) or high temperatures (>90 °C) to ensure product safety or to produce powdered products such as infant formula. Maillard reaction (MR)-induced glycation, as well as protein aggregation, are the most abundant protein modifications during milk processing. The formation of aggregates and the degree of glycation strongly depends on the applied processing conditions and may strongly differ between wet and dry heating conditions as they are applied in industrial processing of milk or dried dairy products. Wet heating of β-lactoglobulin (BLG) results in relatively more aggregation and the formation of polymers with a MW >10 kDa compared to dry heating [1]. At the same time, dry heating induces more glycation as low moisture content (water activity (a_w) between 0.2 and 0.8) enhances protein glycation via the MR [2]. These protein modifications are known to change reactivity of food allergens to innate cell surface receptors and immunoglobulin E (IgE) binding affinity by either destruction and masking of epitopes or formation of neo-epitopes via exposure of interior structures and amino acid side chain modifications [3]. The applied processing conditions, such as pH, humidity, and temperature are crucial determinants for epitope destruction/formation. For example, pasteurization of cow's milk (CM) BLG enhances its allergenicity by redirecting its epithelial uptake towards Peyer's patches [4]. At the same time, clinical studies report that "baked milk products" can be tolerated by ~70% of children with IgE-mediated CM allergy and that consumption of these products potentially facilitates the development of oral tolerance towards raw milk [5–7]. Both glycation and aggregation result in decreased binding of IgE from CM allergic children if heated above 90 °C in a wet system [8,9]. At the same time, cellular signalling can be promoted by the formation of glycation structures on the food allergen [10].

The receptor for advanced glycation end products (RAGE) is one of the most studied cell surface receptors in relation to protein glycation. It is known to bind to advanced glycation end products (AGEs) that are formed via the MR. RAGE is expressed by vascular endothelial cells and cells of the innate and adaptive immune system [10]. RAGE-ligand interaction activates an intracellular signalling cascade, resulting in the release of pro-inflammatory cytokines via NF-κB activation. Its soluble isoform (sRAGE) binds to the same ligands; however, it is considered to be a decoy for RAGE in the peripheral system [11]. Besides AGEs, sRAGE binds to several other ligands, such as lipopolysaccharide, amyloid-β, and S100 protein. Those ligands have the common property to act as oligomers [12]. Liu *et al.* [13] indicated that not only the level of glycation but also the aggregation occurring during dry heating of BLG can promote sRAGE binding affinity. Perkins *et al.* [14] recently reported the involvement of RAGE in type 2 cytokine signal transduction in the lungs of mice. Type 2 cytokines, e.g. interleukin (IL)-4 are involved in B-cell class switch and IgE production [15]. These findings indicate the direct involvement of RAGE with the clinical manifestation of the allergic reaction towards the allergen. MR not only leads to the formation of AGEs, as commonly described RAGE ligands, but also promotes protein aggregation [16]. By using controlled processing

conditions, protein modification can be directed towards either the formation of AGEs or the formation of aggregates enabling a distinction between the functional effects of these two distinct protein modifications. This study aimed to investigate the effect of protein aggregation and glycation on sRAGE binding affinity and the potential allergenic impact. Therefore, BLG was heated under controlled wet and dry conditions to investigate whether AGE formation or rather aggregation (glycation or non-glycation-induced) contributes to changes in sRAGE and IgE binding affinity to BLG.

3.2 Materials and Methods

3.2.1 *Chemicals*

Acetonitrile uHPLC-MS grade was purchased from VWR chemicals (Radnor, Pennsylvania, USA). N^{ε}-(carboxymethyl)-l-lysine (CML) and N^{ε}-(carboxy [2H2]methyl)-l-lysine (d2-CML) were purchased from Polypeptide laboratories (Strasbourg, France). NuPAGE® LDS sample buffer (4X conc.), and NuPAGE™ MOPS SDS running buffer (20X), NuPAGE™ 12% Bis-Tris protein gel, 1.0 mm. gels. Soluble AGE Product-Specific Receptor Human *E. coli* (RD172116100) was obtained from Biovendor (Brno, Czech Republic). Anti-RAGE antibody (monoclonal mouse IgG$_2$B clone, MAB11451) purchased from R&D systems (Minneapolis, Minnesota, USA). HRP conjugated anti-mouse polyclonal goat (P0447) was purchased from Dako (Glostrup, Denmark). TMB substrate (3,3′,5,5′-tetramethylbenzidine) for high sensitivity ELISA was purchased from sdt-reagents (Baesweiler, Germany). WesternBright™ Enhanced chemiluminescence (ECL) western blotting detection kit was obtained from Advansta (San Jose, California, USA). Ovalbumin was purchased from InvivoGen (San Diego, California, USA). Bovine Serum Albumin Fraction V was obtained from Roche (Basel, Switzerland). Amyloid-β (1-42) ultrapure HFIP was purchased from Westburg (Leusden, The Netherlands). (N-Epsilon)-Carboxymethyl-Lysine primary antibody was purchased from Nordic-MUbio (Susteren, The Netherlands). Pro-Q ® Emerald 300 Glycoprotein Gel and Blot Stain Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

BlueRay prestained protein marker was obtained from Jena Bioscience GmbH (Jena, Germany). Coomassie brilliant blue R-250 was purchased from Bio-Rad (Hercules, California, USA). Ultrapure water was prepared by an Purelab® Ultra water system from ELGA LabWaters (Celle, Germany).Three Plasma were purchased from PlasmaLab International (Everett, Washington, USA). Two sera were provided by the Queen Beatrix Hospital (Winters Wijk, The Netherlands), all other sera were provided by the archival serumbank of the Erasmus Medical Centre (Rotterdam, The Netherlands). All other chemicals were purchased from Sigma Aldrich (St Louise, Minnesota, USA) unless mentioned otherwise.

3.2.2 *BLG isolation and purification*

Raw bulk milk obtained from the Department of Animal Sciences, Wageningen University & Research (Wageningen, The Netherlands). BLG was purified and isolated as described by

De Jongh *et al.* [17] using anion exchange chromatography DEAE Sepharose C-6B (GE healthcare, Chicago, Illinois, USA). Isolated BLG was lyophilised and a purity >94% was measured as described by Deng *et al.* [1].

3.2.3   *Heat treatment of BLG*

BLG was heated in a wet system above the denaturation temperature by heating it in phosphate buffer saline (PBS) at pH 7.4 applying 100 °C for 90 min in the presence of lactose (W-HT-Lac) and in the absence of lactose (W-HT). Three additional treatments were conducted as described by Deng *et al.* [1]. Briefly, a low temperature (LT) wet heating was conducted by heating BLG below its denaturation temperature in 10 mM PBS (pH 7.4) at 60 °C for 72 h in the presence of lactose (W-LT-Lac) and in the absence of lactose (W-LT). High temperature (HT) dry heating of BLG was conducted at 130 °C for 10 min in the presence of lactose (D-HT-Lac) and in the absence of lactose (D-HT). Prior to heat treatment, the BLG solution was lyophilised and a_w-level was adjusted to 0.53 over saturated sodium bromide solution. For low temperature dry heating, BLG was heated at 50 °C for 9 h at a_w 0.65 in the absence of lactose (D-LT) and the presence of lactose (D-LT-Lac). Humidity was monitored with a humidity control chamber (HCP108, Memmert, Schwabach, Germany). After heat processing, dry-heated samples were dissolved in water to starting protein concentration. All samples were centrifuged at 2900 ×g for 30 min to remove insoluble material and unreacted lactose was removed by dialysis. Protein concentration of samples showing the formation of insoluble material was determined with DUMAS as described by Deng *et al.* [1].

3.2.4   *Quantification of N^ε-carboxymethyllysine (CML) using uHPLC-MS/MS*

CML was quantified using uHPLC-ESI-MS/MS according to a method described by Troise *et al.* [18]. Samples were diluted to 2.5 mg/mL in ultrapure water and mixed with hydrochloric acid to a final ratio of 0.63 mg protein/1 mL 6 M hydrochloric acid. Solutions were saturated with nitrogen and heated for 22 h at 110 °C. Hydrolysates were centrifuged (4500 ×g, 10 min, 20 °C) using a Heraeus multifuge X3R (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and filtered through a 0.2 μm Polytetrafluoroethylene syringe filter (Phenomenex, Torrance, California, USA). An aliquot was dried under nitrogen and dissolved to the same volume in ultrapure water. Samples were centrifuge (10,000 ×g, 20 min, 20 °C) using an Eppendorf multifuge 5430R (Eppendorf, Hamburg, Germany). Subsequently, they were diluted with acetonitrile to reach 50% acetonitrile and spiked with internal standard CML-d2.

Standard solutions were prepared in a concentration range between 25 ng/mL and 750 ng/mL and spiked with CML-d2. Final concentration of CML-d2 in all sample and standard solutions was 250 ng/mL. CML was separated on a Kinetex 2.6 μm HILIC 100A, 100 × 2.1 mm (Phenomenex, Torrance, California, USA) at 35 °C column temperature. Eluent A was ultrapure water with 0.1% formic acid, eluent B was acetonitrile with 0.1%, and eluent C was

50 mM ammonium formate. Flow rate was set to 0.4 mL/min using the following gradient (time [min]/eluent B [%]/eluent C [%]): (0/80/10), (0.8/80/10), (3.5/40/10), (6.5/80/10), (8.0/80/10), (11/80/10). Electron ionisation was conducted in positive mode. Spray voltage was set to 3500 °C, vaporising temperature was 250 °C, and sheath gas pressure was 60 psig. Capillary temperature was set to 290 °C. The parent mass $[M+H]^+$ 205.3 m/z and 207.3 m/z were selected in Q1 for CML and CML-d2, respectively The characteristic product ions 130.0 m/z (collision energy (CE): 12 V; tube lens: 78) and 84.0 m/z (CE: 22 V; tube lens: 78) were recorded in Q3.

3.2.5   Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE)

Gel electrophoresis under non-reducing conditions was performed to monitor protein aggregates formed during heat treatment of BLG. Samples, NuPAGE® LDS sample buffer (4× conc.) and ultrapure water were mixed in a ratio 5/5/10 (v/v/v), centrifuged (1 min, 500 ×g, 20 °C) on an Eppendorf multifuge 5430R (Eppendorf, Hamburg, Germany) and incubated for 10 min at 70 °C. BlueRay prestained protein marker was used as MW marker. Six microgram protein of each sample were loaded on NuPAGE™ 12% Bis-Tris protein gel, 1.0 mm. Gels were run at 120 V for 1.5 h using NuPAGE™ 1× MOPS SDS running buffer and stained with Coomassie Brilliant Blue R-250 or using Pro-Q® Emerald 300 Glycoprotein staining kit according to the manufacturer's instructions. Images of the stained gels were obtained using a Universal Hood III (Bio-Rad, Hercules, California, USA) and Image Lab 4.1 software (Bio-Rad, Hercules, California, USA).

3.2.6   Thioflavin-T (ThT)-assay

ThT-assay was conducted to monitor the formation of fibril structures during heating. Protein concentration was adjusted to 0.25 mg/mL and mixed with 3.9 mM aqueous ThT solution in a ratio 5.8/1.0 (v/v). All samples were prepared in duplicate. The solution was transferred in a 96-well black greiner polystyrene plate (Greiner CELLSTAR®, Kremsmünster, Austria). After incubated for 10 min in the dark, fluorescence emission was measured (excitation wavelength 450 nm, emission wavelength 485 nm, gain 100) using Infinite® 200 PRO NanoQuant with i-control software (Tecan, Männedorf, Switzerland). The fluorescence intensity of heated and glycated BLG was corrected for the blank (PBS at pH 7.5).

3.2.7   Inhibition sRAGE ELISA

Inhibition sRAGE ELISA was conducted to determine sRAGE binding affinity as described by Liu *et al.* [13] with some modifications. Briefly, soy protein extract glycated with glucose (90 min, 100 °C, wet conditions) was used as coating material. Transparent high binding ELISA plate (Greiner Bio-One, Kremsmuenster, Austria) were coated with G90 for 12 h at 4 °C. Sample protein concentration was adjusted to 25 µg/mL with 1.5% bovine serum albumin (BSA) (w/v) in 0.025% tween in 10 mM PBS (PBST). The optimal protein concentration was chosen based on a dilution curve of BLG-NT obtained from a pre-experiment. The samples

were pre-incubated with 1 µg/mL sRAGE in a ratio 1:1 (*v/v*) for 45 min at 37 °C on a NuncTM polystyrene plate (Thermo Fisher Scientific, Massachusetts, USA) before addition to the ELISA plate. The coated ELISA plate was blocked with PBS with 3% BSA (*v/w*) for 1 h at room temperature and washed with PBST. The washing step was repeated after each step of ELISA. After blocking, the pre-incubated sRAGE/sample mixture was transferred into the ELISA plate and incubation was continued for 1 h at 37 °C. After washing, anti-sRAGE antibody was added at a concentration of 0.25 µg/mL and the plate was incubated under shaking for 30 min at room temperature. After washing, anti-mouse polyclonal goat HRP conjugated antibody at a concentration 0.25 µg/mL was added and the incubation was continued for 30 min at room temperature. The signal was detected with TMB. The colour reaction was measured at 450 nm vs. 620 nm reference using a Filter Max F5 multi-mode microplate reader (Molecular Devices, San Jose, California, USA). Each sample was measured in triplicate. Amyloid-β was used as a positive control, while ovalbumin was used as a negative control.

Inhibition was calculated using the following formula:

$$\text{Inhibition } [\%] = (\text{Abs}_{\text{Max}} - (\text{Abs}_{\text{Sample}} - \text{Abs}_{\text{Min}}))/\text{Abs}_{\text{Max}} \times 100$$

where Abs_{Max} is the absorbance obtained from sRAGE without competition agent and Abs_{Min} is the absorbance obtained from blank sample (PBS) without sRAGE, $\text{Abs}_{\text{sample}}$ is the absorbance obtained from the mixture of sRAGE and each sample. High inhibition indicates high sRAGE binding affinity.

3.2.8 *sRAGE western blot*

SDS-PAGE was conducted as described before. However, protein concentration increased to 20 µg protein for wet-heated BLG at HT, 25 µg protein for wet-heated BLG at low temperature, 10 µg protein for dry-heated BLG in the presence of lactose, and 4 µg protein for BLG-NT and BLG heated in a dry system in the absence of lactose, to achieve similar band densities on the gel. Gels were blotted on Amersham™ Protran™ 0.45 µm nitrocellulose membrane (GE Healthcare Life science, Marlborough, Massachusetts, USA). Gels were blotted with semi-dry western blot blotting buffer at 15 V for 35 min. Membranes were washed for 2 × 5 min with 1x Tris buffer saline (TBS) with 0.2% tween (TBST) and incubated for 1 h at room temperature with 3% BSA in TBST. Subsequently, membranes were washed 2 × 10 min with TBST and incubated at 4 °C for 12 h with sRAGE diluted to 1 µg/mL with 1.5% BSA in TBS (w/v). Membranes were washed 4 × 7 min with 1 × TBST/Triton and 2 × 5 min with TBST. Anti-RAGE antibody was diluted to 0.25 µg/mL with 1.5% BSA in TBS and added to the membrane for 1 h at room temperature. Subsequently, the membrane was washed as described before and incubated for 1 h with anti-mouse polyclonal goat HRP conjugated antibody diluted to 0.25 µg/mL with 1.5% BSA in TBST. After incubation, membranes were washed 4 × 7 min with 1 × TBST/Triton and 2 × 5 min with TBS. ECL western blot detection reagent was added for 30 s. Chemiluminescence was visualized in ChemHighsensitivity mode using an Universal

Hood III (Bio-Rad, Hercules, California, USA) and Image Lab 4.1 software (Bio-Rad, Hercules, California, USA)

3.2.9 *CML western blot*

SDS-PAGE western blotting was performed as described before. Blotted membranes were washed 2 × 5 min with TBST and incubated 1 h in TBST with 3% BSA. After washing 2 × 10 min with TBST, membranes were incubated with 0.25 µg/mL anti-CML-antibody with 1% BSA and 0.5% raw whey protein in TBS at 4 °C for 12 h. Membranes were washed 4 × 7 min with TBST/Triton and 2 × 5 min with TBST. Anti-mouse polyclonal goat HRP conjugated antibody was diluted to 0.25 µg/mL with 1% BSA and 1% raw whey protein in TBST. The membrane was incubated for 30 min with the antibody. Subsequently, it was washed 4 × 7 min with TBST/Triton and 2 × 5 min with TBS. ECL chemiluminescence detection was conducted as described before.

3.2.10 *IgE binding dot blot*

IgE dot blot was conducted to screen the available sera and plasma for their binding affinity to BLG heated under dry conditions at HT and BLG heated under wet conditions at HT, each time in the presence of lactose or absence of lactose. Each time, 5 µg protein was spotted on the membranes. The membranes were washed 5 min with TBST and incubated for with 3% BSA in TBST for 1 h at room temperature. Membranes were washed 2 × 5 min in TBST and incubated with sera/plasma diluted in 1% BSA/TBST for 12 h at room temperature. Sera dilutions were prepared in the ratios as shown in Table 3.1. Membranes were washed 4 × 7 min with TBST/Triton and 2 × 5 min with TBST. Mouse anti-human IgE antibody was diluted 0.5 µg/mL with 0.5% non-fat dry milk in TBST and added to the membranes for 1 h at room temperature. Subsequently, the membranes were washed 4 × 7 min with TBST/Triton and 2 × 5 min with TBST. Anti-mouse polyclonal goat HRP conjugated antibody was diluted to 0.25 µg/mL with 0.5% non-fat dry milk in TBST and added to the membranes for 30 min at room temperature. Subsequently, they were washed 4 × 7 min with TBST/Triton and 2 × 5 min with TBS. Chemiluminescence detection was conducted as described before.

Table 3.1: Levels of specific immunoglobulin E (sIgE) of sera from cow's milk allergic patients. sIgE levels as measured by ImmunoCap.

Patient	sIgE-Level Cow's Milk Proteins [kU/L]	Specimen Type	Dilution
1	52.2	Serum	1:5
2	0.73	Serum	1:3
3	0.96	Serum	1:3
4	0.53	Serum	1:3
5	0.96	Serum	1:3
6	1.69	Serum	1:3
7	1.55	Serum	1:3
8	>100	Plasma	1:5
9	91.0	Plasma	1:5
10	94.8	Plasma	1:5
11	28.4	Serum	1:5
12	6.6	Serum	1:5

3.2.11 *IgE binding western blot*

IgE western blot was conducted to directly identify the bands on the SDS-PAGE that show IgE binding. The specimen was selected depending their activity observed in the IgE dot blot and pooled for similar IgE binding according to the scheme in Table 3.2.

Table 3.2: Pooling of sera and plasma for IgE-western blot and used dilution. Membrane number indicates the membrane that was incubated with the specific pooled specimen.

Pool/Serum	Patient Serum/Plasma	Dilution
Pool 1	7, 11, and 12	1:5
Serum 6	6	1:7
Pool 2	8 and 9	1:7
Pool 3	1 and 10	1:5

SDS-PAGE was prepared and blotted as described for sRAGE western blot (paragraph 3.2.8). All other steps were conducted as described for the IgE dot blot (paragraph 3.2.10).

3.2.12 *Statistical analysis*

Statistical analysis was conducted using IBM SPSS version 23. For multiple sample comparison, one-way ANOVA and Tukey post hoc comparison test was used. Results were considered statistical different at $p < 0.05$ if not mentioned otherwise.

3.3 Results

To investigate the effect of different heat treatments of BLG on aggregation, formation of MR products, binding to sRAGE, and binding of IgE, BLG was heated in solution (wet conditions) and in powdered form with controlled a_w (dry conditions). For each humidity condition, BLG was heated in the presence or absence of the milk sugar lactose to distinguish between the effect of glycation and heating. Additionally, each treatment was conducted below the denaturation temperature (LT heating) and above the denaturation temperature (HT heating) of BLG.

3.3.1 *Solubility of BLG after heat treatments*

Solubility of BLG was impaired after heating in a dry system, but only in the presence of lactose at 130 °C. This was observed by the formation of insoluble material after dissolving BLG in PBS. Therefore, the insoluble aggregates were removed by centrifugation and the soluble fraction of was used for further characterization. Analysis of the total nitrogen content with DUMAS showed a recovery of soluble protein of 76.5% of the total protein. BLG heated in a wet system and at low temperature in a dry system, did not show the formation of insoluble material, independent of the presence of lactose.

3.3.2 *Quantification of CML*

CML was quantified in all samples to determine the level of the advanced stage of the MR. Table 3.3 compares CML quantities of BLG heated under different conditions in the presence or absence of lactose. No CML was found in the BLG samples that were heated without lactose, because CML formation requires the presence of either a reducing sugar moiety or polyunsaturated fatty acids. CML quantities were on average ~30% lower when BLG was heated in a wet system than when it was heated in a dry system. The quantities of CML were positively correlated with the temperature of heating in both systems. Additionally, CML levels were relatively higher after dry heating of BLG compared to wet heating.

Table 3.3: CML quantities determined by uHPLC-ESI-MS/MS in the soluble fraction of BLG heated in the presence of lactose (Lac) using wet (W) or dry (D) heat treatment at either low or high temperature (LT/HT), BLG-NT: unheated BLG, ND: quantities below the detection limit. Data are expressed in means of technical duplicates ± SD ($n = 2$). Letters indicate significant differences between groups ($p < 0.05$) as analysed by one-way ANOVA and Tukey post hoc comparison test.

Sample	BLG-NT	W-HT-Lac	W-HT	W-LT-Lac	W-LT	D-HT-Lac	D-HT	D-LT-Lac	D-LT
CML mg/100 g protein	ND	129 ± 10 a	ND	77 ± 4 b	ND	182 ± 7 c	ND	158 ± 9 d	ND

The levels of CML, as a commonly used marker for the "advanced stage" of the MR, indicate that the level of glycation was higher when BLG was heated under dry conditions than under wet conditions. The formation of CML was further facilitated at higher temperature heating within the same humidity conditions.

3.3.3 *SDS-PAGE*

Protein aggregation was monitored using SDS-PAGE under non-reducing conditions, as shown in Figure 3.1.
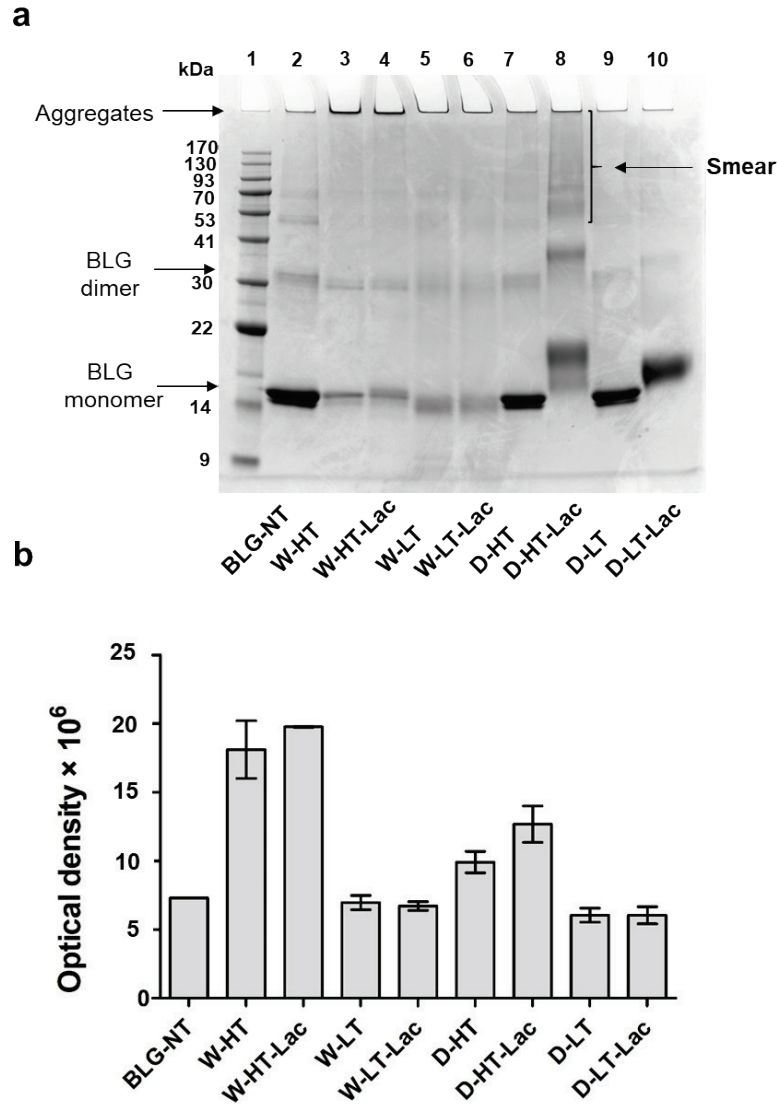
Figure 3.1: (a) Non-reducing SDS-PAGE image of soluble BLG heated and glycated in a wet system (W) and in a dry system (D), respectively in the absence of lactose or in the presence of lactose (Lac) and at either high temperatures (HT) or low temperatures (LT). (b) optical density of the aggregates visible in the top of each lane (>170 kDa). Error bars represent standard deviation of the optical density of two SDS-PAGE gels prepared independently from each other.

When heated in a wet system, BLG formed high-MW aggregates that did not migrate into the gel. Aggregate formation occurred below (60 °C, lanes 5-6) as well as above the denaturation temperature of BLG (100 °C, lanes 3-4); however, they were more abundant when BLG was heated above its denaturation temperature. The relative aggregate quantities, based on the optical density, showed this effect of heating temperature, but not of lactose presence for the wet-heated samples (lanes 3-4 and lanes 5-6 of Figure 3.1a). Dry-heated BLG aggregates showed 23% higher optical density in the presence of lactose compared to the heating in the absence of lactose, however only when BLG was heated at HT.

Heating of BLG in a dry system resulted in protein aggregation showing clear differences between the samples with and without lactose. After heating of BLG with lactose at HT (Figure 3.1a, lane 8), the MW of the BLG monomer increased by approx. 4 kDa while the BLG

dimer showed an increasing MW by approx. 6 kDa. The relative intensity of the BLG dimer increased by 55% compared to the unheated control, indicating a shift of the monomer-dimer equilibrium of BLG. Additionally, D-HT-Lac showed a smear starting from an apparent MW >53 kDa until the top of the gel (lane 8). D-LT (lane 9) showed monomeric BLG at an apparent MW of 14 kDa, which is similar to the non-treated BLG (BLG-NT). However, a faint smear, similar to the one of D-HT-Lac but with much lower intensity, was also observed for this sample. Dry heating at low temperatures did not induce the formation of aggregates, neither in the presence or absence of lactose, in the soluble fraction. However, a shift of apparent MW of the BLG monomer by 2 kDa was observed in D-LT-Lac.

These data indicate that whereas wet heating favours formation of aggregates, dry heating favours MR.

3.3.4   *Detection of glycation structures on SDS-PAGE visible proteins*

To directly identify which of the bands visible on the SDS-PAGE are modified by MR, the Pro-Q™ Emerald 300 glycoprotein staining kit was used. The staining reacts with structures carrying a reducing carbonyl moiety and can therefore indicate the presence of MR products (MRPs) from all three stages of the MR. Stained glycated protein will appear as white bands on the SDS-PAGE image (Figure 3.2).
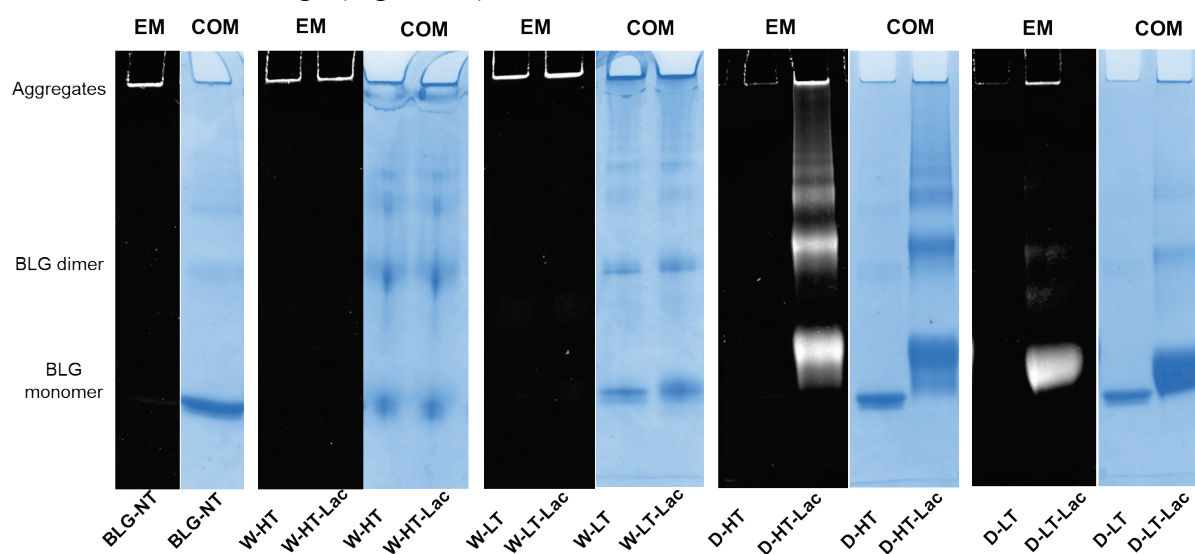
Figure 3.2: Non-reducing SDS-PAGE image of soluble BLG heated and glycated in a wet system (W) and in a dry system (D), respectively in the absence of lactose or in the presence of lactose (Lac) and at either high temperatures (HT) or low temperatures (LT). Gels were stained with Q™ Emerald 300 staining for glycoproteins (EM) and Coomassie staining (COM) for total protein staining.

Wet-heated BLG showed a fluorescence signal of the aggregates in the top of the gel, independent of the presence of lactose and the temperature. Based on CML western blot (Figure 3.2) it can be concluded that under wet heating conditions, the glycation structures are mainly present in the high-MW aggregates; however it was not clearly visualized by glycation staining. It may be explained by the fluorescent specking at the edges of the gel which is an intrinsic property of the dye and/or the fluorescence signal from tryptophan residues of native

BLG. Both effects can contribute to the positive signal for unheated BLG in the top of the gel. It may also partly contribute to the positive signal of the high-MW aggregates of wet-heated BLG making difficult to quantitatively interpret the presence of reducing carbonyl groups in the wet-heated samples.

Dry heating showed high fluorescence when heated in the presence of lactose at high and low temperatures, and not when heated in the absence of lactose. The signal for glycation structures was observed for all proteins that were also visible with Coomassie staining (Figure 3.3). A positive signal with glycation staining was also observed for the BLG monomer of D-HT and D-HT-Lac and the high-MW aggregates. The same is also true for BLG-NT. However, the relative glycation staining intensity of the aggregates from heated and glycated BLG is higher than the high-MW band visible in BLG-NT.
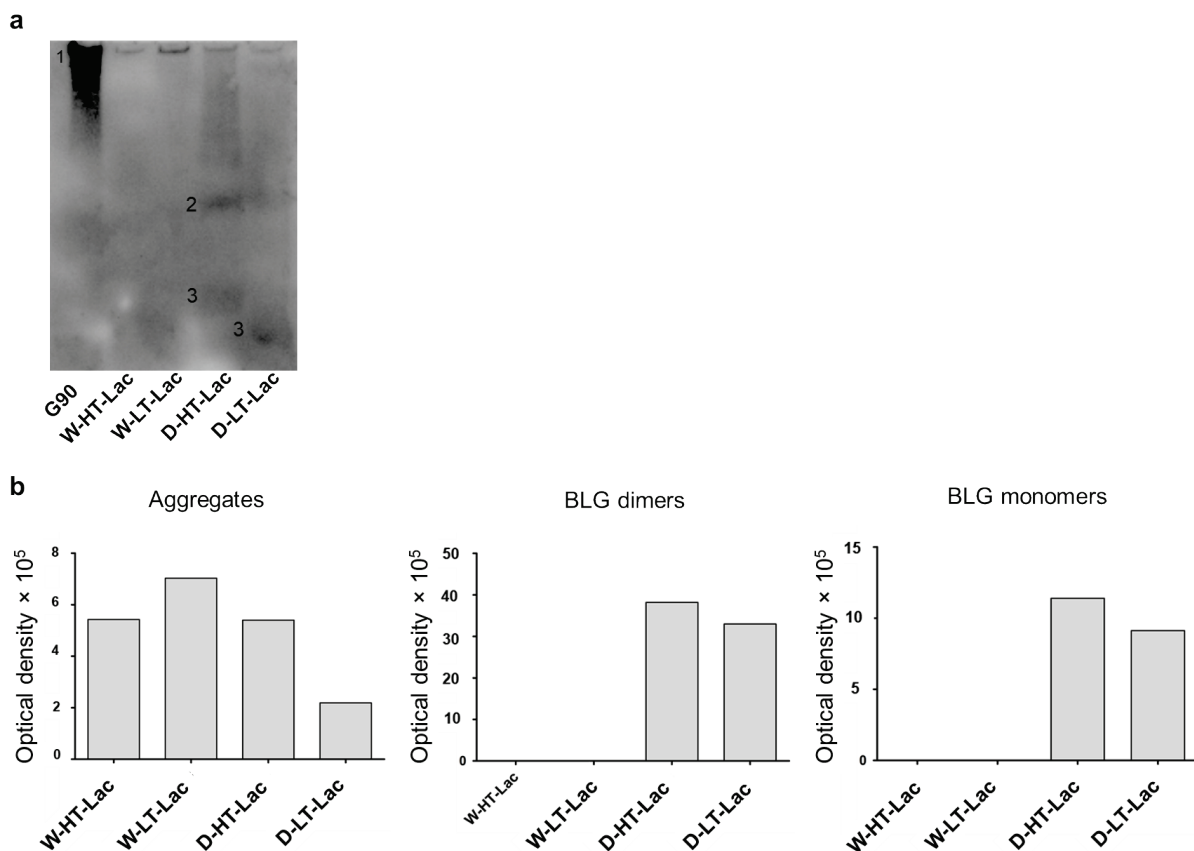
Figure 3.3: (a) Membrane image of CML western blot measured of BLG heated in the presence of lactose (Lac) in a wet system (W) or a dry system (D) at low temperatures (LT) or high temperatures (HT). G90: soy protein extract glycated with glucose for 90 min (positive control). Numbers indicate aggregates (1), BLG dimers (2), and BLG monomers (3). (b) Optical density of bands visible on the CML western blot categorized in high-MW aggregates, BLG dimers, and BLG monomers as indicated on the membrane.

The band of wet-heated BLG at low temperature showed higher intensity than wet-heated BLG at HT, which is in contradiction to the total CML quantities. This can be explained by the lower accessibility of the CML antibody to CML structures if they are buried inside of the aggregate. This effect might have more impact in HT treated samples because of the more compact structure of the aggregates. These data show that under wet heating conditions glycation

structures and CML are mainly detected in high-MW aggregates, while under dry heating conditions they are observed in all protein factions.

3.3.5 *Formation of fibril structures*

The ThT-assay was conducted to monitor the formation of fibril structures and exposure of β-sheet structures upon heating and glycation of BLG. Results are shown in Figure 3.4.
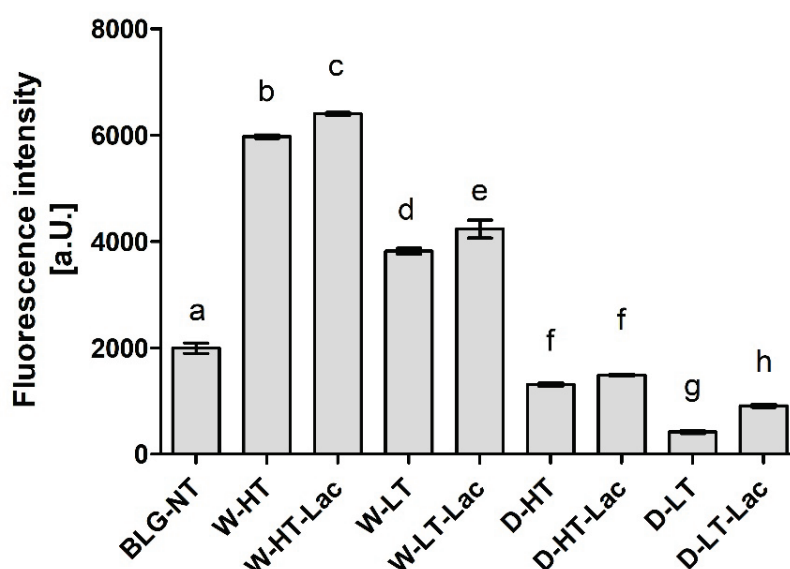
Figure 3.4: ThT fluorescence intensity after incubation with BLG heated in the absence of lactose or in the presence of lactose (Lac) in a wet system (W) or a dry system (D) under low temperatures (LT) or high temperatures (HT). BLG-NT: unheated BLG. Error bars represent standard deviation of technical duplicates. Letters indicate significant differences between the BLG groups. *p*-values < 0.05 are considered statistically significant, as analysed with one-way ANOVA and Tukey post hoc comparison test.

Highest fluorescence intensity was observed for wet-heated BLG and increased with higher heating temperature. When heated under dry conditions, the fluorescence intensity did not increase above the level of BLG-NT, indicating that no additional β-sheet structures were exposed. These results indicate that β-sheet structures are exposed and/or formed when BLG is heated under wet conditions independent of the presence of lactose. At the same time, this effect is not observed under dry heating conditions. This indicates that the exposure of β-sheet structures is facilitated under wet heating conditions while the presence of lactose plays a minor role independent of the humidity conditions.

3.3.6 *Binding of sRAGE to heated and glycated BLG*

Binding of sRAGE to heated and glycated BLG was monitored using sRAGE inhibition ELISA where high inhibition indicates high sRAGE binding affinity (Figure 3.5).

a

b
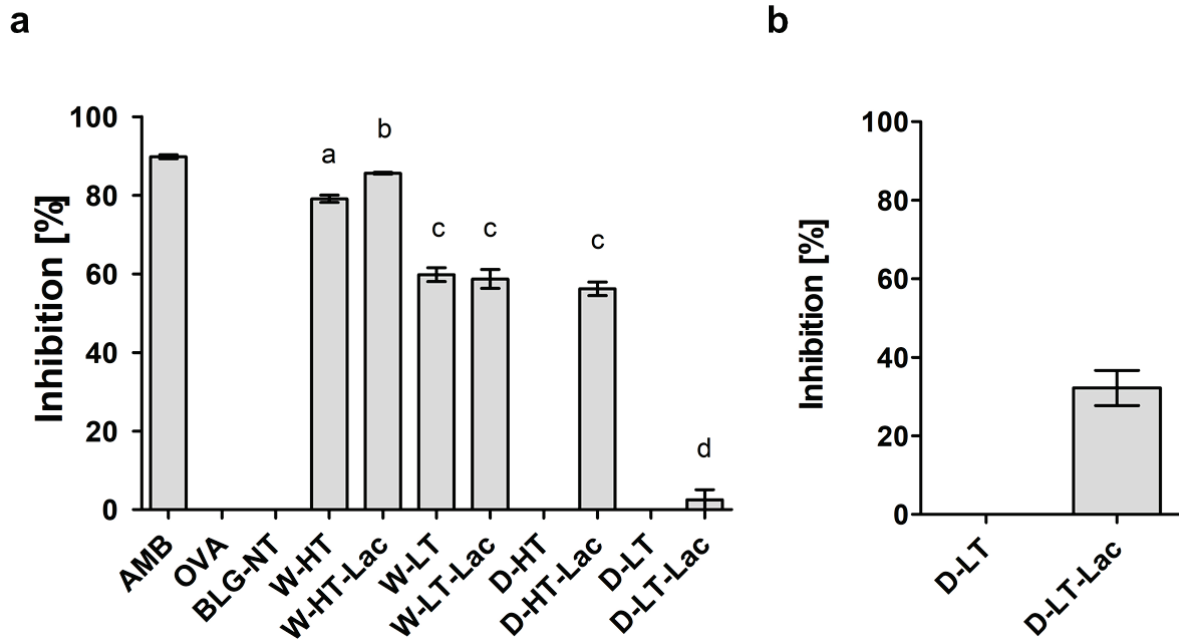
Figure 3.5: (a) Inhibition of sRAGE binding in competition ELISA by BLG heated in wet system (W) and dry system (D) in the absence of lactose or presence of lactose (Lac) at high or low temperature (HT and LT) for all treatments at a protein concentration 25 ug/mL. AMB: amyloid-β (positive control), OVA: ovalbumin (negative control). (b) Results of inhibition sRAGE ELISA for D-BLG-LT and D-LT-La at a protein concentration 100 µg/mL. Error bars represent standard deviation of technical triplicates. Letters indicate significant differences between the BLG groups. p-values < 0.05 are considered statistically significant, as analysed with one-way ANOVA with Tukey post hoc comparison test.

BLG-NT did not show any binding affinity to sRAGE while BLG heated in wet systems, both with and without lactose, showed increased sRAGE binding affinity compared to BLG-NT. Wet heating at higher temperature resulted in 28% higher sRAGE binding affinity than wet heating at lower temperature. At HT, sRAGE showed 8% higher binding to W-HT-Lac than W-HT, while at low temperatures no lactose dependent difference was observed. In contrast to the wet system, BLG heated without lactose in a dry system (at either LT or HT) did not show any binding affinity for sRAGE. D-HT showed a similar sRAGE binding affinity as the LT wet-heated samples. BLG heated in dry system with lactose at low temperature (D-LT-Lac) showed the lowest sRAGE binding compared to the other samples; however the binding increased with higher protein concentration (Figure 3.5b), indicating that sRAGE does show binding affinity but lower than for the other samples.

Western blot analysis was used to directly identify which fractions on the SDS-PAGE were responsible for the sRAGE binding determined for heated and glycated BLG in the sRAGE inhibition ELISA. These results are shown in Figure 3.6.
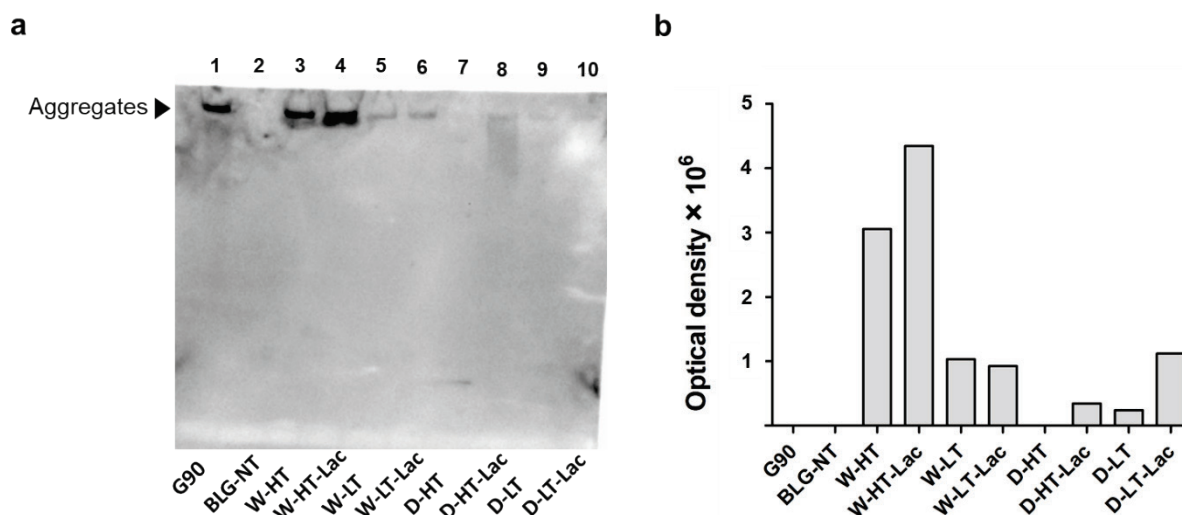
Figure 3.6: Membrane of sRAGE western blot for BLG heated in the absence of lactose or presence of lactose (Lac) under wet conditions (W) or dry conditions (D) at high temperatures (HT) or low temperatures (LT). (b) Optical density of the bands showing sRAGE binding affinity. G90: soy protein extract glycated with glucose for 90 min, 100 °C, wet conditions (positive control).

No binding of sRAGE to the monomeric form of BLG was observed in any of the heat-treated BLG samples except for dry heating at low temperature (Figure 3.6a, lane 9). The faint band in the lower part of lane 7 is most likely an artefact, as no protein was seen at this position on the SDS-PAGE. The fraction showing highest affinity to sRAGE were high-MW aggregates of BLG observed in wet-heated samples, especially those heated at HT (lanes 3-4). Aggregates of wet-heated samples with lactose showed ~30% higher intensity than samples heated without lactose, suggesting that both aggregation but also glycation play a role in sRAGE binding. The same tendency of slightly increasing sRAGE binding in the presence of lactose under wet conditions, was also observed in the ELISA. At LT wet heating, no increased signal intensity was observed when lactose was present. In the dry system at HT, sRAGE binding to the smear formed at MW >53 kDa was observed for lactose-containing sample. At LT dry heating a faint band was observed for aggregates of lactose-free heated BLG. The signal intensity of BLG heated in the presence of lactose under dry conditions, based on the optical density (Figure 3.6b), increased by ~75%. The relative binding intensity of BLG heated under different conditions are in line with the ELISA results (Figure 3.5). These results indicate that aggregation promotes sRAGE binding affinity to a greater extent than glycation.

3.3.7 *IgE binding to heated and glycated BLG*

IgE binding dot blot was conducted to screen the available plasma and sera for their IgE binding affinity to untreated BLG as well as heated and glycated BLG under wet or dry conditions at HT. The results of the 12 different sera/plasma samples from Table 3.1 are shown in Figure 3.7. Based on the dot blot results, the sera were pooled (see Table 3.2) to perform IgE western blots.
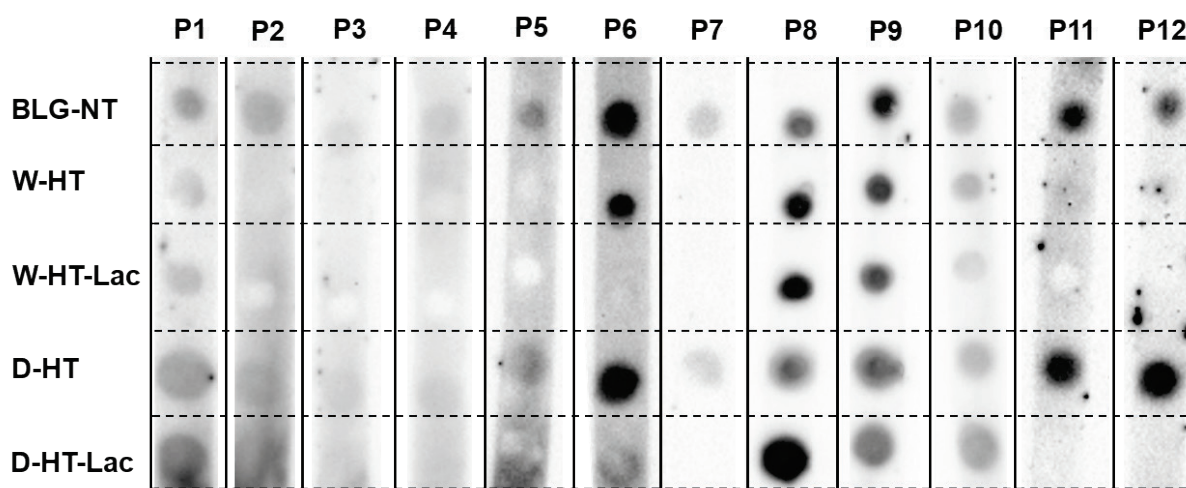
Figure 3.7: IgE dot blot membranes of sera from 12 different patients (P) tested for their binding affinity to unheated BLG (BLG-NT) and BLG heated at high temperatures (HT) either under wet (W) or dry (D) conditions, both in the absence of lactose (BLG) or in the presence of lactose (Lac).

No binding to BLG was observed for the sera of patient 3 and only weak binding to unheated BLG for the sera of patient 4. All other sera showed a positive binding to BLG-NT.

Four different profiles for IgE binding to heated and glycated BLG were observed. The sera described as IgE binding profiles 1-4 were pooled (see Table 3.2 for details) to perform western blots (Figure 3.8). Sera 7, 11, and 12 showed only binding to unheated BLG and BLG heated in the absence of lactose under dry conditions. Serum 2 and serum 5 also showed a similar profile of binding to BLG to sera 7, 11, and 12; however, because of the high background, it could not be excluded that they also show binding to BLG heated in the presence of lactose under dry conditions. Therefore, they were not included in pool 1.
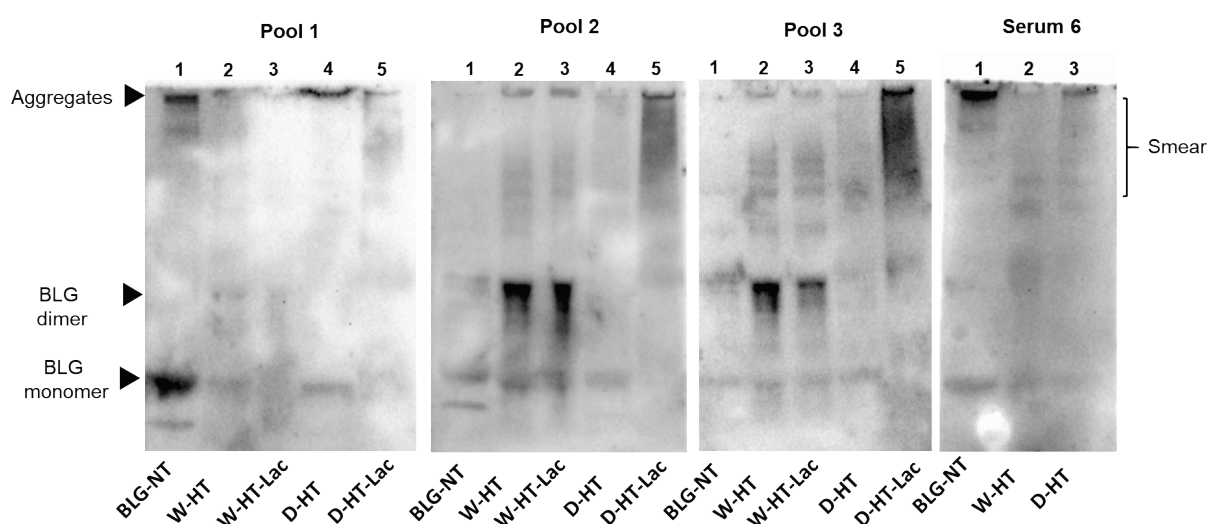
Figure 3.8: IgE western blot membranes of unheated BLG (BLG-NT) as well as BLG heated at high temperatures (HT) either under wet (W) or dry (D) conditions, both in the absence of lactose or in the presence of lactose (Lac). Sera of cow's milk allergic patients were pooled. Pool 1: sera 7, 11, 12. Pool 2: serum 8 and serum 9. Pool 3: serum 1 and serum 10.

Western blots confirmed the results observed in dot blot and indicated that the binding of IgE occurs to both BLG monomer and the aggregates, with strongest binding for BLG-NT and D-HT (pool 1, lane 4 and 5). A band below the BLG monomer band was also observed in unheated BLG although this band was not seen on the SDS-PAGE (Figure 3.1a). While the binding intensity for BLG-NT was equal between the monomer and the aggregates, in D-HT the binding intensity to the aggregates was ~30% higher than to the monomer (Figure 3.8, pool 1, lane 4). Weak binding was also observed for the BLG monomer in samples heated in the absence of lactose under wet conditions and to the smear of BLG heated in the presence of lactose under dry conditions (Figure 3.8, pool 1, lane 2 and 5). Pool 2 showed binding to all samples, with higher binding to wet-heated BLG independent of the presence of lactose and to dry-heated BLG in the presence of lactose. Western blots showed IgE binding to the BLG monomer and the BLG dimer in unheated BLG. When heated under wet conditions (Figure 3.8, pool 2, lane 2 and lane 3), binding was observed to the BLG monomer, BLG dimer, and the aggregates. The dimer showed the highest IgE binding intensity with ~50% and ~60% of the total optical density of W-HT and W-HT-Lac, respectively. BLG heated under dry conditions showed binding to the BLG monomer and to a smear >53 kDa, with higher binding when heated in the presence of lactose (Figure 3.8, pool 2, lane 5) compared to heating in the absence of lactose (Figure 3.8, pool 2, lane 4). Additionally, in the presence of lactose, the aggregates also showed high IgE binding. Pool 3 showed equal binding to unheated and all heated BLG. This sample showed the same IgE binding pattern as pool 2, except for the smear of D-HT-Lac (Figure 3.8, pool 3, lane 5) that showed higher intensity than in pool 2. Serum 6 showed a unique binding pattern with strong binding to unheated BLG, and BLG heated in the absence of lactose for dry and wet heating conditions. A weak binding was also observed for BLG heated under dry conditions in the presence of lactose but not to the equivalent sample heated under wet conditions. Interestingly, the binding intensity was higher to the aggregates than to the monomer, for both BLG-NT (Figure 3.8, serum 6, lane 1) and D-HT (serum 6, lane 3) with ~30% and ~55%, respectively. For BLG heated under wet conditions in the absence of lactose, the binding was also observed to the monomer and dimer but less to the aggregates.

To summarise, the results of IgE dot blot indicate high individual variations in the IgE binding profiles to heated and glycated BLG and allowed the grouping into four binding affinity profiles. Wet-heated BLG showed reduced IgE binding when compared to non-treated BLG for 5 out of 10 sera giving the positive signal, independent of the presence of lactose. Dry heating of BLG without lactose did not affect IgE binding compared to non-treated BLG. On the other hand, BLG dry-heated in the presence of lactose increased IgE binding compared to both untreated BLG and dry-heated BLG without lactose, revealing an effect of glycation on the sIgE binding capacity. Results showed which BLG fractions were responsible for IgE binding observed in the dot blot analysis, indicating a shift of IgE binding towards higher MW fractions (BLG dimers and aggregates) formed upon heating.

3.4   Discussion

Food processing promotes the changes of the immunogenic and antigenic properties of food allergens [3], among others via MR. In this study, we showed that during processing of BLG both the temperature and a_w (wet vs. dry heating) are important factors affecting its immunogenicity. Wet heating of BLG, independent of the presence of lactose in the sample, promotes the formation of aggregates recognised by sRAGE and IgE from CM allergic patients. In contrast to wet heating the aggregates formed upon dry heating showed differences in an immunogenic profile of the samples heated in the presence or absence of lactose, therefore revealing contribution of glycation to the formation of both sRAGE and IgE binding epitopes.

Both glycation and aggregation of BLG depends on the applied heating conditions. Glycation of milk proteins is promoted at low a_w of 0.31-0.98 [19]. At the same time, heating at high humidity conditions facilitates the aggregation of BLG via disulphide linkages [20,21] but leads to a relative lower levels of glycation structures compared to dry heating [22,23]. This effect was also observed in this study where the formation of CML and detection of glycation structures on BLG was significantly higher in dry-heated BLG than in wet-heated BLG. This was confirmed by CML western blot (Figure 3.3) showing highest levels of CML in D-HT-Lac detected in monomers, dimers, and aggregates of BLG while in the wet-heated samples the CML appeared only in the aggregates. Next to that, more glycation structures formed during heating of BLG under dry conditions in the presence of lactose were detected by the glycation staining (Figure 3.2). On the other hand, the formation of high-MW aggregates was independent of the presence of lactose in wet-heated samples compared to dry-heated samples. However, aggregation was also observed in the sample heated under dry conditions with lactose. It has already been described that glycation-induced modifications of lysine and arginine can increase the flexibility of the protein structure and leads to the formation of covalent crosslinking between different amino acid residues. Both facilitates protein denaturation and aggregation [16,24] and influences the morphology of the protein aggregates [25,26]. Additionally, glycation strongly affects the size of these aggregates when heated under dry conditions but may also contributes to structural differences of the formed aggregates under both humidity conditions [1]. However, reactant mobility during the wet heating also affects the aggregation rate of proteins, where higher inter-molecular mobility results in higher protein aggregation [27]. The observed differences in aggregation of wet-heated BLG vs. dry-heated BLG are in line with the findings of Deng *et al*. [1] who showed increased formation of polymers and oligomers in wet-heated BLG compared to dry-heated BLG. The authors also showed that the dry heating of BLG at 130 °C increased its oligomerisation and polymerisation and was mainly affected by the presence of lactose. On the other hand, the wet heating led to more exposure of β-sheet structures in the soluble aggregates than dry heating. These findings stay in line with our results showing a higher content of surface-exposed β-sheets structures in the wet-heated BLG independent of the presence of lactose in the sample. The formation of aggregates in both wet and dry heating conditions was shown to have a great impact of the formation of sRAGE

binding ligands. Three different patterns of sRAGE binding affinity (Figure 3.6) were observed, depending on the heating temperature and the a_w of the system. First, when heated in the wet system high sRAGE binding was observed to the formed aggregates, regardless of the presence of lactose. Secondly, sRAGE binding occurred independent of the presence of CML but increased with higher exposure of β-sheet structures after wet heating (Figure 3.3 and Figure 3.4). Finally, in the dry-heated system, sRAGE binding was observed only to BLG heated in the presence of lactose (high-MW fractions), revealing an impact of glycation under dry heating conditions (Figure 3.6). Being a promiscuous receptor, sRAGE binds not only to AGEs but also to amyloid-β, S100 protein, and HGMB1 [11]. Those ligands share a common property to act as oligomers, highlighting the potential of sRAGE to recognize high-MW structures [12]. Next to the aggregation itself, the glycation-induced aggregation was already pointed out to be responsible for the formation of sRAGE ligands under dry heating conditions; however this was not directly proven [13]. With the western blot analysis of sRAGE binding we confirmed these findings, proving that under the dry heating conditions of BLG glycation without aggregation does not lead to increased sRAGE binding. Consequently, the changes in sRAGE binding were accompanied by the changes in IgE binding affinity by (treatment-dependent) formation of neo-epitopes or destruction of existing epitopes. Several studies indicated that both glycation and aggregation, contribute to changes in cellular recognition and allergic sensitisation [1,4,13,28,29]. The results of dot blot with the sera of CM allergic patients showed high individual variations in the IgE binding to heated and glycated BLG. Four different profiles of IgE binding affinity were observed which showed, depending on the used conditions, either increased or diminished IgE binding to heat-treated BLG. The dot blot analysis revealed that wet heating reduces IgE binding independent of the presence of lactose which was observed in 5 out of 10 sera giving a positive signal with non-treated BLG (Figure 3.7). Therefore, glycation appears to play a minor role in changing IgE binding when BLG is heated under wet conditions. For dry-heated samples, IgE binding was only decreased when lactose was present during heating (Figure 3.7), indicating the masking of epitopes by the attached sugar moiety. These differences indicate that humidity applied during the heat processing as well as glycation are important factors influencing IgE binding affinity. The western blot results (Figure 3.8) obtained for four pools of sera to heated BLG allowed to detect the fractions and bands responsible for IgE reactivity observed in dot blot. Three different fractions present in non-treated BLG were recognised by IgE: BLG monomer, BLG dimer, and oligomers in the top of the gel. Binding of IgE to the oligomers present in non-treated BLG was always accompanied with the positive binding to the oligomers present in dry-heated sample without lactose suggesting the existence of similar epitopes in these two samples (Figure 3.8, pool 1 and serum 6). Interestingly, the aggregates formed during the wet heating showed IgE reactivity only in case of half of the tested sera indicating a lactose-independent decrease of IgE binding affinity. It has been shown in the past that the free cysteine (C121) is strongly involved in the aggregation of BLG when heated in a wet system and this results in the formation of covalently linked aggregates [21]. Due to the higher translational motion in the wet system

compared to the dry system the formation of these covalent linkages is favoured in the wet system leading to higher extend of aggregation. This might also result in the embedding or exposure of different IgE epitopes when comparing the two humidity conditions. The observation of IgE binding for half of the patients stay in line with a previous finding that reported decreased IgE binding to BLG when heated above 90 °C under wet conditions [9,30,31]. Additionally, Kleber and Hinrichs [31] postulated that protein aggregation may decreases IgE binding affinity. However, our study points out that there are different MW fractions formed during wet and dry heating of BLG that can be recognised by IgE of some patients. This was lactose-independent after wet heating of BLG and was mostly found to higher MW fractions > BLG dimers. The same patients also show higher IgE binding to dry-heated BLG in the presence of lactose compared to the lactose-free control. The most immuno-reactive fraction was a high-MW smear of dry-heated BLG in the presence of lactose which also showed the presence of glycation structures and CML (Figure 3.8). This was also true for the BLG monomer and dimer in this sample but IgE binding was not observed to these fractions in the same pool of sera. This indicates that MR may has a double effect in sensitisation. First, it can lead to masking of epitopes in the BLG monomer and dimer as described previously also for other food allergens [8,32,33]. Secondly, the formation of glycation-induced aggregates can enhance IgE binding via neo-epitope formation. The study of Vissers *et al.* [34] showed reduced allergenicity of MR-modified peanut allergen Ara h 1 in IgE binding test while enhanced β-hexominidase release from basophils upon incubation with the same MR-modified allergen. The authors suggested that MR-induced aggregates of Ara h 1 could be responsible for the observed increased capacity of antigen to cross-link the IgE and initiate the mediator release from RBL-2H3 cells. Similar observations were also made for other protein families such as peanut proteins and scallop tropomyosin [8,35–37]. Thus, the influence of MR on IgE binding seems to depend on physicochemical properties of proteins (hydrophobicity, size, amino acid composition, charge) as well as on the conditions of the MR (type of sugar, time, a_w, pH, temperature, presence of salts) [36,38].

Different IgE binding profiles observed in this study indicate the high individual differences between CM allergic patients and demonstrates the importance of involving better characterised CM allergic patients' sera in the IgE binding study. That would allow a prediction to be made towards the influence of aggregation and glycation of BLG on its allergenicity. This would help to optimize processing conditions towards less immunoreactive products which is especially relevant in the production of infant formula. Furthermore, it should be considered that both aggregation and glycation affect protein digestibility. Antigens with resistance to gastrointestinal digestion are commonly understood to be the most potent allergens in terms of IgE binding [39]. It is known that glycation-induced aggregates are more resistant to *in vitro* gastrointestinal digestion [26]. Therefore, aggregates might have higher physiological relevance than aggregates formed in the absence of sugar even though they were as potent in stimulating sRAGE binding and IgE binding as aggregates formed in the absence of sugar.

3.5   Conclusions

sRAGE binding is highest when BLG is heated under wet conditions and is mostly determined by aggregation rather than formation of MRPs. Under dry heating conditions, sRAGE binding is mostly affected by the presence of lactose but a strict distinction between the effect of the formation of MRPs and glycation-induced aggregation could not be made. Also IgE binding seems to be affected by aggregation and glycation; however high inter-individual differences do not allow clear differentiation between the effects of the used heating conditions on allergic sensitisation. Therefore, more individuals need to be screened to get more insight in the role of aggregation and glycation on sRAGE binding and IgE binding affinity of thermally processed BLG. However, this data suggests that different heating conditions of BLG result in the formation of different sRAGE ligands and IgE epitopes and that the a_w during the processing of milk compounds contributes to the pattern of sensitisation.

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Chapter 4

Binding of CML-modified as well as heat-glycated β-lactoglobulin to receptors for AGEs is determined by charge and hydrophobicity

This chapter has been published as:

Abstract:

Intake of dietary advanced glycation end products (AGEs) is associated with inflammation-related health problems. N^ε-carboxymethyllysine (CML) is one of the best characterised AGEs in processed food. AGEs have been described as ligands for receptors present on antigen presenting cells. However, changes in protein secondary and tertiary structure also induce binding to AGE receptors. We aimed to discriminate the role of different protein modifications in binding to AGE receptors. Therefore, β-lactoglobulin (BLG) was chemically modified with glyoxylic acid to produce CML and compared to BLG glycated with lactose. Secondary structure was monitored with circular dichroism, while hydrophobicity and formation of β-sheet structures was measured with ANS-assay and ThT-assay, respectively. Aggregation was monitored using native-PAGE. Binding to sRAGE, CD36, and galectin-3 was measured using inhibition ELISA. Even though no changes in secondary structure were observed in all tested samples, binding to AGE receptors increased with CML concentration of CML-modified BLG. The negative charge of CML was a crucial determinant for the binding of protein bound CML, while binding of glycated BLG was determined by increasing hydrophobicity. This shows that sRAGE, galectin-3, and CD36 bind to protein bound CML and points out the role of negatively charged AGEs in binding to AGE receptors.

4.1   Introduction

β-lactoglobulin (BLG) is the main component of the whey protein fraction that contributes ~10% to the total protein in cow's milk and has been described as one of the major allergens in cow's milk [1]. Moreover, it is one of the few proteins in cow's milk that is not present in human breastmilk. Its amino acid sequence consist of 162 amino acids, including 16 lysine residues, 3 arginine residues, and 1 free cysteine [2], which makes it prone to glycation via the Maillard reaction (MR) as well as heat induced unfolding. This may result in the formation of advanced glycation end products (AGEs) and irreversible structural changes of BLG upon heating. Formation of AGEs as a result of the MR can occur endogenously or exogenously. Exogenous AGEs have a source in thermally processed foods, where protein is heated in the presence of reducing sugars. This is considered to contribute to 10% of the AGE pool in the body [3]. There is a growing body of evidence that AGEs contribute to a number of inflammation-related health issues such as chronic inflammation, atherosclerosis, and allergy [4-6]. In fact, *in vivo* animal studies with glycated food proteins confirmed their potential to activate inflammatory pathways and showed the contribution of high dietary intake of AGEs on inflammation-mediated health implications [7,8]. Furthermore, it was reported that glycation of food proteins enhances their uptake by antigen presenting cells (APCs) and affects T-cell proliferation [9-11].

Antigen uptake and initiation of immunological responses to AGEs is mediated by AGE-receptor/ligand interactions on the surface of APCs [12]. A number of AGE-binding receptors on the surface of innate immune cells have been described in previous studies, such as the receptor for AGEs (RAGE), CD36, galectin-3 (Gal-3) as part of the AGE-R complex, scavenger class A type I and type II [13-17]. Next to AGEs, these pattern recognition receptors can also bind to other structures and some share other ligands such amyloid beta for RAGE and CD36, as well as bacterial lipopolysaccharides for RAGE and Gal-3. [18-20]. Lysine modification with N^{ε}-carboxymethyllysine (CML) is one of the most abundant as well as one of the best characterised AGEs in processed milk [21,22]. CML has been the target of previous studies, determining the effect of AGEs in receptor mediated immune responses [23-25]. Only few studies looked at the role of specific AGEs while limiting 3D-folding modifications during the glycation process [23,26]. However, disentangling these effects is crucial, as protein aggregation, heating and glycation induced increase of hydrophobicity and β-sheet structures have also been reported as determinants for uptake of glycated food proteins by macrophages and the binding to AGE receptors [27-29]. In this study, chemical modification of the milk allergen BLG was performed to obtain a specific modification of lysine to CML, which was then compared to BLG glycated with lactose below the denaturation temperature of BLG, to avoid heat-induced structural changes of the protein. Binding to the soluble form of RAGE (sRAGE), which shows identical binding to the extracellular ligands as RAGE [30], as well as binding to CD36 and Gal-3 was assessed with inhibition ELISA tests to assess the respective role of various protein modifications in receptor binding.

4.2   Materials and Methods

4.2.1   *Chemicals*

Lyophilised BLG from bovine milk (>90% purity) was purchased from Merck (Darmstadt, Germany). Novex™ Wedge Well™ 14% tris-glycine gel, Novex™ tris-glycine native sample buffer (2x), and Novex™ tris-glycine native running buffer (10x) were purchased from Thermo Fischer (Massachusetts, USA). Coomassie brilliant blue R-250 was obtained from Biorad (Hercules, California, USA). CML and CML-d2 were purchased from Polypeptide laboratories (Strasbourg, France). MS grade acetonitrile was purchased from Actu-All chemicals (Oss, Netherlands). Ultra-pure water was prepared by an ELGA LabWater Purelab Plus water system (Celle, Germany). Soluble Advanced Glycation End Product-Specific Receptor Human E. coli (RD172116100) was purchased from Biovendor (Brno, Czech Republic). Anti-RAGE antibody (monoclonal mouse IgG2B clone, MAB11451), recombinant human CD36/SR-BIII Fc chimera, recombinant human galectin-3 protein, and human galectin-3 antibody (Monoclonal mouse IgG2B clone) were purchased from R&D systems (Minneapolis, Minnesota, USA). HRP conjugated anti-mouse polyclonal goat (P0447) and polyclonal rabbit anti-mouse antibody/biotinylated were obtained from Dako (Glostrup, Denmark). Goat Anti-Human IgG/HRP was purchased from SouthernBiotech (Birmingham, Minnesota, USA). TMB substrate (3,3′,5,5′-tetramethylbenzidine) for high sensitivity ELISA and streptavidin-PolyHRP80 were purchased from sdt-reagents (Baesweiler, Germany). Ovalbumin (OVA) was purchased from InvivoGen (San Diego, California, USA). Bovine Serum Albumin Fraction V (BSA) was obtained from Roche (Basel, Switzerland). Amyloid-β (1-42) ultra-pure HFIP was purchased from Westburg (Leusden, the Netherlands). All other chemicals were purchased from Merck (Darmstadt, Germany).

4.2.2   *CML introduction in BLG*

CML was introduced in BLG using the method by Glorieux *et al.* [25] with slight modifications. Briefly, BLG was dissolved in 10 mM phosphate saline buffer (PBS) at pH 7.4. Glyoxylic acid monohydrate was added in three different molar ratios of glyoxylic acid to lysine residues. These ratios were 1:1, 3:1, and 5:1, respectively. Subsequently, sodium cyanoborohydride was added in a ratio 8.8 mmol/g BLG and the solutions were incubated for 20 h at 40 °C in a heating block (Labtherm Graphit, Liebisch, Germany). Incubation of BLG at 40 °C for 20 h was performed as control (C1). After incubation, samples were dialysed at 4 °C using a MWCO of 6-8000 Spectra/Por membrane (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to remove residual glyoxylic acid. All samples were prepared in duplicate. All solutions were freeze dried and dissolved in water.

4.2.3 Glycation of BLG

BLG was glycated with lactose at 60 °C to minimise thermally induced protein aggregation. Incubation was conducted over saturated potassium fluoride solution to keep the water activity (a_w) at 0.23. Heating was conducted for 12 h, 24 h, and 48 h in the presence of lactose. BLG heated under the same conditions in the absence of lactose was taken as heating control. After heating, BLG was immediately cooled down and dissolved in water before storage at -20 °C and stored until further analysis.

4.2.4 Quantification of CML using LC-MS/MS

CML was quantified after borohydride reduction and acidic hydrolysis and solid phase extraction were conducted considering previous methods [31,32]. Quantification was conducted using external calibration with d2-CML as internal standard. Analysis was performed using a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) coupled with a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The UPLC unit consisted of a SIL-30AC autosampler, a LC-20ADXR solvent delivery module, DGU-20ASR degassing unit, a CTO-20AC column oven and a FCV-20AH2 valve unit. The samples (5 µL) were injected on a Kinetex HILIC 2.6 µm, 2.1 mm × 100 mm (Phenomenex, Torrance, California, USA). The flow rate was set at 0.4 mL/min and the column temperature at 30 °C. The mobile phases consisted of 0.1% formic acid (solvent A), acetonitrile with 0.1% formic acid (solvent B) and 50 mmol/L ammonium formate (solvent C) with the following elution profile (time in [min]/[%B]/[%C]): (0/80/10), (0.8/80/10), (3.5/40/10), (6.5/40/10), (8.0/80/10) and (10.0/80/10). MS data were collected for 10 mins. Positive ionisation mode was used for all MS analyses. The voltage of the turbo ion-spray ionization was 4.0 kV. The temperature of electrospray ionization probe, desolvation line, and heat block were set at 300 °C, 250 °C, and 400 °C, respectively. The pressure of the collision-induced dissociation gas was 4 kPa whereas the flow rates of the drying gas, nebulizer gas, and heating gas were set at 10 mL/min, 3 mL/min, and 10 mL/min, respectively. The electrode voltage of Q1 pre bias (collision cell energy (CE) entrance potential), collision cell Q2 (CE), Q3 pre bias (CE exit potential), parent and fragment ion m/z of the multiple reaction monitoring transitions were optimized using support software (Shimadzu Corporation, Kyoto, Japan). Precursor ions were 205.200 m/z and 207.200 m/z for CML and CML-d2, respectively. Product ions were 84.050 m/z, 130.050 m/z, 56.150 m/z and 84.050 m/z, 130.200 m/z, 56.100 m/z for CML and CML-d2, respectively. The second and third product ion yield was selected as structural confirmation based on the optimized selected reaction monitoring transition, whereas the first product ion was used for quantification.

4.2.5 Quantification of free available amino groups

Free amino groups were measured using o-phthaldialdehyde (OPA)-assay to monitor overall formation of MPRs other than CML. OPA-assay was conducted following a method published

by Mulet-Cabero *et al.* [33] with some modifications. Samples were diluted with 10 mM phosphate buffer saline (PBS) and 10 µL were pipetted in a transparent polystyrene 96-well plate (Greiner, Kremsmünster, Germany). To each well, 190 µL OPA reagent was added and the plate was incubated in the dark for 15 min. L-Leucine was used for the calibration curve in a concentration range between 0.4 to 4 mM. Absorbance was measured at 340 nm using Infinite® 200 PRO NanoQuant with i-control software (Tecan, Männedorf, Switzerland).

4.2.6 *Secondary structure*

Secondary structure was analysed with far UV-circular dichroism (CD). Measurements were conducted using a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan). Samples were diluted to 0.15 mg/mL protein concentration. The secondary structure was recorded with far UV between a 180 to 260 nm using a 15 times quartz cuvette with a path length of 0.1 cm at 20 °C.

4.2.7 *Surface hydrophobicity*

Surface hydrophobicity was determined using 8-anilino-1-naphthalenesulfonic acid (ANS)-assay according to a method Alizadeh-Pasdar *et al.* [34] with some modifications. Briefly, samples were diluted to 0.5 mg/mL and mixed with 0.8 mM ANS solution in a ratio 1/20 (v/v). After incubation for 10 min in the dark, fluorescence was measured with an excitation wavelength of 390 nm and emission wavelength of 470 nm.

4.2.8 *Thioflavin T (ThT)-assay*

The ThT-assay was performed to determine a possible increase in amyloid-β structures. Samples were diluted to a protein concentration of 2 mg/mL with 10 mM PBS (pH 7.4). Subsequently 20 µL sample were mixed with 20 µL aqueous ThT-solution (1.25 mg/mL ThT) and mixed with 120 µL PBS. Samples were incubated for 10 min at room temperature in the dark. Fluorescence intensity was measured with an excitation wavelength of 450 nm and emission wavelength of 480 nm. All samples were measured in duplicate and corrected for the blank (PBS with ThT-solution). To correct for autofluorescence samples were measured at the same dilution as in the assay and signal was subtracted when applicable.

4.2.9 *Native gel electrophoresis*

Native gel electrophoresis was performed to monitor possible formation of aggregates in the samples. Samples were diluted to equal protein concentrations in 10 mM PBS (pH 7.4) and mixed with native samples buffer (1:1 (v/v)). Those solutions were loaded onto the gel with a final protein content of 10 µg in each pocket. The gels were run at 120 V for 90 min in 1x native running buffer and subsequently stained with Coomassie brilliant blue R-250. Images were taken using a Universal Hood III (Biorad, Hercules, USA) and Image Lab 4.1 software (Biorad, Hercules, USA).

4.2.10 *Inhibition ELISA assay for receptor binding*

Binding to the receptors for AGEs was determined with inhibition ELISA as described by Zenker *et al.* [29]. Briefly, samples were diluted with 10 mM PBS to 100 µg/mL protein concentration. Free CML was measured at a concentration of 2 mg/mL. Dilutions were incubated for 45 min at 37 °C on a Nunc™ 96-Well Polypropylene MicroWell™ (Thermo Fisher Scientific, Massachusetts, USA) with the receptors at concentrations of 0.5 µg/mL for sRAGE, 0125 µg/mL for CD36, and 0.4 µg/mL for Gal-3, respectively. Subsequently, the solutions were added to a high binding transparent Nunc-Immuno™ MicroWell™ 96-well solid plate (Thermo Fisher Scientific, Massachusetts, USA), blocked with soy protein glycated with glucose for 90 min at 100 °C (G90) and incubated for 1 h at 37 °C. After washing, the plates were incubated for 30 min at room temperature with the specific anti-receptor antibody: anti-RAGE, anti-Gal-3 or anti-his antibody for CD36. Subsequently incubation for 30 min at room temperature was performed with the respective detection antibody: polyclonal goat anti-mouse Antibody/HRP, polyclonal rabbit anti-mouse antibody/ biotinylated, and goat anti-human IgG/HRP. For Gal-3, an additional incubation step was performed with streptavidin for 15 min at room temperature before detection with 3,3′,5,5′-tetramethylbenzidine solution. Absorbance was measured at wavelength 450 nm with wavelength 620 mm as reference wavelength. To evaluate the role of charge in binding to the receptors for AGEs different concentrations of sodium chloride (NaCl) were used. The dilution buffer for the samples contained 0.13 M NaCl. This was supplemented to reach 0.2 M and 0.3 M NaCl in the final solution of the receptor/sample mixture, respectively. All samples were measured in technical triplicates.

4.2.11 *Statistical analysis*

Statistical analysis was conducted using IBM SPSS version 23. For multiple sample comparison, one-way ANOVA and Tukey post hoc comparison test was used. Results were considered statistical different at $p < 0.05$.

4.3 Results

4.3.1 *Chemical modification and glycation*

To analyse binding of CML on BLG to receptors, the protein was chemically modified with glyoxal to CML (BLG-CML). To distinguish the effect of lysine blockage via the MR with lactose, without heat-induced structural changes, BLG was glycated with lactose (BLG-Lac) at 60 °C, at a_w 0.23 for 12, 24, and 48 h. Moreover, subsequent chemical CML modification of glycated BLG was used to evaluate whether this changed receptor binding epitopes. The level of CML modification was determined using LC-MS/MS (Table 4.1).

Table 4.1: Nᵋ-carboxymethyllysine (CML) content of differentially treated β-lactoglobulin (BLG). BLG was non-treated (NT), chemically modified with glyoxal to obtain CML at different degree of modification (1, 3, and 5), glycated with lactose (Lac) by heating at 60 °C for 12 h, 24 h, and 48 h; heated at 60 °C for 12 h, 24 h, and 48 h in the absence of any sugars (H), and glycated by heating with lactose followed by CML induction with glyoxal. CML-modified lysine was calculated based on theoretical levels of lysine available in BLG. Results are given as mean ± standard deviation (n=2). Statistical differences were tested using one-way ANOVA with Tukey post-hoc test. Significant differences are considered at $p < 0.05$, where two variables have different letters if they are significantly different.

Sample	CML content [mg/g protein]	CML-modified lysine [%]
BLG-NT	0.4 ± 0.3 a	0.39 ± 0.3 a
BLG-CML-1	28.0 ± 0.1 b	17 ± 0.0 b
BLG-CML-3	56.1 ± 0.1 cd	34 ± 0.1 de
BLG-CML-5	62.6 ± 1.3 d	38 ± 0.8 d
BLG-Lac-12	2.0 ± 0.2 a	1.2 ± 0.1 a
BLG-Lac-24	3.7 ± 0.4 a	2.2 ± 0.2 a
BLG-Lac-48	4.4 ± 0.3 a	2.7 ± 0.2 a
BLG-H-12	1.6 ± 1.9 a	1.0 ± 1.2 a
BLG-H-24	0.3 ± 0.0 a	0.2 ± 0.0 a
BLG-H-48	0.4 ± 0.2 a	0.2 ± 0.1 a
BLG-Lac-12-CML	46.4 ± 2.9 c	28 ± 1.8 c
BLG-Lac-24-CML	50.3 ± 7.8 c	31 ± 4.7 cd
BLG-Lac-48-CML	34.5 ± 1.9 b	16 ± 0.9 b

The level of CML-modified lysine and arginine residues increased with a higher ratio of glyoxylic acid/BLG, resulting in 17% modified residues in BLG-CML-1, 34 % in BLG-CML-3, and 38% in BLG-CML-5. BLG heated in the absence of lactose (BLG-H) and BLG heated in the presence of lactose (BLG-Lac) contained much lower levels of CML than BLG-CML samples, regardless of the heating time. Modification of BLG with CML after glycation (BLG-Lac-CML) showed higher levels of CML modification with shorter heating time, resulting in 28%, 31%, and 16% CML-modified residues in BLG-Lac-12-CML, BLG-Lac-24-CML, and BLG-Lac-48-CML, respectively. To determine the extent of heat- and glycation-induced modification in BLG-H and BLG-Lac, free amino groups were quantified using the OPA-assay (Figure 4.1).
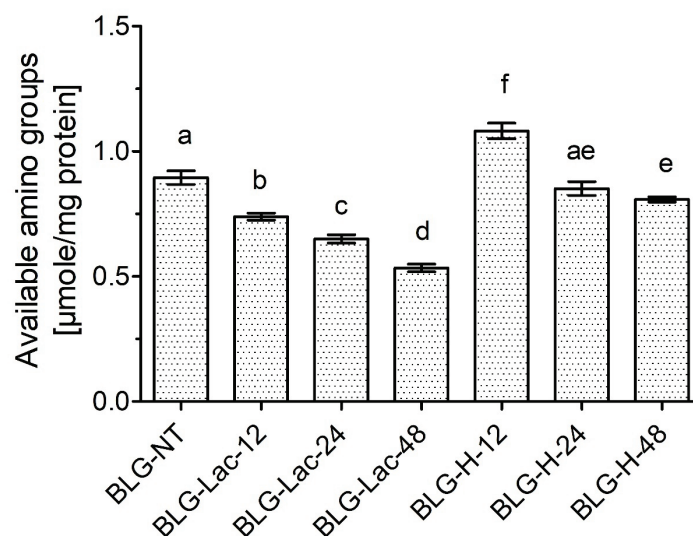
Figure 4.1: Available amino groups of β-lactoglobulin (BLG), non-treated (NT), glycated with lactose (Lac) by heating at 60 °C for 12 h, 24 h, and 48 h, as well as heated at 60 °C for 12 h, 24 h, and 48 h in the absence of any sugars (H). Error bars represent standard deviation of technical replicates (n=3). Statistical differences were tested using one-way ANOVA with Tukey post-hoc test. Significant differences were considered at $p < 0.05$, where two variables have different letters if they are significantly different.

When BLG was heated in the presence of lactose, the number of free amino groups decreased with longer heating time. At the same time, heating in the absence of lactose first increased the levels of free amino groups after 12 h of heating (BLG-H-12), but subsequently decreased with prolonged heating time to the level of BLG-NT.

4.3.2 Structural changes

Hydrophobicity relative to untreated BLG was measured using the ANS-assay (Figure 4.2).
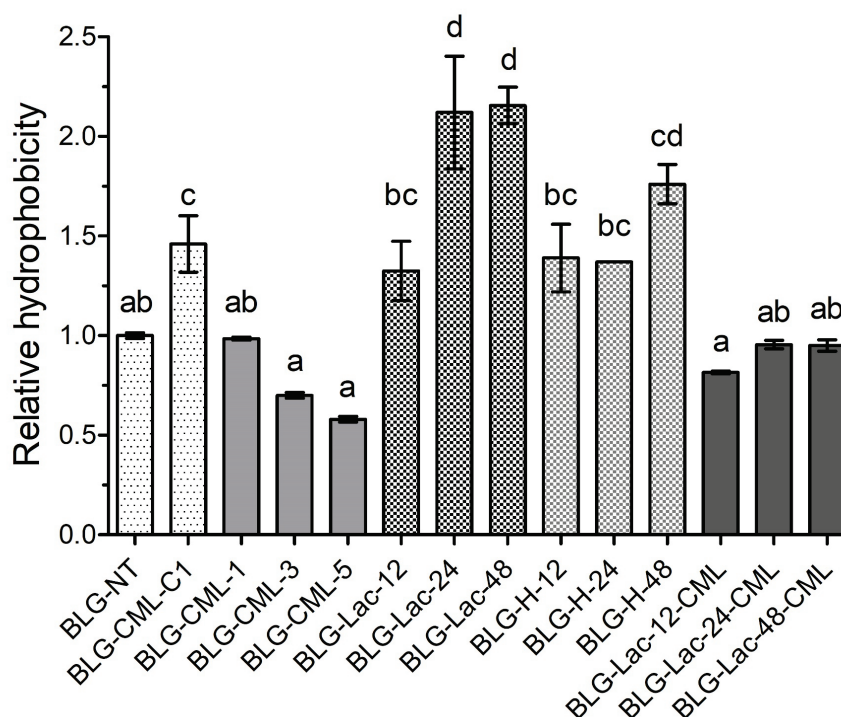
Figure 4.2: Hydrophobicity of β-lactoglobulin (BLG) samples relative to non-treated (NT) BLG, chemical modification control without addition of glyoxylic acid (C1), chemically modified to introduce N$^\varepsilon$-carboxymethyllysine (CML) at different degree of modification (1, 3, and 5), glycated with lactose (Lac) by heating at 60 °C for 12 h, 24 h, and 48 h; heated at 60 °C for 12 h, 24 h, and 48 h in the absence of any sugars (H), and glycated by heating with lactose followed by CML induction with glyoxal. Error bars represent standard deviation of technical replicates (n=2). Statistical differences were tested using one-way ANOVA with Tukey post-hoc test. Significant differences were considered at $p < 0.05$, where two variables have different letters if they are significantly different.

BLG-CML did not show significant differences in relative hydrophobicity compared to BLG-NT, independent of the level of modification. At the same time, BLG-Lac showed significantly higher levels of hydrophobicity compared to BLG-NT after 24 h and 48 h heating time. Heating in the absence of lactose did not change hydrophobicity of BLG until 24 h of heating, however it increased after 48 h heating. Modification of BLG-Lac samples with glyoxylic acid to form additional CML, resulted in decreased hydrophobicity of BLG-Lac-24-CML and BLG-Lac-48-CML compared to the respective BLG-Lac samples and reached comparable levels as for BLG-NT.

CD-spectra were monitored to determine changes in the secondary structure of BLG by heating, glycation, and chemical modification, respectively (supplementary material: Figure 4-S1). The CD-spectra of all samples showed no deviation from the spectra of BLG-NT.

ThT-assay was performed to monitor changes in the level of β-sheets upon treatment of BLG (Figure 4.3). BLG-CML-3 and BLG-CML-5 revealed significantly higher fluorescence intensity compared to BLG-NT, with the tendency of higher fluorescence intensity with increasing level of modification in BLG-CML samples. Also BLG-Lac samples showed a higher signal compared to BLG-NT. BLG-H-24 has significantly higher fluorescence intensity compared to BLG-NT but not compared to the other BLG-H samples. BLG-Lac-CML samples showed higher fluorescence intensity than BLG-NT, however fluorescence intensity did not differ between BLG-Lac-CML samples.
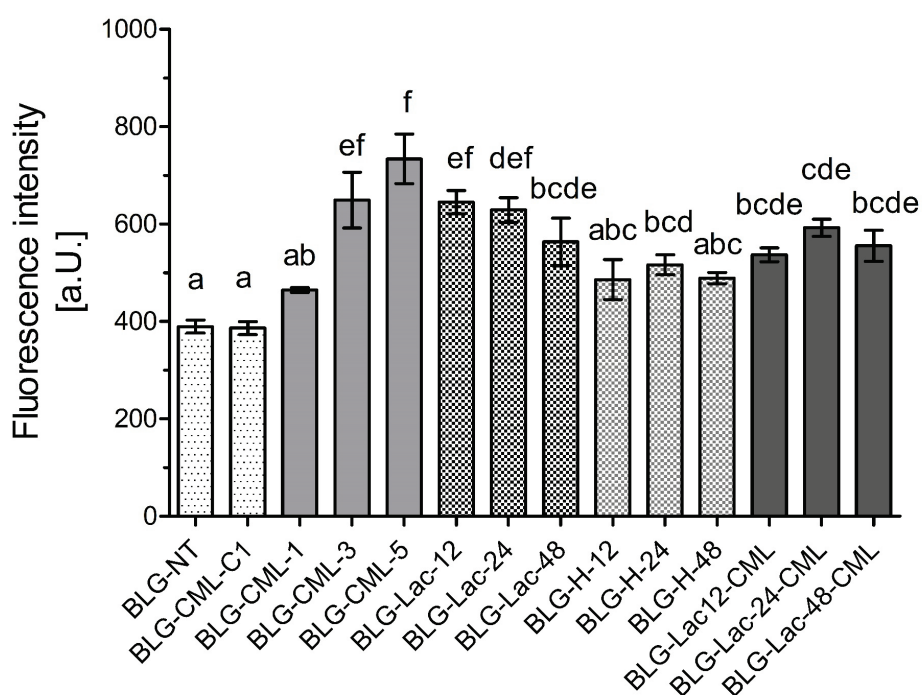
Figure 4.3: Thioflavin-T assay of β-lactoglobulin (BLG), non-treated (NT), control for chemical modification (C1), chemically modified BLG to introduce N^ε-carboxymethyllysine (CML) at different levels of CML, glycated with lactose (Lac) by heating at 60 °C for 12 h, 24 h, and 48 h; heated at 60 °C for 12 h, 24 h, and 48 h in the absence of lactose (H), and glycated by heating with lactose followed by CML induction with glyoxal. Fluorescence intensity was corrected for the autofluorescence of the samples and fluorescence of the blank. Significant differences were tested using one-way ANOVA with Tukey post-hoc test, considered as significant at $p < 0.05$ between technical replicates (n=2), where two variables have different letters if they are significantly different.

Gel-electrophoretic separation was conducted under native conditions (native PAGE) to monitor the occurrence of protein aggregation after the treatments (Figure 4.4). Native PAGE, separates the proteins by a combination by charge and size without changing the structure of the protein. Analysis showed that no aggregation had occurred.
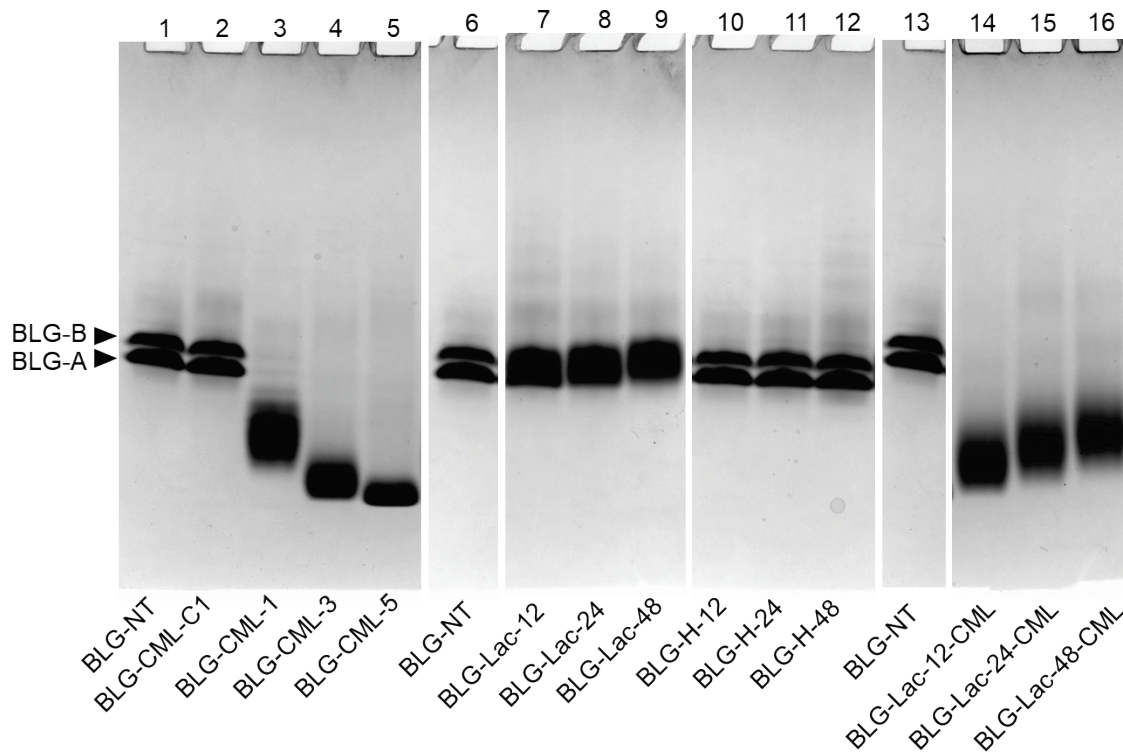
Figure 4.4: Native-PAGE of β-lactoglobulin (BLG), non-treated (NT), chemical modification control (C1), chemically modified to introduce Nᵉ-carboxymethyllysine (CML) at different degree of modification (1, 3, and 5), glycated with lactose (Lac) by heating at 60 °C for 12 h, 24 h, and 48 h; heated at 60 °C for 12 h, 24 h, and 48 h in the absence of any sugars (H), and glycated by heating with lactose followed by CML induction with glyoxal. BLG-NT of lane 1 and 13 are identical and were on the same gel as samples in lane 2-5 and lane 14-16, whereas BLG-NT in lane 6 was run on the same gel as samples in lane 7-12.

BLG-NT and BLG-C1 (lane 1 and lane 2) showed two distinct bands corresponding to the variant A and B that show slight differences of their isoelectric point and therefore can be separated via native gel electrophoresis [35]. BLG-CML samples (lane 3-5) showed one broad band and higher level of CML modification caused further migration through the gel accompanied with decreased broadening of the band. Whereas in SDS-PAGE under reducing conditions, only minimal differences in migration behaviour of BLG-CML samples were observed (data not shown). BLG-Lac (lane 7-9) samples showed one broad band at the same position as the two bands of BLG-NT. Only BLG-Lac-48 showed a slightly lesser migration. BLG-H (lane 10-12) samples showed no difference to BLG-NT, independent from the heating time. BLG-Lac-CML (lane 14-16) samples showed one broad band at a lower position compared to BLG-NT and BLG-Lac samples. As in BLG-CML samples, the BLG moved faster through the gel upon higher level of CML modification.

4.3.3   Receptor binding

Binding of CD36, Gal-3, and sRAGE was determined with inhibition ELISA, where higher inhibition indicates more binding to receptor ligands (Figure 4.5).
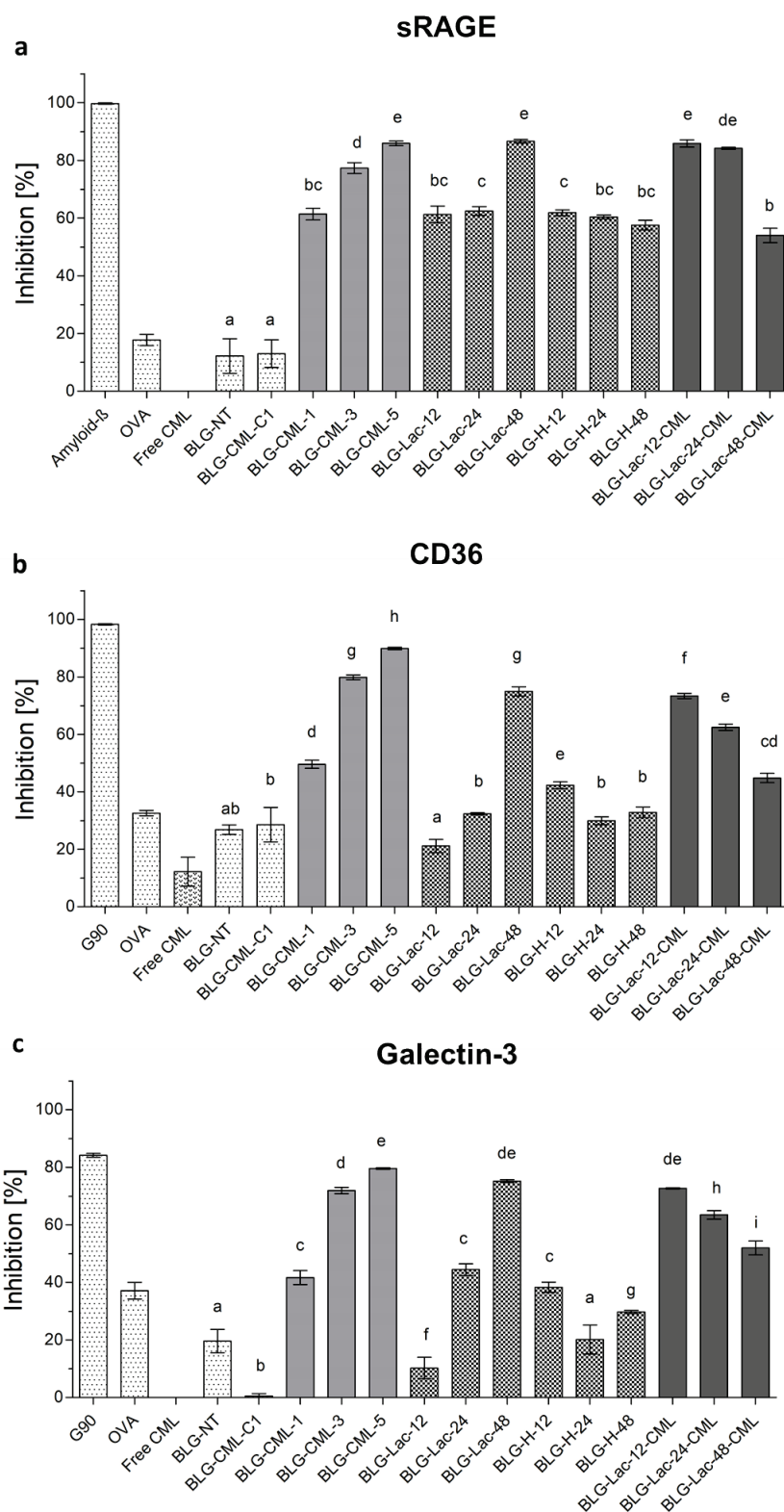
Figure 4.5: Inhibition ELISA for binding of (a) sRAGE, (b) CD36, and (c) galectin-3 to β-lactoglobulin (BLG), non-treated (NT), control for chemical modification (CML-C1), chemically modified BLG with N^ε-carboxymethyllysine (CML) at different levels of CML, glycated with lactose (Lac) by heating at 60 °C for 12 h, 24 h, and 48 h; heated at 60 °C for 12 h, 24 h, and 48 h in the absence of any sugars (H), and glycated by heating with lactose followed by CML induction with glyoxal, with ovalbumin (OVA) as negative control, and glycated soy protein (G90) as positive control for CD36 and galectin-3, as well as amyloid-β as positive control for sRAGE. Protein concentrations were set to 100 μg/mL, while free CML was used at 2 mg/mL. Significant differences were tested using one-way ANOVA with Tukey post-hoc test, considered as significant at p < 0.05 between technical replicates (n=3), where two variables have different letters if they are significantly different.

All receptors showed increased binding to BLG-CML samples compared to BLG-NT which increased with higher CML levels. At the same time binding to free CML was only observed for CD36, however this was lower than the binding to OVA. BLG-Lac samples showed higher binding upon prolonged heating time, however only starting after 24 h for both CD36 and Gal-3. In contrast, sRAGE binding increased already for BLG-Lac-12, remained on the same level in BLG-Lac-24 and then increased again for BLG-Lac-48. BLG-H samples revealed no heating time-dependent trend for binding to all receptors and, overall, displayed either similar or lower binding compared to the BLG-Lac sample heated for the same duration. For CD36, only BLG-H-12 showed higher binding than BLG-NT, while for Gal-3 both BLG-H-12 and BLG-H-48 showed higher levels of binding. For sRAGE, the BLG-H samples all showed a level of binding that was similar to the BLG-Lac samples, and significantly increased compared to BLG-NT. BLG-Lac-CML samples showed higher binding the more CML was present in the sample, which followed a stepwise increase for binding to CD36 and Gal-3, while sRAGE showed similar binding to BLG-Lac-12 and BLG-Lac-24 and only decreased for BLG-Lac-48.

Receptor binding with different concentrations of NaCl was performed to assess the role of charge in binding of BLG-CML-5 and BLG-Lac-48 to AGE receptors (Figure 4.6).
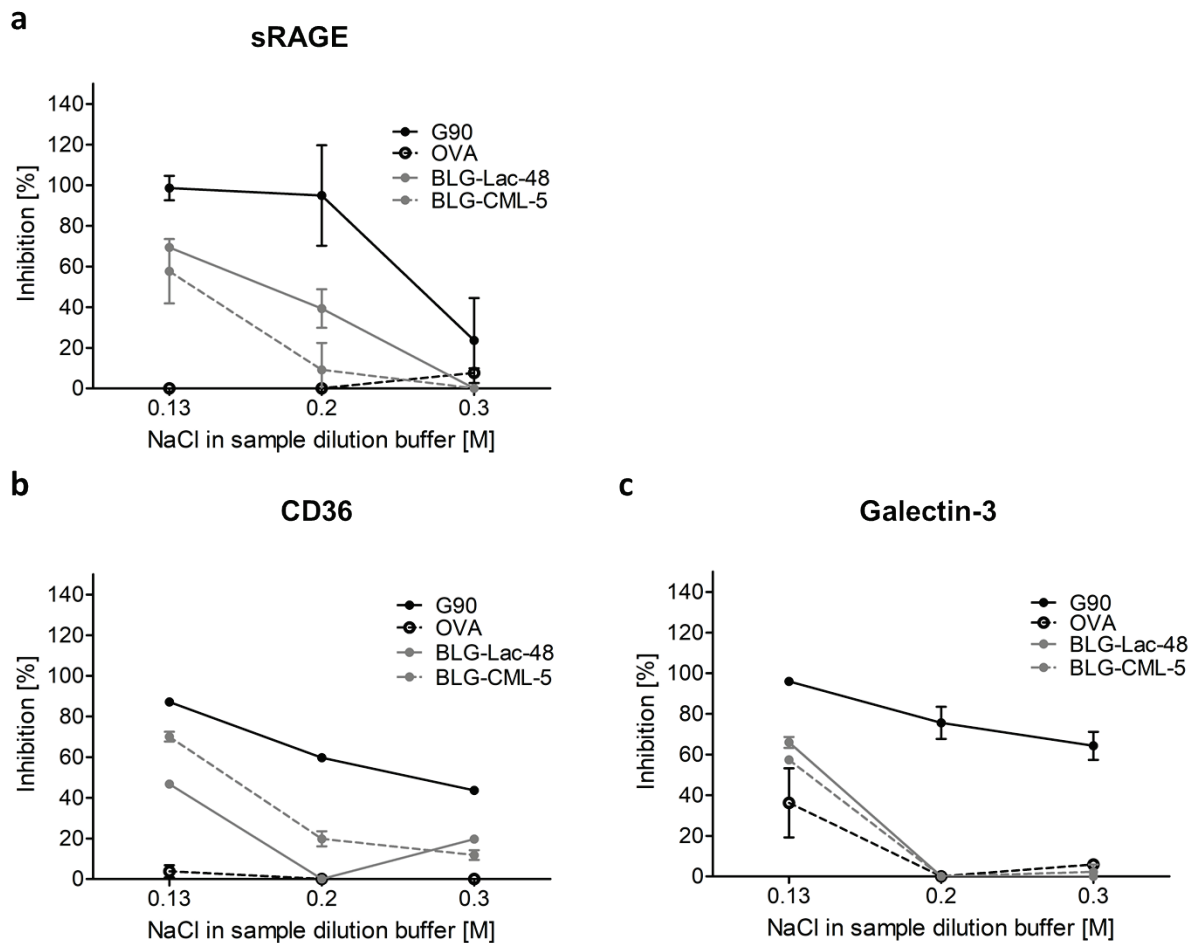
Figure 4.6: Inhibition ELISA for binding of (a) sRAGE, (b) CD36, and (c) galectin-3 to β-lactoglobulin (BLG), chemically modified BLG with N$^\varepsilon$-carboxymethyllysine (BLG-CML-5), BLG dry-heated in the presence of lactose (Lac) for 48 h using different concentrations of sodium chloride (NaCl) in the sample dilution buffer. Protein concentrations were set to 100 μg/mL. Error bars represent standard deviation of technical replicates (n=3).

With increasing salt concentration of the sample dilution buffer to block the negative charge of CML, the binding to all receptors of BLG-Lac-48 and BLG-CML-5 decreased. For CD36 and Gal-3 no binding was observed already at 0.2 M NaCl in the sample dilution buffer, while sRAGE showed a gradual decrease in binding from 0.13 M to 0.3 M NaCl. Also the positive control showed lower binding with increasing NaCl concentration, starting at 0.2 M NaCl for CD36 and Gal-3, while sRAGE showed a decrease only at 0.3 M NaCl.

4.4   Discussion

CML is not only the most commonly used marker of MR in food but also an important biomarker of protein modifications *in vivo* [36,37]. CML modifications *in vivo* were shown to contribute to increased levels of inflammation markers in serum [38,39]. However, disentangling the effect of the presence of AGEs and 3D-changes of the protein structure upon heating induced glycation was often not possible. Recent studies highlighted the importance of separating the effect of aggregation, heating-induced exposure of hydrophobic as well as β-sheet structures and the formation of AGEs in order to explore the role of AGEs in inflammation [27-29]. In this study BLG was chemically modified with glyoxylic acid to induce CML and structural changes were closely monitored. The binding to sRAGE, CD36, and Gal-3 was evaluated to assess the immunoreactive potential of CML-modified proteins.

Modification of BLG with glyoxylic acid resulted in the formation of CML-modified BLG without changing the 3D-structure of the protein. This was illustrated by the increasing CML levels at higher ratio of glyoxylic acid to BLG (Table 4.1), lack of additional structural modifications (supplementary material: Figure 4-S1) and aggregation (Figure 4.4). Levels of CML in liquid and powdered dairy products have been reported in a wide range between 2-210 µg/g protein. Where already small amounts are found in raw milk and increase with heating intensity and prolonged storage at higher temperatures (up to 50 °C for 4 months) [40]. In contrast, during the incubation of BLG in the presence of lactose only low amounts of CML were formed which was expected for the mild heat treatment and the short heating time (60 °C, for maximum 48 h). However, free amino groups decreased with prolonged incubation times (Figure 4.1), indicating that the MR occurred but did not proceed beyond the early stage, most likely resulting in the formation of other MR products (MRPs) than CML. The heating conditions used to induce protein glycation as well as the MR itself can promote protein aggregation [41]. This is a crucial determinant as it has been shown before that heating- and glycation-induced aggregation of BLG results in the formation of sRAGE binding ligands [28,29]. Native PAGE confirmed that in this well-defined system no aggregates were formed in any of the samples (Figure 4.4). However, faster movement through the native PAGE (Figure 4.4) also indicated that protein charge changed, especially in BLG-CML and BLG-Lac-CML. This was confirmed by the same mobility of all BLG-CML samples in SDS-PAGE gels where charge is not affecting mobility of the proteins (data not shown). The different charge seemed to be dependent on the CML content as CML gives an additional

negative charge to each modified lysine. The broadening of the bands, being most distinct in BLG-CML-1 and BLG-Lac samples, indicated the presence of a wide range of molecules modified differently, in extent and/or position.

Further structural analysis revealed that the chemical modification of BLG with CML did not result in CML level-dependent changes in hydrophobicity (Figure 4.2) and of the secondary structure (supplementary material: Figure 4-S1). This is also in line with previous findings on the model protein OVA which was chemically modified following the same procedure [23]. Also BLG-Lac and BLG-H samples showed no changes in secondary structure compared to BLG-NT, which is in line with the results of Enomoto *et al.* [42] who reported that neither glycation nor heating under dry heating conditions at 50 °C significantly affected the secondary structure of BLG. However, the ThT-assay showed an apparent increase of β-sheet structures with increasing level of modification in BLG-CML samples, which is in contrast with the CD measurements (supplementary material: Figure 4-S1). It is most commonly accepted that ThT binds to the cross β-structure of amyloid fibrils and thus is suitable to give an indication about their presence and relative quantities. However, it has also been shown to bind to hydrophobic structures [43]. This could explain the discrepancies between CD measurements (supplementary material: Figure 4-S1) and ThT-assay (Figure 4.3) for BLG-Lac and BLG-H samples as they showed increased hydrophobicity (Figure 4.2). Moreover, ThT is positively charged and the signal intensity of ThT-fibril/protein complexes can be affected by the charge state of the ThT ligand [44]. Native PAGE (Figure 4.4) indicated that the chemically modified samples were more negatively charged the higher the level of CML. This is in line with the data of ThT binding assuming that in this case ThT interacts with the negative charge. This could possibly explain the discrepancies between the results from CD (supplementary material: Figure 4-S1) and ThT measurements (Figure 4.3) for BLG-CML samples. This interpretation is supported by the general opinion that the chemical modification with glyoxylic acid is considered as a very specific method that solely results in CML modification of lysine residues without affecting the secondary structure [23]. BLG-Lac and BLG-H samples showed significant differences in surface hydrophobicity after 24 h incubation and 48 h incubation, respectively (Figure 4.2). This is in line with the findings of Morgan *et al.* [45] who showed that ANS binding increases upon dry heating of BLG (relative humidity of 65%, at 50 °C) and is even higher when BLG is heated in the presence of lactose. Structural changes of BLG-Lac samples were also confirmed by the native PAGE (Figure 4.4), that showed a broader band for BLG-Lac samples. However, this could also be the result of glycation-induced side-chain modifications on BLG-Lac samples, as indicated by the OPA-assay (Figure 4.1). To summarise, chemical modification of BLG did not result in detectable 3D-structural changes but did induce a negative charge to the lysine residues. At the same time, glycation of BLG increased the surface hydrophobicity (Figure 4.2). However, after chemical modification of BLG-Lac samples with CML, hydrophobicity decreased again, which is possibly related to the addition of a negative charge on lysine residues.

Dietary AGEs have previously been described as potent ligands for a number of cell surface receptors expressed on APCs including RAGE, CD36 and Gal-3 as the extracellular unit of the AGE-R complex [46]. However, the knowledge on the particular binding sides of these receptors to MR-modified proteins is very limited because of complexity of the structural rearrangements during the heat treatment and glycation. For instance, heating that is required to form AGEs via the MR, as well as the MR itself, promote protein aggregation which was already shown to induce an interaction with sRAGE [28,29]. Structural analysis of BLG-CML showed that the tertiary and secondary structure of BLG is maintained upon modification. Moreover, no aggregates were observed on the native PAGE excluding protein unfolding or aggregation as the reason of increased binding to the receptors observed for BLG-CML. Both CML-modified samples (BLG-CML and BLG-Lac-CML) showed CML content-dependent increased binding to all tested receptors (Figure 4.5), indicating the role of this specific AGE in promoting the binding to the selected receptors. This is in agreement with previous literature which described CML as ligand for sRAGE *in vivo* and *in vitro* [24,47]. However, glycation of the tested proteins was not selective for CML and structural changes were not monitored.

The investigated receptors are multiligand receptors that show binding to several structural elements. The role of amyloid structures, aggregation and hydrophobicity has previously been discussed in relation to binding to these receptors and cellular uptake in macrophages as well as a wide spectrum of lectin structures for Gal-3 [20,27,29,48,49]. Ohgami *et al.* [14] showed that CD36 in CHO-CD36 cells binds to AGE-OVA that carried a significant amount of CML, while for Gal-3 Vlassara *et al.* [17] reported that it shows high affinity binding to glycated BSA with unknown CML content. The results presented in this study are the first that directly show the role of CML in binding to CD36 and Gal-3. Based on the results of native PAGE and inhibition ELISA with various salt concentrations (Figure 4.6), it is hypothesized that the negative charge of CML is the crucial determinant to induce binding to sRAGE, CD36 and Gal-3. For sRAGE this is in agreement with the findings by Xue *et al.* [50], who showed that CML-modified peptides and CML-modified BSA bind to the positively charged cavity within the V domain of sRAGE. In contrast, a study conducted by Buetler *et al.* [26] showed that BLG which had 10-40% of its lysine chemically modified to introduce CML did not show binding of antibody-captured GST-RAGE by using the Biacore technique. However, to evaluate the binding, a buffer containing 5 mM calcium chloride was used in this study. Comparable to the effect of NaCl, where the positively charged sodium cation can interact with the negative charge of CML, this could decrease sRAGE binding (Figure 4.6). As reviewed by Collot-Teixeira *et al.* [49], CD36 binds to oxidised low density lipoprotein (oxLDL) which is suggested to occur via a positively charged moiety that binds to negatively charged ligands, such as oxLDL. According to these authors, the binding site within CD36 for oxLDL and AGEs are identical. Moreover, the role of charge in binding to murine CD36 ectodomain has also been postulated for diacylglycerol ligands [51], thus supporting the hypothesis that also for CD36 the negatively charged CML moiety of BLG-CML is essential for binding to the receptor. Galectins in general bind to carbohydrates, which is considered to occur as a result of

hydrophilic interactions via hydrogen bonds, and hydrophobic interactions, specifically the CH-π interaction [52]. This explains the binding affinity of Gal-3 to lectins and lipopolysaccharides but not to BLG-CML with the hypothesis of a charge-dependent recognition. The binding mechanism of ligands to Gal-3 is in many cases unknown, emphasising the importance of further studies to better understand the binding affinity of Gal-3 and its interaction with protein-bound CML.

Interestingly, the binding to the AGEs receptors tested here also increased with longer incubation times of BLG-Lac samples, indicating that glycation with other MRPs than CML also plays a role in modulating the receptor recognition of BLG, while CML levels were much lower compared to BLG-CML samples (Table 4.1). This could be explained by the increasing hydrophobicity of BLG-Lac samples (Figure 4.2), which has been shown to promote the uptake of heated and glycated BLG in macrophages [27]. At the same time, BLG-Lac-CML samples showed no increase in hydrophobicity compared to BLG-NT (Figure 4.2) and lower levels of CML compared to BLG-CML samples (Table 4.1), while binding to the receptors was still high (Figure 4.5). This suggests that other MRPs, that induce a negative charge on lysine residues, could be involved in the increased binding to the AGE receptors. This is also supported by the decreasing binding of BLG-Lac-48 with increasing salt concentration and highlights the importance to closely monitor the degree of glycation in processed food.

In this study, it was shown that BLG bound CML is a ligand for the receptors present on APCs: RAGE, CD36 and Gal-3. Although the levels of CML induced in this study are higher than what is normally observed in processed milk and dairy products, this study suggests a role of CML in immunomodulation by dietary AGEs. Moreover, this study demonstrated that not only CML but also other MR-induced modifications result in binding to AGE receptors, because glycated BLG with low levels of CML also induced binding to these receptors. The role of dietary AGEs in inflammatory disease is still under debate due to lacking possibilities to differentiate between exogenous and endogenous AGEs, limited knowledge of the metabolic transit, and possible biases of some *in vivo* studies for instance by high caloric index of the study material as well as the presence of reactive oxidative species [53]. It is possible that smaller MR-modified digestion-derived peptides reach the mucosal immune system in the gastrointestinal tract [54] and that intact BLG is sampled from the intestinal lumen via DCs and thus can be involved in mucosal immunity [55]. However, further studies are needed to evaluate the bioavailability of CML-modified protein as well as CML-modified digestion-derived peptides and their possible immunomodulatory effect *in vivo*.

4.5   Conclusions

Chemical modification of BLG to introduce CML on lysine residues resulted in a CML concentration-dependent increase of binding to sRAGE, CD36, and Gal-3, confirming that protein-bound CML is a ligand for these AGE receptors. Next to BLG-CML, BLG-Lac-48 was also a potent ligand for the AGE receptors, which was positively correlated with increasing

hydrophobicity upon longer heating time of BLG-Lac. The reduced receptor binding upon increased salt concentration indicated that the negative charge induced to the lysine residues by CML was a crucial determinant for binding of BLG-CML samples to AGE receptors. The same effect of salt concentration was also observed for the BLG-Lac-48 sample suggesting that, next to hydrophobicity other MRPs that induce a negative charge to lysine residues can contribute to the binding of glycated proteins. These findings showed that heating and glycation of food proteins results in structural modifications amongst which hydrophobicity and MRPs carrying a negative charge were found to be important determinants to predict possible immunological consequences. This also highlights the possible physiological relevance of CML in processed food as well as other MRPs that induce a negative charge to amino acid residues. At the same time, bioavailability of these MRPs and the relevance in aetiology of adverse inflammation *in vivo* remains to be better investigated.

Supplementary material
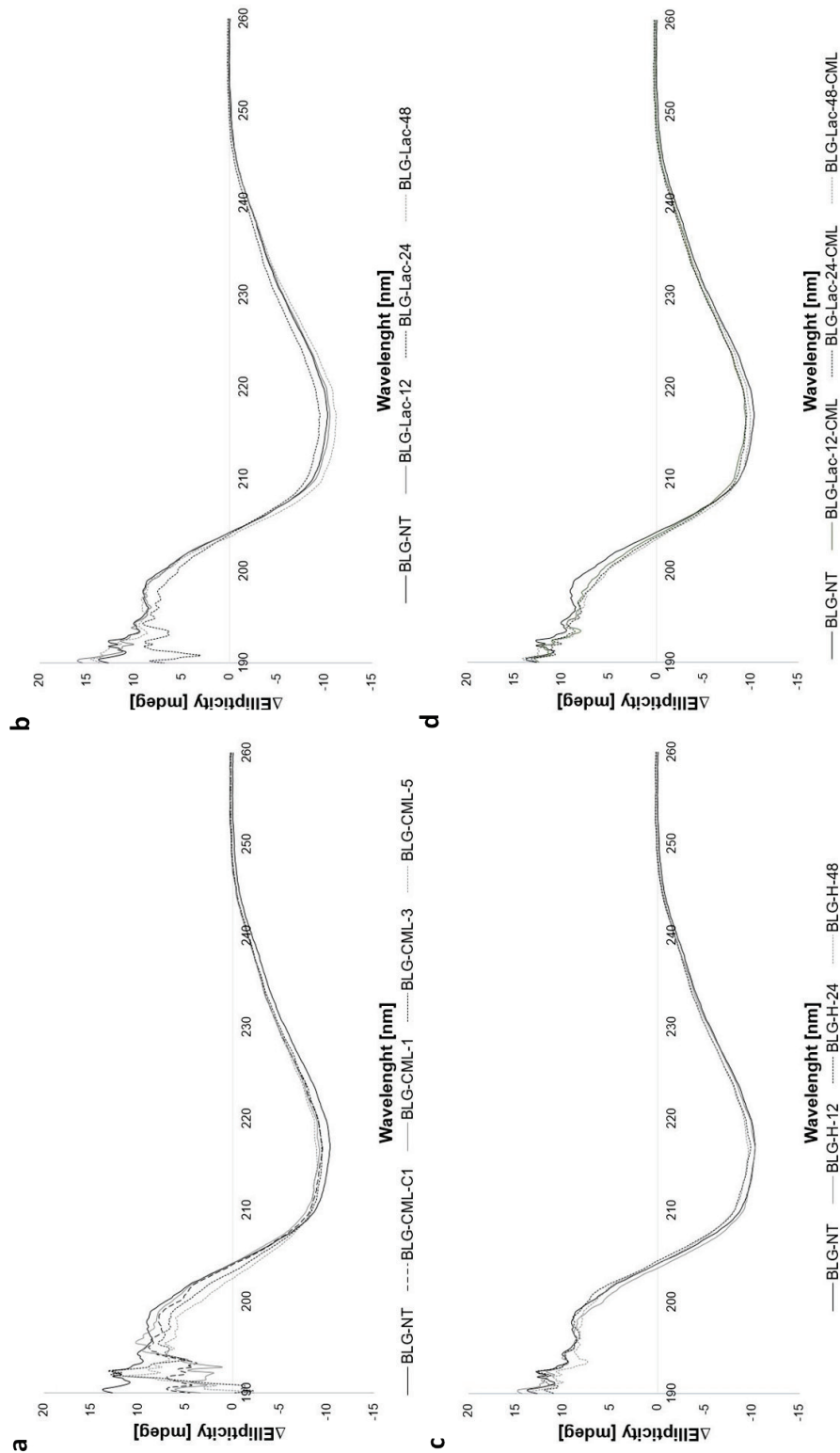
Figure 4-S1: Far-UV circular dichroism spectra of (a) β-lactoglobulin (BLG), non-treated (NT), chemical modification control without addition of glyoxylic acid (C1), chemically modified to introduce N^ε-carboxymethyllysine (CML) at different degree of modification (1, 3, and 5), (b) BLG glycated with lactose (Lac) at 60 °C for 12 h, 24 h, and 48 h, (c) BLG heated in the absence of lactose (H) at 60 °C for 12 h, 24 h, and 48 h, and (d) BLG glycated and subsequently chemically modified.

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Chapter 5

Enhanced uptake of processed bovine β-lactoglobulin by antigen presenting cells: Identification of receptors and implications for immunogenicity

This chapter is based on:

Abstract:

β-lactoglobulin (BLG) is a major cow's milk allergen encountered by the immune system of infants fed with milk-based formulas. To determine the effect of processing on immunogenicity and allergenicity of milk proteins, we characterised the mechanisms by which heated and glycated BLG are recognised and internalised by antigen presenting cells (APCs). Also, the effect of heat-induced structural changes as well as gastrointestinal digestion on immunogenicity of BLG was evaluated. The binding and uptake of BLG isolated from raw cow's milk heated alone (BLG-H) or with lactose or glucose (BLG-Lac and BLG-Glu) to the receptors present on APCs were analysed by ELISA and cell binding assays. Physicochemical analyses characterised the effect of heating and glycation on the structure, while immunogenicity of BLG preparations was evaluated before and after simulated infant *in vitro* digestion. Heated and glycated BLG showed enhanced binding to RAGE, galectin-3 (Gal-3), CD36, and SR-AI, without enhanced secretion of pro-inflammatory cytokines. Gal-3 and CD36 and SR-AI, but not RAGE-binding, induced internalisation of heated and glycated BLG. Receptor affinity of BLG was dependent on aggregation as well as on increased hydrophobicity and exposure of β-sheets. Digested BLG-Lac and BLG-Glu maintained the binding to sRAGE and Gal-3 and were detected on the surface of APCs. Glycation of milk may thus enhance immunogenicity by triggering innate (via RAGE) as well adaptive immune response (via Gal-3), however implications for allergic sensitisation remain unclear.

5.1   Introduction

Cow's milk (CM) is an example of a food that is subjected to specific thermal processing, dependent on the type of product produced from it. Heating provokes structural modifications of milk proteins via amongst others denaturation, aggregation and the Maillard reaction (MR) [1], leading to increased immunogenicity and possibly also allergenicity of milk proteins [2]. β-lactoglobulin (BLG), the most abundant whey protein (WP) that is not present in breast milk, has been described as one of the major allergens causing CM allergy [3]. In infants fed with CM formulas, BLG is thus one of the first foreign antigens encountering the mucosal immune system of the gastrointestinal tract [4]. Therefore, a number of studies have been performed in order to understand the relative importance of heat-induced changes of immunogenicity and allergenicity of BLG [5-10]. Denatured BLG, in contrast to the native form, was shown to induce significantly higher infiltration of mononuclear inflammatory cells in the gastroduodenal mucosa of sensitised rats. This observation indicates a more intense local immunologic response towards the heated form of BLG, compared to its native form [11]. Also, pasteurization-induced aggregates of BLG were shown to play a role in allergic sensitisation. Roth-Walter and colleagues showed that aggregated BLG is predominantly taken up from the intestinal lumen by the Peyer's patches instead of via transcytosis through enterocytes [12]. Similarly, cross-linked BLG was increasingly endocytosed by dendritic cells (DCs), in a food allergy-murine model leading to the development of Th2-associated environment [13]. Lastly, the Maillard reaction (MR) was also shown to play a role in enhanced immunogenicity of processed BLG. Glycated WP increased mRNA expression of pro-inflammatory cytokines tumour necrosis factor α (TNF-α), interleukin (IL) 1β and IL-6, as well as increased phagocytic activity of a murine macrophage cell line [14]. Furthermore, also aggregated BLG appears to be recognised by the receptor for advanced glycation end products (RAGE) expressed on antigen presenting cells (APCs) [15-17]. RAGE has been postulated to play a role in the development of food allergies upon interaction advanced glycation end products (AGEs) formed in the food due to the glycation [18].

Nevertheless, the relative importance of glycation vs. aggregation on immunogenicity and allergenicity of milk proteins, including BLG as one of the major milk allergens, remains unclear. For instance, Perusko *et al*. [8] reported an increased uptake of glycated BLG by mice bone marrow–derived DCs compared to non-treated BLG, but BLG heated in the absence of sugar was not included in their study. In contrast, the study of Deng *et al*. [19] emphasized the heat-induced formation of amyloid-like structures, aggregates and increased hydrophobicity as the most important features predisposing heat processed proteins to be recognised by APCs. The processing-related structural changes that enhance immunogenicity of BLG are therefore not fully understood. Thus, exploring the molecular mechanisms of interaction between heat-treated BLG, as a potential antigen present in infant nutrition, and the human APCs is of particular interest. The aim of the present study was firstly to evaluate and compare heat and glycation induced changes in immunoreactivity of BLG. Secondly, to specify the receptors

involved in binding and internalisation of heated and glycated BLG in order to reveal mechanisms responsible for triggering innate and/or adaptive immunity. Lastly, to evaluate the immunogenicity of heated and glycated BLG after enzymatic digestion in an infant *in vitro* digestion model.

5.2   Materials and Methods

5.2.1   *Chemicals*

The Recombinant Factor C Endotoxin Detection Assay was purchased from Hyglos GmbH (Bernried, Germany). Anti-his antibody (# 148052751) was obtained from Qiagen (Venlo, The Netherlands). Roswell Park Memorial Institute 1640 Medium (RPMI 1640), fetal bovine serum (FBS), Alexa Fluor 647 Protein Labelling Kit, Fluorescein-EX Protein Labelling Kit, oxidised low-density lipoprotein (50-150 µg/mL, #L34357), and cytochalasin D (0.5 µM, #PHZ1063) were purchased from Thermo Fischer Scientific (Waltham, Massachusetts, USA). Anti-BLG antibodies (#ab112893) and 33DFTG (10 mM, #AOB37408) were obtained from Abcam (Cambridge, UK) and Aobius (Gloucester, Massachusetts, USA), respectively. The culture of human acute monocytic leukaemia cell line (THP-1) was obtained from the American Type Culture Collection (Manassas, Verginia, USA). Cytometric bead array (CBA) kit (Human Inflammatory Cytokine Kit was purchased from BD Bioscience (Franklin Lakes, New Jersey, USA). Mini-protean TGX precast gels were purchased from Bio-Rad (Hercules, California, USA). Soluble RAGE (#RD172116100) was purchased from BioVendor (Brno, Czech Republic). Polyclonal goat anti-mouse Antibody/HRP (#P0447) and polyclonal rabbit anti-mouse antibody/biotinylated (#E0464) were purchased from Dako (Jena, Germany). Intracellular Fixation & Permeabilization Buffer Set and Fixable Viability Dye eFluor 506 were obtained from eBioscience (San Diego, California, USA). G3-C12 (400 µM, #RP20329) and ovalbumin were purchased from GenScript (Piscataway, New Jersey, USA) and InvivoGen (San Diego, California, USA), respectively. FPS-ZM1 (100-250 nM, #553030) was purchased from Merck Millipore, Burlington, Massachusetts, USA). IL-4 and GM-CSF protein were purchased from Peprotech (Cranbury, New Jersey, USA). Nε-(carboxy [2H2]methyl)-l-lysine (d2-CML), Nε-(carboxymethyl)-l-lysine (CML), furosine, and furosine-d4 were purchased from Polypeptide laboratories (Strasbourg, France). CD36 (#1955-CD), anti-RAGE antibody (#MAB11451), and anti-Gal-3 antibody (#MAB11541) were purchased from R&D system (Minneapolis, Minnesota, USA). Bovine serum albumin was obtained from Roche (Basel, Switzerland). TMB substrate (3,3′,5,5′-tetramethylbenzidine) for high sensitivity ELISA was purchased from sdt-reagents (Baesweiler, Germany). All other chemicals were purchased from Sigma Aldrich (St. Louise, Missouri, USA).

5.2.2   *Isolation, treatment and fractionation of BLG and WP*

Raw milk was obtained from the Department of Animal Sciences, Wageningen University & Research (Wageningen, The Netherlands). The WP fraction was purified from the raw bovine

milk as described before by Perdijk *at al.* [20]. BLG was purified and isolated as described by De Jongh *et al.* [21] using anion exchange chromatography DEAE Sepharose C-6B (GE healthcare, Chicago, Illinois, USA). Isolated BLG was lyophilised and a purity >94% was measured as described by Deng *et al.* [19]. The lipopolysaccharide (LPS) removal was performed according to the protocol described by Teodorowicz *et al.* [22] and the recombinant factor C endotoxin detection assay was used to monitor the LPS levels. LPS-free BLG and WP were heated in a wet system above the denaturation temperature by heating it in phosphate buffer (PBS) at pH 7.4 applying 100 °C for 90 min in the presence (1:1 w/w) of lactose (BLG/WP-Lac), glucose (BLG/WP-Glu) and in the absence of lactose (BLG/WP-H). After the heating step BLG and WP were fractionated using Amicon Ultracell 100k and Amicon Ultracell 3k filters (Amicon, UFC910024, Millipore Corporation, Bedford, Massachusetts, USA). Proteins were loaded first onto the Amicon Ultracell 100k filter and washed 7 times with PBS followed by centrifugation (3363 ×g, 10 min, 25 °C). The filtrates were collected, pooled and subsequently loaded onto Amicon Ultracell 3k filter in order to concentrate the collected fraction. The protein concentration in both fractions (above and below 100 kDa) was measured using NanoDrop ND1000. Both fractions were aliquoted before storage at -20 °C.

5.2.3   *Thioflavin-T (ThT)-assay*

ThT-assay was conducted to monitor the formation of fibril structures during heating according to the previously described protocol [15]. Briefly, samples were diluted to a protein concentration of 0.25 mg/mL and mixed with 4 mM ThT dissolved in PBS at a ratio 7:1 (v/v). Samples were measured in duplicate using the Infinite® 200 PRO NanoQuant and i-control software (Tecan, Männedorf, Switzerland) at the excitation wavelength 450 nm and the emission wavelength 475 nm.

5.2.4   *Surface hydrophobicity*

Hydrophobicity was measured using 8-anilino-1-naphthalenesulfonic acid (ANS) assay according to the previously described protocol [19] with the addition that samples were diluted to a protein concentration of 0.5 mg/mL. All samples were measured in duplicate in a 96-well black polysterene plate using the Infinite® 200 PRO NanoQuant and i-control software (Tecan, Männedorf, Switzerland) at excitation wavelength 390 nm and emission wavelength 470 nm.

5.2.5   *Quantification of N^ε-carboxymethyllysine and furosine using uHPLC-MS/MS*

N^ε-carboxymethyllysine (CML) was quantified using uHPLC-ESI-MS/MS according to a method described previously [15,23]. Briefly, samples were hydrolysed with 6 M hydrochloric acid at 110 °C for 22 h and hydrochloric acid was removed under nitrogen flow. Dried samples were dissolved with acetonitrile (ACN)/water 1:1 (v/v) and spiked with CML-d2 as well as furosine-d4 to a final concentration of 250 ng/mL. Analytes were separated on a Kinetex 2.6 µ HILCI 100A, 100 × 2.1 mm (Phenomenex, Torrance, California, USA) at 35 °C column temperature. Eluent A was ultrapure water with 0.1% formic acid, eluent B was ACN

with 0.1%, and eluent C was 50 mM ammonium formate. Flow rate was set to 0.4 mL/min using the following gradient (time [min]/eluent B [%]/eluent C [%]): (0/80/10), (0.8/80/10), (3.5/40/10), (6.5/80/10), (8.0/80/10), (11/80/10). Electron ionization was conducted in positive mode. Spray voltage was set to 3500 °C, vaporising temperature was 250 °C, and sheath gas pressure was 60 psig. Capillary temperature was set to 290 °C. For furosine parent ion M + [H+] 255.1 m/z and fragment ion 84.4 m/z were monitored (collision energy (CE) 19 and tube lens offset (TL) 75) as well as 130.0 m/z (CE: 15, TL 75), while d4-furosine was monitored with M + [H+] 259.1 m/z and fragment 88.0 m/z (CE 20, TL 72), 134.0 m/z (CE 13, TL 72), and 196.0 m/z (CE 13, TL 72). CML was monitored using M + [H+] 205.3 m/z, 84.0 m/z (CE 18, TL 75), and 130.0 m/z (CE 15, TL 75) while d2-CML was monitored with M + [H+] 207.3 m/z, 84.0 m/z (CE 19, TL 75), and 130.0 m/z (CE 15, TL 75).

5.2.6 Qualitative screening of non-enzymatic post translational modifications (nePTMs)

For the analysis of nePTMs, all samples were dialysed against demineralised water for 30 h with a molecular cut-off of 3.5 kDa. The samples were freeze dried and reconstituted in tris(hydroxymethyl)aminomethane hydrochloride buffer (100 mmol/L, pH 7.8). After this, reduction and alkylation of disulphides was conducted with 1, 4-dithiothreitol and iodoacetamide. Enzymatic hydrolysis was performed with Glu-C at 37 °C overnight and stopped by adding formic acid. After dilution to a final concentration of 0.1 μg/μL, the peptide mixture was analysed by reversed phase micro liquid chromatography. For this, an Ultimate 3000 RS system (degasser, binary nano flow pump, autosampler, column oven; ThermoFisher, Dreieich, Germany) was coupled to a 6500 + QTrap mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with an electrospray ionization source. Analyst 1.6.3 was applied for instrument control as well as for data acquisition. A YMC Triart C18 (500 μm inner diameter, 100 mm length, 3 μm particle size) column was used at 35 °C with the following gradient: A, formic acid (0.1%); B, ACN with formic acid (0.1%), flow rate 30 μL/min; [time (min)/%B]: 15/2, 5/2, 55 /42.5, 55.5/95, 65/95. An aliquot of 5 μL was injected. All flow eluting before 2.0 min and after 55 min was discarded by a two-position valve prior to MS analysis. All measurements were performed in positive ionization mode. The ion source was operated at 350 °C with a voltage of +5000 V, the curtain gas set to 40 psi, the nebulizer gas to 40 psi, and the heating gas to 60 psi. All data were acquired in the scheduled multiple reaction monitoring mode (sMRM) with three transitions per peptide. Each sample was analysed in triplicate. With this method, 12 modified peptides representing 15 different modifications on 25 different amino acid side chains were monitored [24]. For data evaluation, MultiQuant 3.0 software was used.

5.2.7 Inhibition ELISA with recombinant receptors

Inhibition ELISA was conducted to determine binding of the processed BLG to the following receptors: soluble form of RAGE (sRAGE), galectin-3 (Gal-3) and two receptors from the scavenger receptor family: scavenger receptor class A type I (SR-AI) and CD36 according to the protocol described by Zenker et al. [15]. Briefly, transparent high binding ELISA plate was

coated overnight with glycated soy extract (SE) followed by blocking for 1h with 3% bovine serum albumin (v/w) and washing with 0.05% (v/v) Tween-20 in PBS. The BLG samples in the range of the dilutions were pre-incubated with recombinant receptors 45 min prior to adding to the coated and blocked ELISA plate. The sample/receptor mixture was incubated in the ELISA plate for 1 h at 37 °C. The plate was washed and incubated (30 min at room temperature) with anti-receptor antibodies: anti-RAGE, anti-Gal-3 or anti-his antibody for receptors containing His tag. After washing the plate was incubated with detection antibody: polyclonal goat anti-mouse Antibody/HRP, polyclonal rabbit anti-mouse antibody/ biotinylated, goat anti-human IgG/HRP. The signal was detected with TMB and a colour reaction was measured at 450 nm vs. 620 nm reference using a Filter Max F5 multi-mode microplate reader (Molecular Devices, San Jose, California, USA). Each sample was measured in triplicate. Amyloid-β for sRAGE and CD36 and SE for Gal-3 were used as a positive control, while native ovalbumin was used as a negative control. Percentage of inhibition was calculated using the following formula:

$$Inhibition\ [\%] = (Abs_{Max} - (Abs_{Sample} - Abs_{Min}))/Abs_{Max} \times 100$$

where Abs_{Max} is the absorbance obtained from receptor without competition agent; Abs_{Min} is the absorbance obtained from blank sample (PBS), Abs_{Sample} is the absorbance obtained from the mixture of receptor and each sample.

5.2.8 *Native-PAGE electrophoresis*

Gel electrophoresis under non-reducing conditions was performed to monitor protein aggregates formed during heat treatment of BLG. Samples were mixed with native PAGE sample buffer (62.5 mM Tris, 25% glycerol, 1% bromophenol blue, pH 6.8) and loaded on Mini-protean TGX precast gels. Gels were run at 100 V for 90 min using running buffer containing 25 mM Tris and 192 mM glycine and stained with GelCode Blue Stain Reagent. Images of the stained gels were obtained using a Universal Hood III (Bio-Rad, Hercules, California, USA) and Image Lab 4.1 software (Bio-Rad, Hercules, California, USA).

5.2.9 *Isolation and culturing of monocyte-derived DCs (MoDCs)*

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation from fresh blood obtained from healthy anonymous donors as described previously [20]. In short, CD14+ cells were isolated on a separation magnet (BD IMagnet, BD Biosciences) according to the manufacturer's instructions. Per well 10^5 monocytes were cultured in 96 cells wells flat bottom plates in RPMI 1640 and 10% FBS, normocin (100 µg/ml), penicillin and streptomycin (100 U/ml). Monocytes were differentiated into DCs by culturing them for six days in the presence of 20 ng/mL IL-4 and GM-CSF protein. After six days, immature DCs were used for binding and internalisation assay.

5.2.10 *Culture of human acute monocytic leukaemia cell line (THP-1)*

The THP-1 cells were obtained as described before [25]. Macrophage phenotype was induced by stimulating THP-1 cells with 10 ng/mL PMA for 48 h. Differentiated cells were washed twice in supplemented medium and were allowed to rest by incubating them for another 24 h in supplemented medium. Afterwards, the cells were used directly for the binding and/or uptake assay or incubated 24 h in the presence of BLG (25, 125, 1000 μg/mL) followed by the detection of cytokines in the supernatant. The supernatant was collected and human cytokine concentrations (IL-6, IL-8, IL-1β, TNF-α, and IL-10) were determined using the CBA kit, according to the manufacturer's instructions. The samples were analysed by flow cytometry (CytoFLEX Flow Cytometer, Beckman Coulter, Brea, California, USA). The results were normalised to cytokine levels of unstimulated macrophages cultured in the medium.

5.2.11 *Binding and internalisation assay*

To detect the BLG that was internalised by THP-1 cells, anti-BLG antibodies were labelled using the Alexa Fluor 647 protein labelling kit or Fluorescein-EX protein labelling kit. Initially, the antibodies were concentrated by ultrafiltration (7000×G) using Microsep Advance centrifugal devices with Omega membrane with 3 kDa cut-off (PALL Corporation, New York, USA), to a final concentration of 2 mg/mL. Then, the antibodies were labelled with the Alexa Fluor 647 dye, following the procedure as described by the manufacturer. The binding and internalisation of BLG samples was evaluated using PMA-differentiated THP-1 macrophages and MoDCs. The cells were incubated with BLG or WP samples (125 μg/mL) for 2 h. Then, the cells were washed with FACS buffer, detached from the plate and were transferred to a round-bottom 96-well NUNC plate (Thermo Fisher Scientific, Waltham, Massachusetts, USA), on ice. Subsequently, immunostaining with anti-BLG dye-conjugated antibodies was performed. Initially, the cells were stained extracellularly (30 min, 4 °C, in the dark) with fluorescein isothiocyanate (FITC) conjugated anti-BLG antibodies, to assess the extracellular binding of the protein followed by fixation and permeabilization using intracellular fixation and permeabilization buffer set. After centrifugation (3 min, 300 ×g, 4 °C), the cells were incubated with AlexaFluor 647-conjugated anti-BLG antibodies (30 min, 4 °C, in the dark), to stain intracellularly the internalised BLG. Finally, after two washes with permeabilization buffer, the cells were resuspended in FACS buffer and were analysed by flow cytometry (CytoFLEX LX Flow Cytometer, Beckman Coulter, Brea, California, USA). The viability of the cells was monitored using Fixable Viability Dye eFluor 506.

5.2.12 *Inhibition of binding and internalisation of BLG by THP-1 cells*

To perform the inhibition of binding and internalisation of BLG by THP-1 cells, the cells were differentiated with PMA, as described in paragraph 5.2.10. After the 24 h resting step, the cells were treated with the chosen inhibitors: FPS-ZM1 as RAGE inhibitor, oxidised low-density lipoprotein (oxLDL) as CD36 inhibitor; G3-C12 and 33DTFG as Gal-3 inhibitor and fucoidan

as inhibitor for the SR-A1. Finally, dynasore was used as inhibitor of clathrin-depended endocytosis, while macropinocytosis and phagocytosis were inhibited by cytochalasin D. FPS-ZM1, ox-LDL, G3-C12, 33DFTG, fucoidan, dynasore, and cytochalasin D. After 45 min of incubation with the inhibitors, the BLG protein samples were added and the cells were incubated for two additional hours. Extracellular and intracellular staining followed, as described in paragraph 5.2.11. The samples were analysed by flow cytometry (CytoFLEX LX Flow Cytometer, Beckman Coulter, Brea, California, USA).

5.2.13 *In vitro digestion of BLG in infant gastrointestinal static in vitro model*

An infant gastrointestinal static *in vitro* model was used in order to mimic the digestive conditions of the gastric phase (GP) and the intestinal phase (IP) of full-term infants as described before by Ménard *et al.* [26]. During the GP, simulated gastric fluid (94 mM sodium chloride; 13 mM potassium chloride; pH 5.3) was added and the pH was adjusted to 5.3 with 0.1 M hydrochloric acid followed by the addition of pepsin and lipase. Enzyme activities were set at 268 U/ml of gastric content for pepsin and 19 U/ml of gastric content for lipase. The GP was completed with an incubation at 37 °C for 1 h, while mixing. The IP was initiated with the addition of simulated intestinal fluid (164 mM sodium chloride, 10 mM potassium chloride, 85 mM of sodium bicarbonate pH 6.6) after which the pH was adjusted to 6.6 with 0.1 M sodium bicarbonate. Then, in this order, pancreatin and bovine bile extract. Calcium chloride stock solution was added in the beginning of the IP at a concentration of 3 mM within the volume of the intestinal fluid. Bovine bile extract was added to a final content of 3.1 mM of bile salts and the pancreatin covered the intestinal lipase activity required of 90 U/ml of intestinal content and the trypsin activity needed i.e. 16 U/ml of intestinal content. The IP was completed with an incubation at 37 °C for 2 h, while mixing. At the start and end of both phases, the pH was measured for all samples. At the end of the IP, the pH was adjusted to 6.8. The digestion was finalised by inactivating the digestive enzymes with 1 mM Pefabloc.

5.2.14 *Statistical analysis*

Statistical analysis were conducted using GraphPad Prism. For multiple sample comparison, one-way ANOVA was used and Tukey post hoc comparison test, if not mentioned otherwise. Results were considered statistical different at $p < 0.05$ if not mentioned otherwise.

5.3 Results

5.3.1 *Chemical characterization of processed BLG*

Bovine BLG isolated from raw milk was heated: (1) in the presence of glucose (BLG-Glu), (2) in the presence of lactose (BLG-Lac), and (3) without sugar (BLG-H). WP, isolated from raw bovine milk, was subjected to the same treatment in order to study the effect of presence of all WPs simultaneously on the structural and functional changes of BLG. Prior to the heat treatment, both BLG and WP were depleted from LPS contamination. The remaining levels of

LPS in the samples used in the functional *in vitro* assays were below 2 pg/mL (supplementary material: Figure 5-S1). To describe the influence of heating and glycation on the structure of BLG the exposure of β-sheets as well as hydrophobic structures was measured. Exposure of both, β-sheets and hydrophobicity showed significantly higher levels than non-treated BLG (BLG-NT) already after heating in the absence of reducing sugars (Figure 5.1a and b).
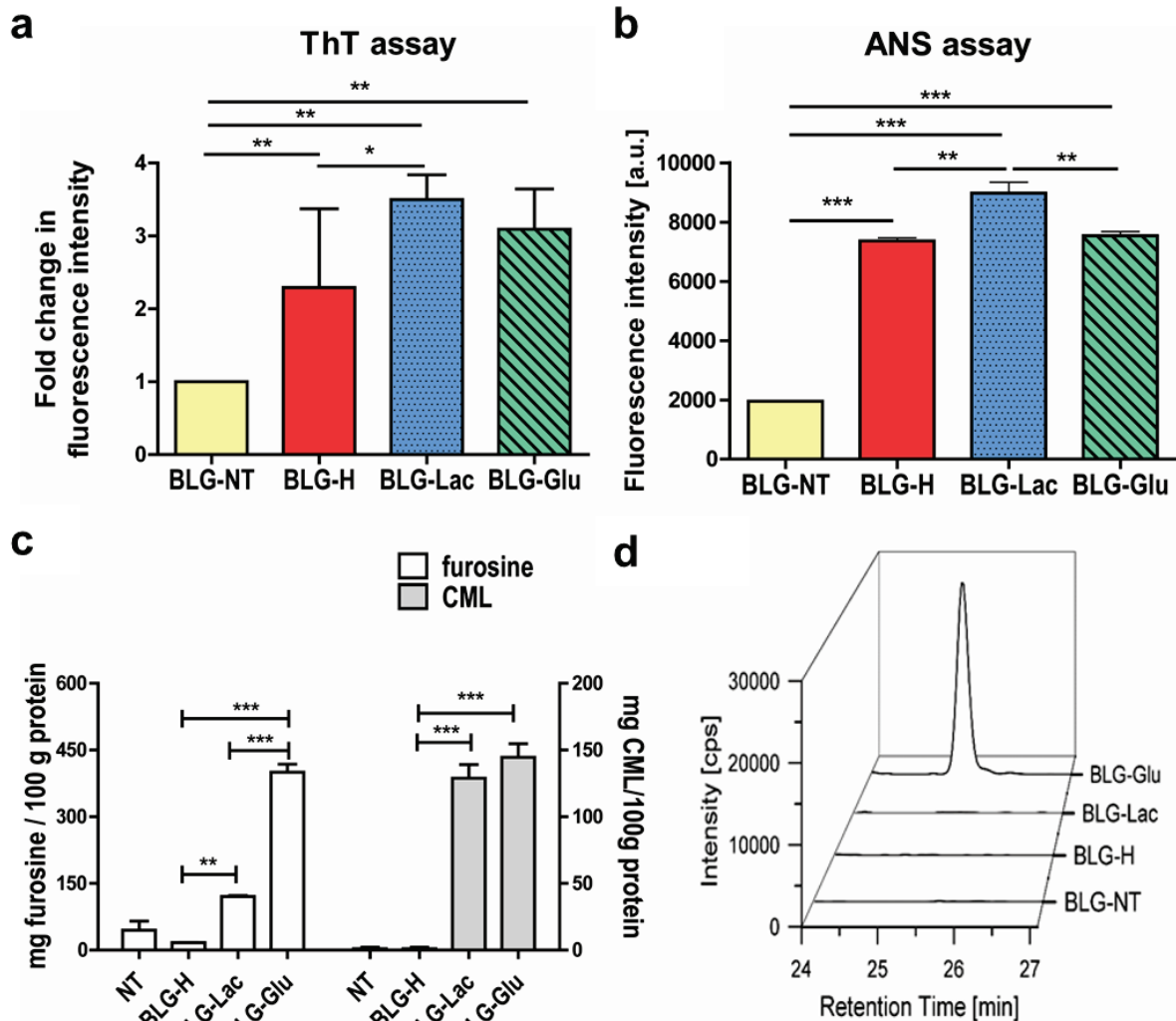
Figure 5.1: Biochemical characteristic of heated and glycated BLG. (a) formation/exposure of the fibril structures measured with ThT-assay, (b) changes in hydrophobicity measured with ANS assay, (c) level of glycation expressed as amount of formed N^ε-carboxymethyllysine (CML) and furosine, (d) LC-sMRM chromatogram of methylglyoxal-dihydroxyimidazoline on AQSAPLR[+72.0]VYVEE in BLG-Glu. Three MRM transitions are displayed. BLG: non-treated (BLG-NT), heated (BLG-H), heated with lactose (BLG-Lac) or heated with glucose (BLG-Glu). Data shown as mean ± SD of triplicate wells and are representative of at least three independent experiments. Significant differences analysed with one-way ANOVA with Tukey post hoc comparison test; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

Hydrophobicity and exposure of β-sheet structures further increased in BLG-Lac compared to BLG-H, whereas BLG-Glu was comparable to BLG-H (Figure 5.1a and b). However, BLG-Lac showed significantly higher fluorescence intensity as compared to BLG-H indicating a more hydrophobic nature of, additionally, glycated proteins. In order to determine the level of the intermediate and advanced MRPs, furosine and CML were quantified in all samples. The level of furosine and CML was significantly higher in the BLG-Lac and BLG-Glu when compared

to BLG-NT and BLG-H (Figure 5.1c). The levels of furosine detected in BLG-Glu were significantly higher than in BLG-Lac, while the CML levels were comparable in both samples. Finally, the post translational modification (nePTM) profile of BLG-Glu vs. BLG-Lac was analysed to screen for modifications, which may be specific for a sample including 15 nePTMs on 25 bindings sites [24]. Only methylglyoxal-dihydroxyimidazoline (MG-DH1) on R40 was exclusively detected in BLG-Glu (Figure 5.1d) while all other nePTMs were either detectable in all or in none of the samples. Therefore, glycation of BLG in the presence of sugars in the wet heating model was confirmed by detection of furosine, CML and nePTM profiling while the heat-induced structural changes were demonstrated by exposure of β-sheets and hydrophobic patterns.

5.3.2 *Heated and glycated BLG and WP bind to sRAGE, Gal-3, CD36 and SR-AI*

The next step in our study was to relate the biochemical changes of BLG and WP to its biological and functional properties, by studying direct interaction with human immune cells. Initially, we aimed to identify the receptors responsible for the binding of processed and non-processed BLG. For that purpose, screening of receptors expressed on APCs, including sRAGE, Gal-3, and two receptors of the scavenger family: CD36 and SR-AI, was performed, with inhibition ELISA (Figure 5.2). The binding of BLG-NT to all four receptors did not differ from the negative control while BLG-H showed a high binding to all studied receptors, which was further increased for the glycated proteins. The differences of receptor inhibition between heated and glycated BLG reached a maximum value of 18% for the CD36 receptor (Figure 5.2a) suggesting high relevance of heat-induced structural changes in formation of the receptors ligands. The type of sugar used for glycation (glucose vs. lactose) had a marginal impact on the level of binding to the studied receptors. Heating of BLG in a presence of other WPs resulted in a significantly lower binding to Gal-3, SR-AI, and CD36 when compared to pure BLG at the same concentration, suggesting that the binding as observed in WP is mainly the result of modification of BLG.
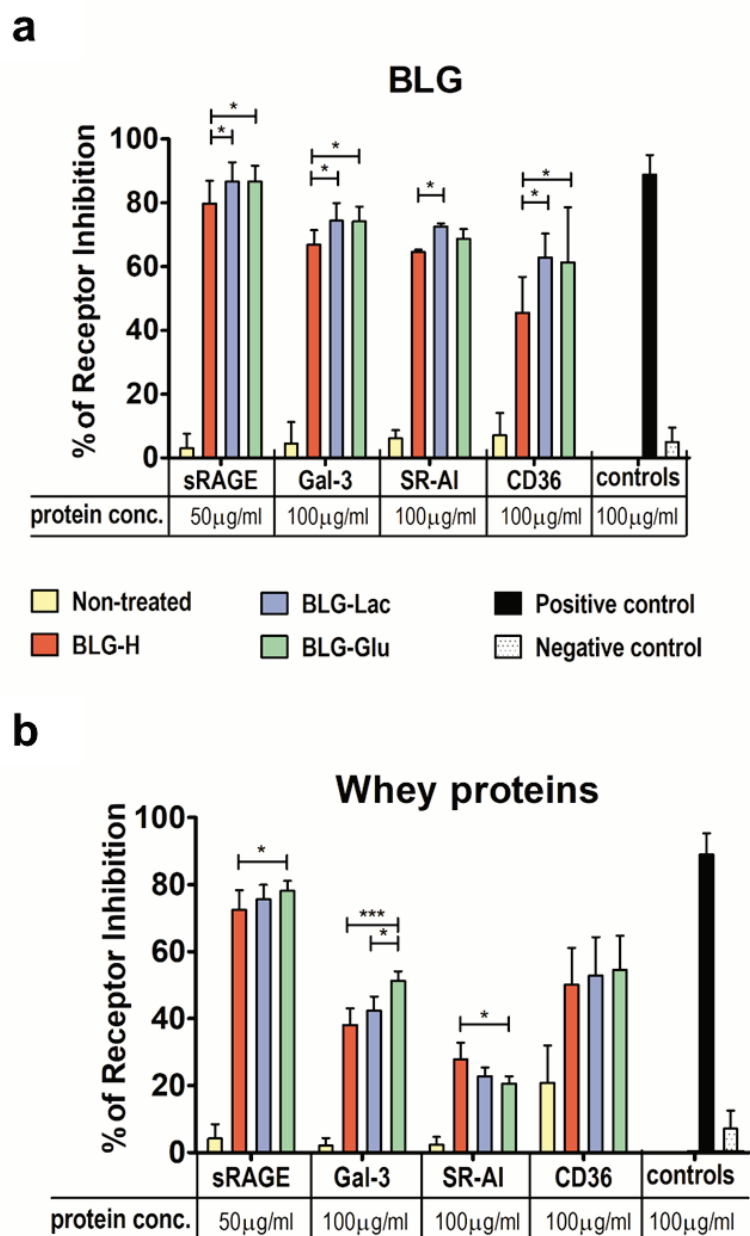
Figure 5.2: Inhibition of sRAGE, Gal-3, CD36 and SR-AI by BLG and whey proteins measured in competition ELISA. BLG (a) and WP (b), non-treated or heated in the absence of sugar (BLG-H) or in the presence of lactose or glucose (BLG-Glu, BLG-Lac) were pre-incubated with the recombinant forms of sRAGE, Gal-3, CD36 and SR-AI prior adding to the ELISA plate coated with glycated soy protein extract. The data are expressed as percentage of inhibition by the use of non-inhibited signal as the maximum value. Positive control: highly glycated soy protein extract; Negative control: ovalbumin. Data shown as mean ± SD of triplicate wells and are representative of at least three independent experiments on three different batches of processed BLG. Significant differences analysed with one-way ANOVA with Tukey post hoc comparison test; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

5.3.3 Heated and glycated BLG and WP bind to THP-1 macrophages and get internalised via both actin- and clathrin-dependent endocytosis mechanisms

To confirm the inhibition ELISA results, we performed binding and uptake assays of non-processed and heated BLG and WP, using THP-1 macrophages and MoDCs (Figure 5.3).

a

Binding THP-1 cells

b

Internalisation THP-1 cells

c

Binding MoDCs

d

Internalisation MoDCs
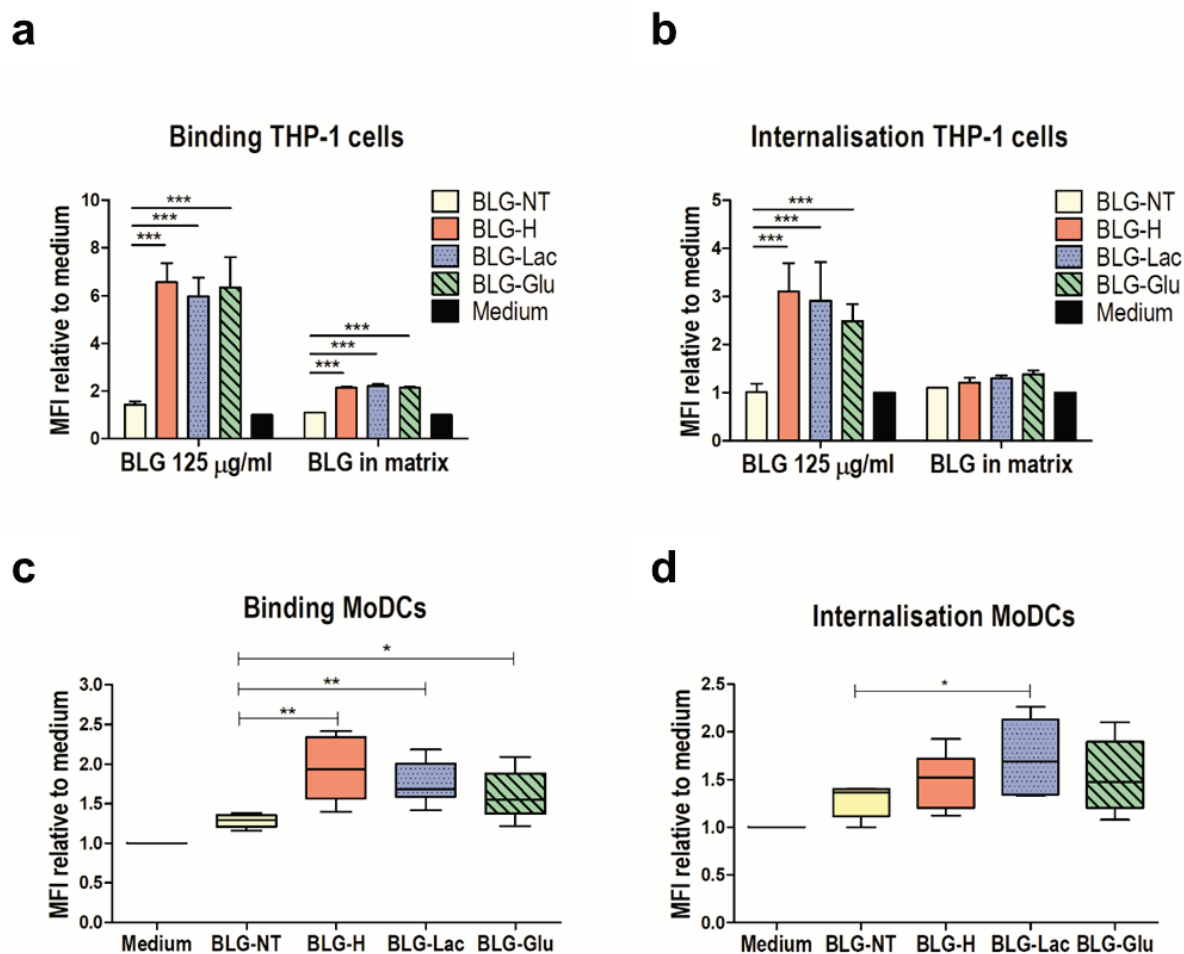
Figure 5.3: Binding and internalisation of processed BLG by human THP-1 macrophages (a, b) and human monocyte derived DCs (c, d). PMA differentiated THP-1 macrophages or MoDCs were incubated 2h in a presence or absence of BLG: non-treated (BLG-NT), heated (BLG-H), heated with lactose (BLG-Lac) or heated with glucose (BLG-Glu) followed by extracellular staining with FITC conjugated anti-BLG antibodies and intracellular staining with Alexa Fluor 647 conjugated anti-BLG antibodies. The level of fluorescence was measured by flow cytometry and expressed as a relative to medium control. Data for THP-1 macrophages (a, b) shown as mean ± SD of triplicate wells and are representative of at least three independent experiments. Data for MoDcs shown as mean ± SD of nine (c) or six (d) healthy donors. Significant differences analysed with one-way ANOVA with Tukey post hoc comparison test; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

To be able to distinguish the binding of BLG from internalisation of BLG, anti-BLG antibodies labelled with two different fluorescent dyes were used, to perform extracellular and intracellular staining. BLG heated in the absence and presence of sugars bound to the surface of THP-1 macrophages, and was also detected intracellularly, but BLG-NT neither bound to nor entered the cells. Moreover, BLG present in WP was equally bound to and internalised by the THP-1 macrophages. The concentration of BLG in WP is approximately 50%; therefore, its binding and internalisation by APCs is lower compared with BLG-NT but detectable with anti-BLG antibodies (Figure 5.3a and b). No significant differences between BLG heated in the presence or absence of sugar were observed in terms of binding and internalisation. This same pattern was noted for binding to and internalisation by human MoDCs, although the level of internalisation was lower when compared to THP-1 macrophages (Figure 5.3c and d). To describe a more detailed mechanism of internalisation of heated and glycated BLG we inhibited

the major endocytosis pathways. Cytochalasin D was used to block the actin-dependent endocytosis mechanisms pinocytosis and phagocytosis, while a dynamin inhibitor, dynasore, was used to block the clathrin-dependent endocytosis mechanism. Both cytochalasin D and dynasore significantly reduced the uptake of heated and glycated BLG, indicating that both the actin-dependent endocytosis mechanisms and the receptor-mediated endocytosis mechanisms are involved in the uptake of processed BLG (Figure 5.4b).
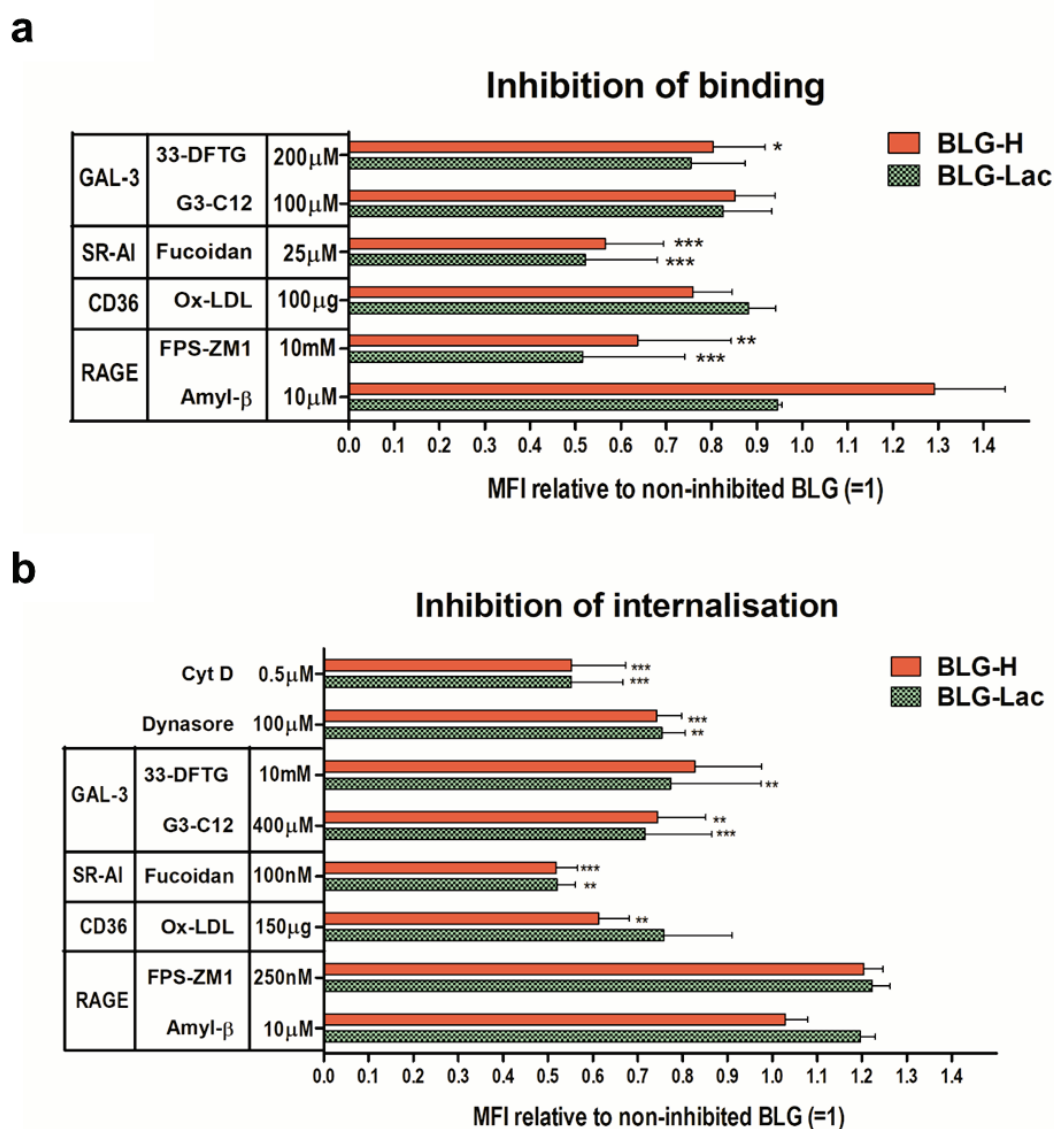
Figure 5.4: Inhibition of binding and internalisation of processed BLG by human THP-1 macrophages. PMA differentiated THP-1 macrophages were pre-incubated with endocytosis inhibitors: dynasore and cytochalasin D (Cyt D) or inhibitors of specific receptors: amyloid-β and FPS-ZM1 for RAGE; Oxidized low-density lipoprotein (Ox-LDL) for CD36; fucoidan for SR-AI, short peptides 33-DFTG and G3-C12 for Gal-3. Followed by pre-incubation with inhibitor the cells were incubated in a presence or absence of BLG: BLG heated in the absence of reducing sugars (BLG-H) or heated with lactose (BLG-Lac) and stained extracellularly (a) or intracellularly (b) with Alexa Fluor 647 conjugated anti-BLG antibodies. The level of fluorescence was measured by flow cytometry and expressed as a relative to non-inhibited THP-1 cells. Data shown as mean ± SD of triplicate wells and are representative of at three independent experiments. Significant differences analysed with one-way ANOVA with Tukey post hoc comparison test; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

Therefore, we blocked the receptors: RAGE, CD36, SR-AI and Gal-3 which expression on the surface of the PMA-differentiated THP-1 cells was first confirmed by flow cytometry (Supplementary Figure 5-S2). We observed the reduced binding of the BLG for the various receptors, demonstrating that the receptor with the highest binding capability was SR-AI followed by RAGE, Gal-3 and finally CD36 (Figure 5.4a). Finally, we used the anti-BLG antibodies labelled with different dyes for extra- and intracellular staining to distinguish between the receptors directly involved in binding and internalisation of heated and glycated BLG. The inhibitors of SR-AI > Gal-3 > CD36 significantly reduced the uptake of both heated and glycated BLG, pointing at the receptors from the SRs family, but also Gal-3, as the main receptors involved in receptor-mediated endocytosis. An inhibitor of RAGE (FPS-ZM1) did not significantly affect uptake of processed BLG (Figure 5.4b) while it was shown to reduce the external binding to RAGE. No significant differences in the blocking efficiency between heated and glycated BLG were observed.

5.3.4 *Internalisation of BLG does not impact the secretion of pro-inflammatory cytokines by THP-1 macrophages*

To study the physiological consequences of the binding and internalisation of heated and glycated BLG by THP-1 macrophages, the levels of the cytokines IL-6, IL-1β, TNF-α, IL-10 were determined in the cell culture medium after 24h incubation with BLG. No significant increase of any of the measured cytokines was observed upon incubation of cells with processed BLG, when compared to the medium control or to BLG-NT (supplementary Figure 5-S3a). These findings demonstrated that uptake of processed BLG does not result in direct release of pro-inflammatory cytokines. However, when THP-1 cells were first pre-incubated with BLG-Lac followed by the re-stimulation of the cells with LPS, enhanced levels of IL-6 and IL-8 were measured compared to the cells pre-incubated with BLG-H and BLG-NT (supplementary material Figure 5-S3b).

5.3.5 *Agglomerates formed during heating and glycation of BLG show higher potential to bind to the receptor present on APCs than the low molecular weight fractions*

As the next step, we aimed to specify which fractions of heated and glycated BLG are responsible for binding and internalisation by THP-1 macrophages. For that reason, we fractionated processed BLG into two fractions with molecular weight (MW) above and below 100 kDa. The purity of the fractions was checked by Native-PAGE, which showed that fraction with MW>100 kDa was not contaminated with monomeric BLG (Figure 5.5c).
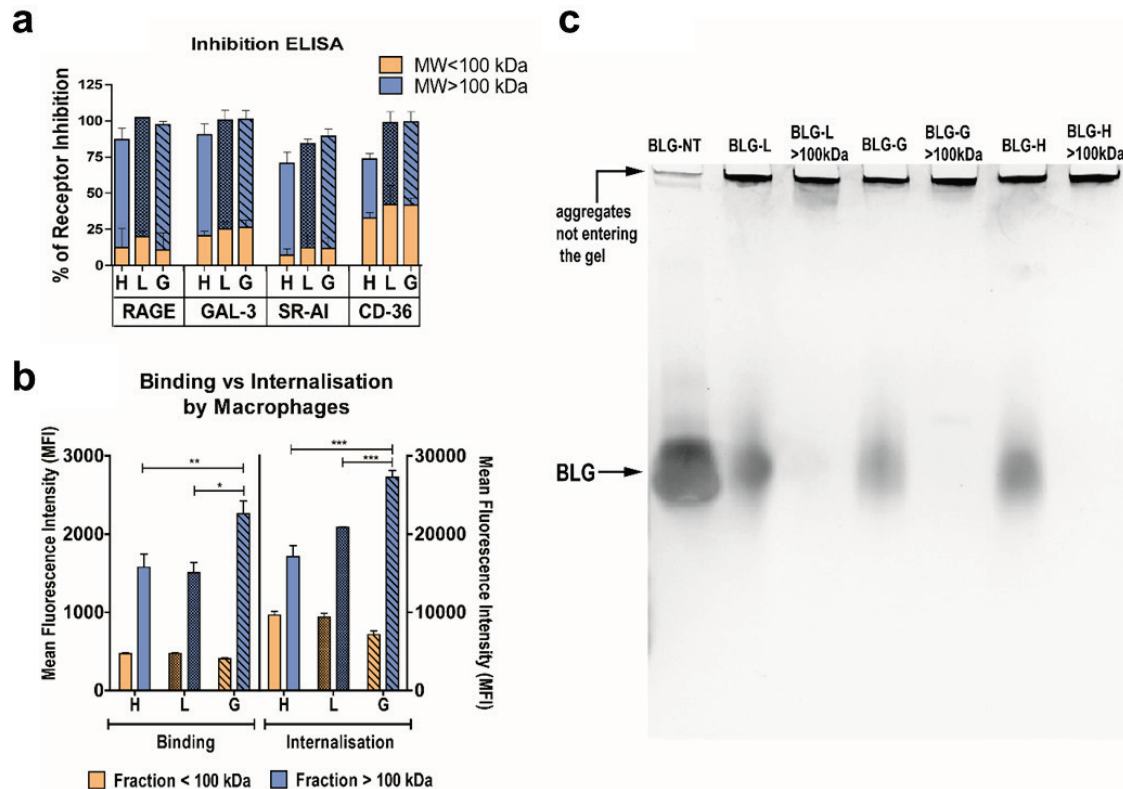
Figure 5.5: Binding and internalisation of aggregates vs. low molecular fractions of heated and glycated BLG. (a) Processed BLG: heated (H), heated with lactose (L) or heated with glucose (G) was fractionated using Amicon-Ultra Centrifugal Filters into two fractions: high molecular weight fraction >100 kDa and low molecular fraction <100 kDa. Both fractions were subjected to (a) receptor binding assay using inhibition ELISA, (b) binding/internalisation assay using THP-1 macrophages. Binding to THP-1 macrophages was detected with extracellular staining using FITC conjugated anti-BLG antibodies (b, left Y axis) and internalised BLG was measured by intracellular staining with Alexa Fluor 647 conjugated anti-BLG antibodies (b, right Y axis). (c) Composition of the BLG samples as well as the purity of the fraction >100 kDa was analysed by native PAGE. Data shown as mean ± SD of triplicate wells and are representative of at three (a) or two (b) independent experiments. Significant differences analysed with one-way ANOVA with Tukey post hoc comparison test; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

Moreover, the protein separation on the gel reveals that heating promotes the formation of aggregates that were visible on the top of the gel in all the samples, independent of the presence of sugar during heating. Subsequently, both fractions were analysed in receptor binding ELISA allowing to identify the fraction with MW >100 kDa as the most potent for binding to all studied receptors (Figure 5.5a). Finally, the binding assay employing THP-1 macrophages showed more potent binding and internalisation of the aggregates (Figure 5.5a). This shows that heat induced aggregation is a major force promoting internalisation of heated and glycated BLG by the receptors present on APCs. Interestingly, aggregates obtained from BLG-Glu were shown to bind and be internalised significantly more efficient than the aggregates obtained from BLG-H and BLG-Lac (Figure 5.5b).

5.3.6 *Glycated BLG agglomerates are partially resistant to digestion*

Lastly, we verified the physiological relevance of BLG aggregates by subjecting them to an *in vitro* digestion model mimicking gastrointestinal digestion in the infant's stomach and small intestine. At first, the binding of the BLG digest to sRAGE, Gal-3, CD36 and SR-AI was evaluated in an ELISA receptor binding test and compared to the binding of enzyme matrix and digest of BLG-NT (Figure 5.6a).
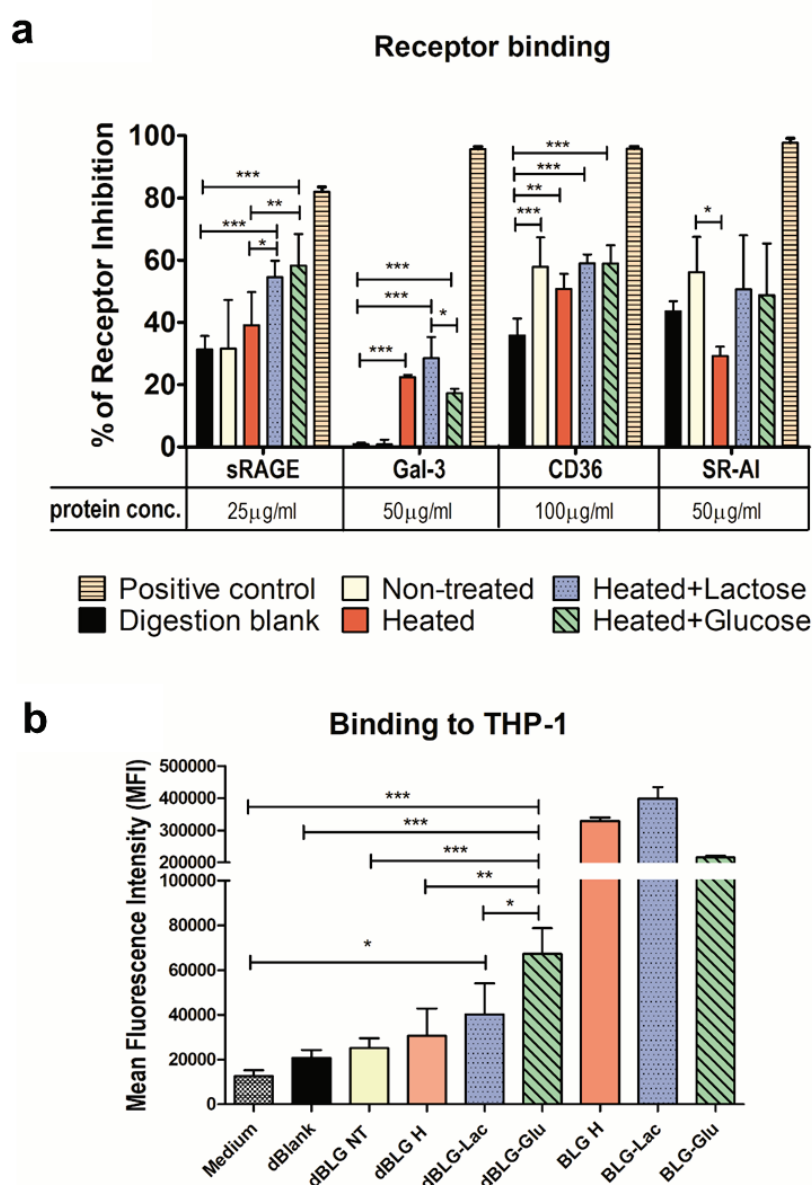
Figure 5.6: Immunoreactivity of heated and glycated BLG after enzymatic digestion in infant gastrointestinal static *in vitro* model. BLG: Non-treated, heated (BLG-H), heated with lactose (BLG-Lac) or heated with glucose (BLG-Glu) were subjected to gastric and intestinal phase of enzymatic digestion and immunogenicity of digested samples was measured as (a) binding to sRAGE, Gal-3, CD36 and SR-AI in inhibition ELISAs, (b) binding assay to THP-1 macrophages using Alexa Fluor 647 conjugated anti-BLG antibodies for flow cytometry detection. Data shown as mean ± SD of triplicate wells and are representative of at three (a) or two (b) independent experiments. Significant differences analysed with one-way ANOVA with Tukey post hoc comparison test; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

Digests of BLG-Glu and BLG-Lac showed a significantly enhanced binding to sRAGE when compared to digests of BLG-H and BLG-NT. Gal-3 showed significantly higher binding to digests of BLG-H, BLG-Glu, and BLG-Lac as compared to BLG-NT. The binding of the digests of BLG-H, BLG-Glu, and BLG-Lac to the scavenger receptors (CD36 and SR-AI) was on a comparable (BLG-Glu and BLG-Lac) or lower (BLG-H) level than the binding to the digest of BLG-NT. Next, the binding of all BLG digests to the THP-1 macrophages was studied. The binding of digested BLG was in general significantly reduced when compared to undigested BLG. However, the digested BLG-Glu was still successfully detected on the surface of THP-1 macrophages by fluorescently labelled anti-BLG antibodies (Figure 5.6b). Peptides released from BLG-Glu retained 30% of the binding capacity of non-digested material showing significantly higher binding compared to BLG-NT, BLG-H, and BLG-Lac. Digested BLG-Lac showed a similar trend which, however, was not statistically significant. This result shows that aggregates formed especially during heating of BLG in the presence of glucose maintain their immunogenicity after *in vitro* gastrointestinal digestion.

5.4 Discussion

CM allergy is one of the most common food allergies in early childhood [27] and heat processing of milk has been implicated in increased allergenicity of CM proteins [2,12,13]. The first step of allergic sensitisation is internalisation of antigen by APCs and presentation to T-cells. Heat-treated BLG was already shown in murine *in vivo* studies to be taken up more efficiently through the Peyer's patches than through absorptive epithelial cells, allowing the protein to interact with APCs [12]. However, the mechanisms by which heated but also glycated BLG is recognised by APCs and the relevance of these interactions to allergic sensitisation remain unclear. In this study we described mechanisms by which heat treated and glycated BLG is recognised by APCs. We evaluated the relative importance of aggregation vs. glycation as well as glycation with glucose vs. lactose in terms of receptor binding and internalisation. Lastly, since an immunogenicity of processed BLG *in vivo* would be modulated by gastrointestinal digestion, we therefore included an infant digestion model of BLG to come closer to the actual *in vivo* situation.

Cellular binding assays using THP-1 macrophages revealed enhanced binding and uptake of heated and glycated BLG but no differences between these two treatments were observed (Figure 5.3). This suggests that enhanced internalisation of non-digested BLG is dominantly caused by heating and to a lesser extent by glycation. Increased binding and uptake of both heated and glycated BLG was observed also in human MoDCs confirming in primary cells the results observed in the THP-1 model. Using receptor specific inhibition ELISA we identified RAGE, Gal-3, CD36 and SR-AI as the receptors recognizing heated and glycated BLG but not BLG-NT (Figure 5.1). This finding was supported by cellular assays showing that pre-incubation of the cells with receptor-specific inhibitors significantly reduced binding of heated and glycated BLG to THP-1 macrophages (Figure 5.4). These results confirm previous

finding which indicated RAGE, Gal-3 and receptors from the scavenger family as the main receptors recognizing glycated food proteins [28-32]. Also BLG heated and glycated in the presence of other WPs showed the binding to the studied receptors in ELISA and was detectable on the surface of THP-1 macrophages using anti-BLG antibodies. Therefore, despite the structural changes and protein crosslinking caused by heating in the presence of WPs, the BLG was recognised by studied receptors. This illustrates a major influential role of BLG, as one of the components of the WP fraction, in interacting with APCs. Interaction of glycated BLG with APC was reported previously [8,19,28], but has never been directly compared with heated BLG from the perspective of binding and internalisation by APCs.

To explore further effects of heating vs. glycation on the immunogenicity of BLG, we tested separately the binding and internalisation of size-separated aggregates vs. low molecular weight fractions obtained from BLG-H, BLG-Glu and BLG-Lac. Aggregated BLG showed higher binding to all studied receptors as well as more efficient uptake by THP-1 macrophages than lower molecular weight fractions (Figure 5.5). Increased exposure of β-sheet structures and hydrophobic patterns detected on BLG-H, BLG-Glu and BLG-Lac suggest that these structures drive the binding of aggregated BLG to studied receptors. The amyloid structure, defined as predominantly containing cross β-sheet patterns [33], may promote binding of heated BLG to RAGE and CD36 since both receptors are already known to bind amyloid-β [34,35]. Next to exposed β-sheets, hydrophobicity is a second physicochemical feature dictating the immune response [36] for instance of the well-known damage-associated molecular patterns like the lipid-A fraction of LPS or peptidoglycan [37]. Hydrophobic patterns exposed on the surface of protein due to the heat treatment may interact non-specifically with each other forming aggregates of different sizes [37,38] subsequently leading to the exposure of additional β-sheets. This processes, as observed also in our study, created all together the immunogenic profile of processed BLG recognised by APCs. This stays in line with the findings of Deng *et al.* [19] who previously suggested that heat-induced structural changes, but not glycation, predispose BLG to be recognised by THP-1 cells.

Although, for non-fractionated BLG, heating seemed to be a driving force for binding to the receptors, the aggregates isolated from BLG-Glu showed enhanced binding and uptake by THP-1 cells compared to the aggregates isolated from BLG-H and BLG-Lac. This indicates that glycation of BLG, especially with glucose, also plays a role in the generation of specific binding ligands for receptors present on APCs. Glucose, having a shorter carbonic chain than lactose, was shown to be more reactive and modifying more amino groups of BLG than lactose [39]. This was confirmed in our study by higher levels of furosine detected in BLG-Glu compared to BLG-Lac. Furosine indicates the level of early MR products (Amadori products) like fructosyllysine or lactulosyllysine. Therefore, the higher level of Amadori products detected in BLG-Glu may further explain the enhanced binding of BLG-Glu to THP-1 cells which may be potentially mediated by fructosyllysine-specific receptors [40]. Another explanation of the enhanced binding of BLG-Glu to THP-1 macrophages provides the MG-DH1

modification detected only in BLG-Glu. Methylglyoxal (MGO), a highly reactive compound formed due to the spontaneous degradation of glucose, may further react with lysine and arginine residues forming MGO-modified proteins [41]. MGO-modified proteins were shown to be highly immuno-reactive [42] possibly also via interaction with RAGE [43]. Therefore, MGO-modifications of BLG-Glu may contribute to its interaction with APCs. This reveals an essential difference between glycation of milk proteins with naturally present lactose during e.g. milk processing and the glycation with glucose typically used to illustrate an effect of glycation of food proteins [8,29,30].

As both heat-induced and glycation-induced aggregates are internalised by APCs via specific receptors (Figure 5.4 and Figure 5.5), we subsequently evaluated if the aggregates retain their receptor binding capacity after *in vitro* enzymatic digestion mimicking the infants' gastrointestinal conditions. Although the binding of glycated proteins to studied receptors was already shown [15,17,29-31], the susceptibility of the binding sites to enzymatic digestion has not been well studied. Our data showed that BLG-Glu and BLG-Lac were still recognised by sRAGE after digestion, demonstrating significantly higher binding compared to BLG-NT and BLG-H. Moreover, digested BLG-Glu was detected on the surface of APCs indicating the binding to the receptors. Blocking experiments with the RAGE antagonist FPS-ZM1 demonstrated that RAGE is not involved in internalisation of processed BLG confirming its role in signal transduction. Internalisation may thus occur through Gal-3, which showed the binding to the digests of BLG-H, BLG-Lac and BLG-Glu. The binding of digests of glycated and heated BLG to scavenger receptors was not higher than BLG-NT and control sample containing enzymes only (Figure 5.6). This suggests that aggregation is a dominant factor determining the binding capacity of BLG to scavenger receptors, although the role of these receptors after digestion is minor. Summarizing, our results indicate that the molecular mechanisms via which digested glycated BLG triggers an immune response are mediated via RAGE on the level of innate immunity and via Gal-3 on the level of the adaptive immune compartment.

RAGE is known to be implicated in a wide range of inflammatory reactions including systemic but also local intestinal and airway inflammation [44,45]. In murine models of asthma and allergic airway inflammation, RAGE has been shown to promote the expression of the type 2 cytokines IL-5 and IL-13 [46], but also IL-33 leading to the accumulation of type 2 innate lymphoid cell in the lungs [47]. These studies demonstrate an essential role of RAGE in allergic airway inflammation, however the link between RAGE and the development of food allergy is not directly proven yet. Smith *et al*. [18] in the false alarm hypothesis, proposed that AGEs present in the diet contribute to the development of food allergies via interaction with RAGE consequently priming immune signalling. RAGE is highly expressed on DCs, macrophages, T-lymphocytes, and B-cells [44] regulating such a processes as migration of DCs to the lymph nodes [48] as well as induction of T-cell proliferation [49]. Therefore, the authors of the false alarm hypothesis suggested that the signalling and priming of immune cells by RAGE-activated

APCs is likely important [18]. Our study supports this hypothesis by showing that RAGE ligands formed as an effect of glycation of BLG retain their activity after enzymatic digestion. This suggests that *in vivo*, the relative importance of glycation is higher than that of heat-induced aggregation. In the used model, the binding and uptake of BLG did not trigger the direct release of pro-inflammatory cytokines, however the re-stimulation of the THP-1 macrophages with LPS lead to enhanced IL-6 and IL-8 responses (supplementary material: Figure 5-S3) suggesting priming of the cells. However, to understand better the role of glycation in allergic sensitisation other models, like human DCs, are needed.

5.5   Conclusions

In conclusion, this study showed that heat treatment of BLG generates ligands for RAGE, Gal-3, CD-36 and SR-AI as a results of both (1) heat treatment with as consequence increased hydrophobicity, exposure of β-sheets, and aggregation, and (2) glycation (predominantly with glucose) possibly connected to the formation of fructosyllysine and/or MGO. The importance of glycation *in vivo* was suggested after gastrointestinal digestion of aggregates, showing that glycated aggregates are less sensitive to digestion and therefore maintain their binding capacity to RAGE and Gal-3. On the basis of these results, we propose that post-digestion retained immunogenicity of glycated BLG may be mediated via RAGE as a signalling pathway, and via Gal-3 as a putative receptor responsible for enhanced antigen uptake and processing, possibly activating the adaptive immune system. However, future studies are needed to confirm this in humans and animal models of allergic sensitisation.

Supplementary material
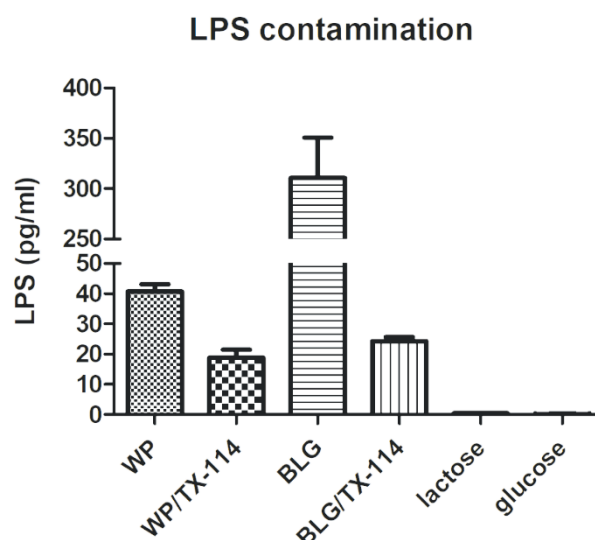
LPS contamination

Figure 5-S1: LPS content in the BLG and WP samples before and after LPS removal. LPS was removed from the samples using Triton-X 114 method and the LPS contamination was measured using Endozyme® II assay. The levels of LPS the samples after Triton-X 114 purification were low: WP 18.83 pg/ml ± 2.674 and BLG 24.32 pg/ml ± 1.320 reaching after dilution in *in vitro* test concentrations below 2 pg/ml. Spike control (data not shown) showed that the matrix does not influence the readout in whey and BLG. Sugars used for glycation of WP and BLG were LPS free.
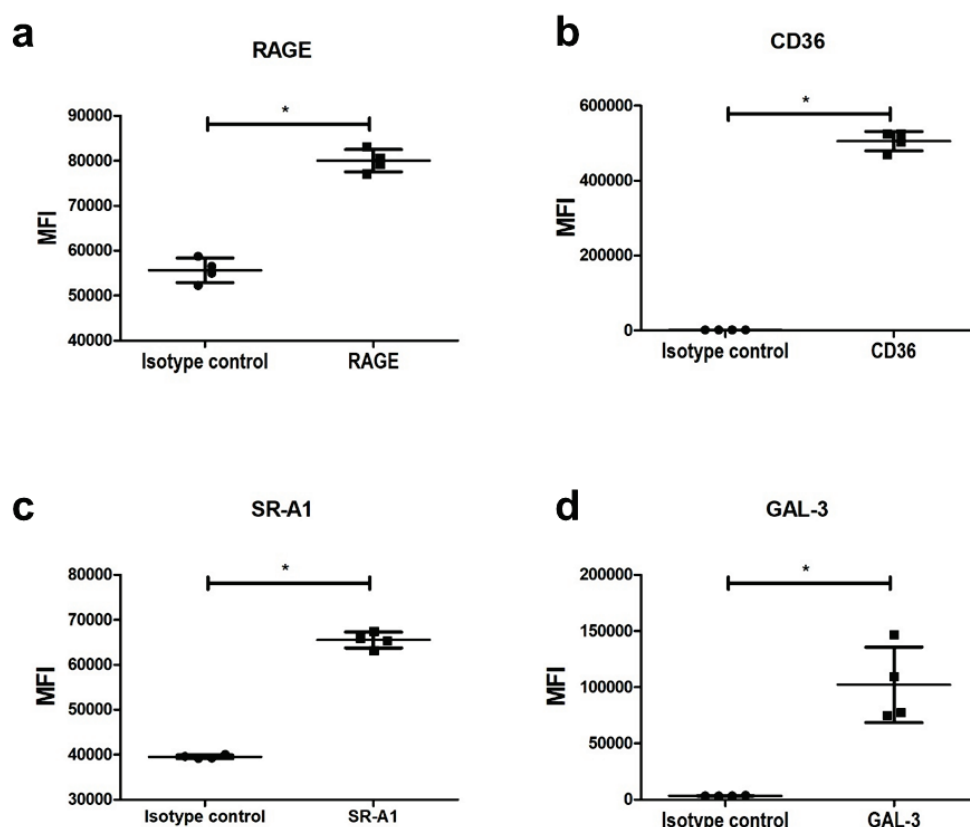
Figure 5-S2: Expression of receptors that are involved in the uptake process of glycated BLG on PMA-differentiated THP-1 macrophages. The samples were analysed with flow cytometry. The vertical scatter plots resemble the MFIs obtained from cells treated either with the specific anti-receptor antibodies or with respective isotype controls. The error bars represent the standard deviation. The statistical significance of the data was evaluated by performing Mann-Whitney, two-tailed statistical analysis. n.s. $p > 0.05$, * $p < 0.05$.
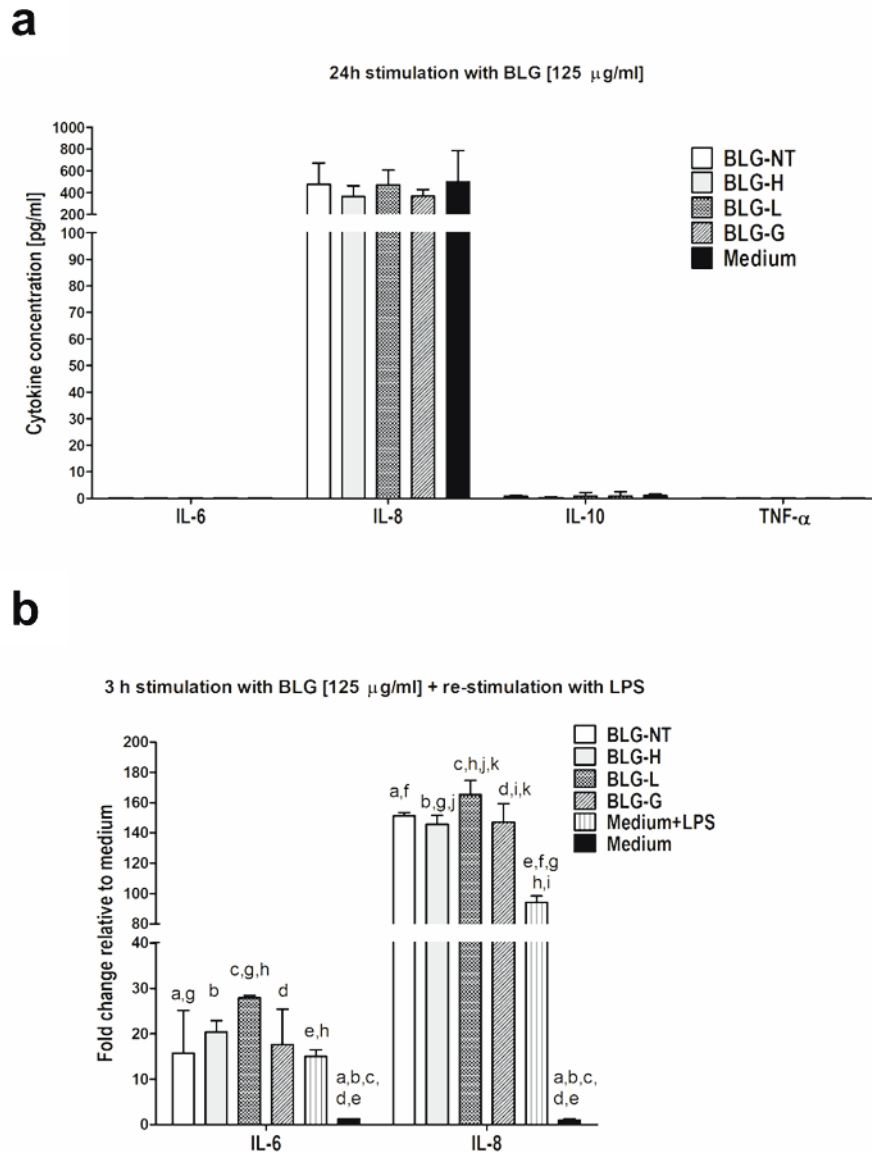
Figure 5-S3: The secretion of pro-inflammatory cytokines by THP-1 macrophages after 24 hours-long incubation with glycated and heated BLG (a) or 3 hours-long incubation with glycated and heated BLG followed with re-stimulation with LPS (b). PMA differentiated THP-1 macrophages were incubated in a presence of BLG: non-treated (BLG-NT), heated (BLG-H), heated with lactose (BLG-Lac) or heated with glucose (BLG-Glu) for 24h (a) or 3h-long followed by re-stimulation with LPS (b). The levels of cytokines in supernatants (IL-1β, IL-6, IL-8, IL-10 and TNF-a) were assessed with flow cytometry. Data shown as mean ± SD of triplicate wells and are representative of two independent experiments. Significant differences analysed with one-way ANOVA with Tukey post hoc comparison test are indicated with the same letters. Statistically significant differences were considered at p < 0.05.

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Chapter 6

Peptide release after simulated infant in vitro digestion of dry-heated cow's milk protein and transport of potentially immunoreactive peptides across the Caco-2 cell monolayer

This chapter is based on:

Abstract:

Dry heating of cow's milk protein, as applied in the production of "baked milk", facilitates the resolution of cow's milk allergy symptoms upon digestion. The heating and glycation-induced changes of the protein structure can affect both digestibility and immunoreactivity. The immunological consequences may be due to changes in the peptide profile of the digested dry-heated milk protein. Therefore, cow's milk protein powder was heated at low temperature (60 °C) and high temperature (130 °C) and applied to simulated infant *in vitro* digestion. Digestion-derived peptides after 10 min and 60 min in the intestinal phase were measured using LC-MS/MS. Moreover, digests after 10 min intestinal digestion were applied to a Caco-2 cell monolayer. T-cell epitopes were analysed using a prediction software, while sIgE binding epitopes were identified based on literature. The largest number of sIgE binding epitopes was found in unheated samples, while T-cell epitopes were equally represented in all samples. Transport of glycated peptide indicated a preference for glucosyllysine and lactosyllysine-modified peptides, while transport of peptides containing epitopes structures was limited. This showed that the release of immunoreactive peptides can be affected by the applied heating conditions, however availability of peptides containing epitopes might be limited.

6.1 Introduction

Cow's milk protein (MP) consists of two major protein fractions, casein and whey protein, and is an important protein source in infant nutrition. Heating and glycation of MP has been shown to alter its digestibility and immunogenicity. Dry heating, as applied in this study, is not commonly used in the dairy industry, however has an important role in mimicking the heat treatment, when MP powder is baked into a muffin or waffle. These products are often referred to as "baked milk" and have been shown to accelerate the resolution of cow's milk allergy symptoms in allergic children [1]. Under these heating conditions (low water activity (a_w), high sugar content, high temperature), protein aggregation and modifications via the Maillard reaction (MR) are favoured [2]. The MR is the reaction between primary amino-groups of proteins, peptides and amino acids and the reactive carbonyl group of reducing sugars, for instance lactose. During the early stage of the MR, the initial condensation to the Amadori product occurs followed by a rearrangement to lactosyllysine or glucosyllysine. In the advanced stage of the MR, a pool of different advanced glycation end products (AGEs) are formed [3]. Amongst these, N^ε-carboxymethyllysine (CML) has been used as a marker for the advanced stage of the MR and is one of the most abundant AGEs in processed dairy products [4,5]. The extent of the MR can affect the digestibility and immunoreactivity of MP. With respect to digestibility, the effect of thermal processing of milk and dairy products on peptides generation during gastrointestinal digestion *in vitro* and *in vivo* has been subject to a several studies [6-11]. Moreover, it was shown that lysine blockage via the MR affects peptide size distribution after simulated infant *in vitro* digestion of infant formula [12] and that glycation of isolated milk proteins changes the composition of the peptides in the digest [13]. Next to this, heating and glycation can also affect immunogenicity and allergenicity of MP [14]. For example, binding of immunoglobulin E (IgE) to either isolated MP or MP in mixture has been shown to be decreased for extensively glycated milk proteins, possibly related to a masking effect on epitopes [15-17]. However, the effects of glycation and protein aggregation under the applied heating conditions are difficult to disentangle and can both affect IgE binding [18]. Moreover, Corzo-Martínez *et al.* [19] also showed that impaired digestibility may increase the residual allergenicity after *in vitro* digestion, comparing heat-glycated and unheated β-lactoglobulin (BLG). Differences in the peptide profiles after ingestion of dry-heated MP vs. unheated MP could affect the immunological response by differential preservation or glycation induced-modification of linear specific IgE (sIgE) binding epitopes. Moreover, the generation of peptides carrying a glycation structure can modulate the inflammatory response by binding to the receptors for AGEs on antigen presenting cells [14,20]. Binding of AGEs to AGE receptors has particularly been shown for protein-bound CML and pyrraline [21,22], while for peptide bound AGEs this was only demonstrated for CML [20]. The availability of AGE-modified peptides to the gastrointestinal immune system by means of translocation across the epithelial barrier is an important determinant in the immunological response to a foreign antigen. The metabolic transit of AGEs has been shown in previous literature on the excretion

of CML and pyrraline in urine [23,24]. Moreover, the translocation across the Caco-2 cell monolayer has been shown for lactosylated and CML-modified dipeptides [25]. However, to our knowledge the transport of food derived glycated peptides larger than two amino acids has not yet been investigated. Transport of larger glycated peptides can be an important factor for the binding of AGE-modified peptides as it has been suggested that CML is more abundant in fractions of *in vitro* digests that are larger than 1 kDa [26]. Therefore, transport of larger AGE-modified peptides could also contribute to the pool of dietary derived AGEs. This could be crucial as it has been shown on the example of CML that binding to AGE receptors is dependent on the concentration in which the CML is present in the vicinity of the receptors [22]. In this study, the peptide profiles of low temperature (LT) and high temperature (HT) heated MP after simulated infant *in vitro* digestion was compared to that of non-treated milk (NT). The most abundant glycation induced post translational modifications (PTMs), including CML and pyrraline as potential AGE receptor ligands, were monitored before and after transport across a Caco-2 cell monolayer. Special attention was given to sIgE binding epitopes and T-cell epitopes to asses immunomodulatory potential of the digest on the peptide level.

6.2 Materials and Methods

6.2.1 Chemicals

Dulbecco's Modified Eagles Medium supplemented with high glucose, HEPES, L-glutamine (42430082), both with and without phenol red as well as trypsin-EDTA (0.25%) with phenol red, and HyClone™ Fetal Bovine Serum were purchased from Thermo Fisher Scientific (Waltham, USA). All other chemicals were obtained from Merck KGaA (Darmstadt, Germany).

6.2.2 Preparation of milk powders and heat treatment

Liquid raw cow's MP concentrate was obtained from FrieslandCampina (Wageningen, Netherlands) and was composed of micellar casein (MCI88 liquid) and whey protein (acid WPC80 liquid) in the ratio 80:20. After the addition of lactose in the ratio 1:1.5 (protein/lactose) the solutions were freeze dried. Heat treatment was applied at two different temperatures and durations as described elsewhere [27]. Briefly, for LT heated MP (LT-MP), the powder was heated for 3 weeks at 60 °C (a_w 0.23) and for HT heated MP (HT-MP) the powder was heated for 10 min at 130 °C (a_w 0.23). An unheated part of the milk powder was as heating control (NT-MP).

6.2.3 Infant in vitro digestion

Simulated infant *in vitro* digestion was conducted in duplicate as published elsewhere and was based on the protocol by Ménard *et al.* [28] with adaptions specific for the type of product described elsewhere [27]. Briefly, protein concentration of the meal was set to 1.2%. Digestion in the gastric phase (GP) was conducted for 60 min with a pepsin activity set to 268 U/mL and

at pH 5.3, however without the use of gastric lipase. Digestion in the intestinal phase (IP) was conducted for 60 min using pancreatin adjusted for its trypsin activity that was set to 16 U/mL digest and at pH 6.6. Samples were taken after 10 min and 60 min in the IP and stopped by the addition of 0.5 mM Pefabloc in the ratio Pefabloc/digest of 1/20 (v/v).

6.2.4   *Caco-2 cell culture*

The Caco-2 cell line was purchased from the American Type Culture Collection (Manassas, Virginia, USA). They were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) at 37 °C and in humidified atmosphere containing 5% CO_2. Cells were sub-cultured weekly upon confluence 85-95% using trypsination. Caco-2 cells were used from passage 30-40 and seeded into 24-well transwells (Greiner Bio-One, Kremsmünster, Austria) at a concentration of 0.225×10^6 cells/mL in DMEM with 10% heat inactivated FBS. The medium was changed, apical (150 µL) and basolateral (700 µL) every 2-3 days and cells were used for the transport experiment after 21 days of incubation. Before transport experiments, the transepithelial electrical resistance (TEER) value was measured and only wells with a TEER value higher than 750 Ω*cm2 were used.

6.2.5   *Transport across the Caco-2 cell monolayer*

Digest were diluted 1:1 with DMEM without phenol red supplemented with 0.1% penicillin-streptomycin (10,000 U/mL) and applied to the apical side of the Caco-2 cell monolayer. TEER was measured at 37 °C using a Millicell-ERS Ω Meter (Millipore, Molshein, France) and samples were incubated for 2 h with the Caco-2 cells at 37 °C and 5% CO_2 saturation. After that, TEER was measured and samples were taken from the basolateral side. Each sample was applied in duplicate. Samples were kept at -20 °C until further analysis.

6.2.6   *Peptide analysis*

Digests after 10 min in the IP contained 3.6 mg/mL, 3.7 mg/mL, 3.6 mg/mL protein in NT-MP, LT-MP, and HT-MP, respectively. Digest after 60 min in the IP contained 4.2 mg/mL, 3.5 mg/mL, 3.8 mg/mL protein in NT-MP, LT-MP, and HT-MP, respectively. Samples were mixed 1:1 with trichloroacetic acid (20%) and centrifuged (10 min, 3500 ×g, 4 °C). The supernatants were cleaned using an in-house stage tip following a protocol described by Dingess *et al.* [29]. All samples were concentrated to compensate for the dilution during trichloroacetic acid precipitation.

Peptides were analysed on a on a Thermo nLC 1000 system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled to a LTQ orbitrap XL (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for peptides in the in vitro digest or Q Exactive HF-X X (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for peptides on the basolateral side as well as glycated peptides. Each sample was measured once. Chromatographic separation was

conducted over a 0.10 * 250 mm ReproSil-Pur 120 C18-AQ 1.9 μm beads analytical column. A gradient consisting of acetonitrile in water, spiked with 0.1% formic acid was used. Acetonitrile increased from 9% to 34% within 50 min using a flow rate of 0.5 μL/min. Full scan positive mode spectra were measured in the Orbitrap between m/z 380 and 1400 using high resolution (60,000). CID LTQ or HCD (Q Exactive HF-X) fragmentation was applied using isolation width of 2 m/z respectively 1.2 m/z and 30% respectively 24% normalised collision energy (CE). MSMS scans were recorded in the data dependent mode for 2-3 respectively 2-5+ charged peaks in the MS scan. For glycated peptides measured by the Q Exactive HF-X, a step CE (20-30-40) was used based on the method published by Liu *et al.* [30]. LC-MS/MS runs were processed using the MaxQuant version 1.6.3.4 with the Andromeda search engine [31]. Digestion mode was set to "unspecific". A fixed modification was set for the formation of propionamide on cysteines, while variable modifications were set for acetylation of the peptide N-terminus, deamidation of asparagine and glutamine, as well as oxidation of methionine.

Peptides were identified using a bovine database from Uniprot (https://www.uniprot.org) that includes all bovine milk proteins observed by Boggs *et.al.* [32] (PRIDE PXD003011) in combination with a database for common contaminants. Peptides with a minimum length of 8 amino acids and maximum peptide length of 25 amino acids were identified. Both peptide and protein false discovery rates were set to 1%. Post translational modifications were included for lactosylation (+324 Da), hexose modification (+162 Da), N^{ε}-carboxymethyllysine modification (+58 Da), and pyrraline modification (+108 Da). For simplicity glucosyllysine was used to referred to the hexose modification, although also other hexoses could result in this mass shift. Phosphorylated and glycated peptides were not included in the quantitation during the MaxQuant search. Due to the limited number of measurements as well as the limitations in obtaining quantitative data from glycated peptides, data were reported as peptide count.

6.2.7 *Data analysis*

Data were filtered for peptides derived from the six major milk proteins: α_{s1}-casein, α_{s2}-casein, β-casein, β-lactoglobulin, and α-lactalbumin. All peptides with score >80 were used for the overall peptide profiles, while for the sIgE binding epitopes and T-cell epitopes, only peptides with a score >100 were used. For total peptide count per sample in the digest, each duplicate digestion was filtered separately for non-modified peptides (intensity >0) and for phosphorylated and glycated peptides (identification by matching and/or by MS/MS). For all further analysis, only peptides identified in both digestions of the same heat treatment were reported.

6.2.8 *sIgE binding epitope identification*

sIgE binding epitopes were identified by comparison of peptide sequences with sIgE binding epitopes as reviewed previously [33]. Peptides were reported as potential sIgE binding epitopes if their sequence matched ≥80% of the sequence of a known sIgE binding epitope.

6.2.9 *T-cell epitope prediction*

T-cell epitopes were predicted using IEDB MHC Class II Binding Prediction software (http://tools.iedb.org/mhcii/, 02.06.2020) where an MHC class II allele reference set was obtained from (https://help.iedb.org/hc/en-us/articles/114094151851, 02.06.2020). The default method "IEDB recommended 2.2" was used for T-cell epitope predictions. All peptides within the size range 15-24 amino acids length, which was previously be reported as the size range for T-cell epitopes, were applied to the prediction software [34]. Peptides were reported as potential T-cell epitopes following the recommendations of the prediction tool, where each peptide reaching a percentile rank <10.0% can be considered as potential T-cell epitope.

6.3   Results

6.3.1 *Identification of peptides in in vitro digests*

Peptides released upon infant *in vitro* digestion after 10 and 60 min in the IP were analysed using LC-MS/MS. Only peptides derived from the six major MPs, α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, BLG, and α-lactalbumin (ALA) were considered in the data analysis. Dry heating of MP decreased the number of peptides released upon infant *in vitro* digestion (Figure 6.1), where HT heating resulted in even less peptides than LT heating after 10 min (315 ± 36 vs. 369 ± 26 peptides) and 60 min (207 ± 1 vs. 246 ± 7 peptides) in the IP.
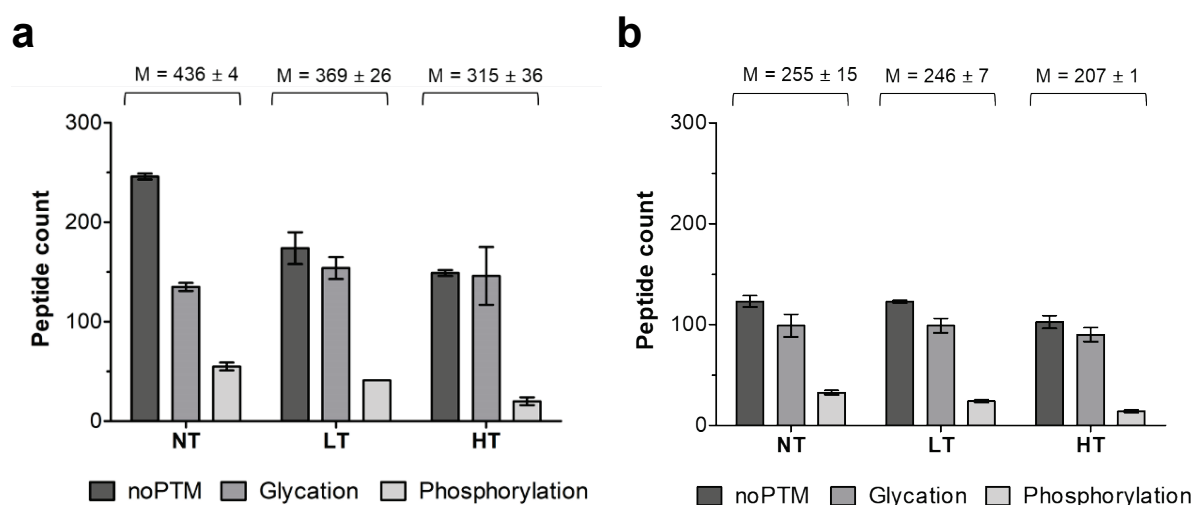
Figure 6.1: Total count of non-modified, glycated and phosphorylated digestion-derived peptides derived from cow's milk protein. Samples were non-treated (NT), heated at low temperatures (LT), and heated at high temperature (HT) (a) after 10 min in the intestinal phase and (b) after 60 min in the intestinal phase. Number of peptides without post translational modification (noPTM), glycated, and phosphorylated peptides were compared. The minimum length for identification was 8 amino acids. Error bars represent the standard deviation of duplicate digestions. The mean (M) of the total count of peptides per treatment and digestion time point ± standard deviation for duplicate digestions is shown above the bars.

Differences in the modification state of the peptides (non-modified vs. glycated vs. phosphorylated peptides) were higher after 10 min than after 60 min in the IP. After 10 min in the IP, heated samples showed comparable levels of glycated and non-modified peptides, while the NT-MP sample had 2-fold more non-modified peptides than glycated peptides. At the same

time, the number of phosphorylated peptides was 4.6-fold lower in HT-MP compared to NT-MP after 10 min in the IP, while LT-MP only showed a 1.6-fold decrease. This trend continued until 60 min in the IP, however to a lesser extent. Most peptides after 10 min in the IP were derived from β-casein, followed by BLG and α_{s1}-casein, while a smaller number of peptides originated from α_{s2}-casein, followed by κ-casein, and ALA (supplementary material: Figure 6-S1a). This trend did not change after 60 min in the IP (supplementary material: Figure 6-S1b).

In line with the number of peptides per protein, peptides generated after 10 min in the IP covered large parts of the protein sequences of β-casein, BLG, and α-caseins (Figure 6.2a).

a
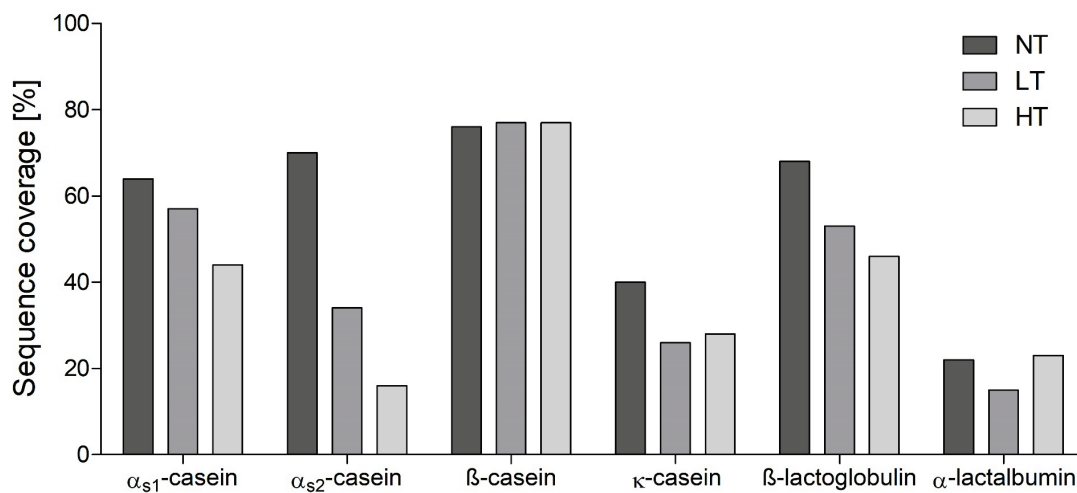
b
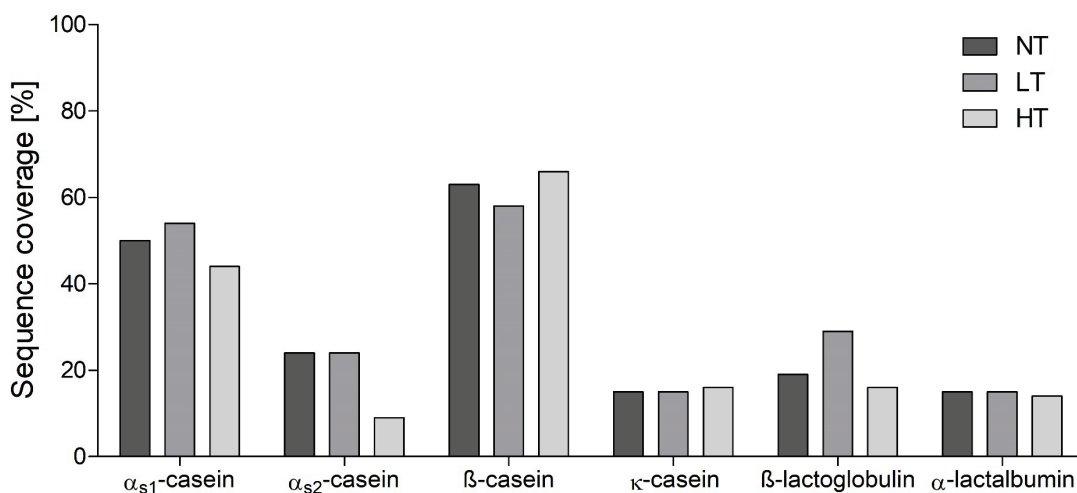
Figure 6.2: Sequence coverage of the 6 main milk proteins by digestion-derived peptides, with and without post translational modification, derived from *in vitro* digests of cow's milk protein (a) after 10 min in the intestinal phase and (b) after 60 min in the intestinal phase. Samples were non-treated (NT), heated at low temperature (LT), and heated at high temperature (HT).

Sequence coverage was higher for NT-MP compared to LT-MP and HT-MP. This difference was highest for the α-caseins and BLG, while β-casein showed no changes. After 60 min in the IP, only α_{s2}-casein showed remarkably lower sequence coverage in HT-MP compared to NT-MP and LT-MP (Figure 6.2b). Due to the larger differences observed after 10 min in the IP, and the possibility of an immune response already at this stage of digestion, we focused mainly on the samples from 10 min in the IP.

In Figure 6.3, peptide sequence alignment is shown for peptides generated after 10 min in the IP. Independent from the heat treatment, all proteins showed specific regions that were similarly covered in all samples, however with different numbers of peptides generated in the same region.

a

α_{s1}-casein

b (continuation Figure 6.3)

α_{s2}-casein


```
         10                20                30              40            50
M K F F I F T C L L A V A L A | K N T M E H V S S S E E S I I S Q E T Y K Q E K N M A I N P S K E N L
        Signal peptide

         60                70                80              90           100
C S T F C K E V V R N A N E E E Y S I G S S S E E S A E V A T E E V K I T V D D K H Y Q K A L N E I

        110               120               130             140          150
N Q F Y Q K F P Q Y L Q Y L Y Q G P I V L N P W D Q V K R N A V P I T P T L N R E Q L S T S E E N S

        160               170               180             190          200
K K T V D M E S T E V F T K K T K L T E E E K N R L N F L K K I S Q R Y Q K F A L P Q Y L K T V Y Q

        210               220
H Q K A M K P W I Q P K T K V I P Y V R Y L
```

c

β-casein


```
         10                20                30              40            50
M K V L I L A C L V A L A L A | R E L E E L N V P G E I V E S L S S S E E S I T R I N K K I E K F Q S
        Signal peptide

         60                70                80              90           100
E E Q Q Q T E D E L Q D K I H P F A Q T Q S L V Y P F P G P I P N S L P Q N I P P L T Q T P V V V P

        110               120               130             140          150
P F L Q P E V M G V S K V K E A M A P K H K E M P F P K Y P V E P F T E S Q S L T L T D V E N L H L

        160               170               180             190          200
P L P L L Q S W M H Q P H Q P L P P T V M F P P Q S V L S L S Q S K V L P V P Q K A V P Y P Q R D M

        210               220
P I Q A F L L Y Q E P V L G P V R G P F P I I V
```

d (continuation Figure 6.3)

κ-casein


```
            10                    20            30      40          50
M M K S F F L V V T I L A L T L P F L G A Q E Q N Q E Q P I R C E K D E R F F S D K I A K Y I P I Q
            Signal peptide

            60                70              80            90            100
Y V L S R Y P S Y G L N Y Y Q Q K P V A L I N N Q F L P Y P Y Y A K P A A V R S P A Q I L Q W Q V L

            110            120          130          140            150
S N T V P A K S C Q A Q P T T M A R H P H P H L S F M A I P P K K N Q D K T E I P T I N T I A S G E

            160          170          180            190
P T S T P T T E A V E S T V A T L E D S P E V I E S P P E I N T V Q V T S T A V
```

e

β-lactoglobulin


```
            10              20          30          40          50
M K C L L L A L A L T C G A Q A L I V T Q T M K G L D I Q K V A G T W Y S L A M A A S D I S L L D A
            Signal peptide

            60              70          80          90        100
Q S A P L R V Y V E E L K P T P E G D L E I L L Q K W E N G E C A Q K K I I A E K T K I P A V F K I

            110          120          130          140          150
D A L N E N K V L V L D T D Y K K Y L L F C M E N S A E P E Q S L A C Q C L V R T P E V D D E A L E

            160          170
K F D K A L K A L P M H I R L S F N P T Q L E E Q C H I
```

f (continuation Figure 6.3)   **α-lactalbumin**

Figure 6.3: Sequence alignment of digestion-derived peptides from the six major milk proteins (a-f) identified after 10 min in the intestinal phase of simulated infant *in vitro* digestion of non-treated cow's milk protein (——), heated at low temperature (-----), and heated at high temperature (- - -). Glycated peptides (orange), phosphorylated peptides (blue), trypsin cleavage sites (⬇), chymotrypsin cleavage sites (⬇). Grey highlights indicated IgE binding epitopes according to [33]. Trypsin and chymotrypsin cleavage sites were determined using Expasy Bioinformatics Resource Portal (https://web.expasy.org/peptide_cutter/ last visited 08.06.2020).

In all proteins, most differences in peptide distribution along the protein sequence were observed between HT-MP and the other samples. The peptides derived from BLG (Figure 6.3e) came from three main regions (f17-39, f56-73, and f138-161) and only minor differences were observed between heat treatments in f56-73, while in f17-39 and f138-161, much less peptides were found in HT-MP compared to NT-MP and LT-MP. In contrast, peptides derived from α_{s2}-casein and β-casein where distributed all over the amino acids chain. Interestingly, most differences in caseins were observed as a result of the absence of phosphorylated peptides in HT-MP. In α_{s1}-casein (Figure 6.3a), the region between f52-92 was mostly covered by phosphorylated peptides in NT-MP and LT-MP, while this region did not lead to the formation of peptides in HT-MP. Moreover, the number of peptides covering the same sequence part f52-92 was much lower in LT-MP than NT-MP. Similar observations, where the number of phosphorylated peptides was lower in at least one of the heated samples compared to NT-MP, were also made for α_{s2}-casein f16-32, f68-84, f141-161(Figure 6.3b), β-casein f22-39 and f48-66 (Figure 6.3c), and κ-casein f165-182 (Figure 6.3d). In contrast, glycated peptides only had a minimal effect on differences in sequence coverage when comparing samples. In α_{s1}-casein (Figure 6.3a), the regions f135-138 and f210-213 and in β-casein (Figure 6.3c) the region 191-197 were covered in LT-MP and HT-MP as a result of the presence of glycated peptides. The presence of glycated peptide however affected the number of peptides which arise from specific areas of the proteins. This was especially seen for the region f140-155 of α_{s1}-casein in NT-MP, f113-128 of α_{s2}-casein, and f179-197 of β-casein in both LT-MP and HT-MP.

With progressing intestinal digestion, only little changes were observed in the peptide alignment along the protein sequence of α_{s1}-casein, β-casein, κ-casein, and ALA (supplementary material: Figure 6-S2). In contrast, BLG two resistant areas f57-73 and f139-154 as well as α_{s1}-casein at f119-134.

6.3.2   *Identification of sIgE epitopes and T-cell epitopes in the in vitro digests*

sIgE binding epitopes were identified by comparison with known epitopes from literature (Table 6.1) [33]. Peptides were reported as potential sIgE binding epitopes when at least 80% of the peptide sequence matched a known sIgE epitope sequence. Peptides derived from BLG contained 18 sIgE epitopes, followed by 16 derived from α_{s1}-casein, 14 from β-casein, 3 from α_{s2}-casein, and 1 from ALA and κ-casein, respectively. The majority of sIgE epitopes were found in peptides derived from NT-MP, however up to 69% of α_{s1}-casein derived sIgE epitopes and 77% of β-casein derived sIgE epitopes were also found in either one or both heated samples. The peptides α_{s1}-casein f189-213, α_{s2}-casein f116-128, and β-casein f96-110 were only found in heated samples, however their length only differed by a maximum of 4 amino acid from a similar peptide found in NT-MP and those 4 amino acids were not covering an additional sequence part of the sIgE binding epitope.

Table 6.1: sIgE binding epitopes identified in digestion-derived peptides after 10 min in the intestinal phase. Peptides were identified in cow's milk protein, non-treated (NT), heated at low temperature (LT), and heated at high temperature (HT), after simulated infant *in vitro* digestion and derived from casein (cn), β-lactoglobulin (BLG), and α-lactalbumin (ALA). Amino acids (AAs) position indicates the position within the proteins including the signal peptide. Peptides carrying a post translational modifications (PTM) are marked with phosphorylation (Phos), where phosphorylated serine (S) and threonine (T) residues are highlighted in bold and underline.

Protein	Sample	Peptide sequence	AAs position	sIgE epitope AAs position	PTM	
α_{s1}-cn	NT,LT,HT	VNEL**S**KDIG**SST**EDQ	52-67	54-63	Phos	
	NT,LT,HT	VNEL**S**KDIG**SST**EDQAMEDIK	52-73	54-63	Phos	
	NT,LT,HT	KVPQLEIVPN**S**AEE	120-133	124-135	Phos	
	NT,LT,HT	KVPQLEIVPN**S**AEER	120-134	124-135	Phos	
	NT,LT	VPQLEIVPN**S**AEER	121-134	124-135	Phos	
	NT,LT,HT	LEIVPN**S**AEE	124-133	124-135	Phos	
	NT,LT,HT	LEIVPN**S**AEER	124-134	124-135	Phos	
	NT	EIVPN**S**AEER	125-134	124-135	Phos	
	NT	KEGIHAQQKEPMIGV	139-153	137-147	N/A	
	NT	EGIHAQQKEPMIGV	140-153	141-155	N/A	
	NT,HT	GTQYTDAPSFSDIPNPI	185-201	186-200	N/A	
	NT,LT,HT	QYTDAPSFSDIPNPI	187-201	186-200	N/A	
	NT	QYTDAPSFSDIPNPIGSENSEK	187-208	188-209	N/A	
	NT,LT,HT	TDAPSFSDIPNPIGSENSEK	189-208	188-209	N/A	
	NT,LT,HT	TDAP**S**F**S**DIPNPIG**S**EN**S**EK	189-208	188-209	Phos	
	NT	TDAPSFSDIPNPIGSENSEKT	189-209	188-209	N/A	
α_{s2}-cn	NT	KNTMEHV**SSS**EE**S**IISQ	16-32	16-35	Phos	
	NT	KNTMEHV**SSS**EE**S**IISQET	16-34	16-35	Phos	
	HT	QGPIVLNPWDQVK	116-128	120-129	N/A	
β-cn	NT,LT	RELEELNVPGEIVE	16-29	16-31	N/A	
	NT	RELEELNVPGEIVE**S**L	16-31	16-31	Phos	*
	NT	ELEELNVPGEIVE**S**L	17-31	16-31	Phos	
	NT	TEDELQDKIHPFA	56-68	60-69	N/A	
	NT,LT,HT	SLVYPFPGPIPNS	72-84	70-85	N/A	
	NT,LT,HT	PVVVPPFLQPE	96-106	98-107	N/A	
	NT,LT,HT	PVVVPPFLQPE	96-107	98-107	N/A	
	NT,LT,HT	PVVVPPFLQPEVMG	96-109	98-107	N/A	
	LT,HT	PVVVPPFLQPEVMGV	96-110	98-107	N/A	
	NT,LT,HT	VVPPFLQPE	98-106	98-107	N/A	
	NT,LT,HT	VVPPFLQPEV	98-107	98-107	N/A	*
	NT,LT,HT	EMPFPKYPVEPF	123-134	122-135	N/A	
	NT,LT	QPLPPTVMFPPQS	164-176	164-179	N/A	
	NT,LT,HT	QPLPPTVMFPPQSV	164-177	164-179	N/A	
κ-cn	NT	KNQDKTEIPTINT	133-145	132-147	N/A	
BLG	NT	LIVTQTMKGLDIQ	17-29	17-32	N/A	
	NT	LIVTQTMKGLDIQKV	17-31	17-32	N/A	
	NT	LIVTQTMKGLDIQKVA	17-32	17-32	N/A	
	NT	LIVTQTMKGLDIQKVAGT	17-34	17-32	N/A	
	NT	LIVTQTMKGLDIQKVAGTWYS	17-37	17-32	N/A	
	NT	LIVTQTMKGLDIQKVAGTWYSLA	17-39	17-32	N/A	
	NT	IVTQTMKGLDIQKVAGT	18-34	17-32	N/A	
	NT	IVTQTMKGLDIQKVAGTWYSLA	18-39	17-32	N/A	
	NT	VTQTMKGLDIQKVAGT	19-34	17-32	N/A	
	NT	VTQTMKGLDIQKVAGTWYSLA	19-39	17-32	N/A	
	NT,LT,HT	VYVEELKPTPEGDLE	57-71	**56-70**	N/A	
	NT,LT,HT	VYVEELKPTPEGDLEI	57-72	**56-70**	N/A	
	NT,LT	VYVEELKPTPEGDLEIL	57-73	56-70	N/A	
	NT,LT,HT	YVEELKPTPEGDLE	58-71	56-70	N/A	
	NT,LT,HT	YVEELKPTPEGDLEI	58-72	56-70	N/A	
	NT,LT	YVEELKPTPEGDLEIL	58-73	56-70	N/A	
	NT	LVRTPEVDDEALEK	138-151	136-150	N/A	
	NT	LVRTPEVDDEALEKFDK	138-154	137-156	N/A	
ALA	NT	KILDKVGIN	113-121	112-121	N/A	

Peptides were reported as sIgE binding epitopes if their sequence contained at least 80% of the sequence of an sIgE binding epitope. Peptides matching exactly the sIgE binding epitope sequence are indicated (*).

Additionally, glycated peptides that matched sequence parts of sIgE binding epitopes were identified after 10 min in the IP (Table 6.2). Most of such peptides were found in BLG, however only 6 of them were exclusively found in heated samples and covered similar sequence parts as peptides that were also found in NT-MP. Glycated peptides matching the sequence of an sIgE binding epitope from α_{s2}-casein and β-casein were exclusively found in heated samples. For α_{s1}-casein, 3 out of 4 glycated peptides with sequence homology to an sIgE binding epitope were only found in HT-MP.

Table 6.2: sIgE binding epitopes[1] identified in glycated digestion-derived peptides after 10 min in the intestinal phase. Peptides were identified in cow's milk protein, non-treated (NT), heated at low temperature (LT), and heated at high temperature (HT), after *in vitro* digestion and derived from casein (cn) and β-lactoglobulin (BLG). Amino acids (AAs) position indicates the position within the proteins including the signal peptide. Peptides with post translational modification (PTM) on lysine (K) to lactosyllysine (Lac), glucosyllysine (Gluc), N^ε-carboxymethyllysine (CML), and pyrraline (Pyr), are reported and modified K residues are highlighted in bold and underline with modification site probability given in brackets if multiple options were identified.

Protein	Sample	Peptide sequence	AAs position	sIgE epitope AAs position	PTM
α_{s1}-cn	NT,LT	EGIHAQQ**K**EPMIGV	140-153	141-155	Lac
	HT	TDAPSFSDIPNPIGSENSE**K**	189-208	188-209	Lac
	HT	TDAPSFSDIPNPIGSENSE**K**TTMPL	189-213	188-209	Gluc
	HT	TDAPSFSDIPNPIGSENSE**K**TTMPL	189-213	188-209	Lac
	NT,LT	EGIHAQQ**K**EPMIGV	140-153	141-155	Lac
α_{s2}-cn	LT,HT	LYQGPIVLNPWDQV**K**	114-128	120-129	Lac
	LT,HT	LYQGPIVLNPWDQV**K**	114-128	120-129	Gluc
	LT,HT	LYQGPIVLNPWDQV**K**	114-128	120-129	CML
	LT,HT	LYQGPIVLNPWDQV**K**	114-128	120-129	Pyr
	LT,HT	YQGPIVLNPWDQV**K**	115-128	120-129	Lac
	LT,HT	YQGPIVLNPWDQV**K**	115-128	120-129	Gluc
	LT,HT	YQGPIVLNPWDQV**K**	115-128	120-129	CML
	HT	QGPIVLNPWDQV**K**	116-128	120-129	Lac
	HT	QGPIVLNPWDQV**K**	116-128	120-129	Gluc
	HT	QGPIVLNPWDQV**K**	116-128	120-129	CML
	HT	QGPIVLNPWDQV**K**	116-128	120-129	Pyr
β-cn	LT,HT	EMPFP**K**YPVEPF	123-134	122-135	Lac
	LT,HT	EMPFP**K**YPVEPF	123-134	122-135	Gluc
	HT	SLSQS**K(1)**VLPVPQ**K(1)**AVPYPQ	179-197	182-199	Lac
BLG	LT,HT	LIVTQTM**K**GLDIQ	17-29	17-32	Lac
	NT	LIVTQTM**K(1)**GLDIQ**K(1)**VAGT	17-34	17-32	Lac
	LT	RVYVEEL**K**PTPEGDLE	56-71	56-70	Lac
	NT	RVYVEEL**K**PTPEGDLEI	56-72	56-70	Lac
	NT	VYVEEL**K**PTPEGDLE	57-70	56-70	Lac
	NT,LT,HT	VYVEEL**K**PTPEGDLE	57-71	56-70	Lac
	NT,LT,HT	VYVEEL**K**PTPEGDLE	57-71	56-70	Gluc
	NT,LT,HT	VYVEEL**K**PTPEGDLE	57-71	56-70	CML
	NT,LT,HT	VYVEEL**K**PTPEGDLE	57-71	56-70	Pyr
	NT,LT,HT	VYVEEL**K**PTPEGDLEI	57-72	56-70	Lac
	NT,LT,HT	VYVEEL**K**PTPEGDLEI	57-72	56-70	Gluc
	NT,LT,HT	YVEEL**K**PTPEGDLE	58-71	56-70	Lac
	NT,LT,HT	YVEEL**K**PTPEGDLE	58-71	56-70	Gluc
	NT,LT,HT	YVEEL**K**PTPEGDLE	58-71	56-70	CML
	NT,LT,HT	YVEEL**K**PTPEGDLE	58-71	56-70	Pyr
	LT,HT	YVEEL**K**PTPEGDLEI	58-72	56-70	Lac
	LT,HT	YVEEL**K**PTPEGDLEI	58-72	56-70	Gluc
	LT,HT	YVEEL**K**PTPEGDLEI	58-72	56-70	CML
	NT,HT	YVEEL**K**PTPEGDLEIL	58-73	56-70	Lac
	NT	LVRTPEVDDEALE**K(1)**FD**K(1)**	138-154	137-156	Lac
	NT	LVRTPEVDDEALE**K(1)**FD**K(1)**	138-154	137-156	Pyr
	NT,LT	LVRTPEVDDEALE**K(1)**FD**K(1)**AL**K(1)**ALPM	138-161	137-156	Lac
	NT,LT	LVRTPEVDDEALEKFD**K(0.8)**AL**K(0.2)**ALPM	138-161	137-156	Gluc
	NT,LT	LVRTPEVDDEALE**K(1)**FDKALKALPM	138-161	137-156	CML
	NT,LT	LVRTPEVDDEALE**K(1)**FD**K(1)**ALKALPM	138-161	137-156	Pyr

[1] Peptides were reported as sIgE binding epitopes if their sequence contained at least 80% of the sequence of an sIgE binding epitope. Peptides matching exactly the sIgE binding epitope sequence are indicated (*)

T-cell epitopes were predicted using IEDB MHC Class II Binding Prediction software. All modified and non-modified peptides that were predicted as potential T-cell binding epitopes are shown in Table 6.3.

Table 6.3: Potential T-cell epitopes identified after 10 min in the intestinal phase. Digestion-derived peptides identified from cow's milk protein, non-treated (NT), dry-heated at low temperature (LT), and dry-heated at high temperature (HT) after *in vitro* digestion, identified from casein (cn) and β-lactoglobulin (BLG). Amino acids (AAs) position indicating the position within the proteins including the signal peptide. Unmodified peptides and peptides with post translational modifications (PTM), via phosphorylation (Phos), as well as modification to glucosyllysine (Gluc), lactosyllysine (Lac), Nᵉ-carboxymethyllysin (CML), and pyrraline (Pyr) were reported. Modified AAs are indicated in bold and underline.

Protein	Sample	Sequence	HLA-allele	AAs position	PTM	Perc. rank
α_{s1}-cn	NT, LT	EAESI**SSS**EEIVPNSVEQ	HLA-DQA1*03:01/ DQB1*03:02;	76-93	Phos	2.5
			HLA-DQA1*04:01/ DQB1*04:02			5.3
	NT, LT	YKVPQLEIVPN**S**AEE	HLA-DRB1*04:05;	119-133	Phos	1.9
			HLA-DQA1*04:01/ DQB1*04:02;			4.1
			HLA-DQA1*03:01/ DQB1*03:02			5.8
	NT, LT, HT	YKVPQLEIVPN**S**AEER	HLA-DRB1*04:05;	119-134	Phos	2.2
			HLA-DQA1*04:01/ DQB1*04:02;			5.9
			HLA-DQA1*03:01/ DQB1*03:02			6.5
	NT, LT, HT	KVPQLEIVPN**S**AEER	HLA-DRB1*04:05;	120-134	Phos	1.9
			HLA-DQA1*04:01/ DQB1*04:02;			5.6
			HLA-DQA1*03:01/ DQB1*03:02			5.9
α_{s2}-cn	NT, LT, HT	SIGSSSEESAEVATEEV	HLA-DQA1*04:01/ DQB1*04:02;	68-84	N/A	0.14
			HLA-DQA1*03:01/ DQB1*03:02			0.18
	LT,HT	LYQGPIVLNPWDQV**K**	HLA-DRB1*13:02	114-128	Gluc	9.7
	LT,HT	LYQGPIVLNPWDQV**K**	HLA-DRB1*13:02	114-128	Lac	9.7
	LT,HT	LYQGPIVLNPWDQV**K**	HLA-DRB1*13:02	114-128	CML	9.7
	LT,HT	LYQGPIVLNPWDQV**K**	HLA-DRB1*13:02	114-128	Pyr	9.7
β-cn	HT	SLTLTDVENLHLPLP	HLA-DPA1*03:01/ DPB1*04:02	139-153	N/A	6.3
BLG	NT	VTQTMKGLDIQKVAGT	HLA-DRB4*01:01	19-34	N/A	7.9
	NT	ASDISLLDAQSAPLRV	HLA-DRB4*01:01;	42-57	N/A	4.0;
			HLA-DRB1*01:01;			6.6;
			HLA-DRB1*13:02;			7.9;
			HLA-DRB1*12:01			8.2;
			HLA-DQA1*03:01/ DQB1*03:02			9.0
	HT	SDISLLDAQSAPLRV	HLA-DRB4*01:01;	43-57	N/A	3.3
			HLA-DRB1*01:01;			4.4
			HLA-DRB1*12:01;			6.2
			HLA-DRB1*13:02;			6.3
			HLA-DRB1*09:01			7.2

Overall, 13 potential T-cell epitopes were found in the digest, with most epitopes deriving from α_{s1}-casein and α_{s2}-casein, followed by BLG, and β-casein. In the digest of NT-MP, 7 T-cell epitopes were found of which 5 were also found in at least one of the heated samples. LT and HT heating resulted in the release of 9 T-cell epitopes, respectively, with 6 solely found in heated samples. Of these, 40% were found in the digest of LT-MP and HT-MP were also glycated.

6.3.3   *Peptides identified at the basolateral compartment of the Caco-2 cell monolayer*

To study the epithelial transport, *in vitro* digests sampled after 10 minutes in the IP were applied to a Caco-2 cell monolayer. The number of peptides found in the basolateral compartment for each sample decreased with heating intensity. Observed were 181, 129, and 121 peptides in NT-MP, LT-MP, and HT-MP, respectively. Moreover, most peptides were derived from α_{s1}-casein, β-casein, and BLG (data not shown). Independent from the heat treatment, the majority of peptides was found in the size range between 8-10 and 11-13 amino acids (Figure 6.4). Compared to the composition in the digest before transport relatively higher numbers of peptides in the size range between 8-10 and 11-13 were found (supplementary material: Figure 6-S3). Interestingly, peptides up to 24 amino acids long were identified on the basolateral side of the Caco-2 cells, however at low numbers.
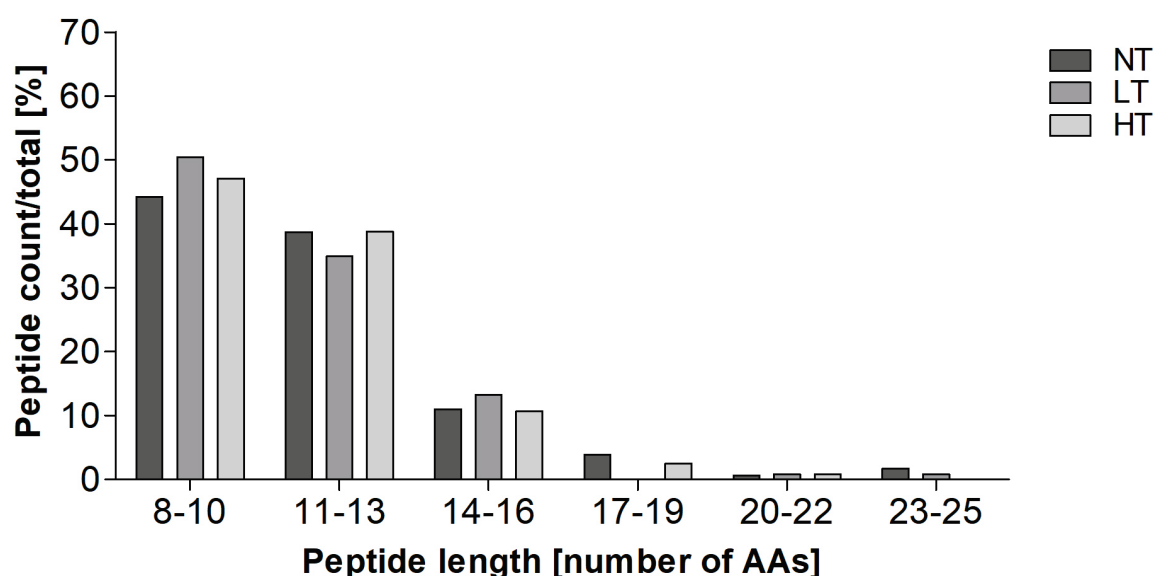
Figure 6.4: Peptide length distribution on the basolateral side of digestion-derived peptides, sampled after 10 min in the intestinal phase from simulated infant *in vitro* digests of cow's milk protein, non-treated (NT), heated in the presence of lactose at low temperature (LT), and high temperature (HT), expressed as peptide count relative to the total number (NT: 181, LT: 129, HT: 121) of peptides in one sample.

In NT-MP less glycated peptides were found on the basolateral side (37%), compared to LT-MP (50%) and HT-MP (56%). The percentage of glycated peptides on the basolateral side increased in all samples compared to the percentage of glycated peptides relative to all glycated

and non-modified peptides in the digest before transport (35 ± 0%, 47 ± 1%, 49 ± 5% for NT-MP, LT-MP, and HT-MP, respectively). In all samples, the majority of those glycated peptides was modified to lactosyllysine, followed by modification to glucosyllysine, pyrraline and CML. Interestingly, the largest increase was observed for the relative number of lactosyllysine and glucosyllysine-modified peptides found on the basolateral side compared to the digest (supplementary material: Figure 6-S3). This effect was larger in heated samples than in NT-MP. Heated samples also showed slightly increasing relative numbers of CML-modified peptides on the basolateral side, while relative numbers of pyrraline-modified peptides showed were comparable to the digest.
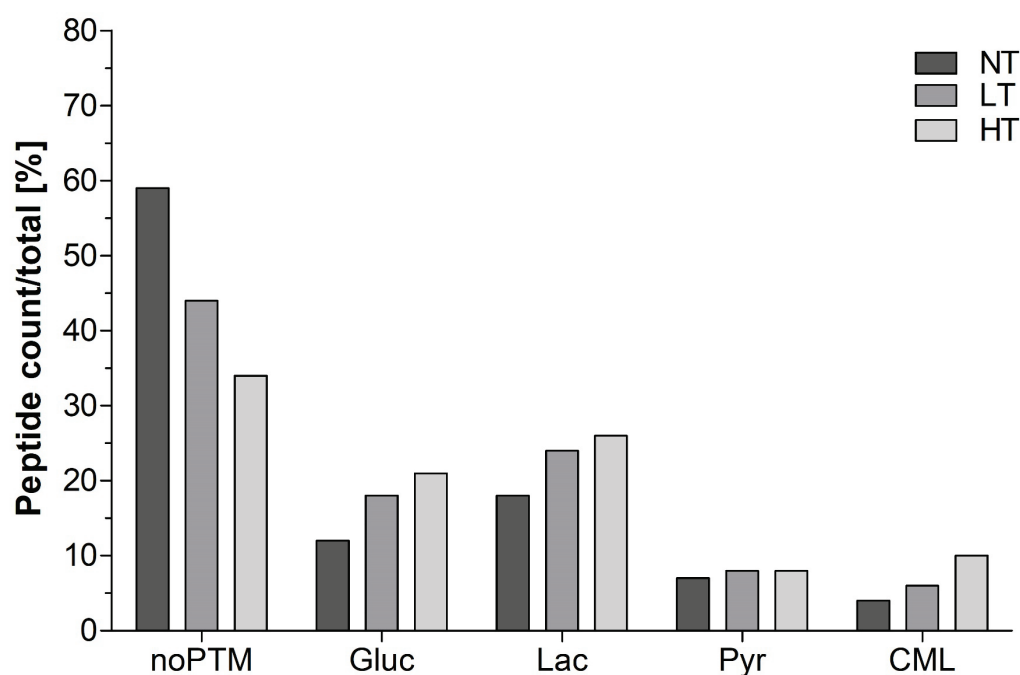
Figure 6.5: Digestion-derived peptides identified on the basolateral side of Caco-2 cells exposed to *in vitro* digests of cow's milk protein, non-treated (NT), heated in the presence of lactose at low temperature (LT), and high temperature (HT). Peptides without posttranslational modification (noPTM), as well as modification to glucosyllysine (Gluc), lactosyllysine (Lac), pyrraline (Pyr), and N^ε-carboxymethyllysine (CML) are shown expressed as peptide count relative to the total number (NT: 181, LT: 129, HT: 121) of peptides in one sample.

6.3.4 *sIgE binding epitopes on the basolateral side of the Caco-2 cell monolayer*

Peptides identified at the basolateral side that carried at least 80% of the sequence of a known sIgE epitope are shown in Table 6.4. Similar to the observations in the digest (Table 6.1) most epitopes were found in peptides derived from BLG, however only two of these peptides were unmodified. Moreover, only 19% of the glycated and non-glycated sIgE binding epitopes found in the digest (Table 6.1 and Table 6.2) were also found on the basolateral side (Table 6.4). Contrastingly, on average the total number of glycated and non-modified peptides found on the basolateral side corresponded to 49% of the number of glycated and non-modified peptides in

the digest. Two of the peptides containing an sIgE epitope derived from α_{s1}-casein (f52-67 and f123-133) were not identified in the digests before the Caco-2 cell experiment (Table 6.1), however these peptides could derive from other precursor peptides (e.g. phosphorylated f56-67 and f124-133). Additionally, a peptide derived from BLG (f57-71) in non-modified and glycated form was previously found in all samples, while identification on the basolateral side was only possible in NT-MP.

Table 6.4: Digestion-derived peptides covering sIgE binding epitope[1] sequences, identified on the basolateral side of the Caco-2 cell monolayer. Peptides were generated after digestion of cow's milk protein, non-treated (NT), heated at low temperature (LT), and heated at high temperature (HT), in an infant *in vitro* model. Amino acids (AAs) position indicates the position within the proteins including the signal peptide. Peptides were identified from α_{s1}-casein (α_{s1}-cn), β-casein (β-cn), and β-lactoglobulin (BLG). Peptides with and without post translational modification (PTM) to lactosyllysine (Lac), glucosyllysine (Gluc), N^{ε}-carboxymethyllysine (CML), and pyrraline (Pyr) are shown, where modified lysine residues (K) are bold and underline.

Protein	Sample	Peptide sequence	AAs position	sIgE epitope AAs position	PTM	
α_{s1}-cn	NT,LT	VNELSKDIGSESTEDQ	52-67	54-63	N/A	
	NT, LT	KVPQLEIVPNSAEE	120-133	124-135	N/A	
	NT, LT	QLEIVPNSAEE	123-133	124-135	N/A	
	NT,LT,HT	LEIVPNSAEER	124-134	124-135	N/A	
β-cn	NT, LT	PVVVPPFLQPEV	96-107	98-107	N/A	
	NT, LT, HT	VVPPFLQPE	98-106	98-107	N/A	
	NT, LT, HT	VVPPFLQPEV	98-107	98-107	N/A	*
BLG	NT, LT, HT	VYVEELKPTPEGDLE	57-71	56-70	N/A	
	NT	VYVEEL**K**PTPEGDLE	57-71	56-70	CML	
	NT	VYVEEL**K**PTPEGDLE	57-71	56-70	Lac	
	NT	VYVEEL**K**PTPEGDLE	57-71	56-70	Pyr	
	NT, HT	YVEELKPTPEGDLE	58-71	56-70	N/A	
	NT, LT, HT	YVEEL**K**PTPEGDLE	58-71	56-70	CML	
	NT, LT, HT	YVEEL**K**PTPEGDLE	58-71	56-70	Lac	
	NT, LT, HT	YVEEL**K**PTPEGDLE	58-71	56-70	Pyr	

[1] Peptides were reported as sIgE binding epitopes if their sequence contained at least 80% of the sequence of an sIgE binding epitope. Digestion-derived peptides covering the exact sequence of an sIgE binding epitope are indicated (*).

From the T-cell epitopes identified in the digest, only one T-cell epitope derived from α_{s1}-casein was found in HT-MP (f68-84). This data suggested an overall low passage of T-cell epitopes and sIgE binding epitope.

6.4 Discussion

6.4.1 *Heat treatment dependent differences in peptide profiles*

Dry-heated MP at LT and HT was subjected to simulated infant *in vitro* digestion and peptides were identified after 10 min and 60 min in the IP. As most differences between heat treatments in the digests were observed after 10 min in the IP, at which time the mucosal immune system in the gastrointestinal tract may already encounter antigens, the focus was set to this digestion time point. Heat treatment of MP resulted in a decreasing number of peptides upon digestion after 10 min in the IP compared to NT-MP which became less distinct after 60 min in the IP (Figure 6.1). The absence of peptides can be a result of both increased and impaired hydrolysis [7]. However, in our previous study we showed that HT dry heating impairs hydrolysis after

10 min and 60 min in the IP suggesting that the absence of peptides results from decreased hydrolysis [27]. Next to this, it is also possible that a larger pool of different linear and crosslinking MR products (MRPs) can result in lower number of peptides as only the most abundant modifications were monitored. At the same time, the relative number of glycated peptides identified in the digest of heated samples was higher compared to NT-MP (Figure 6.1a). Most of the peptides were modified to glucosyllysine and lactosyllysine (supplementary material: Figure 6-S4) and already a large proportion of lactoyslated peptides was observed in NT-MP. The comparison of peptide intensities, however, indicated that the quantities of glycated peptides in the heated samples were higher than in NT-MP (supplementary material: Figure 6-S5). This was in agreement with the findings of Milkovska-Stamenova *et al.* [35], who found 50 lactosylation sites in raw milk which increased to only 70-80 in ultra high temperature treated milk. At the same time, quantification of the glycated peptides in their study showed much lower levels in raw milk compared to processed dairy products.

Most peptides were derived from α_{s1}-casein, β-casein, and BLG (supplementary material: Figure 6-S1). For the two casein this is probably related to the relative higher concentration compared to the other proteins. For BLG this is also related to the larger number of glycated peptides, of which each was counted as a separate peptide (Figure 6.3e). A heat treatment dependent decrease of sequence coverage after 10 min in the IP was especially observed for the two α-caseins and BLG (Figure 6.2 and Figure 6-S1a). For BLG, this originated from the absence or low number of peptides in the regions f17-39, f88-116, and f164-174 (Figure 6.3e). BLG, as a globular protein, is more sensitive to heating induced structural modifications compared to casein [36]. Moreover, it contains 16 lysine residues and 2 arginine residues, which makes it prone to glycation via the MR [37]. The regions f17-39 and f88-116 are rich in lysine residues, explaining the impairment of peptide generation especially in heated samples from this area via tryptic hydrolysis. The region f164-175 is located on the outside of the globular protein, partly incorporated in a β-strand and α-helix structure, which makes it rather easily accessible for digestive enzymes. However, it has been shown that upon heating in solution a α-β transition occurs, contributing to the aggregation of BLG via hydrophobic interactions [38]. This could explain the absence of peptides in the f164-175 region in, as HT heating promotes the aggregation of BLG, but not in LT-MP and NT-MP. For α-caseins, a heating dependent decrease of sequence coverage was mainly reflected by the absence of phosphorylated peptides in HT-MP (Figure 6.3a and b) and is in line with the lower number of phosphorylated peptides (Figure 6.1a). Dephosphorylation has been reported upon heating in solution of caseinate at HT (140 °C) [39]. Both, hydrolysis of phosphoserine as well as β-elimination may induce dephosphorylation of casein. Michael addition subsequently to β-elimination results in the formation of the protein crosslinking [39], which may also explain the lower number of peptides in HT-MP from sequence parts that are more phosphorylated. A study from Wada *et al.* [40] showed that dephosphorylation could decrease digestibility of heated dairy products. This is in line with the low digestibility of HT-MP observed in our previous study [27]. Next to

digestibility, dephosphorylation could also decrease sIgE binding capacity, indicating that overall sIgE binding capacity to linear sIgE binding epitopes could be lower for HT-MP [41]. In contrast to this, the region f22-39 of β-casein, showed increasing number of phosphorylated peptides in HT with progressing digestion (supplementary material: Figure 6-S2c), indicating that a slower release of peptides could be a possible explanation for the absence of phosphorylated peptides after 10 min in the IP. Interestingly, glycated peptides did not result in differences of overall sequence coverage when comparing heat treatments but resulted in a higher number of peptides in some areas (Figure 6.3a and c). This could possibly affect immunoreactivity if present on an epitope or by binding of these peptides to AGE receptors, however it should be noted that quantities of glycated peptides were not measured and that it is not clear which effect the glycation of peptides has for epitope recognition. In summary, dry heating of MP decreases the number of peptides released upon simulated infant *in vitro* digestion and results in lower sequence coverage after 10 min in the IP. Glycated peptides had a minor role in differential sequence coverage. The discrepancies in sequence coverage of specific regions when comparing heat treatments can also be relevant for sIgE binding and T-cell epitope presentation. At the same time, progressing digestion results in less differences, suggesting that digestion kinetics are important determinants for differential release of immunoreactive digestion-derived peptides when comparing heat treatments.

6.4.2 *Hydrolysis resistant areas*

Most regions of κ-casein and ALA from which peptides were released after 10 min in the IP (Figure 6.3d and f) were also detected after 60 min in the IP (supplementary material: Figure 6-S2), however none of them were identified in areas with interest for possible immunological consequences. For α_{s2}-casein, decreasing sequence coverage was only observed for NT-MP. This was related to the disappearance of the phosphorylated peptides (f16-34) and the peptide at f40-50, of which already only low numbers were detected after 10 min in the IP. However, no potential epitopes were identified after 60 min in the IP. For α_{s1}-casein and β-casein, sequence coverage and number of peptides showed only minor decrease with prolonged digestion (Figure 6.2 and supplementary material: Figure 6-S1). For β-casein, the peptide pattern between samples showed only minor differences and therefore also a comparable persistence of peptides carrying an sIgE binding epitope (f96-110, f123-134, and f164-177) (supplementary material: Table 6-S1). For α_{s1}-casein, the region f202-213 was solely covered in heated samples by glycated peptides after 60 min in the IP, suggesting a higher digestion resistance of this area due to glycation. At the same time, f140-155 of α_{s1}-casein which has previously been reported to maintain high residual immunoreactivity after simulated *in vitro* digestion of spray dried milk powder [7], was only partly preserved until the end of the IP (supplementary material: Figure 6-S2a). A higher number of peptides in NT-MP-digests originating from this region could possibly result in a higher immunoreactivity of this sample. Next to this, a larger number of peptides in f119-134, which contained a potential T-cell epitope (Table 6.3) was still found at the end of intestinal digestion in all samples (supplementary

material: Figure 6-S4a). In contrast to the two caseins, BLG showed large decreases of sequence coverage in all samples, whereas for which was related to the disappearance of f17-39 and f42-55. At the same time, the regions f57-73 and f139-154 of BLG were highly resistant to digestion until the end of intestinal digestion (supplementary material: Figure 6-S2e), independent from the heat treatment. This was in line with the findings by Egger *at al.* [42] who observed a high frequency of peptides within particularly these two areas of BLG until 120 min in the IP of a static *in vitro* model. Moreover, the findings for both BLG and α_{s1}-casein were similar to previous findings by Picariello *et al.* [43], who described the sequence part f141-151 of BLG and f119-134 of α_{s1}-casein as highly resistant to gastrointestinal digestion after simulated adult *in vitro* digestion. While there is no direct evidence for the presence of an immunoreactive structure within this region of BLG, f119-134 of α_{s1}-casein partly covers the sequence of and sIgE binding epitope (Figure 6.3a) and was also identified as potential T-cell epitope (Table 6.3). Together with its high resistance until the end of intestinal digestion, this suggest a potential role of f119-134 in sIgE binding to the digest of MP, however independent from the heat treatment. To summarise these findings, caseins generally showed a higher resistance over large parts of their protein sequence until the end of gastrointestinal digestion, which was unaffected by the applied heat treatment. Therefore, no conclusions can be drawn from the resistance of specific areas within the proteins sequence regarding differential immunoreactivity of dry-heated MP compared to NT-MP.

6.4.3   *Effect of heat treatment on identification of sIgE binding epitopes*

Digestion-derived peptides were reported as potential sIgE binding epitopes if they covered at least 80% of the sequence of a linear sIgE binding epitope known from literature [33]. The three proteins showing the highest numbers of digestion-derived peptides, α_{s1}-casein, β-casein, and BLG (supplementary material: Figure 6-S1a) also led to the highest number of sIgE binding epitopes (Table 6.1) after 10 min in the IP. Most sIgE binding epitopes were found in the NT-MP digest, when compared to the heated samples (Table 6.1), which was in line with the higher number of peptides (Figure 6.1a) and the higher sequence coverage of NT-MP (Figure 6.2a). This suggests a higher availability of linear sIgE binding epitopes in NT-MP compared to dry-heated MP. The opposite trend was observed for sIgE binding epitopes carrying a glycation side (Table 6.2). However, this trend did not continue until 60 min in the IP (supplementary material: Table 6-S1). After 60 min in the IP, most sIgE binding epitopes were found in the digest of NT-MP, of which the majority was however present in all samples. This can be explained by the overall smaller differences between samples with progressing digestion, which is possibly related to differences in digestion kinetics especially in the beginning of the IP. The effect of heating and glycation on IgE binding has been subject to previous studies on isolated milk proteins in mixture [15-17,19]. As reviewed by Nowak-Wegrzyn *et al.* [18], milk proteins show reduced IgE binding upon extensive glycation via the MR. However, these observations are based on studies of undigested milk proteins and not of linear epitopes exclusively and thus can probably not be extrapolated for all MRPs and

peptides. A study by Gasparini *et al.* [44] reported an approach to create the basis to study the effect of lactosylation on linear epitopes, however data comparing sIgE binding of lactosylated vs. non-modified peptides is not available at this time. With respect to the predicted T-cell epitopes, ~50% were specifically found in the heated samples, however most of those peptides were glycated. To our knowledge, only data on T-cell epitopes from α_{s1}-casein, BLG, and ALA are available in literature. In a previous study it was shown that a peptide f118-135 of α_{s1}-casein, which is similar to the T-cell epitope identified in our study (f119-135, Table 6.3), is recognised by 1 out of 10 cow's milk allergic children. However, none of the major T-cell epitopes of α_{s1}-casein identified in previous studies have been observed in the digests in our study [45-47]. The T-cell epitopes identified in BLG partly overlapped with the peptide sequences identified previously (f41-55) [48]. All T-cell epitopes from α_{s1}-casein and BLG were predicted as ligands for multiple HLA alleles, indicating that their recognition could be less affected by individual differences in patients. While the majority of α_{s1}-casein derived T-cell epitopes was phosphorylated, which in a previous study did not show consistent differences in epitope recognition [45], most of the α_{s2}-casein derived T-cell epitopes were glycated (Table 6.3). To our knowledge there is no study directly showing the effect of glycation on T-cell epitope recognition. However, as glycation of food proteins has been shown to affect T-cell immunogenicity [21,49], it could be hypothesised that glycation of peptides matching to a T-cell epitope could affect its immunogenicity. To summarise, dry heating of MP resulted in a lower number of peptides that match to known sIgE binding epitopes but a higher number of glycated sIgE binding epitopes. Moreover, T-cell epitopes were identified in the digest and equally distributed between samples, while glycated T-cell epitopes were solely found in heated samples. The consequences of glycation on sIgE epitope and T-cell epitope binding are, however, not clear.

6.4.4 *Identification of peptides on the basolateral side of the Caco-2 cell monolayer*

Transport across the epithelial layer was assessed using a Caco-2 cell monolayer model. Peptide length distribution found on the basolateral side (Figure 6.4) indicated a favoured transport of peptides up to 13 amino acids compared to the distribution in the digest (supplementary material-Figure 6-S3). Interestingly, also peptides with a length up to 24 amino acids were found on the basolateral side (Figure 6.4). The peptides in the larger size ranges (f17-19 and f20-22, and f23-25) were mainly non-glycated peptides derived from β-casein, which originated from hydrophobic patches within the sequence suggesting a passage via transcytosis [50]. Availability of larger peptides increases the possibility to be recognised by the immune system. Moreover, transport of peptides carrying sIgE binding epitope sequences (e.g. f159-177) via transcytosis enables the peptide to reach the lamina propria intact, indicating the importance of monitoring also transport pathways when studying the availability of immunoreactive digestion-derived peptides. Most digestion-derived peptides on the basolateral side were derived from α_{s1}-casein, β-casein, and BLG, which is probably related to the higher number of peptides in the digest (supplementary material: Figure 6-S2a). Consequently, sIgE binding

epitopes found on the basolateral side were only identified for α_{s1}-casein, β-casein, and BLG (Table 6.4). Moreover, sIgE binding epitopes were most abundant in NT-MP which was in line with the total number of digestion-derived peptides between samples (Table 6.1a) and the presence amongst proteins (supplementary material: Figure 6-S1a). However, only 19% of the sIgE binding epitopes (non-modified and glycated) and one T-cell epitope identified in the digests were also found on the basolateral side (Table 6.1, Table 6.2, and Table 6.4), while on average 49% of the number of peptides in the digest were found on the basolateral side, suggesting some sort of epitope-excluding effect of the epithelial layer. For T-cell epitopes it could be hypothesised that this was related to the size as the size ranges 8-10 as well as 11-13 amino acids were preferably transported, while T-cell epitopes normally have a length between 15-24 amino acids [34]. In contrast, most sIgE binding epitopes were identified within the smaller size ranges. Next to peptide size, the transport across the Caco-2 cell monolayer can also be determined by charge and hydrophobicity [50]. However, further studies would be necessary to determine peptides properties to find the reasons for the observed restriction of epitope transport across the Caco-2 cell monolayer. Moreover, it should be noted that *in vivo* a larger number of microfold cells as well as specialised dendritic cells are present in the small intestine, that are able to directly sample antigens from the intestinal lumen [51]. It is thus hypothesised that the translocation of sIgE and T-cell epitopes *in vivo* could be directed towards specialised cells rather than transport via normal enterocytes.

In contrast to this, a relatively higher number of glycated peptides was observed on the basolateral side, e.g. in dry-heated samples, compared to the composition in the digest. Moreover, data indicated a preference for the transport of lactosyllysine and glucosyllysine-modified peptides amongst all samples, when comparing the composition of the digest (supplementary material: Figure 6-S4) and the composition of the basolateral side (Figure 6.5). As reviewed by Moradi *et al.* [52], N- and O-glycosylation with different mono- and polysaccharides of therapeutic peptides has been shown to increase their transport across various biological membranes including Caco-2 cells. For example, Varamini *et al.* [53] observed a 700-fold increased transport across the Caco-2 cell monolayer after modification of the N-terminal amino group from endomorphin-1with lactose and suggested that this transport took place via a lactose-selective transporter. Such transporter-mediated translocation could be a possible explanation for the facilitated migration of glucosyllysine and lactosyllysine-modified digestion-derived peptides across the Caco-2 cell monolayer. However, it should be noted that the position and type of linkage (N- or O-linked) can strongly affect the structure, functionality and transporter mediated uptake of the peptides [52,54]. Therefore, an extrapolation of these findings to any peptide and any kind of modification is probably not possible. With respect to the potential immunological consequences, it is suggested that glycation if present on a linear sIgE binding epitope can affect the interaction between the peptide and the antibody [44]. Moreover, AGEs themselves have also been reported to modulate inflammatory pathways by binding to receptors for AGEs [14]. For the example of peptide-bound CML, it has been shown that it is a potent ligand for the receptor for AGEs and

thus possibly affects inflammatory pathways [20]. This study showed that glycated peptides larger than 7 amino acids are transported independent of the type of modification (Figure 6.5). The findings of this study, suggested that diets with high AGE content can also result in higher uptake of AGE-modified peptides. As recently shown, the binding of AGE receptors depends on the concentration of food protein bound AGEs [22]. Therefore, quantitative data would be necessary to better judge the impact of the transport of AGE-modified peptides on the gastrointestinal immune system as well as the involved transport pathways. To summarise, results indicated that several potentially immunoreactive peptides are transported across a model epithelial barrier. In general, the presence of peptides on the basolateral side is more affected by the overall composition of the digest rather than the selective transport of specific peptides. However, observations for lactosylated and glucosyllysine-modified peptides indicated a facilitated transport of these peptides. Transport seemed to be favoured for smaller peptides (up to 13 amino acids) as well as peptides modified to lactosyllysine and glucosyllysine. However, data also showed that CML and pyrraline-modified peptides are transported comparable to the composition in the digest. At the same, time transport of sIgE binding epitopes and T-cell epitopes was limited, which is possibly related to some intrinsic properties of these peptides.

This study gave an overview of the composition and transport of peptides derived after simulated infant *in vitro* digestion of differentially dry-heated MP. However, it also had in some limitations, as only qualitative data were presented and allergenicity as well as immunogenicity was not measured directly. Moreover, other structures that could affect immunogenicity as well as allergenicity, such as aggregated protein that might also resist *in vitro* digestion, has not been considered [55,56].

6.5   Conclusions

This study showed that different peptide profiles are generated during simulated infant *in vitro* digestion of milk that was dry-heated in the presence of lactose. HT dry heating had the largest effects on peptide generation, resulting in much lower numbers of peptides and a lower sequence coverage. Moreover, a much lower number of sIgE binding epitopes but a larger proportion of glycated sIgE binding epitopes and T-cell epitopes in heated samples indicated that immunogenicity and allergenicity of these samples could be affected. Transport studies showed that the transport of sIgE epitopes and T-cell epitopes across the Caco-2 cell monolayer is limited, highlighting the importance to evaluate different transport pathways. Although, transport of lactosyllysine and glucosyllysine-modified peptides was favoured, also CML and pyrraline-modified peptides were transported depending on their presence in the digest. This resulted in relatively more glycated peptides on the basolateral side in heated samples, indicating that if the initial level of MR is high, this will also increase the transport of glycated peptides and can thereby possibly affect immunoreactivity via interaction with AGE receptors.

Supplementary material

a
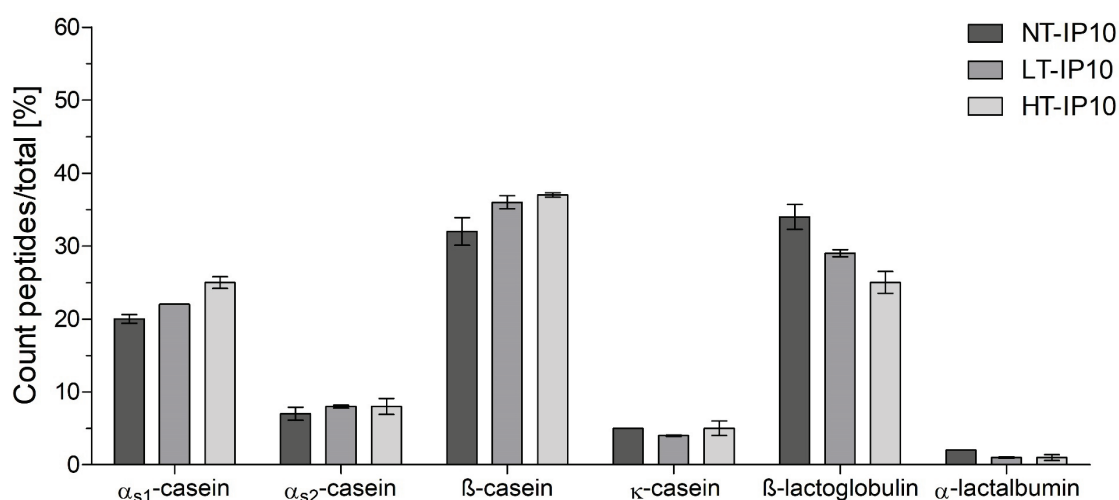
b
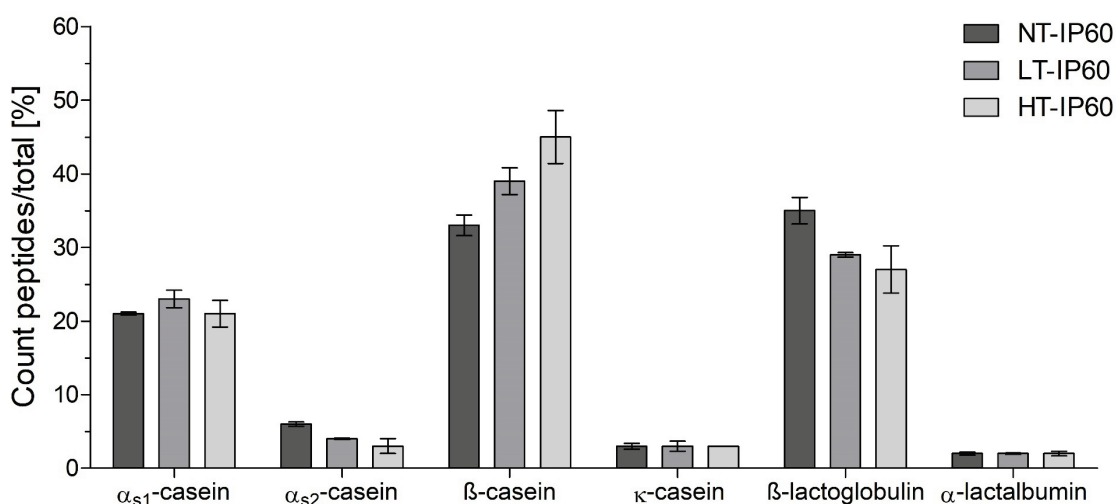
Figure 6-S1: Peptides derived from each of the five major milk proteins after infant *in vitro* digestion of cow's milk protein. Samples were non-treated (NT), heated at low temperature (LT), and heated at high temperature (HT), with data shown as percentage peptide count to total within the same sample after (a) 10 min in the intestinal phase (IP) and (b) after 60 min in the IP. Total number of peptides after 10 min in the IP was 436 ± 4, 369 ± 26, and 315 ± 36 for NT, LT, and HT, respectively. Total number of peptides after 60 min in the IP was 255 ± 15, 246 ± 7, and 207 ± 1 for NT, LT, and HT, respectively. Data are shown in mean $\pm$ standard deviation from duplicate digestion experiments.

a

α$_{s1}$-casein

```
            10              20              30              40              50
M K L L I L T C L V A V A L A R P K H P I K H Q G L P Q E V L N E N L L R F F V A P F P E V F G K E
          Signal peptide

            60              70              80              90             100
K V N E L S K D I G S E S T E D Q A M E D I K Q M E A E S I S S S E E I V P N S V E Q K H I Q K E D

           110             120             130             140             150
V P S E R Y L G Y L E Q L L R L K K Y K V P Q L E I V P N S A E E R L H S M K E G I H A Q Q K E P M

           160             170             180             190             200
I G V N Q E L A Y F Y P E L F R Q F Y Q L D A Y P S G A W Y Y V P L G T Q Y T D A P S F S D I P N P

           210
I G S E N S E K T T M P L W
```

b

α$_{s2}$-casein

```
            10              20              30              40              50
M K F F I F T C L L A V A L A K N T M E H V S S S E E S I I S Q E T Y K Q E K N M A I N P S K E N L
          Signal peptide

            60              70              80              90             100
C S T F C K E V V R N A N E E E Y S I G S S S E E S A E V A T E E V K I T V D D K H Y Q K A L N E I

           110             120             130             140             150
N Q F Y Q K F P Q Y L Q Y L Y Q G P I V L N P W D Q V K R N A V P I T P T L N R E Q L S T S E E N S

           160             170             180             190             200
K K T V D M E S T E V F T K K T K L T E E E K N R L N F L K K I S Q R Y Q K F A L P Q Y L K T V Y Q

           210             220
H Q K A M K P W I Q P K T K V I P Y V R Y L
```

C (continuation Figure 6-S2)

β-casein

d

κ-casein

e (continuation Figure 6-S2)

β-lactoglobulin
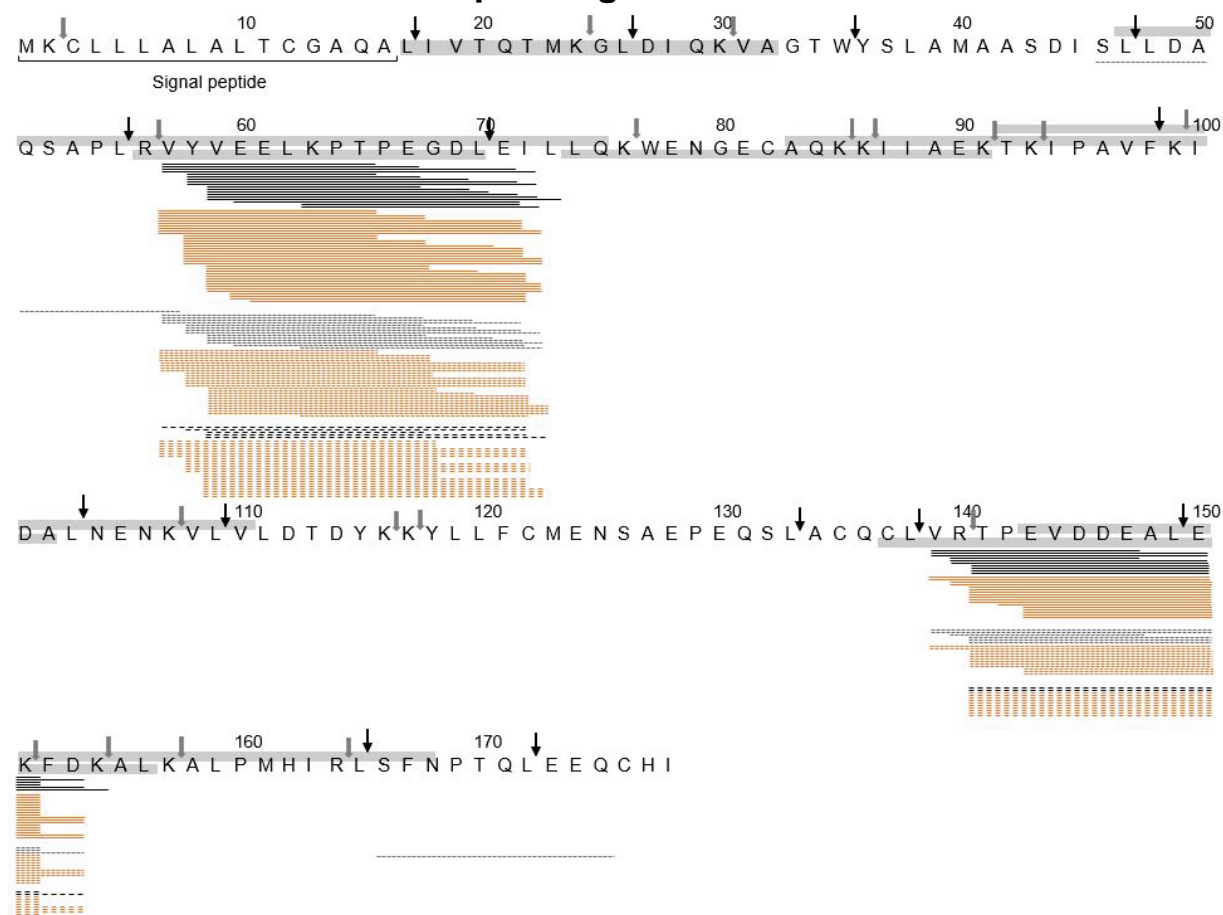
α-lactalbumin
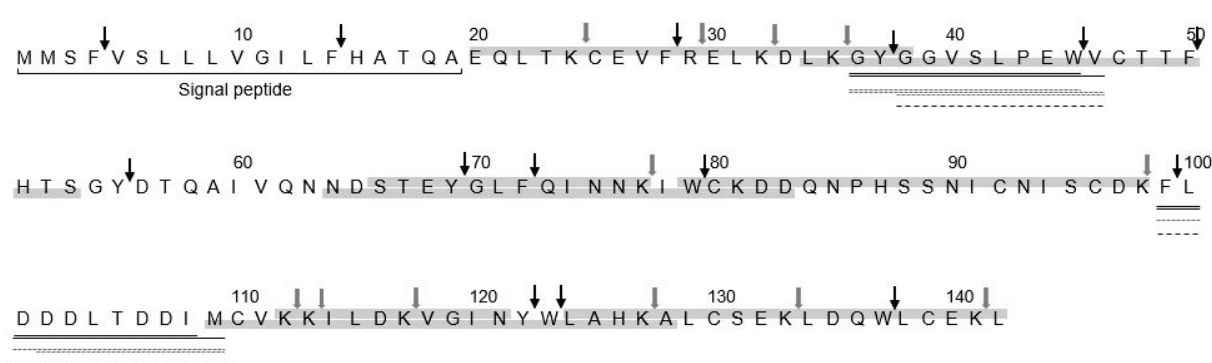
Figure 6-S2: Sequence alignment of digestion-derived peptides from the six major milk proteins (a-f) identified after 60 min in the intestinal phase of simulated infant *in vitro* digestion of non-treated cow's milk protein (——), heated at low temperature (------), and heated at high temperature (- - -). Glycated peptides (orange), phosphorylated peptides (blue), trypsin cleavage sites (↓), chymotrypsin cleavage sites (↓). Grey highlights indicated IgE binding epitopes according to [33]. Trypsin and chymotrypsin cleavage sites were determined using Expasy Bioinformatics Resource Portal (https://web.expasy.org/peptide_cutter/ last visited 08.06.2020).

Table 6-S1. sIgE binding epitopes[1] identified in digestion-derived peptides derived from cow's milk protein after 60 min in the intestinal phase. Samples were non-treated (NT), heated at low temperature (LT), and heated at high temperature (HT) in the presence of lactose. Peptides derived from caseins (cn) and β-lactoglobulin (BLG). Amino acids (AAs) position indicates the position within the proteins including the signal peptide. Peptides carrying a post translational modifications (PTM) are marked with phosphorylation (Phos), where phosphorylated serine (S) and threonine (T) as we as lysine residues (K) modified to glucosyllysine (Gluc), lactosyllysine (Lac), pyrraline (Pyr), and N^{ε}-carboxymethyllysine are highlighted in bold and underline. Localisation probabilities are given in brackets if multiple options were identified.

| Protein	Sample	Peptide sequence	AAs position	sIgE epitope AAs position	PTM	
α_{s1}-cn	LT	VNEL**S(1)**KDIG**S(1)**E**S(0.87)T(0.13)**EDQ	52-67	54-63	Phos	
	NT,LT,HT	KVPQLEIVPN**S**AEE	120-133	124-135	Phos	
	LT	KVPQLEIVPN**S**AEER	120-134	124-135	Phos	
	NT,LT,HT	VPQLEIVPN**S**AEER	121-134	124-135	Phos	
	NT,LT,HT	LEIVPN**S**AEE	124-133	124-135	Phos	
	NT,LT,HT	LEIVPN**S**AEER	124-134	124-135	Phos	
	NT,LT,HT	EIVPN**S**AEER	125-134	124-135	Phos	
	NT,LT,HT	QYTDAPSFSDIPNPI	187-201	186-200	N/A	
β-cn	LT	LVYPFPGPIPNSLPQ	73-87	70-85	N/A	
	NT,LT,HT	PVVVPPFLQPE	96-106	98-107	N/A	
	NT,LT,HT	PVVVPPFLQPEV	96-107	98-107	N/A	
	LT,HT	PVVVPPFLQPEVMG	96-109	98-107	N/A	
	HT	PVVVPPFLQPEVMGV	96-110	98-107	N/A	
	NT,LT,HT	VVPPFLQPE	98-106	98-107	N/A	
	NT,LT,HT	VVPPFLQPEV	98-107	98-107	N/A	*
	NT,LT,HT	EMPFP**K**YPVEP	123-133	122-135	Lac	
	NT,LT,HT	EMPFP**K**YPVEP	123-133	122-135	Gluc	
	NT,LT,HT	EMPFP**K**YPVEP	123-133	122-135	CML	
	NT,LT,HT	EMPFP**K**YPVEP	123-133	122-135	Pyr	
	LT,HT	EMPFP**K**YPVEPF	123-134	122-135	Lac	
	LT,HT	EMPFP**K**YPVEPF	123-134	122-135	Gluc	
	NT,LT,HT	QPLPPTVMFPPQS	164-176	164-179	N/A	
BLG	NT,LT,HT	VYVEELKPTPEGDLE	57-71	56-70	N/A	
	NT,LT,HT	VYVEEL**K**PTPEGDLE	57-71	56-70	Lac	
	NT,LT,HT	VYVEEL**K**PTPEGDLE	57-71	56-70	Gluc	
	NT,LT,HT	VYVEEL**K**PTPEGDLE	57-71	56-70	CML	
	NT,LT,HT	VYVEEL**K**PTPEGDLE	57-71	56-70	Pyr	
	NT	VYVEELKPTPEGDLEI	57-72	56-70	N/A	
	NT	VYVEEL**K**PTPEGDLEI	57-72	56-70	Lac	
	NT	VYVEEL**K**PTPEGDLEI	57-72	56-70	Gluc	
	NT	YVEEL**K**PTPEGDL	58-70	56-70	Lac	
	NT,LT,HT	YVEELKPTPEGDLE	58-71	56-70	N/A	
	NT,LT,HT	YVEEL**K**PTPEGDLE	58-71	56-70	Lac	
	NT,LT,HT	YVEEL**K**PTPEGDLE	58-71	56-70	Gluc	
	NT,LT,HT	YVEEL**K**PTPEGDLE	58-71	56-70	CML	
	NT,LT,HT	YVEEL**K**PTPEGDLE	58-71	56-70	Pyr	
	NT,LT	YVEELKPTPEGDLEI	58-72	56-70	N/A	
	NT	YVEEL**K**PTPEGDLEI	58-72	56-70	Lac	
	NT	YVEEL**K**PTPEGDLEI	58-72	56-70	Gluc	
	NT	YVEEL**K**PTPEGDLEI	58-72	56-70	CML	

[1] Peptides were reported as sIgE binding epitopes if their sequence contained at least 80% of the sequence of an sIgE binding epitope. sIgE binding epitopes of the Peptides matching exactly the sIgE binding epitope sequence are indicated (*).

Table 6-S2: Potential T-cell epitopes identified after 60 min in the intestinal phase. Peptides were identified as potential T-cell epitopes using IEDB MHC Class II Binding Prediction software (http://tools.iedb.org/mhcii/). Digestion-derived peptides identified from cow's milk protein, non-treated (NT), dry-heated at low temperature (LT), and dry-heated at high temperature (HT) applied to simulated infant *in vitro* digestion, derived from casein (cn). Amino acids (AAs) position indicating the position within the proteins including the signal peptide. Unmodified peptides and peptides with post translational modifications (PTM), via phosphorylation (Phos), as well as modification to glucosyllysine (Gluc), lactosyllysine (Lac), N^{ε}-carboxymethyllysin (CML), and pyrraline (Pyr) were reported if applicable.

Protein	Sample	Sequence	HLA-allele	AAs position	PTM	Perc. rank
α_{s1}-cn	NT, LT	EAESI**SSS**EEIVPNSVEQ	HLA-DQA1*03:01/ DQB1*03:02;	76-93	Phos	2.5
			HLA-DQA1*04:01/ DQB1*04:02			5.3
	NT, LT	YKVPQLEIVPN**S**AEE	HLA-DRB1*04:05;	119-133	Phos	1.9
			HLA-DQA1*04:01/ DQB1*04:02;			4.1
			HLA-DQA1*03:01/ DQB1*03:02			5.8
	LT	KVPQLEIVPN**S**AEER	HLA-DRB1*04:05;	120-134	Phos	1.9
			HLA-DQA1*04:01/ DQB1*04:02;			5.6
			HLA-DQA1*03:01/ DQB1*03:02			5.9
α_{s2}-cn	NT, LT	SIGSSSEESAEVATEEV	HLA-DQA1*04:01/ DQB1*04:02;	68-84	Phos	0.14
			HLA-DQA1*03:01/ DQB1*03:02			0.18
β-cn	NT,HT	NVPGEIVESLSSSEES	HLA-DQA1*04:01/ DQB1*04:02	22-37	Phos	5.43
		NVPGEIVESLSSSEESI	HLA-DRB1*04:05	22-38	Phos	5.54
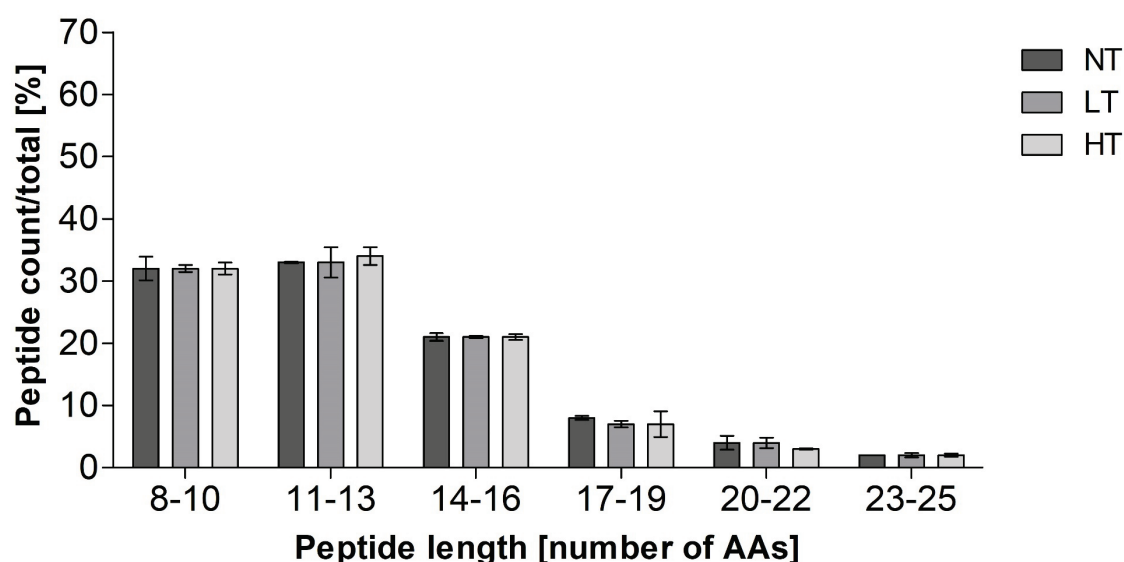
Figure 6-S3: Peptide length distribution identified in the *in vitro* digests of cow's milk protein after 10 min in the intestinal phase. Samples were non-treated (NT), heated at low temperature (LT) as well as at high temperature (HT) in the presence of lactose and applied to simulated infant in vitro digestion. Data are, expressed as percentage to total number of peptides in the same sample (NT: 436 ± 4, LT: 369 ± 26, HT: 315 ± 36).
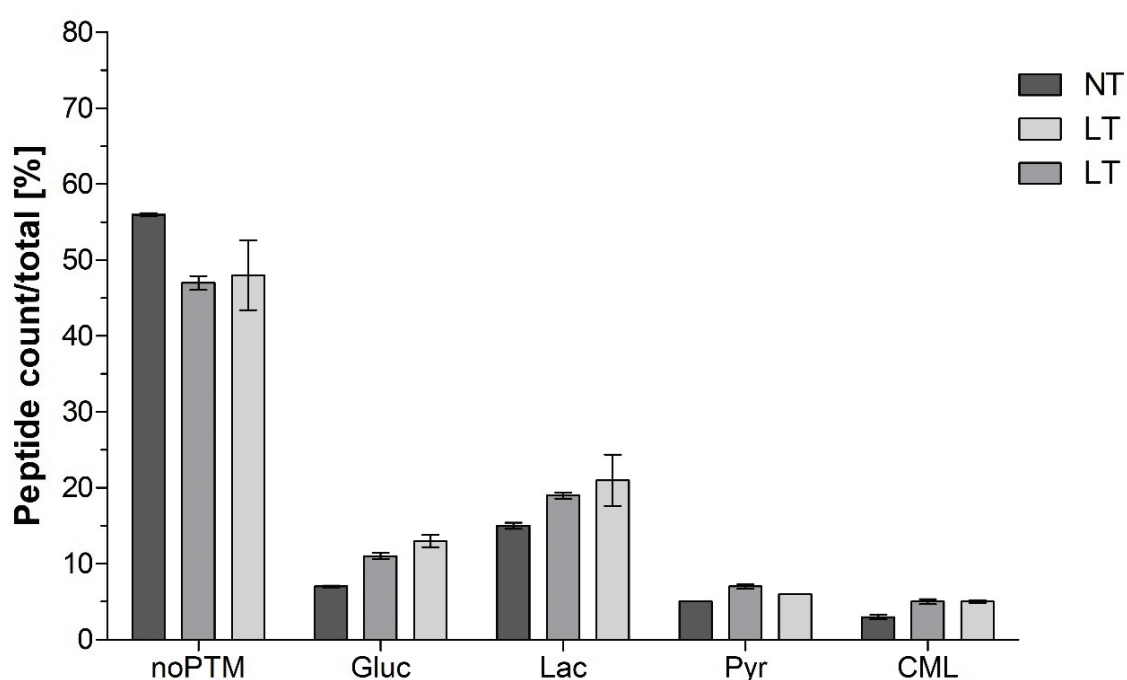
Figure 6-S4: Non-modified vs. glycated digestion-derived peptides identified after 10 min in the intestinal phase. Comparison of peptides without posttranslational modification (noPTM), as well as modified to glucosyllysine (Gluc), lactosyllysine (Lac), pyrraline (Pyr), and N^ε-carboxymethyllysine (CML) that were identified in the *in vitro* digests of cow's milk protein, non-treated (NT), heated in the presence of lactose at low temperature (LT), and high temperature (HT), expressed as peptide count relative to the total number of peptides in the samples (NT: 436 ± 4, LT: 369 ± 26, HT: 315 ± 36). Error bars represent standard deviations of duplicate digestions.

a

Glucosyllysine-modified peptides

Protein name	Mod. site	NT-IP10 DG1	NT-IP10 DG2	LT-IP10 DG1	LT-IP10 DG2	HT-IP10 DG1	HT-IP10 DG2	NT-IP60 DG1	NT-IP60 DG2	LT-IP60 DG1	LT-IP60 DG2	HT-IP60 DG1	HT-IP60 DG2
β-lactoglobulin	1												
β-lactoglobulin	2												
β-lactoglobulin	3												
β-lactoglobulin	4												
αs1-casein	5												
αs1-casein	6												
αs1-casein	7												
αs1-casein	8												
αs2-casein	9												
β-casein	10												
β-casein	11												
β-casein	12												

b (continuation Figure 6-S5)

Lactosyllysine-modified peptides

Protein name	Mod. site	NT-IP10 DG1	NT-IP10 DG2	LT-IP10 DG1	LT-IP10 DG2	HT-IP10 DG1	HT-IP10 DG2	NT-IP60 DG1	NT-IP60 DG2	LT-IP60 DG1	LT-IP60 DG2	HT-IP60 DG1	HT-IP60 DG2
β-lactoglobulin	1												
β-lactoglobulin	2												
β-lactoglobulin	3												
β-lactoglobulin	4												
β-lactoglobulin	5												
β-lactoglobulin	6												
β-lactoglobulin	7												
α_{s1}-casein	8												
α_{s1}-casein	9												
α_{s1}-casein	10												
α_{s1}-casein	11												
α_{s1}-casein	12												
α_{s1}-casein	13												
α_{s1}-casein	14												
α_{s2}-casein	15												
α_{s2}-casein	16												
α_{s2}-casein	17												
β-casein	18												
β-casein	19												
β-casein	20												
β-casein	21												
β-casein	22												
β-casein	23												
κ-casein	24												
κ-casein	25												
κ-casein	26												

c (continuation Figure 6-S5)

Carboxymethyllysine-modified peptides

Protein name	Mod. site	NT-IP10 DG1	NT-IP10 DG2	LT-IP10 DG1	LT-IP10 DG2	HT-IP10 DG1	HT-IP10 DG2	NT-IP60 DG1	NT-IP60 DG2	LT-IP60 DG1	LT-IP60 DG2	HT-IP60 DG1	HT-IP60 DG2
β-lactoglobulin	1												
β-lactoglobulin	2												
α_{s1}-casein	3												
α_{s2}-casein	4												
β-casein	5												
β-casein	6												
β-casein	7												

d

Pyrraline-modified peptides

Protein name	Mod. site	NT-IP10 DG1	NT-IP10 DG2	LT-IP10 DG1	LT-IP10 DG2	HT-IP10 DG1	HT-IP10 DG2	NT-IP60 DG1	NT-IP60 DG2	LT-IP60 DG1	LT-IP60 DG2	HT-IP60 DG1	HT-IP60 DG2
β-lactoglobulin	1												
β-lactoglobulin	2												
β-lactoglobulin	3												
α_{s1}-casein	4												
α_{s1}-casein	5												
α_{s1}-casein	6												
α_{s2}-casein	7												
α_{s2}-casein	8												
β-casein	9												
β-casein	10												

Figure 6-S5: Summed intensities of peptides associated with a specific modification, within the same amino acid sequence. Modification to glucosyllysine (Gluc), lactosyllysine (Lac), N^{ε}-carboxymethyllysine (CML), and pyrraline (Pyr) found in peptides generated after simulated infant *in vitro* digestion of cow's milk protein, non-treated (NT), dry-heated at low temperature (LT), and dry-heated at high temperature (HT), sampled after 10 min in the intestinal phase (IP10) and after 60 min in the intestinal phase (IP60) are shown. Duplicate digestion experiments (DG1 and DG2) for the same sample are shown, where green indicates the lowest intensity (0.0 for Lac, Gluc, CML, and Pyr) and red indicates highest intensity (Gluc:1.3E+9, Lac: 7.5E+9, CML: 5.1E+7, Pyr: 2.7E+8).

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Chapter 7

General discussion

7.1 Project summary

This thesis was part of a NWO-TTW project entitled: "Immunogenic Properties of Heated and Glycated Cow's Milk Protein – Effect on Initiation and Resolution of Cow's Milk Allergy" (short title "iAGE") that was divided in 4 work packages. The outcomes of work package 1 are described in this thesis, while an outline of the results from work package 2, work package 3, and work package 4 is presented below:

- Work package 2: immunological *in vitro* methods were developed to investigate the effect of heated milk protein (MP) on immunogenicity, including all immunoassays used in chapter 5. Chapter 5 and chapter 3 describe the published outcomes of this work package.

- Work package 3: a product of heated and glycated cow's milk protein was developed by FrieslandCampina and included in the clinical trial.

- Work package 4: cow's milk allergic children were recruited in several children's hospitals all over the Netherlands and were challenged with the product developed in work package 3 if they showed an initial tolerance to baked milk. After repeated challenge with the product for a period between 8-23 months, 73% of the children that were recruited at this time (26) tolerated the product without adverse effects. Eight children successfully introduced cow's milk in their diet.

7.2 Background

Thermal processing of cow's milk protein (MP) has previously been shown to result in both increased and decreased immunoreactivity of the protein. The consumption of dry-heated milk ("baked milk") accelerated the resolution of cow's milk allergy symptoms [1], whereas industrial heat treatments, as for instance applied in ultra high temperature (UHT) treated milk, appears to lead to pro-inflammatory immune responses [2]. The heating conditions of "baked milk" strongly differ from industrial heating processes in terms of heating load, water activity (a_w), and reactant mobility. It is therefore considered that the structural modifications differ between the dry vs. wet heating and that the Maillard reaction (MR) is considerably higher in dry-heated MP due to the promoting effect of low a_w on the MR [3]. All major MPs are considered to be potential allergens, however heat induced denaturation/aggregation may alter their immunogenicity and allergenicity. Next to this, advanced glycation end products (AGEs) resulting from the MR have been described as pro-inflammatory if formed endogenously but have also been described as potentially immunomodulatory if derived from exogenous sources [4,5]. The promoting role of dry heating on the formation of AGEs highlights the importance to investigate their potential role in modulating the immunological response towards dry-heated MP. However, it is not clear whether this is a distinctive effect of AGEs themselves or the accompanying protein aggregation. Moreover, aggregation as well as lysine blockage via the MR affects the digestibility of the MP [6,7]. If coming from exogenous sources, for instance after oral ingestion, protein digestion and thus the availability of

immunoreactive structures in the gastrointestinal tract is an important determinant for potential immunological consequences. In the past, a higher resistance of a protein towards digestive enzymes has been linked to its allergenic potential. However, this opinion has been reconsidered as analytical approaches are lacking to establish a direct link between these two factors [8]. Nevertheless, that carry an immunoreactive structure, can give an indication of the immunomodulatory potential of the differentially treated cow's MP. The aim of this thesis was to investigate the structural changes that occur upon heating of MP and to combine these with digestion experiments as well as immuno-assays. This provides better insights in the role of heat- and glycation-induced changes on digestibility and immunoreactivity.

7.3   Structural modifications of cow's MP upon wet vs. dry heating

Chapter 2 showed that casein and whey protein (WP) in mixture loses solubility upon heat treatment under dry conditions at both low temperature (LT) and high temperature (HT), indicating severe structural changes of the protein. The main component of the insoluble fraction was casein, which has previously been suggested to be a result of loss of electrostatic repulsion [9] as well as inter- and intramolecular interactions e.g. the MR [10-12]. As shown in chapter 2, not only the MR but also other inter- and intramolecular interactions promote the loss of solubility (Figure 2.3). Nevertheless, HT heating resulted in relatively more (MR-induced) covalent crosslinking as well as aggregation compared to LT heating, pointing out the role of (MR-induced) covalent crosslinking in loss of solubility and protein aggregation in this model system (Figure 2.2 and Figure 2.5). However, a limitation of the study in chapter 2 was that the large loss of solubility upon dry heating of casein and WP in mixture did not allow a more extensive investigation of the 3D-changes of the proteins. Therefore chapter 3 used isolated β-lactoglobulin (BLG) to monitor structural changes and the possible immunological consequences induced by differential heating conditions. Chapter 3 showed that under dry heating conditions aggregation is promoted by the presence of lactose (Figure 3.1), which was also observed by Cardoso *et al.* [13]. In contrast, aggregation of BLG heated in solution was independent from the presence of lactose. Chapter 3-5 showed that both dry and wet heating of isolated BLG also results in increased hydrophobicity and formation of β-sheet structures. Both, aggregation and increased hydrophobicity of heated and glycated BLG has previously been shown to increase uptake by THP1-macrophages and thus possibly modulate immunological responses [14]. Moreover, Roth-Walter *et al*. [2] showed that pasteurisation-induced aggregation of BLG enhanced the uptake via Peyer's patches in mice and promotes the Th2 pathway. Nevertheless, it should be noted that upon wet heating in the presence of casein micelles and α-lactalbumin, BLG interacts with these proteins [15]. The findings of chapter 2, however, indicated that the formation of the κ-casein/BLG complex on the casein micelle, does not occur upon dry heating of MP (Figure 2.3 and Figure 2.5) and that dry heating of MP in mixture rather promotes changes of the protein structure via other inter- and intramolecular interactions, possibly due to the decreased reactant's mobility. This

suggested that changes of protein-protein interactions when comparing dry vs. wet heating could also result in the formation of different immunoreactive structures.

Next to the changes of the 3D-structure of the protein, the level of N^ε-carboxymethyllysine (CML) as well as the abundance of other glycation structures was higher in dry-heated BLG compared to wet heated samples and increased depending on the heating temperature (Table 3.3). This was expected as low a_w-levels and high temperatures promote the MR [3]. High levels of AGEs could affect the immunogenicity of MP as several cell surface receptors on antigen presenting cells (APCs) have been described as receptors for AGEs indicating a potential role of the MR in inflammation [16]. In summary, the findings on structural modifications of BLG upon dry heating showed increased hydrophobicity and aggregation while the level of MR differed depending on the heating conditions. Moreover, dry heating of the mixture of protein resulted in different intermolecular interactions compared to heating in solution (chapter 2). The observed structural changes e.g. formation of AGEs and aggregation can possibly affect protein immunogenicity and allergenicity. However, the two determinants need to be disentangled to better understand their role in sensitisation or resolution of allergic symptoms to cow's milk.

7.4   The effect of aggregation vs. MR on allergenicity and immunoreactivity

One of the hypotheses underlying this thesis was that heat-induced modifications of the protein structure, and amongst those especially the formation of AGEs, is responsible for the acceleration of cow's milk allergy resolution. Chapter 3 showed that both aggregation and the presence of AGEs can affect immunogenicity and allergenicity of MP. Moreover, it was demonstrated that allergenicity was affected by both aggregation and glycation. Previous studies showed, that aggregation decreases Immunoglobulin E (IgE) binding of WPs after heating at >80 ºC in solution [17]. In contrast, IgE binding to casein remained unchanged when heated in solution at 90 ºC for 60 min, which is possibly related to their higher heat stability and higher abundance of linear epitopes rather than conformational epitopes [18,19]. Next to aggregation, high levels of MR can have a masking effect on both, linear and conformational epitopes. However, literature looking at the effect of either aggregation or glycation in relation to changes in allergenicity is limited, as the reaction conditions for AGE formation during the MR also facilitate protein aggregation, and even MR itself promotes aggregation [13]. Chapter 3, aimed to disentangle the effect of aggregation and glycation of IgE binding to BLG, however high individual differences (Figure 3.7) did not allow a distinction between the two factors or a conclusion regarding the general direction given by either one of these determinants.

Next to this, chapter 3 and 5 also showed that when BLG was heated in solution, protein aggregation appeared to be the crucial determinant for binding of BLG to AGE receptors. However, both chapters also showed that glycation still has an additional promoting effect on AGE receptor binding (Figure 3.5 and Figure 5.2), which could also be related to slightly higher exposure of hydrophobic structures and β-sheet structures as shown in chapter 4 and chapter 5

(Figure 4.2 and Figure 5.1). A previous study by Pinto *et al.* [6] reported different morphology of wet heating induced aggregates of BLG depending on the presence of sugars, which possibly explains these observations. Similarly, dry heating of BLG at both LT (60 ºC) and HT (130 ºC) heating resulted in increased binding to AGE receptors, however in this case binding only occurred to a minimal extent in the absence of lactose. Similarly, also aggregation occurred to a much higher extend when lactose was present during dry heating. Deng *et al.* [14] previously showed that aggregation is an important determinant for the receptor mediated uptake of heated and glycated BLG by THP1-macrophages. However, also the presence of AGEs has been described in relation to inflammatory health implications and it has been reported that AGEs are ligands for AGE receptors expressed on antigen presenting cells such as the receptor for advanced glycation end products (RAGE), galectin-3 (Gal-3), and scavenger receptor class A type I and II [4,20]. In chapter 3, the presence of CML was observed on only some of the protein structures in the western blot that were recognised by the soluble form of RAGE (sRAGE) (Figure 3.6) but could not directly be proven in all structures that showed binding to sRAGE (Figure 3.3). Therefore chapter 4, aimed to understand the role of protein-bound CML in binding to AGE receptors. It was shown that such protein-bound CML is indeed a ligand for the three receptors for AGEs: sRAGE, Gal-3, and CD36 (Figure 4.5). Next to this, chapter 4 showed that the negative charge induced to the lysine residue via modification to CML is an important determinant for the binding to all tested receptors, which was evaluated by changing the concentration of sodium chloride in the ELISA assay (Figure 4.6). The presence of CML on peptides can also result in binding to sRAGE indicating that even after digestion, high initial levels of CML can still modulate the immunological response [21]. At the same time, AGE ligands formed due to aggregation and hydrophobicity may (partly or completely) lose their ability to bind to AGE receptors after gastrointestinal digestion, as conformational epitopes are easier destroyed upon digestion.

7.5   Effect of heating and glycation on protein digestion and immunological consequences

During gastrointestinal digestion, proteins are hydrolysed into peptides and amino acids. Heat treatment of MP can affect their digestibility depending on the structural modifications that occurred during heating [22]. While denaturation increases the accessibility for digestive enzymes, aggregation and side modifications of specific amino acids can reduce enzymatic hydrolysis [23]. In chapter 2, the effects of LT heating and HT heating of MP on infant *in vitro* digestion were investigated. The results showed that dry heating at HT impaired overall digestibility (Figure 2.7), especially due to decreased tryptic hydrolysis (supplementary material: Figure 2-S2). This indicated that MR is an important determinant for digestibility of HT heated MP (HT-MP) as trypsin specifically cleaves after lysine residues [24].

Moreover, chapter 2 showed that aggregated proteins are released from the insoluble material upon gastric digestion and eventually enter the small intestine, thus possibly becoming available

to the immune system (Figure 2.8). Already after 10 min in the intestinal phase (IP) these aggregates are hydrolysed to smaller structures, that however persist until 60 min in the IP (Figure 2.10). Although there is no direct link between resistance of a protein to digestion and its potential to act as an antigen, it is hypothesised that the longer survival of immunoreactive structures during digestion could affect the immunological response. This was also addressed in chapter 5 where the binding of heated and glycated BLG before and after simulated infant *in vitro* digestion was compared. Chapter 5 showed, that binding to the AGE receptor of BLG hydrolysates is decreased compared to undigested wet heated and in-solution glycated BLG (Figure 5.2). However, the hydrolysate of glycated BLG still showed binding to sRAGE and Gal-3 after gastrointestinal digestion (Figure 5.6), which is possibly related to a higher resistance to hydrolysis of glycation-induced BLG aggregates compared to heat-induced BLG aggregates [6]. Similar observations were also made by Liu *et al.* [25] who still observed binding of HT dry-heated BLG to sRAGE after simulated infant *in vitro* digestion. However, immunoreactivity and aggregation behaviour may change when MPs are present in mixture. For example, a study of Bogahawaththa *et. al.* [26] showed that the production of interleukin (IL)-10, IL17A, and interferon-γ in human PBMCs is significantly decreased after exposure to unheated MP as a mixture compared to isolated proteins. Moreover, the presence of casein can decrease aggregation of BLG and results in the formation of the κ-casein/ BLG complex on the micelle surface when heated in solution [15,27]. The findings of chapter 2 did not indicate the same protein-protein interactions upon dry heating as observed during wet heating. Moreover, BLG aggregation upon dry heating in the mixture with casein was limited compared to the extent of aggregation for HT dry-heated isolated BLG (Figure 2.3 and Figure 3.1). This indicated that if WP is heated together with casein aggregates of different morphology and composition are formed. These aggregates might show differential digestibility if applied to *in vitro* digestion and survival in the IP, which can result in a dissimilar immunological response compared to aggregates formed upon heating of isolated protein.

This was also indicated by the results from the ELISA binding assays performed to assess the binding of sRAGE, CD36, and Gal-3 to digested non-treated MP (NT-MP), LT heated MP (LT-MP), and HT-MP (Figure 7.1), which showed that binding of all three receptors was high directly after the GP and was strongly decreased after the IP. All receptors showed higher binding to HT-MP in the GP compared to NT-MP and LT-MP, which is possibly related to the aggregates observed after 60 min in the GP on sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) (Figure 2.8) and scanning electron microscopy images (Figure 2.10). In the IP, binding was lower to HT-MP compared to NT-MP for CD36 and sRAGE suggesting that with further hydrolysis of these aggregates their immunoreactive potential decreases.

a
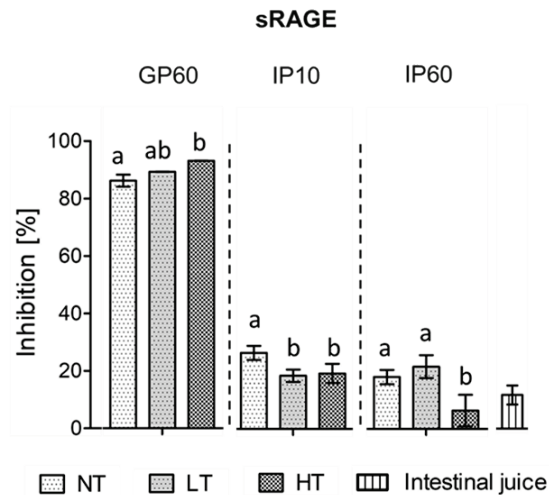
b
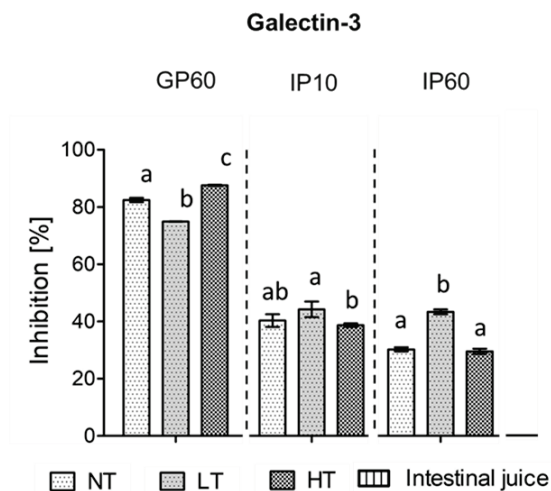
c
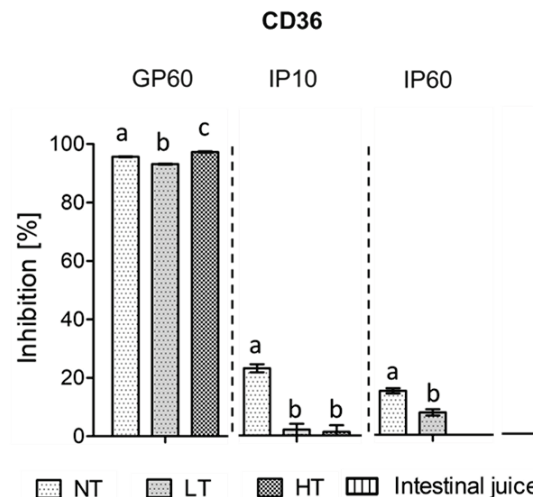
Figure 7.1: Binding of (a) sRAGE, (b) galectin-3, and (c) CD36 to simulated infant *in vitro* digests of cow's milk protein, non-treated (NT), heated at low temperature (LT), and heated at high temperature (HT) after 60 min in the gastric phase (GP60), 10 min in the intestinal phase (IP10), and 60 min in the intestinal phase (IP60) at 100 µg protein/mL. Statistically significant differences of technical replicates between samples of different heat treatments at the same sampling point of digestion are indicated by different letters within the sample sets with $p < 0.05$ (n=2 for GP60 and n=3 for IP10 and IP60). Statistically significant differences were tested using one-way ANOVA (SPSS) with Tukey post hoc test.

Interestingly, the relative higher proportion of larger particles observed in the IP for HT-MP did not affect binding to the receptors for AGEs. A recent study showed for the example of macrophage uptake, that not only aggregation but also hydrophobicity played a role in the response of the innate immune system to heated MPs [14]. However, sample-dependent differences in hydrophobicity (Figure 7.2) could explain the higher binding when comparing GP with IP but did not explain differences between heat-treatments.

a

b   **c**
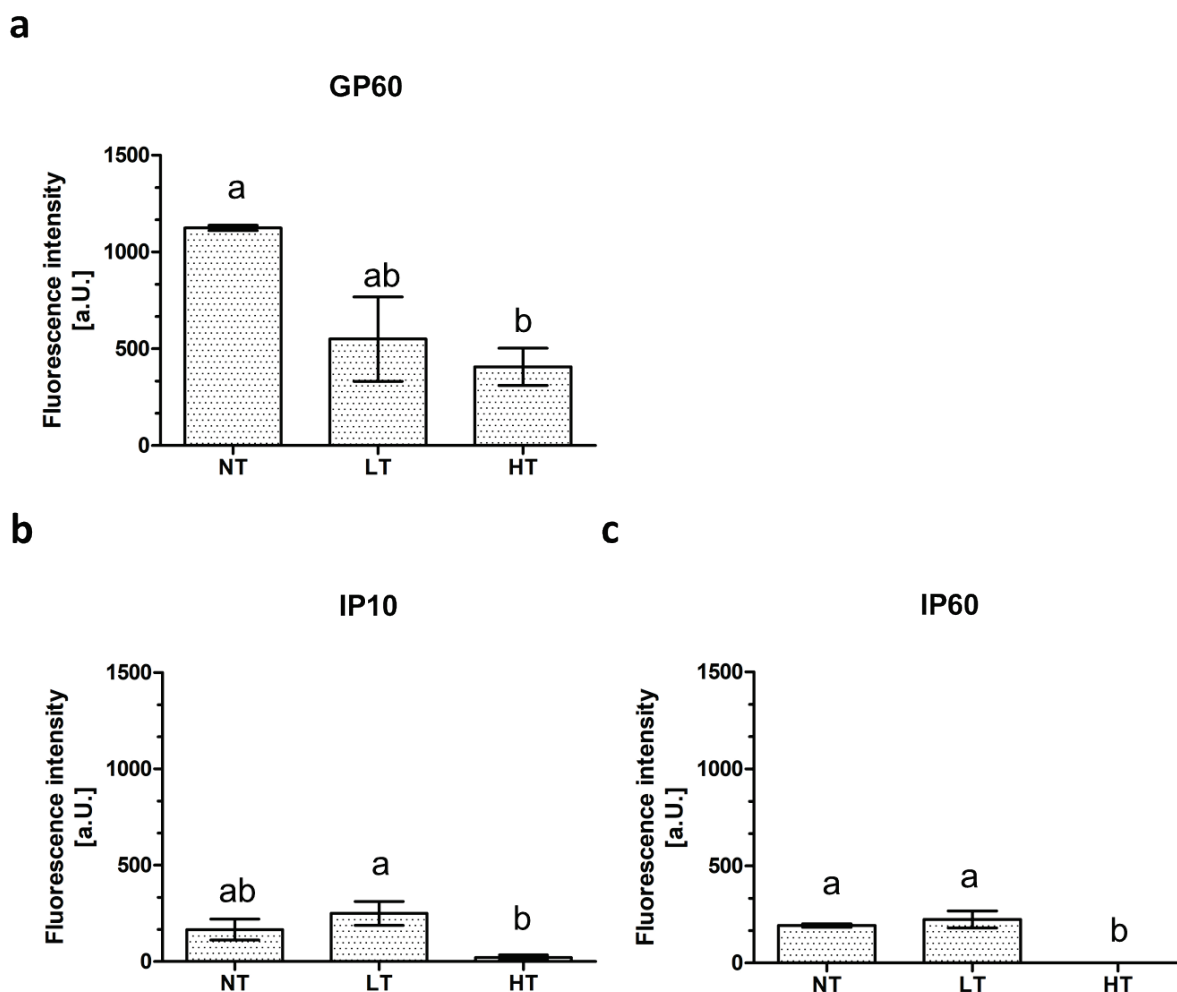
Figure 7.2: ANS-assay on cow's milk protein non-treated (NT), heated at low temperature (LT), and heated at high temperature (HT), applied to an infant *in vitro* digestion and sampled after 60 min in the gastric phase (a), 10 min in the intestinal phase (b) and 60 min in the intestinal phase (c). Error bars represent standard deviation of technical duplicates. Statistically significant differences were tested using one-way ANOVA (SPSS) with Tukey post hoc test and were considered as significantly different at $p < 0.05$, where two variables have different letters if they are significantly different.

Moreover, the higher residual immunoreactivity as shown for glycation-induced aggregates of wet heated BLG after gastrointestinal digestion was not observed for aggregates formed after dry heating in a mixture of casein and WPs. With respect to the different a_w-level of these two systems, it has been shown that WPs, dry-heated at 130 °C, preserve residual binding of sRAGE after gastrointestinal *in vitro* digestion [25]. It is thus hypothesised, that residual binding to AGE receptors after *in vitro* digestion is not only related to differential aggregation behaviour comparing dry vs. wet heating but also to the presence of other proteins during heating and *in vitro* digestion. These findings as well as the findings of chapter 5 indicate, that recognition of heated and glycated MP by APCs is strongly affected by the enzymatic hydrolysis in the gastrointestinal tract and that the resistance of initially present immunoreactive structures to digestion could be a crucial determinant for the immunoreactivity *in vivo*. However, it remains unknown which factors resulted in the differential binding of these specific receptors to heated and unheated casein and WP in mixture after *in vitro* digestion. As neither hydrophobicity nor

relative proportion of aggregates after intestinal digestion could be related to the differences in AGE receptor binding between samples, it is hypothesised that peptides might as well play a role.

7.6 Peptide patterns and their potential relevance in predicting immunological effects

The two major MP fractions show different susceptibility to gastric and intestinal digestion, which is related to their natural structure. WPs are relatively resistant to gastric digestion but rapidly hydrolysed upon intestinal digestion, while caseins are hydrolysed to larger peptides upon gastric digestion and show a slower hydrolysis in the IP compared to WPs [28,29]. As reviewed by Bogahawaththa *et al.* [26], it was suggested that caseins contain relatively more linear IgE epitopes than conformational epitopes. Linear epitopes would still be recognised on the peptide level, while conformational IgE binding epitopes are easier destroyed upon digestion. It is thus hypothesised that a longer persistence of peptides in the gastrointestinal tract can also affect the immunological response if these peptides carry immunoreactive structures. Extensive glycation can decrease IgE binding due to a masking effect on linear as well as conformational epitopes and can also affect the recognition of peptides by AGE receptors [21,26]. Therefore, a higher level of glycated digestion-derived peptides may also alter the immunological response towards digested MP.

In chapter 4, it was demonstrated that HT-MP showed the highest percentage of glycated peptides after 10 min in the IP (Figure 6.1). Moreover, it was confirmed that these glycated peptides were able to pass through a Caco-2 cell monolayer (Figure 6.5), thus suggesting that they are accessible for immune cells in the *lamina propria*. Xue *et al.* [21] demonstrated that the modification of the synthetic peptides FKDLGEE and DEFKADE with CML and N^ε-carboxyethyllysine increases their binding to sRAGE compared to the non-modified form. This shows that for these specific modifications and peptide sequences, the MR can be a crucial determinant of the cellular response even on the peptide level. Moreover, it highlights the importance to gain more insights into the peptide patterns and post translational modifications of the peptides formed upon digestion of differentially heated MP. It should be noted that the concentrations of these peptides are often too low to show a significant change in receptor binding. However, the role of other MR products has not been investigated and it could be hypothesised that an increasing total concentration of peptides carrying any MR products with similar properties, such as a negative charge (chapter 4), could also show physiologically relevant binding to AGE receptors. Although receptor binding to the *in vitro* digest was tested (Figure 7.1), it does not allow any conclusion to underline this hypothesis, as quantification of the glycated peptides was not possible and interfering substrates, such as protein aggregates and substrates of the intestinal juice, are present in the digests.

Changes of peptide patterns upon digestion of differentially heated cow's milk and dairy products has been the subject of a number of studies [7,30-35]. The prediction of the immunological consequences in relation to digestion-derived peptides is however still

challenging. Nevertheless, the screening of linear IgE binding and T-cell binding epitopes in the *in vitro* digest, in combination with their availability to the immune system in the gastrointestinal tract, can indicate the potential of a specific heat treatment to modulate immunoreactivity. Chapter 6, demonstrated that after 10 min in the IP more IgE binding epitopes are found in NT-MP compared to dry-heated samples. This has previously been demonstrated by Picariello *et al.* [36] who observed that the peptides derived from BLG (f127-135), which showed remarkable resistance upon *in vitro* digestion and trans-epithelial translocation, were also recognised by IgE of allergic patients. Next to this, chapter 6 also demonstrated that the majority of specific IgE (sIgE) binding epitopes and T-cell epitopes found in dry-heated samples were glycated. It was suggested by Gasparini *et al.* [37] that lactosylation could have an effect on binding to linear sIgE epitopes, however studies that directly show an effect of glycation on either sIgE or T-cell epitope binding are limited. Moreover, the transport of epitopes across the Caco-2 cell monolayer was limited, which suggested that other transport pathways, such as direct sampling via dendritic cells or translocation via microfold cells (M-cells) could play a pivotal role in availability of immunoreactive digestion-derived peptides.

7.7 Transport of peptides across the Caco-2 cell monolayer

The passage of the intestinal epithelial barrier is important to make the antigen available for APCs in the *lamina propria*. Moreover, the form in which an antigen is presented to the immune system, after heat induced modifications and enzymatic hydrolysis upon digestion, is crucial for the immunological response. In chapter 6, it was demonstrated that peptides of a length up to 24 amino acids, including glycated peptides, were able to pass through a Caco-2 cell monolayer. Although it is not clear whether the transport of glycated peptides occurs in immunological relevant dosages, the proof that they are able to pass the epithelial layer gives first insights and points out the relevance of further studies. It also has to be noted that next to epithelial cells, direct sampling as well as the uptake via M-cells and the Peyer's Patches (Figure 1.2) has a pivotal role in the subsequent immunological reaction cascade. Roth-Walter *et al.* [2] showed that UHT-treated BLG is preferably taken up via Peyer's Patches, thus pointing out the importance of studying different uptake routes. Moreover, Deng *et al.* [22] demonstrated that heated and glycated BLG can pass through the Caco-2 cell monolayer, pointing out that also the digestion persistent aggregates could be accessible for the immune system in the *lamina propria*. Next to this, allergic sensitisation can also result in increased permeability in the intestine ("leaky gut") which might increase the uptake of specific peptides and larger particles that can possibly affect the immunological response to the digested protein. Further studies, with both intact proteins but also their digestion-derived peptides, including also studies on different transport mechanisms, would improve the knowledge on the effects of heat treatment on immunoreactivity of cow's MP.

7.8 Immunological consequences of dietary derived AGE ligand interaction with AGE receptors

Chapter 5 showed that due to a higher resistance of glycated aggregates of BLG to simulated infant *in vitro* digestion, they maintain their binding ability to sRAGE and Gal-3 (Figure 5.6). Both sRAGE and Gal-3 play a role in inflammation. With respect to AGEs as ligands, this has mostly been described in relation with high serum levels of AGEs, however so far little was known on whether binding occurs to diet-derived AGEs and how APCs process the antigen after recognition. Chapter 5 showed that Gal-3 mediates internalisation of glycated BLG, whereas RAGE only showed binding but no involvement in the internalisation of glycated BLG (Figure 5.4). This indicated a differential role of the two receptors in allergic sensitisation to glycated MPs. However, it remains to be further investigated whether the binding to these receptors will result in a pro- or an anti-inflammatory response. Previous studies showed that receptor mediated recognition and uptake by APCs is a key point for cytokine expression in subsequent T-cell cocultures resulting in the increased expression of either pro- or anti-inflammatory cytokines [38-42]. Perusko *et al*. [38] suggested that the intercellular fate of BLG affects cytokine expression, where faster degradation results in lower expression of pro-inflammatory cytokines. However, as shown in chapter 3 and 5, differential treatment of BLG results in different ligands for AGE receptors and thus possibly modulates the uptake via ACPs and the immunological response. Moreover, the sensitisation pathway may affect the cellular response after re-exposure to the antigen or its thermally modulated form. The exposure to "baked milk" normally occurs in the already sensitised patient, highlighting the importance of further investigations of the immunomodulatory properties of heat induced structural modifications to pre-exposed APCs. As the immune system of infants still develops, infant formulas generally experience intensive heat treatment to ensure a safe consumption. This treatment as well as the high content of lactose results in relatively higher levels of MR products compared to most other dairy products [43]. This suggests that sensitisation may already occur against glycated MPs. However, the heating conditions strongly differ from the ones applied in "baked milk" products. Therefore, the structure of the heat-modulated proteins may not be comparable to the one in commercially available infant formula and results in a differential immune response. One of the hypothesise of the iAGE-project was that children that are able to consume baked milk are sensitised to conformational epitopes which are destroyed upon dry heating of the MP, either via protein aggregation or by the masking effect of the MR. A role of 3D-modification as well as the MR was shown in this thesis (chapter 3-5). However, data also suggested that the role of 3D-modifications decreases when the MP is applied to simulated infant *in vitro* digestion. It was thus hypothesised that digestion-derived peptides carrying a immunoreactive structure could play a role in tolerance to "baked milk" and that MR-induced modifications on the peptide level could also be a determinant in the resolution of cow's milk allergy symptoms upon "baked milk" consumption (chapter 6).

7.9   Overall conclusions

In summary, the research presented in this thesis showed that heat-induced changes of the protein structure affect digestibility and immunoreactivity of cow's MPs. The applied heat treatment (dry vs. wet and LT vs. HT) is a crucial determinant for the generation of structural modification of MP that can affect their digestibility, immunoreactivity, and allergenicity. In addition, it was demonstrated that the presence of specific AGEs can modulate binding to the receptors of AGEs by adding an additional negative charge to the modified amino acids. This also indicates that any other amino acid side chain modification that has comparable properties could possibly result in similar effects. However, it was also shown that next to the presence of AGEs, hydrophobicity and aggregation play a pivotal role in modulating the immunological response towards MPs. The persistence of these immunoreactive structures upon enzymatic hydrolysis as shown on the example of aggregated BLG can still induce an immunological response after *in vitro* digestion. This highlights the importance of combining digestion experiments with immuno-assays to better judge the physiological relevance of heat-induced structural changes. Next to the persistence of immunoreactive aggregates upon digestion also the release of potentially immunoreactive peptides could modulate the immunological response. It was shown that glycated peptides were able to cross the Caco-2 cell monolayer and thus are possibly accessible for the immune system. The quantities in which these peptides arrive at the basolateral side might be crucial for the immunological response to these peptides. Overall, the findings of this thesis indicate that the immunological response can be manipulated depending on the chosen heating conditions.

7.10 Future perspectives

This thesis demonstrated that CML-modified BLG is a potent ligand for the receptors for AGEs and that peptides carrying a CML modification are able to pass the epithelial barrier. However, further studies would be needed that include quantitative data of the glycated peptides that are transported across this barrier. This could, for example, be achieved using isotopic labelling which can display statistical significant differences already at small differences, while label free approaches less repeatable and thus not suitable for comparison of samples with only little differences [44]. The most accurate option would be the use of a synthetic peptide as MR-induced modifications of peptides can largely affect the ionisation of the peptide and thus the signal intensity. This is however difficult to obtain for each peptide and including every possible modification.

Next to the limitations of a quantitative analysis of glycated peptides, it is also still a debate whether or not the site modifications of a peptide by MR would affect the functionality of an sIgE-binding or T-cell epitope. Therefore, testing of specific peptides in modified vs. non-modified form in immunological assays would strongly improve the understanding of the role of the MR on the peptide level. Gaining more insights in the quantities of these peptides and the threshold at which they possibly induce an immunological response is an important factor to determine at which level the MR is able to stimulate immunological responses after *in vitro* digestion.

Chapter 5 showed that heated and glycated BLG is take up by APCs, however, further studies on DC-T-cell interaction would be needed to understand whether the receptor mediated recognition and internalisation of glycated aggregates of BLG would result in a pro- or anti-inflammatory immunological response. Moreover, an extension of this research to the effect of dry heating on isolated BLG that, as shown in chapter 3, results in the formation of different sRAGE ligands compared to wet heated BLG, would help to understand the role of dry heating in general.

Finally, immunological assays should be extended to WPs and caseins in a mixture, to stepwise develop the model system towards the situation in "baked milk" as both MP fractions can induce immunological responses and influence the aggregation behaviour and digestion kinetics of each other. Knowing the underlying immunological mechanisms and the structural modifications on both MP fractions will help to develop dry-heated MP products (i.e. "baked milk").

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Summary

The consumption of baked milk products has been described in relation to an accelerated resolution of cow's milk allergic symptoms in cow's milk allergic children that can tolerate baked milk. This thesis was part of a larger project, consisting of 4 work packages. This project aimed to understand the underlying mechanism of this accelerated resolution, by combining a physicochemical characterisation of the heat-induced protein modifications (work package 1) with immunoreactivity characterisation (work package 2). The obtained knowledge should then be used to support the production of a baked milk product (work package 3) which was used in a clinical trial (work package 4) to assess the tolerance-inducing ability and clinical reactions towards this product. This thesis focused on the physicochemical changes that occur upon heating of cow's milk protein (MP) and connect this to their possible effects on immunoreactivity. The underlying hypothesis of this work was that 3D-modifications of the protein structure that occur upon dry heating as well as the Maillard reaction (MR), in particular the formation of advanced glycation end products (AGEs), affect the immunoreactivity of the protein. These heat-induced modifications also affect digestibility. Furthermore, the form in which an antigen encounters the immune system in the gastrointestinal tract is crucial for its recognition by the immune system. Therefore, the assessment of digestibility was also part of the investigations.

In **chapter 2**, a mixture of casein and whey protein was dry-heated in the presence of the reducing milk sugar lactose at high temperature and low temperature to assess the impact of aggregation and MR vs. minimal aggregation and MR on protein digestibility. Both heat treatments resulted in loss of solubility. Therefore, investigations on the inter- and intramolecular interactions that lead to insolubility were performed to determine their role in both the decrease of protein solubility as well as digestibility. Results indicated that mainly caseins end up in the insoluble fraction. Different inter- and intramolecular interactions were responsible for the loss of solubility after both heat treatments. Among all changes occurring, after high temperature heating, both MR-induced crosslinking and other mechanisms of crosslinking were suggested to play a crucial role in decreasing both solubility and digestibility of MP. This also resulted in the release of larger particles from the insoluble material upon gastric digestion and a slower degradation in the intestinal phase. This, as well as the high levels of AGEs, was proposed to affect immunoreactivity of MP.

To disentangle the role of aggregation and the MR on immunoreactivity, **chapter 3** used a simplified model system containing only isolated β-lactoglobulin (BLG). Differential heat treatments comparing low vs. high temperature, dry vs. wet heating, as well as presence and absence of lactose were applied. By using a combination of western blot and ELISA assays, the binding of immunoglobulin E (IgE) as well as the soluble form of the receptor for AGEs (sRAGE) towards the different protein structures (high and low molecular weight) that are visible on the SDS-PAGE was determined. Results showed that protein aggregation plays an

important role in the formation of IgE-binding epitopes and sRAGE binding ligands. While high individual differences between patients did not allow a clear distinction between the effect of aggregation and glycation on IgE binding, it was observed that sRAGE binding strongly increased in the presence of lactose. The promoting effect of glycation on protein aggregation as well as the formation of glycation structures were found as possible explanations for this. In contradiction to literature, sRAGE binding was not observed to all structures visible on the SDS-PAGE, that contained N^{ε}-carboxymethyllysine (CML). Moreover, it is unclear whether these ligands would encounter the immune system in the gastrointestinal tract in the same form after *in vitro* digestion.

In **chapter 4**, BLG was chemically modified to create CML-BLG, to gain more insights in the role of CML in binding to three different AGE receptors: sRAGE, galectin-3 (Gal-3), and CD36. By using this approach, other 3D-modifications of the protein were minimised and allowed a better assessment of the specific role of CML. This was additionally compared to low temperature glycated BLG. Results showed that protein-bound CML was indeed a ligand for AGE receptors which was related to the negative charge of CML. At the same time, binding to low temperature glycated BLG seemed to be related to its increasing hydrophobicity, however results also suggested a possible role of other MR products which may have affected the charge state of the protein and thus its binding to AGE receptors.

The immunological mechanisms following the binding of heated and glycated BLG to AGE receptors was studied in **chapter 5**. BLG wet heated in the presence and absence of two reducing sugars (glucose and lactose) was incubated with THP-1-derived macrophages and monocyte-derived dendritic cells, respectively. The receptor binding was assessed using ELISA and the role of different AGE receptors in internalisation as well as cytokine production was measured. Results showed that especially aggregation led to the formation of AGE-binding ligands and internalisation via CD36, Gal-3 and scavenger receptor class A type I by both cell types. After *in vitro* digestion of heated and glycated BLG, binding to sRAGE and Gal-3 was higher to the digest of glycated BLG compared to other samples. This suggested that even after digestion, glycated milk protein could still trigger immunological responses that could possibly lead to T-cell activation. Moreover it was suggested that immnological responses that are induced by binding to AGE receptors occur via different pathways, with sRAGE functioning in signalling pathways and Gal-3 as a putative receptor responsible for antigen uptake.

Next to aggregates that survive *in vitro* digestion, also digestion-derived peptides can carry immunoreactive structures. In **chapter 6**, peptides generated after simulated infant *in vitro* digestion of dry-heated MP were monitored. sIgE binding epitopes were identified based on literature, while potential T-cell epitopes were predicted using a software tool. Similar numbers of epitopes were found in all samples, however in heated samples more of these peptides were glycated. This suggested that epitope recognition could be altered in heated samples. Overall, relatively more glycated peptides were generated in heated samples and Caco-2 cell experiments indicated a preferred transport of especially lactosyllysine and

glucosyllysine- modified peptides, but also CML-modified peptides in high temperature heated MP. This suggests that relatively more AGE receptor ligands are available in heated samples. On the other hand, only a low number of peptides carrying an epitope structure were transported, suggesting that the availability of T-cell and sIgE binding epitopes following transport via normal enterocytes might be limited.

This thesis showed that several heat-induced structural modifications e.g. increased hydrophobicity, protein aggregation, and presence of AGEs, can affect immunoreactivity of MP. Moreover, it suggested that especially those immunoreactive structures that are able to survive gastrointestinal digestion could also modulate the immunological responses *in vivo*.

List of abbreviations

Abs_{Max}	Absorbance obtained from receptor without competition agent
Abs_{Min}	Absorbance obtained blank sample
Abs_{Sample}	Absorbance obtained the mixture of the receptor and the sample
ACN	Acetonitrile
AGEs	Advanced glycation end products
ALA	α-lactalbumin
ANS	8-anilino-1-naphthalenesulfonic acid
APCs	Antigen presenting cells
a_w	Water activity
BLG	β-lactoglobulin
BSA	Bovine serum albumin
CBA	Cytometric bead array
CD	Circular dichroism
CE	Collision energy
CEL	N^ε-carboxyethyllysine
CM	Cow's milk
CMA	Cow's milk allergy
CML	N^ε-carboxymethyllysine
CV	Cone voltage
DCs	Dendritic cells
DDT	Dithiothreitol
DH	Degree of hydrolysis
DMEM	Dulbecco's modified eagles medium
ECL	Enhanced chemiluminescence
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Gal-3	Galectin-3
GP	Gastric phase
HT	High temperature
HT-MP	High temperature heated milk protein
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IP	Intestinal phase
LFQ	Label free quantification
LPS	Lipopolysaccharide
LT	Low temperature
LT-MP	Low temperature heated milk protein
M-cells	Microfold cells
MG-DH1	Methylglyoxal-dihydroxyimidazoline

MGO	Methylglyoxal
MoDCs	Monocyte-derived dendritic cells
MP	Milk protein
MHC-II	Major histocompatibility complex class II
MR	Maillard reaction
MRPs	Maillard reaction products
MW	Molecular weight
NaCl	Sodium chloride
nePTMs	Non-enzymatic post translational modification
NH_2 (acid)	Total concentration of free amino groups in the sample after acid hydrolysis
NH_2 (final)	Concentration of free amino groups in the digested sample
NH_2 (initial)	Concentration of free amino groups in the sample before digestion
$N_{measured}$	The nitrogen concentration in the soluble fraction
$N_{theoretical}$	The maximum possible concentration of nitrogen that can go into solution
$N_{transfer}$	Nitrogen transferred into in solution
NT	Non-treated
NT-MP	Non-treated milk protein
OPA	o-phthaldialdehyde
Ovalbumin	Ovalbumin
oxLDL	Oxidised low density lipoprotein
PBS	Phosphate buffer saline
PMA	Phorbol 12-myristate 13-acetate
PMCS	Peripheral blood mononuclear cells
PTMs	Post translational modifications
RAGE	Receptor for advanced glycation end products
RPMI 1640	Roswell park memorial institute 1640 medium
SDS-PAGE	Sodium dodecyl sulfate gel electrophoresis
SE	Glycated soy extract
SEM	Scanning electron microscopy
sIgE	Specific immunoglobulin E
sMRM	scheduled multiple reaction monitoring mode
SMUF	Simulated milk ultra-filtrate
SR-AI	Scavenger receptor class A type I
sRAGE	Soluble receptor for advanced glycation end products
TEER	Transepithelial electrical resistance
TBS	Tris buffer saline
TBST	Tris buffer saline with tween
Th	T-helper
ThT	Thioflavin-T
TL	Tube lens offset
TMB	3,3′,5,5′-tetramethylbenzidine
TNF-α	Tumor necrosis factor α

T_{reg}	Regulatory T-cells
q	Transitions used for quantification
Q	Transitions used for confirmation
UHT	Ultra high temperature
WP	Whey protein

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About the author

Curriculum vitae

Hannah Elisabeth Zenker was born on the 28th of November 1992 in Augsburg, Germany.

In 2011, she started her studies of food chemistry at the Friedrich-Alexander University Erlangen-Nuremberg.

For her scientific dissertation from October 2015 to March 2016 she spent 6 months at Abertay University in Dundee, Scotland under the supervision of Alberto Fiore. In this project she focused on the mitigation of Maillard reaction products in cookies by the phenolic compounds, fibre, and protein derived from rapeseed press-cake. She finished the first part of the state exam for a state examined food chemist in April 2016. In October 2016, she started her PhD project in the Food Quality and Design Group at Wageningen University and Research in Wageningen, the Netherlands. The results of her research are presented in this thesis.

List of publications

This Thesis:

- **Hannah E. Zenker**, Jana Raupbach, Sjef Boeren, Harry J. Wichers, Kasper A. Hettinga (2020). The effect of low vs. high temperature dry heating on solubility and digestibility of cow's milk protein. Food Hydrocolloids. (in press)

- **Hannah E. Zenker**, Arifa Ewaz, Ying Deng, Huub F. J. Savelkoul, R. J. Joost van Neerven, Nicolette W. De Jong, Harry J. Wichers, Kasper A. Hettinga, Malgorzata Teodorowicz. (2019). Differential effects of dry vs. wet heating of β-lactoglobulin on formation of sRAGE binding ligands and sIgE epitope recognition. Nutrients, 11(6):1432.

- **Hannah E. Zenker**, **Malgorzata Teodorowicz**, Arifa Ewaz, R. J. Joost van Neerven, Huub F. J. Savelkoul, Nicolette W. De Jong, Harry J. Wichers, Kasper A. Hettinga (2020). Binding of CML-modified as well as heat-glycated β-lactoglobulin to receptors for AGEs is determined by charge and hydrophobicity. International Journal of Molecular Sciences, 21, 4567.

- **Hannah E. Zenker**, Harry J. Wichers, Monic M. M. Tomassen, Sjef Boeren, Nicolette W. De Jong, Kasper A. Hettinga (2020). Peptide release after simulated infant *in vitro* digestion of dry heated cow's milk protein and transport of potentially immunoreactive peptides across the caco-2 cell monolayer. Nutrients, 12, 2483.

Others:

Hannah E. Zenker, **Glenn A. A. van Lieshout**, Martine P. van Gool, Marjolijn C. E. Bragt, Kasper A. Hettinga (2020) Lysine blockage of milk proteins in infant formula impairs overall protein digestibility and peptide release. Food and Function, 11, 358369.

Overview of complete training activities

Discipline specific activities

Courses

Advance Food analysis (VLAG, Wageningen-Netherlands 2017)

Advanced proteomics (VLAG, Wageningen-Netherlands, 2017)

Advanced Immunology (IIU, Utrecht-Netherlands, 2018)

Reaction Kinetics (VLAG, Wageningen-Netherlands, 2018)

Dairy Biochemistry Masterclass (Aarhus University & VLAG, Aarhus-Denmark & Wageningen-Netherlands, 2019)

Conferences and meetings

Young AGErs symposium (University of Berlin, Berlin-Germany, 2017)

Young AGErs symposium (Wageningen University, Wageningen-Netherlands, 2018)

Young AGErs symposium (Maastricht University, Maastricht-Netherlands, 2019)

International conference of food digestion (Infogest, Granada-Spain, 2019)

German food chemistry days (TU Dresden, Dresden-Germany, 2019)

International symposium of molecular allergology (EAACI, Amsterdam-Netherlands, 2019)

General courses

Applied statistics (VLAG, Wageningen-Netherlands, 2017)

Introduction to R (WGS, Wageningen-Netherlands, 2017)

Brain Training (WGS, Wageningen-Netherlands, 2017)

Competence assessment (WGS, Wageningen-Netherlands, 2017)

Scientific publishing (WGS, Wageningen-Netherlands, 2018)

Scientific writing (Wageningen in'to language, Wageningen-Netherlands, 2018)

Reviewing a scientific paper (WUR library, Wageningen-Netherlands, 2018)

Research data management (WUR library, Wageningen-Netherlands, 2018)

Debate training and practice tournament (WGS, Wageningen-Netherlands, 2018)

Scientific artwork vector and images (WGS, Wageningen-Netherlands, 2018)

Searching and organising literature for PhD candidate (WGS, Wageningen-Netherlands, 2018)

Optional activities

Preparation of research proposal (Wageningen-Netherlands, 2016)

PhD study tour to Australia (Australia, 2018)

Preparing PhD study tour to Australia (Australia, 2018)

Organising young AGErs symposium (Wageningen-Netherlands, 2018)

Member of the VLAG PhD council (Wageningen-Netherlands 2016-2019)

Meetings and colloquia (Wageningen, 2016-2020)